Studies into Electroactive Microbial Communities

by

Alexander C. Neocleous, MChem

Submitted for the degree of Doctor of Philosophy

Department of Chemistry
The University of Surrey

September 2014

© Alexander C. Neocleous 2014
Abstract

Microbial fuel cells (MFCs) are an evolving technology built around the idea of treating waste water while simultaneously generating a sustained power output. A large focus has been placed on known electrogens such as *Shewanella oneidensis* and *Geobacter sulfurreducens*, while very little is understood about how species interact together in a microbial community. While inoculating MFCs with naturally occurring communities is one method of studying fuel cells, this work took a different approach and attempted to build a synthetic community based upon a naturally occurring community. This will be important in the future of MFCs since being able to tailor a community to the influent feed will theoretically allow a more efficient use of the substrates and a higher level of treatment to be achieved. The synthetic community was made up of 5 different species, modelled on a naturally occurring microbial community. This was carried out through a number of single species, dual-species and 5-species MFC experiments. This will allow the electrogenic ability and the community’s ability to treat wastewater to be compared between single species and communities.

The single-species MFCs demonstrated the electrogenic potential of three previously unstudied species. It was also observed that the single species MFCs produced higher power than the dual-species and 5-species communities. This contradicts the commonly held view that as biodiversity increases, power output increases. In the dual-community MFCs, a trend emerged where the species which produced the highest power outputs were associated with MFCs containing a fermentative species and an anaerobic respirator, while the lowest powers were observed in dual-cultures made up of two fermentative species. Cyclic voltammetry showed that the studied species did not produce extracellular mediators and the electrogenic activity occurred in the biofilm. It was also shown that as the biodiversity of the community increased, so did the level of chemical oxygen demand (COD) removal, with the 5-species community achieving the highest COD removal of 90% compared to the 70% of the single species.

Metabolic experiments were carried out using a Biolog Omnilog on the individual species and 10 dual species combinations to try and understand the different substrates utilised by individual species. This was done by comparing respiration rates and lag times for the individual substrates which are relevant to MFC experiments. It was observed that faster growth rates but longer lag times were generally observed when two species tried to utilise the same substrate, but the results did not correlate with the results generated from the MFC experiments.
Declaration of Originality

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, in the bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree of professional qualification. I agree that the University of Surrey has the right to submit my work to the plagiarism detection service Turnitin® UK for originality checks. Whether or not drafts have been so-assessed, the University of Surrey reserves the right to require an electronic version of the final document (as submitted) for assessment as above.
Acknowledgements

I thank the EPSRC for funding my PhD research within the Energy Storage Consortium and I gratefully acknowledge my supervisors Prof Robert Slade and Dr Claudio Avignone-Rossa for their support, guidance and supervision.

I would also like to thank Dr Noel Wardell for all of his help solving the variety of problems I encountered in the laboratory and Jane Newcombe for her help, guidance and assistance when it came to conducting the Biolog experiments.

Finally, I would like to thank my family for their support and belief in me and a special thanks to Sarah Mallinson for her support, encouragement and tolerance during the rather hectic write up period.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABE</td>
<td>Acetone, butanol, ethanol</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ASP</td>
<td>Activated sludge process</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BES</td>
<td>Bioelectrochemical systems</td>
</tr>
<tr>
<td>CE</td>
<td>Coulombic efficiency</td>
</tr>
<tr>
<td>CMM</td>
<td>Chopped meat medium</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EMF</td>
<td>Electromotive force</td>
</tr>
<tr>
<td>FAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>IC</td>
<td>Ion chromatography</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirits</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropyl alcohol</td>
</tr>
<tr>
<td>MB17</td>
<td>Mordant Black 17</td>
</tr>
<tr>
<td>MDC</td>
<td>Microbial desalination cell</td>
</tr>
<tr>
<td>MFC</td>
<td>Microbial fuel cell</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitriloacetic acid</td>
</tr>
<tr>
<td>OCV</td>
<td>Open circuit voltage</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OFN</td>
<td>Oxygen-free nitrogen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEM</td>
<td>Proton exchange membrane</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate theylene-diamine-tetraacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acids</td>
</tr>
</tbody>
</table>
Aims and Objectives

The main objective of this project was to build a synthetic community, which was assembled from combinations of individual species that were observed in microbial fuel cells (MFCs) using anaerobic digester sludge. The selected species are *Serratia plymuthica*, *Ochrobactrum anthropi*, *Comamonas denitrificans*, *Clostridium indolis* and *Bacteroides graminisolvens*. This project investigated the electrochemical and metabolic properties of the individual species as well as characterising the metabolism of a synthetic community composed of combinations of the five species.

Metabolic Studies

How will the respiration rates change when pairing the individual species, to form different co-cultures?

The metabolic studies were used to try and determine how the presence of different species altered the respiration rates of the different species utilising different substrates. A series of growth curves were plotted for the individual species to mimic the conditions of the MFCs, which were used to predict which species would grow at the fastest rate in the MFCs. In an attempt to study the selected species, the novel use of the Biolog Omnilog™ was trialled to measure the metabolic profile of the selected five species, and the ten possible co-culture combinations. The experiments looked at metabolites or substrates commonly associated with MFCs (sugars, VFAs and amino acids) and the lag times and maximum respiration rates associated with them. The lag times and maximum respiration rates were measured to determine which species are able to utilise the individual substrates in an attempt to get an understanding of the roles the different species will fulfil in a synthetic community.

Electrogenic Studies

How will the power output change when comparing the individual species to the different co-cultures and 5-species community?

The electrogenic studies aimed to characterise how the power output varied when comparing single species, dual-species and the 5-specie synthetic community. Two MFC repeats were run under identical conditions for each of the five species listed in the previous statement. After the power output had stabilised, polarisation curves were
carried out on each MFC to determine the maximum power output of the individual species. The experiments were then repeated for each of the 10 unique co-species combinations of the five aforementioned species. Finally, the experiments were applied to a synthetic community made up of each of the five species. The electrogenic profiles for the individual species were compared to the co-culture experiments to determine which species paired synergistically and which species paired antagonistically. The profiles of the co-culture MFCs were compared to the five species MFCs in an attempt to determine which species are contributing to power generation.

Samples were taken continuously for denaturing gradient gel electrophoresis (DGGE) experiments to check for purity and to monitor if a species was ever eliminated from the community. Samples were also taken for cyclic voltammetry (CV) experiments which were used to determine if any metabolites, excreted by a species, are redox active which could suggest a synergistic relationship where one species produces a mediator which can be utilised by the other species present. The ability of the different compositions to remove chemical oxygen demand (COD) from the feed was also measured and compared in an attempt to explain power generation observed.

Finally, the results from the metabolic studies were examined in the context of the electrogenic studies in order to explain the differences in the various co-culture MFCs as well as to ultimately define each species’ role in the 5-species community.
List of Contents

Chapter 1 – Introduction and Background Information

1.1 Current State of Power Generation

1.1.1 Power Generation

1.1.2 Green Alternatives

1.1.3 Fuel Cells

1.1.4 Bioelectrochemical Systems

1.2 Microbial Fuel Cell Development

1.2.1 History of Microbial Fuel Cells

1.2.2 Concept of a Microbial Fuel Cell

1.2.3 Problems with Microbial Fuel Cells

1.3 Microbial Fuel Cell Designs

1.3.1 Materials

1.3.2 Architecture

1.3.3 Scale Up

1.4 Electrogenic Theory

1.5 Electron Transfer Mechanisms

1.5.1 Direct Electron Transfer

1.5.2 Mediated Electron Transfer

1.6 Waste Water Treatment with Microbial Fuel Cells

1.7 Microbial Aspects of Microbial Fuel Cells

1.7.1 Microbial Metabolism

1.7.2 Effect of Commonly Used Substrates

1.7.3 Effect of Other Substrates

1.7.4 Improving the Electrogenic Properties of Bacteria

1.8 Community Studies

1.8.1 Comamonas denitrificans
Chapter 2 – Materials and Methods

2.1 Microorganism Cultivation

2.1.1 *Serratia plymuthica* and *Ochrobactrum anthropi* …………………………… 48

2.1.2 *Comamonas denitrificans* ………………………………………………………… 49

2.1.3 *Clostridium indolis* and *Bacteroides graminisolvens* …………………… 49

2.1.4 Synthetic Waste Water…………………………………………………………… 50

2.1.4.1 Vitamin Solution…………………………………………………………… 50

2.1.4.2 Trace Mineral Solution………………………………………………… 50

2.1.5 Agar Plates……………………………………………………………………… 51

2.1.5.1 Modified Cooked Meat Agar…………………………………………… 51

2.1.5.2 Synthetic Waste Water Agar…………………………………………… 51

2.1.6 Growth Curves………………………………………………………………… 51

2.2 Microbial Fuel Cell Set Up and Operation

2.2.1 Microbial Fuel Cell Configuration…………………………………………… 52

2.2.2 Continuously Fed Batch Mode Microbial Fuel Cell Operation………… 56

2.2.3.1 Sampling Methods……………………………………………………… 58

2.3 Chemical Analysis………………………………………………………………… 58
Chapter 3 – Metabolic Analysis of the Different Microbial Species

3.1 Chapter Overview .............................................................71

3.2 Characterisation of the Individual Species ................................71
  3.2.1 Characterisation of *Comamonas denitrificans* Growth ............72
  3.2.2 Characterisation of *Serratia plymuthica* Growth ......................74
  3.2.3 Characterisation of *Ochrobactrum anthropi* Growth ................75
  3.2.4 Characterisation of *Bacteroides graminisolvens* Growth ..........77
  3.2.5 Characterisation of *Clostridium indolis* Growth .....................80
  3.2.6 Discussion ......................................................................81

3.3 Biolog Metabolic Studies ....................................................82
  3.3.1 Single Species Biolog Experiments ....................................82
  3.3.2 Volatile Fatty Acids .......................................................82
  3.3.3 Other Organic Acids .....................................................85
  3.3.4 Sugars .......................................................................88
    3.3.4.1 Commonly Used Sugars ..............................................88
    3.3.4.2 Disaccharides and Polysaccharides ..............................90
Chapter 4 Electrochemical Studies into Microbial Fuel Cells

4.1 Chapter Overview.................................................................124

4.2 Power Generation in Single Species MFCs..................................124
  4.2.1 Power Generation by *Comamonas denitrificans*......................125
  4.2.2 Power Generation by *Serratia plymuthica*.............................127
  4.2.3 Power Generation by *Ochrobactrum anthropi*.......................129
  4.2.4 Power Generation by *Bacteroides graminisolvens*..................130
  4.2.5 Power Generation by *Clostridium indolis*..............................132
  4.2.6 Comparison of the Electrogenic Activity of the Individual Species...133

4.3 Power Generation in Mixed Community MFCs.............................135
  4.3.1 Dual-Species MFCs Involving *Comamonas denitrificans*...........136
  4.3.2 Dual-Species MFCs Involving *Serratia plymuthica*..................138
  4.3.3 Dual-Species MFCs Involving *Ochrobactrum anthropi*..............142
  4.3.4 Dual-Species MFCs Involving *Bacteroides graminisolvens*.........145
  4.3.5 Dual-Species MFCs Involving *Clostridium indolis*..................149
Chapter 5 – Conclusions and Further Work

5.1 Conclusions.................................................................175
5.2 Further Work............................................................183
5.3 Review of Project Aims................................................185
5.4 Bibliography..............................................................187

Appendix I   Biolog Titre Plates.............................................189
Appendix II  Full Range CV Scans..........................................191
Appendix III Single and Co-culture Respiration Rates Comparison...........196
Chapter 1 - Introduction and Background Information

1.1 Current State of Power Generation

1.1.1 Power Generation

In 2005 it was reported that 86% of the world’s energy comes from fossil fuels [1]. This is problematic since not only are fossil fuels finite but also, upon combustion, release carbon dioxide into the atmosphere [1]. In 2009 it was estimated that known coal reserves will be depleted by 2112, whilst oil and natural gas will be depleted by 2040 and 2042 respectively [2].

1.1.2 Green Alternatives

The utilization of conventional fossil fuels leads to emissions with dire environmental consequences; the emissions consist of greenhouse pollutants including CO\textsubscript{x}, NO\textsubscript{x}, SO\textsubscript{x}, C\textsubscript{x}H\textsubscript{y}, soot, ash, droplets or tars and other organic compounds [3]. Greenhouse gases are characterised by their absorption of infra-red radiation, which is then re-emitted at a lower frequency in all directions, including back into the lower atmosphere in what is called the “greenhouse effect”. The greenhouse effect has led to shifts in the climate as the average global temperature increases. In order to try and mitigate the effect of climate change there has been a focus on sustainable energy solutions, which includes “green energy”. Green energy is the production of electricity through sustainable sources which can be utilised with little or no pollution, such as fuel cells.

1.1.3 Fuel Cells

Fuel cells are devices used to convert chemical energy into electrical energy, the most common being the hydrogen-oxygen system. Hydrogen is oxidised to form protons at the anode, producing protons and electrons. The electrons migrate through an external circuit to the cathode while the protons move through the electrolyte and the membrane. At the cathode, oxygen is reduced to form water.
1.1.4 Bioelectrochemical Systems

An extension of the conventional chemical fuel cell is the bioelectrochemical system (BES), where microorganisms are used to oxidise a biodegradable substance to generate an electrical current. The two main categories are microbial fuel cells (MFCs) and enzymatic fuel cells, where the former uses bacteria to oxidise a substrate while in the latter, enzymes located on the electrode surface are used to oxidise a substrate.

1.2 Microbial Fuel Cell Development

1.2.1 History of Microbial Fuel Cells

The link between metabolic processes and electricity was first reported in the 18\textsuperscript{th} century where Italian physician and physicist Luigi Galvani observed what he referred to as “animal electricity” during the dissection of a frog. The contact of the scalpel with frog caused the frog’s leg to twitch. The next major step in the MFC research did not occur until 1911, when the first report of bacteria producing an electrical current was published by Michael Potter. A platinum electrode was placed in a culture of \textit{Escherichia coli} and he deduced that the electrons were generated through the degradation of food by the organisms [4].

The next step forward occurred 20 years later when Barnet Cohen re-invigorated some interest in the area when he achieved a voltage of over 35 volts, using a series of microbial half cells. He was only able to achieve a current of 2 mA and research slowed down again. In 1963 DelDuca \textit{et al.} attempted to use glucose fermentation to generate hydrogen which could be used in a hydrogen and air fuel cell. Despite the functionality of the cell, it was shown to be unreliable due to the inconsistent respiration of the bacteria [5].

Until the 1970s, very little was known about how a MFC functioned despite the first paper being published 60 years previously. During the 1970s however, the area started to gain more attention with work carried out by Suzuki \textit{et al.} which saw the development of what is now seen as the standard design for an MFC [6]. The next breakthrough occurred at King College London, in the 1980s when H. Bennetto saw the potential for MFCs to generate power in developing nations [7]. His research group showed that MFCs where able to generate sustained currents for weeks if a mediator
was present. This meant that the bacteria were able to use the mediator as a terminal electron acceptor, this mediator (in its reduced form) would then be oxidised by the anode which in turn generates power. The most recent major breakthrough was made in 1999 when Kim et al. discovered that a mediator molecule was not required for electron transfer [8]. This was an important step forward because the mediators used such as methyl blue were toxic to the bacterial communities [9]. This also was the first time bacteria were demonstrated to have the ability to directly transfer electrons to the electrode [8].

1.2.2 Concept of a Microbial Fuel Cell

In an MFC, an organic carbon source is oxidised and electrons are donated to the electrode, known as the anode. The electrons produced at the anode, travel round the circuit to the cathode. At this electrode, electrons are used to reduce a substrate to complete the electrical circuit. Both chambers are separated by a membrane which allows protons to move from the anodic chamber to the cathodic chamber to balance the charge. Figure 1.1 demonstrates the basic design of a MFC where oxygen is used to complete the reduction at the cathode.

The oxidation of the substrate is carried out by either a single species or a community of bacteria located in the anodic chamber. The bacteria can be either free floating in the media forming a suspension or they will adhere to the electrode forming what is known as a biofilm. The term exoelectrogen has been given to microbes capable of directly donating electrons to the electrode [8].

MFC performance is often monitored through different electrochemical parameters including current, voltage and power. The electrical current is proportional to the rate of free electrons being transferred from one atom to another, which is measured using current in amps, where 1 amp is the equivalent of $6.241 \times 10^{18}$ electrons per second. The voltage of the system is the difference in the electrical potential between the anode and the cathode. While chemical and enzymatic fuel cells systems operate at a set voltage (since the electricity generated is through a singular reaction), bacteria are able to use a variety of different substrates. Therefore, as the food source in the feed is metabolised, a change in chemical composition of the suspension will be observed which in turn will lead to a change in voltage due to the different potential of the metabolites in
comparison to the original composition. Since the bacteria are able to replicate and form a biofilm on the surface of the electrode, an improved power will be observed as better electrical contact between the bacteria and electrode is achieved, the number of bacterial cells in contact with the electrode will also cause a change in power generated. Both of these factors are due to increasing the rate and number of electrons transferred to the electrode, improving the current.

![Diagram of MFC](image)

*Figure 1.1 – Showing the basic design concept of an air-breathing MFC*

Power refers to the amount of energy generated by the system per unit time and is measured in watts where 1 watt is defined as 1 joule per second. The power generated by the MFCs is often expressed with respect to either the volume of the anodic chamber (W m$^{-3}$) or the area of the anode (W m$^{-2}$). Resistance is the opposition to electron transport through a material and is present whenever a current is generated or applied to a system. The relationship between the resistance, voltage and current is shown in Equation 1.1.
The resistance generated from the flow of electrons is not the only form of resistance observed in MFCs. Additional resistances in the system arise from the individual reaction rates, membrane and electrode materials. The sum of all these resistances is known as the internal resistance. The reaction rates determine the rate at which electrons are generated and consumed. The membrane composition and thickness will affect the rate at which protons migrate to the cathode; balancing the charges and driving the cathodic reaction.

1.2.3 Problems with Microbial Fuel Cells

The power output of MFCs is very low compared to other established electricity generation methods. The MFC electromotive force (Emf, EMF) is defined as the difference in potential between the cathode and the anode. The work (W) done by the MFC is related to the EMF which is also related to Gibbs free energy (Gr) (Equation 1.2 and 1.3).

\[ W = E_{\text{emf}}Q = -\Delta G_r \]

Equation 1.2 – The relationship of EMF (Emf) to work (W) and Gibbs free energy (Gr), where Q is the charge transferred.

\[ Q = nF \]

Equation 1.3 – The equation to calculate the charge transferred (Q) shown in Equation 1.2, where n is the number of electrons per mole, and F is Faraday’s constant (96 485.34 C mol⁻¹).

\[ E_{\text{emf}} = E_{\text{cat}} - E_{\text{an}} \]

Equation 1.4 – The relationship between cell EMF, the potential of the cathode (Ecat) and the potential of the anode (Ean).
Using Equation 1.4 and the half-cell equations shown in Table 1.1, it can be seen that the EMF for a MFC using acetate as the substrate is 1.101 V (vs standard hydrogen electrode (SHE) in standard conditions).

**Table 1.1 – The electrode potentials for the half-cell reactions of an acetate – oxygen fuel cell.**

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Reaction</th>
<th>E (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anode</td>
<td>(2\text{HCO}_3^- + 9 \text{H}^+ + 8 \text{e}^- \rightarrow \text{CH}_3\text{COO}^- + 4 \text{H}_2\text{O})</td>
<td>-0.296</td>
</tr>
<tr>
<td>Cathode</td>
<td>(\text{O}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightarrow 2\text{H}_2\text{O})</td>
<td>0.805</td>
</tr>
</tbody>
</table>

The EMF is an ideal value and, in reality, is lower than the theoretical value due to the internal resistance of the system. The open circuit voltage (OCV) of a MFC should be close to the EMF but this is not the case due to overpotential. The term overpotential is used to refer to the loss of voltage. Experimentally, at pH 7, a voltage of 0.2 V is observed for the reduction of oxygen as opposed to the theoretical value of 0.805 V, due to the oxygen reduction reaction having an overpotential of approximately 0.6 V.

It has been suggested that MFCs will never be able to exceed an OCV of 1.14 V as determined by the NADH (-0.32 V) and pure oxygen (0.805 V) redox potentials [10]. This is because, during cell respiration, NADH is used as the primary electron carrier inside the cell. The oxidation at the anode is not directly due to the substrate being oxidised, but the oxidation of NADH.

The low power has been attributed to slow reaction kinetics and high internal resistive losses [11]. Work using electrical impedance spectroscopy and current interruption techniques have shown that the majority of the internal resistance is caused by the cathodic reaction. Despite that, it has also been shown that the internal resistance of the system decreases with time; a study using a 2-chambered glucose fed system had an internal resistance of 6200 ohms after 9 hours and 2800 ohms after 36 hours [12].

Oxygen diffusion through the membrane can lead to biofouling (the accumulation of microorganisms on a wetted surface) on the anode-facing side of the membrane. This occurs due to bacteria (which are capable of aerobic respiration) competing to utilise the oxygen that diffuses through the membrane. This can cause a barrier to proton transfer therefore causing an increase in the internal resistance of the MFC. The biofilm which
forms is composed of a microbial community encased in a hydrated extracellular polymeric substance (EPS) matrix that is made up of polysaccharides, proteins, nucleic acids and lipids [13]. Two methods are commonly used during membrane modification to prevent a biofilm forming. The first method involves using biocides such as TiO$_2$, copper or silver particles which are fixed to the surface of the membrane or trapped in membrane structure [2]. There is a chance that these biocides will leach from the membrane into the anode which will cause bacterial cell death which can compromise the power generation of the MFC. The second method focuses on altering the hydrophilicity of the membrane or membrane surface, through the incorporation of compounds such as poly(ethylene glycol) to prevent initial biofouling. However, once a membrane starts to foul, the process in MFCs cannot be prevented or reversed according to previous studies [2]. In other fields of synthetic biology, enzymatically active bacteriophages have been developed to remove biofilms which can form and contaminate a variety of different surfaces including medical devices and water pipes. These bacteriophages work by immobilising hydrolases on the proteins of a capsid which can break down the EPS matrix disrupting the biofilm [13]. The biofilm formation is not the only form of fouling which can impact the performance of the MFC; it is also possible for positively charged metal ions present in the wastewater feed to bond to the negatively charged sites on the sulphonic acid head groups. This prevents the protons associating with the sulphonic acid groups thereby inhibiting the proton transport mechanism through the membrane [4].

1.3 Microbial Fuel Cell Design

1.3.1 Materials

Air-breathing electrodes require a catalyst to assist with the reduction of oxygen to water. These are normally precious metals, with platinum being the most common choice. Nafion (a perfluorosulphonic acid polymer) is the most widely used membrane in MFCs due to the fact that it is already used in a variety of industrial processes and has been shown to generate relatively high powers. When using an air-breathing cathode, an ionomer is required to facilitate ion transport to the catalyst. When Nafion is used as the membrane, the ionomer used is a Nafion solution (perfluorosulphonic acid oligomers). It is believed that cathode performance is limited by the properties of the
three-phase boundary between gaseous oxygen, protons in solution and the solid electrocatalyst [12].

Another problem that has been reported is the presence of a liquid-liquid junction overpotential, which is caused by the adsorption of sulphonates to the active sites hindering proton migration to the electrocatalytic surface [14]. Different research groups have attempted to replace the perflurosulphonic acid ionomer with a variety of different ionomers as well as pure water which led to similar power outputs. The use of ethylenediaminetetraacetic acid (EDTA) improved power density by 42% [14]. It was posited that this was due to the EDTA binding to the active sites with the free acetic acid groups providing an alternate route for the protons, increasing their mobility [14].

As mentioned earlier, oxygen diffusion through the air-breathing cathode is a problem for MFCs. A review from Leong et al. concluded that, at present, there are no known membranes capable of preventing oxygen diffusion [2]. A possible solution is through the addition of oxygen scavenging molecules, such as cysteine, which can be used to remove oxygen from the anodic chamber [2]. Diffusion of oxygen through the membrane is not the only concern; it is also possible for substrates to crossover through the membrane. When an anion exchange membrane is used, negatively charged substrates (such as acetate and butyrate) can be transported across the membrane from the anode chamber to the cathode chamber. This can cause problems because aerobic bacteria on the cathode can use the electrode as an electron acceptor, creating an internal short circuit reducing coulombic efficiency (CE) [2].

One method of improving the efficiency of electron transfer from the electroge to the anode is through the treatment and functionalization of the electrode [15]. This has been carried out by soaking the graphite electrode in a 3:1 v/v mix of 1 mol L$^{-1}$ sulphuric and nitric acid while sonicating for 6 hours. Cytochrome c obtained from Desulfovibrio desulfuricans was then bound to the electrode. Cytochrome c is a redox active protein which is involved in cell respiration. When used in a MFC inoculated with D. desulfuricans, the current density was seen to increase by 41% and achieved a greater electron transfer rate than Geobacter sulfurreducens, one of the more commonly studied exoelectrogenic species [15].

Other groups have focussed on using non-carbon based materials for the electrodes; Ti-TiO$_2$ electrodes have attracted attention with reported improvements to performance
of 250% relative to graphite electrodes [16]. Reducing the cost of the catalyst is seen as an important step in improving the economic viability of MFCs, with MnO$_2$ being a promising choice [17][18].

1.3.2 Architecture

MFCs originally started as 2-chambered systems made up of an anodic chamber and cathodic chamber each containing the respective electrode. The chambers are separated by a membrane to facilitate proton conduction. These MFCs use electron acceptors, such as ferricyanide, which are shown to produce high power densities. These types of MFC are not feasible in the long term because of the need of replacing the electron acceptors once they have been electrochemically reduced, a costly procedure [19].

Liu and Logan were the first to work on a single-chambered MFC, using oxygen (from the air) as the electron acceptor, inspired by chemical fuel cells. In this system, the main chamber contains the anode, with the cathode bound to a membrane on one side of the chamber [19]. When compared to a 2-chambered cell where the cathode was submerged in water and purged with air, the air-cathode showed higher power densities than the aqueous cathode [19].

Microfluidic MFCs are an attempt to maximise the power production through increasing the surface area to volume ratio. This is carried out by using channels where the cross-sectional length scale is in the order of tens of microns, producing power densities of 90 mW m$^{-2}$ [20][21].

1.3.3 Scale Up

Due to the relatively low power output and small size of MFCs, various studies have been carried out looking at different ways to scale up the power and treatment abilities to increase their economic viability. One avenue of research that has been undertaken is to use additional electrodes in the anode chamber to increase the surface area to volume ratio [22][23]. Studies have shown that simply increasing the reactor size leads to decreased volumetric power output when compared to smaller bench scale reactors [24].
The most promising method of scaling up MFCs is to use a stack of MFCs, electrically connected in series. Scalable MFCs are focussed on increasing power output and power recovery while reducing material costs. Zhuang et al. used a stack consisting of 5 tubular MFCs (connected in series) to treat wastewater from a pig farm, where a power density of 175.7 W m\(^{-2}\) was obtained with a chemical oxygen demand (COD) removal of 77.1\% [10].

Usually connecting multiple batteries or fuel cells in series electrically, will cause the voltage to increase in proportion to the number of individual units. In the case of MFCs, connecting multiples together in series can cause a voltage reversal; adding additional cells may lead to smaller gains and potentially elimination of power production [25][10]. The voltage reversal has been attributed to a phenomenon known as cell starvation where insufficient substrate is present at one of the anodes in series; this in turn causes the anode to assume the voltage of a neighbouring cathode. Eventually this causes the MFC to act as a parasitic internal load on the series [26]. Work by Borghani et al. has shown it is possible to by-pass the malfunctioning MFC by connecting the MFCs in a series which appears as bridging throughout the stack [26].

Ieropoulos et al. have produced a fully autonomous robot (EcoBot) which is powered by a MFC stack. The EcoBot is powered by the degradation of organic matter (dead insects and organic waste) as a food source as opposed to wastewater [27].

1.4 Electrogenic Theory

It is well documented that certain species of bacteria are able to convert chemical energy into electricity through the oxidation of a variety of organic carbon sources. When a microorganism respires, electrons are liberated from the substrate in a series of reactions, to be finally transferred (through a chain of intracellular redox active compounds) to a terminal electron acceptor. In the case of aerobic respiration, the terminal electron acceptor is oxygen, while for anaerobic organisms a variety of compounds can act as the terminal electron acceptor [28][29][30]. The chemical compounds nicotinamide adenine dinucleotide (NAD\(^+\)) (in its reduced form NADH) and flavin adenine dinucleotide (FAD) (in its reduced form FADH) (and other electron carriers) transport the electrons released from the substrate by the metabolic reactions to a series of redox enzymes which are found in the cytoplasmic membrane; these can
include NADH hydrogenases, flavoproteins and cytochromes. Many species have been found to have the ability to transfer electrons to the anode in MFCs: the most studied species at present are Shewanella oneidensis and Geobacter sulfurreducens [31][32].

MFCs can utilise a variety of substrates while operating over a wide range of conditions if they are inoculated with a suitable microbial species or with a microbial community. Most research is carried out at ambient temperatures and at near neutral pHs but by altering the community it is possible to tailor the performance to the desired conditions [33]. Throughout MFC operation, the pH in the anodic chamber will decrease but it has been shown that acidic conditions suppress the oxidation carried out by the bacteria and the optimum power generation has been observed at between pH 6.3 and 7.0 [2].

1.5 Electron Transfer Mechanisms

The method by which a microorganism transfers an electron to the anode is extremely important in the operation of MFCs. There are two main classes of electron transfer: direct electron transfer, which involves using membrane-bound redox proteins to transfer the electron, and mediated electron transfer, which requires the microorganism to use a dissolved redox mediator to transfer the electron to the anode.

1.5.1 Direct Electron Transfer

For a microorganism to be able to directly transfer an electron to the anode, physical contact between the microorganism and anode must be present. This can be either through the bacterial cell membrane or through pilus-like structures, known as nanowires [34][35].

The electron transfer from the cell membrane to the anode has been attributed to C-type cytochromes found in Geobacter sulfurreducens, Shewanella oneidensis, Rhodoferax ferrireducens [36][37][38] and Desulfovibrio desulfuricans [39]. This is commonly the case in metal-reducing species due to the fact that, in nature, they use naturally occurring metal oxides (e.g. Fe₂O₃) as their terminal electron acceptor. When present in MFCs it was shown that these species would use the anode as an electron acceptor for the metal oxides [37], effectively replacing the metal oxide. A variety of different
species of bacteria have been shown to be able to utilise pilus-like structures commonly referred to as “nanowires” [34]. Recent work by Pirbadian et al. has asserted that the nanowires are extensions of the outer membrane and periplasm as opposed to the previously suggested pilin structures. The electrical conductivity has been attributed to the multi-heme cytochromes MtrC and OmcA [40][41][42]. These nanowires are electrically conductive and form a long range bond between the bacteria and the terminal electron acceptor (in this case the MFC anode). This allows bacteria whose membrane is not in direct contact with the surface of the anode to contribute to the power production of the MFCs. The species and classes of bacteria which have been shown to be capable of producing nanowires include the metal-reducing species (G. sulfurreducens and S. oneidensis.) [35], the cyanobacterium (Synechocystis PCC6803) and the thermophilic fermentative bacterium (Pelotomaculum thermopropionicum) [36].

It has been suggested by some sources that gram-positive bacteria are not electrochemically active, due to their cell wall electrically insulating the cell [43]. However, recent work at the University of Surrey has shown that the gram-positive species Bacillus subtilis demonstrated electrogenic activity (unpublished work, C. Avignone-Rossa and F. Zhao, 2014).

1.5.2 Mediated Electron Transfer

Direct contact with the anode is not the only method of electron transfer present in MFCs. It is possible for a species to transfer an electron to an external redox active compound which can act as an electron shuttle between the microbial cell and the anode. Three different types of mediator have been identified: exogenous redox mediators, mediators secreted by the microbe and primary metabolites [44]. To allow efficient electron transfer, the mediator should have a negative redox potential, higher than the potential of the substrate, but lower than the anode potential.

Exogenous mediators can be added to the MFC to assist with electron transfer to the anode. Examples of these mediators include neutral red and methylene blue. Research has moved away from the use of exogenous mediators since some of them, like methylene blue, have been reported to be toxic to the microbial community, while also driving up costs through essential periodic replenishment [9][45].
It has been shown that some species’ metabolic products are redox active therefore allowing them to act as a mediator, enabling the microorganism to use the mediator as a terminal electron acceptor instead of the anode. Members of the genus *Pseudomonas* produce pyocyanine and phenazine, which have been demonstrated to act as mediators in MFCs [43][46]. Riboflavins and quinones have also been proposed as electron shuttles generated by *S. oneidensis* and *Escherichia coli* [47][48].

*D. desulfuricans* MFCs operate on the sulphate/sulphide redox couple, where *D. desulfuricans* cells reduce sulphates present in the medium to hydrogen sulphide. This is then oxidised at the anode liberating the electrons used by the bacteria for anaerobic respiration [49]. This replenishes the sulphates allowing them to act as the terminal acceptor again. Fermentation products such as hydrogen, lactate and formate can also be oxidised at the anode making use of the metabolic products, but catalysts are required for the process to be carried out [50][51].

Species belonging to the gram-positive genus *Brevibacillus*, which is not electrochemically active in single species MFCs, was shown to be electrochemically active in co-cultures with *P. aeruginosa*. This was attributed to the production of phenazine [43] indicating that gram-positive species are able to use mediators to contribute to power generation. It was also suggested that rhamnolipids are believed to be essential in the electrogenic activity in *Brevibacillus* when interacting with phenazine [43]. Other gram-positive species have also been reported to be electrochemically active in pure cultures, including *Clostridium butyricum*, *Thermincola* sp, and *Lactococcus lactis* [51]. *Lactococcus* have been shown to secrete soluble quinones to which the electricity generation has been linked.

### 1.6 Wastewater Treatment with Microbial Fuel Cells

The activated sludge process (ASP) has been the primary method of wastewater treatment over the past century, where the primary goal is to remove pollutants from the wastewater stream to allow safe discharge back into the environment. The ASP method is a very energy intensive process, and in 2002 it was reported that just to aerate the sludge in USA, 2% of the total US electricity consumption was required [52]. The process also generates approximately 0.4 kg of sludge for each 1 kg of COD removed.
The sludge has specific disposal conditions and disposal costs of approximately $900 per tonne dry mass [53].

In an effort to reduce the costs associated with wastewater treatment, a focus has been placed on using MFCs to treat wastewater while simultaneously generating a sustained power. While MFCs are not able to produce large amounts of power, a sizable amount of energy will be made available by removing the need for some industries to treat their waste which, as stated earlier, is an energy intensive process. The MFCs are able to produce sustained small amounts of power over a large period of time, therefore allowing reserves of energy to be accumulated. This provides an advantage over other renewable energy sources such as solar and wind which are intermittent.

High levels of nitrates and phosphates are a concern as wastewater pollutants since the accumulation of these nutrients can lead to eutrophication; the sudden increase in the growth of plant life (usually in the form of simple algae), leading to negative environmental effects such as hypoxia (depletion of oxygen). Phosphates and nitrates are, however, important nutrients for the growth of plants and are generally the limiting factor for growth in simple plant organisms like algae. When present in high levels they cause an explosive growth, known as an algal bloom, as the algae die they sink to the bottom of the water body and decompose which consumes any oxygen present.

Denitrification is carried out at the cathode where nitrate is reduced to nitrite before being further reduced to nitrogen gas in a process which consumes 8 electrons for every molecule of NO$_3^-$ reduced. To balance the charge, 8 protons, generated by anaerobic respiration, are required to migrate through the membrane to the cathode chamber. This process requires the addition of sodium bicarbonate to the cathodic influent to maintain the pH since the reduction of nitrate causes the electrolyte to gradually become alkaline. Magnesium hydroxide and calcium carbonate deposits have been shown to develop in high alkaline environments around the cathode [54].

Other studies have examined the treatment of ammonia-contaminated wastewater. The ammonia can be used as an electron source at the anode where complete oxidation of 1 mole of ammonia leads to 8 moles of electrons; the final product in this oxidation is nitrate [55]. This could potentially be used in a conjunction with denitrification cells where the nitrate, generated through the reduction of the ammonia, is used as the catholyte where it is reduced to nitrogen gas.
The main method used for the removal of phosphates is through Struvite (\(\text{NH}_4\text{MgPO}_4\cdot6\text{H}_2\text{O}\)) crystallisation. Throughout crystallization an increase in pH is observed due to the consumption of protons to reduce hydrogen. The solubility of Struvite decreases as pH increases and it can be found deposited on or around the cathode. *Simplicissima* and *Acinetobacter* have been implicated in the removal of phosphorus for waste streams [56].

Work carried out by Tao *et al.* [57] looked at using a 2-chambered MFC to treat synthetic wastewater high in ammonia and phosphate. It was found that no phosphate removal took place in the anode but at the cathode, phosphate removal efficiency was around 90%. It was found that denitrification was linked to the dissolved oxygen (DO) level. At high DO levels (2.8 mg \(\text{L}^{-1}\) and higher), the ammonium was converted to nitrate but the total nitrogen did not markedly decrease, while at a DO level below 2.5 mg \(\text{L}^{-1}\) the nitrogen removal efficiency was over 85% [57]. It was also noted that high DO levels correlated with higher voltages, this means that treatment and power generation had to be balanced for optimum efficiency [57].

Nitrates and phosphates are not the only contaminants of concern present in wastewater: many others pose a greater problem for MFCs. Contaminants such as iron(III), sulphates and azo-dyes can act as electron sinks, competing with the anode and lowering the power output of the system [58]. Yang *et al.* looked into the effects of different electron acceptors on the behaviour of the MFC and found the presence of fumarate and \(\text{Fe}_2\text{O}_3\) increased the CE of the system, whereas nitrate, the azo-dye Mordant Black 17 (MB17), and a mixture of nitrate, azo-dye, \(\text{Fe}_2\text{O}_3\) and fumarate caused a decrease in the CE [59]. The decrease was expected because reduction of these species diverts electrons from electricity generation. It was noted, however, that the highest power densities were obtained from the MFCs inoculated with the MB17, suggesting that naphthols are generated through the reduction of the azo-bond in the dye. It has been suggested that these intermediates act as electron mediators, allowing a higher percentage of the microorganisms to contribute towards power generation [59].

Since microbes have been found in a wide variety of environments, it is possible that a microbial community could be tailored to treat specific contaminants that are found in waste streams. An example of this is the case of *Shewanella oneidensis* which has been shown to be able to reduce water soluble uranium(VI) to the insoluble uraninite (\(\text{UO}_2\)) which can then be reclaimed [60]. Another example is in the case of chloroaniline
which has been found in wastewater containing azo dyes and has been known to be degraded by *Comamonas denitrificans* [61].

1.7 Microbial Aspects of Microbial Fuel Cells

1.7.1 Microbial Metabolism

Microbial metabolism takes place through a variety of pathways depending on the species and the substrates. Not all microorganisms are able to utilise the same pathways. Therefore, a microbial community will have an expanded access to a wider range of pathways and an increased flexibility when it comes to utilise them. Different metabolic pathways are used for both aerobic and anaerobic respiration. Figure 1.2 shows a simplified schematic showing the relationships between different metabolic pathways and power generation in an MFC.

![Microbial Metabolism Diagram](image_url)

*Figure 1.2 - A simplified schematic showing the various degradation pathways and stages in MFCs, adapted from Velasquez-Orta et al. [60].*
Glycolysis (shown in Figure 1.3) is the metabolic process in organisms which converts glucose into pyruvate. 1 molecule of glucose is broken down, generating 2 molecules of adenosine triphosphate (ATP), the main metabolic energy carrier and 2 molecules of pyruvate. In the presence of oxygen, pyruvate is mainly consumed in the Krebs cycle whilst in anaerobic conditions a variety of different processes are utilised depending on the species.

Fermentation is the biochemical breakdown of organic compounds where the products retain their oxidation state but with reduced energy content [62]. Fermentation leads to a variety of different products including volatile fatty acids (carboxylic acids which consist of 6 carbons or less, VFAs), hydrogen and other organic compounds like alcohols and lactate. Lactate and the VFAs are often used as organic carbon sources in fuel cells linking their consumption with electricity generation [62]. As butyrate and propionate are utilised, acetate can be generated in a side reaction [38]. Fermentation is a complex process and there are a wide range of different possible pathways which can be used by fermentative species. Figure 1.4 shows an example schematic outlining the fermentation of glucose in a heterofermentative species.
Figure 1.3 - A non-stoichiometric schematic showing the individual steps and enzymes required for glycolysis [63]
Chapter 1: Introduction and Background Information

Hydrolysis of disaccharides and polysaccharides, and fermentation both lead to energy losses, reflected by the decrease in CE as the substrates become more complex [66]. This is due to a large range of metabolites being generated (including gases such as hydrogen) which cannot be utilised by species for power production. It is important to note, however, that fermentation is important for converting molecules (such as glucose) which cannot be directly used for power production into substrates (such as lactate and acetate), which are commonly used as carbon sources in MFCs.

Studies have shown that the rate constant for fermentation can be 10 times higher than for that of hydrolysis [66]. This can be used to explain the long lag times required for the MFC power output to stabilise, commonly associated with MFCs inoculated with complex organics, as opposed to VFAs and lactate which generally have shorter lag times before peak power productions [66].

It has been reported that when working with air-breathing MFCs, significant oxygen diffusion in the anode chamber can occur even when using Nafion (which is a proton exchange membrane (PEM)). This means that even if the feed is purged, there is still the possibility of aerobic respiration occurring in the anode chamber [10][67], albeit to a lesser extent. As mentioned earlier, when oxygen is present, aerobic respiration is

Figure 1.4 - A schematic showing the fermentation of glucose into ethanol and lactate by a heterofermentative species.
possible where pyruvate is converted to citrate in the “Krebs cycle” shown in Figure 1.5. While aerobic respiration reduces CE by diverting electrons to oxygen as opposed to the electrode. Due to the individual stages of the cycles occurring at different rates; it is possible that substrates, such as fumarate and malate, could be excreted due to overflow metabolism [68].

Methanogenesis is a metabolic process associated with the domain of single cell organisms known as archaea. Despite being phylogenetically distinct from bacteria, they are commonly found associated with anaerobic communities meaning they are often observed in naturally occurring microbial communities. Methanogenesis is seen as detrimental in microbial communities since methanogens use carbon in the form of carbon dioxide or acetate as an electron sink, as the carbon is reduced to methane.
Work has been carried out using different methods to suppress methanogenesis. One method used 2-bromoethanesulphonate to suppress the growth of methanogens, which lead to an increase in hydrogen production [66]. While it was also reported that methanogenesis decreases over time as a more electrogenic community is selected for [66].

1.7.2 Effect of Commonly Used Substrates

Acetate is the most common substrate of choice for MFC studies, due to its inertness towards alternative microbial conversions (fermentations and methanogenesis) at room temperature. This increases the power density by diverting all electrons to the anode opposed to the other processes which act as electron sinks [69]. Another benefit from studying acetate is that it is the end product in several metabolic pathways, including the Entner-Doudoroff pathway for glucose [69].

It has been reported that power density is more sensitive to VFA composition than the CE [67]. An investigation studied the effects of acetate, propionate and acetate on a MFC inoculated with anaerobic sludge. It showed that acetate produced the highest power and CE while propionate showed an antagonist effect on CE when it exceeded 19% of the total VFAs in a MFC fed with mixed VFAs [67]. Butyrate was found to have a negative impact on both power density and CE as well as having a larger effect on the community composition [67]. Power in the community was attributed to two species closely related to Geobacter, but neither of these species was found in the MFC fed with butyrate. The denaturing gradient gel electrophoresis (DGGE) showed potential competition between these species as it was observed that as the intensity of the band associated with one of the species increased, a decrease in the band for the corresponding species decreased [67].

Another study looking at the effect of VFAs on the community showed that power decreased with an increase in the number of carbons in the chain of a VFA with acetate producing the highest power, followed by propionate and finally butyrate with the lowest power produced. The community also changed depending on the VFA used, with Geobacter being found in high amounts in the acetate MFC but not found in either the propionate or butyrate MFC. Firmicutes (a phylum of gram-positive bacteria which contains the classes Clostridia and Bacilli) made up the majority of the community in
the propionate MFC while the butyrate MFC shared a similar composition to the acetate MFC, with *Clostridium* and *Comamonas* being found in the community [68].

Glucose is commonly used as an organic carbon source in MFCs, due to it being a universal carbon and energy source for microorganisms. It has been noted in a variety of papers that MFCs fed with glucose often have a lower CE than MFCs operated using VFAs as the carbon source. This is expected since glucose metabolism leads to a variety of fermentation products [70]. Another reason for the lower CE is methanogenesis, where carbon is reduced to methane by archaea (methanogens) found in the microbial community; this process acts as an electron sink diverting electrons from power generation [71].

Glucose-fed MFCs operated in batch mode (no influent or effluent) have also been shown to have long lag times before reaching maximum power production while VFA-fed batch MFCs quickly reach peak power. Despite this, power production decreases relatively quickly in the VFA-fed MFCs, whereas the glucose MFCs manage to produce sustained power for a longer period of time.

The delay in the glucose MFCs is due to the time it requires fermentative bacteria to convert the glucose into a useable form for power production [72]. This lag time reportedly gives methanogens time to acclimatise which is not available in VFA-fed MFCs [71]. Acetate and propionate are utilised at a slower rate when other organic substrates are present which depending on flow rate can lead to lower CEs [73].

Communities acclimatised to glucose saw an increase in power when acetate was added while the reverse was not true. This suggests a symbiotic relationship between the communities; with fermentative species producing VFAs for the exoelectrogenic species while in the acetate-acclimatised MFCs, the microbes present were unable to ferment the glucose [70]. Other studies have shown that glucose-fed MFCs will select for *Geobacter* if present in the community even though it has been shown that *Geobacter* is unable to utilise glucose as a substrate [71].

The biofilm formed by the bacteria has also been shown to differ when exposed to either acetate or glucose. MFCs fed with acetate lead to biofilms consisting of a monolayer of cells. This indicates that all bacteria present in the biofilm are using the anode as an electron acceptor as they are all in direct electrical contact with the anode. In the glucose MFC, the biofilm consisted of a thick matrix where 95% of the species
were not in contact with anode. The majority of the cells in the glucose biofilm were involved in fermentation and methanogenesis. In both cases, replacing the anode in a MFC consisting of fresh media led to the power quickly returning to its original state [72].

1.7.3 Effect of Other Substrates

Hydrolysis in MFCs is usually associated with polysaccharides and disaccharides. In this step, monosaccharide units are released from the polymer allowing them to be utilised through glycolysis.

Cellulose is a naturally occurring linear polymer of glucose connected through β-1,4-linkages which has been researched as a carbon source for MFCs. A community obtained from the rumen of a cow were used to utilize the cellulose for power generation. This substrate favoured a community in which the majority of the species belonged to the phylum firmicutes or deferrribacters (58% and 29%) [45]. Starch, which shares a similar structure to cellulose (only differing in bond positions and branching), led to a predominance of β-proteobacteria in the community [70]. The suspension showed no redox mediators; suggesting that the species present in the suspension performed a fermentative role and was not involved in any exoelectrogenic activity even though the biofilm was shown to be electrochemically active [70].

Amino acids have also been used as food sources on acetate-acclimatised MFCs and it was noted that polar amino acids had shorter adaptation times than their non-polar counterparts. It was also noted that the adaptation time was proportional to the molecular weight of the amino acid and no relationship was observed between charge and power density [74].

Studies have been carried out utilising real wastewater as opposed to synthetic wastewater (loaded with an organic carbon source such as acetate or glucose). In all cases it has been revealed that the power densities obtained are lower and also that the communities associated with the MFC differ in composition. Work by Velasquez et al. compared synthetic wastewater to domestic wastewater both with a COD loading of 100 mg L⁻¹ [75]. The power densities associated with the synthetic wastewater ranged from 191 to 754 mW m⁻² while the domestic wastewater had power densities ranging
from 116 to 149 mW m$^{-2}$ [59]. This difference highlights one of the problems MFC research is facing, with focus on design to optimise power output by working with single species as simple compounds for the carbon source such as acetate, opposed to complex naturally-occurring communities and carbohydrates.

### 1.7.4 Improving the Electrogenic Properties of Bacteria

A variety of work has been carried out to improve the power production of the MFC through the manipulation of organisms of their community. It has been reported by Kouzuma et al. that a mutant strain, *Shewanella oneidensis MR-1*, which has a deficit in its ability to biosynthesise cell-surface polysaccharides, has shown an increased ability to adhere to a graphite anode which led to an increase in the current generated [76].

Cofactors are non-protein chemical compounds which are often required to assist the proteins (usually enzymes) in biochemical transformations. They are also able to act as redox carriers in energy transfer within the cells [77]. NAD$^+$ and its reduced form NADH are primarily involved with cellular electron transfer and metabolic pathways. It has been shown in *Pseudomonas aeruginosa* that overexpression of the genes involved with the NAD$^+$ salvage pathway (pathway to convert preformed nicotinamide, as opposed to synthesis from simple amino acids) would increase the NADH/NAD$^+$ ratio, which in turn favoured the synthesis of pyocyanin and improved the power from 10.86 µW cm$^{-2}$ to 40.13 µW cm$^{-2}$ [78].

### 1.8 Community Studies

It has been shown that when communities are fed with different carbon sources, different phyla and classes of bacteria will dominate the community. Sediments rich in acetate have been shown to favour delta-proteobacteria [79], ethanol-fed MFCs lead to an enrichment of beta-proteobacteria [80], and gamma-proteobacteria are predominant in cysteine-fed MFCs [80]. Glucose, on the other hand, leads to a diverse microbial community [81][82].

Dilution to extinction studies carried out by Xing et al. reported that as diversity of species present on the biofilm decreased, the overall power generated by the system
decreased. This shows that essential members of the community are lost or altered through repeated dilution and transfers [22]. This also indicates that power generation is not due to a single member of the community.

When a bacterial cell is near starvation, it will still only metabolise a subset of the available substrates. A community of microorganisms made up of species with differing metabolic activities will allow a community to make use of a wider variety of substrates [83]. This expanded metabolism should allow for communities to better reduce the COD of wastewater allowing better treatment of water.

Work from Zhang et al. looked at the effect of pH on the operation of MFCs inoculated with anaerobic sludge. It was found that there were significant changes in the community at pHs between 4 and 7. At lower pHs, the power generated was shown to be lower while COD removal occurred at a faster rate [56].

It has been shown that across the biofilm, a proton concentration gradient emerges with pH decreasing as proximity to the biofilm decreased. This can lead to adverse effects since different species have different tolerances when it comes to the local pH. An example of this is G. sulfurreducens, one of the primary electrogens found on the electrode, which becomes metabolically inert under pH 6.0 [84].

The work in this project is built upon the work by Beecroft et al. [85]. A MFC inoculated with anaerobic digester sludge fed with a carbon source (sucrose) was operated for 100 days. The maximum power densities obtained for the three repeats were 1.03, 1.20 and 1.79 mW m\(^{-2}\). The community was analysed and the most abundant species, which had been sequenced, were selected for the focus of this project. The selected species all belong to different classes of bacteria whilst also showing a variety of different respiration methods. This suggests potential synergistic relationships between the species in the community. The selected species are Comamonas denitrificans, Serratia plymuthica, Ochrobactrum anthropi, Bacteroides graminisolvens and Clostridium indolis [82].

1.8.1 Comamonas denitrificans

Comamonas denitrificans (C. denitrificans) is a gram-negative facultative anaerobic bacterium (able to respire aerobically and anaerobically) which belongs to the class
beta-proteobacteria. *C. denitrificans* and other *Comamonas* species have been found in a wide variety of MFC communities suggesting it plays a role in power generation [45][70][73][86]. These communities all have come from different waste sources including, but not limited to, anaerobic sewage sludge from a pharmaceutical factory and anaerobic digesters [56]. In MFCs fed with cellulose, *C. denitrificans* was found to make up the majority of the suspension [45]. *C. denitrificans* was also shown to appear in communities in MFCs fed with glucose and propionate, while the same research group found that it was not present in acetate or butyrate MFC communities [70]. Other *Comamonas* species were found in MFCs fed with acetate and hexanoic acids, while being found in lower concentrations or absent from MFCs fed with other VFAs [53]. While it was shown that *C. denitrificans* is capable of low power production in 2-chamber MFCs, it was also found that it was unable to produce power in single-chamber systems. This was attributed to aerobic respiration taking place due to diffusion of oxygen through the membrane [87]. It was also noted that in a mixed-species MFC this problem would also be reduced by other species scavenging the oxygen, leaving *C. denitrificans* to use the anode as the terminal acceptor [87].

It is normally reported that *C. denitrificans* carries out denitrification (nitrate reduction) in the community [88][89]. The addition of nitrate into *C. denitrificans* systems leads to a decrease in power output demonstrating that an alternate pathway is used which affects power output. Apart from the role *Comamonas* species plays in power generation and denitrification, it has also been shown to degrade chloroaniline [61].

### 1.8.2 *Serratia plymuthica*

*Serratia* is a gram-negative, facultative anaerobe which belongs to the bacterial family *Enterobacteriaceae*. Very little work has been carried out on *Serratia plymuthica* (*S. plymuthica*) and the role it fulfils in a microbial community, but a study was carried out on the ability of *S. plymuthica* to remove organophosphates and radio nuclides from wastewater [90].

Despite the lack of work on *S. plymuthica* in MFCs, it has been noted that *Serratia marcescens* plays a role in the corrosion of AA2024, an aeronautical aluminium alloy. In the study hexadecane was used as the food source, and it was noted that in the presence of *S. marcescens*, an increase in the anodic current was observed whereas in
the presence of *Bacillus cereus* an increase was not observed. This indicates that members of the *Serratia* genus can be potentially electrogenic [91].

The *Enterobacteriaceae* family contains two groups of bacteria based upon the end products of glucose fermentation. Mixed acid fermenters include the genus’s *Escherichia*, *Salmonella* and *Shigella* which mainly produce short chain acids including acetic, formic, lactic and succinic. The other group, which includes *Serratia* and *Klebsiella*, produces minor amounts of acids but at higher concentrations of neutral end products including ethanol, acetoin and 2,3-butanediol. The species utilise the pathway shown in Figure 1.6. There are two hypotheses on the role of the pathway the first being that it is used to avoid the excessive, and potentially lethal, acidification of the environment. The second is that 2,3-butanediol can be used as an organic carbon store and converted to acetoin when oxygen stores have been depleted [92].

![Figure 1.6 - A schematic showing the pathway used for fermentation by *S. plymuthica* [92].](image)

### 1.8.3 *Ochrobactrum anthropi*

*Ochrobactrum anthropi* (*O. anthropi*) is a gram-negative species of bacteria belonging to the class alpha-proteobacteria. Not much work has been reported on *O. anthropi* and its role in MFC communities but Shushkova *et al.* looked into how *O. anthropi* can degrade N-phosphonomethylglycin (Glyphosphate) [93]. Glyphosphate is an organophosphate found in the RoundUP™ pesticide which has been shown to persist in soils for up to 2 years [93]. This, combined with the data alluding to the electrogenic
nature of the species, means that *O. anthropi* could be an important member of synthetic communities tailored to water treatment.

*Ochrobactrum* species have been observed in different MFC communities using glucose and acetate as the carbon source, but not much was known about the exoelectrogenic nature, until dilution to extinction experiments from Zuo *et al.* which demonstrated the exoelectrogenic potential of *O. anthropi* [94]. It was noted however that *O. anthropi* shared a variety of characteristics with the exoelectrogenic species *Pseudomonas aeruginosa* where both species are facultative anaerobes, capable of aerobic respiration as well as anaerobic denitrification. Both species are also found in the rhizosphere of a diverse selection of plants and produce exopolysaccharides while also being opportunistic pathogens. It has been suggested that exoelectrogenesis could be a selective property of opportunistic pathogens [94][95].

1.8.4 *Bacteroides graminisolvens*

*Bacteroides* species are a genus of gram-negative, strictly anaerobic bacteria and have been found in microbial communities using anaerobic digester sludge as an inoculum [86][96]. *Bacteroides* has been suggested to fill a fermentative role in microbial communities, being primarily found within the suspension of the MFC as opposed to in the biofilm on the anode [86]. A study into the development of a microbial community over time noted that *Bacteroides graminisolvens* (*B. graminisolvens*) was a dominant species in the community up until day 97, after which it was no longer observed. The disappearance of *B. graminisolvens* coincided with the appearance of *Paludibacter propionicigenus*, which was believed to fill a similar role in the community, fermenting carbohydrates while producing acetate and propionate as the two main by-products [97].

1.8.5 *Clostridium indolis*

*Clostridium* is a genus of gram-positive belonging to the phylum Firmicutes. They are usually obligate anaerobes but oxygen tolerance varies between the different species. *Clostridium* species are reported as fermentative species which are able to transfer electrons to the anode suggesting it can play an important role in synthetic communities allowing it to generate VFAs for the other electrogens while also contributing towards
power production [1]. Dilution to extinction studies have suggested that not all *Clostridium* species are capable electrogens and it was observed that when an uncharacterised *Clostridium* species was found within the community power generation decreased, while in the same experiment the species *Clostridium aminobutyricum* was concluded to be an electrogen [87].

*Clostridium butyricum* was the first reported gram-positive species capable of electrogenic activity which was previously thought to not be possible due to the cell wall [98]. Different *Clostridium* species have been found in different microbial communities. In MFCs fed with cellulose, firmicutes (closely related to *Clostridium* and *Ruminococcus*) dominated the anode biofilm [45]. *Clostridium* species have also been found alongside *C. denitrificans* in MFCs fed with glucose and butyrate while being absent in both acetate and propionate MFCs [70].

*Clostridium indolis* (*C. indolis*) is commonly found in the intestine of mammals. *C. indolis* has been linked to butyrate production from lactate while other *Clostridium* species have been shown to produce n-butanol from sugars like glucose [99] [100]. Butanol is produced through the acetone, butanol ethanol (ABE) fermentation pathway (Figure 1.7) and through Ehrlich pathway (Figure 1.8) which is used for amino acid degradation [99]. Some research groups have tried to capitalise on the metabolism of *Clostridium* species in a community to produce butanol as well as the production of power [96][99].
Figure 1.7 - A schematic showing the ABE pathway used for fermentation of glucose by *C. indolis* [99].
Chapter 1: Introduction and Background Information

1.8.6 Communities Containing the Selected Species

In community studies it has been noted that firmicutes, closely related to Clostridia (Clostridium) and Bacteroidetes (Bacteroides), and beta-proteobacteria (Comamonas) are often present together in microbial communities [45][70][86][87][89][101]. This suggests that these families of bacteria fulfil different niches in the community and possibly have synergistic relationships. It has been noted that fermentative species may be able to produce power due to a metabolic switchover which favours a more energy efficient metabolism as the fermentable substrate is consumed [97].

1.9 Alternative Applications

Various studies have been carried out using MFCs to recover metals from wastewater streams or industrial processes. One example is the recovery of cobalt from lithium ion batteries. Using a 2-chamber tubular MFC, Huang et al. were able to achieve “complete” cobalt recovery, converting LiCoO$_2$ to Co$^{2+}$(aq) and finally Co(s) [102].

Desalination is another potential application for MFCs which has drawn a lot of attention. Desalination is the process of removing salt from brackish water and seawater to create potable water. Current desalination processes are expensive and energy intensive, so research has focussed on using a modified MFC called a microbial desalination cell (MDC) to simultaneously degrade organic waste, using the energy generated to remove salt from the water being treated. The design differs from a MFC by having an additional third chamber between the anodic and cathodic chambers.

**Figure 1.8 - A schematic showing the Ehrlich pathway used for fermentation of amino acids by C. indolis [96].**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>2-Keto acid</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>transaminase</td>
<td>decarboxylase</td>
<td>dehydrogenase</td>
</tr>
<tr>
<td>Valine</td>
<td>Isovalerate</td>
<td>Isobutanol</td>
</tr>
<tr>
<td>Glycine</td>
<td>Ketivalerate</td>
<td>Butanal</td>
</tr>
<tr>
<td>Threonine</td>
<td>Ketobutyrate</td>
<td>Butanol</td>
</tr>
</tbody>
</table>

Valine, Glycine, and Threonine are used as the starting substrates, which are converted into 2-Keto acid and further into Alcohol.
Currently, MDCs have achieved a salt removal efficiency between 43-68% while achieving a maximum power density of 3.1 mW m$^{-2}$ [103][104].

Microbial electrolysis cells – a type of modified MFC - have been used for hydrogen generation [105][106]. Microbes have been shown to produce hydrogen through the fermentation of glucose but are limited to 4 moles of hydrogen for each mole of glucose. The limitations are due to the formation of “dead end” products like acetate which require additional energy for further conversion. With the addition of a small voltage, on top of that produced by the MFC, it is possible to aid the conversion of the “dead end” products, improving the overall yield of hydrogen gas [107].

Hydrogen is not the only metabolite generated from a microbial community which can be used to increase the economic viability of MFCs. Ethanol, butanol and pentanoic acid have all been studied as possible products of interest. The species *Saccharomyces cerevisiae* has been used to convert cellulose and xylose found in biomass to ethanol [108]. Studies have also looked at using the species *Klebsiella, Enterobacter, Bacillus* and *Saccharomyces* to produce 2,3-butanediol, a compound of great interest due to its versatility with applications in the pharmaceutical and food industries [109].

Another focus is using photosynthetic microalgae to replace the catalyst at the cathode. The microalgae release oxygen during respiration which will act as the terminal electron acceptor as with air-breathing cathodes. A benefit of using microalgae is through carbon fixation, with the algae converting carbon dioxide into sugars [8][96]. This leads to the accumulation of microalgal biomass. The microalgal biomass generated can be used as a substrate for the anodic community in a MFC; this biomass contains approximately 32% proteins and 51% carbohydrates which are readily degradable by the electrogens [110]. Carbon dioxide generated through microbial respiration can be fed into the cathodic chamber providing the food source for the cathodic community [8].

### 1.10 Methods

#### 1.10.1 Voltage and Power Measurements

The voltage of the MFC is often measured through the use of voltmeters, multimeters or battery testers. Cell voltage is measured between the anode and the cathode, while the potential of an electrode can be measured against a reference electrode. External load
(resistance), expressed as a function of current through that load, leads to the generation of a polarization curve. As the load is decreased, the voltage is measured and the current is calculated according to Ohm’s law (Equation 1.1) [99][111]. Power output is normally used to evaluate the performance of MFCs; this can be achieved through the use of a power curve, where power density is a function of current, and output.

1.10.2 Cyclic Voltammetry

Cyclic voltammetry (CV) is a technique which allows the “redox potential” of a given sample to be analysed. In the case of MFCs, CV can be performed on the suspension and will give information on any redox active compounds in the suspension which can indicate the presence of mediators excreted by the microbes in the MFCs. It can also be carried out on the anode of the MFC to give information on the redox properties of the biofilm [112]. For instance, CV experiments on the cell suspension of MFCs working with \( P. \) aeruginosa have shown a redox peak at \(-0.20\ \text{V}\) (vs. Ag/AgCl electrode) which has been attributed to pyocyanin redox electrochemistry [46].

In biofilms containing \( \textit{Shewanella} \), two peaks of note have been observed. The first is a peak at \(-0.23\ \text{V}\) (vs standard hydrogen electrode (SHE)) which has been suggested to arise from flavins secreted by the microorganism. The second peak (located at \(0.01\ \text{V}\) (vs SHE)) has been attributed to outer membrane cytochrome c [112][113][114]. Work from Yang \textit{et al.} showed that that addition of various electron acceptors to the MFC caused some alterations in the CVs of \( \textit{Shewanella} \) MFCs. The addition of \( \text{Fe}_2\text{O}_3 \) and fumarate, which were shown to enhance CE, enhanced the peaks associated with the outer membrane cytochromes [59]. The addition of nitrates caused the peak associated with outer membrane cytochromes to disappear while enhancing the peak associated with flavins [59].

CVs carried out on the biofilm in MFCs operated between 5-10°C showed a previously unobserved peak at \(-0.35\ \text{V}\). It was suggested that the peak arose from the prevalence of light-induced psychrophilic bacteria which were either absent or found in low concentrations in MFCs operated at ambient temperatures.

Work by Kaur \textit{et al.} looked at using a biofilm, acclimatised to acetate, propionate and butyrate, for CV experiments to work as a biosensor to detect the concentrations of
VFAs. It was noted that a different CV profile was observed for each acclimatised anode depending on which VFA was present. The acetate biofilm showed an oxidation peak at -0.205 V and a reduction at -0.226 V, the propionate biofilm showed oxidation peaks at -0.378 V and -0.231 V as well as reduction peaks at -0.219 V and -0.408 V, while the butyrate biofilm showed an oxidation peak of 0.0373 V and a reduction peaks at -0.1645 and -0.2430 V respectively [115]. This suggests that the redox chemistry of the biofilm changes depending on the substrate being used for respiration.
1.12 Bibliography


Chapter 2 - Materials and Methods

Unless otherwise stated microorganism strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany), culture media from Oxoid (UK) and chemicals from Sigma Aldrich (UK).

2.1 Microorganism Cultivation

All anoxic counterparts of aerobic media were converted by boiling the desired medium and allowing it to cool under a nitrogen atmosphere.

2.1.1 Serratia plymuthica and Ochrobactrum anthropi

The initial *Serratia plymuthica* (DSM 4540) and *Ochrobactrum anthropi* (DM 14396) stocks were resuspended using 5 mL Nutrient Broth (CM0001). After the microbial cells were resuspended, they were transferred to a serum vial (100 mL) containing 70 mL Nutrient Broth and incubated for 33 hours at 25°C in an orbital shaker at 150 rpm (*O. anthropi*) or for 20 hours at 30°C (*S. plymuthica*) [1][2]. The purity of stocks was confirmed through microscopic observation as well as a Nutrient Agar (CM0003) streak plate using 0.1 mL of culture and incubated for 72 hours at 25°C (*S. plymuthica*) and at 30°C (*O. anthropi*). The purities were checked using a microscope (Vickers Instruments M170431) to observe the plate to make sure the individual cultures were homogenous; an example streak plate is shown in Figure 2.1.

Working stocks were prepared by adding 1 mL of culture to a cryotube containing 0.8 mL Brain Heart Infusion (CM0225) dissolved in a glycerol solution (aq, 20% w/v) and stored at -80°C until needed. When the species were cultivated for microbial fuel cell (MFC) experiments, anoxic media was used.
2.1.2 Comamonas denitrificans

*Comamonas denitrificans* (DSM 17887) was resuspended in 1 mL Tryptone Soya Broth (CM0129) [3]. After the microbial cells were resuspended, they were transferred into a serum vial (100 mL) containing 70 mL Tryptone Soya Broth and incubated for 34 hours at 30°C, in an orbital shaker at 150 rpm. The purity of stocks was confirmed through microscopic observation as well as a Tryptone Soya Agar (CM0131) streak plate using 0.1 mL of culture and incubated for 72 hours at 30°C. The purity was checked by observing the plate under the microscope to make sure the individual cultures were homogenous (as previously described in Section 2.1.1). Working stocks were prepared by adding 1 mL of culture to a cryotube containing 0.8 mL of Brain Heart Infusion dissolved in a glycerol solution (aq, 20% w/v) and stored at -80°C until needed.

2.1.3 Clostridium indolis and Bacteroides graminisolvens

For both species, a modified chopped meat medium (CMM) with carbohydrates was required. CMM (1 L, CM0081) was prepared following manufacturer instructions and autoclaved at 121°C for 15 minutes. The medium was then filtered to remove the cooked meat particles, and water added to bring the volume back up to 1 L. The following compounds were added: casitone: 30.0 g; yeast extract: 5.0 g; K$_2$HPO$_4$: 5.0 g; glucose: 4.0 g; cellobiose: 1.0 g; maltose: 1.0 g; starch (soluble): 1.0 g; resazurin: 1.0 mg. The solution was made anoxic and cysteine (0.5 g) was added (pH 7.0). The
medium was distributed anaerobically in serum vials (50 mL) [4]. The initial *Clostridium indolis* (DSM 755) and *Bacteroides graminisolvens* (DSM 19988) were re-suspended using 5 mL of modified CMM. When re-suspended, the culture was transferred to a serum vial (100 mL) containing 50 mL CMM and both were incubated at 37°C for 17 hours in the case of *C. indolis* and 15 hours in the case of *B. graminisolvens* in an orbital shaker set to 150 rpm [5][6].

The purity of stocks was confirmed through microscopic observation as well as a modified cooked meat agar (Section 2.1.5) streak plate using 0.1 mL of culture and incubated for 72 hours at 37°C. The purity was checked by observing the plate to make sure the individual cultures were homogenous. Working stocks were prepared by adding 1 mL of culture to a cryotube containing 0.8 mL Brain Heart Infusion dissolved in a glycerol solution (aq, 20% w/v) and stored at -80°C until needed.

### 2.1.4 Synthetic Waste Water

Synthetic wastewater was used as the medium for all MFC runs [7], containing (g L⁻¹): NH₄Cl: 0.31; NaH₂PO₄·H₂O: 2.69; Na₂HPO₄: 4.33; KCl: 0.13, (pH 7.0). After being autoclaved, the medium was supplemented with trace mineral (12.5 mL) and vitamin (12.5 mL) solutions. As a carbon source, 15 mL of glucose (aq, 200 g L⁻¹) was added.

#### 2.1.4.1 Vitamin Solution

The vitamin solution [7] contained (mg L⁻¹): biotin: 2.0; folic acid: 2.0; pyridoxine–HCl: 10.0; riboflavin: 5.0; thiamine–HCl: 5.0; nicotinic acid: 5.0; pantothenic acid: 5.0; vitamin B-12: 0.1; p-aminobenzoic acid: 5.0; thioctic acid: 5.0, (pH 7.0). The biotin was dissolved in water before adding the other components; several hours were needed for the vitamins to fully dissolve.

#### 2.1.4.2 Trace Mineral Solution

The trace mineral solution [7] contained (g L⁻¹): nitriloacetic acid (NTA): 1.5; MgSO₄·7H₂O: 3.0; MnSO₄·H₂O: 0.45; NaCl: 1.0; FeSO₄·7H₂O: 0.1; CaCl₂·2H₂O: 0.1; CoCl₂·6H₂O: 0.18; ZnCl₂: 0.13; CuSO₄·5H₂O: 0.01; AlK(SO₄)₂·12H₂O: 0.018; H₃BO₃: 0.01; Na₂MoO₄: 0.025; NiCl₂·6H₂O: 0.024; Na₂WO₄·2H₂O 0.025. The NTA was
dissolved first and the pH was adjusted to 6.5, using KOH (aq, 1 mol L\(^{-1}\)) solution, before adding the other minerals. The final pH was adjusted to 7.0 using KOH (aq, 1 mol L\(^{-1}\)) solution.

All finalised mediums were autoclaved at 121°C for 15 minutes, except the vitamin, mineral and the glucose solutions which were filter-sterilised (0.22 μm) into a sterile vessel

### 2.1.5 Agar Plates

#### 2.1.5.1 Modified Cooked Meat Agar

For solid-phase cultivation of *C. indolis* and *B. graminisolvens*, 14 g Agar Bacteriological (Agar No1) was added to 1 L of the medium made in Section 2.1.3. The agar preparations were autoclaved at 121°C for 15 minutes.

#### 2.1.5.2 Synthetic Waste Water Agar

A synthetic wastewater agar was also produced by adding 28 g Agar No1 to 970 mL of medium without the addition of the minerals, vitamins and glucose. This was autoclaved for 15 minutes at 121°C, and allowed to cool down to 60°C before vitamins, minerals and glucose were added.

### 2.1.6 Growth Curves

Growth curves using the recommended growth media were generated for *S. plymuthica, O. anthropi, C. denitrificans, C. indolis* and *B. graminisolvens*. Liquid cultures were grown according to Section 2.1 then a 0.5 mL sample was taken every hour and the optical density (at a wavelength of 600 nm) was measured using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Sweden). All experiments were repeated in triplicate.

For the synthetic waste water growth curves cell suspensions for inoculation were prepared by adding the working stock solutions to the corresponding medium for each species as specified above (Section 2.1). The cultures were grown until the late
exponential growth phase, as determined by comparing the optical density (OD) readings at a wavelength of 600 nm with the standard growth curves (Chapter 3, Section 3.1). The ODs used were C. denitrificans: 1.9; S. plymuthica: 0.6; O. anthropi: 0.7; B. graminisolvens: 2.8 and C. indolis: 2.1. The cells were harvested using a Labofuge 400 (Haraeus Instruments) (3500 g for 20 min), washed three times with 95 mL of phosphate buffer (aq, 0.1 mol L$^{-1}$, pH 7.5) and re-suspended in a further 95 mL of phosphate buffer to obtain a final OD$_{600}$ of 3.7. The phosphate buffer was composed of NaH$_2$PO$_4$ (aq, 16 mmol L$^{-1}$) and Na$_2$HPO$_4$ (aq, 84 mmol L$^{-1}$).

### 2.2 Microbial Fuel Cell Set Up and Operation

#### 2.2.1 Microbial Fuel Cell Configuration

A single chambered MFC (Figure 2.2) was employed for the experiments detailed in this thesis because it was found, in previous work, to be suitable for obtaining reliable and repeatable results [8]. The cell was made up of a Perspex anode chamber (140 cm$^3$) with a Perspex plate on either side (Figure 2.3). The plate on the cathode side had four 0.5 cm by 4 cm sections removed to allow the air to come into contact with the cathode (Figure 2.5). Two stainless steel plates were placed on top of the Perspex plates for support and to prevent the plates from warping. A rubber gasket (low density PVDM) was placed between the anodic chamber and backing Perspex, and a rubber gasket frame was placed on either side of the membrane to prevent leaking.
Figure 2.2 – A photograph of the single chambered MFC used for this project. Three views are highlighted (A, B & C) which are shown separate schematics (Figures 2.3, 2.4 & 2.5).

Figure 2.3 – View A (from Figure 2.2) from outside the cell, detailing the layers of components that make up the MFC.
The anode consisted of a 7 cm by 11 cm piece of carbon veil (National Centre for Biotechnology Education, United Kingdom) wrapped around a Perspex rod. A length of nickel/chromium wire (11 cm, diameter of 25 µm, resistance 0.6 ohms, Advent Research Materials, UK) was threaded through and around the carbon veil and rod (Figure 2.4). The length of wire selected was the minimum length required to thread the anode as well as having 2-3 cm outside the MFC to allow an electrical connection to the Arbin to be made (Figure 2.4). In the fully assembled system, the anodic suspension volume is 151 cm$^3$. The large anodic volume was selected to allow frequent samples to be taken from the MFC without disrupting power generation through normal operation.

**Figure 2.4** – View B (from Figure 2.2) shows a cross-sectional view through the anodic chamber.
Figure 2.5 – View C (from Figure 2.2) shows a view of the air-breathing cathode from outside the MFC.

Fuel Cells Etc, United States supplied the air-breathing cathode which was bought in ready-made and cut into 8 cm by 4.5 cm pieces. The ready-made cathode consisted of a piece of Vulcan cloth, one side of which is coated with a layer of platinum (0.5 mg cm\(^{-2}\)) and a layer of Nafion perfluorinated ion-exchange ionomer (0.5-1.0 mg cm\(^{-2}\)); the other side of the cloth is coated with a polytetrafluoroethylene (PTFE) gas-diffusion layer. Before MFC assembly, the ready-made cathode was hot-pressed to a piece of Nafion ion-exchange membrane using a hot-press at 135°C under 0.5 tonnes for 3 minutes. The Nafion 115 proton exchange membrane (PEM) (9 cm by 7 cm, Du Pont) had to be pre-treated before use; this was carried out by boiling it for 1 hour in each of 3% w/v H\(_2\)O\(_2\), 1 mol L\(^{-1}\) H\(_2\)SO\(_4\) and then deionised H\(_2\)O [9]. After activation the membrane was stored in deionised water in the dark until required. Figure 2.6 illustrates the fully assembled air-breathing cathode electrode which is then orientated in the MFC with the gas-diffusion layer side in contact with the air and the membrane side forming one of the ‘walls’ of the anodic chamber inside the MFC.
2.2.2 Continuously-Fed Batch Mode Microbial Fuel Cell Operation

Prior to MFC assembly, to ensure all the individual components were sterile, all components (apart from the electrodes (anode and cathode) and membrane) were submerged in Virkon (aq, 1% w/v) overnight, and during assembly the individual components were sprayed with industrial methylated spirits (IMS) and left to dry inside a laminar flow unit. The anode was soaked in IMS (aq, 70% w/v) and allowed to dry before being soaked in synthetic wastewater and then the MFC was then assembled as described above.

All tubing was autoclaved prior to use and assembled as shown in Figure 2.6. A length of Masterflex Tygon Size 14 tubing (E-3603, Cole Parmer, United Kingdom) was run from the medium bottle to a polypropylene barbed T-connector 1/16” (Cole Parmer). From each branch, a length of Masterflex Tygon Size 13 tubing (E-3603, Cole Parmer) was run through a Masterflex L/S Digital drive (Cole-Parmer) pump before being connected via a polypropylene reducing connector 1/8” to 1/16” (Cole Parmer) to a length of silicone tubing (5mm diameter) attached to the MFC. A length of Masterflex Tygon Size 16 (E-3603, Cole Parmer) tubing ran from each MFC to the waste bottle.
Cell suspensions for inoculation were prepared by adding the working stock solutions to the corresponding medium for each species as specified above (Section 2.1). The cultures were grown until the late exponential growth phase, as determined by comparing the optical density (OD) readings at a wavelength of 600 nm with the standard growth curves (Chapter 3, Section 3.1). The cells were harvested using a Labofuge 400 (Haraeus Instruments) (3500 g for 20 min), washed three times with 95 mL of phosphate buffer (aq, 0.1 mol L\(^{-1}\), pH 7.5) and re-suspended in a further 95 mL of phosphate buffer to obtain a final OD\(_{600}\) of 3.7. The phosphate buffer was composed of NaH\(_2\)PO\(_4\) (aq, 16 mmol L\(^{-1}\)) and Na\(_2\)HPO\(_4\) (aq, 84 mmol L\(^{-1}\)). The ODs used were C. denitrificans: 1.9; S. plymuthica: 0.6; O. anthropi: 0.7; B. graminisolvens; 2.8 and C. indolis: 2.1. For all runs consisting of a single microbial species, 25 mL of inoculum was used; for dual-cultures, 12.5 mL of each species was used and for the synthetic community consisting of all 5 species, 5 mL of each was used.

The synthetic wastewater and cell suspension was purged with nitrogen for 2 hours prior to starting an experiment and the medium was purged throughout operation. The MFCs were operated in batch mode for 48 hours before the pumps were activated (0.1 mL min\(^{-1}\)). The bottle containing the influent medium was replaced every 7 days and the waste effluent was discarded. Two control experiments were conducted: one using sterile medium and one which included inoculating the MFC with all 5 species but no
carbon source being added to the medium. Each experiment had 2 repeats. All MFCs were run at room temperature (approximately 22°C).

2.2.3.1 Sampling Methods

Every 3 days, 3 repeat samples were taken from each MFC for Denaturing Gradient Gel Electrophoresis (DGGE); each consisted of 1 mL of anodic suspension suspended in 0.8 mL glycerol (aq, 20% w/v) and stored at -80°C. Upon completion of the experiment, 1 cm² samples of the anode were taken as shown in Figure 2.8. These samples were stored in glycerol (aq, 20% w/v) at -80°C until required for DGGE analysis.

![Figure 2.8](image)

**Figure 2.8** – A photograph showing the samples points used on the anode. The different colours show the three different layers when the anode is assembled.

Upon completion of the experiment, 20 mL samples of the anodic suspension were taken, filtered and stored at -20°C for use in cyclic voltammetry (CV) studies. A further set of samples were also taken (2 mL) which were filtered (Millex GP polyether sulfone membrane filter, 0.22 µm) to remove the bacteria from the sample. The filtrate samples were frozen at -20°C for total carbohydrate analysis.

2.3 Chemical Analysis

2.3.1 Total Carbohydrate Analysis

MFCs are also used to treat wastewater while generating power, this means that it is important to measure the community’s ability to reduce the level of organics present in the feed. To do this the total carbohydrate concentration was measured for the influent and effluent (at the termination of the experiment).
The total carbohydrate concentration of the influent and effluent was determined using the colorimetric phenol/sulphuric acid method [10]. Samples of both the influent and effluent were analysed. Samples were prepared by adding 200 µL of phenol (aq, 5% w/v) to 200 µL of sample followed by the addition of 1 mL concentrated sulphuric acid (aq, 18 mol L\(^{-1}\)). Samples were vortexed and left to stand for 15 minutes before the absorbance (at 490 nm) was measured (Ultrospec 2000 UV/visible spectrophotometer, Pharmacia Biotech, Sweden). Calibration curves were prepared using glucose solutions of 5 different concentrations (aq, between 0.25 – 3.5 g L\(^{-1}\)) and all results were expressed as glucose equivalents. All measurements were conducted in triplicate. The total carbohydrate consumption (%) was calculated using Equation 2.1 and the carbohydrate consumption over a specific time period was presented as mean ± SEM (standard error mean), where \(n\) is the number of samples during that time period, measured in triplicate.

\[
C = \left( \frac{c_i - c_e}{c_i} \right) \times 100
\]

**Equation 2.1** – Calculation of the carbohydrate consumption where \(C\) (%) is the carbohydrate consumption, \(c_i\) (g L\(^{-1}\)) is the carbohydrate concentration in the influent; \(c_e\) (g L\(^{-1}\)) is the carbohydrate concentration in the effluent.

The chemical oxygen demand (COD) is defined as the amount of oxygen fully required to oxidise the organic components of a feed. It is used in MFCs to determine how effective the microbial community is at treating wastewater steams. The theoretical COD per unit mass of glucose in the feed was calculated according to Equation 2.2 [11]. The same calculation was also used to analyse the samples taken from the MFC.

\[
COD_t = \frac{8 \times (4x + y - 2z)}{(12x + y + 16z)}
\]

**Equation 2.2** – Calculation of the theoretical COD (mg L\(^{-1}\)), where \(x\), \(y\) and \(z\) are determined from \(C_\text{H}_\text{O}_\text{2}\) which in the case of glucose are 6, 12 and 6 respectively.
2.3.2 pH and Temperature Measurements

The pH and temperature of the MFC effluent was monitored every 3 days using a Mettler Toledo MP220 pH meter (United Kingdom). The pH was monitored to make sure it was consistent for each species.

2.4 Electrochemical Techniques

2.4.1 Electrochemical Methods

All mono-culture and mixed-species electrochemical measurements were studied either using a 1480 Multistat System (Solartron Analytical, UK) controlled by a computer with CellTest™ software, or using an Arbin BT2000 Battery Tester (Arbin Instruments, USA) controlled by a computer with MITS Pro software (Arbin Instruments).

The single- and multi-species MFC experiments were run on open circuit voltage (OCV) for 3 hours before switching to a load of 40 000 ohms. This resistance was determined by running a polarization curve of the anaerobic sludge (the work this project is based upon [11]). The resistance was held at 40 000 ohms until the power output of the cell reached a stable value. To determine when the MFCs had reached a steady state, a 3 point moving average was applied to the data. It was assumed that a steady state had been reached when 3 consecutive averaged data points had a variation of less than 1% of the maximum power output of the MFC (At 40 000 ohms as described in Section 2.4.1). Polarisation curves were used to determine the maximum power generated by the MFC and were generally carried out when both replicates had stabilised.

When a stable power output was observed, a polarization curve was determined. The first stage of the polarization curve was to run the MFC at OCV for 2 hours to allow the MFC to reach its maximum voltage. A series of decreasing external loads were applied to the system and the system was left to acclimatise for 5 minutes before the resistance was decreased again. The resistances used were: 700 000, 300 000, 100 000, 70 000, 40 000, 25 000, 18 000, 12 000, 8 000, 6 000, 4 000, 3 000, 2 000, 1 000 and 500 ohms.

The volumetric power density (µW L⁻¹) was calculated using Equation 2.3 and the anodic power density (µW m⁻²) was calculated using Equation 2.4.
It should be noted, however, that the power per unit anode area assumes to that all 3 layers of anode have a biofilm forming on them, therefore all values in this thesis are conservative estimates of the power generated by the MFC.

### 2.4.2 Cyclic Voltammetry

Cyclic voltammetry (CV) is a technique where the working electrode potential is ramped linearly versus time, and when the set voltage is achieved, it will ramp in the opposite direction. When a substrate, which can be oxidised or reduced, comes into contact with the electrode a change in current is measured. The 3 electrodes – working, counter and reference – are placed in the sample solution. The potential is applied between the working electrode and the reference electrode whilst the current is measured between the working electrode and counter electrode. This set up ensures that the flow of current is not through the reference electrode itself, where application of current could change its potential due to unwanted redox reactions.

In these experiments, CV was used to observe the redox properties of the anodic suspension to determine if extracellular mediators were generated by the microbes. A glassy carbon electrode was used as the working electrode; platinum wire was used as a
counter electrode and an Ag/AgCl electrode (BASi, 3.0 mol L\(^{-1}\) NaCl, +0.196 V versus standard hydrogen electrode at 25\(^\circ\)C) was used as the reference electrode.

All solutions were purged with Oxygen Free Nitrogen (OFN) for 30 minutes before analysis. The potential was cycled between -1.5 V to 1.5 V with a scan rate of 10 mV s\(^{-1}\) using an Autolab potentiostat/galvanostat system (EcoChemie, The Netherlands) controlled by a computer with Nova 1.9 software.

![Figure 2.9](image_url)

**Figure 2.9** – The CV profile for the potassium hexacyanoferrate standard showing a peak splitting of 0.086 V (between the reduction peak at 0.2 V and the oxidation peak at 0.286).

Each day the working electrode was cleaned using a diamond abrasive pad, followed by an aluminium silicate cleaning pad. The electrode was then sonicated for 5 minutes. Potassium hexacyanoferrate (aq, 0.10 mmol L\(^{-1}\)) was used as a standard, and to make sure the electrode was cleaned to the same degree, the process was repeated until a difference between the peak of the oxidation and reduction peaks was 0.086 V as shown in Figure 2.9.

Scans were carried on the synthetic wastewater and synthetic wastewater with added supplements (vitamin, mineral and glucose solutions) which were used as blanks, and the filtered anodic suspension samples from each MFC. The volume of the sample was 10 mL and each sample was run in duplicate with a fresh sample in case of irreversible oxidation or reductions occurring in the sample.
2.5 Analysis of Microbial Communities

DGGE analysis of polymerase chain reaction (PCR) amplified genes coding for partial 16S ribosomal ribonucleic acid (rRNA) was carried out on anode samples and the suspension of the MFCs. These techniques were used to determine if, in certain multi-species cells, there is a difference in the species which colonise the anode and those which are found in the suspension. The technique was also used to determine if any cross contamination had occurred between the MFCs or if a bacterial species from the environment had infiltrated the community.

2.5.1 Sample Preparation and DNA Extraction for PCR-DGGE

Total deoxyribonucleic acid (DNA) was extracted from either the anodic suspension sample (1 mL), anode electrode sample (1 cm²) or from the pure cultures in the corresponding growth medium using a FastDNA Spin Kit for Soil (MP Biomedical). The samples were pre-treated by centrifugation (10 000 g, 5 min; Eppendorf Centrifuge 5415 R, UK) and washed through 3 times with 1 mL phosphate-buffered saline (PBS (g L⁻¹): 8.0 NaCl, 0.2 KCl, 1.15 Na₂HPO₄, 0.2 KH₂PO₄, pH 7.3; Oxoid, UK) and re-suspended in 100 µL of nuclease-free water (Promega, UK). The sample, 978 µL of sodium phosphate buffer and 122 µL of MT buffer were added to a 2 mL tube containing a lysing matrix (mixture of ceramic and silica particles) and the tube processed in FastPrep instrument (MP Biomedicals, UK) for 30 s at speed 5.5 (setting on the FastPrep), followed by being centrifuged for 30 s at 14 000 x g. The supernatant was transferred to a clean 15 mL tube to which 250 µL of protein precipitation solution reagent was added and then mixed by shaking by hand for 10 s. The solution was centrifuged (10 000 g, 5 min), the supernatant was again transferred to a clean tube and 1 mL of binding matrix was added (prior to addition the binding matrix solution was re-suspended by shaking by hand for 30 s). To allow for the binding of the DNA to the matrix, the tube was inverted for 2 minutes and then left for 3 minutes to allow the silica matrix to settle. 500 µL of the supernatant was removed and discarded before re-suspending that binding matrix in the remaining supernatant.

600 µL of the re-suspended binding matrix was transferred to a clean catch tube with a spin filter, and centrifuged (10 000 g, 1 minute). The supernatant in the catch tube was
discarded and the process was repeated twice more. 500 µL of salt/ethanol wash solution was added to the spin filter, which was then centrifuged (10 000 g, 1 minute) and the flow-through was decanted. The spin filter was then dried through centrifuging (10 000 g, 2 minutes) the sample to remove the residual salt/ethanol wash from the matrix. The spin filter was then transferred into a fresh catch tube and allowed to air-dry for 5 minutes at ambient temperature. The DNA was eluted though the addition of 50 µL of DNAase/pyrogen free water and the matrix gently with a pipette tip to re-suspend the silica for efficient elution of the DNA. The DNA was eluted in a catch tube by centrifugation (10 000 g, 1 minute) and stored at -20°C.

2.5.2 Polymerase Chain Reaction

The partial bacterial 16S rRNA genes (550 bp) were amplified using bacteria-specific forward primer 341F (E. coli 16S rRNA positions 341-357) [13] and universal reverse primer 907R (E. coli 16S rRNA position 907-926) [13] (Table 2.1). A GC-clamp was added to the forward primer at the 5’-end to stabilize the melting behaviours of the DNA fragments in the DGGE [13] (Table 2.1).

The PCR reaction mixture contained 1 x Taq PCR buffer (10 mM Tris-HCl, 1.5 mmol L⁻¹ MgCl₂, 50 mmol L⁻¹ KCl, pH 8.3 at 20°C), 200 µmol L⁻¹ dNTP, 0.2 µmol L⁻¹ each primer, 0.025 U µL⁻¹ of Taq DNA polymerase (Roche, UK), 400 ng µL⁻¹ of bovine serum albumin (BAS, Fermentes Canada) and nuclease-free water (Promega, UK) to a final volume of 50 µL, to which 1 µL of template was added. The PCR was conducted using a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) with the following amplification program: initial denaturation at 95°C for 5 minutes; 25 cycles of denaturation at 94°C for 0.5 minutes, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes; this was followed by final extension at 72°C for 7 minutes. The PCR products were analysed by gel electrophoresis through a 1% w/v agarose gel stained with GelRed (Cambridge Bioscience, UK) by incorporating 5 µL of 10 000x water-based stock reagent to a 80 mL agarose gel (product protocol; Biotium, USA).
Table 2.1 - Target positions of the PCR and sequencing primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5’ to 3’)</th>
<th>Target</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>341F</td>
<td>341-357</td>
<td>CCT ACG GGA GGC AGC AG</td>
<td>Bacteria</td>
<td>54</td>
<td>[13]</td>
</tr>
<tr>
<td>341F-GC&lt;sup&gt;3&lt;/sup&gt;</td>
<td>341-357</td>
<td>CGC CGG CCG CGC GCG GGC GGC GGG GGG GGG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG</td>
<td>Bacteria</td>
<td>90</td>
<td>[13]</td>
</tr>
<tr>
<td>907R</td>
<td>907-926</td>
<td>CCG TCA ATT CCT TTR AGT TT</td>
<td>Universal</td>
<td>45</td>
<td>[13]</td>
</tr>
</tbody>
</table>

<sup>1</sup> F and R correspond to forward and reverse primer, respectively.

<sup>2</sup> according to nucleotide numbering of *E. coli*.

<sup>3</sup> GC is a 40-nucleotide GC-rich sequence, called GC clamp, attached to 5’ end of the primer 341F.

### 2.5.3 Denaturing Gradient Gel Electrophoresis

DGGE experiments were conducted utilising an INGENY phorU system (Ingeny Internationl BV, The Netherlands) using 8% w/v polyacrylamide gels (acrylamide:bisacrylamide, 37/5:1, gel stock solution; Sigma, USA) in 1 x tris-acetate ethylene-diamine-tetraacetic acid (TAE; 40 mmol L<sup>-1</sup> Tris, 20 mmol L<sup>-1</sup> acetic acid, 1 mmol L<sup>-1</sup> EDTA, pH 8.3), containing 0.04 w/v ammonium persulphate (APS) and 0.02% v/v N,N,N’N’-tetramethylethylenediamine (TEMED). The denaturing gradient ranged from 40 to 70% (100% denaturant contains 7 mol L<sup>-1</sup> urea and 40% v/v formamide).

The manufacturer’s method was modified according to Dopson *et al.* [14] to make the surface of the gel smoother. During the polymerisation step, 3 mL of isopropyl alcohol (IPA) was added to the top of the gradient gel. Polymerisation takes approximately 2 hours, after which the IPA is removed and 5 mL of the stacking gel (8% w/v polyacrylamide in 1 x TAE, containing 0.2% w/v APS and 0.06% v/v TEMED) is cast on the gradient gel and allowed to polymerise for 30 minutes.

The gels were run in 1 x TAE at 60°C at 100 V for 21 hours and stained for 1 hour in 3 x GelRed solution (Cambridge Bioscience, UK) (product protocol; Biotium, USA) containing NaCl (aq, 0.1 mol L<sup>-1</sup>). The gel was washed with 1 x TAE for 10 minutes and visualised under UV-light.
2.6 Biolog

An anaerobic metabolic profile was generated for *S. plymuthica*, *O. anthropi*, *C. denitrificans*, *B. graminisolvens* and *C. indolis* using an Omnilog instrument (Biolog, United States). Biolog is an experimental technique for determining the ability of a microorganism to respire using a variety of different organic substrates. The substrates analysed were the organic carbon sources found on the PM1 and PM2A (a full listing of the substrates on PM1 and PM2A plates is given in Appendix 1). Each titre plate contains 96 wells, each with a different organic substrate immobilised at the bottom. The species being studied was added to each well alongside a redox active tetrazolium dye. Since the dye is the only terminal electron acceptor present in the well, when a species can respire using the substrate present the dye is oxidised going from colourless to magenta. The Biolog records the absorbance (580 nm) every 15 minutes allowing the respiration rate to be calculated. The data obtained will be used to attempt to ascertain which role the species fulfils in the community and compared to the data obtained from the MFC runs.

The Biolog titre plates were placed in an anaerobic bag (heat sealed) containing an ageless sachet and left for 3 days to ensure anaerobic conditions. One portion of Anaerobic Mix B was prepared for each plate being used (Anaerobic Mix B contains AN IF-0a; 18.2072 mL, Dye mix D; 0.240 mL, 1 mol L\(^{-1}\) potassium ferricyanide; 0.0288, 1 mmol L\(^{-1}\) menadione; 0.024 mL). All materials were made anaerobic by bubbling nitrogen gas through them for 1 hour prior to use [15].

Each species to be analysed was grown on the corresponding agar plate as described in Section 2.1. The colonies on the agar plates were re-suspended (using a sterile swab) in 18.0 mL of AN IF-0a until a 40% transmission was observed using a turbidity meter. Next, 1.5 mL of this mixture was transferred to a sterile tube containing 22.5 mL of Mix B and the transmission was checked again to make ensure a transmission of 85%. This mixture was then used to inoculate the plates which were then incubated for 4 days at 37°C.

Every experiment was carried out in duplicate and the readings were averaged before being plotted. The results were processed by plotting the natural log of the absorbance
against time to generate a respiration curve. Respiration curves are a good representation of the physiological state of a microorganism. The Biolog can only measure the respiration rate for the selected substrate and not the growth rate. Respiration rate is the rate at which electrons are transferred from a substrate to a terminal electron acceptor and growth rate is the rate at which a bacterial cell divides. For the following section it will be assumed that maximum growth rate of a given substrate is directly proportional to the maximum respiration rate [16]. It should be noted that in some cases a species is able to respire using a substrate without growing but a species cannot grow without respiring using the substrate present [17]. To measure the variability, a linear regression is going to be applied to the data (R² value). The R² value was calculated for the maximum respiration rate (Section B in Figure 2.10) and in the cases where a value below 0.81 was observed the experiments were repeated.

In Figure 2.10, the portion of the respiration curve indicated by A represents the lag phase, which is the period during which no growth is observed, and which can be linked to the re-adaptation of the cells to the respiration medium (for instance, by expressing enzymes needed to metabolize the substrates in the medium). At the end of the lag phase, cells enter a period of fast respiration, known as the exponential respiration phase. The maximum respiration rate (h⁻¹) can be calculated as the gradient of the line obtained by plotting the natural log of absorbance) vs time (indicated as line B in Figure 2.10) [18].

![Figure 2.10](image)

**Figure 2.10** – An example of the respiration curves generated using the Biolog showing the metabolism of glucose by *C. denitrificans*. Section A represents the lag time, while the gradient (labelled B) shows the maximum respiration rate (*μ*_max, h⁻¹).
In some cases, the Biolog results did not show any lag phase. This means that the maximum respiration phase started before the Biolog had taken its first reading. These species might exhibit a very short lag phase between inoculation of the plates and loading them into the Biolog incubation chamber. In these cases, a lag time of 0 was entered and the gradient of the line which intersected the y axis was calculated (Figure 2.11). The metabolic profiles of the different strains show a wide variation, and all species were able to utilise a large range of substrates.

**Figure 2.11** – An example of the respiration curves generated using the Biolog highlighting an example where growth had begun before the first reading. Section A representing the lag time is not present, while the gradient (labelled section B) shows the maximum respiration rate (\( \mu_{\text{max}} \), h\(^{-1} \)).
2.7 Bibliography


Chapter 3 - Metabolic Analysis of the Different Microbial Species

3.1 Chapter Overview

The work in this chapter examines the respiration rates and metabolic activity of the focal species of the project. Each of the selected species belongs to a different class of bacteria (Comamonas denitrificans, beta-proteobacteria; Serratia plymuthica, gamma-proteobacteria; Ochrobactrum anthropi, alpha-proteobacteria; Clostridium indolis, Clostridia; Bacteroides graminisolvens, Bacteroidetes), which will potentially allow them to fulfil different roles in communities through the utilization and excretion of different carbon compounds.

Growth curves will be created for each species, first using the recommended growth media and conditions and then a second set using synthetic wastewater which will be used for all microbial fuel cell (MFC) experiments at room temperature (to mimic natural operation conditions). The growth curves will be used to determine the optimum point at which the cells should be harvested which could then be used to seed the MFCs. The growth curves will also show the respiration rates for each species which can give insight into which species will propagate faster in the mixed species MFCs.

The selected species will be studied using a Biolog Omnilog to determine their utilisation of a variety of organic carbon sources found upon the Biolog PM1 and PM2A plates. Experiments analysing the individual pairings of species will also be carried out to study any variations between the respiration rates and lag times for each carbon source of interest.

3.2 Characterisation of the Individual Species Growth

This section shows the growth characteristics observed for the individual species being studied in this project. The first set of growth curves were generated using the optimum growth media and conditions as recommended by the supplier, while the second set of
growth curves were carried out using the synthetic wastewater at room temperature to mimic the conditions in the MFC experiments.

Growth curves plot absorbance (measured at 600 nm) against time and the gradient (at the steepest point on the line) was used to determine the maximum growth rate ($\mu_{\text{max}}$, h$^{-1}$) of the species. The data generated from these experiments would be then used to determine at what point the cultures grown from stocks should be harvested and used to seed the MFCs, as well as to establish the experimental conditions (for example flow rates) to be used in the multi-species MFCs.

### 3.2.1 Characterisation of *Comamonas denitrificans* Growth

The tryptone soy broth growth curves for *Comamonas denitrificans* (*C. denitrificans*) in Figure 3.1 show very little variation between the different replicates, all of which required 36 hours to reach the stationary growth phase. The exponential growth phase began between 19 and 23 hours in all cases, and a growth rate of 0.09 h$^{-1}$ was observed.

The synthetic wastewater growth curves were less reproducible than the tryptone soy broth counterparts with a large variation in optical density of the stationary growth phase. However, the maximum growth rates observed were similar: 0.085, 0.094 and 0.100 h$^{-1}$ for *C. denitrificans* 1, 2 and 3 respectively. The time required to reach the exponential growth phase differed drastically for *C. denitrificans* 1 with only 14 hours required while the other replicates required 32 hours.
Figure 3.1 – Growth curves for *C. denitrificans*. With A) showing the curves carried out at 30°C in tryptone soy broth, while B) was carried out at room temperature using synthetic wastewater.
3.2.2 Characterisation of *Serratia plymuthica* Growth

The two growth curves (Figure 3.2) showed a variation in growth pattern under the different conditions, but it should be noted that all replicates required similar times to reach the stationary growth phase (approximately 33 hours). The major difference between the two growth curves was the change in maximum growth rate. The growth curve carried out under optimum conditions reached the exponential growth phase (0.04 h\(^{-1}\)) after around 6 hours and quickly fell off after 9 hours following that growth was linear until 33 hours. This may suggest there is some other limitation. The linear growth, with a rate of 0.01 h\(^{-1}\) persisted until the stationary growth phase was reached.

A greater variation was observed in the growth curves using synthetic wastewater but very little growth was observed for the first 12 hours, while an additional 7 hours were required for *Serratia plymuthica* (*S. plymuthica*) 1 to enter the exponential growth phase (0.05 h\(^{-1}\)). The other 2 replicates entered the exponential growth phase at 27 and 24 hours for *S. plymuthica* 2 and *S. plymuthica* 3 which reached maximum growth rates of 0.15 and 0.147 h\(^{-1}\) respectively.

The temperature for the recommended growth media (25°C) was similar to the temperature the microbes would be exposed to in the microbial fuel cells (27°C) which is reflected by a similar time required to reach the stationary growth phase in the synthetic wastewater. The difference in lag times is likely attributed to the species acclimatising to the new growth media and expressing enzymes to metabolise the newly available substrates [1][2][3].
3.2.3 Characterisation of *Ochrobactrum anthropi* Growth

The *Ochrobactrum anthropi* (*O. anthropi*) nutrient broth growth curves (Figure 3.3) showed that each repeat reached a similar optical density for the stationary growth phase. Replicates 1 and 3 required 7 and 8 hours to reach the exponential growth phase and reached similar maximum growth rates (0.099 and 0.100 h⁻¹). Replicate 2 also took
7 hours to reach the exponential growth phase which only persisted between 7 and 8 hours.

The synthetic wastewater required a longer period of time to reach the stationary growth phase with the shortest being replicate 1 which required 27 hours, while replicate 3 required 31 hours which was the longest time observed. Replicate 1 reached the exponential growth phase after 7 hours and a maximum growth rate of 0.07 h\(^{-1}\) which was sustained until the stationary growth stage was reached. The other replicates required 20 and 21 hours to reach the exponential growth phase, but drastically different maximum growth rates were observed with replicate 2 reaching 0.17 h\(^{-1}\) while replicate 3 had a maximum growth rate of 0.10 h\(^{-1}\).
3.2.4 Characterisation of Bacteroides graminisolvens Growth

The Bacteroides graminisolvens (B. graminisolvens) growth curve in cooked meat media (CMM) showed good repeatability with all replicates reaching a similar optical
density at the stationary growth phase (Figure 3.4). The replicates all required a similar amount of time to reach the exponential growth phase was reached after 5, 6 and 7 hours for replicates 1, 2 and 3 respectively, replicates 1 and 2 also produced similar maximum growth rates (0.23 h\(^{-1}\) and 0.24 h\(^{-1}\)) while replicate 3 had a much lower growth rate of 0.17 h\(^{-1}\).

The synthetic waste growth curves showed longer lag times before the exponential growth curve was reached, requiring 17, 18 and 23 hours for replicate 1, 2 and 3 respectively. Despite replicate 2 taking the longest time to reach the exponential growth phase, it reached the stationary growth phase at the same time as replicate 1 (30 hours). Each replicate showed a different maximum growth rate with replicate 1 showing the lowest rate at 0.08 h\(^{-1}\), followed by replicate 3 at 0.10 h\(^{-1}\), while the highest rate shown by replicate 2 at 0.14 h\(^{-1}\).

The growth curves showed that *B. graminisolvens* had faster growth rates but much longer growth times in the synthetic wastewater medium as opposed to the CMM. This could be attributed to the lower temperatures (27\(^\circ\)C compared to 37\(^\circ\)C) during the synthetic wastewater growth curves as opposed to the carbon sources present in the medium.
Figure 3.4 – Displaying the different growth curves for *B. graminisolvens* with A) showing the curves carried out at 37°C in cooked meat medium, while B) was carried out at room temperature using synthetic wastewater.
3.2.5 Characterisation of *Clostridium indolis* Growth

The *Clostridium indolis* (*C. indolis*) growth curves in CMM (Figure 3.5) show all replicates reaching a similar optical density during the stationary growth phase, but all reached it after a different period of time, with the shortest coming from replicate 3 at 17 hours while replicate 1 required the longest time at 27 hours. Replicate 2 and 3 began the exponential growth phase after 9 hours while replicate 1 required 11 hours. The maximum growth rates were similar for all 3 replicates (0.21, 0.19, 0.19 h\(^{-1}\)).

![Growth curves of *Clostridium indolis*](image)

**Figure 3.5** – Displaying the different growth curves for *C. indolis* with A) showing the curves carried out at 37°C in cooked meat medium, while B) was carried out at room temperature using synthetic wastewater.
Chapter 3 – Metabolic Analysis of the Different Microbial Species

The synthetic wastewater growth curves showed a higher degree of variation, with replicate 3 showing a very different curve to replicate 1 and 2. The optical density of the species also showed a high degree of variation (1.05, 1.16 and 1.36) between the replicates. Replicate 3 had the lowest maximum growth rate of 0.09 h\(^{-1}\), while the other two replicates produced similar maximum growth rates of 0.13 and 0.11 h\(^{-1}\). The synthetic wastewater curves showed faster growth rates, but longer lag times relative to those observed under optimum conditions.

3.2.6 Discussion

The differences in lag times and growth rates observed when comparing the recommended growth media and synthetic wastewater can be explained by the different conditions the two experiments were conducted under. Examples of this are the different temperatures the experiments are carried out under (25°C, 30°C and 37°C compared to 27°C which was the temperature of the laboratory). Another difference is the concentration of organic carbon sources present in the media with the synthetic wastewater having approximately 3 g of glucose while the recommended growth medias had varying concentrations of organic carbon sources (Nutrient broth; 3 g peptone and Tryptone Soy Broth; 3 g Soybean meal, 2.5 g dextrose).

Since the cultures were grown prior to the growth curve, the species will have acclimatised to the temperatures of the recommended growth media. The decreased temperature of the synthetic wastewater experiments could also account for the longer lag times and growth periods.

The growth curves were inoculated using the same optical densities (using the same number of washes) as the single species MFCs in an attempt to predict the speed the individual species in the MFCs will acclimatisethe system. The data generated suggest that *O. anthropi* will potentially require the shortest time to acclimatise and reach a stable power output in the MFCs. The data also suggest that *C. denitrificans* will require the longest time to reach a stable power output because it required the longest period of time to reach the stationary growth phase.
3.3 Biolog Metabolic Studies

3.3.1 Single Species Biolog experiments

Due to the species being able to utilise a wide range of substrates, the following section has been organised by substrates as opposed to species.

3.3.2 Volatile Fatty Acids

Volatile fatty acids (VFAs) are known to play an important role in the electrogenic activity of MFCs with reports in the literature suggesting that they are the final electron donors from which certain species such as *Geobacter* and *Shewanella* will transfer electrons to the anode [7]. Of these substrates the most important are seen to be acetate, propionate and butyrate which have all been intensively studied [8][9][10][11]. Due to the substrates on the Biolog plates not using salts, the acid form had to be used so acetic acid was analysed opposed to acetate.

Catal *et al.* monitored the concentration of acetate, propionate and butyrate as different combinations of monosaccharides were utilised by a microbial community and found that the concentrations of acetate and propionate were always higher than that of butyrate [12]. This suggests that acetate and propionate are produced more frequently during respiration and fermentation which suggests that they are more relevant to power generation than butyrate. This could also suggest that the species utilising acetate and propionate are more important to power generation than the species utilising butyrate, since higher concentrations of acetate and propionate are more likely to be present in the MFC. Table 3.1 shows the various half equations for the reduction of different VFAs. Assuming complete oxidation of one mole of the substrates, acetate generates 8 moles of electrons, propionate 14 moles of electrons and butyrate 20 moles of electrons [13]. This suggests that despite being in lower concentrations, butyrate (which produces over 3 times the number of electrons per mole, when compared to acetate, assuming complete oxidation) can still be an important substrate when it comes to power generation.
Chapter 3 – Metabolic Analysis of the Different Microbial Species

**Table 3.1** – The half reactions for the complete reductions of acetate, propionate and butyrate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Half Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>$\frac{1}{8} \text{CO}_2 + \frac{1}{8}\text{HCO}_3^- + \text{H}^+ + \text{e}^- \rightarrow \frac{1}{8} \text{Acetate} + \frac{3}{8} \text{H}_2\text{O}$</td>
</tr>
<tr>
<td>Propionate</td>
<td>$\frac{1}{7} \text{CO}_2 + \frac{1}{14}\text{HCO}_3^- + \text{H}^+ + \text{e}^- \rightarrow \frac{1}{14} \text{Propionate} + \frac{5}{14} \text{H}_2\text{O}$</td>
</tr>
<tr>
<td>Butyrate</td>
<td>$\frac{3}{20} \text{CO}_2 + \frac{1}{20}\text{HCO}_3^- + \text{H}^+ + \text{e}^- \rightarrow \frac{1}{20} \text{Butyrate} + \frac{7}{20} \text{H}_2\text{O}$</td>
</tr>
</tbody>
</table>

The Biolog results (Table 3.2) showed that no single species could utilise all three of the VFAs, with *O. anthropi*, *C. indolis*, and *C. denitrificans* being able to utilise two of the three. Only *S. plymuthica* and *B. graminisolvens* were able to utilise acetic acid. It was also observed that acetic acid was the most utilised substrate, followed by butyric acid and propionic acid which was only metabolised by *O. anthropi*.

In almost all cases, a lag time was observed before the substrate was metabolised, with the exception of *O. anthropi* which showed colour development from $t = 0$ for butyric acid. A lag time of 33 hours was observed before *O. anthropi* was able to utilise propionic acid. In the case of *C. indolis* and *C. denitrificans* the lag times were shorter for the 2-carbon acetic acid (37 and 18 hours respectively) than the 4-carbon butyric acid (56 and 25 hours respectively) (Table 3.2).
Table 3.2 - The maximum respiration rate and lag times for the different species utilisation of different VFAs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Acetic Acid</th>
<th>Propionic Acid</th>
<th>Butyric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. plymuthica</strong></td>
<td>Lag Time (h)</td>
<td>36</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.67</td>
<td>-</td>
</tr>
<tr>
<td><strong>O. anthropi</strong></td>
<td>Lag Time (h)</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>-</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>C. indolis</strong></td>
<td>Lag Time (h)</td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.29</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. graminisolvens</strong></td>
<td>Lag Time (h)</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.80</td>
<td>-</td>
</tr>
<tr>
<td><strong>C. denitrificans</strong></td>
<td>Lag Time (h)</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.74</td>
<td>-</td>
</tr>
</tbody>
</table>

For a community involving all 5 of these species based on maximum respiration rates and lag times (Table 3.2), these results suggest that there would be competition to utilise acetate and butyrate. It is possible that *B. graminisolvens* would adapt most rapidly to the presence of acetate which could explain the early dominance observed in some fuel cells [14][15]. In terms of maximum respiration rate, both *B. graminisolvens* and *C. denitrificans* produced higher rates than *S. plymuthica* and *C. indolis* suggesting that they would not compete for acetate and utilise different substrates. It has been shown that the *Comamonas* species is highly abundant in the biofilm of a MFC and has been implied to be an exoelectrogenic species, which indicates that *C. denitrificans* will be able to compete with *B. graminisolvens* for the acetate [15][16]. As discussed earlier, only *O. anthropi* has been shown to be able to utilise the propionic acid generated, but it is also able to utilise butyric acid without any lag phase. The next shortest lag time is *C. denitrificans* but the maximum respiration rate is much lower than that of *O.*
which indicates that *O. anthropi* could potentially out compete the other species when it comes to utilising butyric acid. *C. indolis* had a relatively long lag time (56 hours) but a much faster respiration rate than *O. anthropi*, indicating over time *C. indolis* could potentially out compete the other species and possibly dominate the culture.

### 3.3.3 Other Organic Acids

While studies with acetate, propionate and butyrate make up the majority of the literature, they are not the only carboxylates that are expected to be produced by a microbial community [17]. One of these acids is lactate which is also used as a carbon source in fuel cells inoculated with *Desulfovibrio* and *Shewanella*. The reason for this is that lactate is the primary product of glycolysis when cells grow under anaerobic conditions [17]. Another reason for this may be that oxygen diffusion through the membrane could have generated a microaerobic environment in the anodic chamber. This presence of oxygen can lead to the activation of aerobic respiration, but due to the relatively low concentrations of oxygen, there would be a mismatch between the flux of metabolites through glycolysis and the flux through the Krebs cycle, with glycolysis causing what is called “overflow metabolism”. This leads to excess production and excretion of metabolites such as citrate, malate and succinate, among others [18][19]. An understanding of how these potential substrates are metabolised by the community can help determine the relationships between species, and eventually help optimise the community for power generation.

When looking at the organic acids, it can be seen that *S. plymuthica*, *O. anthropi* and *B. graminisolvens* can all utilise six of the eight acids analysed, while *C. indolis* and *C. denitrificans* are only able to metabolise three of the eight species (Table 3.3). In all cases *S. plymuthica* had one of the longest lag times but also showed lower maximum respiration rates than most of the other species. The one exception to this was in the case of acetoacetic acid, where *B. graminisolvens* and *O. anthropi* both had the shortest lag times (10 and 12 hours) but *B. graminisolvens* showed a higher maximum respiration rate, suggesting that *O. anthropi* will not metabolise acetoacetic acid. The other 3 species all had lag times of approximately 25 hours but *S. plymuthica* showed a
higher maximum respiration rate which suggests that it is the more likely species to compete with \textit{B. graminisolvans} for acetoacetic acid utilisation.

Lactic acid is the product of glycolysis under anaerobic conditions, as well as a product of respiration using acetate [20], therefore it is expected to be a relatively abundant substrate in the MFCs. It has also been widely used as a carbon source in MFCs and has been shown to produce higher cumblic efficiencies and powers than other substrates [21]. When looking at lactate (Table 3.3), \textit{B. graminisolvans} and \textit{C. denitrificans} both showed the shortest lag times (5 and 9 hours respectively), with \textit{C. denitrificans} showing a $\mu_{\text{max}}$ of 1.72 h\(^{-1}\) compared to \textit{B. graminisolvans} $\mu_{\text{max}}$ of 1.44 h\(^{-1}\). Since \textit{B. graminisolvans} is able to utilise a large number of other substrates at relatively high rates it is possible that \textit{C. denitrificans} is the species primarily responsible for utilisation of lactate in the anodic community, which could explain its predominance in MFCs.

Due to the gas permeable nature of nafion, oxygen is likely to be present in the low concentrations around the membrane. This means that there is a good chance that the facultative anaerobes present in the anodic chamber will respire using the oxygen. This can potentially lead to metabolites like succinate, malate and citrate being excreted by the anodic microbial community. In the case of citric and maleic acid, the fastest maximum respiration rate (0.89 and 1.18 h\(^{-1}\) respectively) was shown by \textit{O. anthropi} (Table 3.3). In the case of succinic acid both \textit{O. anthropi} and \textit{B. graminisolvans} showed short lag times of 14 and 11 hours respectively, but \textit{B. graminisolvans} showed a higher maximum respiration rate of 1.84 h\(^{-1}\) compared to \textit{O. anthropi}’s 1.23 h\(^{-1}\).

Fumaric, formic and oxalic acids are all utilised to a lower extent than the other acids, with only two or three species able to utilise them (Table 3.3). Oxalic acid was only metabolised by \textit{C. indolis} but was associated with a lag time of 43 hours. Formic acid was utilised by \textit{S. plymuthica} and \textit{B. graminisolvans}, however there was a large difference between the lag times with \textit{B. graminisolvans} showing a shorter lag time of 8 hours compared to the 39 hours for \textit{S. plymuthica} suggesting \textit{B. graminisolvans} will utilise the formic acid generated. Finally, fumaric acid was utilised by \textit{O. anthropi} and \textit{B. graminisolvans}, both of which had the same lag times and similar maximum respiration rates.
Table 3.3 – The maximum growth rate and lag times for the different species utilisation of different carboxylic acids corresponding to the carboxylates generated through metabolic reactions.

<table>
<thead>
<tr>
<th>Carboxylic Acid</th>
<th>S. plymuthica</th>
<th>O. anthropi</th>
<th>C. indolis</th>
<th>B. graminisolvens</th>
<th>C. denitrificans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric Acid</td>
<td>0.17</td>
<td>0.89</td>
<td>0.08</td>
<td>0.16</td>
<td>-</td>
</tr>
<tr>
<td>Succinic Acid</td>
<td>0.55</td>
<td>1.23</td>
<td>0.49</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Lactic Acid</td>
<td>0.03</td>
<td>0.49</td>
<td>-</td>
<td>1.84</td>
<td>-</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>0.22</td>
<td>-</td>
<td>-</td>
<td>1.44</td>
<td>-</td>
</tr>
<tr>
<td>D,L-Malic Acid</td>
<td>0.63</td>
<td>1.18</td>
<td>0.65</td>
<td>1.56</td>
<td>-</td>
</tr>
<tr>
<td>Fumaric Acid</td>
<td>0.63</td>
<td>0.96</td>
<td>0.65</td>
<td>0.64</td>
<td>-</td>
</tr>
<tr>
<td>Acetoacetic Acid</td>
<td>-</td>
<td>1.18</td>
<td>0.65</td>
<td>0.99</td>
<td>-</td>
</tr>
<tr>
<td>Oxalic Acid</td>
<td>1.11</td>
<td>0.68</td>
<td>1.28</td>
<td>1.29</td>
<td>-</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>17</td>
<td>5</td>
<td>10</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Lag Time (h): 5
Maximum Respiration Rate (h⁻¹): 1.56
3.3.4 Sugars

Sugars are good substrates in MFCs because as they are metabolised they generate other organic compounds which can be used as a carbon source, such as lactate, acetate and propionate [22]. This diversity of carbon sources allows the generation of a more diverse community, thus allowing the community to break down more substrates in water treatment. A large number of studies have shown that inoculation of a fuel cell with substrates, such as glucose, led to a higher power density even though lower coulombic efficiency (CE) was observed [23].

3.3.4.1 Commonly Used Sugars

Glucose and sucrose are commonly used as the substrate in MFCs. Table 3.4 shows the metabolic profile of the different species when cultured using sucrose and glucose as carbon sources.

**Table 3.4 – The maximum growth rate and lag times for the different species using sucrose and glucose.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sucrose</th>
<th>a-D-Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (h)</td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.40</td>
<td>0.07</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.74</td>
<td>1.46</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>-</td>
<td>1.60</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>2.26</td>
<td>1.98</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.94</td>
<td>1.90</td>
</tr>
</tbody>
</table>
Glucose is often seen as the universal substrate, which is reflected in the fact that all species were able to utilise glucose to some degree [24]. *S. plymuthica* was able to utilise glucose but showed a longer lag time than the other substrates and also produced the slowest respiration rate, which suggests that when glucose is used as a substrate, *S. plymuthica* will not be involved in the initial stages and most likely uses the metabolites generated by other species (Table 3.4). This can also be seen with sucrose where *S. plymuthica* had a longer lag time and slow maximum respiration rate. The fact that *S. plymuthica* is found in MFCs using sucrose as the carbon source, but shows slow lag times and low respiration rates using the Biolog experiments, suggests that it is not primarily involved with sucrose metabolism in the MFCs, when a microbial community is present but relies upon other metabolic products generated [25]. It is possible that *S. plymuthica* is primarily used hydrolysed glucose for fermentation into acetoin and 2,3-butanediol but it is also possible that it uses pyruvate, which some species sometimes excrete when metabolising certain substrates [26][27].

Even though *C. indolis* is a fermentative bacterium, it was unable to metabolise sucrose but despite this, *C. indolis* has been detected in communities of fuel cells using sucrose as a substrate, where it was believed to ferment the sucrose [9][11][15]. The inability to metabolise sucrose suggests that *Clostridium* may fulfil a different role in the community than the fermentative role it was originally believed to have and would consume other substrates present in the MFC.

The species with the shortest lag time required for sucrose utilisation was *O. anthropi* which had already undergone the exponential respiration phase before the first reading was taken. After 5 hours both *B. graminisolvens* (respiration rate: 2.26 h⁻¹) and *C. denitrificans* (respiration rate: 0.94 h⁻¹) were able to metabolise sucrose, with *B. graminisolvens* having the faster respiration (Table 3.4). This suggests that *O. anthropi* and *B. graminisolvens* may be the primary fermenters of sucrose in this community.

Glucose, which is one of the products of the metabolism or hydrolysis of sucrose, can be utilised by all species with *C. denitrificans* (respiration rate: 1.90 h⁻¹) and *B. graminisolvens* (respiration rate: 1.98 h⁻¹) exhibiting the shortest lag times (4 hours), while *O. anthropi* (respiration rate: 1.46 h⁻¹) and *C. indolis* (respiration rate: 1.60 h⁻¹) both had lag times of around 10 hours (Table 3.4). This suggests that all species (except...
for *S. plymuthica*, lag time: 31 hours, respiration rate: 0.07 h⁻¹) will utilise glucose to some degree in the system. However further work is required to develop a better picture of the interactions within a MFC.

### 3.3.4.2 Disaccharides and Polysaccharides

Disaccharides and polysaccharides are promising organic sources for use in MFCs using a microbial community as opposed to single a species. Table 3.5 shows the metabolic profile for the disaccharides and polysaccharides found on the Biolog PM1 and PM2A plates (See Appendix A for complete Biolog PM1 and 2A titre plates).

Table 3.5 shows that all species were able to utilise all disaccharides present on the Biolog PM1 and PM2A plates, with the exception of *S. plymuthica* which was unable to metabolise sucrose. Both maltose and lactose are very similar in the metabolic profiles generated from the Biolog, with species having similar lag times and rates. The major difference observed is for *O. anthropi* which metabolises lactose at a much slower rate than maltose and with a longer lag time. In these cases, both *B. graminisolvens* and *C. denitrificans* have short lag times of around 5 hours and present a faster respiration rate than the other species present. *C. indolis* had a lag time of around 14 hours in both cases, as well as showing a similar respiration rate to *C. denitrificans* and *B. graminisolvens*. Cellobiose was quickly metabolised by *S. plymuthica* and *B. graminisolvens*, which had both passed their exponential respiration phase before the first reading was taken.

In the case of polysaccharides glycogen and pectin (Table 3.5) the only species able to utilise either were *C. indolis* and *B. graminisolvens*. For both glycogen and pectin, *C. indolis* had long lag times (44 and 63 hours respectively) associated with the metabolism of the polysaccharides, while *B. graminisolvens* had a lag time of 5 hours for glycogen, and pectin had already been metabolised before the Biolog first reading was taken.
Table 3.5 – The maximum growth rate and lag times for the different species using the disaccharides and polysaccharides found on the Biolog PM1 and PM2A plates.

<table>
<thead>
<tr>
<th>Disaccharide/Polysaccharide</th>
<th>S. plymuthica</th>
<th>O. anthropi</th>
<th>C. indolis</th>
<th>B. graminisolvens</th>
<th>C. denitrificans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>32</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>a-D-Lactose</td>
<td>39</td>
<td>20</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>D-Cellobiose</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>14</td>
<td>15</td>
<td>0</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>Glycogen</td>
<td>14</td>
<td>15</td>
<td>0</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>Pectin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.19</td>
<td>0.84</td>
<td>0.94</td>
<td>1.17</td>
<td>1.25</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.05</td>
<td>0.57</td>
<td>0.23</td>
<td>1.55</td>
<td>1.58</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>14</td>
<td>40</td>
<td>40</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.74</td>
<td>0.23</td>
<td>1.15</td>
<td>0.67</td>
<td>0.22</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.07</td>
<td>-</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>-</td>
<td>-</td>
<td>1.26</td>
<td>2.26</td>
<td>-</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.94</td>
<td>-</td>
</tr>
</tbody>
</table>

Chapter 3 – Metabolic Analysis of the Different Microbial Species
3.3.4.3 Monosaccharides

From Table 3.6 it can be seen that all species showed the ability to metabolise all pentose sugars analysed with the exception of *B. graminisolvens*, which was unable to metabolise D-arabinose. Furthermore *O. anthropi* had a lag time associated with the metabolism of D-arabinose suggesting that, if present, as a food source it will probably not be utilised by *O. anthropi*. It is problematic to reach further conclusions since the respiration rates were very high.

**Table 3.6 – The maximum growth rate and lag times for the different species using the different pentose sugars found on the Biolog PM1 and PM2A.**

<table>
<thead>
<tr>
<th>Species</th>
<th>L-Arabinose</th>
<th>D-Xylose</th>
<th>D-Ribose</th>
<th>D-Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. plymuthica</em></td>
<td>Lag Time (h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>0.59</td>
<td>0.50</td>
<td>0.27</td>
</tr>
<tr>
<td><em>O. anthropi</em></td>
<td>Lag Time (h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>0.61</td>
<td>0.47</td>
<td>0.40</td>
</tr>
<tr>
<td><em>C. indolis</em></td>
<td>Lag Time (h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>0.08</td>
<td>0.23</td>
<td>0.21</td>
</tr>
<tr>
<td><em>B. graminisolvens</em></td>
<td>Lag Time (h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>0.24</td>
<td>0.19</td>
<td>0.12</td>
</tr>
<tr>
<td><em>C. denitrificans</em></td>
<td>Lag Time (h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>0.22</td>
<td>0.16</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Table 3.7 shows that the different species had higher activity for the metabolism of pentoses than hexoses. This is demonstrated by the fact all the sugars required a lag time before utilising the substrate. Mannose and galactose were both utilised by all five species, while only *S. plymuthica* was unable to metabolise fructose. In the case of mannose, four out of the five species showed shorter lag times when compared to glucose, but the maximum respiration rate was lower in all cases.
Galactose was similar to glucose but showed shorter lag times and lower maximum respiration rates. The data suggests that *B. graminisolvens*, *S. plymuthica* and *C. indolis* would all compete to utilise galactose, which could work better in a MFC since it is possible that *C. denitrificans* will be out competed by other species and thus able focus on utilising organic acids, potentially improving the electrogenic nature of the community. Fructose (one of the monosaccharides which are combined in sucrose) is of interest here since it is likely to be present in a fuel cell using sucrose as the carbon source. When comparing the lag times of species utilising glucose or fructose, they showed similar times, but those utilising fructose were unable to reach the same maximum respiration rate as those utilising glucose. It is difficult to hypothesise which species will utilise fructose since all species displayed similar respiration curves.

Based on Tables 3.5, 3.6 and 3.7, it is possible that lactose could be a better organic carbon source to use than sucrose, because lactose is utilised by a higher number of
species. Furthermore, galactose replaces fructose as the second monosaccharide and is better utilised than fructose by the species selected [12].

A study, by Catal et al., was conducted into the effect of different monosaccharides (glucose, galactose, fructose, xylose, arabinose and ribose) on power generation [12]. Of all the combinations, glucose showed the shortest adaptation time (1 hour) with arabinose showing the longest (25 hours). All the pentose sugars tested had adaptation periods of between 15 and 25 hours, in disagreement with the observations in the Biolog, where the species analysed started metabolising the sugars before the Biolog’s initial reading was taken. The communities in that study were not analysed, so it cannot be confirmed that both communities will work in a similar way. This inconsistency was also observed in the cases of the hexose sugars where galactose showed shorter lag times than glucose, but in the cell data longer adaptation periods were observed.

A further study conducted by Catal et al. looked at different combinations of monosaccharides (glucose, galactose, mannose, arabinose and xylose), all of which showed similar voltage outputs (ranging from 0.5 to 0.59 V) which lasted for similar periods (11 – 15 hours) [23]. These experiments did not include fructose so a comparison to sucrose cannot be made. A cell containing all 5 sugars was also tested, which showed a lower maximum voltage (0.5 V) but was sustained over a longer period of time (22 hours) due to the higher abundance of organic substrates [23].

### 3.3.5 Amino Acids

Studies have been carried out by Yang et al. showing the effect of 8 different amino acids on the power generation of a MFC and it was concluded that polar amino acids produced higher power densities than their non-polar counterparts; it was also determined that as the size of the R group increases, the power output decreases [28]. The cells were inoculated with wastewater but the study did not analyse the community (thus the species composition is unknown). However, the species being analysed in this work have been shown to commonly occur in a large variety of MFCs run with wastewater. The behaviour of communities may therefore be assumed to be the same until MFC cells studies with the selected species have been carried out.
The literature showed that serine (Ser), which was shown to produce the highest power density (7.68 mW m$^{-2}$) was the only amino acid that was utilised by all 5 species and generally had a short lag time [28]. The shortest lag time was 8 hours (in the case of *C. denitrificans*) and the longest was 34 hours (for *C. indolis*). Despite all the species being able to utilise Ser, the maximum respiration rates associated with it differed, with *C. denitrificans* and *B. graminisolvans* having the highest respiration rates and the shortest lag times (Table 3.8). This could potentially mean that *C. denitrificans*, a known electrogenic species, would be able to proliferate more and lead to a higher power output.

In the data from Yang *et al.*, Alanine (Ala) produced the lowest power density (556 mW m$^{-2}$) and was utilised by three species: *O. anthropi*, *B. graminisolvans* and *C. denitrificans* [28]. The shortest lag time is *B. graminisolvans* (10 hours), which also has the highest maximum respiration rate ($2.5 \text{ h}^{-1}$) when compared to *O. anthropi* (14 hours and $0.37 \text{ h}^{-1}$) and *C. denitrificans* (17 hours and $0.40 \text{ h}^{-1}$) (Table 3.8); this suggests that *B. graminisolvans* would be more prolific in an Ala fed system. *Bacteroides* has not been associated with power production in MFCs, so the slow respiration of *Comamonas* compared to *Bacteroides* could explain the low power output.

Histidine (His), Arginine (Arg) and Glutamic acid (Glu) also showed relatively high power densities (718 mW m$^{-2}$, 727 mW m$^{-2}$ and 686 mW m$^{-2}$) [28]. In the case of Glu both *O. anthropi* and *C. denitrificans* showed no lag time and had already passed the exponential respiration phase before the initial reading was taken, when compared to *B. graminisolvans* which had a lag time of 4 hours. It is likely that the community would favour *O. anthropi* and *C. denitrificans* as opposed to *B. graminisolvans*. Similar to Glu, Arg was also only metabolised by three species but this time *C. indolis* replaced *B. graminisolvans*. All species exhibited a lag time, with *C. denitrificans* and *O. anthropi* showing the shortest lag time (17 hours); and *C. denitrificans* had a higher maximum respiration rate ($1.83 \text{ h}^{-1}$ compared against $0.51 \text{ h}^{-1}$) suggesting that *C. denitrificans* would be more prevalent in the system.

Of the amino acids on the Biolog PM1 and PM2A plates but not included in the study conducted by Yang *et al.* [28], it can be seen that isoleucine, leucine and glycine will promote respiration of *O. anthropi* and that glutamine and proline are likely to
encourage the respiration of *B. graminisolvens*, another species which has been associated with electrogenic activity.

Putrescine is not an amino acid but is generated through utilisation of amino acids. Only *C. indolis* (a fermentative species) is able to metabolise it, but even then a relatively long lag period has to be overcome before it is slowly utilised. This indicates that the other species excrete putrescine, leading to a build-up in cells run with protein rich sludge and suggesting that *C. indolis* could be useful for running cells primarily concentrating upon water treatment.
**Table 3.8** – The maximum growth rate and lag times for the different species using the different amino acids found on the Biolog PM1 and PM2A. The pink shaded boxes indicate the amino acids studied by Yang *et al.* [28].

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Glycine</th>
<th>D-Alanine</th>
<th>L-Serine</th>
<th>L-Proline</th>
<th>L-Valine</th>
<th>L-Isoleucine</th>
<th>L-Leucine</th>
<th>L-Aspartic Acid</th>
<th>L-Lysine</th>
<th>L-Glutamic Acid</th>
<th>L-Histidine</th>
<th>L-Arginine</th>
<th>Putrescine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lag Time (h)</td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>Lag Time (h)</td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>Lag Time (h)</td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>Lag Time (h)</td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>Lag Time (h)</td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>Lag Time (h)</td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>Lag Time (h)</td>
</tr>
<tr>
<td><strong>C. denitrificans</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>S. plymuthica</strong></td>
<td>14</td>
<td>0.37</td>
<td>14</td>
<td>0.93</td>
<td>0.37</td>
<td>1.10</td>
<td>0</td>
<td>0.04</td>
<td>0.37</td>
<td>0.04</td>
<td>0.37</td>
<td>0.04</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>O. anthropi</strong></td>
<td>1002</td>
<td>0.95</td>
<td>13</td>
<td>0.79</td>
<td>0.95</td>
<td>0.77</td>
<td>15</td>
<td>0.48</td>
<td>15</td>
<td>0.48</td>
<td>15</td>
<td>0.48</td>
<td>15</td>
</tr>
<tr>
<td><strong>B. graminisolvens</strong></td>
<td>1450</td>
<td>0.93</td>
<td>14</td>
<td>0.79</td>
<td>0.93</td>
<td>0.77</td>
<td>15</td>
<td>0.48</td>
<td>15</td>
<td>0.48</td>
<td>15</td>
<td>0.48</td>
<td>15</td>
</tr>
</tbody>
</table>

*Note: The data is presented in a tabular format with columns for lag time and maximum respiration rate for different species and amino acids.*
3.4 Dual-Species Biolog experiments

3.4.1 Volatile Fatty Acids

The dual-cultures showed very little variation in their ability to utilise substrates when combined with another species. In no instance was a combination of species able to utilise a substrate which both species were unable to utilise in the single species experiment. Another trend observed was that when only one species could utilise the substrate in the single species experiments, the community could also utilise the substrate. The one exception was the *S. plymuthica* and *C. denitrificans* dual-culture which was not able to utilise butyric acid despite *C. denitrificans* being able to utilise it in the single-species MFC (Figure 3.8 and Table 3.10). There are no references in the literature of these two species having metabolic interactions, but is possible that *S. plymuthica* excretes a substance which inhibits *C. denitrificans* ability to respire utilising butyrate, the species *Serratia marcescens* has been shown to produce a compound called serratamolide which can inhibit the growth of certain gram-positive strains such as *Staphylococcus aureus* but no link was found with gram-negative strains, such as *C. denitrificans* [29]. This substance is unlikely to be a respiration or metabolism product because the dye in the Biolog is the only electron acceptor available so a colour change would be observed if any metabolites were generated. It should also be noted that when both species were able to utilise a substrate, the dual-culture would also be able to use the substrate.

In the single species butyric acid experiments, it was observed that no lag time was associated with *O. anthropi*, which had already begun utilising the substrate before the first reading was taken. The shortest lag time observed by a dual-culture was the *O. anthropi* and *B. graminisolvens* pairing, at 3 hours, closely followed by the *O. anthropi* and *S. plymuthica* pairing at 4 hours (Table 3.10). The longest lag time in the single species experiments was that of *C. indolis* at 56 hours, but all dual-cultures showed a shorter lag with the longest being the *C. indolis* and *S. plymuthica* dual-culture at 43 hours.
Figure 3.8 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate butyric acid. Species with no result shown were found to be unable to respire using the substrate. Side by side comparison can be found in Appendix III.
When looking at the lag times and respiration rates associated with acetic acid (Figure 3.9 and Table 3.9), only two (O. anthropi and C. indolis, O. anthropi and C. denitrificans) of the dual-cultures showed a shorter lag time than the individual components. On the other hand, most dual-cultures showed an increased respiration rate compared to the single species experiments. The only dual-cultures that exhibited a decrease in respiration rate were B. graminisolvens with either C. indolis or C. denitrificans. It should also be noted that B. graminisolvens did produce the highest respiration rate (1.8 h⁻¹) in the single species experiments (Figure 3.9 and Table 3.9). Four of the dual-cultures showed respiration rates greater than the highest respiration rate achieved in the single species experiments, and only two of them contained B. graminisolvens. Three of the four communities included O. anthropi as one of the species which was unable to utilise acetate in the single species MFCs.

As expected from the single species experiments, only communities involving O. anthropi were able to respire utilising propionic acid (Figure 3.10 and Table 3.9). In three of the four dual-cultures, the lag times were shorter and the respiration rate was higher than the single species experiments (33 hours, 0.27 h⁻¹). The exception was the O. anthropi and B. graminisolvens dual-culture (42 hours, 0.5 h⁻¹).

The dual-cultures generally showed increased respiration rates which could arise from the competition arising from a second species being present. It is also possible that during the respiration process a species may generate a metabolite which is utilised by the species. The converse is also true where a metabolite is excreted, which can inhibit the metabolism of another substrate, decreasing the overall respiration rate.

With regards to the utilisation of various VFAs, it appears that O. anthropi is the ablest to capitalise on the presence of a second species. The O. anthropi communities were the only community able to utilise all VFAs, suggesting that these communities could provide the highest CEs and provide the most effective COD treatment. While the S. plymuthica with B. graminisolvens and C. denitrificans pairings will produce the lowest CEs and reductions in COD due to their inability to utilise propionic acid and butyric acid.
Table 3.9 – The maximum respiration rates and lag time for different dual-cultures ability to utilise different VFAs. The purple shaded boxes indicate that only one of the individual species was able to utilise a substrate, and the red shaded box represents when neither species could utilise a substrate.

<table>
<thead>
<tr>
<th></th>
<th>Acetic Acid</th>
<th>Propionic Acid</th>
<th>Butyric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. plymuthica + O. anthropi</td>
<td>Lag Time (h)</td>
<td>41</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.90</td>
<td>2.70</td>
</tr>
<tr>
<td>S. plymuthica + C. indolis</td>
<td>Lag Time (h)</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.10</td>
<td>-</td>
</tr>
<tr>
<td>S. plymuthica + B. graminisolvens</td>
<td>Lag Time (h)</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>2.20</td>
<td>-</td>
</tr>
<tr>
<td>S. plymuthica + C. denitrificans</td>
<td>Lag Time (h)</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>O. anthropi + C. indolis</td>
<td>Lag Time (h)</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>3.40</td>
<td>1.40</td>
</tr>
<tr>
<td>O. anthropi + B. graminisolvens</td>
<td>Lag Time (h)</td>
<td>12</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>3.70</td>
<td>0.50</td>
</tr>
<tr>
<td>O. anthropi + C. denitrificans</td>
<td>Lag Time (h)</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>3.30</td>
<td>3.90</td>
</tr>
<tr>
<td>C. indolis + B. graminisolvens</td>
<td>Lag Time (h)</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.10</td>
<td>-</td>
</tr>
<tr>
<td>C. indolis + C. denitrificans</td>
<td>Lag Time (h)</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.30</td>
<td>-</td>
</tr>
<tr>
<td>B. graminisolvens + C. denitrificans</td>
<td>Lag Time (h)</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.20</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.9 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate acetic acid. Species with no result shown were found to be unable to respire using the substrate. Side by side comparison can be found in Appendix III.
Figure 3.10 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate propionic acid. Species with no result shown were found to be unable to respire using the substrate. Side by side comparison can be found in Appendix III.
3.4.2 Other Organic Acids

The results for other organic acids (Table 3.10) show similar trends to those highlighted in Section 3.4.1 (Table 3.9), the dual-cultures were not able to respire using a substrate which neither species were able to utilise in the single species experiments. The one exception to this was the *S. plymuthica* and *C. denitrificans* co-culture which were able to metabolise fumaric acid despite neither being able to utilise it in the single species experiments. In the majority of cases where one species was able to utilise the substrate, the dual-culture would also be able to respire using the substrate. In all cases where both species were able to utilise the substrate the dual-culture was also able to utilise it.

Lactic acid, which is commonly used a food source in MFCs, was utilised by every dual-culture. The shortest observed lag time in the single species experiments was *B. graminisolvens* at 5 hours, followed by *C. denitrificans* at 9 hours, while the longest times were 43 and 14 hours and occurred in the *S. plymuthica* and *O. anthropi* experiments (Table 3.10). The *S. plymuthica* dual-cultures showed a shorter lag time in two of the four communities but in all communities a higher maximum respiration rate was observed (Figure 3.11 and Table 3.10). The same trend was observed in the *O. anthropi* dual-cultures where two of the four pairing showed a shorter lag time, but all combinations showed an improvement in the maximum respiration rate.

The *C. denitrificans* communities showed the lowest utilisation of the organic acids, with the *C. indolis* pairing being unable to use four of the eight substrates. The presence of *C. denitrificans* in the community seemed to prevent *S. plymuthica* and *O. anthropi* from being able to utilise oxalic acid while there was an increase in *C. indolis* maximum respiration rate. Despite the *C. denitrificans* pairings being the least able to utilise the substrates, the longest lag times and lowest respiration rates were generally associated with *S. plymuthica* and *C. indolis*. The fact that both species are fermentative in nature could explain the generally slower rates and lag times (Table 3.10)

The least variation between the different dual-cultures was observed in the *B. graminisolvens* pairings, where the difference between the shortest and longest lag times was approximately 10 hours. The same trend was noticed in the maximum respiration rates between the *B. graminisolvens* pairings. The dual-cultures involving *O. anthropi* also often showed an improved lag time and maximum respiration rate.
compared to their single-species counterparts, whilst the presence of a second species generally increased *O. anthropi*’s lag times as well as the maximum respiration rate.

**Table 3.10** – The maximum respiration rates and lag time for different dual-cultures ability to utilise different organic acids. The purple shaded boxes indicate that one of the individual species was able to utilise a substrate, and the red shaded box represents when neither species could utilise a substrate.

<table>
<thead>
<tr>
<th></th>
<th>Citric Acid</th>
<th>Succinic Acid</th>
<th>L-Lactic Acid</th>
<th>Formic Acid</th>
<th>D,L-Malic Acid</th>
<th>Fumaric Acid</th>
<th>Acetoacetic Acid</th>
<th>Oxalic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. plymuthica + O. anthropi</strong></td>
<td>Lag Time (h)</td>
<td>12</td>
<td>25</td>
<td>22</td>
<td>48</td>
<td>24</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.20</td>
<td>1.70</td>
<td>2.20</td>
<td>0.80</td>
<td>2.70</td>
<td>1.10</td>
<td>3.20</td>
</tr>
<tr>
<td><strong>S. plymuthica + C. indolis</strong></td>
<td>Lag Time (h)</td>
<td>19</td>
<td>56</td>
<td>47</td>
<td>42</td>
<td>26</td>
<td>-</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.50</td>
<td>0.80</td>
<td>0.40</td>
<td>0.50</td>
<td>0.80</td>
<td>-</td>
<td>1.30</td>
</tr>
<tr>
<td><strong>S. plymuthica + B. graminisolvens</strong></td>
<td>Lag Time (h)</td>
<td>21</td>
<td>21</td>
<td>9</td>
<td>17</td>
<td>16</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.90</td>
<td>0.80</td>
<td>3.80</td>
<td>2.10</td>
<td>0.80</td>
<td>2.10</td>
<td>3.30</td>
</tr>
<tr>
<td><strong>S. plymuthica + C. denitrificans</strong></td>
<td>Lag Time (h)</td>
<td>32</td>
<td>64</td>
<td>88</td>
<td>-</td>
<td>33</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.20</td>
<td>0.30</td>
<td>1.10</td>
<td>-</td>
<td>4.40</td>
<td>2.30</td>
<td>1.40</td>
</tr>
<tr>
<td><strong>O. anthropi + C. indolis</strong></td>
<td>Lag Time (h)</td>
<td>6</td>
<td>10</td>
<td>9</td>
<td>-</td>
<td>7</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>3.70</td>
<td>5.60</td>
<td>4.70</td>
<td>-</td>
<td>2.20</td>
<td>4.60</td>
<td>4.30</td>
</tr>
<tr>
<td><strong>O. anthropi + B. graminisolvens</strong></td>
<td>Lag Time (h)</td>
<td>7</td>
<td>18</td>
<td>21</td>
<td>19</td>
<td>14</td>
<td>21</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.10</td>
<td>2.20</td>
<td>2.60</td>
<td>4.70</td>
<td>3.30</td>
<td>1.90</td>
<td>3.50</td>
</tr>
<tr>
<td><strong>O. anthropi + C. denitrificans</strong></td>
<td>Lag Time (h)</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>-</td>
<td>6</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>3.20</td>
<td>1.20</td>
<td>5.10</td>
<td>-</td>
<td>4.20</td>
<td>6.80</td>
<td>6.20</td>
</tr>
<tr>
<td><strong>C. indolis + B. graminisolvens</strong></td>
<td>Lag Time (h)</td>
<td>8</td>
<td>19</td>
<td>7</td>
<td>15</td>
<td>19</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.20</td>
<td>0.50</td>
<td>2.20</td>
<td>2.70</td>
<td>3.10</td>
<td>2.20</td>
<td>3.30</td>
</tr>
<tr>
<td><strong>C. indolis + C. denitrificans</strong></td>
<td>Lag Time (h)</td>
<td>11</td>
<td>-</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>4.70</td>
<td>-</td>
<td>2.10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.10</td>
</tr>
<tr>
<td><strong>B. graminisolvens + C. denitrificans</strong></td>
<td>Lag Time (h)</td>
<td>27</td>
<td>15</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>29</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.50</td>
<td>2.10</td>
<td>1.80</td>
<td>2.20</td>
<td>0.80</td>
<td>2.30</td>
<td>1.30</td>
</tr>
</tbody>
</table>
Figure 3.11 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate L-lactic acid. Species with no result shown were found to be unable to respire using the substrate. Side by side comparison can be found in Appendix III.
3.4.3 Sugars

3.4.3.1 Polysaccharides and disaccharides

Every dual-culture was able to utilise the different disaccharides analysed, but when compared to the single species data it can be seen that the lag times are often longer with the dual-cultures (Table 3.11). While C. indolis was unable to utilise sucrose in the single species experiments, it contributed to higher metabolism rates in the different dual-cultures with increases of between 123% and 481% on three of the four co-cultures (Figure 3.12 and Table 3.12). The exception was the C. indolis and B. graminisolvens co-culture which could be explained partly by B. graminisolvens producing the fastest respiration rate in the single species experiments. This suggests that despite not being involved in the hydrolysis of sucrose it was able to metabolise both fructose and glucose and led to the increased respiration rates. Cellobiose generally produced the shortest lag times and fastest maximum respiration rates, suggesting it could be a viable choice of substrate for MFCs due to the potentially faster acclimatisation period. On the whole, the maximum respiration rates measured were faster than those observed in the single species experiments for all of the substrates.

In terms of utilising the different polysaccharides, both the dual- and single-species exhibited similar patterns. S. plymuthica was still the only fermentative species which was unable to utilise glycogen and pectin. Each of the communities involving C. indolis and B. graminisolvens were able to use the polysaccharides for respiration. The lag times for C. indolis were generally shorter than the 44 hours and 63 hours observed in the single species experiments (Table 3.11). The B. graminisolvens dual-cultures generally showed longer lag times than the single species (5 and 0 hours) experiments, but were significantly faster than the C. indolis dual-cultures. In all cases the maximum respiration rate was faster in all the dual-cultures; this could be attributed to the various monosaccharides being generated as the complex polysaccharides are broken down. This would allow the species, which are unable to utilise the polysaccharides, to also respire contributing to the maximum respiration rate.
### Table 3.11 – The maximum respiration rates and lag time for different dual-cultures ability to utilise different disaccharides and polysaccharides. The purple shaded boxes indicate that one of the individual species was able to utilise a substrate, and the red shaded box represents when neither species could utilise a substrate.

<table>
<thead>
<tr>
<th></th>
<th>Maltose</th>
<th>a-D-Lactose</th>
<th>D-Cellobiose</th>
<th>Sucrose</th>
<th>Glycogen</th>
<th>Pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lag Time (h)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. plymuthica + O. anthropi</td>
<td>27</td>
<td>33</td>
<td>6</td>
<td>17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>0.90</td>
<td>0.70</td>
<td>1.90</td>
<td>0.90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. plymuthica + C. indolis</td>
<td>19</td>
<td>23</td>
<td>20</td>
<td>21</td>
<td>45</td>
<td>66</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>1.90</td>
<td>1.60</td>
<td>2.10</td>
<td>1.90</td>
<td>1.50</td>
<td>1.40</td>
</tr>
<tr>
<td>S. plymuthica + B. graminisolvens</td>
<td>9</td>
<td>14</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>1.80</td>
<td>1.00</td>
<td>3.10</td>
<td>2.70</td>
<td>2.10</td>
<td>1.90</td>
</tr>
<tr>
<td>S. plymuthica + C. denitrificans</td>
<td>17</td>
<td>21</td>
<td>0</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>2.10</td>
<td>1.50</td>
<td>1.20</td>
<td>0.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O. anthropi + C. indolis</td>
<td>10</td>
<td>24</td>
<td>8</td>
<td>6</td>
<td>39</td>
<td>35</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>1.40</td>
<td>1.70</td>
<td>3.10</td>
<td>4.30</td>
<td>1.80</td>
<td>1.90</td>
</tr>
<tr>
<td>O. anthropi + B. graminisolvens</td>
<td>17</td>
<td>13</td>
<td>9</td>
<td>3</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>1.40</td>
<td>2.20</td>
<td>2.70</td>
<td>6.10</td>
<td>1.80</td>
<td>2.00</td>
</tr>
<tr>
<td>O. anthropi + C. denitrificans</td>
<td>8</td>
<td>9</td>
<td>7</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>2.20</td>
<td>2.10</td>
<td>3.30</td>
<td>4.40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. indolis + B. graminisolvens</td>
<td>12</td>
<td>8</td>
<td>3</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>1.70</td>
<td>2.10</td>
<td>2.60</td>
<td>2.30</td>
<td>1.30</td>
<td>1.40</td>
</tr>
<tr>
<td>C. indolis + C. denitrificans</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td>29</td>
<td>13</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>2.10</td>
<td>1.40</td>
<td>2.50</td>
<td>2.10</td>
<td>1.50</td>
<td>1.10</td>
</tr>
<tr>
<td>B. graminisolvens + C. denitrificans</td>
<td>14</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Maximum Respiration Rate (h(^{-1}))</td>
<td>1.20</td>
<td>2.20</td>
<td>4.00</td>
<td>2.10</td>
<td>1.60</td>
<td>1.70</td>
</tr>
</tbody>
</table>
Table 3.12 – Comparison of the respiration rates between the single and dual species for the substrate sucrose. The percentage change from the single species to the dual species has also been calculated.

<table>
<thead>
<tr>
<th>Species Combination</th>
<th>Highest Single Species Respiration Rate (h⁻¹)</th>
<th>Dual Species Respiration Rate (h⁻¹)</th>
<th>Percentage Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. plymuthica + O. anthropi</td>
<td>0.74</td>
<td>0.90</td>
<td>21.62</td>
</tr>
<tr>
<td>S. plymuthica + C. indolis</td>
<td>0.40</td>
<td>1.90</td>
<td>375.00</td>
</tr>
<tr>
<td>S. plymuthica + B. graminisolvens</td>
<td>2.26</td>
<td>2.70</td>
<td>19.47</td>
</tr>
<tr>
<td>S. plymuthica + C. denitrificans</td>
<td>0.94</td>
<td>0.30</td>
<td>-68.09</td>
</tr>
<tr>
<td>O. anthropi + C. indolis</td>
<td>0.74</td>
<td>4.30</td>
<td>481.08</td>
</tr>
<tr>
<td>O. anthropi + B. graminisolvens</td>
<td>2.26</td>
<td>6.10</td>
<td>169.91</td>
</tr>
<tr>
<td>O. anthropi + C. denitrificans</td>
<td>0.94</td>
<td>4.40</td>
<td>368.09</td>
</tr>
<tr>
<td>C. indolis + B. graminisolvens</td>
<td>2.26</td>
<td>2.30</td>
<td>1.77</td>
</tr>
<tr>
<td>C. indolis + C. denitrificans</td>
<td>0.94</td>
<td>2.10</td>
<td>123.40</td>
</tr>
<tr>
<td>B. graminisolvens + C. denitrificans</td>
<td>2.26</td>
<td>2.10</td>
<td>-7.08</td>
</tr>
</tbody>
</table>

Despite not being a fermentative species, *C. denitrificans* has demonstrated an ability to utilise disaccharides with short lag times and fast respiration rates which hints at a potential fermentative pathway. When paired with *O. anthropi*, some of the highest respiration rates were observed and it was shown to be very effective at utilising organic acids for respiration. It should also be noted that the *O. anthropi* and *C. denitrificans* co-culture showed a similar metabolic profile to both the *C. indolis* and *O. anthropi* and the *B. graminisolvens* and *O. anthropi* co-cultures both of which feature a known mixed acid fermenter.
Figure 3.12 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate sucrose. Species with no result shown were found to be unable to respire using the substrate. Side by side comparison can be found in Appendix III.
3.4.3.2 Monosacharides

Table 3.13 shows how the different combinations were able to utilise the individual hexose sugars. Each dual-culture was able to utilise all of the hexose sugars, when compared to the single species experiments, longer lag times were observed but in all cases a faster maximum respiration rate was obtained. When compared to the VFA and organic acids the lag times were generally shorter and increased maximum respiration rates were observed.

While the combinations generally showed lag times shorter than 10 hours there was one exception, the *O. anthropi* and *C. denitrificans* dual-culture, which showed long lag times for each substrate with the exception of glucose. While in the *O. anthropi* and *S. plymuthica* dual-culture, four of the five smallest maximum respiration rates were observed. This suggests that *O. anthropi* is unlikely to utilise sugars as a substrate in the mixed culture communities.

The mixed acid fermentative species *C. indolis* and *B. graminisolvens* showed some of the shortest lag times, apart from when paired with *S. plymuthica*, which suggests that there is a potentially antagonistic effect between these species.

As mentioned in Section 3.4.3.1, *C. denitrificans* demonstrated an ability to utilise the different monosaccharides which could potentially demonstrate the ability to act as a mixed acid fermenter.

The most important of the monosaccharides is glucose, which is a constituent in each of the different polysaccharides analysed as well as the carbon source for the MFC experiments. The lag times were generally longer than the other monosaccharides. Four of the five longest lag times for glucose were observed in the *S. plymuthica* pairings, while the shortest lag times showed no pattern. A similar trend was also observed in the respiration rates, again with four of the five lowest respiration rates being attributed to the *S. plymuthica* pairings (Figure 3.13 and Table 3.13).
While no pattern was discerned for the shortest lag times, when looking at the respiration rates it can be seen that 3 of the four highest rates involved a community involving *C. denitrificans*. The *B. graminisolvens* MFCs showed similar respiration rates in all pairings indicating that it is potentially a dominant species in the community.

### Table 3.13 – The maximum respiration rates and lag time for different dual-cultures showing their ability to utilise different monosaccharides. The purple shaded boxes indicate that one of the individual species was able to utilise a substrate.

<table>
<thead>
<tr>
<th>Combination</th>
<th>L-Arabinose</th>
<th>D-Xylose</th>
<th>D-Ribose</th>
<th>D-Arabinose</th>
<th>a-D-Glucose</th>
<th>D-Fructose</th>
<th>D-Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. plymuthica + O. anthropi</strong></td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>12</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>0.80</td>
<td>0.40</td>
<td>0.60</td>
<td>0.90</td>
<td>1.30</td>
<td>2.10</td>
<td>4.10</td>
</tr>
<tr>
<td><strong>S. plymuthica + C. indolis</strong></td>
<td>12</td>
<td>16</td>
<td>16</td>
<td>11</td>
<td>18</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>6.60</td>
<td>4.50</td>
<td>3.10</td>
<td>6.20</td>
<td>2.10</td>
<td>1.80</td>
<td>3.60</td>
</tr>
<tr>
<td><strong>S. plymuthica + B. graminisolvens</strong></td>
<td>7</td>
<td>7</td>
<td>12</td>
<td>15</td>
<td>16</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>4.40</td>
<td>5.20</td>
<td>1.70</td>
<td>2.20</td>
<td>2.30</td>
<td>2.50</td>
<td>2.10</td>
</tr>
<tr>
<td><strong>S. plymuthica + C. denitrificans</strong></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>7.10</td>
<td>5.50</td>
<td>4.40</td>
<td>2.70</td>
<td>2.20</td>
<td>-</td>
<td>6.60</td>
</tr>
<tr>
<td><strong>O. anthropi + C. indolis</strong></td>
<td>7</td>
<td>11</td>
<td>23</td>
<td>7</td>
<td>7</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>7.30</td>
<td>7.30</td>
<td>4.40</td>
<td>7.10</td>
<td>4.30</td>
<td>2.10</td>
<td>4.40</td>
</tr>
<tr>
<td><strong>O. anthropi + B. graminisolvens</strong></td>
<td>4</td>
<td>8</td>
<td>11</td>
<td>9</td>
<td>9</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>4.20</td>
<td>3.10</td>
<td>3.20</td>
<td>4.80</td>
<td>2.40</td>
<td>2.10</td>
<td>2.40</td>
</tr>
<tr>
<td><strong>O. anthropi + C. denitrificans</strong></td>
<td>14</td>
<td>12</td>
<td>22</td>
<td>10</td>
<td>4</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>7.10</td>
<td>8.40</td>
<td>0.50</td>
<td>2.20</td>
<td>3.80</td>
<td>2.30</td>
<td>2.10</td>
</tr>
<tr>
<td><strong>C. indolis + B. graminisolvens</strong></td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>6.70</td>
<td>6.90</td>
<td>4.40</td>
<td>7.20</td>
<td>2.50</td>
<td>3.30</td>
<td>4.40</td>
</tr>
<tr>
<td><strong>C. indolis + C. denitrificans</strong></td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>6.00</td>
<td>5.10</td>
<td>3.30</td>
<td>6.20</td>
<td>2.00</td>
<td>5.40</td>
<td>4.20</td>
</tr>
<tr>
<td><strong>B. graminisolvens + C. denitrificans</strong></td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Lag Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Respiration Rate (h⁻¹)</td>
<td>1.60</td>
<td>0.50</td>
<td>1.20</td>
<td>1.70</td>
<td>2.70</td>
<td>2.30</td>
<td>1.90</td>
</tr>
</tbody>
</table>
Figure 3.13 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate a-D-glucose. Species with no result shown were found to be unable to respire using the substrate. Side by side comparison can be found in Appendix III.
3.5 Cell Suspension Analysis

3.5.1 Carbohydrate Utilisation and COD Removal

One of the main attractions of MFCs is their ability to treat wastewater as power is produced. The chemical oxygen demand (COD) of a solution is used to measure the level of organic pollutants present in a sample. The ability of an MFC to reduce the COD of a system is used to determine how well the microorganisms can use the substrates present. At peak power production, the single species MFCs showed a similar ability to reduce the COD of the feed used for the MFC (Table 3.14). The major exception was the *O. anthropi* MFC which was only able to remove 42% of the COD. This suggests that *O. anthropi* is not as able to effectively utilise the glucose as the other species being studied. This could be mitigated by an electron acceptor in the suspension which acts as a mediator allowing non-biofilm bacteria to contribute to power generation.

Table 3.14 - The COD levels found in the influent and effluent of individual species MFCs. The errors shown in the table are derived from the standard deviation of 3 repeats.

<table>
<thead>
<tr>
<th>Species</th>
<th>MFC</th>
<th>Influent (mg L⁻¹)</th>
<th>Effluent (mg L⁻¹)</th>
<th>% Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. denitrificans</em></td>
<td>1</td>
<td>3184 ± 43</td>
<td>929 ± 5</td>
<td>70.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>938 ± 23</td>
<td>938 ± 23</td>
<td>70.5</td>
</tr>
<tr>
<td><em>S. plymuthica</em></td>
<td>1</td>
<td>3312 ± 35</td>
<td>841 ± 16</td>
<td>74.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>854 ± 31</td>
<td>854 ± 31</td>
<td>74.6</td>
</tr>
<tr>
<td><em>O. anthropi</em></td>
<td>1</td>
<td>3112 ± 50</td>
<td>1804 ± 22</td>
<td>42.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1776 ± 92</td>
<td>1776 ± 92</td>
<td>42.9</td>
</tr>
<tr>
<td><em>B. graminisolvens</em></td>
<td>1</td>
<td>3007 ± 24</td>
<td>769 ± 28</td>
<td>74.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>732 ± 28</td>
<td>732 ± 28</td>
<td>75.7</td>
</tr>
<tr>
<td><em>C. indolis</em></td>
<td>1</td>
<td>3390 ± 46</td>
<td>806 ± 10</td>
<td>76.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>870 ± 15</td>
<td>870 ± 15</td>
<td>74.4</td>
</tr>
</tbody>
</table>

The dual-species MFCs showed an improved ability to reduce the COD level of the medium when compared to the single species MFCs (Table 3.15). There are multiple
factors which could contribute to the improved COD removal efficiencies. It is possible that the presence of another species is causing the metabolic focus of the community to shift, so that the individual species fulfil a niche. An example of this is the *C. indolis* and *O. anthropi* pairing where it is possible that *C. indolis* focuses more on fermenting the glucose present while the *O. anthropi* utilises the butyrate generated through cell respiration. The lowest COD removal occurred in the *C. denitrificans* and *O. anthropi* dual-species MFC, this is the only pairing of species where a fermentative species is not present. It is possible that *C. denitrificans* has fermentative pathways which it is able to utilise to a lower degree.

The pairing of *S. plymuthica* and *O. anthropi* also showed a COD removal efficiency of below 80% (Table 3.15). Despite being a fermentative species, *S. plymuthica* is not a mixed acid fermenter and generates acetoin and 2,3-butanediol instead of short chain organic acids. It is possible that *O. anthropi* is unable to make use of these products thus reducing the overall efficiency of the MFC.

The *B. graminisolvens* and *C. indolis* paring showed the highest level of COD removal which is most likely attributed to the fact that both are fermentative species which are able to utilise a wide range of substrates.
The 5-species community showed the highest level of COD removal out of all the experiments, indicating that as biodiversity increases; the ability to reduce COD also improves (Table 3.16). This hints at different species fulfilling different niches in the

Table 3.15 The COD levels found in the influent and effluent of the different dual-species MFCs. The errors shown in the table are derived from the standard deviation of 3 repeats.

<table>
<thead>
<tr>
<th>Species 1</th>
<th>Species 2</th>
<th>MFC</th>
<th>Influent (mg L⁻¹)</th>
<th>Effluent (mg L⁻¹)</th>
<th>% Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. denitrificans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. plymuthica</td>
<td>1</td>
<td>3210 ± 35</td>
<td>646 ± 23</td>
<td>79.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3214 ± 26</td>
<td>645 ± 26</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3100 ± 11</td>
<td>864 ± 54</td>
<td>72.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3132 ± 45</td>
<td>888 ± 84</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td>O. anthropi</td>
<td>1</td>
<td>3341 ± 21</td>
<td>545 ± 54</td>
<td>83.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3339 ± 32</td>
<td>513 ± 32</td>
<td>84.6</td>
<td></td>
</tr>
<tr>
<td>B. graminisolvens</td>
<td>1</td>
<td>3119 ± 32</td>
<td>426 ± 22</td>
<td>86.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3119 ± 32</td>
<td>527 ± 31</td>
<td>83.1</td>
<td></td>
</tr>
<tr>
<td>C. indolis</td>
<td>1</td>
<td>347 ± 12</td>
<td>64 ± 12</td>
<td>88.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>331 ± 24</td>
<td>74 ± 24</td>
<td>89.1</td>
<td></td>
</tr>
<tr>
<td>O. anthropi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. graminisolvens</td>
<td>1</td>
<td>3054 ± 22</td>
<td>413 ± 13</td>
<td>86.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3054 ± 22</td>
<td>471 ± 47</td>
<td>85.5</td>
<td></td>
</tr>
<tr>
<td>C. indolis</td>
<td>1</td>
<td>2998 ± 11</td>
<td>496 ± 64</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2998 ± 11</td>
<td>427 ± 61</td>
<td>85.5</td>
<td></td>
</tr>
<tr>
<td>S. plymuthica</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. anthropi</td>
<td>1</td>
<td>3121 ± 21</td>
<td>764 ± 12</td>
<td>75.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3121 ± 21</td>
<td>714 ± 23</td>
<td>77.1</td>
<td></td>
</tr>
<tr>
<td>B. graminisolvens</td>
<td>1</td>
<td>3205 ± 45</td>
<td>512 ± 12</td>
<td>84.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3205 ± 45</td>
<td>535 ± 45</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td>C. indolis</td>
<td>1</td>
<td>3021 ± 12</td>
<td>480 ± 12</td>
<td>84.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3021 ± 12</td>
<td>444 ± 67</td>
<td>85.3</td>
<td></td>
</tr>
<tr>
<td>B. graminisolvens</td>
<td>C. indolis</td>
<td>1</td>
<td>3056 ± 53</td>
<td>347 ± 12</td>
<td>88.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3056 ± 53</td>
<td>331 ± 24</td>
<td>89.1</td>
<td></td>
</tr>
</tbody>
</table>
community and therefore allowing the community to take advantage of the various different metabolites available.

It would be expected that the higher the COD removal should correlate to a higher power being produced since the community is able to utilise a larger percentage of the available carbon sources present. It is possible this will not always be the case though, in the instance of the species utilising specific substrates in non-electrogenic respiration or fermentation processes. It is also possible that potentially electrogenic reactions taking place in the suspension are using alternative electron acceptors to the electrode for respiration, an example of this is oxygen which has diffused through the membrane.

**Table 3.16** The COD levels found in the influent and effluent of the 5-species MFCs. The errors shown in the table are derived from the standard deviation of 3 repeats.

<table>
<thead>
<tr>
<th>MFC</th>
<th>Influent (mg L(^{-1}))</th>
<th>Effluent (mg L(^{-1}))</th>
<th>% Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3320 ± 70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>270 ± 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>234 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>91.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>92.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.6 Proposed Metabolic Interactions and Conclusions

In almost all cases the lag times are generally lower in the dual-cultures, which could be attributed to the lower number cells for each individual bacterial species used for inoculation, despite the overall number of cells being equal. In some cases, this could also potentially explain the increased respiration rates observed for the majority of the species, since if one species is unable to utilise a substrate then that substrate is available for the other species. In the cases of the sugars, the higher respiration rates are likely due to metabolites being used by the individual species. When both species are able to utilise a substrate, the resulting competition between individual species is likely to increase the respiration rate. It is also possible that a higher respiration rate will be observed due to the sum of both of the maximum respiration rates. Other possible factors that could increase the overall rate include synergistic and antagonistic relationships between the species present. This can only occur when respiration has occurred due to the nature of the Biolog where the dye is the only terminal electron acceptor present. One form of antagonistic relationship is through secondary inhibitory metabolites which reduce a species’ ability to utilise a substrate, in turn reducing the respiration rate [30]. Conversely, an increased respiration rate can occur from species being able to utilise a secondary metabolite as a food source. An example of synergistic relationships that was observed was pairing any species with \( C. \text{indolis} \) for sucrose metabolism where despite \( C. \text{indolis} \) not being able to utilise it in the single species experiments, it enhanced the rate of three of the four co-cultures.

A constant theme throughout the different Biolog experiments suggests that \( B. \text{graminisolvens} \) is the species most likely to thrive in a microbial community, due to the wide variety of substrates which it is able to utilise. Another piece of evidence pointing to \( B. \text{graminisolvens} \) dominance is that it generally showed the lowest levels of variance between the maximum respiration rates and lag times observed in the dual-culture experiments.

\( O. \text{anthropi} \) dual-cultures were the only combinations able to utilise propionic acid, which would suggest an increased power observed in MFCs which contain \( O. \text{anthropi} \). It was also observed that \( O. \text{anthropi} \)-containing pairs generally demonstrated an increased ability to utilise acetic acid, again hinting at the potential increase in MFC power observable on addition of \( O. \text{anthropi} \) to a community.
The addition of *B. graminisolvans* and *C. indolis* allowed the microbial communities to utilise glycogen and pectin, which would be beneficial in the creation of a synthetic community to treat wastewaters, known to contain complex organic compounds which are otherwise difficult to break down.
3.7 Bibliography


[20] Adaptation of soil microbes during establishment of microbial fuel cell consortium fed with lactate, H. Futamata, O. Bretschger, A. Cheung, J. Kan, R. Owen,


[29] Identification of a methicillin-resistant Staphylococcus aureus inhibitory compound isolated from Serratia marcescens, D. Kadouri, R. Shanks, 2013, Research
in Microbiology, Vol. 164, pp. 821-826.

Chapter 4 - Electrochemical Studies into Microbial Fuel Cells

4.1 Chapter Overview

The work described in the first section of this chapter (sections 4.2 to 4.6) was conducted using a single-chamber, air-breathing cathode microbial fuel cell (MFC). The MFCs used have a relatively large volume (140 ml) to allow straightforward sampling of the suspension and anode with minimal disruption to the microbes developing in the suspension and the biofilm. An air-breathing cathode was selected since it requires no aeration or the addition of an external electron acceptor, while also eliminating substrate cross-over; since the bacteria selected are facultative anaerobes, the oxygen diffusion through the membrane should not lead to the build-up of toxic levels of oxygen.

The synthetic community being modelled was based upon five of the most abundant species identified in a naturally occurring community found in a MFC inoculated with anaerobic digester sludge and using sucrose as a food source [1]. To determine the exoelectrogenic capability of the individual species, MFCs inoculated with a single species were operated as a batch flow reactor for the first 2 days of operation before being switched to a continuous flow system, fed with synthetic wastewater loaded with glucose. The MFCs were operated until the power density stabilised (with a 3 day moving average applied to the data), indicating that the maximum power generation in this set up. After the power had stabilised, a polarization curve was run to determine the maximum power at different resistances.

The same set up was used to run MFCs inoculated with each available combination of two species, as well as a synthetic community made up of all 5 species. These data were used to determine how the exoelectrogenic properties of the bacterial species differed when other species were present.

To determine if any redox active compounds (which could be used by the bacteria as a mediator for electron transfer to the anode, discussed in chapter 1) were generated during MFC operation, samples of the suspension were analysed using cyclic voltammetry (CV).
4.2 Power Generation in Single Species MFCs

Due to the nature of the electrode (wrapped around a plastic rod submerged in the chamber), it is possible that only the more external parts of the electrode were utilised, as microorganisms would be impeded reaching the internal layers. All power densities quoted make the assumption that the full anodic surface was colonised, so the power density expressed is considered to be the minimum possible in this particular MFC set up. It should also be noted that the duplicate MFC results presented in this chapter show very good repeatability for this type of experiment, allowing more meaningful conclusions to be drawn.

To determine when the MFCs had reached a steady state, a 3 point moving average was applied to the data. It was assumed that a steady state had been reached when 3 consecutive averaged data points had a variation of less than 1% of the maximum power output of the MFC (At 40 000 ohms as described in Section 2.4.1). Polarisation curves were used to determine the maximum power generated by the MFC and were generally carried out when both replicates had stabilised.

4.2.1 Power Generation by *Comamonas denitrificans*

*Comamonas denitrificans* (*C. denitrificans*) is a gram-negative species which has been reported in a number of microbial communities in MFCs inoculated with activated sludge. Power output was observed from day 1 in both MFCs inoculated with *C. denitrificans* when compared to the blank control (Blank 1, 23 mV; Blank 2, 27 mV; *C. denitrificans* MFC1, 43 mV; *C. denitrificans* MFC2, 88 mV). Figure 4.1 shows the power generation growing at a steady rate (the power decreases at day 8 and 14 due to a technical failure in the pumping system). In both MFCs the power output stabilised on day 23 at 0.64 and 0.79 mW m⁻² for MFCs 1 and 2 respectively.
**Figure 4.1** – The power produced over time by the *C. denitrificans* single species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.

**Figure 4.2** – The polarisation curve produced by the *C. denitrificans* single species MFCs under standard laboratory conditions.
The maximum power outputs (shown in Figure 4.2) were 2.24 mW m\(^{-2}\) at 53 µA for MFC1 and 2.50 mW m\(^{-2}\) at 69 µA for MFC2. Previous work by Xing et al. [2] conducted experiments using *C. denitrificans* in single chamber MFCs where they did not observe any power generation. The lack of power generation was attributed to oxygen diffusion through the membrane, diverting electrons to aerobic respiration [2]. The contradicting data could potentially be ascribed to the larger anodic chamber volume (144 cm\(^3\) compared to approximately 64 cm\(^3\)) being used. Bacteria in the suspension may be able to completely utilise the oxygen that diffuses through the membrane, allowing the bacteria on the biofilm to carry out anaerobic respiration. A thick biofilm (not shown) was also observed on the anodic surface of the membrane and could potentially help keep the chamber anaerobic by consuming the dissolved oxygen which diffuses in, therefore reducing the overall oxygen concentration present in the anodic chamber.

### 4.2.2 Power Generation by *Serratia plymuthica*

*Serratia plymuthica* (*S. plymuthica*) is a gram-negative species which was found in the naturally occurring community this work is based on [1] but no work has been carried out on the exoelectrogenic nature of the species. However, in a study into the microbial corrosion of an aluminium alloy, an increased current was observed when *S. plymuthica* was present [3], implying that the species will be capable of exoelectrogenic activity in MFCs. In the MFCs tested here, power generation started slowly with only a slight variation in voltage compared to the blank MFCs until day 4. Both MFCs reached their maximum power output on day 14 at around 0.59 mW m\(^{-2}\) in MFC1 and 0.64 mW m\(^{-2}\) in MFC2 (Figure 4.3). It was also noted that a biofilm developed on the anodic chamber surface of the membrane. Due to the gas permeable nature of Nafion, it is likely that aerobic respiration was occurring on the membrane surface thus, helping maintain an anaerobic environment in the anodic chamber (as noted in Section 4.2.1).
Figure 4.4 shows the polarization curves for both *S. plymuthica* MFCs with the maximum power densities being 1.39 mW m$^{-2}$ and 1.29 mW m$^{-2}$ for MFC1 and MFC2 respectively, both observed at 42 and 40 µA respectively.

Figure 4.3 – The power produced over time by the *S. plymuthica* single species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.

Figure 4.4 – The polarisation curve produced by the *S. plymuthica* single species MFCs under standard laboratory conditions.
4.2.3 Power Generation by *Ochrobactrum anthropi*

*Ochrobactrum anthropi* (*O. anthropi*) is a gram-negative bacterium which has been proven to be an exoelectrogenic species but no detailed work has been carried out on the species [4]. It has been observed in a high abundance in the community which this work is based on but it has not been reported in other communities [3][5].

Figure 4.5 shows that the species reached maximum power output on day 6 in both MFCs, generating a power density of 0.89 mW m\(^{-2}\) and 0.92 mW m\(^{-2}\) for MFC1 and MFC2 respectively. In both MFCs, a biofilm was observed on the membrane surface as noted in section 4.2.1 and 4.2.2.

![Power Density over Time](image)

**Figure 4.5** – The power produced over time by the *O. anthropi* single species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.

Figure 4.6 shows the polarisation curves for both MFCs. Maximum power density was observed at 10 µA with MFC1 and MFC2 peaking at 0.30 mW m\(^{-2}\) and 0.33 mW m\(^{-2}\) respectively.
4.2.4 Power Generation by \textit{Bacteroides graminisolvens}

\textit{Bacteroides graminisolvens} (\textit{B. graminisolvens}) is a strictly anaerobic, gram-negative species [6], and no electrogenic potential has been reported to date. The peak power output was observed after 7 days at 1.14 mW m$^{-2}$ in MFC1 while MFC2 peaked at 1.21 mW m$^{-2}$ after 9 days (Figure 4.7). No biofilm was observed on the surface of the membrane indicating that no aerobic respiration was occurring. This is due to \textit{B. graminisolvens} being unable to use oxygen for respiration, so it cannot colonise the membrane surface due to oxygen diffusing through the Nafion membrane.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{polarisation_curve.png}
\caption{The polarisation curve produced by the \textit{O. anthropi} single species MFCs under standard laboratory conditions.}
\end{figure}
The polarisation curve showed that both MFCs produced their maximum power at approximately 60 µA with MFC1 reaching 2.74 mW m\(^{-2}\) and MFC2 generating 3.09 mW m\(^{-2}\) (Figure 4.8).

**Figure 4.7** – The power produced over time by the *B. graminisolvens* single species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.

**Figure 4.8** – The polarisation curve produced by the *B. graminisolvens* single species MFCs under standard laboratory conditions.
4.2.5 Power Generation by *Clostridium indolis*

*Clostridium* species are gram-positive bacteria commonly found in a variety of microbial communities used in MFCs [7][8]. So far, only *C. butyricum* and *C. aminobutyricum* have been demonstrated to be electrogenic species [5][9][10], but work on *Clostridium indolis* (*C. indolis*) (the species used in this work) is very limited. Both MFCs showed a rapid increase in power with both cells reaching a short plateau after 7 days followed by a slow and relatively steady increase until the maximum power was reached. The difference between the two MFCs (approximately 0.30 mW m$^{-2}$) was unable to be explained, possible explanations which were dismissed after being investigated were temperature, influent flow rate and rate of agitation from the magnetic stirrer. Despite the relatively large difference in power output after the first plateau both MFCs stabilised at approximately 0.74 mW m$^{-2}$ (Figure 4.9). No biofilm was observed on the surface of the membrane indicating that *C. indolis* is unable to respire using the oxygen which diffuses through the membrane like other species in the community (as discussed in section 4.2.4). The polarisation curves were carried out on different days due to the extended period of time MFC1 took to stabilise. In all other cases, the polarisation curves were conducted approximately 3-4 days after stabilisation, and at the time it was unknown how long MFC1 would require to stabilise. This would have introduced further variables into the analysis of the results since it was not determined how long a stable power output could be achieved before factors like biofouling caused a decrease in the output.

![Figure 4.9](image_url)

*Figure 4.9 –* The power produced over time by the *C. indolis* single species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.
Despite reaching almost identical power outputs after stabilisation, MFC1 reached a higher maximum power output, as shown by the polarisation curve (Figure 4.10). The maximum power generated from MFC1 was 1.55 mW m$^{-2}$ while MFC2 reached 1.29 mW m$^{-2}$, both at approximately 30 µA.

**Figure 4.10** – The polarisation curve produced by the *C. indolis* single species MFCs under standard laboratory conditions.

4.2.6 **Comparison of the Electrogenic Activity of the Individual Species**

From the data shown in Table 4.1, it can be observed that the time taken to reach peak power outputs differs for each species, but there is no clear trend associated with the duration of the lag time period. Considering the main metabolic features of the species, the fermentative species, *B. graminisolvens*, *C. indolis*, and *S. plymuthica*, produced a variety of times to reach max power at 7, 18 and 13 days respectively, while for the non-fermentative species, *C. denitrificans* and *O. anthropi*, maximum power was reached at 23 and 6 days respectively. Similarly, there is also no link between the nature of respiration and the time required to reach maximum output - the strict anaerobes, *B. graminisolvens* and *C. indolis*, required 7 and 18 days whereas the facultative
anaerobes, *C. denitrificans*, *S. plymuthica* and *O. anthropi*, required 23, 13 and 6 days. When compared to the metabolic data obtained in Chapter 3 for the metabolism of glucose, both *C. denitrificans* and *B. graminisolsven* showed lag times of 4 hours before respiration became detectable using glucose as a substrate. *B. graminisolsven* showed one of the shortest lag times, and *C. denitrificans* demonstrated the longest. *O. anthropi* and *C. indolis* both had similar lag periods of 12 and 10 hours respectively, while it took 6 and 18 days to reach maximum power output. *S. plymuthica* had the longest lag time of 31 hours but the time it required to reach maximum power output was 13 hours. The same trend followed respiration rates using glucose with *B. graminisolsven* and *C. denitrificans* having the highest rates, followed by *C. indolis* and *O. anthropi* and finally *S. plymuthica* with the lowest respiration rate. All these data suggest that there is no primary governing factor associated to the time required for the species to reach the maximum power output.

![Table 4.1 – The lag times and maximum power densities obtained from the individual single species MFCs.](image)

<table>
<thead>
<tr>
<th>Species</th>
<th>MFC 1</th>
<th>MFC 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Power Density (mW m(^{-2}))</td>
</tr>
<tr>
<td><em>Comamonas denitrificans</em></td>
<td>23</td>
<td>0.64</td>
</tr>
<tr>
<td><em>Serratia plymuthica</em></td>
<td>13</td>
<td>0.58</td>
</tr>
<tr>
<td><em>Ochrobactrum anthropi</em></td>
<td>6</td>
<td>0.73</td>
</tr>
<tr>
<td><em>Bacteroides graminisolsven</em></td>
<td>7</td>
<td>1.14</td>
</tr>
<tr>
<td><em>Clostridium indolis</em></td>
<td>18</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Figure 4.11 shows the averages of the 2 duplicate experiments for easier comparison. It can be seen that the lowest maximum power generated was by *O. anthropi* whose maximum power output was 0.31 mW m\(^{-2}\) at 9 µA. The duplicate experiments for both *C. indolis* and *S. plymuthica* showed high levels of repeatability in terms of power outputs and polarisation curves. However, the duplicate experiments for *C. denitrificans* and *B. graminisolsven* were less reproducible but both of these species produced significantly higher powers than the other 3 species. Since all species were capable of power generation, it can be concluded that all species are capable of anaerobic respiration where the electrode or a compound in the suspension acts as a terminal electron acceptor,
unexpectedly including the known fermentative species *S. plymuthica*, *B. graminisolvens* and *C. indolis*.

**Figure 4.11** – The polarisation curves produced by the individual single species MFCs plotted using the averages from the two experiments carried out under standard laboratory conditions.

### 4.3 Power Generation in Mixed Community MFCs

To determine any potential interaction between the individual species in a microbial community, MFCs were run using each possible combination to determine if any synergistic or antagonistic interactions were present. Upon completion of the polarisation curves, samples of the anode and anodic suspension were taken for DNA extraction and DGGE analysis. The results (not shown) showed no contamination of bacteria from external sources and in all MFCs; the species inoculated were recovered from the suspension and the anodic biofilm confirming the identity of the consortia tested. While the DGGE experiments showed that all 5 species were present in the mixed species MFCs
the technique is not quantitative so determination of the relative abundances was not possible.

### 4.3.1 Dual-species MFCs Involving *Comamonas denitrificans*

Figure 4.12 shows that all dual-species consortia, where *C. denitrificans* was present, followed a similar trend when compared to the results obtained from the single species consortia (Section 4.2.6). The dual-species community involving *C. denitrificans* and *O. anthropi* produced the lowest overall power output, while the community with *C. denitrificans* and *B. graminisolvans* produced the overall highest power. It took a similar amount of time in all MFCs for the power output to stabilise (between 22 and 26 days). The power output was not particularly stable throughout operation with numerous peaks and troughs which could indicate competition between the species.

![Figure 4.12](image-url)

**Figure 4.12** – The power produced over time by the different *C. denitrificans* dual-species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.
None of the dual-species *C. denitrificans* community MFCs generated a higher power than the *C. denitrificans* single species MFC. Figure 4.13 shows that, overall, the highest power output came from the *C. denitrificans* and *B. graminisolvens* but the results were less repeatable than the single species MFCs. Overlaps were observed between the polarization curves for three of the four co-species communities, with the exception being the community involving *O. anthropi* which produced a significantly lower maximum power than the other species pairs, with MFC1 producing a power of 0.51 mW m\(^{-2}\) and MFC2 producing a power of 0.56 mW m\(^{-2}\) at 15 µA. The dual-culture with *O. anthropi* was the only combination measured which exceeded that of the *O. anthropi* single species MFCs. This is more likely due to *C. denitrificans* being a more active electrogenic species as opposed to any form of synergistic relationship between the species. This could indicate that the species fulfil a similar role within the community, as both *C. denitrificans* and *O. anthropi* are non-fermentative species; both species are facultative anaerobes capable of respiratory metabolism.

![Figure 4.13](image)

*Figure 4.13 – The polarisation curves produced by the by the different *C. denitrificans* dual-species MFCs under standard laboratory conditions.*
As previously mentioned, the other pairs showed less reproducible results, with the biggest variation occurring in the *B. graminisolvens* pair with MFC1 producing 1.17 mW m\(^{-2}\) at 22 µA and MFC2 producing a power of 1.50 mW m\(^{-2}\) at 25 µA. As it can be seen in Figure 4.13, *C. indolis* MFC2 produced a higher maximum power output of 1.18 mW m\(^{-2}\) at 30 µA, which was slightly higher than the maximum output of *B. graminisolvens* MFC1. On the other hand, *C. indolis* MFC1 produced power densities lower than the combinations involving *B. graminisolvens* and *S. plymuthica* and produced a maximum power of 0.84 mW m\(^{-2}\) at 23 µA. The *S. plymuthica* MFCs showed more reproducible results, with MFC1 reaching a maximum power output of 1.00 mW m\(^{-2}\) and MFC2 reaching a peak power of 1.11 mW m\(^{-2}\), both achieved at 27 µA. Despite producing lower powers than *B. graminisolvens* MFCs plus *C. indolis* MFC2, both *S. plymuthica* MFCs reached the highest maximum currents obtained in all *C. denitrificans* dual-culture MFCs.

*B. graminisolvens*, *C. indolis* and *S. plymuthica* are all fermentative species which could explain the similar power production between the communities involving these species and *C. denitrificans*. It is possible that the fermentative species are converting glucose into short chain metabolites which *C. denitrificans* is able to utilise for power generation. It should be noted that all the species being studied in this work have been shown to be capable of anaerobic respiration when in single-species MFCs. In all MFCs, a biofilm was observed on the anodic surface of the membrane. This is most likely due to each pairing containing a facultative anaerobe which is able to utilise the oxygen permeating through the membrane for respiration.

### 4.3.2 Dual-Species MFCs Involving *Serratia plymuthica*

Figure 4.14 shows how power generation increased over time in dual-species MFCs involving *S. plymuthica*. It can be seen that the dual-communities involving *C. denitrificans* and *C. indolis* produced significantly higher powers than those involving *B. graminisolvens* and *O. anthropi*. The first MFCs to reach a stabilised power output were the MFCs inoculated with *S. plymuthica* and *B. graminisolvens*, which required 16 and 13 days in the case of MFC1 and MFC2. On day 6, the *B. graminisolvens* MFC peaked at a maximum power density of 0.11 mW m\(^{-2}\) but the power output quickly dropped before stabilising at a maximum power density of 0.07 mW m\(^{-2}\), while
B. graminisolvens MFC1 peaked on day 6 and day 11 at 0.76 mW m\(^{-2}\) and 0.65 mW m\(^{-2}\) respectively (in both cases the power output quickly decreased before stabilising at 0.65 mW m\(^{-2}\) on day 16).

The other MFCs had similar times required for power stabilisation with the C. denitrificans MFCs requiring 29 and 23 days for MFC1 and MFC2 respectively, which was the largest variation between stabilisation times. The C. indolis MFCs required 25 and 20 days for MFC1 and MFC2. In both cases, there was a relatively large variation (approximately 0.12 mW m\(^{-2}\)) between the two MFCs after the power stabilised. Despite producing an overall lower power than C. indolis MFC1, it should be noted that C. indolis MFC2 was generating higher power between day 10 and day 22 (apart from day 16 where the power output dropped to below that of C. indolis MFC1). The O. anthropi MFCs took 27 and 31 days for the power outputs of MFC 1 and MFC2 to stabilise. Between day 6 and day 27, MFC2 produced a higher power but stabilised at the same overall power at 0.06 mW m\(^{-2}\) which was the lowest overall power generated during the continuous flow experiment.

![Figure 4.14](image-url) – The power produced over time by the different S. plymuthica dual-species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.
Figure 4.15 shows the polarization curves generated for each species combined with *S. plymuthica*. There is a very clear divide where the pair involving *S. plymuthica* combined with *C. indolis* and *C. denitrificans* produced substantially higher power densities than that of the *S. plymuthica* and *B. graminisolvens* and *S. plymuthica* and *O. anthropi* MFCs (for clarity Figure 4.16 shows Figure 4.15 with *C. denitrificans* and *C. indolis* removed).

The highest power produced was the combination involving *C. denitrificans*, where a maximum power output of 1.11 mW m\(^{-2}\) was achieved in MFC2 while the power density of MFC1 was 1.00 mW m\(^{-2}\) at 27 µA. The *C. indolis* MFCs produced similar polarisation curves, with MFC1 and MFC2 generating a power densities of 0.89 mW m\(^{-2}\) at 17 µA and 0.87 mW m\(^{-2}\) at 19 µA respectively.

When comparing the data in Figure 4.14 and Figure 4.15, it is seen that, despite producing a higher power during the continuous flow experiments, when a polarisation curve was carried out the power produced by the *B. graminisolvens* dual-community was lower than that of the *O. anthropi* dual-community. In the *B. graminisolvens* MFCs the maximum power output was 0.12 mW m\(^{-2}\) at currents of 4.7 µA for both MFCs. The *O. anthropi* MFCs followed a similar trend with *O. anthropi* MFC2 produced a maximum power density 0.15 mW m\(^{-2}\) at 5.4 µA while *O. anthropi* MFC2 generated 0.17 mW m\(^{-2}\) at both 5.7 µA.

**Figure 4.15** – The polarisation curves produced by the by the different *S. plymuthica* dual-species MFCs under standard laboratory conditions.
The results in the *S. plymuthica* dual-communities differ quite drastically from the single species MFCs. All of the powers generated were lower than the maximum power obtained from the *S. plymuthica* single species MFC (1.29 and 1.39 mW m$^{-2}$). The major difference is the dual-community where *B. graminisolvens* produced the lowest overall power while in the single species MFCs, it generated the highest powers observed. Both species are fermentative but generate different metabolites through fermentation: *B. graminisolvens* is a mixed-acid fermenter while *S. plymuthica* produces butane-2,3-diol and acetoin [11][12]. The lower power suggests that there is competition occurring between the species with the glucose being converted into organic substrates which cannot be utilised by its competitor hindering the electrogenic potential of both species.

Metabolically, *C. indolis* MFCs produced a lower power than the combination with *C. denitrificans* which corresponds with the data generated from the single species MFCs, where *C. denitrificans* produced higher powers than *C. indolis*.

Metabolically, *C. indolis* and *S. plymuthica* share very little in common with one another. *S. plymuthica* is able to carry out aerobic respiration, which hints at a potential synergy.
between the species as *S. plymuthica* is able to use any oxygen which diffuses into the MFC. Both species are also fermentative but, as with *B. graminisolvens*, they produce different metabolites using differing pathways. *C. indolis* has a similar metabolism to *B. graminisolvens*, where both species are mixed acid fermentors while *S. plymuthica* produces acetotin and butane-2,3-diol. The MFCs paired with *B. graminisolvens* produced extremely low powers with *S. plymuthica*, while *C. indolis* was able to produce a relatively high power compared with the other *S. plymuthica* pairs; this indicates that potentially both *S. plymuthica* and *C. indolis* are able to utilise the metabolites produced by the each other to produce power.

A biofilm was observed on the anodic surface of the membrane of all MFCs which suggests aerobic respiration. In the *B. graminisolvens* and *C. indolis* MFC the biofilm is probably made up entirely of *S. plymuthica* since, in the corresponding single species MFCs, no biofilm was observed. In the other pairs of species, the biofilm was likely a combination of species due to the facultative nature of *S. plymuthica, C. denitrificans* and *O. anthropi*.

### 4.3.3 Dual-Species MFCs Involving *Ochrobactrum anthropi*

The single species MFC inoculated with *O. anthropi* reached the maximum power generation in the shortest time (at 6 days, which is shorter than each of the pairs shown in Figure 4.17 and Figure 4.18). The shortest time required was 12 days for *B. graminisolvens* MFC2, but the replicate *B. graminisolvens* MFC1 required 24 days, which is comparatively much longer than the other MFCs. Despite the variation between the stabilisation periods, both MFCs peaked on reaching similar overall power densities (0.11 and 0.10 mW m$^{-2}$ in the case of *B. graminisolvens* MFC1 and MFC2 respectively). On day 10, *B. graminisolvens* MFC1 peaked at 0.14 mW m$^{-2}$, which was higher than both *B. graminisolvens* MFCs.

The *C. indolis* MFCs were fairly reproducible with similar times required to reach maximum power while also stabilising at similar powers (*C. indolis* MFC1, 23 days, 0.8 mW m$^{-2}$; *C. indolis* MFC2 18 days, 0.84 mW m$^{-2}$). *C. indolis* MFC1 was a lot less stable than MFC2 throughout operation, with multiple peaks and troughs, but overall none of the peaks produced a power output higher than the stable output.
The *C. denitrificans* MFCs required 27 days and 25 days for the power to stabilise in MFC1 and MFC2 respectively. Both MFCs had multiple peaks and troughs throughout operation with MFC2 being more stable overall and producing a higher power overall, but it can be seen that up to day 10 MFC1 generated a higher power. The longest time required for stabilisation was associated with the *S. plymuthica* pairing, where after 27 and 30 days the power output stabilised.

![Figure 4.17](image)

**Figure 4.17** – The power produced over time by the different *O. anthropi* dual-species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.
The polarisation curves (shown in Figure 4.19) show distinct differences compared to the single species MFC polarisation curves, with *B. graminisolvens* (the highest power producer on its own), producing the second lowest power output in combination with *O. anthropi*. The maximum power outputs in this combination were 0.23 mW m$^{-2}$ and 0.24 mW m$^{-2}$ for *B. graminisolvens* MFC1 and MFC2, both at approximately 6.7 µA.

Another distinction from the single species polarisation curves was the fact that *C. indolis* and *S. plymuthica* produced significantly different powers, while in the single species MFCs the polarisation curves were very similar. The combination with *S. plymuthica* produced the overall lowest outputs, generating 0.15 mW m$^{-2}$ and 0.17 mW m$^{-2}$ for MFC1 and MFC2 at approximately 5.6 µA.
In the case of *C. denitrificans* and *C. indolis*, both combinations produced a higher maximum power density than the single species *O. anthropi* MFC (0.35 mW m$^{-2}$). The *C. denitrificans* combination produced a significantly lower power than the single species MFC (>2 mW m$^{-2}$). The maximum power output was obtained at 12.5 µA for MFC1 (0.54 mW m$^{-2}$) and 17.4 µA for MFC2 (0.56 mW m$^{-2}$). The *C. indolis* MFCs produced powers close to that generated in the *C. indolis* single species MFCs (1.29 mW m$^{-2}$ and 1.55 mW m$^{-2}$). The maximum power generated was 1.13 mW m$^{-2}$ at 26.9 µA for MFC1 and 1.05 mW m$^{-2}$ at 21.2 µA for MFC2.

All MFCs produced a biofilm on the anodic surface of the membrane suggesting that aerobic respiration was taking place to some extent. It is safe to assume that in the *B. graminisolvens* and *C. indolis* MFCs, the biofilm only consisted of *O. anthropi* while the other biofilms probably consisted of a mixture of the two species present.

### 4.3.4 Dual-Species MFCs Involving *Bacteroides graminisolvens*  

The *B. graminisolvens* single species MFCs reached maximum power output after 7 days while it took between 12 and 24 days for the dual-culture MFCs to stabilise. The
*O. anthropi* MFCs had the largest difference between stabilisation times requiring 12 and 24 days. The *C. denitrificans* MFCs stabilised at the same time (day 22) but were generally slower than the other combinations (shown in Figure 4.20 and in Figure 4.21, the latter of which shows the data with the *C. denitrificans* experiments omitted for clarity). Figure 4.22 shows the stabilisation period required and Figure 4.23 shows the polarisation curves with *C. denitrificans* MFCs omitted for clarity. *S. plymuthica* MFC1 produced the most unusual stabilisation pattern peaking at 0.12 mW m\(^{-2}\) after 4 days, which continuously dropped until day 13, when it began increasing again. The MFC eventually stabilised at 0.79 mW m\(^{-2}\) but never did regain the maximum power obtained on day 4. A similar trend was observed in *O. anthropi* MFC1 and *C. indolis* MFC1, which both produced a peak power which was higher than the sustained power achieved.

![Figure 4.20](image_url)

*Figure 4.20* – The power produced over time by the different *B. graminisolvens* dual-species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.
The polarisation curve in Figure 4.2 shows that the most electrogenic combination involving B. graminisolvens is the dual-culture with C. denitrificans. The variation in maximum power densities between the two repeats is relatively large with MFC1 generating a maximum power density of 1.17 mW m\(^{-2}\) and MFC2 generated 1.50 mW m\(^{-2}\), obtained at 22.4 and 25.3 µA respectively. This data also corresponds with the single species MFCs where, after B. graminisolvens, the C. denitrificans MFCs produced the highest power. Figure 4.23 shows the polarisation curves with the C. denitrificans MFCs omitted to clearly show the other species power curves. The O. anthropi dual-culture produces the highest power of the remaining three species, which does not correspond with the single species MFC studies where O. anthropi produced lower powers than the other species. The results were conservative, with a maximum power of 0.23 and 0.24 mW m\(^{-2}\) at approximately 6.6 µA for MFC1 and MFC2 respectively. The C. indolis and S. plymuthica MFCs showed a similar trend to that seen single species MFCs; both combinations produced similar powers within the margin of

![Figure 4.21](image) – The power produced over time by the different B. graminisolvens dual-species MFCs under standard laboratory conditions with a 3 day moving average applied to the data. For clarity the C. denitrificans dual-species MFCs have been omitted.
error. The *C. indolis* MFCs produced the largest variation with MFC2, generating 0.20 mW m\(^{-2}\) at 6.2 µA, while MFC1 generated 0.11 mW m\(^{-2}\) at 3.4 µA. It should also be noted that MFC2 produced higher powers at resistances between 100,000 ohms and 18,000 ohms than the maximum power of MFC1. The *S. plymuthica* MFCs produced 0.12 mW m\(^{-2}\) at a 4.7 µA in both MFCs.

When looking at the metabolisms of the individual species it appears that *B. graminisolvens* produced lower power when paired with another fermentative species (*C. indolis* and *S. plymuthica*), while the species which are more commonly associated with anaerobic respiration produced a slightly higher power (approximately +0.1 mW m\(^{-2}\)) in the case of *O. anthropi* and a significantly higher power (approximately +0.9 mW m\(^{-2}\)) in *C. denitrificans*.

All MFCs, apart from the dual-culture involving *B. graminisolvens* and *C. indolis*, had a biofilm on the anodic surface of the membrane. This is most likely attributed to the fact that both these species are strict anaerobes, indicating that they are not able to utilise the oxygen present for aerobic respiration. In the other MFCs it is likely that the biofilm is primarily colonised by *S. plymuthica, O. anthropi* and *C. denitrificans* in the respective MFCs since these species are capable of aerobic respiration. It is possible that low levels
of oxygen present in the *B. graminisolvens* and *C. indolis* MFCs could be toxic to the species, thus reducing their growth rates for the species.

![Polarisation curves for different MFCs](image)

**Figure 4.23** – The polarisation curves produced by the by the different *B. graminisolvens* dual-species MFCs under standard laboratory conditions. For clarity the *C. denitrificans* dual-species MFCs have been omitted.

### 4.3.5 Dual-Species MFCs Involving *Clostridium indolis*

By examining Figure 4.24, the times required for power output to stabilise for dual-cultures involving *C. indolis* can be compared, the *B. graminisolvens* MFCs stabilised in the shortest time but produced the lower overall powers when compared to the other species paired with *C. indolis*. On day 14, MFC2 stabilised while MFC1 stabilised on day 15. The next shortest stabilisation period required was for *O. anthropi* MFC2 which required 18 days whilst MFC1, on the other hand, required a slightly longer period of 22 days. When looking at the *S. plymuthica* MFCs, it can be seen that up until day 10, MFC1 produced a higher power which was maintained up until the power output stabilised on day 20. On day 22, MFC1 started producing a higher power than MFC2 and it stabilised on day 25 which was the longest stabilisation period required out of the *B. graminisolvens* dual-cultures. The final pair, which involved *C. denitrificans*, required 22 and 19 days for MFC1 and MFC2 to stabilise, while MFC2 stabilised at the highest power it produced.
Figure 4.24 – The power produced over time by the different *C. indolis* dual-species MFCs under standard laboratory conditions with a 3 day moving average applied to the data.

Figure 4.25 shows the polarisation curves of the individual MFCs which vary slightly from the trends demonstrated in Figure 4.24. While *O. anthropi* MFC2 produced the highest power during continuous flow operation, it was only able to produce the third highest power overall (1.05 mW m$^{-2}$ at 21.2 µA). *O. anthropi* MFC1 produced the second highest power in both continuous flow operation and during the polarisation curve producing 1.13 mW m$^{-2}$ at 26.9 µA while also producing 1.07 mW m$^{-2}$ at 21.2 µA similar to MFC2. The *C. denitrificans* MFCs produced the largest variation between MFCs; MFC2 produced the highest observed power (1.18 mW m$^{-2}$ at 30 µA) while MFC1 produced the third lowest maximum power (0.08 mW m$^{-2}$ at 23 µA).
The *S. plymuthica* MFCs produced relatively similar polarisation curves and generated 0.89 and 0.87 mW m\(^{-2}\) at 16.5 and 19.3 µA respectively. The power generation in *S. plymuthica* was comparative or greater than the power generation occurring in the *C. denitrificans* and *O. anthropi* MFCs until the current exceeded approximately 16 µA after which it started to decrease thus generating less power, even when compared to *C. denitrificans* MFC1 which produced a lower maximum power density.

The *B. graminisolvens* MFCs produced the lowest powers observed when combined with *C. indolis* where powers of 0.01 and 0.02 mW m\(^{-2}\) at 4.4 and 6.2 µA. The highest powers were observed in MFCs which combined a fermentative species and species normally associated with anaerobic respiration. While *S. plymuthica* is also a fermentative species, it uses different pathways than *C. indolis* so it is possible that both species are able to utilise the respiration products instead of competing for organic carbon sources. *B. graminisolvens* and *C. indolis* have similar metabolisms which could suggest competition between the species. In all MFCs, aside from the *C. indolis* and *B. graminisolvens* pairing, a biofilm was observed on the membrane surface which is expected since neither of those species are capable of aerobic respiration and will not grow if oxygen is present in the growth medium. In each other pairing, a facultative anaerobe is present, so it would attempt to colonise the membrane to utilise any oxygen.

**Figure 4.25** – The polarisation curves produced by the by the different *C. indolis* dual-species MFCs under standard laboratory conditions.
which diffuses into the anodic chamber. An example of dual-culture microbial synergy is in polymicrobial infections where *Aggregatibacter actinomycetemcomitans* is only able utilise L-lactate when in the presence of *Streptococcus gordonii* [12]. Another instance of microbial co-culture synergy is where the presence of *Fusobacterium nucleatum* supported the growth of *Porphyromonas gingivalis* in moderately oxygenated conditions where it would not survive in a monoculture [13].

### 4.4 Power Generation in a Synthetic Community

The power generation in the synthetic community was more repeatable than expected when compared to some of the mixed species MFCs. It can be seen in Figure 4.26 that throughout operation MFC2 produced a higher power than its counterpart, but when the power had stabilised they both produced the same output. Both MFCs took around 35 days to stabilise which was the longest period required for any of the MFCs. This suggests as a community becomes more complex the time required for a stable output increases, which is confirmed by the dual-culture pairings generally taking longer to stabilise than the single species equivalents. While in batch flow operation, MFC2 ended up reaching a higher power which was maintained until day 21; this could indicate different species establishing a foothold in the community in the first few days.

![Figure 4.26](image)

*Figure 4.26 – The power produced over time by the 5-species synthetic community MFCs under standard laboratory conditions with a 3 day moving average applied to the data.*
The 5 species synthetic community has been compared to the polarisation curves generated by the *C. denitrificans* dual-culture MFCs (Figure 4.27) and the *C. indolis* dual-culture MFCs (Figure 4.28). The 5 species synthetic community generated higher powers than all communities which did not involve *C. denitrificans* or *C. indolis*. The powers generated by the synthetic community were 0.98 and 0.86 mW m$^{-2}$ at 24 μA.

The polarisation curve of the synthetic community closely resembled the power curves generated by the *C. denitrificans* with *S. plymuthica* and *C. indolis* dual-culture. This could suggest that these three species were more dominant in the synthetic community, and became the primary electrogens present. It also shared similarities to the *C. indolis* and *O. anthropi* communities. This suggests that *B. graminisolvens* is the least relevant species in the synthetic community, or it could imply that it fulfils a fermentative role in the anodic suspension. These results suggest that as the biodiversity of the community increases, an improvement in the power output is observed.

**Figure 4.27** – The polarisation curves produced by the 5-species synthetic community MFCs compared against the *C. denitrificans* dual-species MFCs under standard laboratory conditions.
4.5 Variation of pH in Dual-Species MFCs.

As discussed in the introduction (Section 1.8.2) *S. plymuthica* ferments organic carbon sources to produce generally neutral end products opposed to VFAs. One of the proposed hypotheses for the use of this pathway is to regulate the pH of the environment and prevent acidification which can be detrimental to the growth of the microorganisms [14]. Taking this into consideration, the pH of the different co-culture MFCs was monitored. The pH of each MFC was recorded when CV samples were taken after the polarisation curve was carried out (Table 4.2). The lowest pH measured was that of the dual-culture containing *C. indolis* and *B. graminisolvans*, where pHs of 6.15 and 6.09 were observed. The combinations involving *C. indolis* and *B. graminisolvans* generally produced lower pHs, with the exception of the pairs using *S. plymuthica*. The lowest pH measured in an MFC not containing *C. indolis* or *B. graminisolvans* was 6.31, which is similar to the *O. anthropi* and *B. graminisolvans* MFC2 which had a pH of 6.30. This pH trend is explained by the fact that both *C. indolis* and *B. graminisolvans* are both mixed acid fermenters.

![Figure 4.28 – The polarisation curves produced by the 5-species synthetic community MFCs compared against the *C. indolis* dual-species MFCs under standard laboratory conditions](image)
The MFCs inoculated with *S. plymuthica* produced the highest pHs observed out of all the different pairings. The lowest pH involving *S. plymuthica* was the dual-culture with *C. indolis* at 6.70 which were still higher than all pairings excluding *S. plymuthica*. The high pHs associated with *S. plymuthica* pairings can be attributed to the fermentation pathway utilised. *S. plymuthica* produces acetoin and butan-2,3-diol opposed to the short chain organic acids produced by the other fermentative species.

The 5 species MFCs produced pHs which fell in the middle of the range generated from the dual-culture MFCs. The pHs were higher than generally produced by the mixed acid fermenters, while lower than the *S. plymuthica* dual-cultures. This suggests that the pH can potentially be controlled by changing the composition of the microbial community and introducing species such as *S. plymuthica* to a community.

### Table 4.2 – The different pHs for the different dual-species and the 5-species synthetic community MFCs. The starting media had a pH of 7.

<table>
<thead>
<tr>
<th>Species</th>
<th>MFC1</th>
<th>MFC2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. denitrificans</em> and <em>C. indolis</em></td>
<td>6.18</td>
<td>6.21</td>
</tr>
<tr>
<td><em>C. denitrificans</em> and <em>O. anthropi</em></td>
<td>6.31</td>
<td>6.35</td>
</tr>
<tr>
<td><em>C. denitrificans</em> and <em>S. plymuthica</em></td>
<td>6.81</td>
<td>6.80</td>
</tr>
<tr>
<td><em>C. denitrificans</em> and <em>B. graminisolvens</em></td>
<td>6.29</td>
<td>6.24</td>
</tr>
<tr>
<td><em>C. indolis</em> and <em>O. anthropi</em></td>
<td>6.41</td>
<td>6.42</td>
</tr>
<tr>
<td><em>C. indolis</em> and <em>S. plymuthica</em></td>
<td>6.70</td>
<td>6.76</td>
</tr>
<tr>
<td><em>C. indolis</em> and <em>B. graminisolvens</em></td>
<td>6.15</td>
<td>6.09</td>
</tr>
<tr>
<td><em>O. anthropi</em> and <em>S. plymuthica</em></td>
<td>6.88</td>
<td>6.84</td>
</tr>
<tr>
<td><em>O. anthropi</em> and <em>B. graminisolvens</em></td>
<td>6.28</td>
<td>6.30</td>
</tr>
<tr>
<td><em>S. plymuthica</em> and <em>B. graminisolvens</em></td>
<td>6.78</td>
<td>6.76</td>
</tr>
<tr>
<td><strong>5 Species</strong></td>
<td>6.66</td>
<td>6.59</td>
</tr>
</tbody>
</table>

### 4.6 MFC Data Conclusions

The MFC trials showed that the highest power densities were observed in the single species MFCs. All species were found to be capable of anaerobic respiration, even the species commonly regarded as fermenters and not previously reported as electrogenic, as well as those conflicting and contradictory results. While in the single species MFC *B. graminisolvens* produced the highest maximum power density, a substantially lower
power was observed when paired with another species. In three of the four pairs, it produced either the lowest or second lowest maximum power density; with the exception being the dual-culture with *C. denitrificans* which produced the second highest power in the single species MFCs. *O. anthropi* produced the lowest power in the single species MFCs as well as producing the lowest powers in the mixed species alongside *B. graminisolvens*. The one exception to this is the dual-culture with *C. indolis* where one of the highest power outputs was observed.

Looking at the dual-cultures, it can be seen that the best combinations appear to be when a mixed acid fermentative species is paired with a species normally associated with anaerobic respiration. An example of this is the *C. denitrificans* dual-cultures where the lowest power observed was when it was combined with *O. anthropi* - another traditional anaerobic respirator (the other three species were all fermentative in nature). The same holds true for *C. indolis* where the dual-culture involving *B. graminisolvens* produced significantly lower power than the other pairs. An exception is observed in the *C. indolis* and *S. plymuthica* dual-culture, where both species are fermentative but produce a power output similar to the *C. indolis* and *C. denitrificans* dual-culture. This is most likely attributed due to the fact that *C. indolis* is a mixed acid fermenter while *S. plymuthica* is a butan-2,3-diol fermenter. The sample size in this project is relatively small, however, and further work should be undertaken to see if this type of analysis and discussion applies to a wider range of species [12][15].

These experiments have shown that it is not possible to predict the maximum power generation of a co-culture based on single species experiments, as shown by the *B. graminisolvens* co-cultures. Despite being unable to accurately predict which combinations will produce the highest powers, it should be noted that generally when a respirative species was paired with a fermentative species higher powers were obtained, compared to co-cultures made of two fermentative or two respirative species. This is possibly due to the respirative strains using secondary metabolites generated from the fermentation of glucose as a carbon source for power generation.

The difference in powers generated in the single species MFCs suggests potential antagonistic effects occurring between the individual species which reduces the powers generated while extending the lag times.
4.7 Cyclic-Voltammetry Studies

Cyclic voltammetry experiments were carried out on filtered anodic suspension samples using a glassy carbon working electrode, Ag/AgCl reference electrode and a platinum wire counter electrode. The goal of these experiments was to determine if any of the individual species were producing metabolites which could act as redox active mediators. The presence of mediators in the suspension would indicate a synergistic relationship which allows microbes in the suspension to contribute to power generation. By studying the cyclic voltammogram we can observe any oxidation or reduction peaks and the potential at which they occur. Blank experiments were carried out using the synthetic wastewater with and without the glucose, vitamin and mineral supplement solutions. Enlarged copies of the full CV scans can be found in Appendix 2.

4.7.1 Single Species CVs

The CV shown in Figure 4.29A allows the comparison of the individual species over the full range of the scan (-1.5 V to 1.5 V). Due to the large range of both current and voltage, it is quite hard to determine anything from this graph; the exception is the samples obtained from the S. plymuthica MFCs. A sharp current increase is observed, which begins at around 0.76 V which continues until the cycle finished at 1.5 V, both MFCs produced similar power outputs with MFC1 generating 396 µA while MFC2 reached 362 µA. This oxidation peak is likely due to the splitting of water and could be a shifted version of the oxidation observed at approximately 1.05 V in the other MFCs.

No discernible oxidation peaks can be observed in the blank synthetic wastewater samples shown in Figure 4.29B. The blank solutions also showed a lower peak current than was observed in the individual species MFCs. The peak current of the species did not correlate with the maximum power produced by each MFC, with B. graminisolvens producing the highest powers but had a lower peak current than both S. plymuthica MFCs and C. indolis MFC1. Both S. plymuthica and C. indolis produced similar power outputs while they produced different CVs. It should be noted that C. indolis MFC1 produced approximately 0.25 V more than C. indolis MFC2 which was reflected in the CV studies. A similar trend was also observed in the C. denitrificans MFCs with
\textit{C. denitrificans} MFC2 showing a larger peak current and producing a higher power output.

Two small oxidation peaks can be observed at 0.14 V and 0.75 V in all single species suspension samples, with the exception of \textit{S. plymuthica}. The current of the oxidation peak at 0.14 V correlates with the overall peak current of the system with the clearest definition observed from \textit{C. indolis} MFC1 which had the highest peak current from the remaining samples excluding \textit{S. plymuthica}. A slightly different trend is observed at the oxidation at 0.75 V where \textit{C. indolis} MFC2 despite having a lower peak current than \textit{C. denitrificans} MFC2 a higher current was generated. Two peaks were also observed in the \textit{S. plymuthica} MFCs but at different voltages the first at -0.11 V, while very small in \textit{S. plymuthica} MFC1 it is more clearly defined in MFC1. The second oxidation peak can be observed at 0.4 V, as with the 0.11 V oxidation the peak is clearer in MFC1 than MFC2.

After the addition of supplements to the synthetic wastewater, two small reduction peaks can be observed at -0.05 V and – 0.56 V (Figure 4.29C), which is most likely due to the reduction of transition metals in the mineral solution. A clear reduction at -0.7 V is observed in the \textit{S. plymuthica} MFCs, this peak could possibly correspond with the oxidation at -0.11 V but this is unlikely due to the different intensities and the relatively large voltage difference between the two. This indicates that the reduction is a non-reversible process indicating that the compound responsible is unlikely to act as a mediator in \textit{S. plymuthica} MFCs. A reduction peak is observed at -0.5 V in \textit{C. indolis} MFC1 which could correspond to the oxidation at 0.14 V, both peaks share a similar intensity which could indicate a redox active compound. It should be noted that this reduction is not present in the other MFCs which suggests that the oxidation and reduction are unrelated.
Figure 4.29 – The CV for the single species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V where A. depicts the full scan, B. shows the key oxidative peaks and C. shows the key reductive peaks.
4.7.2 Dual-Species CVs

4.7.2.1 Dual-Species CVs Involving Comamonas denitrificans

The CV profiles for the *C. denitrificans* dual-culture MFCs are all very similar with each MFC having two oxidation peaks present at 0.17 V and 0.73 V which can be seen in Figure 4.30A and B. The oxidation at 0.17 V is similar between all the MFCs with the apex of the peak ranging between 3.15 and 3.19 µA. The oxidation at 0.73 V produces a larger variance between MFCs with the *O. anthropi* and *S. plymuthica* MFCs reaching 5.48 µA and the *C. indolis* and *B. graminisolvens* MFCs 4.5 µA. This does not concur with the power generation where the *O. anthropi* dual-cultures generated the lowest power and the other 3 species generated similar powers. This shows that this peak is very unlikely to be important for power generation with in the MFCs.

Two small reduction peaks can be observed at around 0 V and -0.5 V. The *C. indolis* MFCs produce the smallest peaks with only a slight change in current while the peak appears to be most pronounced in *B. graminisolvens* (Figure 4.30C). It should also be noted that the *C. denitrificans* MFCs contained a pale yellow pigment which was not observed in any other MFCs, nor was present in the *C. denitrificans* single species MFC.
Figure 4.30 – The CV for the C. *denitrificans* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V where A. depicts the full scan, B. shows the key oxidative peaks and C. shows the key reductive peaks.
4.7.2.2 Dual-Species CVs Involving *Serratia plymuthica*

The CV profiles for the *S. plymuthica* dual-cultures (Figure 4.31) are all relatively similar but differ quite drastically from the single species profile. The first point of note is that the peak current is a lot lower in the dual-cultures than in the single species MFCs. The oxidation at 0.25 V was 4.43 µA in the single species MFC but only 2.5 µA in the highest peak current dual-culture (Figure 4.31B). The lowest reduction current observed at -0.5 V in the dual-culture suspensions was 4.35 µA (Figure 4.31C), while the current observed in the single species suspension was -8.8 µA.

All dual-culture MFCs showed an oxidation peak at approximately 0.8 V with the peak in the *C. denitrificans* MFCs appearing at 0.7 V. In most cases the CV profile was similar for both MFC1 and MFC2, with the one major exception being the *O. anthropi* MFCs. *O. anthropi* MFC2 produced a clearer oxidation peak which reached 6.2 µA opposed to MFC1, which only produced 4.2 µA. During the MFC studies *O. anthropi* MFC2 produced a higher maximum power density than *O. anthropi* MFC1 which could indicate that the compound responsible for the peak is involved in power production in *O. anthropi* and *S. plymuthica* dual-cultures. It should be noted that the *O. anthropi* and *B. graminisolvens* MFCs produced the lowest overall powers.

The biggest difference between the *S. plymuthica* single species MFCs and the dual-cultures was the absence of the reduction peak at -0.7 V. A potential reason for this could be that whichever compound was responsible for the peak was being utilised as a carbon source by the other species in the MFC. It could also indicate that the pathway used for the production of the compound is suppressed by the other species presence, this could occur through the generation of secondary inhibitory metabolites.

A slight reduction peak can be observed at around -0.5 V in the *B. graminisolvens* and *O. anthropi* MFC2. It is unlikely that this reduction is important to power production in the MFC due to its low intensity, and that it only appears in the species combinations which were linked to low power.

The CV profile for the *C. indolis* MFCs is very similar to that of the synthetic wastewater (supplements) blank. The only difference is the disappearance of the reduction peaks at -0.05 V and 0.65 V again suggesting that these peaks are associated with the vitamin and mineral solutions, and are consumed during MFC operation.
Figure 4.31 – The CV for the *S. plymuthica* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V where A. depicts the full scan, B. shows the key oxidative peaks and C. shows the key reductive peaks.
4.7.2.3 Dual-Species CVs Involving *Ochrobactrum anthropi*

The CV oxidation profiles for all *O. anthropi* dual-cultures produce a higher oxidation potential than the blank synthetic waste water blanks (Figure 4.32). In general, the two repeats produce similar profiles with only the *S. plymuthica* MFCs producing distinct profiles. While *S. plymuthica* MFC2 showed a generally higher current (approximately 1 µA), a clearly defined oxidation peak is also observed at -0.8 V (Figure 4.32B). Each other dual-culture showed a subtler oxidation peak between 0.75 V and 0.85 V. Another very slight increase in current can be observed between 0 and 0.4 V which can be attributed to a much smaller intensity oxidation. *S. plymuthica* MFC2 also produced a more defined peak at 0.36 V.

The reduction profiles for the different dual-cultures are very similar, with the main difference being the variations in peak current (Figure 4.32C). The reduction peak associated with the supplements is no longer visible, with the only potential reduction peak occurring at -0.5 V which is only observed in *S. plymuthica* MFC2.

Neither the oxidation or reduction peak currents are linked to power output of the MFC. The *O. anthropi* and *C. indolis* dual-culture MFCs produced the highest observed power but had the lowest peak current and smallest reduction and oxidation peaks observed during the CV experiments.
Figure 4.32 – The CV for the *O. anthropi* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V where A. depicts the full scan, B. shows the key oxidative peaks and C. shows the key reductive peaks.
4.7.2.4 Dual-Species CVs Involving *Bacteroides graminisolvens*

The *B. graminisolvens* dual-culture MFCs all showed an improvement in peak current from the blank synthetic water samples (Figure 4.33). All dual-cultures produced a similar oxidation profile with very little variation between the repeats. All MFCs showed two oxidation peaks (Figure 4.33B), the first being observed at around 0.35 V which only showed a current increase of approximately 0.5 µA in all MFCs. The second peak observed at around 0.8 V is more defined but there is little variation in the current increase between the different dual-cultures.

The reduction profiles show more variation between the different dual-cultures with each MFC showing a higher peak current than the blanks. The dual-cultures each showed a variance in peak current with the *C. indolis* MFCs and *S. plymuthica* MFC1 producing the lowest peak currents while the other MFCs all showed similar values. The reduction peak (Figure 4.33C) associated with the supplements at -0.02 V was not observed but the reduction peak at -0.55 V was reduced in a number of the MFCs but was more pronounced in the *S. plymuthica* MFC2.
Figure 4.33 – The CV for the *B. graminisolvens* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V where A. depicts the full scan, B. shows the key oxidative peaks and C. shows the key reductive peaks.
4.7.2.5 Dual-Species CVs Involving *Clostridium indolis*

The *C. indolis* dual-cultures oxidation CV profiles were all relatively similar with the different between the individual MFCs being a change in peak current (Figure 4.34). Excluding the blanks all MFCs displayed 2 oxidation peaks (Figure 4.34B) a broad shallow peak observed at around 0.3 V and a more defined peak at around 0.8 V.

The reduction profiles (Figure 4.34C) show an increase in the peak current in all of the MFCs with the exception of *S. plymuthica* MFC1 which shows a similar profile to that of the blank with supplements. The reduction peak at -0.02 V in the supplements solution is no longer present in any of the dual-cultures. A broad shallow peak can be observed at approximately -0.55 V in all dual-culture MFCs which is also present in the MFC with supplements blank.
Figure 4.34 – The CV for the *C. indolis* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V where A. depicts the full scan, B. shows the key oxidative peaks and C. shows the key reductive peaks.
4.7.3 5 Species CVs

The 5 species community produced a CV distinct from that produced from the blanks, with increased peak current and the appearance of two oxidation peaks at 0.2 V and 0.75 V (Figure 4.35A and B). These peaks are consistent with the data obtained from the single species and dual-culture MFCs which suggest the peaks arise from either a common metabolite excreted from the bacteria, or it could also be attributed a protein present in the suspension. The peaks do not match any observed peaks in literature. Due to the lack of a reduction counterpart these compounds do not act like a mediator, which again confirms the lack of contribution of power generation from respiration occurring in the suspension.

The reduction profile shows an increased peak current and removal of the reduction peaks observed at -0.02 V and -0.55 V which suggests the compounds responsible for the peaks are consumed throughout MFC operation (Figure 4.35C).
Figure 4.35 – The CV for the 5-species synthetic community MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V where A. depicts the full scan, B. shows the key oxidative peaks and C. shows the key reductive peaks.
4.7.4 CV Conclusions

Despite the presence of a variety of oxidation and reduction peaks present in the MFCs, it is highly unlikely that any of them play a major role in power production or act as mediators to facilitate the transfer of electrons from the bacteria to the electrode. The large separation between oxidation and reduction peaks means that they are unlikely to be attributed to a redox active compound and are more than likely separate metabolites or proteins which are generated through MFC operation. The peaks at 0.25 V (vs Ag/AgCl electrode) could be related to outer membrane cytochromes which have been shown to produce a peak at 0.01 V (vs SHE, (0.24 vs Ag/AgCl electrode)) which would explain the presence in all the MFCs.

The major difference between all the different MFCs was the reduction peak at -0.75 V observed in the S. plymuthica single species MFCs which is not present in any of the dual-culture MFCs involving S. plymuthica. This suggests that the presence of other species could be suppressing the production of the metabolite (potentially pyrimine, a rose coloured pigment secreted by Serratia species [16]), or utilising the substrate as an organic carbon source.

These data suggest that in all the MFCs, the majority of electrogenic activity is occurring in the biofilm (which forms on the anode) due to the lack of substantial redox peaks. This also hints that any non-fermentative metabolism which occurs in the suspension is likely to decrease the power output of the community since the carbon sources will be consumed but the electrons will not be diverted to the anode. Fermentation processes will also potentially reduce the power output through consumption of organic carbon sources, but potentially useful metabolites such as acetate and propionate will be consumed. Therefore in an effort to improve this community further addition of a species capable of excreting a redox active metabolite could potentially increase the power output by allowing the suspension to donate electrons to the anode.
4.8 Bibliography


[12] 2,3-Butanediol fermentation promotes growth of *Serratia plymuthica* at low pH but not survival of extreme acid challenge. **B. Vivija, P. Moons, A. Geeraerd,**
A. Aertsen, C. Michiels. 2014, International Journal of Food Microbiology, Vol. 175, pp. 36-44.


Chapter 5 - Conclusions and Further Work

5.1 Conclusions

The work in this project aimed to provide the ground work to help further understand the dynamics of a microbial community. The first stage of the project was analysing the individual species electrogenic potential by running two repeats of each species using single species microbial fuel cells (MFCs).

These experiments showed that each of the selected species was capable of power generation in a single chamber air-breathing cathode system. Throughout the course of these investigations the electrogenic potential of three new species was reported. The first being *Serratia plymuthica* (*S. plymuthica*) which had been observed in naturally occurring electrogenic microbial communities but these experiments were the first to show its electrogenic potential. Previous experiments with different *Clostridium* species have shown the species is capable of electrogenic activity but that not all were capable of power generation [1][2][3]. This work showed the first documented case of *Clostridium indolis* (*C. indolis*) being capable of power production in a MFC. *Bacteroides graminisolvens* (*B. graminisolvens*) has also been observed in a variety of microbial communities, this was the first study which demonstrated the ability of *B. graminisolvens* to produce power.

The fact that all these species (*S. plymuthica, C. indolis and B. graminisolvens*) are also known fermentative species suggests that a metabolic switchover may be more common than previously thought, occurring when the fermentative species adapt towards a more energy efficient metabolism using the electrode as a terminal electron acceptor [4]. A previous study, which demonstrated the electrogenic nature of *Comamonas denitrificans* (*C. denitrificans*), concluded that power production was not viable in a single chamber MFC due to oxygen permeation through the membrane causing aerobic respiration to be favoured over anaerobic respiration [2]. The data generated in the experiments in this thesis demonstrated that this is not in fact the case and *C. denitrificans* is capable of power production in single chamber MFCs. This could be attributed to the biofilm which was observed on the surface of the membrane,
preventing oxygen from penetrating the MFC which, in turn, impacted metabolism in the anode biofilm. This would be due to the bacteria (located in the membrane biofilm) which will utilise the oxygen that diffuses through for aerobic respiration, preventing it reaching the community in the anode biofilm. This mechanism would be detrimental to power generation due to it providing an alternate terminal electron acceptor. This could also be contributed to by the large anodic volume lowering the concentration of any oxygen present in the system. While the concentration of oxygen was not monitored during these experiments, any further work carried out on facultative anaerobes should pay attention to the concentration of oxygen.

Table 5.1 – Comparing the different single species MFCs lag times and maximum power outputs to the Biolog respiration rate and lag time data generated for glucose.

<table>
<thead>
<tr>
<th>Species</th>
<th>Microbial Fuel Cell Lag Time (d)</th>
<th>Maximum Power Output (mW m⁻²)</th>
<th>Biolog Lag Time for Glucose (h)</th>
<th>Biolog Respiration Rate for Glucose (h⁻¹)</th>
<th>Reported Metabolism</th>
<th>Membrane Surface Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides graminisolvens</td>
<td>7</td>
<td>3.09</td>
<td>4</td>
<td>2.26</td>
<td>Mixed Acid Fermentor</td>
<td>No</td>
</tr>
<tr>
<td>Comamonas denitrificans</td>
<td>23</td>
<td>2.50</td>
<td>4</td>
<td>1.90</td>
<td>Anaerobic Respirator</td>
<td>Yes</td>
</tr>
<tr>
<td>Clostridium indolis</td>
<td>15</td>
<td>1.55</td>
<td>10</td>
<td>1.60</td>
<td>Mixed Acid Fermentor</td>
<td>No</td>
</tr>
<tr>
<td>Serratia plymuthica</td>
<td>13</td>
<td>1.39</td>
<td>31</td>
<td>0.07</td>
<td>Alcohol Fermentor</td>
<td>Yes</td>
</tr>
<tr>
<td>Ochrobactrum anthropi</td>
<td>5</td>
<td>0.33</td>
<td>12</td>
<td>1.46</td>
<td>Anaerobic Respirator</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The single species data (Table 5.1) showed that *Ochrobactrum anthropi* (*O. anthropi*) produced substantially lower MFC power (0.33 mW m⁻²) than the other single species, while the highest powers observed occurred in the *B. graminisolvens* MFC (3.09 mW m⁻²). When looking at the powers generated and the associated respiration, it can be seen that no pattern can be observed for these 5 species. This contradicts the general assumption normally made that the fermentative species are not expected to contribute to power generation, while the anaerobic respirators are expected to be the primary power generators. The Biolog experiments showed that *O. anthropi* was better able to utilise glucose as a substrate (than *S. plymuthica*) and to a similar level as *C. indolis* in terms of both lag time and maximum respiration rate. The shortest lag times
and growth rates observed in the Biolog experiments were attributed to *B. graminisolvens* and *C. denitrificans*, together with the fact that they produced the highest powers in the single species MFCs (3.09 and 2.50 mW m\(^{-2}\) respectively), shows a possible link between respiration rate and maximum power output. This indicates that when trying to create a synthetic community, species with short lag times and high respiration rates may result in higher power output. No link was observed between the time required for the power to stabilise using the lag times and respirations rates for glucose from the Biolog.

When it came to carbohydrate utilisation, four out of the five species were able to reduce the chemical oxygen demand (COD) of the influent by approximately 70%, the exception being the *O. anthropi* MFCs (40%) which were drastically lower. The highest levels of COD removal were observed in the *B. graminisolvens* and *C. indolis* MFCs. This trend approximately matches with the power output of the MFCs where *O. anthropi* showed the lowest power output and lowest COD treatment. This indicates that *O. anthropi*’s low power is related to its inability to efficiently make use of the substrates available and fully oxidise all carbon present. This suggests that it might not be a viable option for synthetic communities in future studies.

The ability of the microorganisms to reduce COD increased as the biodiversity of the system increased, with the highest level of COD removal occurring in the 5-species MFC (90%). In all cases, the dual-species MFCs displayed a better ability to remove COD from the feed than the single species MFCs. In the dual-species MFCs the highest levels of COD removal were observed in the communities involving *C. indolis* and *B. graminisolvens*. Since the highest power outputs were observed in the single species MFCs, it is unlikely that power output is related to the level of COD removal. This suggests a large number of electrons are diverted away from the anode to other terminal electron acceptors. It is also possible that the majority of the treatment occurs in the suspension where the bacterial cells are not in contact with the electrode. This means that in this system, a compromise has to be made between COD removal and power generation. A potential solution to this is to greatly reduce the anodic volume so that the anode biofilm utilises the carbon sources. Another possible alternative is to introduce a mediator to allow respiration occurring in the suspension to contribute to power generation.
The cyclic voltammetry (CV) experiments conducted on the suspensions showed an increase in capacitance but no major redox or oxidation peaks which indicates that all the power generation in the MFC is occurring in the biofilm. A small oxidation peak was observed in all MFCs at 0.25 V (vs Ag/AgCl electrode) which could potentially correlate with outer membrane cytochromes which are present in the suspension. The small current associated with the peak and lack of reduction counterpart suggests that it is unlikely that this peak is involved in power production. This suggests that in this system, having a large anodic volume is detrimental to the performance of the MFC since the respiration occurring in the suspension is not contributing to the power generation of the MFC. Through the addition of a mediator to the system, it should be possible to increase the power density of the MFC due to the presence of a vehicle to shuttle electrons from the suspension to the electrode. The biofilm observed on the surface of the membrane may indicate that aerobic respiration is occurring, utilising carbon sources without contributing to power generation.

In all the dual-species MFCs examined in this work, lower power outputs were observed than were expected (based on the single species MFCs), which contradicts the assumption commonly made that an increase in diversity leads to an improvement in the performance of the MFC for power production [5]. A potential reason for this is that the biodiversity found in these experiments (5 species), is far lower than that observed in naturally occurring communities with approximately 80 found in the community which this work is based upon [4]. Since the 5 species chosen for this work were present in the highest concentrations in the naturally occurring community, this would suggest that species found in low concentrations have a larger than anticipated effect on the electrogenic nature of the community, possibly by producing a larger range of metabolites than those generated by the 5 species alone. [6]. This also suggests that, generally, the individual species compete against each other, opposed to the synergistic effects which would be expected since the species have been found together in naturally occurring communities. The biggest difference between the MFCs was the fact that, except for the co-culture with *C. denitrificans, B. graminisolvens* produced some of the lowest observed powers (with *S. plymuthica*, 0.12 mW m$^{-2}$; *C. indolis*, 0.20 mW m$^{-2}$ and with *O. anthropi*, 0.20 mW m$^{-2}$). The *C. denitrificans* and *B. graminisolvens* co-culture produced the highest observed power (1.50 mW m$^{-2}$) out of any co-species MFC. This suggests that *O. anthropi, S. plymuthica* and *C. indolis* all have antagonistic effects.
when paired with *B. graminisolvens* which could be due to secondary metabolites that inhibit its ability to utilise substrates. This is unlikely because the COD treatment of the co-cultures exceeded that of the single species. Another possible explanation is that the presence of other species causes *B. graminisolvens* to favour non-electrogenic pathways. No reported literature has mentioned any metabolites which can inhibit these individual species, nor have any of the selected species been noted to have antibacterial properties.

Out of the ten different dual-cultures, the six with the highest power outputs all contained either *C. denitrificans* or *B. graminisolvens* (Table 5.2). The most significant trend was that the highest powers produced by single species often involved systems where the metabolisms of the species complimented each other when compared to the other co-culture experiments. This could explain why, in high biodiversity systems, increased power is observed; since, with a larger number of species, it is possible that a wider range of utilisable substrates are generated. As previously stated, the highest power outputs were observed in the *B. graminisolvens* and *C. denitrificans* dual-culture, a mixed acid fermenter and an anaerobic respirator. The next highest powers were observed in the dual-culture of *C. denitrificans* and *C. indolis*, and *C. indolis* and *O. anthropi*. Both of these dual-cultures show the same pattern of complementary metabolisms leading to the highest co-culture powers. The converse pattern was also observed when the fermentative species were paired together, *B. graminisolvens* and *C. indolis* produced one of the lowest observed powers (0.20 mW m⁻²), as did the *B. graminisolvens* and *S. plymuthica* co-culture (0.12 mW m⁻²). There were some exceptions including the *C. indolis* and *S. plymuthica* co-culture (0.89 mW m⁻²), which produced a power close to that of the *S. plymuthica* and *C. denitrificans*. Despite both species being fermenters, they utilise different pathways so it is possible that they are both capable of utilising the metabolites of the other species, providing an example of synergy.

The dual-cultures (Table 5.2) showed longer acclimatisation periods than their single species counterparts (Table 5.1), a trend which was also observed in the Biolog experiments. The Biolog data showed that *B. graminisolvens* was able to utilise a wide range of substrates and was often the more dominant species. It would be expected that this would make the *B. graminisolvens* dual-cultures follow the *B. graminisolvens*
single species electrogenic profile, but instead the opposite happened: *B. graminisolvens*-containing MFCs showed the biggest decrease in power output. The fact that the dual culture Biolog experiments featuring *B. graminisolvens* showed enhanced respiration rates indicates that the decline in power may not be due to antagonistic effects, such as that of secondary inhibitory metabolites. It has been noted previously that species such as *E. coli*, which have a range of metabolic pathways (aerobic respiration, anaerobic respiration and fermentation), will switch from fermentation to respiration based on the conditions so a similar process could be occurring in the co-cultures with species switching to non-electrogenic pathways [7]. There was also no observable pattern that emerged between the power generated and the maximum respiration rates which were observed during the Biolog experiments, but an interesting note was that the respirator co-culture MFC showed one of highest respiration rates utilising glucose (3.8 h\(^{-1}\)) which could indicate that as previously suggested *C. denitrificans* (which demonstrated one of the shortest lag times and highest respiration rates utilising glucose in the single species Biolog experiments, 4 h and 1.90 h\(^{-1}\) respectively) has available fermentative pathways which can be used.

It was observed that the presence of *S. plymuthica* caused an increase in the pH (from approximately 6.2 to 6.8) of the anodic suspension. This indicates that *S. plymuthica* has the potential to be used to raise the pH in communities which are too acidic. This could be important because some species of bacteria are only able to operate efficiently in a specific pH range.
Dual Species MFCs | Microbial Fuel Cell Lag Time (d) | Maximum Power Output (mW m²) | Biolog Lag Time for Glucose (h) | Biolog Respiration Rate for Glucose (h⁻¹) | Reported Species 1 Metabolism | Reported Species 2 Metabolism | Membrane Surface Biofilm
---|---|---|---|---|---|---|---
*B. graminisolvens + C. denitrificans* | 24 | 1.50 | 6 | 2.7 | Fermentor | Respirator | Yes
*C. indolis + C. denitrificans* | 22 | 1.18 | 8 | 2 | Fermentor | Respirator | Yes
*O. anthropi + C. indolis* | 22 | 1.13 | 7 | 4.3 | Respirator | Fermentor | Yes
*S. plymuthica + C. denitrificans* | 25 | 1.11 | 8 | 2.2 | Fermentor | Respirator | Yes
*S. plymuthica + C. indolis* | 25 | 0.89 | 18 | 2.1 | Fermentor | Fermentor | Yes
*O. anthropi + C. denitrificans* | 27 | 0.56 | 4 | 3.8 | Respirator | Respirator | Yes
*O. anthropi + B. graminisolvens* | 22 | 0.20 | 9 | 2.4 | Respirator | Fermentor | Yes
*C. indolis + B. graminisolvens* | 15 | 0.20 | 5 | 2.5 | Fermentor | Fermentor | No
*S. plymuthica + O. anthropi* | 29 | 0.17 | 12 | 1.3 | Fermentor | Respirator | Yes
*S. plymuthica + B. graminisolvens* | 16 | 0.12 | 16 | 2.3 | Fermentor | Fermentor | Yes

Table 5.2 – Comparing the different dual-species MFC lag times and maximum power outputs to the Biolog respiration rate and lag time data generated for glucose.
The 5-species synthetic community demonstrated the longest time required for the power output to stabilise, which was also observed with the dual-species MFCs. The 5-species synthetic community showed a lower power output than the dual-combinations which produced the highest power outputs. When looking at the dual-species MFCs it can be observed that *S. plymuthica* appears in 2 out of the 3 MFCs that produce the lowest power output. This suggests that *S. plymuthica* is one of the species associated with the drop in power observed in the synthetic community. The polarisation curve for the synthetic community closely matches the polarisation curves for the following dual-species MFCs - *C. denitrificans* with *C. indolis*, *C. denitrificans* with *S. plymuthica* and *C. indolis* with *O. anthropi*. This data indicates that *B. graminisolvens* is not involved in the power production of the synthetic community. Due to the ability of *B. graminisolvens* to utilise a wide range of substrates, it is possible that it fulfils a fermentative role in the anodic suspension, supplying substrates that can be used by the electrogenically active species. To determine if either of these possibilities are occurring, three species and four species MFCs would be required.

It was also observed that the 5-species synthetic community demonstrated the best ability for COD removal, which is likely due to the ability of the community to utilise a wide variety of different metabolic pathways. Despite the improvement observed for COD treatment compared to the other MFCs, the power did not improve. This combined with the lack of mediators found in the suspension through CV experiments shows that the majority of the treatment is occurring in the suspension and not contributing to the power output. An MFC with a reduced suspension volume is likely to lead to increase in observed power output.

Synthetic communities show potential but the interspecies interactions are more complex than previously thought with apparent antagonistic relationships occurring between the species. Now the weaknesses of the studied synthetic community have been noted, steps can be taken to further optimise the community. The presence of *S. plymuthica* caused an increase in the observed pH which makes it a good candidate for future communities which have species which cannot tolerate low pHs. Another optimisation that can be made to the synthetic community is adding species which are able to produce redox active metabolites which could potentially allow bacteria not present in the biofilm to contribute to power output. While the Biolog studies were
useful for determining substrate utilisation and looking at potential synergies in species metabolism, the data did not align with the power outputs of the MFCs.

5.2 Further Work

The first and most obvious experiments for future work is to study the performance of three- and four-species communities MFCs. The single species MFCs generally produced higher powers than those observed in the dual-cultures, while the 5-species MFCs produced higher powers than the MFCs which did not contain C. indolis and C. denitrificans. This suggests that the addition of one or more species to the dual-cultures will cause an increase in the observed power output. Once all the possible permutations have been carried out, two different paths are available.

One route is through adding additional species to the community and slowly growing and expanding the community while removing species which are seen as detrimental to the performance of the MFC. Over time this would lead to the manufacture of a truly synthetic microbial community as opposed to a synthetic community modelled upon a naturally occurring community which was the focus of this project. An obvious starting point is through the addition of known electrogenic species like Shewanella oneidensis and Geobacter sulfurreducens [8][9]. Another possibility is the fermentative species Pelotomaculum thermopropionicum which has demonstrated the ability to use nanowires [10].

The second route is to experiment using the same community but to change the substrates. The Biolog experiments revealed a variety of different possible substrates which could be trialled as the organic carbon source in the MFC. Examples of possible substrates for the community include lactose, a disaccharide which not only were all species able to utilise, but the two constituent monosaccharides are also able to be used for respiration by all species analysed in this work. Lactose is also a major constituent in dairy farm wastewater which would allow for more industry-focused research [11]. Another possible substrate is using complex polysaccharides such as pectin, glycogen and starch, C. indolis and B. graminisolvens were both able to utilise these substrates and in the dual-culture experiments, high respiration rates were observed which suggested synergistic relationships between the species.
The focus of the MFC studies could also be altered, to study the generation of high value organic products such as 1-butanol, 2,3-butandiol and acetoin, which are all fermentation products from species present in the community (notably *clostridium*). This, combined with the low levels of power generation, could increase the economic viability of the MFCs.

The CV work carried out in this project suggested that the majority of the power produced from the bacteria in the MFC came from the biofilm. This suggests that the having a large anodic-chamber volume is detrimental to the performance of the MFC. By designing a series of MFCs which only vary in anodic chamber size, the relationship between anodic-chamber volume and power generation could be determined.

Another avenue of research is to put a larger focus on investigating the biofilm and how it contributes to the power generated by the community. This could be conducted using CV to scan for redox reactions which take place in the biofilm. Once the redox nature of the biofilm has been determined, scanning electron microscopy (SEM) can be used to study the structure of biofilm. While this research showed that the fermentative species are capable of anaerobic respiration and able to use the electrode as a terminal electron acceptor, it is possible that in microbial communities, a larger focus is placed on fermentation so the majority of the bacterial cells will be found in the suspension.

A different approach is to enhance the electroactive nature of the suspension. This can be attempted through building a community around a known mediator-producing species such as *Pseudomonas aeruginosa*, which produces phenazine and pyocyanin, and *Shewanella oneidensis* which is able to produce quinones and riboflavins which have been suggested to act as an electron shuttle [12][13][14][15]. The presence of a mediator-generating species would allow the other species in the suspension to be able to contributed to the overall power production.

Finally, instead of allowing a community to naturally develop over time, research could focus on shifting the dynamics of an already existing community. A route to achieve this would be to alter the conditions to favour one species over another; for example, with the community used in this research, increasing the temperature to 37°C to favour *C. indolis* and *B. graminisolvens*. Another possible route is by adding a species directly to an already established community. This could be achieved by acclimatising the anode in a pure culture of the designed species and then transferring it to the desired
community. Another method would be simply adding the liquid culture to the MFC and monitoring the community composition over time using denaturing gradient gel electrophoresis (DGGE).

As has been highlighted in the previous discussions, there is still room for microbial fuels cells to develop, with a large potential being found within synthetic communities. Tailoring communities to the feed the waste streams being used should allow high power output, coulombic efficiencies and COD removal to be achieved. Also understanding the interactions between species in synthetic communities will lead to a better understanding of how both synthetic and naturally occurring communities contribute to power generation through MFCs.

### 5.3 Review of Project Aims

The overall aim of this project was to build a synthetic community based on the 5 most common species observed in anaerobic digester sludge MFCs in order to examine the metabolic and electrogenic properties to try to define each species’ role in the 5-species community.

**Single-species MFCs**

- Each of the 5-species was proven to be capable of power generation in a single chamber air-breathing cathode system.
- The novel electrogenic potential of 3 species was observed – *S. plymuthica, C. indolis* and *B. graminisolvens*.
- Contrary to reported literature, *C. denitrificans* was found to be capable of power production in single chamber MFCs.
- The highest powers were generated by *B. graminisolvens* whilst the lowest powers were produced by *O. anthropi*.
- *O. anthropi* was not able to make efficient use of the substrates available, resulting in the lowest carbohydrate utilisation. This is likely to the cause of the low power production of the corresponding MFC.
Dual-species MFCs

- All dual-species MFCs displayed a better ability to remove COD from the feed (compared to the single species), particularly communities involving *C. indolis* and *B. graminisolvens*.
- It was determined that power output seems to be unrelated to COD removal.
- Lower powers were observed in the dual-species MFCs than in the corresponding single-species MFCs, therefore single-species MFCs are not useful predictors for multi-species MFC power generation.
- Contrary to the single-species MFCs, *B. graminisolvens* co-cultures produced some of the lowest observed powers in the dual-species MFCs. This could indicate that the presence of other species causes it to favour non-electrogenic pathways.

5-species MFCs

- Produced lower power output than predicted by the dual-species MFCs.
- Data indicated that *B. graminisolvens* may not contribute to power production in the 5-species MFC which concurs with the findings from the dual-species MFCs. It is able to fulfil a fermentative role in the community.
- Cyclic voltammetry showed that mediators were not present, indicating that power generation occurs in the anode biofilm using direct electron transfer (which was also found to be the case for the single- and dual-species MFCs).

Novel use of the Biolog to measure the metabolic profile of each of the 5 bacteria species individually and the 10 unique dual-species combinations.

- Link between lag time and respiration rate was found.
- *B. graminisolvens* had the shortest lag time and growth rate. In the single-species MFCs, it was found to produce the highest power.
- In single species MFCs a link between lag time, respiration rate and power output was observed, but this didn’t hold up for the dual species MFCs.
5.4 Bibliography


**Appendix I – Biolog Titre Plates**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PM</td>
<td>01</td>
<td>02</td>
<td>03</td>
<td>04</td>
<td>05</td>
<td>06</td>
<td>07</td>
</tr>
<tr>
<td>2</td>
<td>Positive Control</td>
<td>D-Serine</td>
<td>D-Mannose</td>
<td>Glucose</td>
<td>Phosphate</td>
<td>L-Tyrosine</td>
<td>L-Arginine</td>
<td>L-Histidine</td>
</tr>
<tr>
<td>3</td>
<td>Negative Control</td>
<td>L-Amino</td>
<td>D-Galacturonic Acid</td>
<td>Tween 80</td>
<td>D-Fructose</td>
<td>D-Formic Acid</td>
<td>2,6-Dihydroxy-6-methyl-4-pyrimidinone</td>
<td>L-Threonine</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>D-Maltose</td>
<td>D-Mucic Acid</td>
<td>Tween 40</td>
<td>D-Fructose</td>
<td>D-Formic Acid</td>
<td>2,6-Dihydroxy-6-methyl-4-pyrimidinone</td>
<td>D-Threonine</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>D-Maltose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure A1.1** – The different substrates found on a Biolog PM1 titre plate.
<table>
<thead>
<tr>
<th>Figure A.1.2</th>
<th>The different substrates found on a Biolog PM2a titre plate.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Reaction Type</td>
</tr>
<tr>
<td>1. L-arabinose</td>
<td>2-Hydroxy-1-deoxyribulose</td>
</tr>
<tr>
<td>2. D-arabinose</td>
<td>2-Aldehyde-1-thiouracil</td>
</tr>
<tr>
<td>3. L-arabinose</td>
<td>2-Hydroxy-1-deoxyribulose</td>
</tr>
<tr>
<td>4. D-arabinose</td>
<td>2-Aldehyde-1-thiouracil</td>
</tr>
<tr>
<td>5. L-rhamnose</td>
<td>3-O-Acetyl-2-keto-3-deoxy-erythrose</td>
</tr>
<tr>
<td>6. D-rhamnose</td>
<td>3-O-Acetyl-2-keto-3-deoxy-erythrose</td>
</tr>
<tr>
<td>7. L-rhamnose</td>
<td>3-O-Acetyl-2-keto-3-deoxy-erythrose</td>
</tr>
<tr>
<td>8. D-rhamnose</td>
<td>3-O-Acetyl-2-keto-3-deoxy-erythrose</td>
</tr>
<tr>
<td>9. L-ribose</td>
<td>2-Keto-3-deoxy-erythrose</td>
</tr>
<tr>
<td>10. D-ribose</td>
<td>2-Keto-3-deoxy-erythrose</td>
</tr>
<tr>
<td>11. L-arabinose</td>
<td>2-Hydroxy-1-deoxyribulose</td>
</tr>
<tr>
<td>12. D-arabinose</td>
<td>2-Aldehyde-1-thiouracil</td>
</tr>
</tbody>
</table>

**Legend:**
- **L:** L-configuration
- **D:** D-configuration
- **2-Hydroxy-1-deoxyribulose:** 2-Hydroxy-1-deoxyribulose
- **2-Aldehyde-1-thiouracil:** 2-Aldehyde-1-thiouracil
- **3-O-Acetyl-2-keto-3-deoxy-erythrose:** 3-O-Acetyl-2-keto-3-deoxy-erythrose
- **2-Keto-3-deoxy-erythrose:** 2-Keto-3-deoxy-erythrose

*PM2a titre plate:* 12 substrates, each with a unique reaction type and product type.
Figure A3.1 – The CV for the *C. denitrificans* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V
Figure A3.2 – The CV for the *B. graminisolvans* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V
Figure A3.3 – The CV for the *O. anthropi* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V
Figure A3.4 – The CV for the *S. plymuthica* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V
Figure A3.5 – The CV for the *C. indolis* dual-species MFCs under standard laboratory conditions over a range of -1.5 – 1.5 V
Appendix III – Single and Co-Culture Respiration Rates Comparison.

**Figure A3.1** – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate butyric acid. Species with
Figure A3.2 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate acetic acid. Species with no result shown were found to be unable to respire using the substrate.
Figure A3.3 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate propionic acid. Species with no result shown were found to be unable to respire using the substrate.
Figure A3.4 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate L-lactic acid. Species with no result shown were found to be unable to respire using the substrate.
Figure A3.5 - The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate sucrose. Species with no result shown were found to be unable to respire using the substrate.
Figure A3.6 – The maximum respiration rates for the single species are compared to that of the dual species Biolog experiments for the substrate a-D-glucose. Species with no result shown were found to be unable to respire using the substrate.