THE FEASIBILITY OF METHANE AS A FEED-SOURCE FOR A MICROBIAL FUEL CELL

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Abstract

Microbial fuel cells (MFCs) directly use the energy contained in organic matter to generate electricity. This work investigated the potential of using methane-oxidizing bacteria as biocatalysts in a microbial fuel cell with mediators to facilitate charge transfer. Mediators enhance the power output of MFCs by acting as intermediate electron shuttles between the bacterial cell and the anode. The ability of methanotrophic bacteria to use mediators as the terminal electron acceptor in place of oxygen during methane oxidation has been speculated. However, the existing literature is inconclusive.

An initial attempt to isolate the pure methanotroph which previously indicated an ability to reduce the mediator potassium ferricyanide was unsuccessful. A mixed methane-oxidizing culture was subsequently enriched from soil in the Canterbury region and cultivated in a mineral medium using a fed-batch bioreactor operated at 30 ^{o}C and pH 6.8. Through online monitoring of carbon dioxide production and sodium hydroxide addition, it was confirmed that the mixed culture reduced potassium ferricyanide with methane as the input energy source. In an anoxic environment with 5% methane and 95% nitrogen and an initial ferricyanide concentration of 2 mM, the maximum specific reduction rate was 0.4 mmol/g·h using cells in the exponential phase (DCW 0.2 g/L). Reduction of ferricyanide also occurred in the absence of methane (100% nitrogen) at a rate of 1.3 mmol/g·h. Several scenarios were identified to explain this behaviour, all of which could be applied to a methane-fed MFC:

- A novel oxidation pathway was operational where the methanotrophs directly reduced ferricyanide during methane utilisation. This was unlikely as ferricyanide could not be used as a growth substrate in place of oxygen. However, the ferricyanide also had an inhibitory effect on the cells which may have hindered growth.
- A microbial consortium existed which used intermediate compounds produced by the methanotrophs as a carbon source and ferricyanide as the terminal electron acceptor. This was identified as the most likely scenario, with either ethanol or acetate produced during aerobic methane oxidation as the reducing species.
- The methanotrophs reduced ferricyanide by oxidising internal storage polymers such as glycogen or PHA. This was less likely, as the mixed culture reduced ferricyanide during the exponential growth phase before these polymers tend to accumulate.

Methylene blue, thionine acetate, resorufin and neutral red were also tested as external electron acceptors. In serum vials, methylene blue was reduced only in the presence of methane. However, this result was inconclusive, as reduction did not occur in the bioreactor. Thionine acetate was reduced in serum vials in

the presence and absence of methane at 0.6 mmol/g·h. Resorufin and neutral red were not reduced by the mixed culture.

At the current mediator reduction rates, the estimated power density of a methane-fed MFC was 7 W/ m^3 (anode volume) using ferricyanide and 200 W/m^3 with thionine acetate. This is similar to traditional, liquid-fed MFCs and would be limited to low power consuming devices such as remote biosensors. If future work demonstrates an ability to generate meaningful power densities (> 1kW/m^3), methane-fed MFCs could be an option for small agricultural farms producing biogas via anaerobic digestion. This would compete with gas combustion engines which are limited by high operating costs and low energy efficiencies.

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Table of contents

List of figures

List of tables

Chapter 1: Introduction

1.1 Background

Microbial fuel cells (MFCs) use microorganisms to convert the energy contained in organic matter to electricity. Compared to traditional fuel cells, MFCs are an attractive technology due to moderate operating conditions and the wide range of potential biological feedstocks (Logan, 2008). Promising areas of application include small electrical devices, biological sensors and wastewater treatment. At present, the power density of MFCs is limited due to poor electrical contact between the biofilm and the anode (Schroder, 2007). This can be partially overcome by using soluble mediators which shuttle electrons directly from the bacterial cell and allow the entire culture to be electrically active. The main drawback of using mediators with a liquid feedstock is that the mediators are lost with the waste products (Logan, 2008). Gaseous feedstocks bypass this issue, as the mediators can be retained in the anode chamber during substrate degradation.

Methane is a valuable energy resource and a significant contributor to global warming; thus, efficient capture and use of this gas from natural and anthropogenic environments is becoming increasingly important. Traditional electricity generation from methane involves combustion to drive gas turbines or engines, a process that wastes a significant portion of the available energy as heat (Gür, 2016). Methane-fed MFCs represent an alternative oxidation route which can harness this wasted heat energy and potentially deliver more electricity per unit of fuel consumed. This is a novel concept that has not yet been fully explored.

Successful operation of a MFC requires the microorganism to anaerobically use the anode as the electron acceptor. This can be achieved by many species, as oxygen is usually only required as the electron 'sink' at the end of the electron transport chain. However, aerobic methane-oxidizing bacteria or 'methanotrophs' also require oxygen to activate the methane monooxygenase enzyme which converts methane to methanol in the first step of methane oxidation (Hanson & Hanson, 1996). The ability to anaerobically oxidize methane is limited to certain slow-growing species which at present are difficult to isolate.

Evidence of methane oxidation by an aerobic methanotroph in the absence of oxygen was first presented by Evelyn (2012). In this work, it was speculated that the mediator ferricyanide could be reduced to ferrocyanide in a novel, oxygen-free methane oxidation pathway. If true, a MFC using mediators to transfer charge to an electrode with methane as the energy source could be implemented. This would overcome several limitations faced by internal combustion engines and simultaneously curb emissions of this potent greenhouse gas.

1.2 Research objectives

This research project will build on the hypothesis that an alternative electron acceptor can be used by aerobic methane-oxidizers to facilitate charge transfer in a MFC. The overall objectives are to:

1. Confirm the oxidation of methane to carbon dioxide by an aerobic methanotroph culture and the simultaneous reduction of mediator.

2. If reduction is possible, determine the viability of the mediator as a growth substrate. Bioprocessing parameters such as growth rate, cell yield and mediator reduction rate will be evaluated.

3. Investigate the engineering feasibility of a methane-fed MFC.

Experiments will be conducted in a bench scale bioreactor with control over environmental conditions such as pH and temperature. If the hypothesis is proven, it will justify development of a system that can achieve high methane conversion and power density. Identification of a novel methane-oxidation pathway will also provide the impetus for pursuing fundamental scientific research regarding the metabolic flexibility of aerobic methanotrophs.

1.3 Thesis organization

This thesis has been divided into seven chapters. Chapter one introduced the topic of interest and stated the key objectives of the project. The remainder of the thesis is organised as follows:

Chapter 2: A general overview of methane, methanotrophic bacteria and microbial fuel cells. The advantages and disadvantages of methane-fed MFCs are also discussed.

Chapter 3: Describes the materials and methods used for investigating the hypothesis.

Chapter 4: Discusses the growth dynamics of the mixed methane-oxidizing culture used to investigate the hypothesis and the challenges faced with isolation of a pure methanotroph. The proof-of-concept study then determines the ability of the mixed culture to reduce ferricyanide and describes the control experiments performed and the resulting implications. Finally, the mechanism of reduction is investigated and alternative mediators explored.

Chapter 5: Discusses the practical implications of a methane-fed MFC including estimates of power output based on mediator reduction rates and the potential application in New Zealand.

Chapters 6 & 7: Summarises the key findings from the report and recommends future directions.

Chapter 2: Literature review

2.1 Energy in the 21st century

With the population of the earth expected to reach 10 billion by 2050, enormous pressure will be placed on our resources; resources which are already dwindling as a result of the past century's rapid economic development. Consumption of energy in 2014 was close to 13,000 million tonnes of oil equivalent (Mtoe), nearly double the consumption in the 1970s (IEA, 2015). The population has also nearly doubled in the same period of time. Although fossil fuels have allowed us to flourish in the 20th and 21st centuries, an economy built on non-renewable resources is unsustainable and a shift in our way of living is required.

According to a report published in 2015, oil remains the leading fuel with 35.4% of the Total Primary Energy Supply, followed by natural gas at 25.6% and coal at 19.3%. Nuclear and renewables (biofuels, hydro-electric, solar and wind) make up 9.9% and 9.5% respectively (IEA, 2015). The 2015 climate talks in Paris emphasized the need to move away from conventional energy sources if we are to limit global temperature rises to 2 ^{o}C (Willis, 2014). This is mainly due to the vast quantities of carbon dioxide and environmental pollutants which are released into the atmosphere when fossil fuels are burned.

Production of commodities via biological processes is a promising alternative, with industries such as pharmaceuticals and food/beverage processing already exploiting microorganisms to generate high value end products. The main advantages over conventional fuel sources include the carbon neutrality of biological processes and the widespread availability of carbon based waste streams (Li & Khanal, 2016). Fuels which can be produced include methanol, ethanol, hydrogen and methane, all of which can be used in transportation, heating or electricity generation. Other examples include bio-plastics, animal feed supplements and chemicals such as acetic acid (Wall et al., 2008). The coming decades will see a greater number of bio-based products available on the market, fuelled by advancements in synthetic biology and a growing trend away from fossil fuels.

2.2 Methane as a biological resource

Methane is widely regarded as the next generation carbon feedstock. It is primarily sourced from natural gas and has recently surpassed coal as an energy supplier in developed countries such as the Unites States. The main advantage of natural gas over coal and oil is that less carbon dioxide is produced for the same amount of energy, as well as fewer environmental pollutants such as nitrous oxides. Industrial uses include heating and electricity generation via combustion, steam reforming for syngas production and as a hydrogen supplier for the Haber-Bosch process. Chemicals such as methanol and acetic acid are also produced using methane as the carbon feedstock (EIA, 2017).

Methane is a particularly potent greenhouse gas, estimated to be 20 times more effective at trapping heat in the atmosphere than carbon dioxide (Knittel & Boetius, 2009). Although a significant amount of the available methane is captured, approximately 800 Tg is released into the environment each year, with 60% coming from anthropogenic sources (Strong et al., 2015). Current mitigation strategies from sectors such as oil and gas extraction include flaring or reinjection to enhance recovery which is a tremendous waste of a valuable resource (Labinger & Bercaw, 2002). Methane produced from the decomposition of organic matter in landfills and on farms can also be captured and utilized much more efficiently.

The major hurdle of a gas-driven economy is lack of infrastructure for transporting and distributing gas from production sites to populated areas. Conversion of gas to liquid fuels via chemical processes is a potential stepping stone. However, for technologies such as Fischer-Tropsch, the carbon conversion efficiency is less than 50% and an input of energy is required (Fei et al., 2014). Thus, in many cases, chemical conversion of methane is both uneconomical and harmful to the environment.

Bioconversion has been identified as an alternative to chemical based synthesis (Fei et al., 2014). Methanotrophs are the bacteria esponsible for the oxidation of methane in the environment and can produce fuels such as methanol and butanol, as well as other useful commodities such as single celled proteins and bio-plastics (Strong et al., 2015). Advantages over traditional pathways include the self - sustaining nature of biological process, lower temperature requirements and a more direct conversion route. The major drawbacks preventing commercialization include the poor solubility of methane in fermentation broths and low productivity (Strong et al., 2016a). This is being addressed by development of new reactors such as the U-loop fermenter, a technology currently employed by companies such as *Calysta* and *UniBio* for production of animal feed proteins using natural gas (Calysta, 2017; Unibio, 2017). Future advancements in metabolic engineering will also improve product yields.

2.3 Anaerobic digestion

Natural gas from underground reservoirs will provide a significant portion of future energy needs as well as a platform for investigating methane-bioconversion. However, it is not the answer to long term energy sustainability. This is particularly relevant for countries such as New Zealand, with 14% of the total consumer energy demand supplied by natural gas and only 1-2 decades remaining in the reserves (MBIE, 2015). ‘Biogas’ is a renewable source of methane produced anaerobically during the decay of organic matter in a process called methanogenesis. This is an important part of the carbon cycle and occurs naturally in wetlands, oceans and lakes, as well as more extreme environments such as hydrothermal vents and hot springs. Methane produced during methanogenesis is oxidized by methanotrophs, hence closing the carbon cycle (Fig. 2-1)

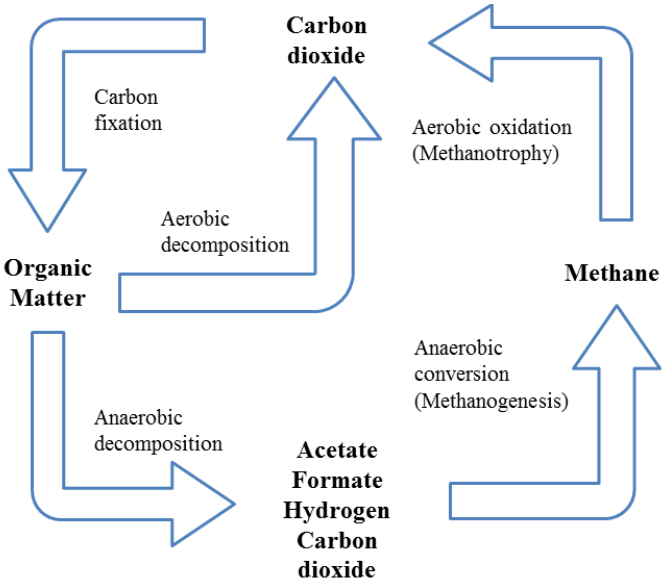


Figure 2-1: Carbon cycle in nature (Wall et al., 2008)

Anaerobic digestion replicates part of the carbon cycle in a controlled environment. Complex feedstocks such as municipal, agricultural and food waste are broken down by a microbial consortium to produce organic acids which ultimately get converted to methane-enriched biogas via methanogenesis (Khanal, 2011). This process significantly reduces the solid waste ending up in landfills and has found worldwide commercial success. Through proper waste management strategies, the biogas can also be collected and used as a renewable fuel and/or feedstock. Large scale waste treatment plants recycle the gas to maintain digestion temperature, with the excess simply being flared to the atmosphere or used to generate electricity (Bioenergy Association, 2016).

One of the major drawbacks of biogas is the cost and technical difficulties associated with removing contaminants (particularly hydrogen sulfide and siloxanes); unfortunately, this removal is necessary for use in gas turbines and combustion engines. However, with increasing energy prices, anaerobic digestion is likely to become more favorable in the future and could potentially offset natural gas usage by 20%-30% (Khanal, 2009). Biogas could also be used in place of sugar- based crops as a biological feedstock; this would have a two- fold benefit of mitigating greenhouse gas emissions and increasing the land available for food production.

2.4 Aerobic methanotrophs

Methane oxidation occurs aerobically or anaerobically in the environment, with remarkably different pathways of carbon assimilation. Aerobic methanotrophs are the most widely studied form and show the most promise for biocatalysis.

2.4.1 Metabolic pathways

The overall reaction for biological methane oxidation to carbon dioxide using oxygen as the terminal electron acceptor is described by Eq. 1 (Hanson & Hanson, 1996).

$$CH_4 + 2O_2 + Nutrients \rightarrow CO_2 + H_2O + Biomass + Energy \quad (1)$$

The process is catalyzed by several enzymes, the most unique being the methane mono-oxygenase enzyme (MMO) which converts methane to methanol by splitting molecular oxygen and inserting an atom into methane, with the other being used to form water (Gorgen et al., 2005). It occurs in two forms; particulate (pMMO) and soluble (sMMO). The pMMO enzyme is membrane bound and contains copper at the active site whereas sMMO contains iron; environmental conditions therefore play a key role in determining which form of the enzyme is used. It has been reported that methanotrophs which express the pMMO form have higher growth yields and higher affinity for methane (Hanson & Hanson, 1996). The sMMO form requires NAD(P)H as a source of electrons, however the physiological electron donor for the pMMO form has yet to be uncovered (Kalyuzhnaya et al., 2015).

Following methanol formation, the methanol dehydrogenase enzyme catalyzes the removal of hydrogen to produce formaldehyde (Hwang et al., 2014). This can be further oxidized into carbon dioxide to produce reducing equivalents or converted into biomass via several pathways (Fig. 2-2). Two major groups of aerobic methanotrophs, based on their physiology and pathway for formaldehyde assimilation, have been identified:

Type I**:** Belong to the gamma subunit of proteobacteria and utilise the Ribulose Monophosphate Pathway RuMP for carbon assimilation. Growth is generally more efficient than Type II methanotrophs and is favoured by low levels of methane and high nitrogen/copper levels. These organisms tend to exhibit the particulate form of the MMO enzyme. Within this group, a separate Type X (consisting of the *Methylocaldum* and *Methylomonas* genus) has been distinguished. These species contain enzymes from the serine cycle and grow at higher temperatures than other Type I and II methanotrophs (Hanson & Hanson, 1996).

Type II: Belong to the alpha subunit of the proteobacteria and utilise the Serine pathway for carbon assimilation. In contrast to Type I Methanotrophs, these species generally grow better in higher methane concentrations and lower copper/nitrogen levels. They tend to exhibit the soluble form of the MMO enzyme (Hanson & Hanson, 1996).

A further genus '*Methylacidiphilum'* belonging to the Verrucomicrobia phylum has also been described which oxidize methane in extreme conditions (high temperature, low pH) and fix CO_2 via the Calvin Benson Bassham (CBB) cycle (Khadem et al., 2011).

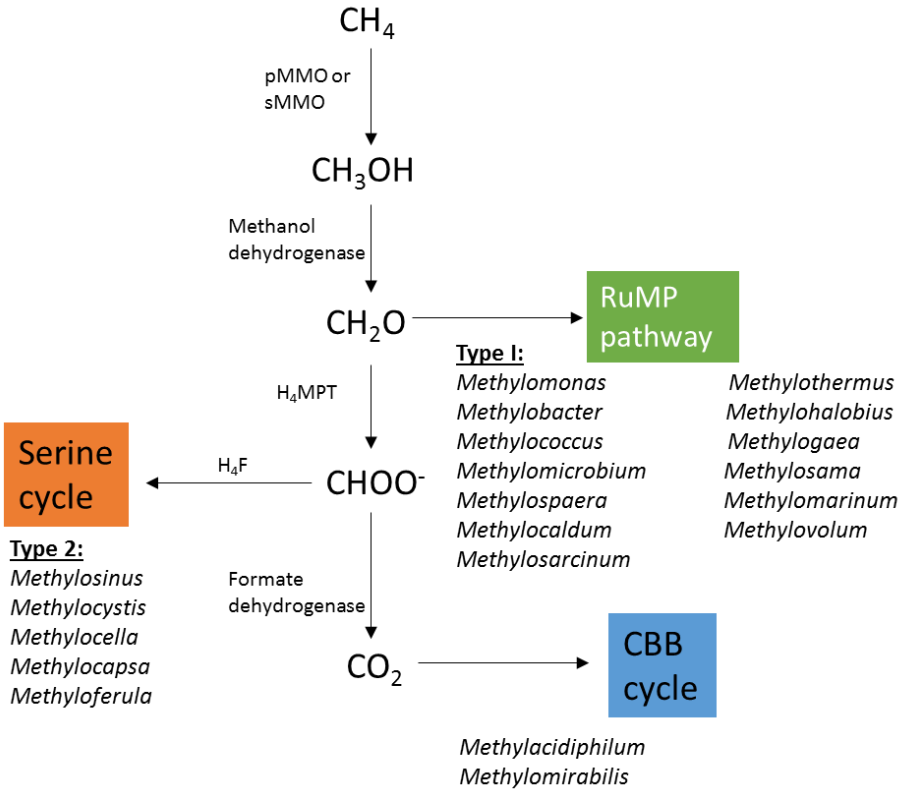


Figure 2-2: Pathways and enzymes for aerobic methane oxidation (Hwang et al., 2014).

An ecologically important reaction is methane oxidation coupled to denitrification (AME-D) which plays a significant role in the global carbon and nitrogen cycles. AME-D traditionally involves a microbial consortium containing nitrate-reducing bacteria which consume organic intermediates of methane oxidation such as methanol or acetate (Zhu et al., 2016). Interestingly, recent studies have shown direct nitrate reduction by the novel strain *Methylomonas denitrifcans sp.* FJG1during methane oxidation (Kits et al. 2015b). Similar studies of *Methylomicrobium album* strain BG8 also demonstrated the coupling of C_1 and C_2 compounds to nitrite reduction in hypoxic conditions (Kits et al., 2015a). Thus, AME-D may not be dependent on a syntrophic relationship with nitrate-reducers as previously assumed which represents a significant metabolic diversity of aerobic methanotrophs. This process has gained attention due to the potential combination of methane bioremediation with denitrification of wastewater and waterways (Zhu et al., 2016).

2.4.2 Alternative substrates

Most aerobic methanotrophs have been reported as obligate methylotrophs which grow primarily on methane and in some cases are able to consume other products generated during the metabolism such as methanol and formaldehyde (Bowman, 2011). The speculative reason for the obligatory nature of

methanotrophs is the absence of key enzymes and assimilatory pathways required to utilize complex sugars and organic acids. The first report on multicarbon substrate consumption of the Type II methanotroph *Methylocella* was by Dedysh et al. (2005). The culture grew preferentially using acetate and switched to methane uptake upon acetate depletion. In addition, all of the species tested could grow using compounds such as ethanol and malate. Facultative methanotrophy of a novel H2s strain from the *Methylocystis spp*. using acetate as the secondary carbon source was further demonstrated by Belova et al. (2011), indicating that this survival technique may be widespread amongst Type II methanotrophs.

Co-oxidation of ammonia with methane (Stein & Klotz, 2011) is well-established amongst aerobic methanotrophs and occurs due to the similarity between MMO and the equivalent ammonia mono-oxygenase (AMO) (Stein et al., 2001; Whittenbury et al., 1970). Despite the ability of the MMO enzyme to convert ammonia to hydroxylamine, methanotrophs are unable to derive energy from ammonia oxidation. The competition of ammonia for the same enzyme and the production of toxic intermediates such as nitrite can also have inhibitory effects on methane oxidation (Nyerges & Stein, 2009).

2.5 Anaerobic methanotrophs

Anaerobic oxidation of methane (AOM) occurs in anoxic sediments and seawater and is estimated to consume 7%-25% of environmental methane production (Knittel & Boetius, 2009). Literature describes three pathways; sulfate dependent, nitrate/nitrite dependent and metal ion dependent methane oxidation. Commercial applications have not been explored, most likely due to slow growth rates and an incomplete understanding of the metabolism.

2.5.1 AOM coupled to sulfate reduction

It was initially proposed that anaerobic methane oxidation was carried out by methanogens in a consortium with sulfate reducing bacteria (SRB) under low hydrogen concentrations (Eq. 2) (Caldwell et al., 2008).

$$CH_4 + {SO_4}^{2-} \rightarrow {HCO_3}^{-} + HS^{-} + H_2 \quad (2)$$

The free energy of this reaction is very low (-16 kJ/mol) and despite efforts to prove this theory, methanogens operating in the back reaction have yet to be isolated. In the late 1990's, Hinrichs et al. (1999) discovered that a group phylogenetically related to known methanogens were indeed carrying out methane oxidation in cooperation with sulfate reducing bacteria. Three groups of anaerobic methanotroph (ANME) archaea have subsequently been identified; ANME-1, ANME-2, ANME-3. Due to the necessary coupling to SRB as a source of electrons, pure cultures of anaerobic methanotrophs are very difficult to isolate (Cui et al., 2015). Recent advancements showed the decoupling of certain ANME-2 organisms

from SRB using extracellular electron acceptors such as AQDS, ferric citrate and humic acids which may be a step towards successful isolation (Scheller et al., 2016).

2.5.2 AOM coupled to nitrate reduction

The prevalent bacterial division associated with anaerobic methane oxidation coupled to denitrification is NC10. Ettwig et al. (2010) proposed a new species *'Methylomiribilis oxyfera'* as the dominant species within the NC10 phylum. Although the mechanism is unclear, it has been postulated that oxidation occurs via an intra aerobic pathway where nitrite is ultimately decomposed into dinitrogen and oxygen (Eq. 3).

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad (3)$$

The oxygen produced during the breakdown of nitrite can then be used by methane mono-oxygenase in the conventional pathway which is supported by the presence of a complete genome for aerobic methane oxidation. This bacteria is also able to assimilate carbon via the Calvin Benson cycle, suggesting that autotrophy is more common with methanotrophs than previously assumed (Rasigraf et al., 2014). *Methanoperedens nitroreducens* is another recently identified species from the ANME-2d group which couples methane oxidation directly with nitrate reduction to nitrite. The nitrite is then used by a partner organism which reduces it to nitrogen (Eq. 4) (Haroon et al., 2013).

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \quad (4)$$

In this case it is speculated that denitrification genes were transferred from a bacterial donor, allowing for a completely novel pathway of methane oxidation.

Although there are still unanswered questions regarding this process, it is evident that, in addition to AME-D, anaerobic methane oxidation coupled to denitrification also plays an important role in the cycling of carbon and nitrogen in natural ecosystems (Caldwell et al., 2008).

2.5.3 AOM coupled to metal ion reduction

Metal ions such as Mn^{2+} and Fe^{3+} as a terminal electron acceptor of AOM was first presented by Beal et al. (2009) (Eq. 5,6).

$$CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O \quad (5)$$

$$CH_4 + 8Fe(OH)_3 + 15H^+ \rightarrow HCO_3^- + 8Fe^{2+} + 21H_2O \quad (6)$$

Although members from all three ANME groups were identified in the consortium, the microbes responsible for methane oxidation could not be pinpointed. It has been suggested that anaerobic methanotrophs may have evolved from using metal oxides prior to forming a syntrophic relationship with SRB which overall is a more thermodynamically favorable reaction (Cui et al., 2015). Metal ion reduction has subsequently been identified in environments ranging from freshwater to brackish sediments.

2.6 Microbial fuel cells

Electricity production accounts for 18% of the total global energy consumed each year. Although the use of renewables and nuclear power is on the rise, more than 60% comes from combustion of fossils fuels (IEA, 2015). Thus, development of technologies which can generate electricity more efficiently or use alternative energy sources (e.g. solar or biomass) is an important area of energy research.

2.6.1 General overview

Microbial fuel cells (MFCs) use microorganisms as biocatalysts to convert the chemical energy stored in organic matter to electrical energy. During cellular respiration, electrons produced from substrate degradation flow through various complexes which causes protons to be pumped out of the intermembrane space into the cell cytosol. This creates an electrochemical gradient which forces hydrogen ions to flow back through the adenosine triphosphate (ATP) synthase complex, transforming adenosine diphosphate ADP and phosphate into ATP (oxidative phosphorylation). The electrons are transferred to the terminal electron acceptor (usually oxygen) which diffuses into the cell from the external environment (Hogg, 2013). MFCs exploit this process by transferring the electrons to an anode prior to reduction of the terminal electron acceptor at the cathode (Fig. 2-3).

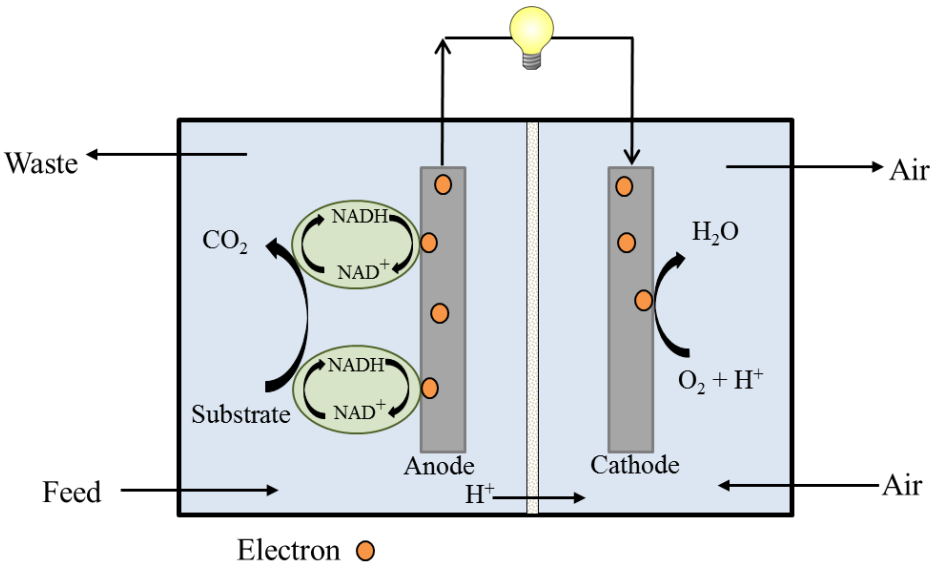


Figure 2-3: Microbial fuel cell schematic (Schroder, 2007).

Substrate degradation occurs anaerobically in the anode compartment which allows the anode to act as the electron acceptor. As the electrons flow through an external circuit, the excess energy usually lost as heat during the normal respiratory process is captured in the form of electricity (Rahimnejad et al., 2015).

MFCs are gaining attention in the scientific community due to the mild operating conditions, self-regulating nature of bacteria and the wide variety of substrates which can be used; this ranges from simple organics such as glucose to more complex feedstocks such as industrial/domestic wastewater (Pant et al., 2010). Expensive catalysts for driving the anode reaction are also not required. At present, the power density of MFCs is limited, with an average output of 40 W/m^3 (anode volume) reported using wastewater (Pham et al., 2006). The highest value achieved was 2.15 kW/m^3 using *G. sulfurreducens* grown on acetate (Nevin et al., 2008).

Most of the MFC research to date has focussed on aqueous waste streams. Direct gaseous substrate conversion has not been extensively studied, most likely due to the difficulties associated with gas/liquid mass transfer. The most recent efforts were made by Li et al. (2013) using toluene, a common environmental pollutant. By optimizing the design of the anode, a removal rate comparable to traditional biofilters was achieved with a power density of 4 W/m^3. Additionally, Kim and Chang (2009) achieved 6.4 W/m^3 using syngas, a by-product from industries such as steelmaking; in this case, carbon monoxide was first converted into organic intermediates and then oxidized by electroactive bacteria at the anode. Potential carbon feedstocks that could be used in a gas-fed MFC include ethanol from the brewing industry, gasified and pyrolyzed biomass and biogas (Evelyn et al. 2013).

2.6.2 Electron transfer

Harnessing of electrical energy *from Saccharomyces cerevisiae* and *Escherichia coli* was first identified by Potter (1911) using glucose as a substrate. However, low power densities meant that MFCs were disregarded until the 1990s when it was discovered that certain compounds or 'mediators' could be added to the anode to facilitate electron transfer (Logan, 2008). Further breakthroughs identified certain 'self-sufficient' species and it is now generally accepted that electron transfer occurs via three mechanisms (Khanal, 2009):

1. Outer membrane electron carriers such as cytochrome c, quinones and flavins. This has been shown in certain bacterial species such as *G. sulfurreducens* and is dependent on direct contact of the cells with the electrode (i.e. biofilm formation) (Magnuson et al., 2001).

2. Electron shuttling via self-produced mediators. Following the initial discovery by Kim et al. (2002), several species capable of excreting soluble electron shuttles have been identified, for example *Geothrix fermentans, Shewanella Spp. and Pseudomonas aeruginosa* (Bond & Lovley, 2005; Jain et al., 2012). Further to this, *P. aeruginosis* was shown by Rabaey et al. (2005) to

produce the secondary metabolite pycocyanin, which not only enhanced electron transfer in *P. aeruginosa* but also other bacteria species present in the consortium.

3. Self-formed bacterial nanowires. It has been reported by Gorby et al. (2006) that the metal reducing species *Geobacter* and *Shewanella* can form conductive bacterial nanowires. These nanowires attach from a distance to the electrode and are an efficient form of electron transfer. Several other species have been identified such as the thermophilic *Pelotomaculum thermopropionicum*, showing that nanowires are not limited to metal reducing bacteria.

Traditional MFCs require the formation of an active biofilm on the anode surface which is the major bottleneck preventing widespread application (Schroder, 2007). The electrical contact can be enhanced by selecting more electroactive organisms or by using mixed cultures which allow for interspecies electron transfer (Gorby et al., 2006). New reactor configurations and materials for the anode are also being investigated (Du et al., 2007; Logan, 2008).

2.6.3 Thermodynamic considerations

The thermodynamic feasibility of a MFC depends on the spontaneity of the redox reaction occurring at the electrodes, as determined by the Gibbs energy change (Eq. 7) (Logan et al., 2006).

$$\Delta G_R = -nFE^o + RTln(\Pi) \quad (7)$$

Where ΔG_R is the Gibbs energy change (J/mol), n is the number of electrons involved in the reaction, F is Faradays constant (96,485 C/mol) and E^o is the standard cell potential (V). R is the universal gas constant (8.314 J/mol.K), T is temperature (K) and Π is the reaction quotient (activity of the products divided by the reactants).

The Gibbs energy change represents the maximum amount of energy which can be converted into electricity and must be negative for a reaction to occur. A dictating factor is the standard cell potential, determined by the difference in the reduction potential between the reducing agent at the anode and the oxidizing agent at the cathode (Eq. 8) (Logan, 2008).

$$E^o{}_{cell} = E^o{}_{cathode} - E^o{}_{anode} \quad (8)$$

As shown in Eq. 7, the actual cell potential depends on additional factors such as temperature and activity of the redox species. Energy loss will also occur, caused by several 'polarization' effects (Logan et al., 2006):

- Activation overpotential: Energy required for overcoming the slow reaction rates at the electrodes.

- Concentration overpotential: Formation of a diffusion layer at the electrode surface which limits the rate of electron transfer.
- Resistance overpotential: Ohmic losses in the electrolyte and/or electrodes.

Without taking these factors into consideration, the cell voltage $E^o{}_{cell}$ can be used to estimate the power output of the fuel cell under standard conditions (Eq. 9)

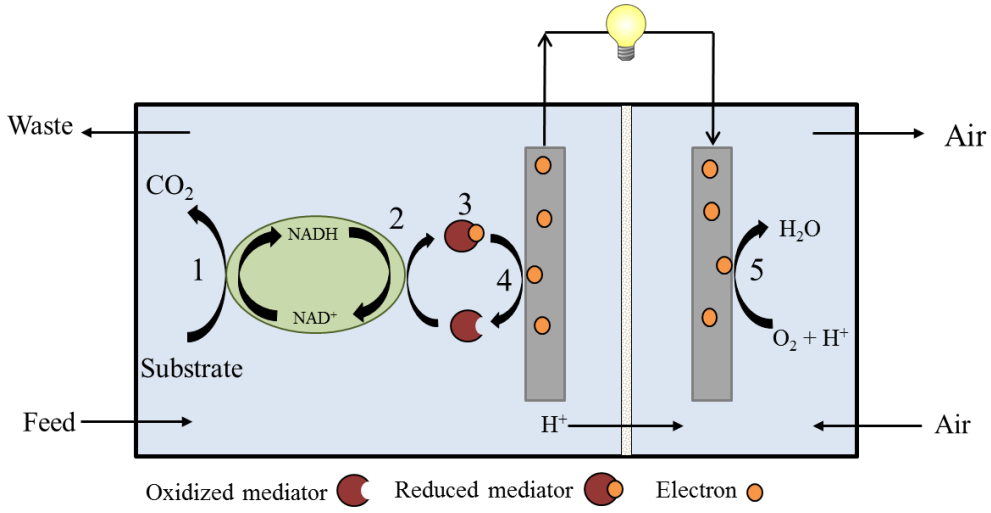
$$P = \frac{{E^o{}_{cell}}^2}{R_{ext}} \tag{9}$$

Where R_{ext} is the external resistance, calculated from ohms law (V=IR). For comparison between MFCs, power output is often normalized to the volume of the anode compartment or surface area of the anode (Logan et al., 2006).

2.6.4 Mediators

2.6.4.1 Background

Mediators are externally added, intermediate compounds which oxidize charge carrying compounds such as NADH and shuttle electrons directly to the anode (Rahimnejad et al., 2012) (Fig. 2-4).



1. Electron transfer from fuel to bacterial cell
2. Transfer of electrons from bacterial cell to the oxidant/mediator
3. Transport of charge carrying species through electrolyte to anode
4. Adsorption of charge carrying species on the anode
5. Transfer of electrons to the oxidant at the cathode

Figure 2-4: Electron transfer in a MFC with NADH oxidation coupled to mediator reduction. Electrochemical and biochemical resistances to electron transfer are also shown (Logan et al., 2006).

Mediators allow the entire culture to be electrically active which can significantly improve the power density, as charge transfer is not limited to the anodic biofilm. For example, Babanova et al. (2011) reported a power density of 22 W/m^3 using *Candida melibiosica 2491* with methylene blue as the electron mediator compared to 0.7 W/m^3 in a mediator-less fuel cell. Mediators such as ferricyanide have also been used in the cathode compartment in place of oxygen, which is limited by the solubility of oxygen in the electrolyte (Logan, 2008).

The major drawback of using mediators in a liquid feed stream is that constant replenishment is required otherwise the mediator, which is potentially toxic to the environment, is flushed out with the waste products. Due to this, mediators have been largely disregarded in MFC research. Recently, Fujita et al. (2014) investigated the immobilization of mediators on the anode and it was shown that the cell could be refuelled multiple times without loss of activity. However, charge transfer was still limited to the bacteria in direct contact with the electrode.

Evelyn et al. (2013) identified the potential of using mediators with gaseous feedstocks in a 'flow-battery' arrangement. An anaerobic, bio-trickling filter was suggested for degradation of the gaseous contaminant using mediators as the electron acceptor. The mediators then re-oxidize in a fuel cell and are not lost with the gaseous effluent (Fig. 2-5).

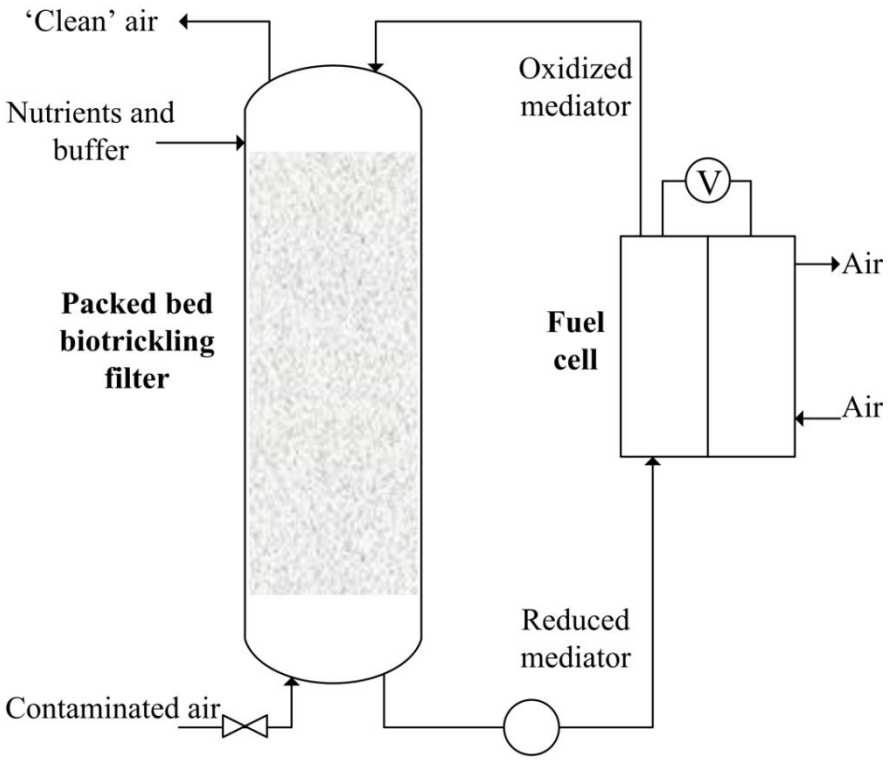


Figure 2-5: A 'flow battery' arrangement for generating electricity from gaseous feedstocks using mediators to facilitate charge transfer (Evelyn et al., 2013).

Gaseous contaminants which have been successfully implemented in bio-filters include alcohols, VFA's, VOC's and methane (Devinny et al., 1998). A drawback is the accumulation of biomass in the reactor greatly impedes gas flow. This can be combated by periodically washing the biofilm and replacing with fresh nutrients. Alternative process strategies would need to be investigated to limit the loss of mediators while maintaining a high level of substrate conversion.

2.6.4.2 Selection criteria

As with all redox reactions, the ability of a mediator to extract electrons from a charge carrying species depends on the Gibbs energy change (J/mol) (Eq. 7). For a reaction to occur, the reduction potential of the mediator must be higher than the biological electron carrier to give a negative Gibbs energy. In nature, bacteria maximize energy/ATP production by using oxygen as the terminal electron acceptor which has a high reduction potential (+0.82 V) (Hogg, 2013). Other common electron acceptors include nitrate (+0.74V) and metal ions such as Fe^{3+} (+0.77 V). For mediated-MFCs, it is important that species with more positive reduction potentials such as oxygen are not present in the environment to prevent competing reactions. A wide range of mediators exist with different reduction potentials exist for use in biological applications (Table 2-1).

Table 2-1: Reduction potential of commonly used exogenous mediators (Katz et al., 2003).

Mediator	**Reduction potential (V)**
AQDS	-0.184
Methylene blue	-0.021
Neutral red	-0.325
Potassium ferricyanide	+0.36
Prussian blue	+0.38
Resorufin	-0.051
Thionine acetate	+0.064
Toluidine blue	+0.034

From a thermodynamic stand-point, potassium ferricyanide and prussian blue are the most favourable for driving redox reactions due to more positive reduction potentials. However, the viability is dependent on the point at which electrons are removed from the electron transfer chain (Schaetzle et al., 2008).There are many intracellular electron carriers which can be used and regulated by the cells to give favourable reactions (Table 2-2).

Table 2-2: Reduction potential of species involved with the electron transport chain (Schaetzle et al., 2008).

Redox species	**Reduction potential (V)**
$NAD^+/NADH + H^+$	-0.32
$FAD^+/FADH_2$	-0.22
Cytochrome b (+3) / Cytochrome b (+2)	0.07
Ubiquinone (ox) / Ubiquinone (red)	0.10
Cytochrome c (+3) / Cytochrome c (+2)	0.22
Fe^{3+}/ Fe^{2+}	0.77
$½ O_2 + 2H^+/ H_2O$	0.82

The feasibility of a particular mediator is difficult to predict without knowing the identity of the biological electron carrier. The mechanism by which the mediator interacts with the cell is also important. They may be hydrophilic, unable to cross the cell membrane or lipophilic, able to enter the cell and interact with intracellular redox couples. In most prokaryotes, electron carriers such as cytochrome c are accessible extracellularly by hydrophilic mediators such as ferricyanide. In contrast, eukaryotes such as *S.cerevisiae* have the majority of their reductive species located within the cytoplasm and mitochondria and therefore need mediators such as toluidine blue which can cross the cell membrane. They often exist in double mediated systems, shuttling electrons to transport systems in the periplasm which transfer the electrons to a hydrophilic mediator located outside the cell (Rawson et al., 2014). In addition, mediators must be stable in a range of operating conditions, soluble in the aqueous phase and non-toxic to living cells (Park & Zeikus, 2000).

Regardless of the mechanism for driving mediator reduction, the maximum energy available for capture from a mediated- MFC depends on the difference between the reduction potential of the mediator and the oxidizing species at the cathode (usually oxygen). In this regard, mediators with more negative reduction potentials are favourable. However, the potential must still be positive enough to be reduced by the biological electron carrier.

2.6.5 Methane in a MFC

Generation of electricity from methane traditionally occurs via combustion, with the energy from the high temperature exhaust gases used to drive an engine or turbine. Industrial power plants often use a Combined Cycle Gas Turbine (CCGT) which captures the excess energy produced during combustion to heat steam which powers an additional turbine. The average efficiency of gas-fired plants is 44.8%, with many operating at efficiencies as low as 30% (Gür, 2016). In addition, gas fired engines/turbines are sensitive to changes in the inlet gas composition and require heavy maintenance. The gas must often be

stripped of corrosive species such as hydrogen sulphide using costly absorption methods prior to combustion (Abbott et al., 2012). Methane/natural gas can also be used in a non-microbial fuel cell. Recent efforts were made by Joglekar et al. (2016) for low temperature (80 ^{O}C) bond activation but the power density of the fuel cell was still too low for commercial application; this makes combustion the preferred oxidation route.

A methane-fed MFC is attractive as the excess heat energy lost during combustion can theoretically be captured; this means a higher amount of usable energy produced per unit of fuel consumed. Coulombic efficiencies greater than 80% have been reported for MFCs (Devasahayam & Masih, 2012; Rabaey et al., 2003), with the remaining energy used by the bacteria for growth and maintenance (Logan et al., 2006). In addition, a MFC would not require pre-treatment of the inlet gas and with fewer mechanical parts and lower temperature requirements, the operating costs would be lower than a combustion engine or turbine.

Although the use of methane as a fuel for electricity generation in a MFC has been patented by Girguis and Reimers (2011), electrogenic activity from a methanotroph culture has yet to be reported (Popov et al., 2012). This is most likely due to the oxygen requirement of the MMO enzyme which prevents the anaerobic transfer of electrons to an electrode. The low solubility of methane is another major issue which will limits the rate of substrate conversion to carbon dioxide; for this reason, methane-fed MFCs are unlikely to compete with gas turbines for large scale electricity generation. Smaller operations with a consistent supply of methane such as coal seams or biogas produced from anaerobic digestion may have potential, as gas turbines are generally less economical at this scale (EPA, 2011).

Chapter 3: Materials and methods

3.1 Culturing of microorganisms

3.1.1 Nutrient preparation

The nutrient medium was prepared according to Bowman (2011) in deionized water and contained, in g/L: NH_4Cl 0.5, $MgSO_4.7H_2O$ 1, Na_2HPO_4 0.284, KH_2PO_4 0.272, $CaCl_2.2H_2O$ 0.134, $FeCl_3$ 0.002, EDTA 0.004, yeast extract (where specified) 0.01, cycloheximide (where specified) 0.1. The medium was adjusted to pH 6.8 using 0.25 M NaOH or 0.25 M H_2SO_4.

Trace element solution was added to 0.1% v/v and contained, in g/L: EDTA 0.5, $FeSO_4.7H_2O$ 0.2, H_3BO_3 0.03, $ZnSO_4.7H_2O$ 0.01, $MnCl_2.4H_2O$ 0.003, $CoCl_2.6H_2O$ 0.02, $CuSO_4.5H_2O$ 0.1, $NiCl_2.6H_2O$ 0.002, $Na_2MoO_4.2H_2O$ 0.004,

3.1.2 Stock revival

The previous stock culture used by Evelyn (2012) was rapidly thawed from -80 ^oC to room temperature (~25 ^oC) and cultivated in liquid media as follows:

1. A volume of 12 ml of nutrient medium was added to 60-mL serum vials, sealed with butyl rubber stoppers and autoclaved in a New Brunswick AC-48 autoclave at 121 ^oC for 20 minutes. The vials were then crimp-sealed with aluminium caps.

2. A 1-L Tedlar bag was filled with Ultra-High-Purity methane from a gas cylinder through a 0.22 µm air filter using an Alicat mass flow controller. A volume of 12 mL of methane was then injected into the headspace of each serum vial using a needle, syringe and a three way valve.

3. Serum vials were inoculated in a laminar flow bio-cabinet with 1.2 mL of thawed culture using a needle and syringe and placed in an incubator/shaker at 120 rpm and 30 ^oC.

3.1.3 Enrichment of mixed culture from soil

A 25-g sample of top soil supplied by Parkhouse Garden Supplies in Canterbury, NZ (July 2016), was mixed in a 250-mL conical flask of sterile nutrient solution using a magnetic stirrer and refrigerated at 4 ^oC to allow the solids to settle. The resulting supernatant was then filtered through cheesecloth to remove any coarse particles. The regime outlined above for serum vial cultivation was repeated using a 10% v/v inoculum of decanted soil extract.

An alternative cultivation method was also used where gas containing 5% methane and 95% air was supplied continuously using a double stacked peristaltic pump with LS14 and LS16 pump heads to give a total flowrate of 1 mL/min. Methane was supplied from a Tedlar gas bag and air directly from the atmosphere through a 0.22 m filter to prevent contamination. The reactor vessels were 250-mL Schott bottles sealed with a rubber bung. The rubber bung had two identical holes with 1/8" stainless steel piping in each. One pipe was immersed in the solution to allow for aseptic sampling and gas bubbling and the other was positioned in the headspace for the exhaust gas. The culture was mixed on a magnetic stirrer/heater at room temperature (~25 ^{o}C) (Fig. 3-1).

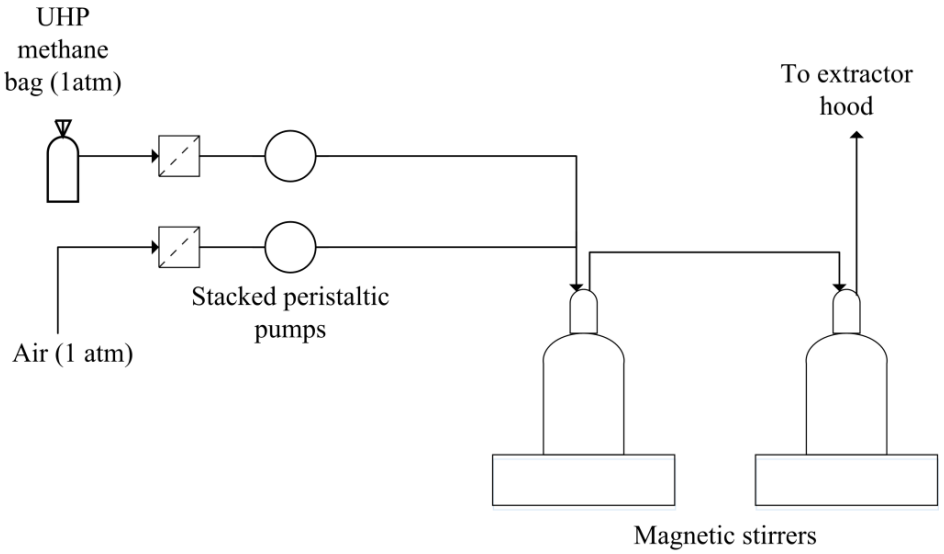


Figure 3-1: Methanotroph culturing method in 250-mL Schott bottles using 5% CH_4 and 95% air at 1 mL/min.

3.1.4 Isolation of aerobic methanotrophs

Serial dilution: 1 mL of cells cultivated in serum vials were removed and inoculated into 9 mL of fresh medium, producing a 10% diluted solution. The culture was agitated on a vortex mixer and used as the inoculum for the next dilution; this was repeated 10 times to give a final dilution of 10^{-9}. Incubation occurred at 30 ^{o}C and 120 rpm.

Plating: Nutrient medium containing 15 g/L of agar was autoclaved and after reaching a temperature of ~60 ^{o}C was poured evenly into petri dishes in a laminar flow bio-cabinet and left to solidify. The plates were then streaked using a wire loop from the culture of interest. Incubation occurred in a 2-L closed vessel at room temperature (~25 ^{o}C) with a continuous supply of gas containing 5% methane and 95% air using a double stacked peristaltic pump (Fig. 3-2). The vessel was initially flushed with three volumes of the gas mixture to reach a steady state concentration.

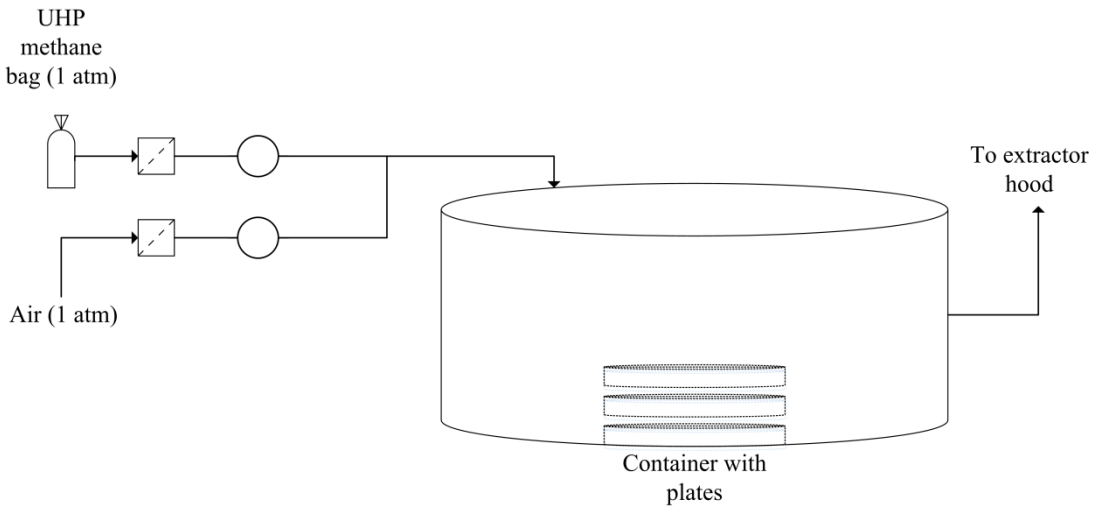


Figure 3-2: Setup used to incubate methanotrophs on solid medium.

3.2 Cultivation in bioreactor

3.2.1 Scale-up

Serum vial cultivations were scaled in to 100 mL of medium in 250-mL Schott bottles (10% v/v inoculum) sealed with the rubber bungs described in Sec.3.1.3. Norprene tubing was attached to each pipe, with a one way syringe valve on the tube immersed in the liquid and a 0.22 μm filter with a three-way valve on the other for methane addition. Air was removed from the headspace and replaced with the same volume of methane. An extra clamp was placed on the tubing to minimize leakage (Fig. 3-3).

(a) (b) (c)

Figure 3-3: Reactor vessels used for successive scale-up of mixed methanotroph culture: (a) 60-mL serum vial, b) 250-mL Schott bottle, c) 3.6-L bioreactor

3.2.2 Schematic

A 3.6-L (working volume between 1.5 L and 2 L) Labfors 3 fermenter operated at 30 ^{o}C, pH 6.8 and agitated at 200 rpm was used for bench scale cultivation of the methanotroph culture (Fig. 3-4). Air and nitrogen flowrates were controlled by internal flowmeters and the methane (UHP) through an external rotameter calibrated using a bubble flow meter (Appendix 1). Methane was mixed with the incoming gas stream to a final concentration of 5%- 20% v/v where specified and the mixture fed to the reactor through a 0.22-µm filter at 0.14 vessel volumes per minute (vvm).

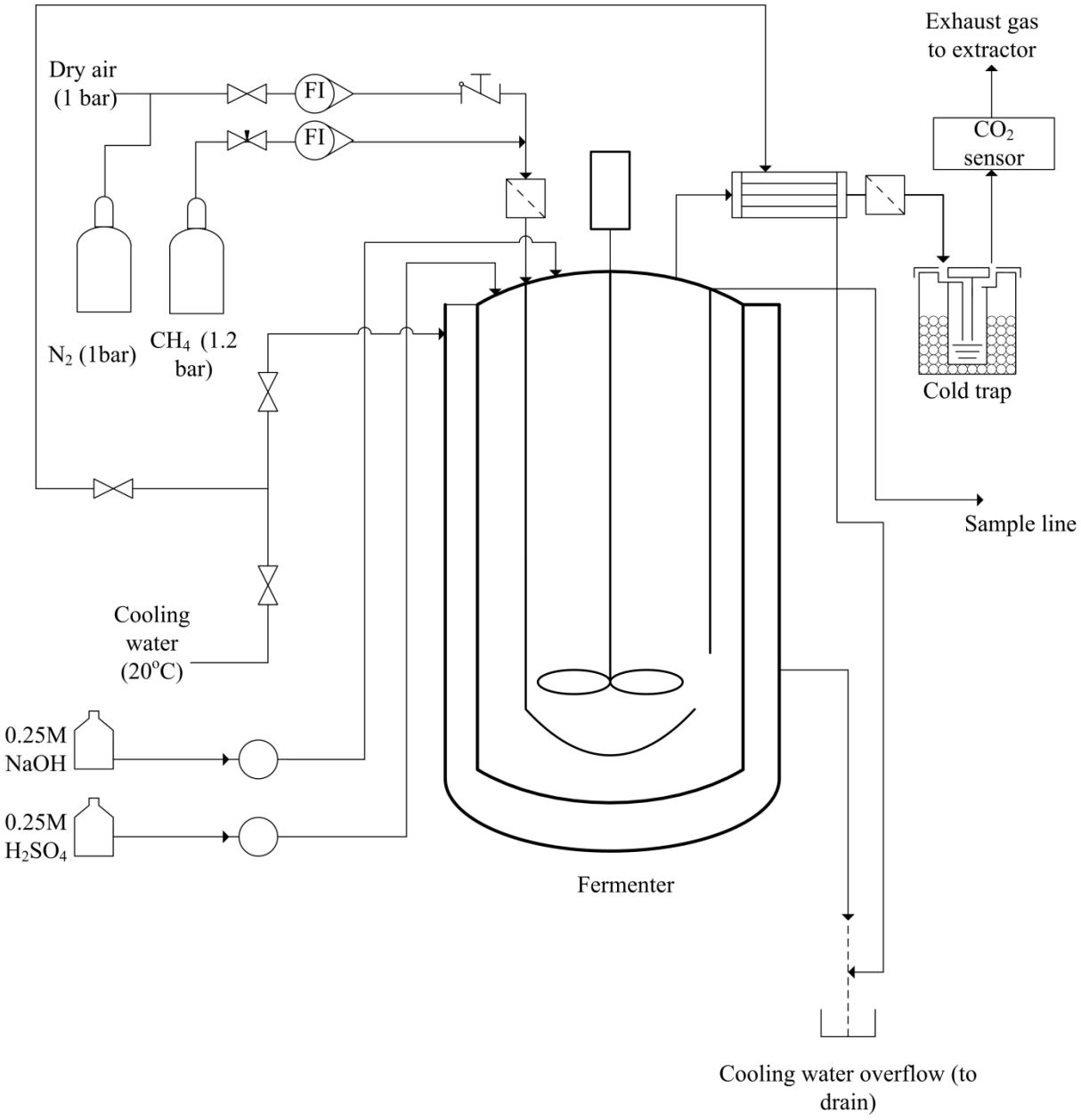


Figure 3-4: Schematic drawing of the 3.6-L bioreactor used for cultivation of methanotrophs.

3.2.3 Preparation and inoculation

The bioreactor was thoroughly cleaned, dried and filled with 1.5 L- 2.5 L of freshly prepared nutrient medium. Baffles were fitted with a Rushton impeller, along with pH and dissolved oxygen (DO) probes. Care was taken to ensure all necessary O-rings were included and that the mechanical seal did not run dry by supplying fresh lubricant (glycerine) as required.

Autoclaving took place for 45 minutes at 121 ^{o}C. Note that all of the inlet/outlet lines were clamped except for the main outlet gas line for pressure relief during sterilization. Following autoclaving, the piping for cooling water, air and methane was connected according to Fig. 3-4 and the unit switched on for five hours to allow the DO probe to polarize. To calibrate the DO probe, 100% air was fed at a flowrate of 210 mL/min and stirrer speed 200 rpm to fully saturate the media with oxygen, followed by a pure nitrogen purge for the zero-point calibration.

The bioreactor was inoculated with 10% of the working volume from a Schott bottle culture in mid-exponential phase using a sterile syringe and one way valve. During the growth phase, 50 mL of media was removed into three centrifuge tubes and placed in the refrigerator at 4 ^{o}C for future cultivations. Separate samples were also stored for future revival in 30% glycerol at -80 ^{o}C.

3.2.4 Monitoring of key parameters

The key parameters monitored and/or controlled during fermentation were pH, temperature, dissolved oxygen and carbon dioxide concentration.

- pH: A Metler Toledo autoclavable pH probe (length 225 mm) with a glass reference electrode design continuously monitored and maintained pH at 6.8. The probe was calibrated using two points in a suitable range (4-7) prior to autoclaving. An internal on-off controller with a peristaltic pump fed 0.25 M sodium hydroxide to the system as required. For this system, sulfuric acid was not required as the pH did not increase above 7.

- Temperature: A thermistor temperature probe measured temperature. The Infors controller used cooling water supplied at ~15-20 ^{O}C to maintain the fermenter temperature at 30 ^{o}C. In addition to this, the exhaust gas was cooled to minimize evaporation.

- Dissolved oxygen (DO): A Hamilton oxygen sensor with oxylyte electrolyte was fitted with a clamping adaptor and monitored DO during aerobic growth experiments. This parameter was not necessary to control as air was supplied in excess to the bioreactor (Jiang et al., 2016).

- Carbon dioxide: A Vaisala GMP343 sensor which measures the infrared absorption of carbon dioxide was connected to the exhaust; a five point calibration was performed prior to use by

feeding known concentrations of CO_2 in air and recording the observed values (Appendix 1). As methane has a similar infrared absorption spectrum, the effect on absorption at different methane concentrations was also determined. For levels above 5% methane, methane absorption interfered with the CO_2 readings; thus, a 5% concentration of methane in air (or nitrogen) at the inlet was chosen when measuring carbon dioxide production. A cold trap was also fitted to the exhaust to prevent condensation in the measurement chamber. CO_2 measurements are reported in ppm (v/v).

Foam was not an issue in this system.

3.2.5 Sampling and cell density measurements

Aseptic sampling occurred at regular intervals using a sampler with an air gap and one way syringe valve (dipped in ethanol) (Fig. 3-5). This provided multiple layers of protection against contaminants.

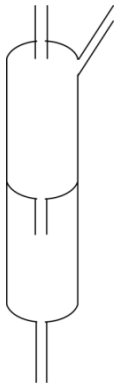


Figure 3-5: Device used for aseptic sampling from the bioreactor.

Cell growth was monitored off-line with a Shimadzu UV-Vis spectrophotometer at a wavelength of 600 nm. Dry cell weight was calibrated against OD600 by taking samples at various stages during aerobic growth (Appendix 1). Samples were collected in 50-mL (pre-weighed) centrifuge tubes and spun down in a Heraeus Multifuge X3 Centrifuge Series at 4000 g for 20 minutes. The supernatant was removed and the resulting cell pellet dried at 80 ^{o}C until the balance reading was stable. Where indicated, cell density values are the average of triplicate biological measurements plus or minus the standard deviation. Means were compared using a two tailed unpaired Student's t-test. Results were considered significant for p-values less than 0.05.

3.2.6 Specific growth rate

The specific growth rate, μ (h^{-1}) was used to characterize the growth kinetics of the population. The maximum growth rate μ_{max} occurred during the exponential growth phase and was calculated using two OD measurements from the growth curve (Eq. 10)

$$\mu = \frac{\ln(OD_2) - \ln(OD_1)}{t_2 - t_1} \tag{10}$$

The cell doubling time (hr) was estimated using Eq. 11.

$$t_d = \frac{\ln(2)}{\mu} \tag{11}$$

3.2.7 Carbon dioxide

To measure the total amount of CO_2 produced and the resulting specific production rate, Matlab software was used to estimate the area under the CO_2 concentration curve. The CO_2 concentration in ppm was converted to mol/L using the density of CO_2 (1.98 g/L assuming an ideal gas), with the assumption that the total volumetric flowrate at the inlet was equal to that at the outlet (0.21 L/ min). This was then divided by the time interval of interest and normalized using dry cell weight.

3.2.8 Organic acid analysis

High performance liquid chromatography (HPLC) was performed by Carlo Carere of GNS Science. To prepare samples, 5 mL was withdrawn from the bioreactor, spun down at 4000 g for 20 minutes and decanted into fresh vials. Growth was inhibited by adding 0.25 µL of Proclin 300 and storing the samples at 4 ^{o}C. The cell pellets were washed with deionized water, centrifuged and stored at -80 ^{o}C.

3.3 Mediator study

For mediator reduction experiments performed in the bioreactor, oxygen and carbon dioxide were purged using nitrogen at a flowrate of 0.21 L/min until the concentrations reached zero. In serum vials, the rubber bung was pierced with two needles and purged with nitrogen through a 0.22 µm filter for ten minutes. The atmosphere was then adjusted accordingly. Mediator was prepared at the desired concentration in nutrient medium and injected through a 0.22 µm syringe filter.

3.3.1 Reduction kinetics

The biotic reduction of ferricyanide was followed using UV-Vis spectrophotometry at 420 nm. In this instance, reduction refers to the decrease in concentration of ferricyanide as it gains electrons and is

transformed to ferrocyanide. Samples of volume 50 mL were collected and centrifuged at 4000 g for 20 minutes and the supernatant removed prior to absorption measurements. The absorption of ferricyanide was correlated to concentration by preparing a number of standard solutions of known concentration and measuring the absorption (Appendix 1). For ferricyanide, up to an absorption of 2 the correlation followed Beers law (i.e. linear relationship between concentration and absorption); for higher concentrations the solution was suitably diluted. The specific reduction rate was calculated by taking the slope of the linear region from the reduction curve and dividing by the dry cell weight.

Due to the sensitivity of methylene blue and thionine acetate to oxygen, the reduction of these mediators was monitored visually over time as an oxygen-free spectrophotometer system was not available.

3.3.2 Determination of reduced mediator concentration

Ferricyanide has an additional peak at 300 nm which shifts to 289 nm in ferrocyanide. This measurement was used as a simple method to detect ferrocyanide which could then be compared against the amount of oxidized mediator injected. A standard calibration curve at various ferrocyanide concentrations was prepared (Appendix 1). Solutions were diluted using deionized water when the concentration was greater than 2 mM.

Chapter 4: Results and discussion

4.1 Analysis of aerobic growth

In order to investigate the hypothesis that external mediators could be used as the terminal electron acceptor by aerobic methanotrophs, a stable culture with reproducible growth dynamics was required. This section outlines the attempted isolation of a pure methanotroph culture and the development of a mixed methane-oxidizing culture used in the mediator reduction experiments. Key parameters of the mixed culture are evaluated, including specific growth rate and carbon dioxide production.

4.1.1 Inoculum development

4.1.1.1 Pure culture

The initial methanotroph stock culture recovered from -80 ^{o}C storage was believed to be a methanotroph isolate. However, the sequence data was unavailable (Evelyn, 2012). Two sets of experiment were performed in triplicate serum vials with 20% CH_4 and 80% air in the headspace: one with 0.01 g/L yeast extract and one defined medium (Sec. 3.1.1). Yeast extract provided an additional source of carbon, amino acids and vitamins and is commonly used to stimulate bacterial growth. Control experiments were also performed without methane addition to determine if the culture was dependent on methane oxidation, as well as a non-inoculated control to verify aseptic technique (Table 4-1).

Table 4-1: Experiments and controls performed in serum vials at 30 ^{o}C to determine if methanotrophs were present in the initial stock culture.

Group	Description	Expected outcome	Result
Experiments	Cells with methane, with 0.01g/L yeast extract	Cell growth	✓
	Cells with methane	Cell growth	✓
Controls	Methane without cells	No cell growth	✓
	Cells without methane	No cell growth	✓
	Cells without methane, with 0.01 g/L yeast extract	No cell growth	×

✓ = Result consistent with expected outcome

× = Result not consistent with expected outcome

From an initial optical density (OD) of 0.03, after 24 hours of cultivation growth occurred in the experiment with yeast extract to an OD of 0.16 ± 0.03 and without yeast extract to 0.12 ± 0.02. Based on a two-tailed Student's t-test, the yeast extract did not have a significant effect on cell growth (p. value 0.2)

(Appendix 2). Growth was not observed in the control without methane or yeast extract, with a very small OD increase of 0.01 in the control containing yeast extract which indicated methanotroph abundance.

Holwerda et al. (2012) reported a total carbon content of 0.4 g/g of yeast extract. Assuming a dry cell composition of $CH_{1.8}O_{0.5}N_{0.2}$ (Nielsen et al., 1995) and a cell carbon yield of 0.62 g/g substrate carbon (based on a standard biomass yield of 0.5 g/g substrate), at a concentration of 0.01 g/L the maximum biomass increase was 0.004 g/L corresponding to an OD increase of 0.01. The growth observed in the control was therefore attributed to consumption of the yeast extract, either by a facultative methanotroph (no reports available) or a contaminating species.

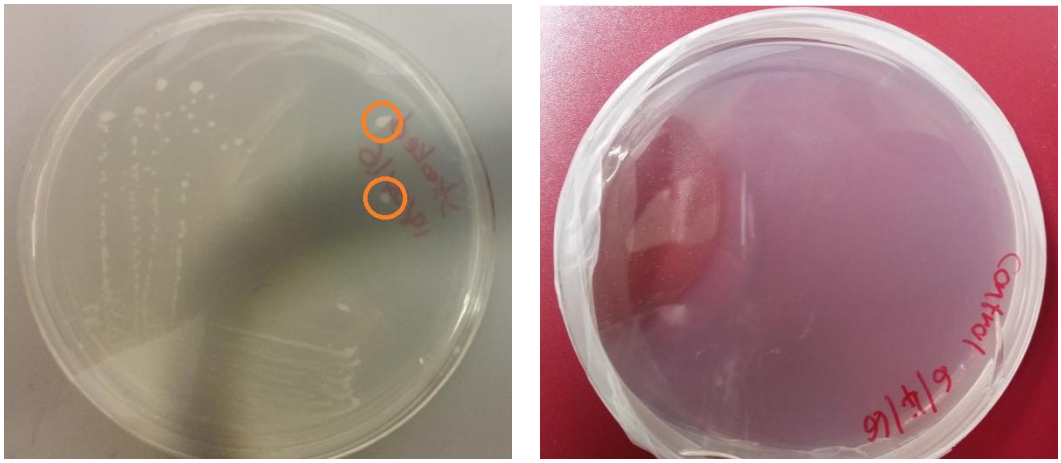
Growth observed in the experiment without yeast extract indicated a methane-dependent community. However, it was possible that the methanotrophs released by- products such as methanol which served as a carbon/energy source for non-methanotrophic contaminants. This is common in nature and can be beneficial for the dominant organism by removing toxic by-products which hinder growth (Helm et al., 2006). Presence of cocci and rods was confirmed by microscopic examination, indicating a pleomorphic culture, multiple methanotroph species or a syntrophic consortium containing several different species.

After performing three (10% v/v) passages, the growth initially observed could no longer be sustained (maximum observed OD < 0.04). The purpose of sub-culturing was to eliminate slow growing and/or low concentrations of contaminating species. Thus, an essential organism which promoted methanotrophy may have been diluted to a point where it could no longer provide support to the community. In addition, the initial inoculum may have contained an alternative carbon source such as glycerol (used as storage medium) which was co-metabolised by the dominant methanotroph.

The possibility of methane leakage was investigated by manually injecting a 1 mL sample of the headspace into a gas chromatograph. Although the concentration of methane was not monitored over time, methane was detected in each sample after > 2 weeks. Thus, methane depletion was not the cause of the poor growth observed in the sub-cultures.

Before abandoning the culture, it was streaked onto triplicate plates with defined medium and 15 g/L of agar and incubated in a 5% methane and 95% air atmosphere at room temperature (20 ^{o}C- 25 ^{o}C) (Fig. 4-1). Two controls plates were also prepared:

1. Steaked and incubated in 100% air.
2. Not streaked and incubated in a 5% methane and 95% air atmosphere.



(a) (b)

Figure 4-1: Methanotroph culture streaked onto solid medium incubated in (a) 20% CH4 and 80% air, (b) 100% air (control)

After one week of incubation, single colonies appeared on the plates (circled orange, Fig. 4-1a) inoculated with methane. The colonies were flat and circular with an approximate diameter of 3 mm and a white, opaque appearance. Negligible growth occurred on the control plates (Fig. 4-1b) indicating a pure methanotroph culture. Colonies were selected from these plates for inoculation into defined liquid medium. After prolonged incubation at 30 ^{o}C (> 2 weeks), growth was non- existent in these liquid cultures. Several possibilities were identified:

1. The isolated culture was extremely slow growing.
2. The culture grown on the plates was not a methanotroph (the agar provides an additional source of carbohydrates). This was unlikely as growth did not occur on the control plates.
3. The selected colony was a heterotroph reliant on methanotrophs or a byproduct of methanotrophy such as methanol.

Another approach to promote methanotrophy would have been the addition of a small amount of methanol (0.05% v/v) and yeast extract (0.05% w/v) to the medium, followed by serial dilution and re-streaking. This is a very time consuming procedure with a high probability of a strictly methylotrophic contaminant predominating (Bowman, 2011).

In addition to plating, a 10^{-9} serial dilution was performed. Turbidity was observed in the first three dilutions within two days of incubation and after one week in dilution 9. The 10^{-9} dilution was used as the inoculum for a 250-mL Schott bottle cultivation (Sec. 3.2.1) which grew to a maximum OD of 0.08 after 8 days. The culture was then inoculated into the bioreactor and the cell growth monitored. After one week of cultivation, zero growth was observed as indicated by an unchanging OD and pH. This was most likely due to a weak inoculum and/or a contaminating species which was severely diluted upon addition to the bioreactor.

Previous reports have highlighted the difficulties of isolating and growing pure methanotroph cultures; reasons include resistance of certain strains to growth on agar, the inevitability of heterotrophic contaminants (Bowman, 2011) and excretion of toxic by-products (Burlage, 1998). The slow growth rates of pure methanotrophs can be partially overcome by using mixed cultures which generally exhibit faster growth kinetics and stability at high cell densities. From an industrial biotechnology standpoint, mixed cultures are appealing as sterilization is not an issue, a wider range of substrates can be used and the microbial consortia are able to adapt easier to changes in environmental conditions and/or feedstocks (Yang et al., 2012).

A mixed culture where methanotrophs dominate and use methane as the energy source could be implemented in a mediated MFC. The pure culture study was subsequently abandoned and enrichment of a mixed methane-oxidizing culture from the environment attempted.

4.1.1.2 Mixed culture

A mixed methane-oxidizing culture was successfully enriched from fresh top-soil (Parklands, NZ) using a defined medium. Two cultivations were attempted for comparison (Sec. 3.1.3):

1. Continuous addition of methane and air into a 250-mL Schott bottle with 75 mL of nutrient medium. The culture was agitated using a magnetic stirrer and maintained between ~25 ^{o}C and ~30 ^{o}C. A maximum OD of 0.6 was obtained after 5 days of cultivation.

2. Three 60-mL serum vials with 12 mL of medium. The cell density reached a maximum OD of 0.48 ± 0.04 after 5 days of cultivation.

To minimise growth of non-methanotrophs present in soil, yeast extract was excluded from the medium. The major energy sources were ammonia and methane as well as a small amount of energy provided from the soil. However, growth did not occur in the control without methane, indicating that methanotrophs were abundant in the consortium.

In both cultivation techniques, the pH dropped from 6.8 to 5.4. Partial oxidation of the organic substrate as a result of imbalanced conditions can lead to the excretion of acidic compounds such as lactate or citrate (Papagianni, 2011). With ammonium as the nitrogen source, hydrogen ions were also liberated during uptake; these factors could therefore have accounted for the pH change. This may also have limited cell growth as most mesophiles have an optimum pH range between 6.5 and 7.5 (Hogg, 2013). The concentration of phosphate could have been increased to improve the pH buffering capacity of the medium.

The higher cell density obtained in the continuously-fed culture was likely due to increased availability of methane and oxygen to the cells. This was a result of the gas being fed directly to the liquid and the

improved mixing for bubble dispersion. For simplicity and better temperature control, serum vials were used for cultivation from herein. After 10% v/v sub-culturing numerous times (> 5), growth was reproducible, with observable turbidity (OD > 0.1) occurring within 24 hours.

Based on these findings, it was concluded that a mixed, mesophilic culture with dependence on methane oxidation was successfully cultivated. The culture was stable and the growth dynamics were investigated in the bioreactor.

4.1.2 Bioreactor cultivation

Serum vials containing the mixed culture were successfully scaled up into 250-mL Schott bottles and allowed to reach exponential phase (OD 0.2, approximately 24 hours after inoculation). The culture was then grown aerobically in the bioreactor in 1.5 L of defined medium and the dry cell weight (DCW) (g/L) recorded at various time intervals following inoculation (Fig. 4-2). Two experiments were performed; the first without addition of cycloheximide and the second with cycloheximide at a concentration of 100 mg/L. Cycloheximide is an inhibitor of protein synthesis in eukaryotes such as protozoa, predators within soil that can consume bacteria as a source of nutrients (Bailey & Ollis, 1976).

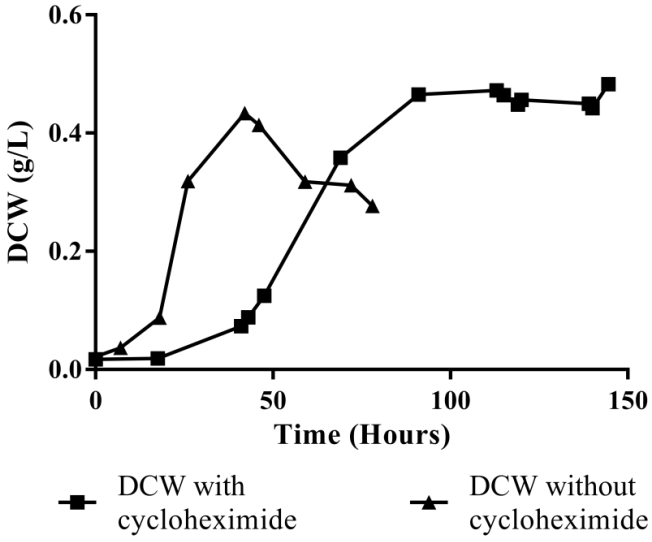


Figure 4-2: Change in dry weight biomass concentration (g/L) of a mixed methane-oxidizing culture grown in 1.5 L of nutrient medium with 20% methane, 80% air at 0.14 vvm and 250 rpm. Temperature and pH set point were 30 ^{o}C and 6.8 pH. The cycloheximide concentration was 100 mg/L.

In the experiment without cycloheximide, growth began immediately from an initial DCW of 0.02 g/L (i.e. no lag phase). Exponential growth occurred for ~25 hours before reaching stationary phase at a maximum of 0.42 g/L after ~45 hours of cultivation. The cell density then decreased which was indicative of predation, cell lysis or consumption of intracellular polymers during nutrient starvation.

In the experiment with cycloheximide addition, the maximum DCW of 0.48 g/L was reached after 90 hours from an initial DCW of 0.017 g/L. The cell density did not decrease once stationary phase was reached, thus predation was the most likely cause of decreasing cell density in the experiment without cycloheximide. The maximum specific growth rate was 70% lower when cycloheximide was present (Table 4-2) which also indicated inhibition of an organism that fed on by-products excreted during methane oxidation. The specific growth rate aligned with previously reported rates of *Methylosinus trichosporium* OB3B, a well characterized Type II methanotroph (Park et al., 1991; Xing et al., 2006).

Table 4-2: Maximum biomass concentrations, growth rates and doubling times for aerobic growth of mixed methane-oxidizing culture on a mineral medium at 30 ^{o}C.

Experiment	**Max. DCW (g/L)**	**μ_{max} (hr^{-1})**	**t_d (hours)**
With cycloheximide	0.48	0.05	14
Without cycloheximide	0.43	0.16	4.3

4.1.3 Increasing cell density

The higher cell density obtained in the bioreactor compared to serum vials was most likely due to improved gas-liquid mass transfer and control of the pH at 6.8. However, the maximum cell density was low in comparison to other reports. For example, Jiang et al. (2016) reported a maximum cell density of 2.75 g/L after 33 hours of batch cultivation in a 5-L bioreactor using nitrate mineral salts. In this case, 95% of the micro-organisms in the culture were related to Type I or Type II methanotrophs. For pure cultures, high cell densities between 15 g/L and 18 g/L for *M.trichosporium* OB3B have been reported (Han et al., 2009; Shah et al., 1996).

Several factors may have caused the cessation of growth at 0.48 g/L (Bailey & Ollis, 1976):

1. Essential nutrient limitation (often nitrogen). Based on the amount of ammonium present in the nutrient medium, a maximum cell density of 0.4 g/L would imply that only 35% of the available nitrogen was assimilated as biomass. If nitrogen limited ,the remaining nitrogen must have been oxidized by another species or a methanotroph via the MMO enzyme (Bédard & Knowles, 1989).

2. Poor solubility of methane and oxygen in the broth. This can be enhanced using organic solvents such as paraffin oil as demonstrated in the study of Han et al. (2009). Environmental factors such as temperature and pH, as well as feed rates/ratios, mixing speeds and reactor configurations also affect the gas solubility.

3. Limitation of the carbon/ energy source as the population increases. This can lead to endogenous metabolism (i.e. consumption of internal storage polymers and/or living cells consuming dead cells) which also decreases the cell density.

4. Accumulation of toxic metabolites; this is usually due to nutrient or substrate limitation when the carbon source is unable to be fully oxidized to carbon dioxide.

5. Space limitations as the cell density increases.

Specifically for methanotrophs, the potential of MMO inhibition due to the competition of ammonium for the active site and the production of nitrite as a toxic intermediate of ammonium oxidation was a potential cause of the lower cell density (Nyerges et al., 2010). For this reason, nitrate is often the preferred source of nitrogen when cultivating methanotrophs and this may have led to the higher cell density observed in the study of Jiang et al. (2016).

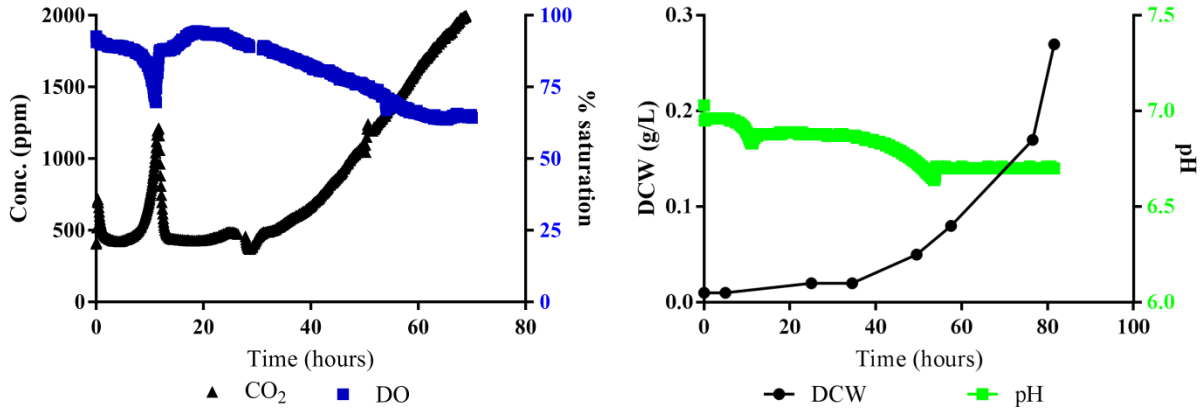
Growth of the mixed culture using 1 g/L of potassium nitrate was compared in serum vials concurrently with those grown in 0.5 g/L of ammonium chloride. At this nitrogen level, the theoretical amount of biomass that could be supported in both cases (assuming 100% assimilation) was 1.2 g/L. The maximum cell densities obtained were 0.156 ± 0.020 g/L with nitrate and 0.160 ± 0.025 g/L with ammonium which implied that the nitrogen source did not have a significant effect on the maximum achievable cell density (p. value 0.43) (Appendix 2). However, this was not conclusive evidence as the drop in pH from 6.8 to 5.4 or accumulation of a metabolite such as methanol or formaldehyde may have caused the culture to cease growth at this point. The rate of growth was not monitored in this experiment; this may have given a better indication on the inhibitory effects of ammonia on methane oxidation.

Disregarding the nature of the nitrogen source, with a continuous supply of carbon and oxygen it was hypothesized that the system was nitrogen limited. This was investigated in the bioreactor by allowing the culture to reach stationary phase and injecting 150 mL of 5 g/L ammonium chloride solution to give an overall concentration of 0.5 g/L. Assuming nitrogen fixation was not occurring, if 35% of the added nitrogen was assimilated as biomass, the expected cell density increase was 0.4 g/L. However, further growth did not ensue, indicating that this system was not nitrogen deficient. Upon addition of 100 mL of the original nutrient solution, an increase of 0.05 g/L was observed. This indicated deficiency of a macro-nutrient such as phosphorus or sulfur, or a trace element such as iron or copper required for enzyme activation. For the purpose of this study, the identity of the limiting nutrient was not pursued further.

To optimize the environmental conditions, the mixed culture could be grown at a series of different pH and temperature levels for comparison. Analysis of the bioreactor exhaust gas using an on-line gas chromatograph would also be beneficial for determining if the culture was methane-limited. The reactor conditions (stirrer speed, gas flow-rate) could then be optimized accordingly.

4.1.4 Carbon dioxide, dissolved oxygen and pH

Carbon dioxide production, dissolved oxygen (DO) and pH were monitored continuously and used as indicators of both methane oxidation and cellular activity. Fig. 4-3(a) demonstrates the CO_2 and DO profiles of the mixed culture during aerobic growth with 5% CH_4 and 95% air. Following inoculation, the CO_2 concentration remained at 400 ppm for ~4 hours before increasing rapidly over the next 8 hours. This corresponded with a decline in DO from 90 % to 70 % saturation, an increase in DCW from 0.01 g/L to 0.02 g/L and a decrease in pH from 6.96 to 6.84 (Fig. 4-3b). A second 'lag' period then occurred from hr 13- 25 where zero activity was observed in all of the measured parameters. After this period, the system functioned at a slower rate, with increasing CO_2 production, decreasing DO to 60% and exponentially increasing cell growth. The pH decreased to 6.65 over 30 hours as the reaction continued. At hour 50, the pH control was resumed at pH 6.8.



(a) (b)

Figure 4-3: (a) Carbon dioxide concentration (ppm) and dissolved oxygen (% saturation) profiles, (b) dry weight biomass concentration (g/L) and pH profile, during aerobic growth of a mixed methane-oxidizing culture with 5 % CH_4 and 95 % air in the inlet at 0.14 vvm. Temperature and pH set-points were 30 ^oC and pH 6.8. The cycloheximide concentration was 100 mg/L.

The coupling of cell growth with increasing carbon dioxide and decreasing pH was expected. Since the dissolved oxygen was not controlled, the decrease in DO was also expected as the demand for oxygen is directly proportional to the cell density. However, the two distinct phases of cellular activity was uncharacteristic of ordinary bacterial growth and may have been caused by one of the following scenarios:

1. Prior to inoculation, the medium was fully saturated with methane and oxygen. Upon addition of cells the methane was rapidly consumed and became limiting at hr 12, causing a lower CO_2 production rate. It is unlikely that the culture was oxygen limited as the typical O_2: CH_4 consumption ratio of methantrophs is 1.34 and air was supplied in excess to the bioreactor (Bailey & Ollis, 1976).

2. The inoculum carried over organic compounds excreted during methane oxidation which served as a carbon/energy source for either the methanotrophs or a partnering heterotroph with a higher growth rate.

HPLC analysis of the bioreactor culture (DCW 0.2 g/L) revealed the presence of acetate and ethanol at concentrations of 1.5 mM and 1.2 mM respectively after 30 hours of cultivation. Eq. 12 and 13 represent the aerobic oxidation of these compounds.

$$C_2H_3O_2^- + 2O_2 + H^+ \rightarrow 2CO_2 + 2H_2O \quad (12)$$

$$C_2H_6O + 3O_2 \rightarrow 2CO_2 + 3H_2O \quad (13)$$

The total amount of carbon dioxide produced from hr 4-12 was 0.96 mmoles, calculated by taking the area under the curve in Fig. 4-3(a) over this time period and multiplying by 0.21 L/min (Sec. 3.2.7). As the stoichiometric ratio of acetate and ethanol to CO_2 was identical, the total amount of these compounds required to produce 0.96 mmoles of CO_2 from aerobic oxidation was 0.5 mmoles.

An inoculum size of 135 mL at a DCW of 0.2 g/L was used for the experiment demonstrated in Fig. 4-3. Based on the concentration values stated above, approximately 0.2 mmoles of acetate and 0.16 mmoles of ethanol would have been present in the inoculum, with a combined total of 0.36 mmoles. As the inoculum had reached stationary phase and the HPLC analysis was conducted on a bioreactor culture in mid-growth phase, it is likely that a larger amount of organic by-products had formed; an organic assay of the inoculum would verify this. Simultaneous methane oxidation by methanotrophs may also account for the CO_2 discrepancy.

4.1.5 Summary

This section focussed on the development of a methane-oxidizing culture which was used in succeeding experiments. The specific findings were:

1. Pure methanotroph cultures were difficult to maintain over time. A methanotroph isolate was revived from -80 ^oC freezer stocks and initially grew rapidly in defined liquid medium to an OD of 0.12 ± 0.02. Addition of yeast extract in the medium did not have a significant effect on cell growth. After sub-culturing numerous times and performing isolation techniques such as serial dilution and incubation on solid medium, the culture was deemed unstable and could not be used to test the hypothesis.

2. Mixed methane-oxidizing cultures were readily obtained from fresh top-soil. The cell density achieved in serum vials was greater than the initial pure culture (0.48 ± 0.04) and was successfully maintained after sub-culturing. Continuous addition of methane and air was an

alternative enrichment technique which produced a higher cell density (OD 0.6) than serum vial cultivations.

3. A mixed culture using methane as the input energy source is equally feasible for a methane-fed MFC. This would have several advantages over pure cultures such as improved stability and lower susceptibility to heterotrophic contamination. Thus, the mixed culture was suitable for testing with external mediators in succeeding experiments.

4. The maximum cell density of the mixed culture obtained in the bioreactor was 0.48 g/L. This was lower than reported literature and was likely due to limitation of an essential nutrient or build-up of a toxic metabolite. The culture was not nitrogen-limited. Soil enrichments with cycloheximide prevented biomass decreases in stationary phase which was attributed to growth of protozoa.

5. Two phases of aerobic growth were observed following inoculation of the mixed culture into a bioreactor. The initial phase was most likely due to consumption of ethanol or acetate carried over from the inoculum, both of which were produced during aerobic methane oxidation. The second phase proceeded at a slower rate due to the low solubility of methane. As expected, cell growth corresponded with production of carbon dioxide and a decrease in dissolved oxygen and pH.

4.2 Proof-of-concept

This section investigates the ability of the mixed methane-oxidizing culture described above to utilize an external mediator as the electron acceptor in place of oxygen during methane oxidation. A set of control experiments were performed to confirm the oxidation of methane to carbon dioxide and the implications of these experiments will be discussed.

4.2.1 Ferricyanide as a model compound

Evelyn (2012) identified potassium ferricyanide as a promising mediator for facilitating the oxidation of methane by a methanotroph culture. It is a hydrophilic compound which accepts electrons extracellularly and is often used to investigate biochemical reactions due to the characteristic yellow colour, which upon the addition of an electron turns colourless (Eq.14) (Wilson & Walker, 2000). The stability of the oxidized and reduced forms in the presence of oxygen and the characteristic UV absorption peak at 420 nm makes this compound ideal for studying the reduction kinetics using spectrophotometry.

$$Fe(CN)_6{}^{3-} + e^- \rightarrow Fe(CN)_6{}^{4-} \quad (14)$$

Ferricyande (yellow) Ferrocyanide (colourless)

$E^0 = +0.36$ V

The theoretical coupling of ferricyanide reduction with methane oxidation to carbon dioxide can be described by Eq. 15 (Logan, 2008). The Gibbs energy value was calculated based on standard biological conditions (pH 7, 298 K, 1 atm).

$$CH_4 + 8FeCN_6{}^{3-} + 2H_2O \rightarrow 8FeCN_6{}^{4-} + CO_2 + 8H^+ \quad (15)$$

$\Delta G = -463$ kJ/mol

With methanotrophs acting as the biocatalyst to drive this reaction, the stated Gibbs energy value is the amount available for bacterial growth and maintenance. The actual energy for conversion to electricity in a MFC is determined by the potential difference between the mediator and the oxidizing species at the cathode (usually oxygen) (Sec 2.6.3). As the reduction potential is less than that of oxygen (+ 0.82V), ferricyanide is feasible for use in a mediated- MFC.

4.2.2 Reduction of ferricyanide

Triplicate serum vials of the mixed methane-oxidizing culture were grown in 12 mL of defined nutrient medium to stationary phase (DCW 0.2 ± 0.02 g/L), injected with 1 mM of ferricyanide and purged using nitrogen. The cultures were incubated at 30 ^{o}C and 120 rpm in a 20% CH_4 and 80% N_2 atmosphere. The characteristic yellow colour decreased over 80 hours and spectrophotometry confirmed a ferricyanide reduction from 1 mM to 0.3 mM, corresponding to a specific reduction rate of 0.04 mmol/ g·h. Incomplete reduction could have been due to depletion of an essential nutrient, a low pH value (which reduced from 6.8 to 5.5 prior to ferricyanide addition) or production of toxic by-products which halted cellular activity. Oxygen ingress was also possible which would prevent reduction as oxygen is the preferred substrate for energy production.

The ability of the mixed culture to reduce ferricyanide was subsequently investigated in the bioreactor. The culture was grown aerobically with 5% CH_4 and 95% air in 1.5 L of defined medium until stationary phase was reached (DCW 0.28 g/L without cycloheximide) followed by a nitrogen purge for 2 hours to remove the dissolved oxygen. A 5% CH_4 and 95% N_2 gas mixture was then fed to the reactor and ferricyanide injected aseptically to a concentration of 1 mM.

(a) (b)

Figure 4-4: Colour change of bioreactor containing mixed culture in stationary phase (DCW 0.28 g/L) after the addition of ferricyanide to a concentration of 1 mM at, (a) t = 0, (b) t = 20 h during the addition of 5% CH_4 and 95% N_2.

The yellow colour disappeared after 20 hours (Fig. 4-4), corresponding to a decrease in the ferricyanide concentration from 1 mM to 0 mM (Fig. 4-5). The specific reduction rate was 0.2 mmol/g·h over the linear region which was close to the maximum rate initially reported by Evelyn (2012) of 0.18 mmoles/g·h. This was 5 times higher than the rate observed in serum vials and was most likely due to

improved mass transfer of methane into the broth, control of the pH at 6.8 and lower susceptibility to oxygen leakage. The unchanging cell density was expected as the culture had reached stationary phase prior to ferricyanide injection.

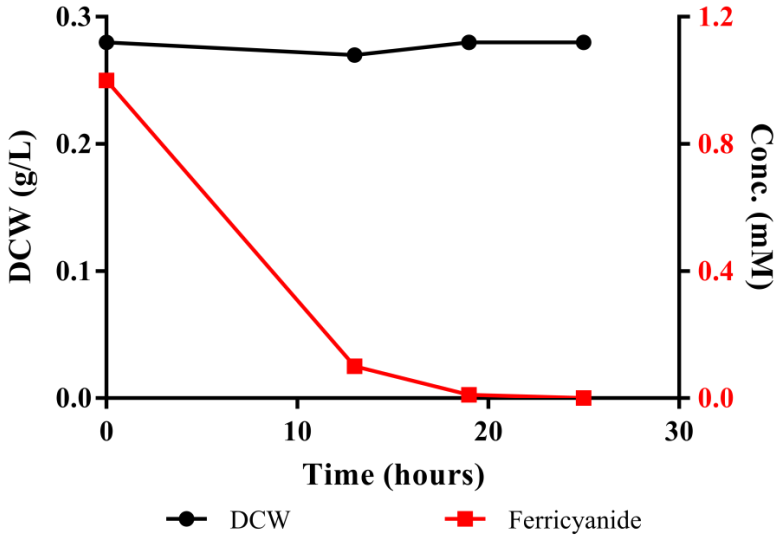


Figure 4-5: Change in ferricyanide concentration (mM) and dry weight biomass concentration (g/L) due to the reduction by a mixed methanotroph culture in a bioreactor fed with 5% CH_4.and 95% N_2. Temperature and pH set-points were 30 ^oC and 6.8.

Evelyn (2012) also described the partial re-oxidation of ferrocyanide following contact with molecular oxygen. After purging the bioreactor with 100% air for 24 hours, the ferricyanide concentration increased from 0 mM to 0.2 mM and was accompanied by a decrease in cell density of 0.1g/L when cycloheximide was absent (not shown) which indicated predation. With cycloheximide present, the ferrocyanide oxidation was observed and the cell density remained unchanged. The partial oxidation of ferrocyanide showed that the reverse action was possible in this system, which is important for a working MFC.

4.2.3 Control experiments

In order to confirm the hypothesis that ferricyanide was facilitating methane oxidation, several control experiments were conducted in serum vials and the bioreactor (Table 4-3). The advantage of using serum vials was that triplicate experiments could be performed simultaneously; the disadvantage was that the environment was more difficult to control and real time monitoring of carbon dioxide production was not possible. The vessel chosen for each control was based on if the measurement of carbon dioxide was required, which was more practical using the bioreactor. Specifically, these controls were designed to confirm that:

1. Mediator reduction was directly coupled to methane oxidation

2. The colour change was due to the reduction of ferricyanide (FCN) to ferrocyanide and not a result of chemical or biological degradation.
3. Mediator reduction was solely due to biological activity.
4. Carbon dioxide was being produced as a result of methane oxidation coupled to mediator reduction.

The processing conditions (temperature, pH, mixing) for the control experiments were identical to the ferricyanide reduction experiments.

Table 4-3: Control experiments performed in serum vials and bioreactor to confirm ferricyanide (FCN) reduction by mixed methane-oxidizing culture.

	Control #	Description	Expected outcome	Result
Serum vials	1	(5% methane, 95% N_2) without cells, with FCN	No FCN reduction	✓
	2	(5% methane, 95% N_2) with autoclaved cells, with FCN	No FCN reduction	✓
Bioreactor	3	(5% methane, 95% N_2) with cells, without FCN	No CO_2 production and no cell growth	✓
	4	100% N_2 with cells, with FCN	No FCN reduction and no CO_2 production	×
	5	(5% methane, 95% N_2) with cells, with FCN → remove cells and measure reduced FCN	Reduced FCN conc. equivalent to initial FCN conc.	✓

✓ = Result consistent with expected outcome

× = Result not consistent with expected outcome

The results from the control experiments confirmed that ferricyanide was reduced biologically by the mixed culture. However, the reduction was not dependent on the presence of methane, as indicated by control 4 (Table 4-3). There were two possibilities to explain this: A microorganism in the consortium oxidized a portion of the ferricyanide to carbon dioxide, or oxidation of a carbon compound produced during methane oxidation. The success of control 5 indicated that the former scenario was unlikely; however, this cannot be fully discounted, as there was a slight discrepancy (5%-10%) between the initial ferricyanide and the final ferrocyanide concentrations. A rigorous confirmation technique would be ^{13}C-methane labelling to track the carbon throughout the experiment. The latter scenario was more likely, as production of ferrocyanide would not have occurred without a simultaneous oxidation reaction.

4.2.4 Carbon dioxide production and base addition

As demonstrated above, CO_2 production was an important variable for confirming/rejecting the primary hypothesis. Prior to ferricyanide addition, CO_2 was purged from the bioreactor using 5% CH_4 and 95% N_2 until the concentration stabilized at ~0 ppm, indicating the absence of alternative electron acceptors such as nitrate which can be a by-product of ammonia oxidation. The addition of sodium hydroxide (base) was also monitored as an indicator of activity; during oxidation of carbon-based compounds, acidic by-products are often excreted into the medium causing base addition to maintain the pH set-point. In addition, ferricyanide does not gain a proton when it is reduced which would cause an accumulation of hydrogen ions and a subsequent decrease in the pH.

Carbon dioxide production, ferricyanide reduction and base addition were measured concurrently during the experiment (5% CH_4 and 95% N_2 in the feed) and the control without methane (100% N_2 in the feed) (Fig. 4-6).

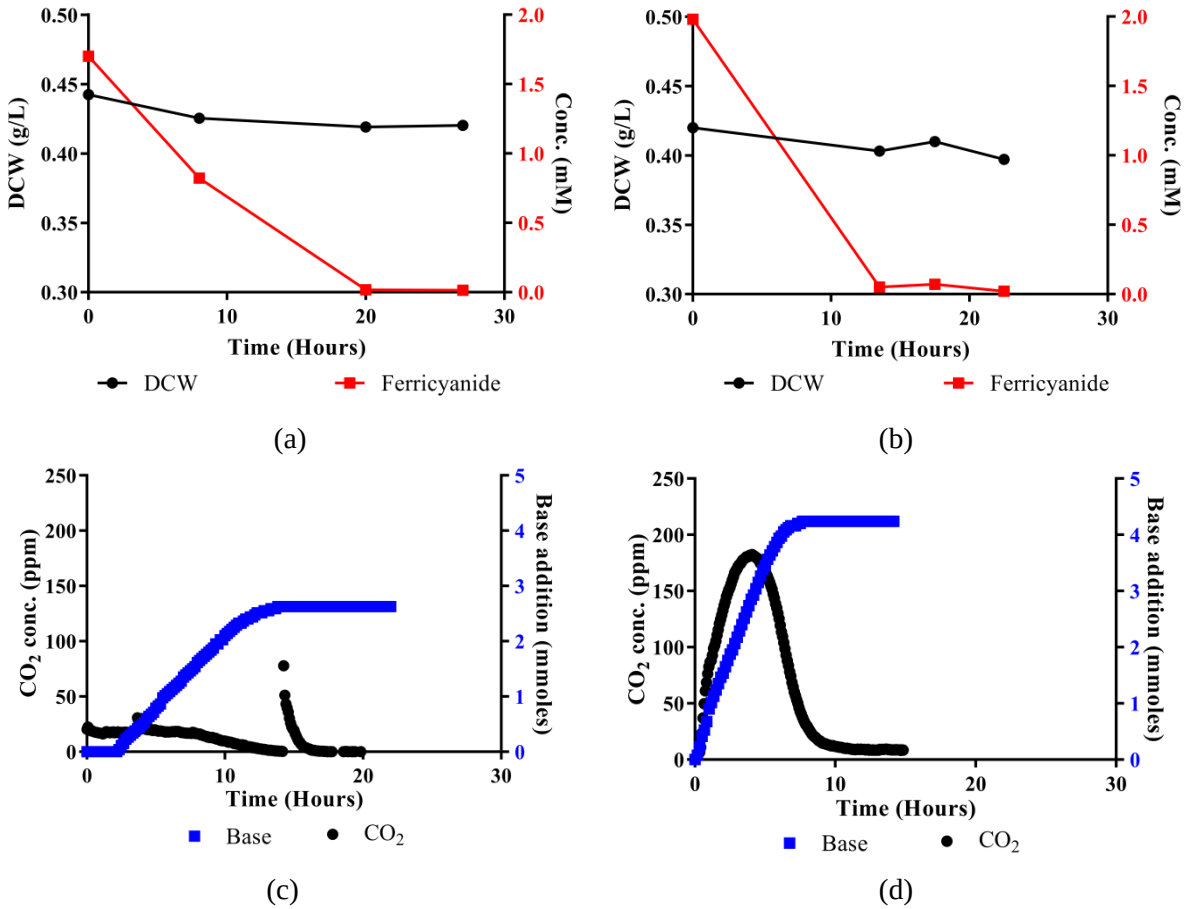


Figure 4-6: The change in ferricyanide concentration (mM) and dry weight biomass concentration (g/L) and the corresponding CO_2 production (ppm) and base addition (mmoles) with (a,c) 5% CH_4 and 95% N_2 in the feed (experiment), (b,d) 100% N_2 in the feed (control). Cells were maintained in the stationary phase (DCW ~0.42 g/L) and the temperature and pH set-points were 30 ^{o}C and 6.8 pH.

The CO_2 profile in the experiment (Fig. 4-6c) was irregular, with a lag period of approximately 3-4 hours between the measured time of complete ferricyanide reduction and the time the CO_2 concentration reached 0 ppm. This may have been caused by a low sampling frequency in this experiment. In the control (Fig. 4-6d), the CO_2 concentration peaked at 180 ppm after 5 hours and decreased to 10ppm over the following 8 hours. The average rates of ferricyanide reduction and CO_2 production in the experiment and control are shown in Table 4-4. The ferricyanide reduction rate was calculated by approximating the concentration curves in Fig. 4-6(a) and Fig. 4-6(b) as linear. However, the ferricyanide sampling frequency limited the accuracy of the estimate.

Table 4-4: Specific ferricyanide (FCN) reduction rate and CO_2 production rate using cells in stationary phase.

	Specific FCN reduction rate (mmol/ g·h)	**Specific CO_2 production rate (mmol/g·h)**
Experiment (5% CH_4 and 95% N_2 in feed)	0.20	0.01
Control (100% N_2 in feed)	0.36	0.13

The pH was maintained by the addition of 2.6 mmoles of base over 12 hours in the experiments and 4.2 mmoles over 8 hours in the control. In both cases, the point at which the CO_2 concentration reached 0 ppm was 2 hours after the time that base addition stopped; this may have been the time required for carbon dioxide to completely purge from the system. The actual time for compete ferricyanide reduction was estimated as 13.5 hours in the experiment and 8 hours in the control, corresponding to reduction rates of 0.3 mmol/g·h and 0.6 mmol/g·h respectively. Repeated experiments confirmed the coupling of mediator reduction with CO_2 production and base addition; these values were therefore better indicators of the true reduction rates. The addition of base/decrease in pH provided further evidence for the role of ferricyanide as the terminal electron acceptor.

The slower reduction rate of the experiment compared to the control indicated that anaerobic oxidation of methane may have been occurring. If ferricyanide was acting as the electron acceptor, the reduction rate (and similarly the carbon dioxide production) would have been affected by the poor solubility of methane in the broth. Based on stoichiometry (Eq. 15), if 1.8 mM of ferricyanide directly oxidized methane, the expected amount of CO_2 production was 0.3 mmoles (working volume 1.6 L). From an estimate of the peak area under the CO_2 production curve in Fig. 4-6(b), 0.1 mmoles of CO_2 was produced over the reduction period; this discrepancy may have indicated production of primary metabolites other than CO_2 or oxidation of another organic compound synthesized during aerobic methane oxidation.

4.2.5 Mechanism of reduction

The ability of a microbial culture to reduce ferricyanide in the presence and absence of methane at different rates indicated separate mechanisms depending on the environmental conditions. Three potential scenarios to explain this behaviour were formulated based on these findings:

1. Other heterotrophic bacteria present in the mixed culture consumed organic compounds (such as methanol) excreted by the methanotrophs during aerobic cell growth using ferricyanide as the terminal electron acceptor. This has been reported as the primary mechanism responsible for AME-D (Sec. 2.4.1) (Stein & Klotz, 2011). Compounds such as lactate, acetate and formate have been produced by aerobic methanotrophs and could serve as the carbon source for a wide range of species (Gilman et al., 2015). Certain compounds such as acetate can also be co-metabolized by methanotrophs as a growth substrate (Dedysh et al., 2005).

2. Methanotrophs were utilizing internal storage polymers such as glycogen or polyhydroxyalkanoates (PHA) with ferricyanide as the terminal electron acceptor. Organic polymers such as PHA and glycogen are synthesized by bacteria as a method of storing carbon for use when the substrate is limited. PHA synthesis has been well documented in both Type I and II methanotrophs, with reported yields of 0.5 g_{PHB}/g_{CH4} for a mixed culture dominated by *Methylocyctis sp.* (Wendlandt et al., 2001). PHB has mainly been shown as a source of reducing equivalents in type II methanotrophs as opposed to a growth substrate in methane limited conditions (Strong et al., 2016b). There have also been numerous reports of glycogen production by aerobic methanotrophs (Cai et al., 2016; Eshinimaev et al., 2002; Khadem et al., 2012). Thus, when oxygen was not present and the MMO enzyme was unable to catalyze the first step of methane oxidation, it is possible these polymers were oxidized as a survival mechanism. This has been demonstrated by Roslev and King (1995), with energy provided from the catabolism of endogenous proteins, polysaccharides and lipids during aerobic starvation.

3. Methanotrophs directly used ferricyanide in place of oxygen during the metabolism (i.e. novel metabolic pathway). This may either have been an aerobic methanotroph which had an anoxic methane oxidation pathway (no reports available) or a novel anaerobic methanotroph present in the consortium. A recent example of the latter scenario was *M. nitroreducens* which coupled nitrate reduction directly with methane oxidation (Haroon et al., 2013).

These scenarios may have occurred simultaneously and could all be applied to a methane-fed MFC. Elucidation of the compound responsible for driving ferricyanide reduction became an additional objective of this research.

4.2.6 Summary

This section showed that it was possible for a mixed methane-oxidizing culture to use ferricyanide as the terminal electron acceptor with methane as the energy source. The specific findings were:

1. Reduction of ferricyanide was observed consistently in serum vials and the bioreactor using the mixed culture maintained in stationary phase. The reduction rate was 0.04 mmol/ g·h in serum vials and 0.2 mmol/g·h in the bioreactor using an initial ferricyanide concentration of 2 mM. This was expected as the environmental conditions such as pH and inlet gas composition were more rigorously controlled in the bioreactor.

2. With methane present in the feed, ferricyanide reduction was coupled with carbon dioxide production and sodium hydroxide addition. This provided strong evidence of ferricyanide acting as the terminal electron acceptor during the oxidation of a carbon based compound. The carbon dioxide production rate was 0.01 mmol/g·h.

3. Ferricyanide reduction was not dependent on the presence of methane. Reduction also occurred in the control without methane at 0.36 mmol/g·h, with a corresponding carbon dioxide production rate of 0.13 mmol/g·h. The carbon dioxide may have been produced from the oxidation of an organic intermediate or a storage polymer such as glycogen or PHA produced during aerobic methane oxidation.

4. The slower rate of ferricyanide reduction in the experiment indicated a separate pathway, potentially limited by the poor solubility of methane (i.e. a novel methane oxidation pathway). The theoretical carbon dioxide production from direct oxidation of methane using ferricyanide did not match the observed value which could have been due to production of organic intermediates. Thus, the mixed culture may have directly oxidized methane when it was available using ferricyanide as the terminal electron acceptor and switched to an alternative substrate during methane starvation.

4.3 Investigating the microbe-mediator interaction

The results from the previous section demonstrated that it was possible for a microbial consortium composed of methane oxidizing bacteria to use ferricyanide instead of oxygen as the terminal electron acceptor. However, for the reason that reduction could proceed in the absence of methane, this section investigates the nature of the reducing compound and the possibility of a novel methane oxidizing pathway. Alternative mediators are also tested and the reduction kinetics compared against ferricyanide.

4.3.1 Ferricyanide reduction during exponential growth phase

The mixed culture was grown aerobically with 5% CH_4 and 95% air and halted at a dry cell weight of ~0.2 g/L by switching the air with 100% N_2. The reduction experiment (5% CH_4 and 95% N_2 in the feed) and the control (100% N_2 in the feed) were repeated using an initial ferricyanide concentration of 2 mM (Fig. 4-7).

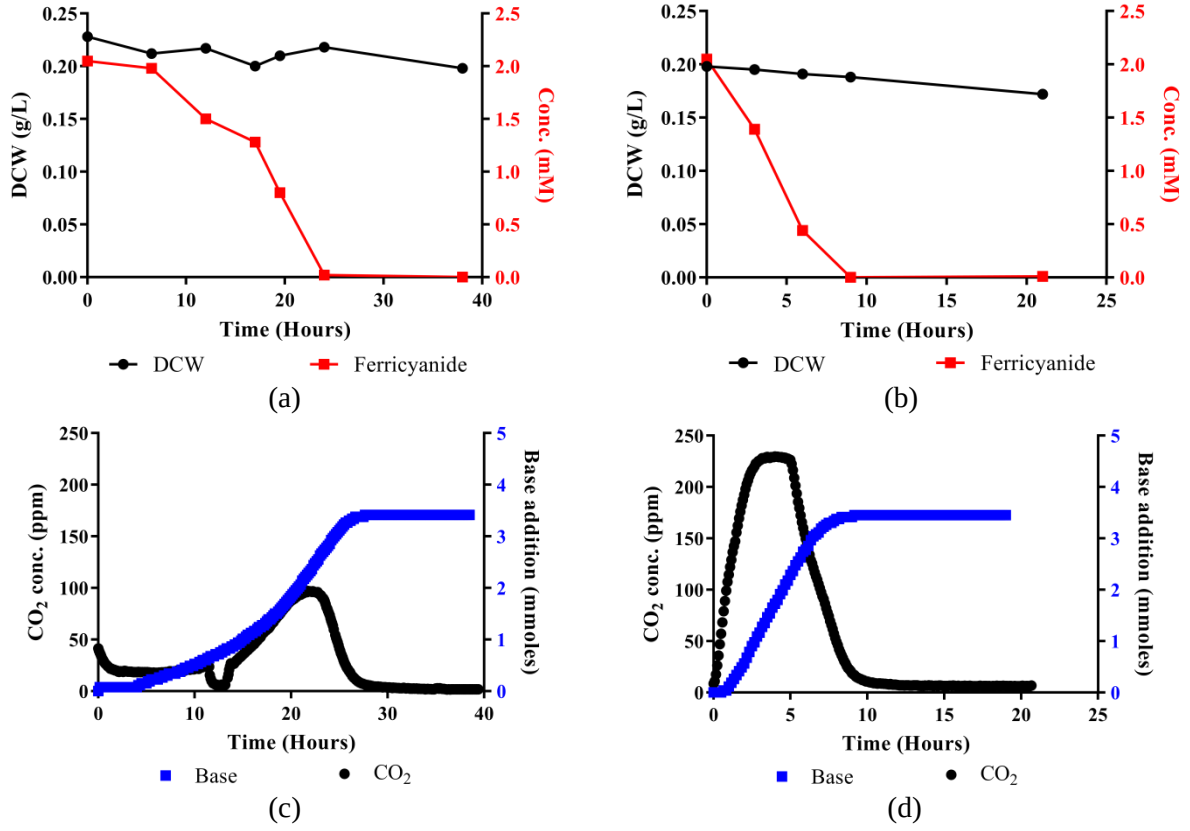


Figure 4-7: The change in ferricyanide concentration (mM) and dry weight biomass concentration (g/L) and the corresponding CO_2 production (ppm) and base addition (mmoles) with (a,c) 5% CH_4 and 95% N_2 in the feed (experiment), (b,d) 100% N_2 in the feed (control). Cells were initially grown aerobically to mid-exponential phase (DCW ~0.2 g/L). Temperature and pH set-points were 30 ^oC and 6.8 pH.

Reduction of ferricyanide during the experiment (Fig. 4-7a) was non- linear, with the rate increasing from 0.04 mmol/g·h between hr 0-8 to a maximum of 0.9 mmol/g·h between hr 18-23. This may have indicated

an optimal ferricyanide concentration of ~1.4 mM or a period of acclimatization as the cells adjusted to an alternative form of metabolism. The total CO_2 production (Fig.4-7c) was 0.58 mmoles over this period which was 45% larger than the theoretical value (0.4 mmoles) calculated for direct methane oxidation by 2 mM of ferricyanide. In the control (Fig. 4-7b), ferricyanide reduction occurred over 10 hrs and was approximately linear. A steady CO_2 concentration of 230 ppm was maintained with a total production of 0.76 mmoles (Fig.4-7d). As previously observed with stationary phase cultures, base addition occurred concurrently with ferricyanide reduction and CO_2 production. The average rates are reported in Table 4-5.

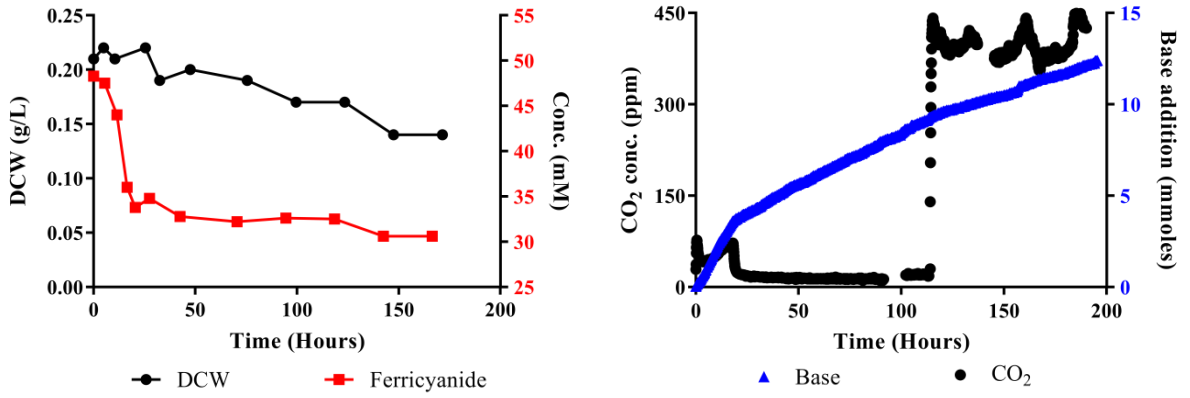
Table 4-5: Average specific ferricyanide reduction and CO_2 production rates by the mixed methane-oxidizing culture in mid-exponential phase.

	Average FCN reduction rate (mmol/g·h)	**Specific CO_2 production rate (mmol/g·h)**
Experiment (5% CH_4, 95% N_2 in feed)	0.40	0.07
Control (100% N_2 in feed)	1.31	0.28

Compared to stationary phase cultures, the ferricyanide reduction rate was 1.7 times higher in the experiment and 3 times higher in the control. In normal aerobic respiration, the specific oxygen uptake is greatest during the exponential phase, as the cells require more energy for growth compared to stationary phase which mainly requires energy for maintenance purposes (Garcia-Ochoa et al., 2010). Although cell growth was not observed, a parallel can be drawn between the ferricyanide reduction rate and oxygen uptake. This provided further evidence for the central role of ferricyanide as the terminal electron acceptor in a respiratory-associated process.

Investigating the ability of the mixed culture to grow anaerobically, using ferricyanide instead of oxygen, was a key objective of this study. According to stoichiometry, the theoretical molar requirement of ferricyanide required for complete methane oxidation is four times that of oxygen in aerobic methane oxidation. A biomass yield of 0.2 g/g_{O2} has been reported for *Methylococcus sp.*, a well characterized Type I methanotroph (Bailey & Ollis, 1976). Using this yield it was estimated that, at an initial ferricyanide concentration of 2 mM, the expected cell density increase would be less than 0.01 g/L which could not be easily detected using spectrophotometry (Appendix 3).

The experiment was subsequently repeated using an initial ferricyanide concentration of 50 mM (Fig. 4-8). Based on the calculation performed above, this would result in a theoretical increase in DCW of 0.1 g/L if methane was being oxidized by ferricyanide.



(a) (b)

Figure 4-8: (a) Ferricyanide concentration (mM) and dry weight biomass concentration (DCW) (g/L), (b) CO_2 production (ppm) and base addition (mmoles), using an initial ferricyanide concentration of 50 mM with cells in mid-exponential growth phase (~0.2g/L). The inlet gas composition was 5% CH_4 and 95% N_2 from hr 0-110 and 5% CH_4 and 95% air from hr 110-190.

Ferricyanide reduction occurred at a rate of 3.2 mmol/g·hr from hr 0-20.5, with a total CO_2 production of 1.5 mmoles. The ferricyanide concentration then decreased slowly from 33.8 mM to 32.8 mM and the CO_2 concentration from 20 ppm to 10 ppm from hr 20.5-110. The cell density decreased from 0.22 g/L to 0.18 g/L over the entire period, implying that a novel, oxygen-free methane oxidation pathway was not operational using ferricyanide as a growth substrate. This was in agreement with previous literature on aerobic methane-oxidation, which to date has not reported the ability of an aerobic methanotroph to convert to an anaerobic form of metabolism using an alternative electron acceptor. The exception is *M. oxyfera* which produces its own oxygen for MMO activation from nitrite; however, this is inherently an anaerobic mechanism (Ettwig et al., 2010). The initial enrichment procedure did not select for such an organism and was unlikely to exist in the consortium.

Base addition occurred over the entire period, with a clear distinction between the addition rate during the initial reduction period (hr 0-20.5) and the time of decreasing carbon dioxide production (hr 20.5-110). The base addition during the second period indicated ongoing cellular activity or leaching of acidic products from the cells due to cell lysis. The effect of ferricyanide on *E. coli* cells has been previously studied by Liu et al. (2009). Below a concentration of 25 mM, negligible effects on cell growth were observed. However, a 50% decrease in the maximum cell density was observed at 50 mM, and upon exposure to a concentration of 100 mM there was visible destruction of the cell wall. A similar effect may have caused the continued addition of base during the second period.

The inhibitory effect of ferricyanide on the mixed culture was investigated by reintroducing air in place of nitrogen at hr 110; this was important, as the power density of a mediated-MFC is dependent on both the availability of mediator to the cells (initial concentration) and the long-term cell viability. As the culture was in mid-exponential phase, if ferricyanide had zero effect on the cell metabolism, it was expected that

growth would continue until stationary phase was reached (DCW ~0.42 g/L). However, the cell density decreased from 0.18 g/L to 0.14 g/L and the CO_2 concentration fluctuated between 380 ppm and 420 ppm which indicated inhibition (400 ppm is the baseline value of CO_2 in air (UCAR, 2017). This may have caused the incomplete reduction and prevented growth of the culture; the ability to use ferricyanide in place of oxygen during methane oxidation was thus not fully rejected.

Experiments performed on exponential-phase cultures also provided insight into the reducing compound responsible for driving ferricyanide reduction in the control. Storage polymers such as PHA and glycogen tend to accumulate in the stationary phase when bacteria lack an essential nutrient and are supplied with excess carbon (Chee et al., 2010). Ferricyanide reduction using internal storage polymers was therefore unlikely, as full reduction occurred in nutrient sufficient conditions before the polymers had time to accumulate. A more rigorous technique to confirm this would involve extraction of PHA using solvents and subsequent FTIR/ spectrophotometric analysis. PHA could also be stained and qualitatively examined under a microscope (Kunasundari & Sudesh, 2011).

On the other hand, production of organic compounds can occur concurrently with growth, for example in the *Acetobacter* and *Glucanobacter sp*. used commercially to produce acetic acid (Raspor & Goranovič, 2008). Certain methanotrophs such as *Methylomicrobium buryatense* 5GB1 have also been shown to produce compounds such as formate and lactate during unrestricted batch growth on methane (Gilman et al., 2015). This was therefore identified as the more likely scenario for driving ferricyanide reduction.

4.3.2 Production of acetate and ethanol

High performance liquid chromatography (HPLC) analysis detected the presence of organic compounds in the broth. The initial sample was collected after 29 hours of aerobic growth on 5% CH_4 and 95% air and the following samples before and after ferricyanide reduction in the experiment (5% CH_4 and 95% N_2 in the feed) and the control (100% N_2 in the feed) (Fig. 4-9).

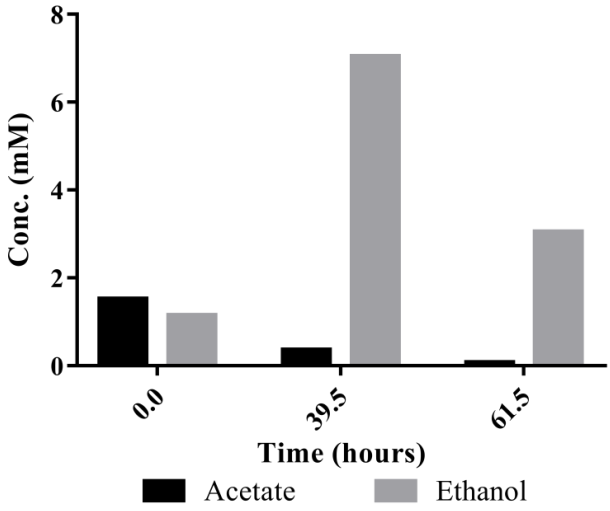


Figure 4-9: The change in concentration of ethanol and acetate throughout the course of mediator reduction in the experiment (5% CH_4, 95% N_2 in the feed) (hr 0-39.5) and the control (100% N_2 in the feed) (hr 39.5-61.5). The culture was initially grown aerobically with 5% CH_4 and 95% air for 29 hrs which represents the concentration at hr 0.

Acetate was initially detected at ~1.5 mM and ethanol at~1.2 mM. Acetate is a derivative of acetyl- CoA and has been reported as a product of methane metabolism in species such as *M. buryatense 5GB1* (Gilman et al. (2015). In contrast, ethanol production by aerobic methanotrophs has not been widely reported; in this case, another species capable of producing ethanol from an organic intermediate of methane oxidation was most likely responsible.

In the experiment (hr 0-39.5), the concentration of ethanol increased by 5.8 mM which indicated a potential fermentation during oxygen starvation. Ethanol could have been formed directly from methane (no reports available) or from the oxidation of internal storage polymers such as glycogen. The ethanol concentration decreased from 7 mM to 3 mM in the control (hr 39.5-61.5) which makes a methane-dependent mechanism more likely. Acetate production was dependent on aerobic conditions as the concentration did not increase in the absence of oxygen.

The presence of acetate and ethanol in the broth meant that ferricyanide reduction could have been driven by oxidation of these compounds. The reactions were thermodynamically feasible according to the Gibbs energy change (Eq. 16 & 17).

$$C_2H_3O_2^- + 2H_2O + 8FeCN_6^{3-} \rightarrow 2CO_2 + 7H^+ + 8FeCN_6^{4-} \quad (16)$$

ΔG = -494 kJ/mol

$$C_2H_6O + 3H_2O + 12FeCN_6^{3-} \rightarrow 2CO_2 + 12H^+ + 12FeCN_6^{4-} \quad (17)$$

ΔG = -799 kJ/mol

Using these equations, the theoretical CO_2 production and amount of ethanol and acetate required to reduce the injected amount of ferricyanide were calculated and compared against the observed values (Table 4-6). These calculations were based on the compound which exhibited the largest decrease in concentration: acetate in the experiment and ethanol in the control.

Table 4-6: Comparison between theoretical and observed values of CO_2 production and concentration of organic compounds if these were responsible for ferricyanide reduction.

	Compound	**FCN used (mmoles)**	**Theoretical CO_2 production (mmoles)**	**Actual CO_2 production (mmol)**	**Theoretical organic required (mmoles)**	**Actual organic consumed (mmoles)**
Experiment (5% CH_4, 95% N_2 in feed)	Acetate	3.2	0.80	0.56	0.40	1.8
Control (100% N_2 in feed)	Ethanol	3.5	0.58	0.78	0.30	6.4

There was a 30% difference in the carbon dioxide production and a 70% difference in the amount of acetate consumed in the experiment compared to the theoretical values. The reported biomass yield for a common acetate consumer *Aerobacter aerogenes* is 10.5 g/mol (Bailey & Ollis, 1976). If the difference between the observed and theoretical amount of acetate (1.4 mmoles) was accumulated as biomass, the increase in cell density would be less than 0.01 g/L. Certain Type II methanotrophs can metabolise acetate in the absence of methane, with reported cell yields of 20.5 g/mol (Sec. 2.4.2) (Dedysh et al., 2005). In this case, the cell density increase would also be less than 0.02 g/L. Thus, acetate may have been responsible for driving mediator reduction in the experiment, with the discrepancy between the observed and theoretical values due to partial assimilation of acetate as biomass.

The amount of ethanol consumed in the control was nearly 20 times larger than the theoretical value, with a 35% discrepancy in the CO_2 values. If the theoretical 0.3 mmoles of ethanol was used for driving ferricyanide reduction, the remaining 6 mmoles must have been assimilated as biomass, lost to evaporation or partially oxidized to another organic intermediate.

The amount of ethanol evaporation from the bioreactor was predicted using Henry's law (Eq. 18):

$$C_g = \frac{K_H . C_L}{RT} \quad (18)$$

Where K_H = Henrys constant (0.005 L.atm/mol), R = Ideal gas constant (0.082057 L.atm/mol.K) and T = Temperature (K) (Sander, 1999)

Conducting a mass balance, the change in moles of the volatile compound over time can then be calculated (Eq. 19):

$$\frac{dn_L}{dt} = \frac{-F_{out} . K_H . n_L}{R.T.V} \quad (19)$$

Where F_{out} = Outlet flowrate (L/min) and V = Reactor volume (L).

Assuming nitrogen fixation was not occurring and the inlet flowrate was equal to the outlet flowrate, after 22 hours of continuous gas flow at 0.21 L/min the amount of ethanol lost to evaporation would be 0.32 mmoles. Therefore, evaporation did not account for the difference in concentration over this period.

A typical biomass yield for an ethanol consumer is 31.2 g/mol (Bailey & Ollis, 1976); if the ethanol was accumulated as biomass, an increase in cell density of 0.13 g/L was expected. This did not align with the experimental values, with the DCW decreasing slightly from 0.2 g/L to 0.19 g/L. Based on the observed CO_2 production, the remaining ethanol must therefore have been partially oxidized to another organic compound, which was possible as there were unidentified compounds in the HPLC spectra.

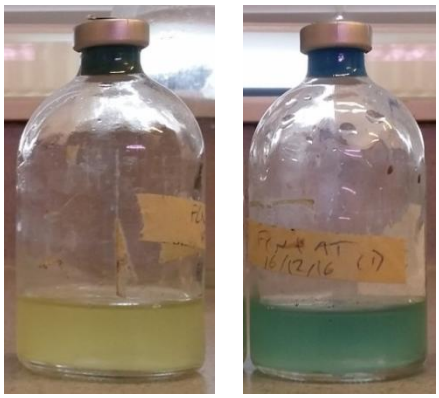
Although not repeated due to time constraints, the results showed that organic compounds could be synthesized by the mixed methane-oxidizing culture using methane as the input energy source. Oxidation of these compounds by a partnering organisms or a facultative methanotroph was the most likely scenario for driving ferricyanide reduction when methane was absent from the feed, and may also have caused the reduction with methane present. Replicate measurements should be conducted in the future, as well as a community analysis using 16s amplicon sequencing before and after mediator reduction to determine how the community composition changed over time.

4.3.3 MMO inhibition

If a novel methane-oxidizing pathway by an aerobic methanotroph was occurring, it was expected that in the presence of a known MMO inhibitor, ferricyanide reduction would continue to be observed. If the mediator remained oxidized then this would indicate that reduction was dependent on the presence of a conventional MMO enzyme/pathway. In this case, oxygen would have to be produced by the culture to activate the enzyme, for example by splitting water. As described previously, the anaerobic *M.oxyfera* is an example, where nitrite is decomposed into nitrogen and oxygen, allowing methane oxidation to

proceed through the normal respiratory pathway (Ettwig et al., 2010). These pathways (Serine cycle or RumP) are not dependent on the presence of oxygen and could use an alternative electron acceptor such as ferricyanide. The issue with applying this rationale to a mixed methane-oxidizing culture is that there could be numerous organisms within the consortium capable of utilizing ferricyanide. The inhibitor may also target enzymes from other species involved with the reduction. However, with assumed methanotroph abundance, the hypothesis was tested.

Allylthiourea was identified from the literature as a promising MMO inhibitor as this compound specifically targets the MMO enzyme. It is a chelating agent with a minimum inhibitory concentration of 100 µM which binds with metals such as copper required for enzyme activation (Bédard & Knowles, 1989). Allylthiourea and ferricyanide were added to a final concentration of 1 mM and 2 mM respectively in triplicate serum vials containing cells in mid-exponential growth phase (DCW 0.2 g/L) and the atmosphere adjusted to 20% CH_4 and 80% N_2.After incubation for 30 hrs, the solution turned aqua blue; the ferricyanide reduction could therefore not be measured by spectrophotometry (Fig. 4-10).



(a) (b)

Figure 4-10: Colour change of serum vials after addition of 2 mM ferricyanide and 1mM allylthiourea at, (a) t = 0 h, (b) t = 15 h in a 20% CH_4 and 80% N_2 atmosphere.

The observed colour change was most likely due to a reaction between ferricyanide and allylthiourea; this has been previously reported by Mukhopadhyay and Bhattacharyya (1983). The allylthiourea appeared to inhibit the abundant organism as the biomass did not increase when added to a vial containing the growing culture. During normal aerobic growth without allylthiourea, the biomass reached a maximum of 0.3 g/L.

The role of the MMO enzyme in reduction of ferricyanide was not determined in this experiment and thus the results do not provide evidence against the hypothesis of a novel methane oxidation pathway. An alternative compound which could be tested in the future is acetylene, a 'suicide' inhibitor of both the particulate and soluble form of MMO. A full list of inhibitors is provided by Bédard and Knowles (1989).

If future works reveals that ferricyanide can still be reduced without an active MMO enzyme, methane consumption should also be monitored to confirm the hypothesis.

4.3.4 Mediator screening

The ability of a redox mediator to extract electrons depends on the potential difference between the mediator and the charge carrying intermediate within the cell (Sec.2.6.4); this can directly influence the power output of a MFC. In general, mediators with positive reduction potentials are more favourable for facilitating charge transfer (Sec.2.6.4). However, the mechanism by which the mediators interact with the cell is also important; for example, neutral red is highly permeable to the lipid bilayer and may accept electrons from different (intracellular) redox species along the respiratory chain (Fathey et al., 2016). Four alternative mediators which have been used in previous MFC research (Li & Khanal, 2016; Logan, 2008) were tested for comparison against ferricyanide (FCN, E^o +0.36 V):

- Methylene blue (MB), E^o -0.021 V
- Neutral red (NR), E^o -0.325 V
- Thionine acetate (TA), E^o +0.064 V
- Resorufin (RS), E^o -0.051 V

In descending order of thermodynamic feasibility, FCN > TA > MB > RS > NR. The colour of each mediator is shown in Fig. 4-11.

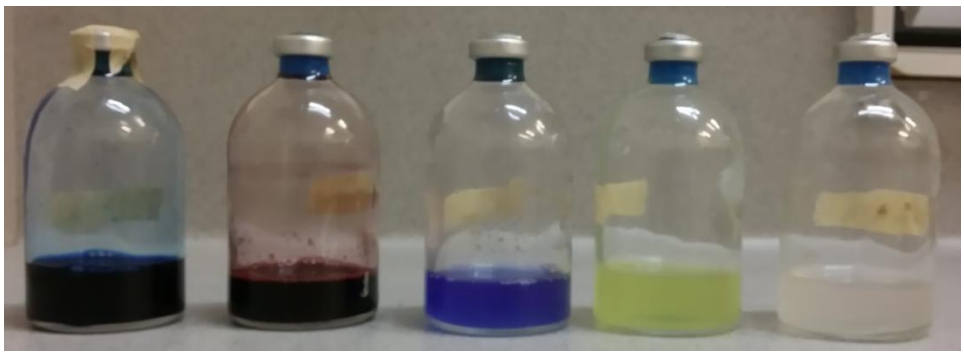


Figure 4-11: Colour of oxidized mediators. From left to right: Methylene blue, neutral red, thionine acetate, ferricyanide and resorufin.

Cells were grown to mid-exponential phase in the bioreactor (DCW ~0.2 g/L), withdrawn and placed in 120 mL serum vials. Each mediator was then added to a final concentration of 2 mM and the atmosphere adjusted according to Table 4-7. Due to the instability of the reduced form of these mediators when in contact with oxygen, the reduction was monitored visually over time.

Table 4-7: Results of experiment and controls from mediator screening study in serum vials.

	Description	Expected outcome	Result
Experiment	(20% CH_4, 80% N_2) with cells, with		
	a) 2 mM NR	a) NR reduction	×
	b) 2 mM MB	b) MB reduction	✓
	c) 2 mM TA	c) TA reduction	✓
	d) 2 mM RS	d) TS reduction	×
Controls	(100% N_2) with cells, with		
	a) 2 mM NR	a) No NR reduction	✓
	b) 2 mM MB	b) No MB reduction	✓
	c) 2 mM TA	c) No TA reduction	×
	d) 2 mM RS	d) No RS reduction	✓
	(20% CH_4, 80% N_2) without cells, with		
	a) 2mM NR	a) No NR reduction	✓
	b) 2 mM MB	b) No MB reduction	✓
	c) 2 mM TA	c) No TA reduction	✓
	d) 2 mM RS	d) No RS reduction	✓

✓ = Result consistent with expected outcome

× = Result not consistent with expected outcome

Resorufin and neutral red were not reduced by the mixed culture. These mediators were ranked the lowest in terms of thermodynamic driving force, thus the electron carrier was likely a species in the electron transport chain with a higher reduction potential such as ubiquinone (E^o +0.045 V) or cytochrome b (E^o +0.07 V) (Logan, 2008).

Full reduction of thionine acetate occurred over approximately 15 hours in the presence and absence of methane, corresponding to a specific reduction rate of 0.6 mmol/ g·hr. This was observed as a colour change from purple to colourless as thionine was reduced to leucothionine (Fig. 4-12). After exposing the culture to air, the reduced mediator oxidised rapidly back to its original colour which implied that it had not been consumed by the culture. The effectiveness of thionine for power generation in MFCs has been well documented (Delaney et al., 1984; Rahimnejad et al., 2012). It is a lipophilic compound which can cross the outer layer of the cell membrane to extract electrons. In contrast, ferricyanide has a low permeability to the cell membrane due to its hydrophilic nature which prevents access to intracellular

electron sites; electrons must be transported to the cell membrane by another species in the electron transport chain (Rawson et al., 2014).

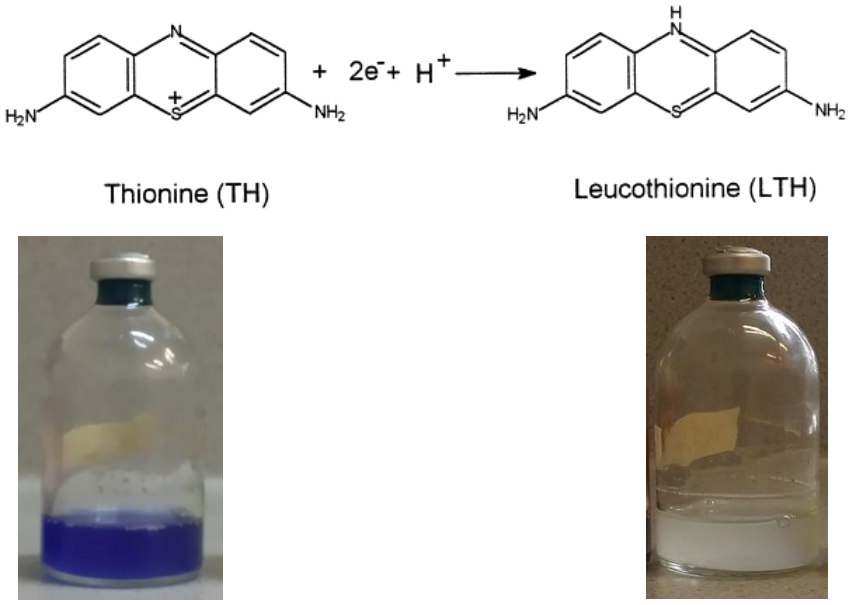
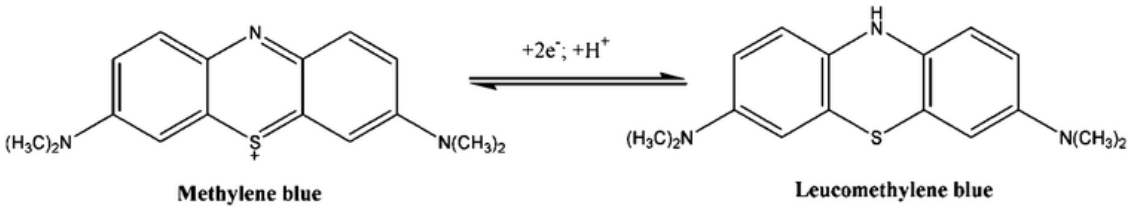


Figure 4-12: Half equation for reduction of thionine to leucothionine (Yang et al., 1999) and the observed colour change during incubation of mixed methane-oxidizing culture with 2 mM of thionine in a 20% CH_4 and 80% N_2 atmosphere for 15 hours.

The rate of thionine reduction was approximately 15 times higher than the ferricyanide reduction rate in serum vials (0.04 mmol/g·hr). Thus, although the driving force for ferricyanide reduction was higher, the structural difference between these mediators appeared to have an impact on the reduction rate. Time constraints did not permit testing of thionine in the bioreactor, however, based on the maximum recorded ferricyanide reduction rate of 3.2 mmol/g·hr, a thionine reduction rate of 50 mmol/g·h was predicted. This would greatly improve the power output of a MFC and should be investigated in future work.

Reduction of methylene blue occurred over a period of 14 days corresponding to a reduction rate of 0.03 mmol/g·hr, observed as a colour change from blue to a colorless leucomethylene blue (Fig. 4-13). Reduction did not occur in the control without methane which implied that reduction was directly coupled to biological methane oxidation. Similar to thionine, upon exposure to air, the mediator oxidized rapidly back to its original colour meaning that it had not been consumed by the cells. The slower reduction rate may have been caused by several factors:

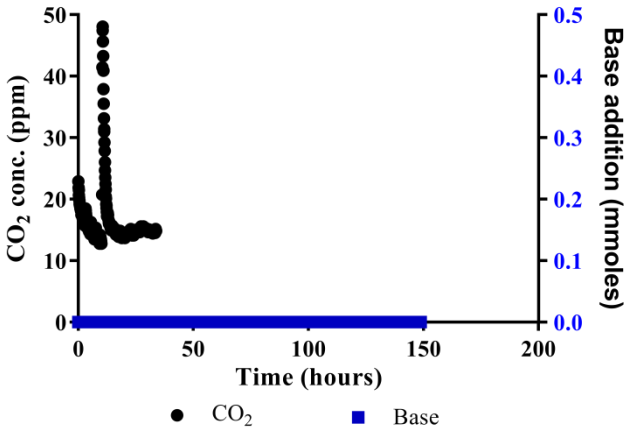
- A lower themodynamic driving force.
- Although MB belongs to the same group as thionine (phenothiazines), it is a larger molecule (MW 319.85 g/mol) which means a greater resistance to diffusion.
- Poor solubility of methane (if reduction was driven by methane oxidation).



t = 0 t = 168 hrs t = 336 hrs

Figure 4-13: Half equation for reduction of methylene blue (MB) to leucomethylene blue (Farjami et al., 2010) and the observed colour change during incubation of mixed methane-oxidizing culture with 2 mM of MB in a 20% CH_4 and 80% N_2 atmosphere for 10 days. In each image, the control vial without methane is shown on the left and the vial with methane on the right.

The experiment was subsequently repeated in the bioreactor with methylene blue at identical conditions to those performed with ferricyanide (30 ^{o}C, pH 6.8). Cells were grown to ~0.2 g/L prior to injecting MB to a concentration of 2 mM and incubated with 5% CH_4 and 95% N_2 (Fig. 4-14).



(a) (b)

Figure 4-14: (a) Colour of mixed methane-oxidizing culture in bioreactor with methylene blue (MB) at a concentration of 2 mM after 168 hrs of cultivation in a 5% CH_4 and 95% N_2 atmosphere, (b) CO_2 production (ppm) and base addition (mmoles) during the reduction experiment.

After incubating for 168 hrs following MB addition, there was no observable change in the colour (Fig. 4-14a). CO_2 was monitored for 45 hours and base addition measured over the entire period (Fig. 4-14b).

The CO_2 concentration increased rapidly at hr 24 from 13 ppm to 50 ppm; however, the peak was not clearly defined and decreased quickly back to 13 ppm. Zero mmoles of base was added which indicated zero activity.

Based on these results, methane oxidation by the mixed culture using methylene blue as the mediator was not possible. The culture may have needed more time to acclimatize to the new conditions which caused the low level of CO_2 production, however, based on the experiment conducted in serum vials, a visible decrease in the blue pigment was expected within 168 hrs. The reduction observed in serum vials was possibly due to the presence of another species that was not present in the original culture.

4.3.5 Summary

The initial study proved that a methane-fed MFC could be developed using a mixed methane-oxidizing culture with mediators to facilitate charge transfer. However, the study also indicated a reduction pathway independent of methane oxidation. The presence of this pathway could influence future development of a MFC and was the key focus of this section. The specific findings were:

1. A mixed methane-oxidizing culture, halted in mid- exponential growth phase, reduced ferricyanide in the presence of methane at 0.4 mmol/g·h which was two times higher than the rate observed in stationary phase. Reduction also occurred in absence of methane (control) at 1.31 mmol/g·h which was nearly four times higher than the value recorded in stationary phase. This provided further evidence of ferricyanide acting as the terminal electron acceptor, as the substrate demand is greatest during exponential growth.
2. At an initial ferricyanide concentration of 2 mM, if ferricyanide was taking the place of oxygen during methane oxidation, the observed cell growth would have been undetectable using UV-Vis spectroscopy. At an initial concentration of 50 mM, the expected cell density increase was 0.1 g/L. However, this concentration did not appear to support cell growth. After re-exposing the culture to methane and air, cellular activity did not continue; thus, it appeared that the elevated ferricyanide concentration had an inhibitory effect on the cells. Due to this, the hypothesis of a novel methane oxidation pathway using ferricyanide in place of oxygen was not fully rejected.
3. As internal storage polymers such as PHA and glycogen tend to accumulate during stationary phase, oxidation of these compounds was the least likely scenario due to the ability of exponential-phase cultures to reduce ferricyanide.
4. Mediator reduction in the absence of methane was most likely driven by the oxidation of acetate or ethanol synthesized during aerobic methane oxidation. Ethanol was also produced with oxygen absent which indicated a fermentation of methane. The observed production of carbon dioxide did not match the theoretical values for oxidation of these compounds using ferricyanide

as the terminal electron acceptor; these discrepancies may have been caused by assimilation as biomass or partial oxidation to other organics. Additional work is required to confirm the responsible compound and to determine if this caused the reduction with methane present.

5. To investigate the role of the MMO enzyme in ferricyanide reduction, a known MMO inhibitor, allylthiourea, was added to serum vials containing the mixed culture with ferricyanide. However, the presence of allythiourea caused the solution to turn aqua blue which meant that the colour change of ferricyanide could not be monitored over time. Thus, the role of MMO in the mediator reduction was not identified.

6. The alternative mediators, resorufin and neutral red, were not reduced by the culture. Methylene blue was reduced in serum vials only in the presence of methane at 0.03 mmol/ g·hr and was not reduced in the bioreactor. The ability of methylene blue to act as the terminal electron acceptor for the mixed culture was therefore inconclusive. Thionine acetate was promising and reduced in the presence and absence of methane in serum vials at a rate of 0.6 mmol/ g·hr.

Chapter 5: Methane-fed MFCs: An engineering perspective

This project has shown that it is fundamentally possible to generate electricity from a mixed methanotroph culture using potassium ferricyanide as the charge carrier. In this chapter, the feasibility of a methane-fed MFC in terms of power density and process design will be examined for two separate scenarios: direct oxidation of methane by ferricyanide and oxidation of organic based compounds produced during aerobic methane oxidation. This will be consolidated by discussing the relevance and potential application in New Zealand.

5.1 Scenario A: Direct methane oxidation

5.1.1 Power generation

The power generating capacity of a direct methane-oxidizing MFC is dictated by the cell potential and the rate limiting step of current production. Eq. 20 demonstrates the oxidation of ferrocyanide to ferricyanide at the anode (E^o +0.36) and reduction of oxygen to water at the cathode (E^o +0.82 V).

$$4Fe(CN)_6{}^{4-} + O_2 + 4H^+ \rightarrow 4Fe(CN)_6{}^{3-} + 2H_2O \quad (20)$$

Based on standard potentials, the maximum cell voltage using these redox species is +0.45 V (Sec. 2.6.3). To estimate the current, it was assumed that electron transfer was limited by the ferricyanide reduction rate. Each mole of ferricyanide reduced requires one mole of electrons; as 1amp (A) is equivalent to one coulomb per second (C/s), the reduction rate is converted to current by multiplying with the Faraday constant (96,485 C/mole). The power density is then estimated using Eq. 21.

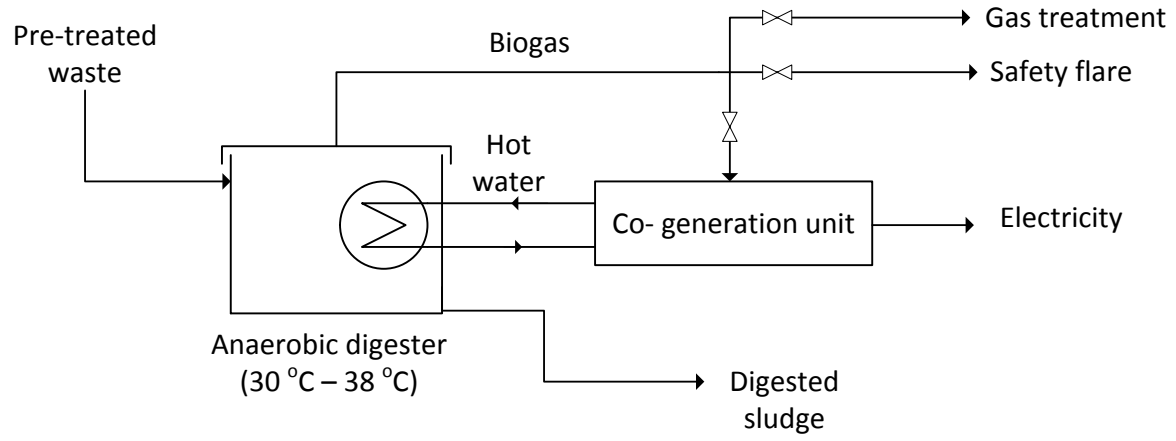
$$P = \frac{VI}{\nu} \quad (21)$$

Where V is the cell voltage, I is the current (A) and ν is the bioreactor working volume (m^3).

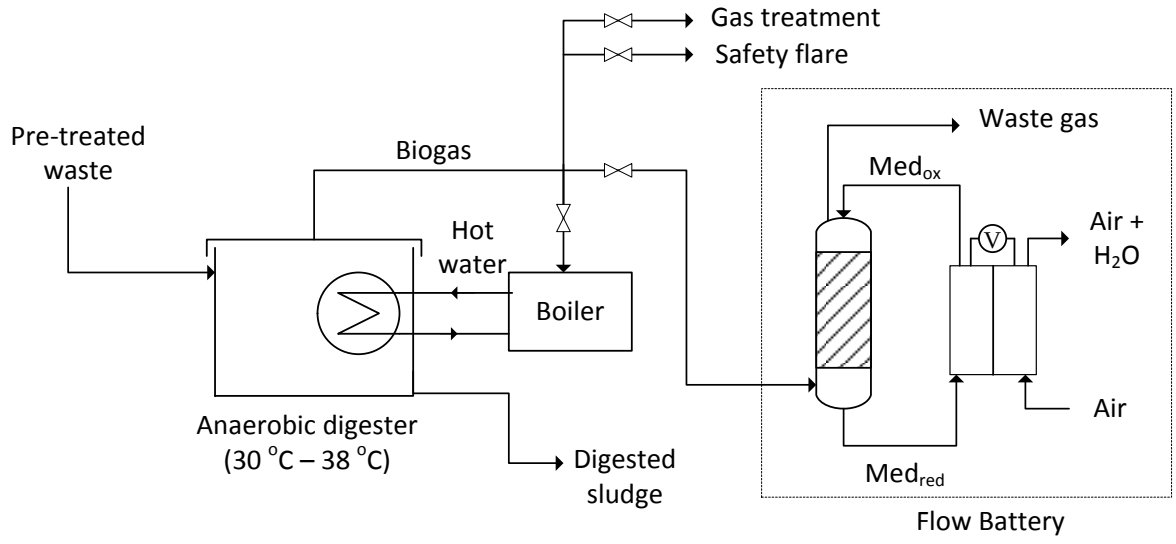
For modelling purposes, the maximum ferricyanide reduction rate is assumed to be 3.2 mmol/g·hr (Sec. 4.3.1). Using Eq. 21 and a bioreactor working volume of 0.0016 m^3, the maximum power density at this reduction rate is 7 W/m^3. To put this in perspective, in order to power a 60 W light bulb, an anode volume of approximately 8 m^3 is required. The practical applications are therefore limited to devices with very low power requirements such as biosensors; this could be used to monitor biological activity or methane levels in environments such as coal mines. However, as with most MFCs, in order to be commercially viable and competitive with existing technologies, a large improvement in the electron transfer rate is required.

5.1.2 Practical application

One of the most extensively researched areas for MFC implementation is wastewater treatment (WWT), a process which generally requires an external input of energy to meet effluent discharge standards. The energy demand can be reduced using anaerobic digestion of the pre-treated sludge to produce biogas which is combusted for electricity and heat generation (Fig. 5-1a) (Khanal, 2011). MFCs exploit the energy contained in organic waste more efficiently than AD; however, the power densities at present are too low to be competitive. Rabaey and Verstraete (2005) have identified a target of 1 kW/m^3 (anode volume) for self- sufficient operation of WWT plants using MFCs



(a)



(b)

Figure 5-1: Process flow diagram of (a) Conventional combined-cycle AD plant (Khanal, 2011), (b) AD coupled with a methane-fed MFC for electricity production.

The main limitation of using biogas from AD in combustion engines is the low energy efficiency and the presence of corrosive contaminants (Sec.2.6.5). A direct methane-oxidizing MFC (Fig. 5-1b) would overcome these issues, and without depending on the anodic biofilm to transfer charge as traditional MFCs, the 1 kW/m^3 target identified by Rabaey and Verstraete (2005) may be more achievable.

Modelling the bioreactor as the anode of a methane-fed MFC, in order to generate 1 kW/m^3 at a cell density of 0.2 g/L, the required specific mediator reduction rate is 0.4 moles/g·hr, which is 125 times larger than the current ferricyanide reduction rate of 3.2 mmol/g·hr (Appendix 3). The main considerations for improving this rate are conversion of substrate to CO_2 and cell density. In most bioreactor applications using sparingly soluble substrates such as oxygen and methane, there are several major rate limiting steps (Bailey & Ollis, 1976):

- Mass transfer from the gas phase into the liquid phase.
- Movement through the gas-liquid interface and diffusion to the cell.
- Diffusion through the cell wall to the reaction site.

These resistances can be decreased by optimizing agitation rate, inlet gas flowrate, impeller design and temperature (Bailey & Ollis, 1976). However, improvements usually come at a cost in terms of power requirements and may not be economical for increasing the reduction rate.

Increasing the cell density is an alternative option. If the reduction rate is kept constant at 3.2 mmol/g.h, the cell density required to produce 1 kW/m^3 is 25 g/L (Appendix 3). Although the maximum cell density obtained in this study is ~0.4 g/L, values as high as 18 g/L and 61 g/L are reported for *Methylosinus Trichosporium OB3b* grown on methane and methanol (Adegbola, 2008). High cell density in a batch reactor requires optimization of parameters such as pH, temperature and inlet gas composition, as well as identification of limiting nutrients (Sec. 4.1.3).

In reality, a well-mixed suspended cell bioreactor is a poor model for the anode of a methane-fed MFC. Practical implementation would require a methanotroph culture (pure or mixed) entrapped in porous beads or immobilized on a surface inside a packed bed biotrickling filter, with a continuous upward flow of methane and down- flow of recycled mediator/nutrients (Sec. 2.6.4). This design improves the contact area of the gas with the cells and decreases the power requirement compared to traditional batch reactors (Devinny et al., 1998).

Conversion efficiency and elimination capacity (EC) are terms used to characterise biofilters and refer to the amount of contaminant removed from the inlet stream and the rate of degradation. For methane filters, conversions as high as 90% and ECs ranging from 30-60 g CH_4/m^3·hr are reported (Streese & Stegmann, 2003). If the mediator could be reduced at the same rate as oxygen and assuming 90% conversion of CH_4

to CO_2, a trickle bed MFC could produce a maximum power density of 830 W/m^3 (Appendix 3). In reality, part of the carbon is accumulated as biomass or extracellular polymeric substances (EPS), leading to conversions between 60% to 70% (Nikiema & Heitz, 2010). Improvements can be made by consideration of design factors such as moisture content, temperature, pH, surface area, packing material and gas/liquid flowrates (Devinny et al., 1998).

Based on the results from this study, it is unlikely that ferricyanide could be reduced at a sufficient rate to achieve a high EC unless a higher cell density (> 10 g/L) is used. However, in a packed bed bioreactor, accumulation of biomass is a major issue as this hinders gas flow resulting in poor conversion (Deshusses & Cox, 2003). Alternative mediators such as thionine acetate may prove more successful for generating meaningful power densities (> 1 kW/m^3). The results indicate a specific reduction rate of 50 mmol/g·h (Sec. 4.3.4), corresponding to a power density of 200 W/m^3. Thus, this mediator is more likely to oxidize methane at a rate similar to that observed in bio-filters.

5.2 Scenario B: Oxidation of organic compounds

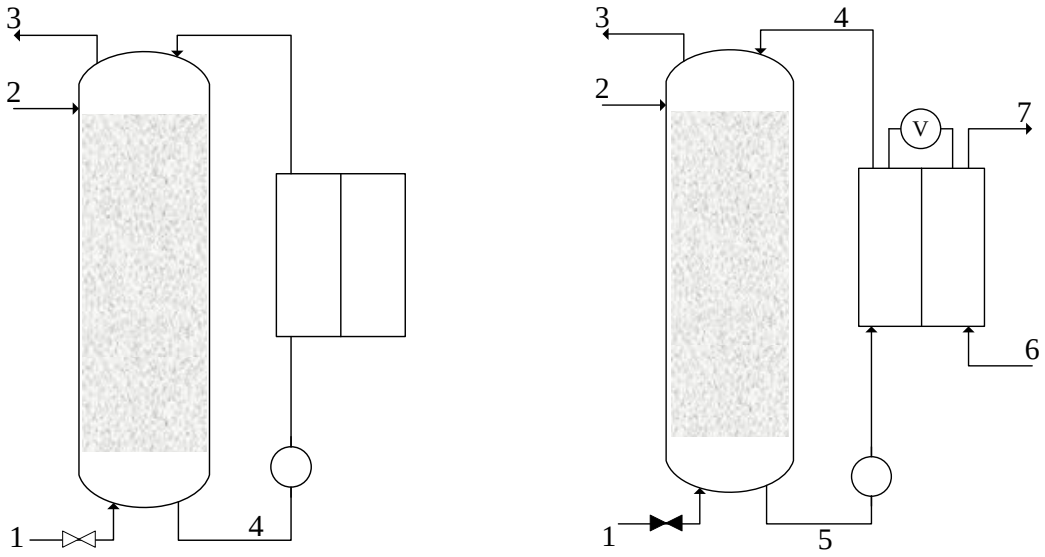
Oxidation of ethanol or acetate synthesized during aerobic methane oxidation was identified as the most likely scenario for driving ferricyanide reduction in the absence of methane and could also be used for electricity generation. A similar concept was explored by Kim and Chang (2009) with syngas as the carbon source; in this case, the electroactive bacteria were grown directly on the anode which limited power density to 6.4 W/m^3. Although mediators help to overcome the low power density issue, a different design approach compared to that outlined for direct methane oxidation would be required.

5.2.1 Alternate growth cycles

As described above, a biotrickling filter could be employed which alternates between aerobic growth on methane to produce the organic intermediate (Fig. 5-2a) and anaerobic growth on the organic using the mediator as the terminal electron acceptor (Fig. 5-2b). Two of these reactors operating simultaneously would maintain constant power generation.

If it assumed that ethanol is the source of reducing power and oxidation can only occur anaerobically, in order to generate a power density of 1 kW/m^3 at a cell density of 0.2 g/L, according to stoichiometry (Eq. 17) the required rate of ethanol oxidation to CO_2 by ferricyanide is 0.03 mol/g·h. To maintain power generation, the rate at which ethanol is synthesized during aerobic growth on methane must also equal the oxidation rate by the mediator. Environmental parameters which can be optimized to enhance the synthesis rate include temperature, pH and substrate availability. The possibility of methane fermentation for ethanol production should also be further explored; this would prevent the simultaneous production and consumption of ethanol during aerobic growth which may limit the ethanol-producing capacity. In addition, if the synthesis rate can reach the level required for commercial application, the value of

generating electricity would need to be compared against the market value of the organic compound. For example, use of methane as a carbon feedstock for ethanol fuel production would be more attractive than fermentation of sugar based crops if the production rate was at a competitive level.



(a) (b)

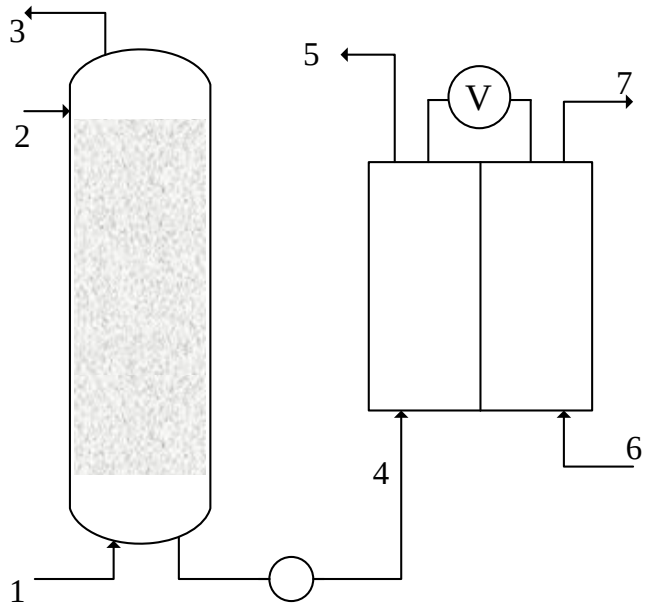
Stream	1	2	3	4	5	6	7
Description	CH_4 + air	Nutrients	CO_2	Oxidized mediator	Reduced mediator	Air	Air + H_2O

Figure 5-2: Single stage reactor design for electricity generation from organic compounds produced during methane oxidation. Two cycles would be required, (a) Aerobic synthesis using methane, (b) Mediator reduction using the organic compound as the carbon source.

This processing strategy could also be applied to PHA/glycogen driven mediator reduction. Although earlier discounted, future research may show that this is possible and more suitable for long term power generation. Cells could be maintained in stationary phase/nutrient depletion to accumulate storage polymers before switching to anaerobic conditions to oxidize the polymers using mediators. In either case, constant switching between aerobic and anaerobic growth on different substrates may not be feasible for maintaining a stable culture over time and would need to be carefully assessed. Organic compounds such as ethanol also tend to have inhibitory effects on cells at higher concentrations which would negatively affect the performance of a MFC (Wieczorek et al., 2011).

5.2.2 Continuous process

An alternative design is a continuous process with organics being produced by the mixed methane-oxidizing culture in a biotrickling filter and the resulting product oxidized directly in a fuel cell (Fig. 5-3). The fuel cell may employ mediators or use a traditional approach with a biofilm grown directly on the anode. The advantage of this design is that the reactor conditions can be optimized separately, for example a high cell density or nutrient deficiency in the biotrickling filter, or selection of an organism which can preferentially oxidize the organic compound at a high rate in the fuel cell. The culture also does not need to continuously alternate between aerobic/anaerobic metabolism and would potentially be more stable over time.



Stream	1	2	3	4	5	6	7
Description	CH_4 + air	Nutrients	CO_2	Organic-enriched product	Waste	Air	Air + H_2O

Figure 5-3: Two- stage reactor design for electricity generation from organic compounds produced during aerobic methane oxidation.

Employment of a liquid-fed MFC faces the traditional disadvantage of poor electron transfer between the biofilm and anode or the regular replacement of mediators. Until MFCs are improved, this design would be most suitable for off-grid devices which require negligible maintenance. As previously described for direct methane-oxidizing MFCs, a possible application is combined bioremediation and monitoring of methane emissions in natural or anthropogenic environments.

5.3 Application in New Zealand

5.3.1 Methane for electricity production

The two main sources of methane in New Zealand are natural gas and biogas. Approximately 5.4 billion m^3 of natural gas is produced from the fields in the Taranaki region, of which approximately 1 billion m^3 is used to generate electricity (MBIE, 2015). The majority of the electricity is generated at the Huntly power station which has a total capacity of 1453 MW. This plant operates four traditional steam turbines with a generating capacity of 250 MW each, as well as a combined cycle gas turbine with a capacity of 403 MW (Genesis Energy, 2017). With a conversion efficiency of ~40%, this plant would power approximately 480,000 homes. It is estimated that approximately 70 billion m^3 of natural gas are still recoverable from the Taranaki fields; at the current consumption rate there are enough reserves for at least a decade (MBIE, 2015). However, as the supply decreases and natural gas prices increase, alternative options need to be considered.

Approximately 90 million m^3 of biogas is captured in New Zealand each year from anaerobic digestion. Wastewater treatment and landfills in urban centres produce > 90% of the total biogas, most of which is used for internal electricity/heat generation (MBIE, 2015). It is estimated that an additional 50 million m^3 per year could be captured from industries such as dairy and meat processing (Bioenergy Association, 2016). In general, biogas produced from anaerobic digesters on farms is used in internal combustion engines, as the cost of compressing air for a small CHP turbine does not outweigh the benefits gained from off-grid electricity generation. Alternatively, the gas is combusted to provide heat and maintain the digestion temperature. Very few farms in NZ exploit the potential of this technology, most likely due to the high initial capital cost of the anaerobic digestion system resulting in a long payback period.

5.3.2 Small scale application

At the estimated power densities of methane-fed MFCs using mediators, large scale electricity production would not be economically competitive with combustion turbines. However, small scale off-grid electricity production may be feasible and would reduce demand on the existing network.

The average power consumption of a New Zealand household is 1 kW or ~8000 kWh/year (Electricity Authority, 2016). Assuming 70% conversion of methane to electricity, a direct methane-oxidizing MFC using mediators with an EC of 60 $g/m^3 \cdot h$ would require a reactor volume of 1.5 m^3 and approximately 1300 m^3 of methane per year (Appendix 3). On average, a household of four residents produces 0.72 m^3 of wastewater per day with a chemical oxygen demand (COD) of 870 g/m^3 (NZIC, 2017). The maximum amount of methane that can be produced by anaerobic digestion is 0.35 m^3/ kg COD (Khanal, 2011); one household may therefore produce up to 80 m^3 of CH_4 per year which would supply ~5% of the total power requirement. This means that additional methane would need to be distributed to each household which

requires more complex infrastructure than electricity distribution; domestic household use of methane-fed MFCs is therefore unlikely.

An alternative application is agricultural farms which produce large quantities of nutrient- rich effluent; for dairy farms, approximately 0.41 kg of volatile solids (VS) is collected per cow each day. A mesophilic plug flow anaerobic digester utilizing dairy waste can produce approximately 0.54 m^3 of biogas per kg VS (Craggs, 2006). Thus, a farm with an average sized herd of 413 cows (DairyNZ, 2014) could generate between 50-60 m^3 of CH_4 each day (assuming biogas contains 60% CH_4). Electricity consumption on a dairy farm can be divided into irrigation (34%), pumping (32%), water heating (15%), refrigeration (11%), lighting (1%) and other (7%) (DairyNZ, 2015), with a total average consumption of 112,100 kWh/y or 12.8 kW. Assuming that the 2 kW required for water heating is supplied from the biogas (either through a combined heat-power cycle or direct combustion in a boiler), a direct methane-oxidizing MFC with an EC of 60 $g/m^3 \cdot h$ and bed volume of 16 m^3 could produce enough power from captured biogas to achieve 100% self -sufficiency. A gas combustion engine with an electrical efficiency of 35% would produce 7 kW or 65% of the required power output (Appendix 3).

5.3.3 Economics

The economic feasibility of a gas combustion engine producing 6.7 kW is briefly compared with a 16 m^3 methane-oxidizing MFC for use on an average sized dairy farm in NZ. The following assumptions were made in the calculations:

- Continuous production of 90 m^3 CH_4 from effluent throughout the year.
- Average power requirement of 12.8 kW.
- Price of electricity from the grid is 30 c/kWh.
- Labour costs for installation are identical for both systems.
- Electrical efficiency of 35% for gas turbine.
- The MFC system uses a 16 m^3 biotrickling filter with EC 60 $g/m^3 \cdot h$ and 70% conversion efficiency of CH_4 to CO_2.
- The cost of capturing excess heat from a generator is equal to that of a gas-fed boiler.
- The biotrickling filter is the major capital expense in the MFC system.
- Exchange rate of 1.42 NZD to USD

An estimate of the costs for a small gas engine with a 4-8 year lifespan is provided by Craggs (2006). This includes gas scrubbing of contaminants such as H_2S, the generator and controller unit and other electrical requirements, but does not include CO_2 removal which is often necessary to increase power output. Operating costs are based on maintenance requirements and replacement of faulty components.

The capital cost of an inexpensive methane biotrickling filter with a 10-20 year lifetime is estimated using the formula provided by Deshusses and Cox (2003) (Eq. 22). This takes into account the materials of construction, bed packing and instrumentation such as pumps and controllers.

$$Cost\ (NZD) = 27000\ \times (Bed\ volume)^{0.757} \tag{22}$$

Operating costs depend on the system characteristics such as the recycle rate of nutrients/mediator and biomass accumulation. However, without moving parts, the maintenance requirements are significantly less than a gas engine. Typical expenditure ranges from \$1-3 per 1000 m^3 of contaminated air treated (Deshusses & Cox, 2003).

A summary of the expenses and electricity savings per year for each system is presented in Table 5-1. All costs were converted to NZD and adapted to reflect inflation.

Table 5-1: Economic comparison between gas combustion engine and a methane-fed MFC

	Gas combustion engine	**Biotrickling filter/MFC**
Capital cost (NZD)	35300	220000
Operating cost (NZD/ year)	7400	80
Electricity costs saved (NZD/ year)	21000	34000
Replacement time	4-8 years	10-20 years
Time to break even	2-3 years	6-7 years

The break-even time on the initial capital expenditure is 3-4 years longer for a MFC compared to a combustion engine. The lower operating costs and electricity savings means that it takes 8-9 years for the MFC to break even with the gas engine, after which the technology outperforms by \$20K per year. Thus, in the long run, MFCs are a more attractive option. To fully assess the potential cost savings, the fuel cell (anode/cathode) should be accurately modelled; factors to be considered include the size and maintenance of electrodes, materials of construction and compressed air requirements for the cathode chamber. In addition, if the reduction is driven by oxidation of organic compounds, the costs will likely be higher due to greater process complexity and the additional reactors /instrumentation required.

The feasibility of anaerobic digesters coupled with electricity/heat generation has been studied extensively; at present, the long payback period and lack of subsidies makes it uneconomical for many NZ

farms. If future work demonstrates an ability to generate meaningful power densities from methane-fed MFCs, this may provide an incentive for future investment in AD. The benefits are numerous and include reduction of solid waste/odours, reduced dependence on the existing electricity network and a reduced carbon footprint (Khanal, 2011).

Chapter 6: Conclusions

This project investigated an alternative pathway for electricity generation from methane using methane-oxidizing bacteria as biocatalysts in a microbial fuel cell. MFCs generally require a strict anaerobic environment in the anode chamber to enable transfer of electrons from the cell to an electrode. This presented a challenge for methane-fed MFCs, as oxygen is required for conversion of methane to methanol by the MMO enzyme, the first step in the respiratory pathway of all known aerobic methanotrophs. Previous work indicated an ability to use external mediators to drive methane oxidation using a pure methanotroph. The main objective of this project was to prove if this was possible.

To test the hypothesis, the pure methanotroph culture initially shown to have this ability was recovered. However, after streaking onto solid medium and serial diluting, a stable culture could not be maintained. A mixed methane-oxidizing culture which used methane as the input energy source was equally feasible for a MFC and was subsequently enriched from soil. Experiments performed in a 3.6-L bioreactor operated at 30 ^{o}C and 6.8 pH indicated an ability of the mixed culture to reduce potassium ferricyanide in the absence of oxygen. At an initial ferricyanide concentration of 2 mM, the maximum reduction rate was 0.2 mmol/g·h using cells in stationary phase and 0.4 mmol/g·h using cells in the exponential phase. Ferricyanide reduction was directly coupled with the production of carbon dioxide and addition of sodium hydroxide which implied that ferricyanide was acting as the terminal electron acceptor during the oxidation of a carbon-based compound.

Control experiments indicated that ferricyanide reduction was not dependent on the presence of methane. In this case, reduction was most likely driven by oxidation of ethanol and/or acetate which were produced during aerobic methane oxidation. The maximum reduction rates were 0.36 mmol/g·h in stationary phase and 1.31 mmol/g·h in exponential phase. The slower reduction rate when methane was present suggested an oxidation pathway limited by the low solubility of methane. However, the culture was unable to grow at elevated ferricyanide concentrations; this may have been caused by inhibition or by an inability to use ferricyanide in place of oxygen. Thus, the presence of a novel methane oxidation pathway was not confirmed in this project.

Regardless of the compound directly responsible for mediator reduction, with methane as the input energy source, a methane-fed MFC using mediators to facilitate charge transfer is feasible. The engineering implication was assessed based on the maximum recorded ferricyanide reduction rate of 3.2 mmol/ g·hr. A theoretical power density of 7 W/m^3 was estimated which was comparable with existing MFCs using liquid substrates. At this power density, practical application is limited and would not compete with existing technologies for electricity generation. Thionine acetate was identified as a more promising mediator, with an estimated reduction rate of 50 mmol/ g·hr and a theoretical power density of 200 W/m^3.

Methylene blue was inconclusive as reduction occurred in the serum vial with methane present but did not occur in the bioreactor Resorufin and neutral red were not reduced by the culture.

In a broader sense, this project has revealed that direct combustion of methane may not be the only route for electricity generation. A brief feasibility study was conducted to compare the technology with a traditional gas combustion engine. The anode was modelled as a biotrickling filter, a proven technology which can aerobically eliminate 30-60 $g/m^3 \cdot h$ of methane with a conversion efficiency of 70%. If future work demonstrates an ability of external mediators to oxidize methane at a similar rate, this technology may be employed instead of gas engines on small scale farms producing biogas from effluent, a renewable form of methane. This is particularly relevant for countries such as New Zealand which generate large quantities of carbon waste from primary industries such as dairy processing.

Chapter 7: Future work

This project identified three potential scenarios to explain the reduction of mediator by a mixed methane-oxidizing culture, all of which have different practical implications for a MFC. Full elucidation of the reducing compound that was directly responsible should be the immediate focus of future research prior to investigating the engineering application. The ability to use mediators as an electron acceptor may also not be widespread amongst mixed methane oxidizing communities; it is recommended that the experiments are repeated using a fresh culture enriched from a different source.

To determine the presence of a novel methane-oxidation pathway, the following experiments may be performed:

- Injecting mediator directly after inoculation before any organics have time to accumulate; this can be confirmed by HPLC analysis of the inoculum.
- Measurement of methane consumption during mediator reduction using a gas chromatograph. A carbon balance may then be conducted through measurement of carbon dioxide and organic compound production. This could be combined with carbon isotope labelling to determine if the carbon dioxide was coming directly from methane.
- Testing the ability of a pure methanotroph culture to reduce mediator. If reduction can occur, the role of MMO should be determined by performing MMO inhibition experiments and the nature of the electron donor investigated. Anaerobic methanotrophs could also be enriched and tested using alternative electron acceptors. Although much slower growing, these microorganisms are not dependent on the MMO enzyme and may be more effective in a MFC.

The organic compound and organism responsible for mediator reduction in the control without methane should be further investigated by repeating the HPLC experiments and conducting a DNA analysis. If both scenarios can occur (direct methane oxidation and oxidation of organics), the more effective processing strategy should be fully assessed in terms of maximum achievable reduction rate and process complexity. As the reduction rate appeared to be proportional to the initial mediator concentration, the inhibitory effect of the mediators on cell metabolism should be investigated by exposing to a range of different concentrations and inoculating into fresh medium. The growth rate can then be compared against the control not exposed to the mediator and the optimum concentration determined.

Construction of a prototype biotrickling filter with recycling of mediator will determine if the culture can maintain a consistent reduction rate over time, while achieving a high methane conversion. Oxidation of the mediator at an electrode can then be explored; this will involve design considerations such as construction materials and surface area of the electrodes to minimize polarization and improve the

electron transfer rate/power density. Significant improvement in the electrodes will likely occur within the next few years as this is a key area of fuel cell research. In the long term, the effect of a fluctuating inlet gas composition should be explored, for example by coupling the flow battery with a bench scale anaerobic digester using a range of organic feedstocks. This may cause the community in the bio-filter to change over time which is the inherent downside of using mixed cultures. The performance of the biotrickling filter should also be assessed in terms of biomass clogging and the overall accessibility of mediator to the cells.

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Appendices

Appendix 1: Calibration curves

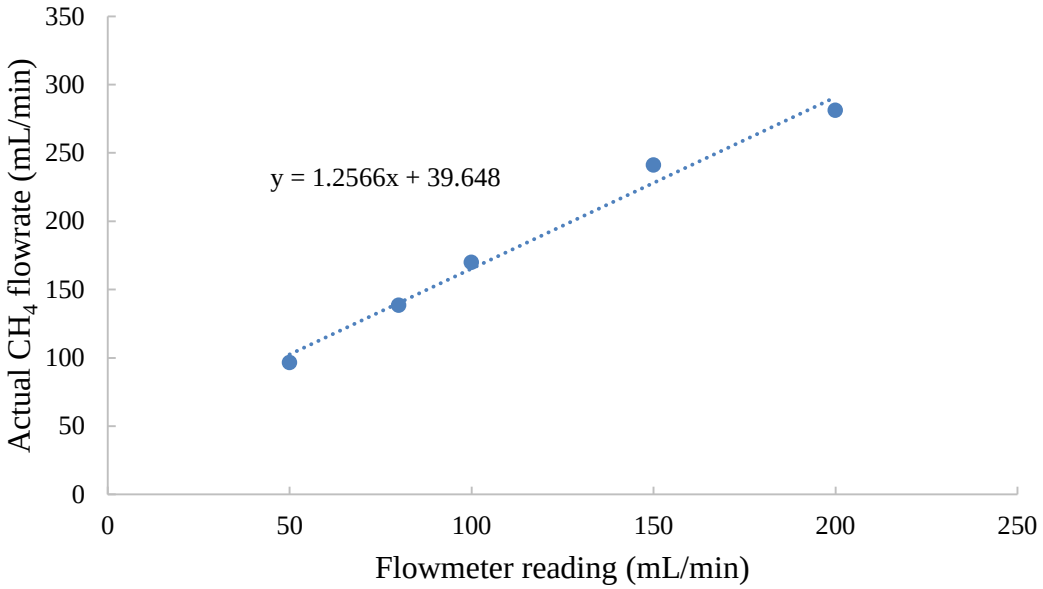

Figure 1-1: Methane flowrate calculated using bubble-flow meter vs. reading on rotameter.

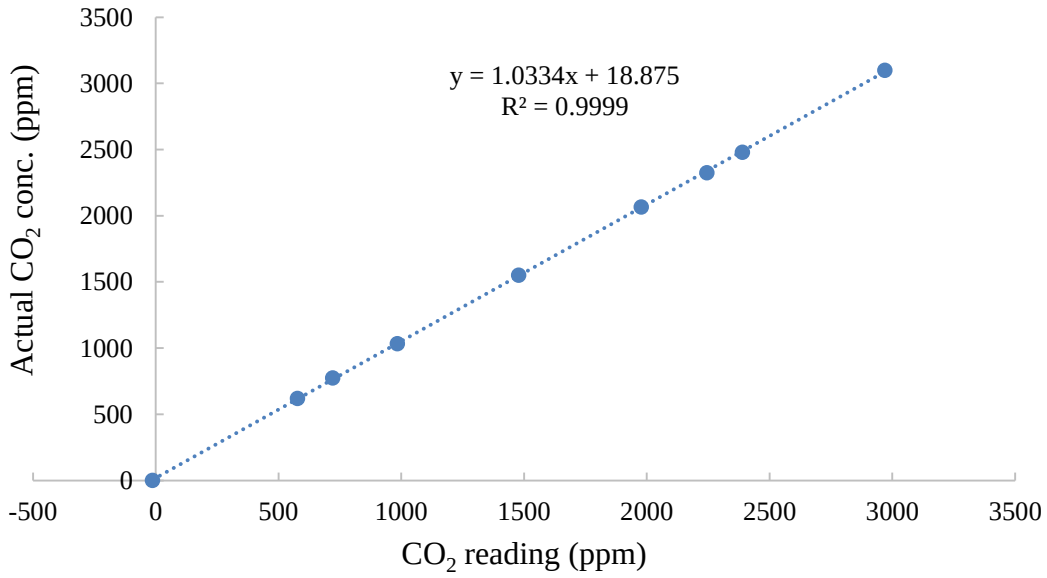

Figure 1-2: Actual carbon dioxide concentration in air vs. reading on Vaisala CO_2 sensor.

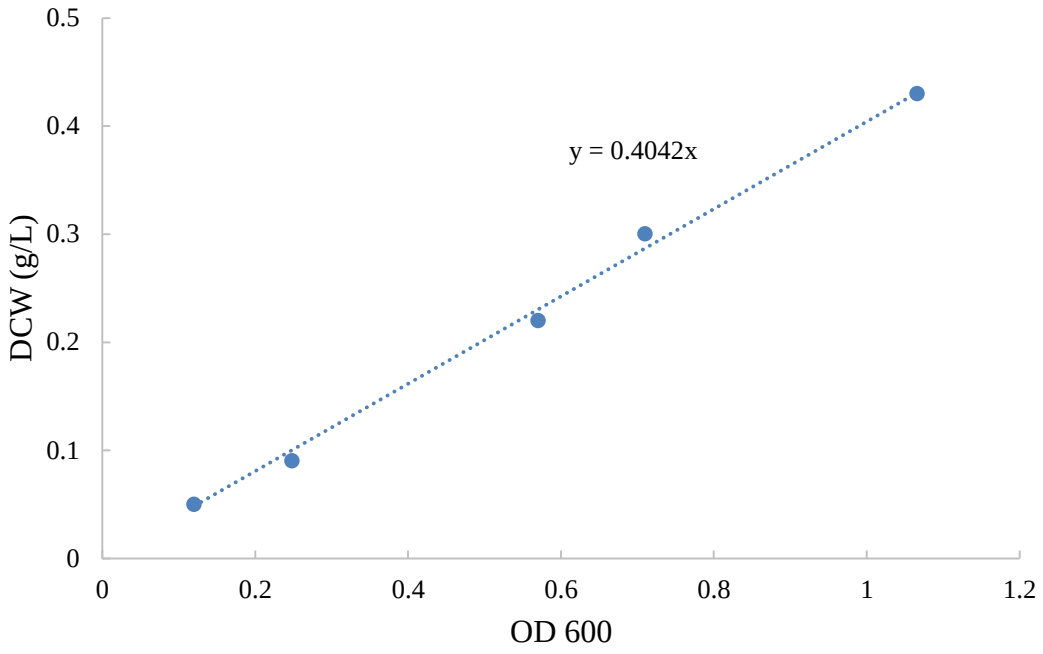

Figure 1-3: Dry cell weight (g/L) of mixed methane-oxidizing culture vs. optical density at wavelength 600 nm.

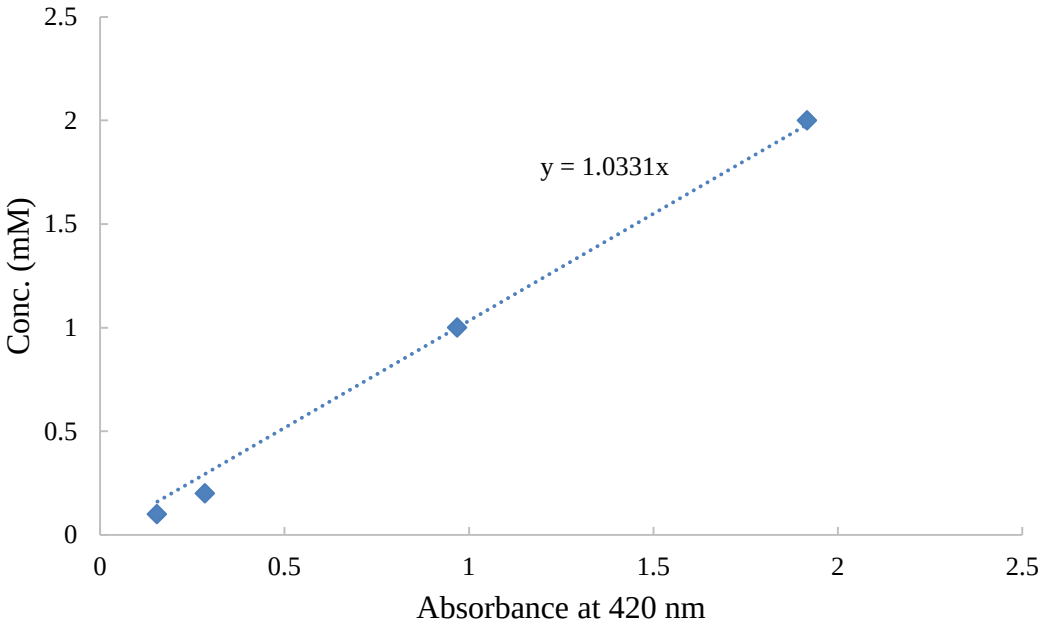

Figure 1-4: Potassium ferricyanide vs. optical density at wavelength 420 nm.

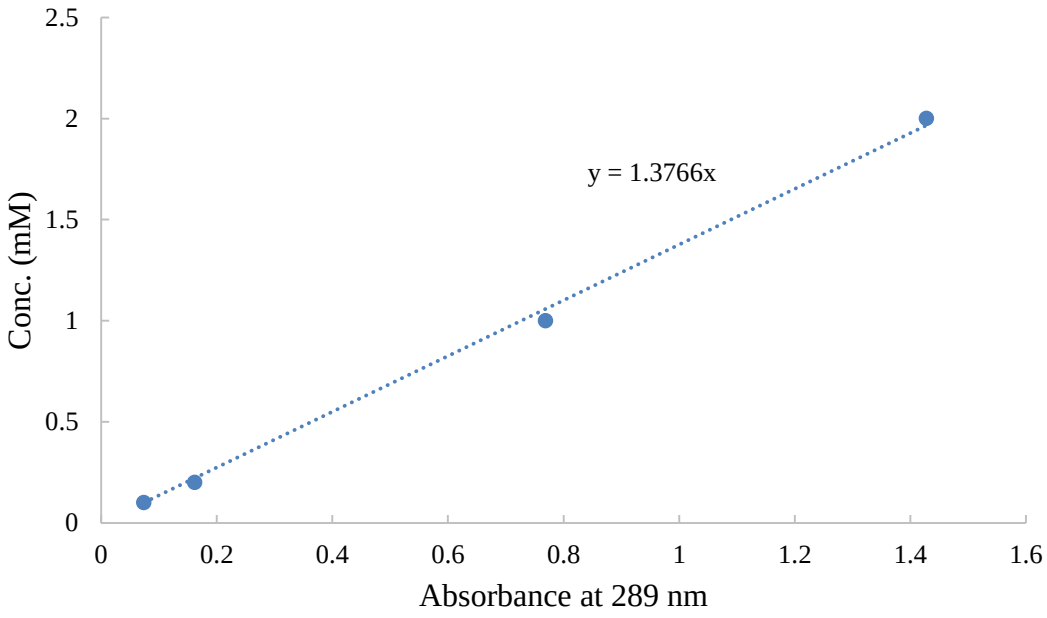

Figure 1-5: Potassium ferrocyanide concentration vs. optical density at wavelength 289 nm

Appendix 2: T-test raw data

Table 2-1: Two-sample t-test for comparing means of maximum optical density (OD) with 0.01 g/L yeast extract (YE) in medium and without yeast extract (assuming equal variances)

	OD with YE	*OD w/out YE*
Mean	0.159	0.121
Variance	0.001	0.001
Observations	3.000	3.000
Pooled Variance	0.001	
Hypothesized Mean Difference	0.000	
df	4.000	
t Stat	1.501	
P(T<=t) one-tail	0.104	
t Critical one-tail	2.132	
P(T<=t) two-tail	0.208	
t Critical two-tail	2.776	

Table 2-2: Two-sample t-test for comparing means of maximum optical density (OD) with nitrate or ammonium as the nitrogen source

	OD with ammonium	*OD with nitrate*
Mean	0.160	0.156
Variance	0.001	0.001
Observations	3.000	3.000
Pooled Variance	0.001	
Hypothesized Mean Difference	0.000	
df	4.000	
t Stat	0.177	
P(T<=t) one-tail	0.434	
t Critical one-tail	2.132	
P(T<=t) two-tail	0.868	
t Critical two-tail	2.776	

Appendix 3: Sample calculations

Expected cell growth using ferricyanide as the electron acceptor:

Methylococcus sp. biomass yield $Y_{x/O2}$ = 0.29 g/g_{O2} = 9.28 g/mol_{O2}

For methane oxidation with oxygen as the terminal electron acceptor:

$$CH_4 + 2O_2 + Nutrients \rightarrow CO_2 + H_2O + Biomass + Energy$$

For methane oxidation with ferricyanide:

$$CH_4 + 8FeCN_6^{3-} + 2H_2O \rightarrow 8FeCN_6^{4-} + CO_2 + 8H^+$$

Oxidation of 1mole of CH_4 requires 4 times more ferricyanide than O_2; consumption of 2 mM ferricyanide thus corresponds to consumption of 0.5 mM oxygen.

Biomass increase: 9.28 g/mol x 0.016g/L = 0.0046g/L

Reduction rate required to generate 1 kW/m^3 at 0.2 g/L:

1 Amp = 1 Coulomb/s

Current density = 1 kW/m^3 ÷ 0.45V = 2.2 A/L

Faradays constant = 96485 C/mol

2.2 A/L ÷ 0.2 g/L = 11.1 C/g·s

(11.1 C/g·s ÷ 96485 C/mol) x 3600 s/hr = 0.4 moles/ g·hr of electrons

Each mole of electrons requires 1 mole of ferricyanide reduced thus the required reduction rate is 0.4 moles/ g·hr.

Cell density required to generate 1 kW/m^3 at a specific reduction rate of 3.2 mmol/g·hr:

1 Amp = 1 Coulomb/s

Current density = 1 kW/m^3/ 0.45 V = 2.2 A/L

Faradays constant = 96485 C/mol

(0.0032 mol/g·hr x 96485 C/mol) ÷ 3600s= 0.086 A/g

Cell density required: 2.2 A/L ÷ 0.086 A/g = 25.6 g/L

Maximum power density of mediated biotrickling filter with methane elimination capacity (EC) of 60 g/m^3 ·hr:

Energy density of methane: 55.5 MJ/kg

If all of the energy contained in methane is transformed to electricity at the given EC (assuming methane is an ideal gas):

(60 g/m^3 ·hr ÷ 3600 s/hr) x 55.5 MJ/kg x 0.001 kg/g = 925 J/m^3·s

At a conversion efficiency of 90%:

925 J/m^3·s x 0.9 = 830 W/m^3

Size of biotrickling filter with EC of 60 g/m^3 ·hr and conversion efficiency 70% required to generate 1 kW for domestic household use and the volume of methane required per house each year.

Energy density of methane: 55.5 MJ/kg

Density of methane: 0.656 kg/m^3

(60 g/m^3 ·hr ÷ 3600 s/hr) x 55500 kJ/kg x 0.001 kg/g x Volume = 1 kW

Rearranging for volume:

Volume = 1.1 m^3

At a conversion efficiency of 70 %:

1.1 m^3/0.7 = 1.6 m^3

Volume of methane required each year:

60 g/m^3 ·hr x 1.6 m^3 x 8760 hrs/ year x 1.5 m^3/ kg x 0.001 kg/g= 1300 m^3

Size of biotrickling filter with EC of 60 g/m^3 ·hr and conversion efficiency 70% required to generate 10.8 kW for use on a dairy farm and the volume of methane required per farm each year:

Energy density of methane: 55.5 MJ/kg

Density of methane: 0.656 kg/m^3

(60 g/m^3 ·hr ÷ 3600 s/hr) x 55500 kJ/kg x 0.001 kg/g x Volume = 10.8 kW

Rearranging for volume:

Volume = 11.7 m^3

At a conversion efficiency of 70 %:

11.7 m^3/0.7 = 16 m^3

Volume of methane required each year:

60 g/m^3 ·hr x 16 m^3 x 8760 hrs/ year x 1.5 m^3/ kg x 0.001 kg/g= 14000 m^3

A farm producing 50 m^3 of CH_4 per day gives a total of 18000 m^3 per year; enough methane is therefore produced for 100% self- sufficient electricity generation.

Maximum power output if all of the biogas generated on a farm is used in a gas combustion engine:

Methane production: 50 m^3 /day

Energy density of methane: 55.5 MJ/kg

Density of methane: 0.656 kg/m^3

Energy available: 50 m^3 /day x 0.656 kg/m^3 x 55500 kJ/kg x 1 day/86400 hrs= 20 kW

At an energy efficiency of 35%, the maximum power output is 0.35 x 20 kW = 7 kW