Fundamental Insights into Propionate Oxidation in Microbial Electrolysis Cells Using a Combination of Electrochemical, Molecular biology and Electron Balance Approaches

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Increasing demand for freshwater and energy is pushing towards the development of alternative technologies that are sustainable. One of the realistic solutions to address this is utilization of the renewable resources like wastewater. Conventional wastewater treatment processes can be highly energy demanding and can fail to recover the full potential of useful resources such as energy in the wastewater. As a consequence, there is an urgent necessity for sustainable wastewater treatment technologies that could harness such resources present in wastewaters. Advanced treatment processes based on microbial electrochemical technologies (METs) such as microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) have a great potential for the resources recovery through a sustainable wastewater treatment process. METs rely on the abilities of microorganisms that are capable of transferring electrons extracellularly by oxidizing the organic matter in the wastewater and producing electrical current for electricity generation (MFC) or H$_2$ and CH$_4$ production (MEC). Propionate is an important volatile fatty acid (VFA) (24-70%) in some wastewaters and accumulation of this VFA can cause a process failure in a conventional anaerobic digestion (AD) system. To address this issue, MECs were explored as a novel, alternative wastewater treatment technology, with a focus on a better understanding of propionate oxidation in the anode of MECs. Having such knowledge could help in the development of more robust and efficient wastewater treatment systems to recover energy and produce high quality effluents. Several studies were conducted to: 1) determine the paths of electron flow in the anode of propionate-fed MECs low (4.5 mM) and high (36 mM) propionate concentrations; 2) examine the effect of different set
anode potentials on the electrochemical performance, propionate degradation, electron fluxes, and microbial community structure in MECs fed propionate; and 3) examine the temporal dynamics of microbial communities in MECs fed with low or high concentration of acetate or propionate relating to the reactor performance. Overall, the findings from these studies provide new knowledge on propionate oxidation in MECs. The discovery of such findings may shed light on the development of an energy positive wastewater treatment process capable of producing a high quality effluent.
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LIST OF ABBREVIATIONS

AD: Anaerobic Digestion
AFBRs: Anaerobic Fluidized Bioreactors
AFMBRS: Anaerobic Fluidized Membrane Bioreactors
AS: Activated Sludge
BES: 2-Bromoethanesulfonate
BLAST: Basic Local Alignment Search Tool
CE: Coulombic efficiency
GC: Gas Chromatograph
HPLC: High-Performance Liquid Chromatograph
MECs: Microbial Electrolysis Cells
me–q: milli electron equivalent
METs: Microbial Electrochemical Technologies
MFCs: Microbial Fuel Cells
mM: milli molar
NCBI: National center for Biotechnology Information
NMDS: Nonmetric Multidimensional Scaling
OTUs: Operational Taxonomic Units
PCoA: Principal Coordinate Analysis
PCR: Polymerase Chain Reaction
QIIME: Quantitative Insights Into Microbial Ecology
rRNA: Ribosomal RNA
SAPs: Set Anode Potentials
SHE: Standard Hydrogen Electrode
TCD: thermal conductivity detector
UPGMA: Unweighted Pair Group Method with Arithmetic mean
VFA: Volatile Fatty Acid
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CHAPTER 1 Introduction and Dissertation Organization

1.1 An outlook of global energy and water crisis

The global population is anticipated to increase to 11 billion by 2100. Providing adequate supply of clean fresh water and energy as the world’s population increases is one of the grand challenges facing society in the current century. Globally, almost 768 million and 2.5 billion people remain without access to safe water and proper sanitation, respectively. Water and energy are deeply interlinked and highly interdependent. For example, energy production is the second largest consumer of freshwater. Similarly, water extraction, delivery, and treatment require energy. Also, the availability and access to water is negatively impacting the energy production around the world. For instance, water shortage caused a shutdown of thermal power plants in India, decreased the energy production in the USA, and threatened the hydropower generation in many countries, including China, Sri Lanka, and Brazil.

According to the United Nations (UN), global water demand will be increased by ~55 percent by 2050 due to growing demands from manufacturing (400 percent), thermal electricity generation (140 percent) and domestic use (130 percent). International Energy Agency (IEA) revealed that fresh water consumption was over 66 billion cubic meters in 2010, and this consumption was estimated to grow by 85% by 2035. Furthermore, global energy demand is likely to be increased by more than one-third by 2035, particularly 50% of the increase will be in China, India, and Middle Eastern countries. In addition, electricity shortage is anticipated to grow by 70% by 2035. The primary source of energy for global electricity generation is coal followed by natural gas (Figure 1.1). It is evident from the above information that the demand for freshwater and
energy will continue to increase over the coming decades, and this increase could damage the limited resources in all the regions of the globe.  

**Figure 1.1** The contribution of energy resources in the electricity generation of the world (redrafted from reference 5).

### 1.2 Renewable energy sources to meet global requirement

The above-mentioned facts highlight a crucial need for developing alternative and sustainable technologies that could manage the energy and water crises. In order to be successful, sustainable technologies should be affordable and consume less energy and water. Renewable energy (RE) sources such as solar, wind, geothermal, hydro and biomass provide a potential solution to the energy crisis. Particularly, solar and wind energy require only a negligible amount of water and these energy sources contributed for a fifth of global electricity output in 2011. IEA revealed that ~13.2% of world energy has been supplied by RE sources in 2012, and anticipated to rise by at least 26% in 2020. Several countries want to obtain most of their energy from renewable sources by integrating RE generation technologies into existing infrastructure to achieve 100% renewable energy. For example, Iceland, Norway and Germany generate nearly 100% of their electricity from hydro, wind and geothermal energy. Also, the USA anticipates to
generate 80% of its energy from renewable sources and likely to increase it to 100% by 2050. However, some of these RE sources are more expensive than fossil fuel and some are environmentally hazardous. For instance, hydro power generates a lot of electricity but such systems can be ecologically damaging, and burning biomass produces copious amount of nitrous oxide, particles and other pollutants into the air. Also, some of the RE sources such as wind, solar, wave and tidal energy are variable and fluctuate during a given day or season. However, stable RE generating capacities like gas and hydropower plants can be integrated to provide flexible renewable technologies.

1.3 Energy requirement of wastewater treatment and resources potential of wastewater

Recovery of energy and water for reuse from wastewater represent one of the solutions for addressing the ever growing water and energy demand. Wastewater is considered more as a resource than waste because it serves as a resource for energy and water for agriculture, irrigation, and domestic purposes. The conventional wastewater treatment process based on activated sludge (AS) process combined with anaerobic sludge digestion recovers only a small fraction of the energy potential in the wastewater. Also, such conventional AS wastewater treatment can be expensive and highly energy intensive, requiring in the order of 0.6 kWh/m³, of which 50% of that energy is used for aeration. Also, ~50% of the current operating costs of the wastewater treatment plants is required for sludge treatment. Strict water quality requirement also likely increases the energy needed for the treatment. For example, wastewater treatment process with aerobic membrane bioreactor produces an effluent with high water quality, but it consumes more energy (0.8-2 kWh/m³) than the AS process. However, the different
types of wastewater including domestic, animal and food processing contains two to four times greater energy (~1.2-2 kWh/m³ or 17 GW) than the amount of energy required to treat it (~0.6-2 kWh/m³ or 15 GW) \(^{10,14,15}\). This suggests that a potential opportunity exists to harness this energy to balance the energy requirements for wastewater treatment. Nevertheless, the challenge is to produce a cost effective and carbon neutral wastewater treatment process capable of harnessing the rich resource of energy from wastewater. A substantial efforts are also being made to upgrade the existing wastewater treatment processes to better manage the wastewater resources, minimize energy requirements, and meet the strict water quality regulations \(^1\).

1.4 Bio-energy generation from wastewater

Different types of the microbial processes such as anaerobic digestion (methanogenesis), fermentative H\(_2\) production, photosynthesis and microbial electrochemical technologies (METs) hold a great promise for converting biodegradable organic biomass and waste streams into bioenergy (CH\(_4\), H\(_2\), and electrical current).

1.4.1 Anaerobic digestion (AD)

Anaerobic digestion (AD) is a well-known and mature technology for the production of CH\(_4\) from wastewater sludge and is considered as one of the low-cost wastewater treatment systems. AD is a sequential step process where organic matter first undergoes hydrolysis followed by acidogenesis, acetogenesis, and methanogenesis, as shown in Figure 1.2 \(^1\). AD is a complex process which requires the coordinated interactions between four major metabolic groups (hydrolyzing organisms, acidogens, acetogens and methanogens) for transfer of organic material to CO\(_2\) and CH\(_4\). Generally, hydrolysis is
considered as a rate limiting step in the AD process. The hydrolysis step degrades both insoluble organic substances and heavy molecular weight compounds like lipids, polysaccharides, proteins and nucleic acids into soluble organic compounds such as amino acids and fatty acids. The components produced during this step are further broken down into VFAs (acetate, propionate, and butyrate) and alcohols, along with NH\textsubscript{3}, CO\textsubscript{2}, H\textsubscript{2}S, and other by-products by the help of acidogenic (or fermentative) bacteria during acidogenesis. The third step in AD is acetogenic, where the produced compounds are digested by acetogenic bacteria to end products of acetate, H\textsubscript{2}, and CO\textsubscript{2}. This step is highly controlled by the partial pressure of H\textsubscript{2} in the mixture. The final stage of AD is methanogenesis, where acetate, H\textsubscript{2}, and CO\textsubscript{2} are converted into CH\textsubscript{4} and CO\textsubscript{2}.

**Figure 1.2.** Steps involved in anaerobic digestion (AD) process (redrafted from reference\textsuperscript{11})
At high substrate loading in AD, such as during the start-up or periods of overload, more VFAs (propionate, acetate, and butyrate) and alcohol (ethanol) get during acidogenesis and acetogenesis. Such VFAs accumulation can lead to a drop in pH resulting in inhibition of methanogenesis and an imbalance of the AD process. In general, AD process requires suitable environmental conditions for stable operation such as high temperature, neutral pH, higher substrate concentration than those present in domestic wastewater and optimum concentration of ammonium, sulphide, sodium, potassium, H₂, volatile fatty acids (VFAs) and heavy metals. Although AD process has many positive features, there are some important issues that need to be addressed. First, CH₄ must not be allowed to be released into the atmosphere because CH₄ is a powerful greenhouse gas with the potential of global warming about 25 times that of CO₂, and thus cost effective and energy efficient CH₄ capture techniques are needed. Another issue is the incomplete removal of organic carbon and nutrients (nitrogen and phosphorus). Therefore, new technologies are needed to completely utilize the organics to produce higher fraction of environmental friendly gases as energy source.

1.4.2 Hydrogen (H₂) production

Hydrogen is cleaner form of energy than methane and has a high energy content. The heat of combustion for H₂ is 142 KJ/e⁻ mol compared to 100 KJ/e⁻ mol for CH₄. H₂ can be generated from fossil fuels such as natural gas and coal (with CO₂ sequestration), nuclear energy, and renewable sources such as biomass, wind, solar, geothermal and hydro-electric power. H₂ production from fossil fuel source is not sustainable, and more energy is required for CO₂ sequestration. Thus, renewable sources of H₂ production are a fruitful pathway which has the benefit of carbon-neutral energy. A
biological route of H$_2$ production is one of the potential options that includes photo-lytic, photo-fermentation, dark fermentation, and microbial electrochemical technologies (METs) $^{19}$. Photo-lytic H$_2$ production uses green algae or cyanobacteria, and sunlight to split water through direct or indirect photolysis route $^{19}$. Direct photolysis route involves green algae, or cyanobacterial photosynthesis capabilities which are used to generate oxygen and hydrogen ions. During this process, hydrogenase or nitrogenase enzymes convert the hydrogen ion and oxygen into hydrogen gas (Fig. 3A). Indirect photolysis occurs when sugars and starches (are produced through photosynthesis driven Calvin cycle) are broken down during dark fermentation. This occurs in the dark or under the condition where photosystem is not actively producing oxygen (Figure 1.3A) $^{19}$. Photofermentative H$_2$ production uses anoxygenic photosynthetic bacteria to produce H$_2$ under nitrogen-deficient conditions. These bacteria lack photosystem II, which is responsible for splitting water into hydrogen and oxygen. In its place, photosynthesis provides energy to completely break down the organic substrates and also drive the nitrogenase reaction that combines protons and electrons generated during organic substrate metabolism (Figure 1.3B). There are some limitations to overcome in photobiological H$_2$ production such as H$_2$ production rate and costs $^{19}$. 
Figure 1.3. Photobiological pathways of H2 production (redrafted from reference 19).

Dark fermentation is another promising route for H2 production through waste biomass and waste streams. Bacteria can produce H2 by using protons as an electron sink during dark fermentation of organic substrates 20. However, a serious limitation of this approach is the low H2 yields. For example, they are thermodynamically and metabolically limited to a maximum of 4 moles of H2 per mole of glucose (representing 17% of the energy), compared to the theoretical value of 12 moles, due to the incomplete oxidation during dark fermentation 20,21. The chemical oxygen demand (COD) of such process remains unchanged due to the incomplete oxidation of organic matter into intermediates such as acetate, propionate, butyrate and lactate. Thus, dark fermentative H2 production requires a subsequent process of microbial electrolysis cells (MECs) to recover more energy (H2) from the intermediate of organic matters (83% of the available energy).
1.4.3 Microbial electrochemical technologies (METs)

METs are considered as one of the sustainable renewable technologies for wastewater treatment and concomitant energy production. METs such as microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) rely on a unique group of microorganisms, called exoelectrogens, that are capable of transferring electrons extracellularly (exoelectrogens) to convert the soluble organic matters in the wastewater into electrical current (MFCs) or H₂ and CH₄ (MECs). In comparison to conventional wastewater treatment, METs have added benefits of minimizing the costs associated with aeration and secondary sludge treatment as it does not require aeration since it is an anaerobic process and also produces lower amount of sludge 22-24. Recently, METs have been integrated with different wastewater treatment systems such as anaerobic digestion and membrane bioreactors for enhancing the performance of wastewater treatment process with increased energy recovery 25-29.

1.4.3.1 Microbial Fuel Cells (MFCs)

In MFCs, bacteria in the anode oxidize the organic matter to CO₂, electrons and protons. The electrons and protons that are generated during oxidation at the anode are captured at the cathode for oxygen reduction reaction (ORR) (Figure 1.4) 30. A wide range of organic substrates have been used in MFCs, such as VFAs, alcohols, carbohydrates, proteins, amino acids and cellulose 30,31.
1.4.3.2 Microbial Electrolysis Cells (MECs)

In MECs, bacteria on the anode oxidize the organic matter to CO$_2$, electrons and protons. The electrons and protons that are generated during oxidation at the anode are captured at the cathode for hydrogen evolution reaction through the addition of minimum voltage (0.6 V) to the circuit (Figure 1.5) $^{31}$. H$_2$ production at the cathode is thermodynamically not favorable reaction under standard conditions, therefore additional energy is required to create thermodynamically favorable conditions for H$_2$ production. The main advantage of MECs is lower applied voltage is required for H$_2$ production compared to water electrolysis (1.23 V).

Figure 1.4. Schematic representation of the principle of a two-chamber microbial fuel cell
Figure 1.5. Schematic representation of the principle of a microbial electrolysis cell

MEC has several merits over other bio-H₂ production processes. First, a variety of substrates such as VFAs (acetate and propionate), alcohol, carbohydrates, proteins and cellulose can be used as fuel sources. Second, organic substrates can be completely oxidized to CO₂, resulting in high conversion yields of 67 to 91% from diverse organic compounds (including cellulose, glucose, propionate, butyrate, propionate, ethanol and acetate), and which is nearly five folds higher than dark fermentation (~17%) ²⁰. Many studies have been conducted to optimize the engineering aspects of the MEC system, which includes reactor configuration, electrode materials, membrane types, medium composition, hydraulic retention times, electrode spacing, and electrode arrangement. Yet, only few studies have been reported on understanding the microbial communities, which are a key component for successful performance of MEC systems ²². As a result, there is
a need to understand the microbial community structure and its functional roles that affect system performance.

In MECs, several groups of microbial communities are present on the anode such as exoelectrogens and non-exoelectrogens (e.g. fermenters, acetogens, and methanogens) (Fig. 5). These groups of organisms interact with each other to convert the substrates to electrical current, \( \text{H}_2 \) or \( \text{CH}_4 \) \(^{32,33} \). The interaction between exoelectrogens and non-exoelectrogens and its relationship to MEC performance is not well understood. Fermentation (breaking down of complex substrates into simple substrates), electrogenesis (substrate conversion to electrical current), and methanogenesis (substrate conversion to \( \text{CH}_4 \)) are three common processes in MECs when fermentable substrates are used as a fuel. The presence of fermenters in the anode is essential as they convert fermentable substrates into fermentation products that is later consumed by exoelectrogens. On the other hand, methanogens are detrimental to the performance of MECs because they compete for the fermentation products (acetate, \( \text{H}_2 / \text{formate} \)) resulting in a coulombic loss (Fig. 5). In hydrogenotrophic methanogenesis, the production of 1 mol of methane consumes 4 mol of hydrogen (\( 4\text{H}_2 + \text{CO}_2 = \text{CH}_4 + \text{H}_2\text{O} \)), as a result, methane generation tremendously decreases the hydrogen yield \(^{34} \). Therefore, the growth of methanogens has to be suppressed to enhance the performance of MEC system.

### 1.5 Research needs

Propionate is a model fermentable substrate to study microbial partnerships in natural and engineered methanogenic systems and an important intermediate in the anaerobic decomposition of organic matter in AD processes. Accumulation of propionate (>20 to 37 mM) at high organic loading rates is detrimental to AD processes \(^{35-37} \). Thus,
propionate removal is necessary for the stable operation of AD. One new and important application of MECs is the addition of electrodes directly into an AD, in order to improve performance and increase the methane concentration in the product gas \(^{38-41}\). However, the impact of high concentrations of fermentable substrates, especially for the case of propionate which is slowly degraded, on MEC performance has not been well examined. Addressing the above knowledge gap, requires more fundamental insights on the paths of electron flow from propionate oxidation in the anode of MECs at low and high propionate concentrations. Such fundamental insight is essential for optimizing these systems for real scale applications.

Setting the anode potential in MECs can impact electrochemical performance, microbial community structure and the theoretical maximum energy gain by exoelectrogens for their growth and maintenance \(^{42}\). The anode potential has been shown to be a critical factor affecting the rate of acetate removal in MECs, but studies on the effect of set anode potentials with propionate are lacking. Therefore, there is a need for further research on MECs fed with propionate and operated at different set anode potentials for improving the performance of this system.

Most of the MEC studies have focused on the engineering aspects, reactor design and material optimization for enhancing the performance of the system. Yet, only a few studies have been reported on understanding the microbial communities, which are a key component for successful performance of MEC systems. Furthermore, most microbial studies on MECs focused on a single sampling event (typically at the end of the MECs operation). Single sampling events does not provide an understanding about the temporal dynamics of microbial communities and its correlation to system performances.
Therefore, a deeper insights into the dynamics of microbial communities over time (multiple sampling events) and linking it with system performances is needed for practical application of MECs for wastewater treatment

1.6 Objectives

My dissertation focuses on providing fundamental insights of propionate oxidation in the anode of MECs using a combination of electrochemical, electron balance, thermodynamic and genome-enable molecular biology approaches. The specific objectives of this dissertation were:

1. Determine the paths of electron flow from propionate oxidation in the anode of MECs operated at an applied voltage of 0.7 V using a power source (i.e. the anode potential in the MECs were not controlled) and fed with low and high concentrations of propionate.

2. Examine the effect of different set anode potentials on the electrochemical performance, propionate degradation rate, electron flux to various sinks, and microbial community structure in MECs fed with high concentrations of propionate.

3. Examine the temporal dynamics of anodic and suspension microbial communities in MECs fed with low or high concentration of acetate or propionate and linking this dynamic to reactor performance.
1.7 Organization of this dissertation

This dissertation is organized into five chapters including the introduction chapter, followed by three chapters (chapters 2, 3 and 4) addressing the above objectives and a conclusion chapter (chapter 5).

Chapter 2 describes the paths of electron flow from propionate oxidation in the anode of MECs at low and high propionate concentrations (objective 1). To evaluate the paths of electron flow from propionate oxidation, two sets of MECs were operated with different concentrations of propionate. In the first set, reactors were operated with low concentration of propionate (4.5 mM) and in the second set, reactors were operated in high concentration of propionate (36 mM). A combination of bioprocess monitoring, combined with electron balances and microbial community analysis (16S rRNA gene pyrosequencing) was employed in this study. The results of this study showed that multiple paths of electron flow to current (via acetate/H₂ or acetate/formate) could occur simultaneously during propionate oxidation in the anode of MECs irrespective of the tested propionate concentrations. The results of this chapter have been published in the Journal *Applied Microbiology and Biotechnology* with authors Hari, A.R., Katuri, K.P., Gorron, E., Logan B.E, and Saikaly, P.E. I devised and conducted all the experiments and wrote the first manuscript draft. All co-authors were involved in the preparation of the final manuscript.

Chapter 3 describes the effect of three different set anode potentials (SAPs; –0.25, 0, and 0.25 V vs. standard hydrogen electrode) on the electrochemical performance, electron flux to various sinks (current, CH₄ and undefined sinks), and anodic microbial
community structure in two-chambered MECs fed with propionate (36 mM) (objective 2). The performance of MEC-SAPs were compared to control reactors, operated in open circuit mode (O.C, as AD reactors). The results showed that (i) SAPs affected the electron fluxes to various electron sinks, where current was a significant electrons sink followed by methane in all the SAPs tested; however, current was relatively higher in the positive SAPs (0 and 0.25 V). In contrast, methane was higher in the negative SAP (–0.25 V); and (ii) SAPs affected the anodic microbial community structure and diversity where higher microbial diversity was detected at SAP of 0.25 V than SAP of 0 and –0.25 V, and the relative abundance of the most dominant members (Geobacter, Smithella and Syntrophobacter) at the anode varied among the tested SAPs. Microbial community structure in the biofilm anode and suspension imply that the degradation of propionate in all the tested SAPs was facilitated by syntrophic interaction between fermenters (Smithella and Syntrophobacter) and Geobacter at the anode and fermenters (Smithella and Syntrophobacter) and methanogens (mainly hydrogeonotrophic methanogens) in suspension. Collectively, these results suggest that anode of MECs operated at a SAP could potentially be integrated with existing anaerobic digestion processes to improve propionate degradation. The results of this chapter have accepted in the Journal Nature Scientific Reports with authors Hari, A.R., Katuri, K.P., Logan B.E, and Saikaly, P.E. I devised and conducted all the experiments and wrote the first manuscript draft. All co-authors involved in the preparation of the final manuscript.

Chapter 4 examines the temporal dynamics of anodic and suspension microbial communities in MECs fed with a low (0.5 g COD/L) or high concentration (4 g COD/L) of acetate or propionate. Reactor performance was continuously monitored during 70
days of batch operation, and anodic and suspension microbial communities were sampled over a time and analyzed using 16S rRNA gene sequencing. The results of this study indicate that the acetate-fed reactors exhibited a more stable (reproducible) and greater performance in terms of electrical current production, coulombic efficiency (CE) and substrate degradation rate than the propionate-fed reactors irrespective of the concentrations tested. Based on 16S rRNA gene sequencing, a relatively similar microbial community composition but with varying relative abundance was observed in all the reactors despite differences in the substrate and concentrations tested. A draft of this manuscript is currently being prepared to be submitted to a high impact, peer-reviewed journal article. I devised and conducted all the experiments and wrote the first manuscript draft.

**Chapter 5** is a summary of the conclusions of the above studies and provides potential implications of integrating MECs with conventional AD process as a next generation wastewater treatment technology.

**1.8 Additional research publications**

The following list includes published research that I contributed as a co-author but is not included in this dissertation.

sequencing data using QIIME platform and sequence data submission to European Nucleotide Archive (ENA). I wrote the molecular biology part of the materials and methods section and also contributed in revising the manuscript.


4. El Chakhtoura, J., El-Fadel, M., Ghanimeh, S., Hari, A.R., Li, D., and Saikaly, P. E. (2014). Electricity generation and microbial community structure of air-cathode MFCs powered with the organic fraction of municipal solid waste and inoculated with different seeds. *Biomass and Bioenergy*, 67, 24-31. I performed the genomic DNA analysis including PCR amplicon preparation for high-throughput sequencing and bioinformatic analysis of the sequencing data using QIIME platform. I also contributed in revising the manuscript along with all the co-authors.

review in *Frontiers in Microbiology*). I performed the genomic DNA analysis including PCR amplicon preparation for high-throughput sequencing, bioinformatic analysis of the sequencing data using QIIME platform and sequence data submission to European Nucleotide Archive (ENA). I also contributed in revising the manuscript along with all the co-authors.

6. Hamdan, H., Salam, D., Hari, A.R., Semarjia, L., & Saikaly, P.E. (2016). Assessment of the performance of SMFCs in the bioremediation of PAHs in contaminated marine sediments under different redox conditions and analysis of the associated microbial communities. (*Science of Total Environment*, 2016). I performed the genomic sequences data analysis using bioinformatics tools. Also, I contributed in revising the manuscript along with all the co-authors.
1.9. References


2. UN. Water-energy crisis threatens earth’s future.


CHAPTER 2 Multiple Paths of Electron Flow to Current in Microbial Electrolysis Cells Fed with Low and High Concentrations of Propionate

This chapter has been published as:

Conference (Platform presentation):
ABSTRACT

Microbial electrolysis cells (MECs) provide a viable approach for bioenergy generation from fermentable substrates such as propionate. However, the paths of electron flow during propionate oxidation in the anode of MECs are unknown. Here, the paths of electron flow involved in propionate oxidation in the anode of two-chambered MECs were examined at low (4.5 mM) and high (36 mM) propionate concentrations. Electron mass balances and microbial community analysis are revealed that multiple paths of electron flow (via acetate/H₂ or acetate/formate) to current could occur simultaneously during propionate oxidation regardless of the concentration tested. Current (57-96%) was the largest electron sink and methane (0-2.3%) production was relatively unimportant at both concentrations based on electron balance. At low propionate concentration, reactors supplemented with 2-bromoethanesulfonate had slightly higher coulombic efficiencies than reactors lacking this methanogenesis inhibitor. However, an opposite trend was observed at high propionate concentration, where reactors supplemented with 2-bromoethanesulfonate had lower coulombic efficiency and there was a greater percentage of electrons lost (23.5%) to undefined sinks compared to reactors without 2-bromoethanesulfonate (11.2%). Propionate removal efficiencies were 98% (low propionate concentration) and 78% (high propionate concentration). Analysis of 16S rRNA gene pyrosequencing revealed the dominance of sequences most similar to G. sulfurreducens PCA and G. sulfurreducens subsp. ethanolicus. Collectively, these results provide new insights on the paths of electron flow during propionate oxidation in the anode of MECs fed with low and high propionate concentrations.
2.1 Introduction

Anaerobic degradation of organic compounds (e.g. fatty acids and alcohol) in methanogenic systems is thermodynamically possible only when the H₂ partial pressure and formate concentration are maintained at extremely low values. Thus, the complete degradation of these organic compounds in methanogenic systems is only sustainable if the electrons (H₂ and formate) produced in the process are removed by other organisms. This critical interdependency between producer and consumer is defined as syntrophy. Such a microbial partnership plays a central role in the microbial degradation of organic compounds in methanogenic systems. In such systems, syntrophy between fermenters (organic compound degraders) and hydrogenotrophic methanogens (H₂ and formate utilizers) is a prerequisite for the complete degradation of fermentable substrates (e.g. ethanol, propionate and butyrate).

Microbial electrochemical cells (MXCs) are anaerobic systems for bioenergy generation from various waste streams. MXCs involve two principal approaches: microbial fuel cells (MFCs) for producing electricity and microbial electrolysis cells (MECs) for producing hydrogen. Similar to methanogenic systems, microbial partnerships play a pivotal role in the degradation of fermentable substrates (e.g. glucose, ethanol and butyrate) in the anode of MXCs where the products of fermentation (e.g. H₂ and acetate) are utilized by exoelectrogens for electrical current generation.

In most of the fermentable substrates fed MXCs studies, methane generation by hydrogenotrophic methanogens represented an important loss of electrons. The H₂ generation during the fermentation process is utilized by hydrogenotrophic methanogens,
which could outcompete other \( \text{H}_2 \)-utilizing organisms (e.g. exoelectrogens and homoacetogens) in MXCs, thus diverting electrons away from current \(^{13-15}\).

Propionate is a model fermentable substrate to study microbial partnerships in natural and engineered methanogenic systems and an important intermediate in the anaerobic decomposition of organic matter in anaerobic digestion processes. Accumulation of propionate (>20 to 37 mM) at high organic loading rates is detrimental to anaerobic digestion processes \(^{16-18}\). Thus, propionate removal is necessary for the stable operation of anaerobic digestion. In these systems, acetogenic bacteria oxidize propionate to acetate, hydrogen (or formate), which are then utilized by acetoclastic (acetate) and hydrogenotrophic methanogens (formate and \( \text{H}_2 \)) to generate methane and \( \text{CO}_2 \) \(^{19}\). In anode of MECs, little is known about the paths of electron flow during propionate degradation. Propionate has been used as a feed substrate in the anode of several MFC \(^{9,20-24}\) and MEC studies \(^{25,26}\), but the main electron flow paths of propionate oxidation have not been examined. In two studies, it was shown that \textit{Bacillus} was the dominant genus on the anode of propionate-fed, two chamber MFCs seeded with anaerobic digester sludge, suggesting direct oxidation and generation of electrical current from propionate \(^{20,22}\). Using a similar configuration and inoculum, de Carcer et al. \(^{21}\), showed that \textit{Geobacter spp.} was the dominant group on the anode suggesting oxidation of propionate by fermenters to fermentation by-products, which are then utilized by \textit{Geobacter}. The conclusions of these studies were based only on microbial characterization of the anodic communities, as there were no data on intermediates, gases produced, or electron balances. Recently, Badalamenti et al. \(^{27}\), have demonstrated that a pure culture of halophilic species, \textit{Geoalkalibacter subterraneus}, can oxidize propionate in anode
directly to current under alkaline conditions without fermentative partners, but bacteria most similar to *Geobacter sulfurreducens* are the dominant exoelectrogens in the anode of most MFC or MEC studies \(^{28}\). *G. sulfurreducens*, cannot oxidize propionate. Therefore, it requires fermentative partners for this substrate in the anode \(^{29}\).

Most of the studies on the degradation of propionate in bioelectrochemical systems were performed with low propionate concentrations (~5 mM), thus, a study that focuses on higher propionate concentrations is much needed as overloaded anaerobic systems always prevail at higher propionate concentrations (>20 mM) \(^{18}\). The objective of this study was to determine the paths of electron flow from propionate oxidation in the anode of MECs at low and high propionate concentrations. A combination of bioprocess monitoring, combined with electron balances and microbial community analysis (16S rRNA gene pyrosequencing) was employed in this study.

### 2.2 Materials and methods

#### 2.2.1 Construction of MECs

Two chambered cube-shaped MECs (each chamber with a 20 mL working volume) were constructed as previously described \(^{30}\). The two chambers were separated by an anion exchange membrane (5 cm\(^2\); AMI 7001, Membranes International, Glen Rock, NJ). A glass gas collection tube (15 mL) was attached to the top of both the anode and cathode chambers. Gas bags (0.1 L Cali -5 -Bond. Calibrate, Inc.) were connected to the top of the glass gas collection tubes to collect more volume of gas. The anodes were graphite fiber brushes (2.5 cm diameter \(\times\) 2 cm long; PANEX 33 fibers, ZOLTEK Inc., St. Louis, MO, USA). The cathodes (projected surface area of 7 cm\(^2\)) were made using carbon cloth
(type B-1B, E-TEK) containing 0.5 mg/cm² of Pt on the side facing the anode, and four polytetrafluoroethylene diffusion layers on other side.

2.2.2 Enrichment and operation

All MEC anodes were enriched initially in single chambered air-cathode MFCs as previously described using anaerobic digester sludge (Manfouha Wastewater Treatment Plant, Riyadh, KSA) as inoculum. The growth medium (pKa 8.34; pH 8.9) consisted of bicarbonate buffer (80 mM), nutrients (6.71 g/L NaH₂CO₃, 0.31 g/L NH₄Cl, 0.05 g/L Na₂HPO₄, 0.03 NaH₂PO₄), Wolfe’s vitamin (10 mL/L) and trace mineral (10 mL/L) solutions. The medium was supplemented with two different concentrations (4.5 mM and 36 mM) of propionate as the sole energy source. The growth medium was boiled and then cooled to room temperature by sparging with N₂: CO₂ (80:20, vol/vol) gas mix for 30 min to remove any dissolved oxygen and was then autoclaved. The MFC anodes were transferred to individual MECs after three cycles of the reproducible voltage (500 mV, over a 1 KΩ external resistor).

A fixed voltage of 0.7 V was applied to the MECs using a power source (3645A, Array, Inc.). A total of eight MECs were operated in a parallel (Figure 2.1). One set of four MECs were operated with a low concentration of propionate (4.5 mM, referred to here as the PL reactors), and another set of four MECs were operated with high propionate concentration (36 mM, are PH reactors). To inhibit methanogenesis in the anode compartment, 10 mM of 2-bromoethane sulfonic acid (BES) was added to all the batches of four MEC reactors designated as PL+BES and PH+BES. This concentration was shown to be inhibitory for methanogens in anaerobic environments. MEC reactors operated without BES addition were labeled as PL-BES and PH-BES (Figure
1.1). All reactor types (i.e. PL-BES, PL+BES, PH-BES, and PH+BES) were run in duplicate and operated in a fed-batch mode at temperature controlled room (30º C). When the current dropped to below 0.3 mA (PL-MECs ~36 hours/cycle; PH-MECs ~4-5 days/cycle), the reactors were refilled with fresh medium and sparged with nitrogen gas (99.999%). The same growth medium was used in the anodic and cathodic compartments; however, propionate was only added to the anode medium.

![Figure 2.1. Schematic of the experimental setup.](image)

2.2.3 Analyzes and calculations

The current in the circuit was determined by measuring the voltage across a resistor (10 Ω) in the circuit using a data acquisition system (Model 2700; Keithely Instruments Inc.), at 20min intervals. The gas generated by the MECs was collected from both chambers in the head space and in a gas bag, and the total gas volume was measured by the gas bag method as previously described. Concentrations of H₂ and CH₄ were measured using a gas chromatograph (GC) (model 310; SRI Instruments) with a thermal conductivity detector (TCD), a 1.83 m molecular sieve packed 5A column, and an argon carrier gas.
CO₂ concentration was measured with a second GC (model 310; SRI Instruments) with a TCD, a 0.91 m silica gel column, and using helium as the carrier gas. The volatile fatty acids (VFAs) profile and its concentrations (propionate, formate, and acetate) were analyzed by high-performance liquid chromatograph (HPLC) (Thermo Scientific, Accela, country) equipped with a photo-diode array (210 nm) and an ultraviolet detector. An Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) was used to separate the VFAs. Sulfuric acid (5 mM) was used as the mobile phase at a flow rate of 650 µL/min, and the pressure was maintained at 9650 kPa. The total elution time was 30 min, and each sample was measured in triplicate and the average concentrations were reported 37.

The performance of the MECs was evaluated on the basis of the hydrogen production rate, \( Q \) (m³ H₂/m³ reactor/day); volumetric current density of the reactor, \( I_V \) (A/m³); hydrogen yield, \( Y_{H_2} \) (mol-H₂/mol-propionate consumed); coulombic efficiency, CE (%); propionate removal (%) as previously described 32.

2.2.4 Electron balance

To establish electron balances during the time course or at the end of a batch experiment, the distribution of electrons in milli e⁻ equivalents (m e⁻eq) in the anode of MECs was followed from the electron donor (propionate) to various measured electron sinks (current, H₂, CH₄, propionate, acetate, and formate). Samples were collected for HPLC analysis during the time course or at the end of the batch. Samples for GC analysis were collected at the end of the batch. Also, the theoretical distribution of electrons from propionate oxidation to various electron sinks was calculated based on stoichiometric equations and the fractions of electrons (\( f^o_s \); Table 2) from the donor substrate that is
utilized for biomass synthesis. Detailed calculations of the theoretical distribution of electrons from propionate oxidation are provided in the Supporting Information.

2.2.5 16S rRNA gene pyrosequencing

At the end of the MEC experiments (day 55), samples were collected from the anode and suspension of each reactor type (i.e. PL-BES, PL+BES, PH-BES, and PH+BES) for microbial community analysis. Genomic DNA was extracted using the PowerSoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. The quality (A260/A280) and quantity (A260) of the extracted genomic DNA was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Triplicate PCR reactions were performed for each sample in a 25 µL reaction volume using the HotStarTaqPlus Master Mix (Qiagen, Valencia, CA), 0.5 µM of each primer, and 100-200 ng of template DNA. Bacterial and archaeal 16S rRNA genes were amplified using domain specific primer sets 38 : 341F (5’-Lib-L/A-Key-Barcode-CA Linker-CCTACGGGNGGCWGCAG-3’) and 785R (5’-Lib-L/A-Key-TC Linker-GACTACHVGGGTATCTAATCC-3’) for bacteria; and 519F (5’-Lib-L/A-Key-Barcode-CA Linker- CAGCMGCCGCGGTAA--3’) and 1041R (5’-Lib-L/A-Key-TC Linker-GGCCATGCACCWCCTCTC-3’) for Archaea. A unique 8–bp error–correcting barcode was used to tag each PCR product. PCR was performed using a C1000 Thermal Cycler (Bio- Rad, Hercules, CA). For bacteria, the PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 27 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at
72° C for 7 min. For archaea, the PCR conditions were as follows: denaturation at 95° C for 5 min, followed by 35 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, extension at 72° C for 1 min and a final extension at 72° C for 10 min.

The triplicate PCR products from each sample were pooled and then purified using the Qiaquick gel extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration of the PCR products was measured with a Qubit® 2.0 Fluorometer using the PicoGreen® dsDNA quantitation assay (Invitrogen, Carlsbad, CA). The purified barcoded amplicons from each sample were pooled in equimolar concentration and pyrosequenced on the Roche 454 FLX Titanium genome sequencer (Roche, Indianapolis, IN) according to manufacturer's instructions.

The bacterial and archaeal 16S rRNA sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME v 1.7.0) pipeline 39. Raw reads were first demultiplexed, trimmed and filtered for quality. The minimum acceptable length was set to 200bp 39. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the uclust algorithm 40. A representative sequence from each OTU was aligned using PyNAST 41, and these were phylogenetically assigned to a taxonomic identity (order, family, and genus level) using the RDP Naive Bayesian rRNA classifier at a confidence threshold of 80% 42. Chimeric sequences were identified and removed from the aligned sequences using chimera Slayer as implemented in QIIME. Ten most abundant OTUs from each sample were further classified at the species level using the BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Rarified OTU tables were used to generate beta diversity metrics. Beta diversity metrics using the unweighted UniFrac distance matrix 43 was calculated and visualized with hierarchical
clustering. Jacknifed beta diversity and hierarchical clustering analysis were performed to
determine the robustness of clustering of samples by the Unweighted Pair Group Method
with Arithmetic mean (UPGMA) was visualized using FastTree. Phylogenetic diversity
of abundant taxa (> 0.1%) was visualized in a heatmap using R ‘vegan scalpel’ program.

A phylogenetic tree was constructed from the dominant Geobacter OTUs and the
closest relatives in the Genebank Database using MEGA 6 software. The phylogenetic
tree was prepared using the neighbor-joining method with Jukes-Cantor distance in
MEGA 6.

2.2.6 Nucleotide sequence accession numbers

The 16S rRNA gene pyrosequencing reads have been deposited in GenBank under the
accession numbers SAMN03177498 for the bacterial sequences and SAMN03288816 to
SAMN03288822 for the archaeal sequences.

2.3 Results

2.3.1 Performance of MECs at low and high concentrations of propionate

The performance of MECs was influenced by the propionate concentration (low or high)
and the presence of BES (Figure 2.2). The PL reactors performed better than the PH
reactors in terms of peak current densities (average of the maximum current density), CE,
H₂ production rates, hydrogen yields and propionate removal (Figure 2.2). The values in
Fig. 2 corresponds to the average of the last three batch cycles of the duplicate reactors (n
= 6). The addition of BES resulted in a different response for the PL and PH reactors. For
example, the peak current density in the PL+BES reactors (92 ± 1) was significantly
higher by 8 ± 1% than the PL-BES reactors (84 ± 1) (P < 0.05, student’s t test for all
comparisons), in contrast the peak current density in PH+BES reactors (53 ± 2) was
significantly lower by $27 \pm 2\%$ compared to PH-BES reactors ($80 \pm 3$) ($P < 0.05$) (Figure 2.2A). Similarly, the $C_E$ was increased by $5 \pm 2\%$ in PL+BES reactors ($P < 0.05$), whereas it was significantly decreased by $9 \pm 1\%$ in PH+BES reactors ($P = 0.01$) (Figure 2.2A). The hydrogen production rate slightly increased from $0.90 \pm 0.01$ m$^3$ H$_2$/m$^3$/d (PL-BES) to $1.08 \pm 0.01$ m$^3$ H$_2$/m$^3$/d (PL+BES) ($P = 0.05$). In contrast, adding BES to PH reactors did not significantly decrease the hydrogen production rates from $0.65 \pm 0.02$ m$^3$ H$_2$/m$^3$/d (PH-BES) to $0.56 \pm 0.01$ m$^3$ H$_2$/m$^3$/d (PH+BES) ($P = 0.15$) (Figure 2.2A).

![Figure 2.2. Performance of MECs (average of last three batch cycles of the duplicate reactors) in terms of (A) current density, coulombic efficiency, H$_2$ production rate (B) H$_2$ yield, and propionate removal](image-url)
The PL reactors produced a two-fold higher hydrogen yield ($Y_{H_2}$) (6.49 ± 0.13) than PH reactors (3.01 ± 0.28) ($P = < 0.05$) (Figure 2.2B). Among all the MECs tested, the PL+BES reactors generated the highest hydrogen yield (6.69 ± 0.22 mol H$_2$/mol propionate). The rate of propionate oxidation was higher in the PL+BES reactors compared to the PL-BES reactors. After 15 h of operation, propionate oxidation reached 90% in the PL+BES reactors compared to 65% in the PL-BES reactors (Figure 2.2B). In PH reactors, the rate of propionate oxidation was similar for the PH-BES and PH+BES reactors (Fig. 2C and D). Propionate removal in PL reactors (98 ± 1%) was significantly higher by 20% than PH reactors (78 ± 2%) ($P = < 0.05$) and addition of BES did not have an effect on propionate removal in PL and PH reactors (Figure 2.2B). The propionate removal rate was 2.82 ± 0.25 mM/d in PL reactors and 6.62 ±0.64 mM/d in PH reactors. The pH of the medium was 7.4±0.6 at the end of the batch for both reactors. This shows the strong buffering capacity of the bicarbonate medium (80 mM; pKa 8.34; pH 8.9) used in this study even at high propionate concentrations (36 mM). A solution with a pH near the pKa of the buffer confers high buffering capacity.

2.3.2 Experimental distribution of electrons from propionate oxidation over the course of a single batch experiment

The distribution of electrons from propionate oxidation to various electron sinks (apart from hydrogen, methane, and undefined sinks) over the course of a batch experiment with matured biofilms (aged 55 days) is shown in Figure 2.3. The gas bag analysis adapted in this study does not allow analysis of multiple points in a single batch cycle. Also, biomass sampling over the course of a single batch might affect the performance of the reactors. Thus, we were unable to quantify electron losses to H$_2$, CH$_4$ and biomass.
over the course of a single batch. During the first three hours of operation of the PL-BES reactors, current had accounted for 25% of the total electrons from propionate (Figure 2.3A) and at the end of the batch experiment (32 hours), current was the dominant electron sink (90%). A higher fraction of electrons for current generation was evident for the PL+BES reactors than PL-BES throughout the batch experiment. In the case of PH reactors, the fraction of electrons for current generation was higher in PH-BES than PH+BES reactors (Figure 2.3C and D). Throughout the batch, acetate and formate accounted for < 1% of the total electrons in the PL and PH reactors (Figure 2.3).

**Figure 2.3.** Distribution of electrons in the anode of PL–BES, PL+BES, PH–BES, and PH+BES MECs. The horizontal dotted line shows the total percentage of electrons in the anode of MECs. “Sum of electrons” as the sum of all measured electron sinks excluding H₂ and CH₄: current, acetate, formate, and
propionate. The gas bag analysis adapted in this study does not allow analysis of multiple points in a single batch cycle. Thus, electron sinks to H₂ and CH₄ were excluded from the analysis. The error bars are not visible as they are smaller than the marker size.

2.3.3 Experimental distribution of electrons from propionate oxidation at the end of the batch experiment

Electron balances at the end of the batch experiment revealed that the current was the largest electron sink for all reactor types (Table 2.1); however, the percentage distributions of the electrons varied between the reactors. In the PL reactors, the electron distribution for the current was significantly higher (91-96%) than PH reactors (57-66%) ($P < 0.05$). Also, it was higher in PL+BES (96%) than PL-BES (91%) ($P < 0.05$), but an opposite trend was observed in PH reactors where current was significantly lower in PH+BES (57%) than PH-BES (66%) ($P < 0.05$). Intermediates of propionate oxidation, such as acetate and formate, accounted < 1% in all the reactors. Interestingly, methane was not a major electron sink (1.2 - 2.3%) in the reactors operated without BES addition. In general, the electron distribution for unconsumed propionate and undefined sinks was relatively higher in PH reactors than PL reactors.
Table 2.1. Distribution of electrons in the anode of MECs at the end of the last batch experiment for all reactor types

<table>
<thead>
<tr>
<th>Electron sinks</th>
<th>Current</th>
<th>Propionate</th>
<th>Acetate</th>
<th>Formate</th>
<th>Hydrogen</th>
<th>Methane</th>
<th>Undefined sinks$^a$ (biomass + soluble microbial products + others)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PL-BES</td>
<td>PL+BES</td>
<td>PH-BES</td>
<td>PH+BES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>91±0.94</td>
<td>96±0.69</td>
<td>66±0.36</td>
<td>57±0.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td>1.1±0.07</td>
<td>0.3±0.06</td>
<td>20.1±0.57</td>
<td>18.6±1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>0.9±0.06</td>
<td>0.05±0.01</td>
<td>0.04±0.006</td>
<td>0.02±0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>0.07±0.001</td>
<td>0.05±0.002</td>
<td>0.01±0.004</td>
<td>0.006±0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen</td>
<td>0.5±0.89</td>
<td>2.5±0.33</td>
<td>0.3±0.05</td>
<td>0.9±0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methane</td>
<td>1.2±0.63</td>
<td>0</td>
<td>2.3±0.36</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undefined sinks$^a$ (biomass + soluble microbial products + others)</td>
<td>5.23±0.14</td>
<td>1.1±0.07</td>
<td>11.2±0.89</td>
<td>23.5±2.11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$e$^-$ (undefined electron sinks) = e$^-$ (propionate) - e$^-$ (current) - e$^-$ (unused propionate) - e$^-$ (acetate) - e$^-$ (formate) - e$^-$ (hydrogen) - e$^-$ (methane), where e$^-$ = electron equivalents
2.3.4 Theoretical distribution of electrons from propionate oxidation to various electron sinks

Three possible routes of propionate oxidation could occur in the anode of MECs at both low (Figure 2.4A) and high propionate concentration (Figure 2.4B). The first possible route involves direct oxidation of propionate to electric current by exoelectrogens. If this is the dominant route, then the highest CE (95%) could be achieved at both low and high propionate concentration due to the fact that the only electron sink is biomass synthesis by exoelectrogens. Routes 2 and 3 involve a microbial partnership between propionate fermenting bacteria and fermentation products (acetate, formate, and H₂) consumers (i.e. exoelectrogens, acetogens, homoacetogens and hydrogenotrophic methanogens). Acetoclastic methanogens were not considered in the analysis as it has been reported that acetate-oxidizing exoelectrogens outcompete acetoclastic methanogens for acetate⁸,¹³. In routes 2 and 3, propionate is oxidized to acetate and hydrogen (route 2) or formate (route 3). The acetate produced could then be oxidized by exoelectrogens to produce current. The hydrogen and formate will be utilized by hydrogenotrophic methanogens to produce methane, by exoelectrogens to produce current or by acetogens or homoacetogens to produce acetate, which is then oxidized by exoelectrogens to produce current. If hydrogenotrophic methanogens outcompete exoelectrogens and acetogens/homoacetogens for hydrogen and formate then 36% of the electrons will be lost to methane, and the maximum flow of electrons to current is 0.64 milli electron (me⁻) equivalents for low propionate (Figure 2.4A) and 4.84 me⁻ equivalents for high propionate (Figure 2.4B). If methanogenesis is not a major sink of electrons, then the maximum flow of electrons to
current is 1.11 me⁻ equivalents for low propionate (Figure 2.4A) and 8.5 me⁻ equivalents for high propionate (Figure 2.4B). Detailed calculations of the values in Figure 2.4 are provided below.
Figure 2.4. Possible theoretical pathways of electron flow in the anode of MECs fed with propionate. (A) low propionate (1.3 milli electron (me\(^-\)) equivalents = 4.5 mM) and (B) high propionate (10 me\(^-\) equivalents = 36 mM). All values in bracket are presented as me\(^-\) equivalents. The electron flow was based on stoichiometric calculations provided in the Supporting Information. Green represents exoelectrogens; blue represents fermenters; red represents methanogens; brown represents homoacetogens using H\(_2\); and black represents acetogens using formate. The electron sinks for the different microbial groups was calculated by multiplying the substrate concentration (i.e. me\(^-\) equivalents values in parenthesis) with the fraction of electrons (f\(_s^o\)) from the donor substrate that is utilized for biomass synthesis \(^{47}\). f\(_s^o\) = 0.05 for G. sulfurreducens, as
representative of exoelectrogens \(^48,49\), \(f_s^o = 0.1\) for fermenters and \(f_s^o = 0.08\) for hydrogenotrophic methanogens \(^47\); and \(f_s^o = 0.1\) for acetogens and homoacetogens \(^50,51\). Detailed calculations of the values in paranthesis are provided below.

**Stoichiometric equations**

The different stoichiometric equations used for constructing Figure 4 are presented below.

**Stoichiometric equations for propionate oxidation**

\[
\Delta G^\circ' \text{ (kJ/mol)} \\
\begin{align*}
a. \quad \text{Propionate}^- + 3\text{H}_2\text{O} & \rightarrow \text{Acetate}^- + \text{CO}_2 + 3\text{H}_2 \quad +76 \\
b. \quad \text{Propionate}^- + 2\text{HCO}_3^- & \rightarrow \text{Acetate}^- + 3 \text{ Formate} + \text{H}^+ \quad +72.2 \\
\end{align*}
\]

**Stoichiometric equations for hydrogenotrophic methanogenesis**

\[
\Delta G^\circ' \text{ (kJ/mol)} \\
\begin{align*}
c. \quad 4\text{H}_2 + \text{CO}_2 & \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \quad -131 \\
d. \quad \text{Formate} + \text{H}^+ & \rightarrow 0.25 \text{CH}_4 + 0.75 \text{CO}_2 + 0.5 \text{ H}_2\text{O} \quad -36.1 \\
\end{align*}
\]

**Stoichiometric equations for homoacetogens**

\[
\Delta G^\circ' \text{ (kJ/mol)} \\
\begin{align*}
e. \quad 4\text{H}_2 + 2\text{CO}_2 & \rightarrow \text{Acetate}^- + \text{H}^+ + 2\text{H}_2\text{O} \quad -95 \\
f. \quad 4 \text{ Formate} + \text{H}^- & \rightarrow \text{Acetate}^- + 2\text{HCO}_3^- \quad -99.1 \\
\end{align*}
\]

**Oxidation half reactions for propionate, acetate, hydrogen and formate in the anode**

\(\Delta G^\circ'\) was calculated using the equation \(\Delta G^\circ' = - nF(E_{\text{anode}} - E^{\circ'}_{\text{substrate}})\) \(^52\).

Where \(\Delta G^\circ\) (kJ/mol) is the Gibbs free energy at standard biological conditions (i.e. reactant and products at 1 M or 1 atm, 298 K, and pH 7), \(n\) is the number of electrons transferred, \(F\) is the Faradays constant (96,485 C/mol e\(^-\)), and \(E_{\text{anode}}\) (V) and \(E^{\circ'}_{\text{substrate}}\) (V) are the anode potential and the standard biological redox potential of the substrate. \(E_{\text{anode}}\)
\[ E^{\circ'}_{\text{substrate}} = - \frac{\Delta G^\circ_r}{nF} \]  

and values from Ref. \(^{54}\).

\[ \Delta G^\circ (kJ/mol) \]

\begin{align*}
g. \text{CH}_3\text{CH}_2\text{COO}^- + 5\text{H}_2\text{O} & \rightarrow 2\text{CO}_2 + \text{HCO}_3^- + 14\text{H}^+ + 14\text{e}^- \quad -72.95 \\
h. \text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} & \rightarrow 2\text{HCO}_3^- + 9\text{H}^+ + 8\text{e}^- \quad -35.5 \\
i. \text{H}_2 & \rightarrow 2\text{H}^+ + 2\text{e}^- \quad -34.9 \\
j. \text{HCOOH} + \text{H}_2\text{O} & \rightarrow \text{HCO}_3^- + 2\text{H}^+ + 2\text{e}^- \quad -49.6
\end{align*}

Conversion of propionate concentration from mM to milli electron \((me^-)\) equivalents

Conversion of propionate concentration from mM to \(me^-\) equivalents =

Propionate concentration (mM) \(\times\) volume of the reactor (Litre) \(\times\) moles of electrons / mole of propionate.

For example in Figure 2.4B: 35.8 mM of propionate is equivalent to 10 \(me^-\) equivalents, calculated as follows:

\[ 35.8 \times 0.02 \times 14 = 10 \text{ } me^- \text{ equivalents} \]

Fraction of electrons utilized for biomass synthesis

The fraction of electrons from the donor substrate that is utilized for biomass synthesis is represented by \(f^o_s\) \(^{47}\). \(f^o_s = 0.05\) for \(G. \text{sulfurreducens}\), as representative of exoelectrogens \(^{48,49}\); \(f^o_s = 0.1\) for fermenters and \(f^o_s = 0.08\) for hydrogenotrophic methanogens \(^{47}\); and \(f^o_s = 0.1\) for acetogens and homoacetogens \(^{50,51}\). The electron sinks for the different microbial groups was
calculated by multiplying the substrate concentration with the $f^o_s$ values of the corresponding organisms.

Example (Figure 2.4B, pathway 1, propionate concentration =10 $me^-$ equivalent): The electron sink for *G. sulfurreducens* biomass (0.5) was calculated by multiplying 10 $me^-$ equivalent by the $f^o_s$ value (0.05) for *G. sulfurreducens* (Table 2.2).

**Table 2.2.** Predicted electron distribution for biomass synthesis at high propionate concentration

<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Substrate</th>
<th>Electrons available (meq) from substrate</th>
<th>$f^o_s$</th>
<th>Electrons utilized for biomass synthesis ($me^-$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct oxidation of propionate by exoelectrogens</td>
<td>Propionate</td>
<td>10</td>
<td>0.05</td>
<td>0.5</td>
<td>47</td>
</tr>
<tr>
<td>Propionate fermenters</td>
<td>Propionate</td>
<td>10</td>
<td>0.1</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>Acetate-utilizing exoelectrogens</td>
<td>Acetate</td>
<td>5.14</td>
<td>0.05</td>
<td>0.3</td>
<td>49</td>
</tr>
<tr>
<td>Acetate-utilizing exoelectrogens</td>
<td>Acetate</td>
<td>3.86</td>
<td>0.05</td>
<td>0.2</td>
<td>49</td>
</tr>
<tr>
<td>H$_2$ utilizing methanogens</td>
<td>H$_2$</td>
<td>3.86</td>
<td>0.08</td>
<td>0.3</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>Homoacetogens</td>
<td>H₂</td>
<td>3.86</td>
<td>0.1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>H₂ utilizing exoelectrogens</td>
<td>H₂</td>
<td>3.86</td>
<td>0.05</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Formate utilizing exoelectrogens</td>
<td>Formate</td>
<td>3.86</td>
<td>0.05</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Formate utilizing homoacetogens</td>
<td>Formate</td>
<td>3.86</td>
<td>0.1</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

**Predicted electron distribution for high propionate concentration** (Figure 2.4B)

**Route 1:**
Propionate (10) is oxidized by *G. sulfurreducens* yielding biomass (0.5) and current (9.5).

**Route 2:**
Propionate (10) undergoes oxidation by propionate fermenters yielding biomass (1), H₂ (3.86) and acetate (5.14). H₂ (3.86) generated from propionate fermentation is utilized by hydrogenotrophic methanogens yielding biomass (0.3) and methane (3.56), or it could be oxidized by *G. sulfurreducens* yielding biomass (0.3) and current (3.66) or it could be utilized by homoacetogens yielding biomass (0.4) and acetate (3.46). The acetate (3.46) generated by homoacetogens is oxidized by *G. sulfurreducens* yielding biomass (0.2) and current (3.26). Also, the acetate (5.14) generated from propionate fermentation is oxidized by *G. sulfurreducens* yielding biomass (0.3) and current (4.84).

**Route 3:**
Propionate (10) undergoes oxidation by propionate fermenters yielding biomass (1), formate (3.86), and acetate (5.14). Formate (3.86) generated from propionate
fermentation is utilized by hydrogenotrophic methanogens yielding biomass (0.3) and methane (3.56), or it could be oxidized by G. sulfurreducens yielding biomass (0.3) and current (3.66) or it could be utilized by homoacetogens yielding biomass (0.4) and acetate (3.46). The acetate (3.46) generated by homoacetogens is oxidized by G. sulfurreducens yielding biomass (0.2) and current (3.26). Also, Acetate (5.14) generated from propionate fermentation is oxidized by G. sulfurreducens yielding biomass (0.3) and current (4.84).

2.3.5 Analysis of microbial community

Pyrosequencing of 16S rRNA gene was used to characterize the bacterial and archaeal communities of biofilm (anode) and suspension samples from four MEC reactors (PL-BES, PL+BES, PH-BES, and PH+BES). Pyrosequencing of eight bacterial and seven archaeal (except S(PL+BES), which failed to amplify) 16S rRNA gene libraries produced 106,533 (bacteria) and 62,538 (archaea) high quality reads (average length of ~400 bp) after denoising, quality filtering and removal of chimeric sequences. The sequences were assigned to 2,660 (bacteria) and 1,737 (archaea) OTUs at a 97% sequence identity threshold. The Good’s coverage ranged from 95 to 99% (data not shown), suggesting that the majority of estimated bacterial OTUs were recovered from each sample.

2.3.5.1 Classification of bacterial communities

Five dominant bacterial phyla (Proteobacteria, Synergistetes, Firmicutes, Bacteroidetes, and Chloroflexi) were identified in all reactor types. The phylum Proteobacteria was the dominant phyla detected on the anode samples (32-77%), whereas the suspension samples were dominated by Proteobacteria (25-55%), Synergistetes (8-36%), Firmicutes (17-26%), and Bacteroidetes (7-19%) (Figure 2.5A). The dominant bacterial phyla detected in the inoculum were Proteobacteria (29.8%), Synergistetes (21.4%),
Chloroflexi (16%), Firmicutes (15%), Bacteroidetes (6%), Thermotogae (2%), Actinobacteria (1.9%), Spirochaetes (1.4%) and Verrucomicrobia (1.4%) (Figure 2.6).

The classification of the bacterial communities at the class level showed that Deltaproteobacteria was higher in the anode samples (25-75%) compared to suspension (6-33%) and more abundant in PL (29-75%) than PH (6-50%) reactors (Figure 2.5B). Also, it was higher at the anode of PH+BES (50%) compared to PH-BES (25%). On the contrary, the class Synergistia was higher in the anode and suspension samples of PH-BES (36%) compared to PH+BES (10-19%). The class Betaproteobacteria was higher in the suspension samples (12-55%) compared to the anode samples (1-7%), and it was more abundant in the suspension of PH+BES (55%) than PH-BES (13%).

The classification of bacterial communities at the family level (Figure 2.5C) showed that Geobacteraceae was the dominant family at the anode (30-77%). Other dominant families detected at the anode of PL and PH reactors include Dethiosulfovibrionaceae (2-12%), Clostridiales (5-12%), Anaerolinaceae (1-14%), Prophyromonadaceae (2-9%), Desulfovibrionaceae (2-12%), and Thermodesulfovibrioaceae (2-5%). The suspension samples of PL and PH reactors were more diverse and dominated by Rhodocyclaceae (11-50%), Geobacteraceae (3-28%), Clostridiales (3-18%), Dethiosulfovibrionaceae (6-16%), Prophyromonadaceae (2-9%), Synergistaceae (2-11%), Desulfovibrionaceae (3-7%), Pseudomonadaceae (1-6%), Bacteroidales (1-6%), Anaerolinaceae (1-3%), and Alcaligenaceae (1-3%).
**Figure 2.5.** Relative abundance of (A) bacterial phyla, (B) bacterial class, and (C) bacterial family. A, anode and S, suspension. Bacterial phyla that represent less than 1% of the total composition are classified as others.

**Figure 2.6.** The relative abundance of the different bacterial phylum in the inoculum. Bacterial phylum that represent less than 1% of the total composition are classified as others.

Hierarchical clustering was used to generate a dendrogram showing dissimilarity in the bacterial community structure among the four reactor types (Figure 2.7). Two clear
clusters were apparent. The anode samples of the PL reactors (PL-BES and PL+BES) formed one cluster that is separated from the remaining samples. Within the second cluster, there were two sub-clusters representing PH+BES reactor (anode and suspension) and PH-BES reactor (anode and suspension).

**Figure 2.7.** Distribution heat map of bacterial genera arranged by hierarchical clustering of anode and suspension samples: A, anode; S, suspension; G, genus; O, order; and F, family. The bootstrap tree on the left shows the primary
clustering of samples. Bacterial phyla representing less than 1% of the total sequences reads are classified as other bacteria.

The classification of the bacterial communities at the phylum, class and family level are provided in the Supporting Information. The dominant genera at the anode of PL and PH reactors were *Geobacter* (31-82%) followed by *HA73* (6-24%) and *T78* (1-18%) (Figure 2.7). The anode of PH reactors also contained *Copothermobacter* (6-8%), *Sedimentibacter* (4%), and *Dechloromonas* (2-7%). The suspension samples contained more diverse genera, such as *Dechloromonas* (6-59%), *HA73* (7-23%), *Geobacter* (4-32%), *Sedimentibacter* (2-20%), *Cloacibacillus* (2-14%), *Prophyromonadaceae* (2-12%), *Pseudomonas* (3-7%), *Desulfovibrio* (3%), *Parabacteroides* and *Copothermobacter* (1-5%), and *Azospira* (1-6%).

Archaeal 16S rRNA gene sequences revealed that the abundant genera in all the reactors were the hydrogenotrophic methanogens, *Methanobacterium* (45-86%) and *Methanobrevibacter* (3-61%), followed by the acetoclastic methanogens, *Methanoseta* (2-10%) and *Methanosarcina* (2%). *Methanobacterium* was more abundant in the MEC reactors without BES addition (i.e. PL-BES and PH-BES) compared to the reactors with BES (PL+BES and PH+BES). They were also more abundant in PH than PL reactors. *Methanobrevibacter* was more abundant in suspension compared to the anode samples. *Methanospirillum* (3%; hydrogenotrophic methanogen) and *Candidatus nitrophaera* (2%) were only present at the anode of PL+BES and PH+BES reactors (Figure 2.8).
**Figure 2.8.** Relative abundance of the (A) archaeal order and (B) genera: A, anode; S, suspension. Archaeal phyla representing less than 1% of the total sequences reads are classified as others. Sample S(PL+BES) failed to amplify.

### 2.4 Discussion

#### 2.4.1 Multiple paths of electron flow during propionate oxidation in the anode of MECs

The paths of electron flow from propionate oxidation and the microbial community structure in the anode of MECs fed with low and high propionate was not studied previously. The presence of propionate fermentation intermediates (acetate, formate, and \( \text{H}_2 \)) at the end of the batch (Table 2.1) suggest that electron flow to current via acetate/\( \text{H}_2 \) (pathway 2 in Figure 2.4) or acetate/formate (pathway 3 in Figure 2.4) could have occurred simultaneously irrespective of the tested concentrations. The presence of propionate fermentation intermediates (acetate, formate, and \( \text{H}_2 \)) during the end of the batch (Table 2.1) suggest that electron flow to current via acetate/\( \text{H}_2 \) (pathway 2 in Figure
2.4) or acetate/formate (pathway 3 in Figure 2.4) could have occurred simultaneously irrespective of the tested concentrations. However, with the experimental results obtained in this study, it could not be determined which pathway was more dominant. Also, it should be noted that the conditions that favor the dominance of one pathway over another was not within the scope of this study. Based on thermodynamics alone (see Supporting Information), direct oxidation of propionate to current (i.e. pathway 1; $\Delta G^\circ' = -72.95 \text{ KJ/mol}$) under standard biochemical conditions (i.e. reactant and products at 1 M or 1 atm, 298 K, and pH 7) should be the dominant pathway followed by formate oxidation (pathway 3; $\Delta G^\circ' = -49.6 \text{ KJ/mol}$) and then hydrogen (pathway 2; $\Delta G^\circ' = -34.9 \text{ KJ/mol}$). However, for pathway 1 to be dominant, it requires the presence of exoelectrogens that can oxidize propionate directly to current. Analysis of the anodic bacterial community with 16S rRNA gene pyrosequencing revealed the dominance of OTUs (OTU1 to OTU4) that were most similar to *G. sulfurreducens* PCA and *G. sulfurreducens subsp. ethanolicus* (Figure 2.9). *G. sulfurreducens* PCA can oxidize acetate, formate, and H$_2$ directly to current, but they cannot oxidize propionate directly to current.$^{29,55}$ Also, *G. sulfurreducens subsp. ethanolicus* can utilize acetate, formate, and H$_2$ but not propionate.$^{56}$ Other *Geobacter*-like exoelectrogens such as *G. metallireducens*, and *Geoalkalibacter subterannus* have been shown to oxidize propionate directly to current.$^{27,57}$, but they were not detected on the anodic biofilms of propionate fed MECs. However, we cannot rule out the possibility that the dominant *Geobacter*-like exoelectrogens found in this study can directly oxidize propionate to current (pathway 1 in Figure 2.4) since our sequences were not long enough (~ 400 bp) to confidently classify sequences down to the species level.
Figure 2.9. Phylogenetic tree based on 16S rRNA gene sequences showing the positions of the dominant Geobacter OTUs (OTU1 to OTU4) retrieved from the anode biofilms of propionate fed MECs among closely related members of the family Geobacteraceae (accession numbers in parentheses). The phylogenetic tree was constructed using the neighbor-joining method with Jukes–Cantor distance in MEGA6. Bootstrap (1,000 replicates) values greater than 70% are shown at the nodes. The scale bar represents 0.02 nucleotide substitution per homologous sequence site. Smithella propionica LYP (X70905) and Syntrophobacter wolinii DB (NR 024989) were used as outgroups.

Earlier studies on anaerobic degradation of propionate in methanogenic systems showed inconsistencies in the interspecies electron transfer mechanisms (i.e. either through formate or hydrogen). For example, in one study it was suggested that interspecies
electron transfer through formate was the predominant mechanism \(^{19}\), whereas in other studies it was suggested that hydrogen transfer is the preferable route \(^{58,59}\). De bok et al. \(^{60}\) showed that the hydrogen transfer mechanism is dominant when the interspecies electron transfer is over a short distance, whereas formate route was dominant when the interspecies electron transfer is over a long distance. Recently, Muller et al. \(^{61}\) reported that interspecies electron transfer could occur simultaneously through formate and \(\text{H}_2\) based on biochemical studies and genome analysis of pure cultures of syntrophic volatile fatty acid degraders. It can be concluded from the aforementioned studies and the current study that the paths of electron flow (either \(\text{H}_2\) or formate) during anaerobic degradation of propionate has not been clearly clarified yet.

2.4.2 Electron sinks in the anode during propionate oxidation

The low electron losses (1.2 - 2.3\%) to methane at low and high propionate concentration (Table 2.1), suggests that methane is not a major sink of electron flow from propionate oxidation otherwise the electron distribution to methane based on theoretical calculations should be around 36\% (Figure 2.4). This observation was consistent with earlier studies on the propionate-fed anode, where a minor loss (0.4 to 3.4\%) of electrons to \(\text{CH}_4\) was reported \(^{9,13}\). In contrast, other MXC studies using fermentable substrates such as glucose, butyrate, and ethanol showed that methanogenesis was an important electron sink (7 to 26\% of the electrons were lost to \(\text{CH}_4\)) \(^{9,11,62}\).

It is not clear why methanogenesis was not an important electron sink in this study. Propionate can be inhibitory to methanogens at concentrations >20 mM \(^{63}\) and it is possible that methanogens in the PH-MECs were inhibited by the high concentration of propionate (36 mM) used in this study. Also, it has been reported that acetate-oxidizing
exoelectrogens (e.g. *G. sulfurreducens*) could outcompete acetoclastic methanogens for acetate because of kinetic benefits\textsuperscript{13,62,64}. For example, acetate-oxidizing exoelectrogens have a half-maximum rate concentration (*K*\textsubscript{s}) of 0.64 mg COD/L and specific substrate utilization rate (q\textsubscript{max}) of 22.7 g COD/g VSS-d, compared to a *K*\textsubscript{s} of 177-427 mg COD/L and a q\textsubscript{max} of 7.6 g COD/g VSS-d for acetoclastic methanogens. In contrast, hydrogenotrophic methanogens could outcompete exoelectrogens and homoacetogens for H\textsubscript{2} because of their thermodynamic and kinetic benefits\textsuperscript{13,15,65}. Also, hydrogenotrophic methanogens contain formate dehydrogenase genes and can utilize formate as their energy and carbon source\textsuperscript{66}. However, *G. sulfurreducens* and homoacetogens also contain formate dehydrogenase genes and have been shown to utilize formate\textsuperscript{55,67,68}. The utilization of hydrogen in the anode of MECs by *G. sulfurreducens* was reported in a number of studies\textsuperscript{32,65,69}. The reasons why hydrogenotrophic methanogens were not enriched in this study are unclear, but may be it is related to the inoculum source (anaerobic digester sludge) used in this study. Anaerobic digester sludge at most domestic wastewater treatment plants predominantly contains acetoclastic methanogens\textsuperscript{70}. In a recent study, it was shown that the presence of hydrogenotrophic methanogens in the inoculum improves methane gas generation in MECs\textsuperscript{70}. The authors tested two inoculum sources and showed that sediment bog samples were a better inoculum than anaerobic digester sludge for both improved current generation and methane gas production in MECs. Also, our experience from other propionate fed MEC studies (36 mM) inoculated with a different inoculum source (effluent from anaerobic membrane bioreactors operated with synthetic municipal wastewater medium containing starch, milk powder, and yeast) showed higher enrichment of hydrogenotrophic methanogens (1-11\%)
and electron distribution to CH$_4$ (21-42%) (unpublished data). Hence, the selection of inoculum source is important in MEC studies and future studies should be conducted to further understand the factors that affect the competition between exoelectrogens, hydrogenotrophic methanogens, and homocetogens for H$_2$ and formate in the anode of single-chamber MECs.

Current was the largest electron sink regardless of the propionate concentration tested (Table 2.1) though at higher propionate concentration the performance of MEC was decreased in terms of $I_V$, CE, and $Y_{H_2}$ (Figure 2.2). The maximum proportion of electrons recovered as current was observed in the PL reactors (Table 2.1), with minimal losses to undefined sinks (1.1-5.2%; biomass, soluble microbial products, others). However, PH-MECs had relatively lower CE (57-66%) than PL-MECs (91-96%) due to the higher losses of electrons to undefined sinks (11.2-23.5%) (Table 2.1). At the end of the batch test, the residual propionate concentration in PL reactors was negligible (<0.04 mM) compared to PH reactors (~7.2 mM).

Parameswaran et al. 62, showed that the inhibition of methanogenesis, which was an important electron sink, by the addition of BES to the anode of ethanol-fed MECs increased the CE by 26% by encouraging new microbial interactions, which ultimately diverted more electrons to current. In the current study, the addition of BES did not improve the CE of the PH reactors (Figure 2.2A) since methanogenesis was not an important sink (Table 1). Moreover, PH reactors supplemented with BES had a lower CE compared to PH-BES reactors (Figure 2.2A) possibly due to the effect of BES on bacteria which was supported by hierarchical clustering that clearly illustrated two separate clusters when BES was added (PH-BES and PH+BES samples) (Figure 2.7). Previous
studies unrelated to bioelectrochemical systems have shown that BES alters the bacterial community composition, in particular affecting the microbial interaction between hydrogen producing fermenters and homoacetogens.\textsuperscript{71,72}

In anaerobic digesters, inhibition of propionate degradation occurs at high propionate concentrations due to the accumulation of the undissociated acid forms of propionate and acetate at pH of 6 to 6.4, and high H\textsubscript{2} partial pressures.\textsuperscript{73} However, in MECs, the reason for unused propionate (20\%) in the anodic suspension of PH reactors remains unclear (Table 2.1) especially that the accumulation of end products (acetate, formate and H\textsubscript{2}) was not noticed and the pH of the medium was \~7.5 at the end of the batch.

2.4.3 Microbial community composition

Although methanogenesis was not a significant electron sink, archaeal 16S rRNA gene pyrosequencing revealed the presence of methanogenic archaea, particularly the hydrogenotrophic methanogen \textit{Methanobacteriales} (55-86\%), on the anodic biofilm and in the suspension of the propionate fed MECs (Figure 2.5).

Bacterial community composition based on 16S rRNA gene pyrosequencing revealed that the classical propionate degraders such as \textit{Syntrophobacter spp.}, \textit{Smithella spp.}, and \textit{Pelotomaculum spp.} that are typically present in methanogenic systems were not detected in the anode, suspension and in the inoculum used in this study (Figure 2.5 and 2.6), they have not been reported in other propionate-fed anode\textsuperscript{9,20-23,74}. It is possible that the conditions in anode favour the presence of other propionate degraders. The possible propionate oxidizers in the anode of propionate fed MECs were members of \textit{Rhodocyclaceae}, \textit{Clostridiales} and \textit{Bacteroidetes} (Figure 2.5), which were reported to oxidize propionate\textsuperscript{75-77} and were abundant in propionate and acetate fed MECs\textsuperscript{74},
propionate enriched soil and anaerobic digester samples \(^{75-78}\). Members of *Rhodocyclaceae* are capable of oxidizing many fatty acids such as propionate and butyrate \(^{74,77}\). Members of *Clostridiales* can also oxidize propionate and have been identified in propionate enriched wetland samples \(^{75}\).

It should be noted that 16S rRNA gene pyrosequencing does not provide information on the metabolically active members in the community. Alternatively, stable isotope probing (SIP) with labelled propionate combined with 16S rRNA gene sequencing should be used in future studies to provide a better insight into the carbon flow during propionate oxidation and to allow the identification of metabolically active members in the community (i.e. core consumers and transformers) that are involved in propionate oxidation \(^{79}\).

### 2.5 Conclusion

Our results showed that multiple paths of electron flow to current could occur simultaneously during propionate oxidation in the anode of MECs irrespective of the tested propionate concentrations. At both concentrations tested, current was a significant sink of electrons, whereas methane was a minor sink of electrons. In methanogenic systems, processing of substrates through multiple routes in parallel is essential for maintaining functional stability in response to a perturbation \(^{80,81}\). In a similar fashion, we hypothesize that multiple paths of electron flow from the substrate in the anode of MECs should result in a higher functional stability of the system. However, this should to be tested in the anode of MECs in future studies. In general, accumulation of high concentrations of propionate (>20 to 37 mM) is detrimental to anaerobic digestion processes \(^{16-18}\). Despite the high concentration (36 mM) of propionate used in this study,
the removal of propionate was still high (78%) in the PH-MEC reactors. Therefore, the anode of MECs could potentially be integrated with existing anaerobic digestion processes to improve propionate degradation. In addition, further studies are needed to elucidate the conditions that favor multiple paths of electron flow and the ones that favor the dominance of one pathway over another.
2.6. References:


57 Lovley, D. R. *et al.* *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch. Microbiol.* 159, 336-344 (1993).


CHAPTER 3 Set Anode Potentials Affect the Electron Fluxes and Microbial Community Structure in Propionate-fed Microbial Electrolysis Cells

This chapter has been accepted to peer reviewed journal article:

Conference (Poster presentation):

Conference (Oral presentation):
ABSTRACT

Anode potential has been shown to be a critical factor in the rate of acetate removal in microbial electrolysis cells (MECs), but studies with fermentable substrates and set potentials are lacking. Here, we examined the impact of three different set anode potentials (SAPs; –0.25, 0, and 0.25 V vs. standard hydrogen electrode) on the electrochemical performance, electron flux to various sinks, and anodic microbial community structure in two-chambered MECs fed with propionate. Electrical current (49-71%) and CH₄ (22.9-41%) were the largest electron sinks regardless of the potentials tested. Among the three SAPs tested, 0 V showed the highest electron flux to electrical current (71±5%) and the lowest flux to CH₄ (22.9±1.2%). In contrast, the SAP of –0.25 V had the lowest electron flux to current (49±6%) and the highest flux to CH₄ (41.1±2%). The most dominant genera detected on the anode of all three SAPs based on 16S rRNA gene sequencing were *Geobacter, Smithella* and *Syntrophobacter*, but their relative abundance varied among the tested SAPs. Microbial community analysis implies that complete degradation of propionate in all the tested SAPs was facilitated by syntrophic interactions between fermenters and *Geobacter* at the anode and fermenters and hydrogenotrophic methanogens in suspension.
3.1 Introduction

Microbial electrochemical technologies (METs) are widely recognized for their potential for recovering energy from organic waste streams. In many different METs, certain microorganisms known as exoelectrogens convert chemical energy in organics to electricity via anaerobic oxidation of wastewater organics at the anode. The electrons and protons that are generated during oxidation at the anode are utilized at the cathode for oxygen reduction reaction in microbial fuel cells (MFCs) or hydrogen evolution reaction in microbial electrolysis cells (MECs). MECs can be operated either by setting the anode potential using a potentiostat, or applying voltage using an external power source or by inserting a reverse electrodialysis stack between the electrodes. Setting different anode potentials can impact the electrochemical performance, microbial community structure and theoretical maximum energy gain (Δ$G^\circ$) by exoelectrogens for their growth and maintenance, according to:

$$\Delta G^\circ = -nF \left( E_{\text{anode}} - E_{\text{donor}}^\circ \right)$$

Eq. 1

Where $\Delta G^\circ$ (J/mol) is the Gibbs free energy at standard biological conditions ($T=25^\circ C$, pH=7 and 1 M of all reactants), $n$ the number of electrons transmitted, $F$ Faradays constant (96,485 C/mol e$^-$), $E_{\text{anode}}$ the anode potential (V), and $E_{\text{donor}}^\circ$ (V) the standard biological redox potential of the electron donor. The real energy gain by exoelectrogens depends on the redox potential of the terminal electron transferring component (e.g., outer membrane protein) serving as the electron donor to the anode. In theory, at low anode potentials, exoelectrogens gain less energy for growth, resulting in lower biomass accumulation, slower development of the anode biofilm community, and
more delayed start-up of current production. At higher anode potentials, exoelectrogens could gain more energy for growth if they have the capability to capture this additional energy.

One new and important application of MECs is the addition of electrodes directly into an anaerobic digester, in order to improve performance and increase the methane concentration in the product gas. Such an integration is not practical using MFCs as the anaerobic digestion (AD) process requires oxygen free environment. However, the impact of a set anode potential (SAP) on MEC performance has not been well examined for such environments where there can be high concentrations of fermentable substrates, especially for the case of propionate which is slowly degraded, as most studies on SAPs in MECs have mainly focused on acetate (Table 3.1). The performance of MECs is well known to be impacted in terms of current production for simple substrates such as acetate, although the community structure is relatively unchanged for different SAPs. When SAPs have been examined in MFCs using fermentable substrates such as xylose, sucrose, formate, and glucose, it was shown that the SAP influenced the biocatalytic activity, electrochemical performance, substrate degradation, and microbial community structure. Also, it altered the syntrophic interactions between the organisms.

In METs fed fermentable substrates, methane generation by hydrogenotrophic methanogens is an important sink of electrons, and hence a coulombic loss. The H₂ generated during the fermentation process is utilized by hydrogenotrophic methanogens, which could outcompete other H₂-utilizing organisms (e.g. exoelectrogens and homoacetogens), thus diverting electrons away from current. The impact of various
approches on methanogenesis has been examined in previous MEC studies such as the use of methanogen inhibitors, reactor exposure to air, the use of short hydraulic retention times and operating the reactors at low temperature of 15 °C. However, the impact of different SAPs in MECs on the electron fluxes to methane versus current has not been examined with fermentable substrates.

Propionate is a model fermentable substrate to study microbial partnerships in natural and engineered methanogenic systems and an important intermediate in the anaerobic decomposition of organic matter in AD processes. Accumulation of propionate (>20 mM) at high organic loading rates is detrimental to AD processes. Thus, propionate removal is necessary for the stable operation of AD. In these systems, propionate is oxidized via a microbial partnership between fermenters and methanogens. Fermenters oxidize propionate to acetate, formate/H₂ and CO₂, which are then utilized by methanogens to produce CH₄. Recently, Hari et al demonstrated that propionate degradation at the anode of MECs operated at an applied voltage of 0.7 V using a power source occurs via a microbial partnership between fermenters and Geobacter. They showed that multiple paths of electron flow to current (via acetate/H₂ or acetate/formate) could occur simultaneously during propionate oxidation regardless of the propionate concentration tested. At a high propionate concentration (36 mM), there was incomplete propionate removal (80%), compared to complete propionate removal at a much lower concentration (4.5 mM). While the use of an applied voltage was useful for studying MEC operation under these different conditions, the anode potentials in the reactors were not controlled, and thus the direct impact of the anode potential could not be determined in these studies separate from other operational conditions.
In order to better understand the impact of anode potential on a fermentable substrate, the effect of three different SAPs (−0.25, 0 and 0.25 V vs. standard hydrogen electrode, SHE) was examined on MEC performance using propionate in terms of its degradation rate, electron fluxes to various sinks (current, CH₄ and undefined sinks), and microbial community structure using two-chamber reactors that minimized hydrogen gas crossover from the cathode.

Table 3.1. Comparative overview of the effect of SAPs in MEC

<table>
<thead>
<tr>
<th>SAPs (V vs. SHE)</th>
<th>MEC configuration</th>
<th>Substrate</th>
<th>Inoculum</th>
<th>Working electrode</th>
<th>Electron fluxes</th>
<th>Microbial communities</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.15, -0.09, 0.02, 0.37</td>
<td>Dual chamber</td>
<td>Acetate (Non-fermentable)</td>
<td>Anaerobic digester sludge</td>
<td>Graphite rod</td>
<td>SAPs of -0.15 V, -0.09 V showed higher electron flux to current than the other tested potentials</td>
<td>Biofilms enriched at lower SAPs (-0.15 V, -0.09 V, 0.02 V) were dominated by G. sulfurreducens. Whereas, biofilm grown under higher SAP (0.37 V) was enriched by a diverse bacterial community</td>
<td>11</td>
</tr>
<tr>
<td>-0.15, -0.09, 0.02</td>
<td>Dual chamber</td>
<td>Acetate (Non-fermentable)</td>
<td>Mixture of soil and activated sludge</td>
<td>Graphite rod</td>
<td>All the potentials tested showed relatively similar electron flux to current</td>
<td>All the potentials were dominated by Geobacter spp. However, 0.02 V dominated by G. lovely and -0.09 and - 0.15 V dominated by G. sulfurreducens</td>
<td>12</td>
</tr>
<tr>
<td>-0.25, -0.09, 0.21, 0.51, 0.81</td>
<td>Single chamber</td>
<td>Acetate (Non-fermentable)</td>
<td>Effluent of primary clarifier</td>
<td>Graphite plate</td>
<td>0.21 V, 0.51 V, -0.09 V showed higher electron flux to current than the other tested SAPs</td>
<td>G. sulfurreducens was dominant in all the tested potentials</td>
<td>1</td>
</tr>
<tr>
<td>-0.2, -0.15, -0.1, 0</td>
<td>Dual chamber</td>
<td>Acetate (Non-fermentable)</td>
<td>Activated sludge/anaerobic sludge</td>
<td>Graphite rod</td>
<td>0 V SAP showed higher electron flux to current and higher acetate degradation rate followed by - 0.1 V, -0.15 V and -0.2 V</td>
<td>No information on microbial community was reported in their study</td>
<td>31</td>
</tr>
</tbody>
</table>
3.2 Materials and Methods

3.2.1 Construction and operation of MECs

Two-chambered, cube-shaped MECs with a working volume of 40 mL (anode chamber) and 20 mL (cathode chamber) were constructed as previously described. The two chambers were separated by an anion exchange membrane (5 cm²; AMI 7001, Membranes International, Glen Rock, NJ). A glass gas collection tube (15 mL) was attached to the top of both the anode and cathode chambers. Gas bag (0.1 L Cali-5-Bond, Calibrate, Inc.) were connected to the top of the glass collection tubes to collect more volume of gas. The anodes were graphite fiber brushes (2.5 cm diameter × 2 cm long; PANEX 33 fibers, ZOLTEK Inc., St. Louis, MO, USA). The cathodes (projected surface area of 7 cm²) were made using carbon cloth (type B-1B, E-TEK) containing 0.5 mg/cm² of Pt on the side facing the anode, and four polytetrafluoroethylene diffusion layers on other side. An Ag/AgCl reference electrode (+210 mV vs SHE; RE-5B; BASi) was
placed between the electrodes. Necessary precautions were taken for positioning the working, counter and reference electrodes in the reactor as recommended by Zhang et al., 2014, to provide uniform potential distribution across the working electrode. All potentials were reported here versus SHE for comparison to other studies.

All MEC anodes were inoculated with effluent from anaerobic membrane bioreactor that had been operated with synthetic municipal wastewater medium (containing starch, milk powder and yeast) for more than one year in our lab. The anaerobic membrane bioreactor was inoculated with camel manure (Jeddah, KSA) and anaerobic digested sludge (Manfouha Wastewater Treatment Plant, Riyadh, KSA). The anode and cathode medium (pH 8.9) consisted of bicarbonate buffer (80 mM), nutrients (0.08 mol/L NaH₂CO₃, 0.006 mol/L NH₄Cl, 0.0004 mol/L Na₂HPO₄, 0.0002 mol/L NaH₂PO₄), Wolfe’s vitamin (10 mL/L) and trace mineral (10 mL/L) solutions. The anode medium was supplemented with propionate (36 mM) as the sole carbon and energy source. The medium was boiled and then cooled to room temperature by sparging with N₂:CO₂ (80:20, vol/vol) gas mix for 30 min to remove any dissolved oxygen and was then autoclaved. The MECs were operated with three different SAPs (−0.25, 0 and 0.25 V) using a potentiostat (VMP3; Biologic, Claix, France) in a temperature controlled room (30 °C), with data recorded at 10-min intervals and analyzed using EC-lab V10.02 software. The control MECs were operated in an open circuit (O.C) mode. All reactor types were run in duplicate and operated in a fed-batch mode. When the current dropped to almost 0 mA (~4-5 days/cycle), the reactors were refilled with fresh medium and sparged with nitrogen gas (99.999%).
3.2.2 Electron balance

To establish electron balances during the time course or at the end of a batch cycle, the distribution of electrons in milli e\textsuperscript{−} equivalents (m e\textsuperscript{−}eq) in the anode of MECs was followed from the electron donor (propionate) to various measured electron sinks (current, H\textsubscript{2}, CH\textsubscript{4}, propionate, acetate, and formate). The concentrations of propionate, formate, and acetate were analyzed by high-performance liquid chromatograph (HPLC) (Thermo Scientific, Accela, country). Samples for HPLC analysis were collected during the time course or at the end of the batch. The concentrations of H\textsubscript{2} and CH\textsubscript{4} were measured using a gas chromatograph (GC) (model 310; SRI Instruments). Samples for GC analysis were collected at the end of the batch. The details of GC and HPLC analysis are provided as follows.

3.2.3 Analyses

The gas generated by the MECs was collected from both chambers in the head space and in a gas bag (0.1 L Cali -5 -Bond. Calibrate, Inc.), and the total gas volume was measured by the gas bag method as previously described\textsuperscript{33}. Concentrations of H\textsubscript{2}, N\textsubscript{2}, CH\textsubscript{4} were measured using a gas chromatograph (GC) (model 310; SRI Instruments) with a thermal conductivity detector (TCD), a 1.83-m molecular sieve packed 5A column, and an argon carrier gas. CO\textsubscript{2} concentration was measured with a second GC (model 310; SRI Instruments) with a TCD, a 0.91-m silica gel column, and using helium as the carrier gas. The volatile fatty acids (VFAs) profile (propionate, formate, and acetate) and their concentrations were analyzed by high-performance liquid chromatograph (HPLC) (Thermo Scientific, Accela, country) equipped with a photo-diode array (210 nm) and an ultraviolet detector. An Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA,
USA) was used to separate the VFAs. Sulfuric acid (5 mM) was used as the mobile phase at a flow rate of 550 µL/min, and the pressure was maintained at 9650 kPa. The total elution time was 30 min, each sample was measured in triplicate, and the average concentrations were reported.  

3.2.4. Calculations  
Hydrogen production rate at the cathode, $Q$ (m$^3$ H$_2$/m$^3$ reactor/day); volumetric current density of the reactor, $I_V$ (A/m$^3$); hydrogen yield, $Y_{H2}$ (mol-H$_2$/mol-propionate consumed); coulombic efficiency, CE (%); propionate removal (%) were determined as previously described. CE is defined as the fraction of the electrons recovered as electrical current compared to the theoretical electrons obtained from the complete oxidation of the substrate (i.e. propionate).  

3.2.5. Cyclic Voltammetry  
Cyclic Voltammetry (CV) was performed immediately after feeding with fresh growth medium containing sodium propionate (36 mM). Scans ranged from –0.5 to 0.5 V (turnover) and –0.5 to 0.3 V (non-turnover) at a rate of 1 mV/s, with the anode as the working electrode, the cathode as the counter electrode and Ag/AgCl as the reference electrode. Nonturnover CVs were performed when the current in MECs decreased to almost 0 mA with depleted sodium propionate growth medium.  

3.2.6. 16S rRNA gene sequencing  
At the end of the experiments (day 155), samples were collected from the anode and suspension of SAP-MECs and O.C reactors for microbial community analysis. Genomic DNA was extracted using the PowerBiofilm DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. The quality (A260/A280)
and quantity (A260) of the extracted genomic DNA was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Triplicate PCR reactions were performed for each sample in a 25 µL reaction volume using the HotStarTaqPlus Master Mix (Qiagen, Valencia, CA), 0.5 µM of each primer, and 100-200 ng of template DNA. The V3-V4 hypervariable region of 16S rRNA genes were amplified using a universal primer set for prokaryotes: Pro 341F (5’-Illumina adapter-Barcode-Linker- CCTACGGGNGGCASCAG-3’) and Pro 805R (5’-5’-Illumina adapter-Linker -GACTACNVGGGTATCTAATCC-3’) [35]. PCR was performed using life technologies veritus thermocycler with the following PCR conditions: initial denaturation at 94°C for 3 min, followed by 28 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 40 seconds, extension at 72°C for 1 min and a final extension at 72°C for 5 min.

Following PCR, all amplicon products from the different samples were mixed in equal concentrations, purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA), and sequenced on the Illumina TruSeq technology (San Diego, CA) according to manufacturer's instructions.

The 16S rRNA sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME v 1.9.0) pipeline [36]. Raw reads were first demultiplexed, trimmed and filtered for quality. The minimum acceptable length was set to 200 bp [36]. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the uclust algorithm [37,38]. A representative sequence from each OTU was aligned using PyNAST [38], and these were phylogenetically assigned to a taxonomic identity (phylum, class and genus level) using the RDP Naive Bayesian rRNA classifier at a confidence
threshold of 80% $^{39}$. Chimeric sequences were identified and removed from the aligned sequences using chimera Slayer as implemented in QIIME. Rarified OTU tables were used to generate alpha and beta diversity metrics by normalizing to the lowest sequence read of the samples. For alpha diversity measurements, both non-phylogenetic based metrics (observed OTUs, Shannon diversity index (H), Simpson diversity index (D) and Chao 1 richness estimator) and phylogenetic based metric (phylogenetic diversity (PD_whole)) were calculated with QIIME at the 3% distance level. Beta diversity metrics using the unweighted UniFrac distance matrix $^{40}$ was calculated and visualized with nonmetric multidimensional scaling (NMDS) using statistical software PRIMER 6 (version 6.1.13).

3.2.7. Nucleotide sequence accession numbers
The 16S r RNA gene sequencing reads have been deposited in European Nucleotide Archive under the accession number PRJEB14918.

3.3 Results

3.3.1 Electrochemical performance of MECs at different SAPs
MECs operated at SAP of 0 V performed significantly better than –0.25 and 0.25 V in terms of current density (average of maximum current density), CE, H$_2$ production rates, and H$_2$ yields ($P < 0.05$, student’s $t$ test for all comparisons) (Figure 3.1 A and B).
Figure 3.1. Performance of the MECs at different SAPs (–0.25, 0, and 0.25 V vs. SHE). (A) current density ([I] A/m³), coulombic efficiency ([CE] %) and H₂ production rate ([Q] m³H₂/m³/day). (B) H₂ yield and propionate removal. Values represent the average of the last five batch cycles of the duplicate reactors.

The values in Figure 1 correspond to the average of the last five batch cycles of the duplicate reactors (n =10). The peak current density was higher at 0 V (103±5 A/m³) than –0.25 V (77±5 A/m³) and 0.25 V (71±6 A/m³) by 25±6% and 31±5% respectively (P < 0.05) (Fig. 1A). The maximum current density (average of duplicate MECs) profile for all the batch cycles of operation is shown in Figure 3.2.
Figure 3.2. Maximum current density (average of duplicate MECs) profile for all batch cycles of operation for reactors operated at SAP of –0.25 V vs. SHE, 0 V vs. SHE, and 0.25 V vs. SHE.

Similarly, CE was higher at 0 V (71±5%), compared to –0.25 V (49±6%) and 0.25 V (56±5%) (P < 0.05) (Figure 3.1A). H₂ production rate was higher at 0 V (0.55±0.01 m³ H₂/m³/day) than 0.25 (0.42±0.02 m³ H₂/m³/day) and –0.25 V (0.28±0.02 m³ H₂/m³/day) by 23% and 49% respectively (P < 0.05) (Figure 3.1A). Although all the SAP-MECs showed nearly complete propionate degradation (99 ±1%), the MECs operated at SAP of 0 V (5.90±0.18 mol H₂/mol propionate) showed significantly higher H₂ yield than –0.25 (2.96±0.21 mol H₂/mol propionate) and 0.25 V (4.31±0.19 mol H₂/mol propionate) (P < 0.05) (Figure 3.1B). The pH of the suspension of SAP-MECs and O.C reactors at the end of the batch was 7.8±0.13 and 8.5±0.2 respectively.
3.3.2 Experimental distribution of electrons from propionate oxidation at the end of the last batch cycle

Electron balances at the end of the last batch cycle with matured biofilms (aged 155 days) indicated that electrical current was the first and CH₄ was the second largest electron sinks among all the tested SAP conditions (Table 3.2).

**Table 3.2.** Distribution of electrons in the anode at the end of the last batch cycle for all SAP and O.C reactors.

<table>
<thead>
<tr>
<th>Electron sinks</th>
<th>Fraction of electrons at the end of batch tests (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–0.25 V</td>
</tr>
<tr>
<td>Electrical current</td>
<td>49±6</td>
</tr>
<tr>
<td>Methane</td>
<td>41.1±2</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>Propionate</td>
<td>b.d.l&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**94**
95

<table>
<thead>
<tr>
<th></th>
<th>Acetate</th>
<th>Formate</th>
<th>Undefined sinks&lt;sup&gt;c&lt;/sup&gt; (biomass + soluble microbial products + others)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b.d.l</td>
<td>b.d.l</td>
<td>9.6±2.8</td>
</tr>
<tr>
<td></td>
<td>b.d.l</td>
<td>b.d.l</td>
<td>6.1±2.7</td>
</tr>
<tr>
<td></td>
<td>b.d.l</td>
<td>b.d.l</td>
<td>15.25±0.9</td>
</tr>
<tr>
<td></td>
<td>b.d.l</td>
<td>b.d.l</td>
<td>27.03±2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent the average of the duplicate reactors  
<sup>b</sup>b.d.l below detection limit  
<sup>c</sup>e<sup>−</sup> (undefined electron sinks) = e<sup>−</sup> (propionate) - e<sup>−</sup> (current) - e<sup>−</sup> (unused propionate) - e<sup>−</sup> (acetate) - e<sup>−</sup> (formate) - e<sup>−</sup> (hydrogen) - e<sup>−</sup> (methane), where e<sup>−</sup> = electron equivalents  
The possibility of propionate and its intermediates crossover to the cathode through the anion exchange membrane was minimal (< 0.5 %). Therefore, we did not considered for distribution of electrons calculation.

The SAP of 0 V showed higher electron distribution to electrical current (71±5%), followed by 0.25 V (56±5%) and −0.25 V (49±6%). In contrast, higher electron distribution to CH₄ was noticed at −0.25 V (41±2%), followed by 0.25 V (29±6%) and 0 V (23±1%). CH₄ was the largest electron sink (73±4%) in the O.C reactors. In general, the electron distribution for undefined sinks was highest in O.C reactors, whereas in the SAP-MECs higher electron distribution to undefined sinks was noticed at 0.25 V (15.25±0.9%) followed by −0.25 V (9.6±2.8%) and 0 V (6.1±2.7%).

3.3.3. Experimental distribution of electrons to acetate and formate during propionate oxidation over the course of the last batch cycle

After 155 days of operation, the distribution of electrons to acetate and formate during the oxidation of propionate was examined over the course of a batch cycle (Figure 3.3).
The gas bag analysis adapted in this study does not allow analysis of H₂ in multiple points in a single batch cycle. Propionate was completely consumed within ~ 60-70 hours in all the SAP-MECs and O.C reactors. The propionate removal rate was approximately similar in all the tested SAP-MECs (10.5±0.4 mM/day) and O.C (9.02±0.5 mM/day) reactors. However, accumulation of acetate (9.9±0.2 mM; 15.85 ±0.6% of electrons) was noticed in the O.C reactors at time ~70 hours of the batch, and it gradually decreased to reach 0% at time 120 hours. No accumulation or minor concentrations of acetate (< 0.4 mM) were measured for the SAP-MECs (Figure 3.3). Formate was below the detection limit throughout the batch of the SAP-MEC and O.C reactors (Figure 3.3).
3.3.4. Cyclic Voltammetry

CV analysis was performed for the biofilms (155 days aged) enriched at different SAPs immediately after feeding with a fresh growth medium (pH 8.9; 36 mM sodium propionate) to examine the influence of imposed anode potential on biofilm redox behavior. All biofilm voltammograms showed catalytic behavior with a rise in oxidation current between the potential window of -0.4 to -0.3 V (Figure 3.4A). Nonturnover CV analysis of the respective biofilms in propionate limited growth medium exhibited three distinct oxidation peaks at, –0.34 V (P1), –0.19 V (P2), and 0.11 V (P3) (Figures 3.4B, C and D). The CV analysis of the virgin anode in propionate growth medium (cell free) and reactor effluent (filtered and unfiltered) did not demonstrate any redox peaks (data not shown). Thus, the observed redox peaks from biofilm-voltammetry were presumably from the anodic biofilm and/or bacterial self-induced mediators (if localized in the biofilm matrix).
Figure 3.4. Cyclic voltammograms (CVs) of the biofilms (155 days) enriched at different SAP (–0.25, 0, and 0.25 V vs. SHE). (A) CV performed at the start of the batch in fresh growth medium with sodium propionate (36 mM) with a scan rate of 1 mV/s. (B, C, and D) CV performed at the end of the batch with depleted sodium propionate growth medium with a scan rate of 1 mV/s.

3.3.5. Analysis of microbial community

A total of 804,695 high-quality reads (average length of ~450 bp) were generated after denoising, quality filtering and removal of chimeric sequences. For alpha diversity measures, the dataset (normalized abundance values) was subsampled to an even depth of 44,170 sequences across the samples to remove inherent heterogeneity of sampling depth. This number was chosen, as it corresponds to the lowest number of sequence reads detected. The diversity values across the anode samples ranged as follows: observed
operational taxonomic units (OTUs: 1,006-1,679), Chao 1 (3,148-5,551), Shannon diversity index (H; 3.3-5.4), Simpson diversity index (D; 0.69-0.92) and phylogenetic diversity (PD; 65-91) (Table 3.3). The highest diversity based on observed OTUs, Chao 1, H, D and PD was observed in the anode of O.C reactors. Whereas, the lowest diversity based on Chao 1, H, D and PD was observed in the SAP-MEC operated at 0 V (Table 3.3).

Table 3.3 Number of observed operational taxonomic units (OTUs) and alpha diversity measures

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number of observed OTUs</th>
<th>Richness estimate (Chao1)</th>
<th>Shannon Diversity Index (H)</th>
<th>Simpson Diversity Index (D)</th>
<th>Phylogenetic diversity (PD)</th>
<th>Good's coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (-0.25 V)</td>
<td>1312</td>
<td>3918</td>
<td>4.7</td>
<td>0.89</td>
<td>75</td>
<td>0.98</td>
</tr>
<tr>
<td>A (0 V)</td>
<td>1006</td>
<td>3148</td>
<td>3.3</td>
<td>0.69</td>
<td>65</td>
<td>0.98</td>
</tr>
<tr>
<td>A (0.25 V)</td>
<td>1344</td>
<td>4100</td>
<td>3.9</td>
<td>0.78</td>
<td>77</td>
<td>0.98</td>
</tr>
<tr>
<td>A (O.C)</td>
<td>1679</td>
<td>5551</td>
<td>5.4</td>
<td>0.92</td>
<td>91</td>
<td>0.97</td>
</tr>
<tr>
<td>S (-0.25 V)</td>
<td>2190</td>
<td>5306</td>
<td>6.0</td>
<td>0.92</td>
<td>112</td>
<td>0.97</td>
</tr>
<tr>
<td>S (0 V)</td>
<td>2484</td>
<td>8010</td>
<td>6.7</td>
<td>0.96</td>
<td>120</td>
<td>0.96</td>
</tr>
<tr>
<td>S (0.25 V)</td>
<td>2499</td>
<td>8085</td>
<td>6.8</td>
<td>0.96</td>
<td>130</td>
<td>0.96</td>
</tr>
<tr>
<td>S (O.C)</td>
<td>2711</td>
<td>8930</td>
<td>5.9</td>
<td>0.93</td>
<td>124</td>
<td>0.96</td>
</tr>
<tr>
<td>Inoculum</td>
<td>1890</td>
<td>6268</td>
<td>5.7</td>
<td>0.93</td>
<td>95</td>
<td>0.97</td>
</tr>
</tbody>
</table>

“A” corresponds to anode and “S” corresponds to suspension

All five indices, that is, observed OTU, Chao 1, H, D and PD, demonstrated that the suspension samples have higher diversity than the anode samples among the four reactor types (i.e. -0.25 V, 0 V, 0.25 V and O.C). Good's coverage (90-94%) revealed that the 16S rRNA gene sequences identified in these samples represent the majority of bacterial diversity present in each sample. A Venn diagram showing the shared OTUs among the three anodes (–0.25, 0 and 0.25 V) was prepared by normalizing the sequence reads to 57,043, which was lowest number of sequence reads detected for the three anodes (Figure
Of the 9,400 total observed OTUs, only 580 OTUs (6.2%), 760 OTUs (8.1%), 932 OTUs (9.9%) and 819 OTUs (8.7%) were shared between the three anodes (−0.25, 0 and 0.25 V), between −0.25 and 0 V, between 0 and 0.25 V, and between 0.25 and −0.25 V (Figure 3.5). On the other hand, the unique OTUs for the −0.25, 0 and 0.25 V correspond to 27.1% (2545 OTUs), 26.5% (2491 OTUs) and 32.1% (3013 OTUs) of the total observed OTUs (Figure 3.5).

Figure 3.5. Venn diagram displaying shared and unique OTUs (3% distance cutoff) of the anode samples enriched at different SAPs.

The phylum level classification of the anodes enriched at different SAPs and O.C conditions showed the dominance of Proteobacteria by 82±5% and 41±3%, respectively (Figure 3.6A).
Figure 3.6. Relative abundance of the microbial communities that developed at (A) phylum and (B) class level for the different SAP (−0.25, 0, and 0.25 V vs. SHE) and O.C reactors. “A” and “S” correspond to the anode and suspension samples. Microbial communities representing less than 1% of the total sequence reads are classified as others.
Additionally, *Firmicutes, Synergistetes, Actinobacteria* and *Bacteroidetes* were detected in all the anodes of SAP-MECs, but to a lesser extent than *Proteobacteria* (Figure 3.6A). *Chloroflexi* and *Euryarchaeota* were present only in the anode of –0.25 V. In addition, *Euryarchaeota* was highly abundant in the anode of O.C (Figure 3.6A). Likewise, the suspension samples of all the reactors were dominated by *Proteobacteria, Firmicutes, Euryarchaeota* and *Synergistetes*, and to a lesser extent by *Chloroflexi, Actinobacteria* and *Bacteroidetes* (Figure 3.6A).

The class level classification of the anodes poised at different SAPs indicated the predominance of *Deltaproteobacteria* (78±5%) and it was significantly lower in the anode of O.C (33±2%) as well as in the suspension (32±6%) of all the samples (*p* < 0.05) (Figure 3.6B). All the anodes and suspension samples contained *Clostridia, Synergistia, Actinobacteria, Betaproteobacteria* and *Bacteroidia* (Figure 3.6B). *Anaerolineae* was present in all the samples (anode and suspension) except the anode of 0.25 V (Figure 3.6B). The methanogenic classes *Methanomicrobia* and *Methanobacteria* were only present in the anode of –0.25 V, but as a minor fraction of the total community. However, they were highly abundant in the anode of O.C and in all the suspension samples (Figure 3.6B).

At the genus level, all the anodes of SAP-MECs were dominated by *Geobacter* with sequences most similar to *Geobacter sulfurreducens* (99.5% similarity). Notably, *Geobacter* was highly abundant in the anode of 0 V (65±5%), followed by –0.25 V (59±3%) and significantly lower in the anode of 0.25 V (45±2.6%) (*P* < 0.05) (Figure 3.7A). The anode of O.C showed absence of *Geobacter*. Moreover, *Smithella* was abundant in the anode of –0.25, 0 V and O.C (8±1.4%) and it was remarkably more
abundant in the anode of 0.25 V (29±3%). *Syntrophobacter* with sequences most similar to *S. sulfatereducens* (99.4% similarity) was observed in all the anodes of SAP-MECs (6±2%) and it was significantly higher in the anode of O.C (27±5%) (Figure 3.7A). Additionally, *Methanobacterium* most similar to *M. formicicum* (99.5% similarity) was detected in the anode of –0.25 V (1.5±0.3%) and it was present at a higher abundance in the anode of O.C (15.4±2.5%). Other methanogens detected in the anode of O.C were *Methanosaeta* (15±2%) most similar to *M. concilii* (99.5% similarity) and *Methanospirillum* (2.5±0.6%), both belonging to the class *Methanomicrobia* (Figure 3.7A).
Figure 3.7. Relative abundance of the microbial communities at the genus level for the different SAP (−0.25, 0, and 0.25 V vs. SHE) and O.C reactors. (A) anode samples and (B) suspension samples. “A” and “S” correspond to the anode and suspension samples. Microbial communities representing less than 1% of the total sequences reads are classified as others.
The suspension samples of SAP-MECs and O.C reactors were highly diverse and dominated by a wide range of microorganisms (Figure 3.7B). *Syntrophobacter* was highly abundant in all the suspension samples (24±7%). Likewise, *Smithella* was abundant in all the suspensions of SAP-MECs (9±2%), but was present in lower abundance in the suspension of O.C (1.2±0.3%). Additionally, all the suspension samples contain high abundance of *Methanobacterium* (9.4±3.3%). *Methanosaeta* was present in low abundance (2±1%) in the suspensions of SAP-MECs, but was highly abundant (17±3%) in the suspension of O.C (Figure 3.7B).

The microbial composition of the inoculum at the phylum level was dominated by *Proteobacteria* (68%), *Firmicutes* (13.5%), *Chloroflexi* (6%), *Bacteroidetes* (6%), and *Euryarchaeota* (1%) (Figure 3.8). The dominant genera detected in the inoculum were *Pseudomonas, Gelria, Proteiniphilum, Longilinea, Azospira, Smithella, and Methanosaeta* (Figure 3.8).

**Figure 3.8.** Relative abundance of the microbial communities of the inoculum (A) at phylum and (B) genus level. Microbial communities representing less than 1% of the total sequence reads are classified as others.
The ratio of Archaea to Bacteria was significantly higher in the anode and suspension of O.C than the anode and suspension of SAP-MECs (Figure 3.9).

Figure 3.9. The ratio of Archaea to Bacteria in the anode and suspension samples of the different SAP (-0.25, 0 and 0.25 V) and open circuit (O.C) reactors. “A” and “S” correspond to the anode and suspension.

Also, the ratio was higher in the suspension than the anode samples of SAP-MECs. Non-metric multidimensional scaling (NMDS) showed that the anode and suspension communities of SAP-MECs differed substantially from the original inoculum source (Figure 3.10). Three clear and distinct clusters can be observed in the NMDS plot. The three clusters correspond to the anode communities in the SAP-MECs, the suspension communities in the SAP-MECs and the anode and suspension communities in the O.C reactors (Figure 3.10).
3.3.6. Standard Gibbs Free Energy Changes for Electricity and Methane Generating Reactions

Electricity generating reactions

$\Delta G^{\circ'}$ was calculated using the equation $\Delta G^{\circ'} = - nF(E_{\text{anode}} - E^{\circ'}_{\text{substrate}})$. Where $\Delta G^{\circ'}$ (kJ/mol) is the Gibbs free energy at standard biological conditions (i.e. reactant and products at 1 M or 1 atm, 298 K, and pH 7), $n$ is the number of electrons transferred, $F$ is the Faradays constant (96,485 C/mol e\textsuperscript{-}), and $E_{\text{anode}}$ (V) and $E^{\circ'}_{\text{substrate}}$ (V) are the anode potential and the standard biological redox potential of the substrate. $E^{\circ'}_{\text{substrate}}$ was calculated using equation $E^{\circ'}_{\text{substrate}} = - \Delta G^{\circ'}_{r} / nF$\textsuperscript{41} and values from Ref. \textsuperscript{42}.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>$\Delta G^{\circ'}$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-0.25 V)</td>
</tr>
<tr>
<td>$\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 9\text{H}^+ + 8\text{e}^-$</td>
<td>-21.6</td>
</tr>
</tbody>
</table>
H$_2$ \rightarrow 2H^+ + 2e^- \hspace{1cm} -31.5 \hspace{1cm} -79.7 \hspace{1cm} -128

HCOOH + H$_2$O \rightarrow HCO$_3^-$ + 2H$^+$ + 2e^- \hspace{1cm} -46.1 \hspace{1cm} -94.4 \hspace{1cm} -142.6

CH$_4$ generating reactions

Standard Gibbs free energy changes of CH$_4$ generating reactions were obtained from $^{43}$

$\Delta G^o'$ (kJ/mol)

CH$_3$COO$^-$ + 4H$_2$O \rightarrow HCO$_3^-$ + CH$_4$ \hspace{1cm} -31 kJ/mol

4H$_2$ + HCO$_3^-$ + H$^+$ \rightarrow CH$_4$ + 3H$_2$O \hspace{1cm} -136 kJ/mol

4 HCOOH + H$^+$ \rightarrow 3HCO$_3^-$ + 2H$_2$O + CH$_4$ \hspace{1cm} -130 kJ/mol

3.3.7. Theoretical Distribution of Electrons from Propionate Oxidation to Various Electron Sinks

Conversion of propionate concentration from mM to milli electron (me$^-$) equivalents

Conversion of propionate concentration from mM to me$^-$ equivalents =

Propionate concentration (mM)×volume of the reactor (Litre)×moles of electrons/mole of propionate.

For example 35.8 mM of propionate used in this study is equivalent to 20 me$^-$ equivalents, calculated as follows:

35.8 ×0.04 ×14 = 20 me$^-$ equivalents

Electrons utilized for biomass synthesis

The fraction of electrons from the donor substrate that is utilized for biomass synthesis is represented by $f_o^s$ $^{44}$. $f_o^s = 0.05$ for *G. sulfurreducens*, as representative of exoelectrogens $^{45,46}$; $f_o^s = 0.1$ for fermenters and $f_o^s = 0.08$ for
hydrogenotrophic methanogens. The electrons utilized for the different microbial groups was calculated by multiplying the substrate concentration in $me^{-}$ equivalent with the $f^0_s$ values of the corresponding organisms.

For example, the electrons utilized for propionate fermenters biomass (2) was calculated by multiplying 20 $me^{-}$ equivalent by the $f^0_s$ value (0.1) for propionate fermenters (Figure 3.11).

**Predicted electron distribution to various electron sinks (Figure 3.11)**

Two possible pathways of propionate oxidation could occur in the anode of MECs. Pathways 1 and 2 involve a microbial partnership between propionate fermenting bacteria and fermentation products (acetate, formate, and $H_2$) consumers (i.e. exoelectrogens and hydrogenotrophic methanogens). In pathways 1 and 2, propionate is oxidized to acetate and hydrogen (pathway 1) or formate (pathway 2). The acetate produced could then be oxidized by exoelectrogens to produce current. The hydrogen and formate will be utilized by hydrogenotrophic methanogens to produce methane or by exolectrogens to produce current.

**Pathway 1 (all values in bracket are presented as me$^{-}$ equivalents):**

Propionate (20) undergoes oxidation by propionate fermenters yielding biomass (2), $H_2$ (7.72) and acetate (10.28). $H_2$ (7.72) generated from propionate fermentation is utilized by hydrogenotrophic methanogens yielding biomass (0.6) and methane (7.12), or it could be oxidized by *G. sulfurreducens* yielding biomass (0.4) and current (7.32). Also, the acetate (10.28) generated from propionate fermentation is oxidized by *G. sulfurreducens* yielding biomass (0.6) and current (9.68). If hydrogenotrophic methanogens outcompete exoelectrogens for hydrogen then 36% of the electrons in propionate will be lost to
methane; and the maximum flow of electrons from propionate to current will be $9.68 \text{ me}^-$ equivalents (i.e. 48% of the electrons in propionate) from acetate oxidation alone.

Pathway 2 (all values in bracket are presented as $\text{me}^-$ equivalents):

Propionate (20) undergoes oxidation by propionate fermenters yielding biomass (2), formate (7.72), and acetate (10.28). Formate (7.72) generated from propionate fermentation is utilized by hydrogenotrophic methanogens yielding biomass (0.6) and methane (7.12), or it could be oxidized by *G. sulfurreducens* yielding biomass (0.4) and current (7.32). Also, Acetate (10.28) generated from propionate fermentation is oxidized by *G. sulfurreducens* yielding biomass (0.6) and current (9.68). If hydrogenotrophic methanogens outcompete exolectrogens for formate then 36% of the electrons in propionate will be lost to methane; and the maximum flow of electrons from propionate to current will be $9.68 \text{ me}^-$ equivalents (i.e. 48% of the electrons in propionate) from acetate oxidation alone.
Figure 3.11. Possible theoretical pathways of electron flow in the anode of MECs fed with propionate (20 me\textsuperscript{–} equivalents =36 mM). All values in bracket are presented as me\textsuperscript{–} equivalents. Blue represents fermenters, red represents methanogens and green represents exoelectrogens.

3.4. Discussion

The results gathered in this study show that SAPs influenced MEC performance as supported by differences in the electron distribution to various sinks (Table 3.2). Electrical current was the largest electron sink regardless of the tested SAPs (Table 3.2) and it was relatively higher in the positive SAPs (0 and 0.25 V) than negative SAP (−0.25 V). In general, at positive SAPs, exoelectrogens can gain more energy for growth and increase their metabolic rate\textsuperscript{47}. At elevated metabolic rates, more substrate is consumed by exoelectrogens for current production and less substrate is available for other competing reactions like methanogenesis (Table 3.2)\textsuperscript{47}. In addition, based on
thermodynamics alone (see Supporting Information), and under standard biochemical conditions (i.e., reactants and products at 1M or 1 atm, 298 K and pH 7), oxidation of acetate to electrical current is more favorable at the positive SAPs tested in this study (0 V: $\Delta G^{\circ'} = -214.6$ kJ/mol and 0.25 V: $\Delta G^{\circ'} = -407.6$ kJ/mol) than the negative SAP (–0.25 V: $\Delta G^{\circ'} = -21.6$ kJ/mol). Similarly, oxidation of H$_2$/formate to current is more favourable at positive SAPs (0 V: $\Delta G^{\circ'} = -79.7-94.4$ kJ/mol and 0.25 V: $\Delta G^{\circ'} = -128-142.6$ kJ/mol) than negative SAP (–0.25 V: $\Delta G^{\circ'} = -31.5-46.1$ kJ/mol). The electron distribution to electrical current at 0 (71%) and 0.25 V (56%) (Table 3.2) was higher than the theoretical electron distribution to current from acetate oxidation alone (48%) (see Supplementary Information), suggesting that both acetate and H$_2$/formate contributed to current generation at these SAPs. Furthermore, at 0.25 V, the current generation from intermediates (acetate: $\Delta G^{\circ'} = -407.6$ kJ/mol, H$_2$: $\Delta G^{\circ'} = -128$ kJ/mol, and formate: $\Delta G^{\circ'} = -142.6$ kJ/mol) is thermodynamically more favorable than 0 V (acetate: $\Delta G^{\circ'} = -407.6$ kJ/mol, H$_2$: $\Delta G^{\circ'} = -214.6$ kJ/mol, and formate: $\Delta G^{\circ'} = -94.4$ kJ/mol) (see Supporting Information). However, results showed that electrical current was a larger electron sink at 0 V than 0.25 V (Table 3.2). Perhaps the additional energy produced at 0.25 V could not be captured by exoelectrogens, and instead it was captured by other microorganisms, which resulted in significant losses to undefined sinks (~2.5 times higher than 0 V) and methane (Table 3.2) ($P < 0.05$).

Methane production occurred at all SAPs and it corresponded to the second largest electron sink (23-41%) (Table 3.2). Most MEC studies showed that methane generation was mainly due to hydrogenotrophic methanogens because acetate-oxidizing exoelectrogens (e.g. G. sulfurreducens) could outcompete acetoclastic methanogens for
acetate because of kinetic benefits. Acetoclastic methanogens were not detected at a SAP of 0 V, and were present at very low abundance (1.7±0.2 %) at a SAP of 0.25 V, suggesting that the methane generated was due to hydrogenotrophic methanogens (Figure 3.7). Interestingly, the MECs operated at SAPs of −0.25V exhibited a higher fraction of electrons distributed to CH$_4$ (41±2%) (Table 3.2) than the theoretical electron distribution to CH$_4$ via hydrogenotrophic methanogenesis (36%) (see Figure 3.11), suggesting that methane generation could have happened through hydrogenotrophic (H$_2$/formate pathways) and acetoclastic methanogenesis (acetate pathway). Indeed, both hydrogenotrophic (M. formicicum and Methanospirillum) and acetoclastic methanogens (M. concilli) were detected in the suspension of −0.25 V (Figure 3.7B). Also, based on thermodynamics alone (see Supplementary Information), and under standard biochemical conditions, oxidation of acetate to CH$_4$ ($\Delta G^\circ = -31$ kJ/mol) is preferable than the direct oxidation of acetate to electrical current ($\Delta G^\circ = -21.6$ kJ/mol). Hence, at more negative SAP (−0.25 V), acetate also likely contributed for CH$_4$ production. Similarly, at SAP of −0.25 V, oxidation of H$_2$/formate to CH$_4$ ($\Delta G^\circ = -136$ and −130 kJ/mol) is thermodynamically more favorable than oxidation of H$_2$/formate to electrical current ($\Delta G^\circ = -31.5$ and −46.1 kJ/mol). Therefore, at more negative SAP (−0.25 V), a major fraction of electrons were lost to CH$_4$ (Table 3.2). As expected, O.C reactors showed a higher fraction of electrons routed for CH$_4$ (73±4) via acetoclastic and hydrogenotrophic methanogens, which were both abundant in the anode and suspension (33±0.4%) (Figure 3.7).

The results revealed that SAPs in propionate-fed MECs affected the microbial community structure of the anode. The most dominant genera detected on the anode of all
three SAPs were Geobacter, Smithella and Syntrophobacter, but their relative abundance varied among the tested SAPs (Figure 3.7A). Also, more positive SAP (0.25 V) showed higher microbial diversity than 0 V and –0.25 V as indicated by higher observed OTUs, Chao 1 and PD (Table 3.3). In an acetate-fed MEC, Torres et al. observed the highest phylogenetic diversity at positive SAP (0.37 V vs. SHE) compared to other tested SAPs (–0.15, 0.09 and 0.02 V vs. SHE), which were mainly dominated by G. sulfurreducens (90%) \(^{11}\). It is likely that the additional energy gain produced at 0.25 V in the current study was not fully captured by Geobacter, and instead it was captured by other diverse microorganisms that interfered with Geobacter to make direct contact with the anode \(^{11}\). This explains why Geobacter was much lower in the anode set at 0.25 V (45%) compared to 0 V (65%) and –0.25 V (59%).

Geobacter (99.5% similarity to G. sulfurreducens) was the dominant genera detected in the anode of the three SAPs tested in this study (average percentage of 56±10%). Previous studies also revealed the dominance of G. sulfurreducens or different species/strains of Geobacter phylotypes at varying SAPs in MXCs fed with acetate \(^{1,6,12,14}\). According to previous studies, G. sulfurreducens can self-adjust their electron transfer pathways (ETP) to adapt to different SAP conditions \(^{1,50,51}\). Zacharood et al. showed that two different types of c-type cytochromes (ImcH and CbcL) were involved in ETP at lower and higher SAPs \(^{52}\). Based on nonturnover CVs, three oxidation peaks (P1, P2 and P3) were observed in the current study (Figures 3.4B, C and D). The redox couple at \(-0.2\) V (P2 position in Figure 3.4) has been observed in previous studies \(^{1}\), \(^{53,54}\), and it is similar to the midpoint potential of the redox couple expressed in G. sulfurreducens matured biofilms for turnover of electrons to the anode \(^{54}\). Also this
potential (-0.2 V) is close to the midpoint potential of periplasmic cytochrome C (PpcA, – 0.17 V), multiheme cytochrome OmcB (–0.19 V), and OmcZ (–0.22 V) 53–56. Peak P1 (-0.34 V) matches previously reported CV of *G. sulfurreducens* biofilm in the potential range of -0.32 V53 37.

Although *G. sulfurreducens* was detected in high abundance at the anodic biofilm, the profile of the voltammograms recorded in this study did not follow the typical shape of *G. sulfurreducens* voltammograms recorded in a previous MEC study using acetate as the fuel substrate and graphite fiber brush as the anode 32 41. Presumably the fermentable nature of the substrate used in this study, which requires syntrophic cooperation between fermenters and exoelectrogens for its degradation, affected the voltammogram measurements in this study. Other dominant genera in the anode were related to *Smithella* and *Syntrophobacter* (99.4% similarity to *S. sulfatereducens*). These bacteria act as the main functional groups responsible for syntrophic propionate degradation in methanogenic bioreactors fed with propionate as the sole carbon source 57–59. Their presence at the anode suggests a syntrophic interaction with *Geobacter* for propionate oxidation. *Smithella* and *Syntrophobacter* were also present at relatively high abundance in the suspension of SAPs along with hydrogenotrophic methanogens. Notably, the relative abundance of *Smithella* was three times higher in the anode of more positive SAP-MECs (0.25 V) than 0 and –0.25 V, and at the moment there is no explanation for this observation. (Figure 3.7A). Recently, a fermentative bacterium (*Thermoanaerobacter pseudethanolicus*) was shown to have the capability to simultaneously ferment sugars (xylose, glucose and cellobiose) and convert fermentation product (acetate) to current in MECs operated at fixed anode potential (0.042 V vs SHE) 60. Therefore, it is tempting to
test if other fermentative organisms like *Smithella* could utilize the anode through acetate during fermentation of propionate when the potential becomes favourable. Thus, future research is needed to provide a deeper insight of the role of *Smithella* at various SAP conditions in MECs fed with propionate.

In this study, complete degradation of propionate (Table 3.2) was observed in all the tested conditions (SAPs and O.C). No accumulation of its intermediates (acetate and formate/H₂) (<0.4 mM) was observed throughout the batch in all SAP-MECs (Figure 3.3). However, accumulation of acetate was noticed in the O.C reactors (Figure 3D). In SAP-MECs, both *Geobacter* and hydrogenotrophic methanogens consumed the intermediates generated by fermenters (*Smithella* and *Syntrophobacter*) (Figure 3.7), and kept their concentrations low resulting in more energetically favourable fermentation, and hence complete removal of propionate. In the O.C reactors, complete removal of propionate was facilitated through microbial partnerships between fermenters (*Smithella* and *Syntrophobacter*) and hydrogenotrophic and acetoclastic methanogens. In our earlier study, we demonstrated that propionate degradation in MECs occurred via a microbial partnership between fermenters and *Geobacter*, and low propionate removal (80% removal) was observed at 36 mM (similar to the concentration used in the current study) compared to complete removal at 4.5 mM. Methanogenesis was not an important electron sink in our earlier study at both propionate concentrations tested, whereas methanogenesis (via hydrogenotrophic methanogens) was an important sink in the current study. In our previous study, MEC anodes were initially enriched (30 days) in single chamber air-cathode MFCs, where oxygen intrusion through the cathode might have affected the growth of methanogens. Whereas in the current study, the reactors
were operated under MEC (anaerobic) mode from the start of the experiment, and this provided a suitable environment for the enrichment of hydrogenotrophic methanogens. Based on the aforementioned studies, it seems that the complete degradation of propionate at elevated concentrations in MECs requires microbial partnerships between fermenters, hydrogenotrophic methanogens and *Geobacter*. Whereas, at low propionate concentration microbial partnership between *Geobacter* and fermenters was sufficient to achieve complete removal of propionate in MEC.

### 3.5. Conclusion

This study is the first to report the effect of SAPs on propionate-fed MECs. The results showed that (i) SAPs affected the electron fluxes to various electron sinks, where current was a significant electrons sink followed by methane in all the SAPs tested; however, current was relatively higher in the positive SAPs (0 and 0.25 V). In contrast, methane was higher in the negative SAP (−0.25 V); and (ii) SAPs affected the anodic microbial community structure and diversity where higher microbial diversity was detected at SAP of 0.25 V than SAP of 0 and −0.25 V, and the relative abundance of the most dominant members (*Geobacter*, *Smithella* and *Syntrophobacter*) at the anode varied among the tested SAPs. A complete oxidation of propionate was observed with no accumulation of its intermediates in all SAP-MECs. Microbial community composition in the biofilm anode and suspension imply that the degradation of propionate in all the tested SAPs was facilitated by syntrophic interactions between fermenters (*Smithella* and *Syntrophobacter*) and *Geobacter* in the anode and fermenters (*Smithella* and *Syntrophobacter*) and methanogens (mainly hydrogenotrophic methanogens) in suspension.
3.6. References


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<table>
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<tr>
<td>60</td>
<td>Lusk, B. G. <em>et al.</em> Characterization of electrical current-generation capabilities from thermophilic bacterium Thermoanaerobacter pseudethanolicus using xylose,</td>
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**CHAPTER 4 Temporal Microbial Community Dynamics in Microbial Electrolysis Cells Fed with Low and High Concentration of Acetate and Propionate**

This chapter in preparation to peer reviewed Journal article:

Microbial electrolysis cells (MECs) are widely considered as a next generation wastewater treatment system; however, fundamental insights on its performance and the temporal dynamics of the associated microbial communities under different organic loadings is still unknown, but it is essential for optimizing this technology for real-scale applications. Here, the temporal dynamics of the anodic microbial communities associated to the performance of MECs was examined at low (0.5 g COD/L) and high (4 g COD/L) concentrations of acetate or propionate, as both of these substrates, are important intermediates in fermentation of municipal wastewaters and sludge. This study indicates that acetate-fed reactors exhibited more stable (reproducible) and greater performance in terms of current production, coulombic efficiency (CE) and substrate degradation rate than the propionate-fed reactors, irrespective of the concentrations tested. Also, acetate-fed reactors attained a nearly complete removal of acetate at both concentrations tested, whereas incomplete propionate removal (75±14%) was observed at high propionate concentration, compared to complete removal at lower concentration. Based on 16S rRNA gene sequencing, a relatively similar microbial community composition but with varying relative abundance was observed in all the reactors despite differences in the substrate and concentrations tested. In addition, the relative abundance
of *Geobacter*, a well-known exoelectrogen, in the anode biofilm of all MEC reactors was high throughout the experimental period. Collectively, these results imply that MECs are functionally stable systems regardless of the carbon source and concentration tested.

### 4.1 Introduction

Conventional anaerobic digestion (AD) is a useful technology for wastewater treatment and renewable energy generation\(^1\). However, the accumulation of volatile fatty acids (VFAs) such as acetate and propionate, main VFAs (24-70%) in municipal wastewater, is an important concern that leads to loss in methane production and process failure of anaerobic treatment systems like AD, anaerobic fluidized bioreactors (AFBRs) and anaerobic fluidized membrane bioreactors (AFMBRs)\(^2\). This issue can be addressed by developing new technologies such as microbial electrolysis cells (MECs). MECs have been widely accepted as an alternative for wastewater treatment technology and bioenergy generation from waste organics\(^4\). In MECs, certain microorganisms known as exoelectrogens convert chemical energy in organics to electricity via anaerobic oxidation of wastewater organics at the anode. The electrons and protons that are generated during oxidation at the anode are captured at the cathode for hydrogen evolution reaction through the addition of minimum voltage (0.6 V) to the circuit\(^5\).

One new and important application of MECs is the addition of electrodes directly into an anaerobic digester, in order to improve performance and increase the methane concentration in the product gas\(^6-9\). Such an integration with AD is not practical with MFC as AD requires operates under an oxygen free environment. So far, attempts to integrate MECs and AD have focused on engineering aspects, reactor design and material
optimization. However, microbial communities involved in the anode are a key component of MECs. Yet, only few studies have been reported on understanding the microbial ecology of MECs towards practical evaluations of the MEC as wastewater treatment technology\textsuperscript{7,10-13}. Furthermore, microbial studies on MECs have usually focused on a single sampling event (typically at the end of the MECs operation)\textsuperscript{7,11}. Single sampling event does not provide an understanding about the temporal dynamics of microbial communities and its correlation to system performances. Therefore, a deeper insights into the dynamics of microbial communities over time (multiple sampling events) and linking it with system performances is needed for practical integration of MEC with AD. In contrast, dynamic studies have been performed in MFC systems\textsuperscript{14 15-18} which are prone for oxygen intrusion to the anode that affects microbial community structure and its metabolic activity. Also, recent studies on integrated MEC-AD system operated with a wide range of carbon sources (VFAs, glucose, proteins, polysaccharides, sludge and its fermentation liquid) and temperature (10 to 40 °C) showed an improved reactor performance in terms of higher stability, enhanced sludge and carbon degradation rates, accelerated methane production rate, and enhanced methane yield\textsuperscript{3,6,7,10,12,19-21}. The presence of enriched exoelectrogens (\textit{Geobacter}, \textit{Ochrobactrum}, and \textit{Rhodoferax ferrireducens}) and hydrogenotrophic methanogens in the electrode enhances the performance of such integrated system in terms of organics degradation rate, methane and hydrogen production rate as stated earlier\textsuperscript{7,10}. The aforementioned studies suggest that presence of synergistic populations belonging to different functional guilds (exoelectrogens, hydrolytic and fermentative bacteria, and methanogens) is essential for the enhanced performance of MEC for practical applications, nevertheless, all these
studies were performed on single sampling event of microbial characterization. However, studies understanding the temporal dynamics of microbial communities in connection with the performance of MECs fed with various VFAs (fermentable and non-fermentable substances), which are typically present in the wastewater have not yet been performed. Importantly, if MEC needs to fulfill its potential as an alternative wastewater treatment process it should be adequately robust to treat those VFAs, which are present in fluctuating concentrations in wastewater. Therefore, the objective of our study was to examine the temporal dynamics of anodic and suspension microbial communities in MECs fed with low (0.5 g COD/L) or high concentration (4 g COD/L) of acetate or propionate. We have chosen these two different concentrations of VFAs (low and high) to mimic the low and high strength wastewater present in AD. To address the above, anodic and suspension microbial communities were sampled periodically during 70 days of batch operation and characterized by 16S rRNA gene sequencing. In addition, reactor performance in terms of current density, CE, and substrate removal rate was continuously monitored over time.

4.2 Materials and methods

4.2.1 Construction of MECs

Two chambered cube-shaped MECs (each chamber with a 20 mL working volume) were constructed as previously described. The two chambers were separated by an anion exchange membrane (5 cm²; AMI 7001, Membranes International, Glen Rock, NJ). A glass gas collection tube (15 mL) was attached to the top of both the anode and cathode chambers. Gasbags (0.1 L Cali-5•Bond. Calibrate, Inc.) were connected to the top of the glass gas collection tubes to collect more volume of gas. The anodes were graphite fiber
brushes (2.5 cm diameter × 2 cm long; PANEX 33 fibers, ZOLTEK Inc., St. Louis, MO, USA). The cathodes (projected surface area of 7 cm²) were made using carbon cloth (type B-1B, E-TEK) containing 0.5 mg/cm² of Pt on the side facing the anode, and four polytetrafluoroethylene diffusion layers on another side.

4.2.2 Enrichment and operation

All MEC anodes were initially enriched in single chambered air-cathode MFCs as previously described using anaerobic digester sludge (Manfouha Wastewater Treatment Plant, Riyadh, KSA) as inoculum. The growth medium (pH 8.9) consisted of bicarbonate buffer (80 mM), nutrients (6.71 g/L NaH₂CO₃, 0.31 g/L NH₄Cl, 0.05 g/L Na₂HPO₄, 0.03 NaH₂PO₄), Wolfe’s vitamin (10 mL/L) and trace mineral (10 mL/L) solutions. The medium was supplemented with two different concentrations (0.5 g COD/L or 4 g COD/L) of propionate or acetate as the energy source. The growth medium was boiled and then cooled to room temperature by sparging with N₂:CO₂ (80:20, vol/vol) gas mix for 30 min to remove any dissolved oxygen and was then autoclaved. The MFC anodes were transferred to individual MECs fed with either acetate or propionate after three cycles of reproducible voltage (500 mV, over a 1 KΩ external resistor).

A fixed voltage of 0.7 V was applied to the MECs using a power source (3645A, Array, Inc.). A total of eight MECs were operated in a parallel. Four MECs were fed only with acetate (referred to as A-reactors) and another four MECs were fed only with propionate (referred to as P-reactors). One set of duplicate MECs were operated with a low concentration of propionate (0.5 g COD/L, referred to as PL-reactors), a second set of duplicate MECs were operated with high propionate concentration (4 g COD/L,
referred to as PH-reactors). The third set of duplicate MECs were operated with a low concentration of acetate (0.5 g COD/L, referred to as AL-reactors) and a fourth set of duplicate MECs were operated with high acetate concentration (4 g COD/L, referred to as AH-reactors). All reactor types (i.e. PL, PH, AL, and AH) were operated in a fed-batch mode in a temperature controlled room (30º C). When the current dropped to below 0.3 mA (PL ~36 hours/cycle; PH ~4-5 days/cycle; AL ~26 hours/cycle; AH ~4-5 days/cycle), the reactors were refilled with fresh medium and sparged with nitrogen gas (99.999%). The same growth medium was used in the anodic and cathodic compartments; however, propionate and acetate were only added to the anode medium.

4.2.3 Analyzes and calculations

The current in the circuit was determined at 20 min intervals by measuring the voltage across a resistor (10 Ω) in the circuit using a data acquisition system (Model 2700; Keithley Instruments Inc.). The gas generated by the MECs was collected from both chambers in the head space and in a gas bag, and the total gas volume was measured by the gas bag method as previously described. Concentrations of H₂ and CH₄ were measured using a gas chromatograph (GC) (model 310; SRI Instruments) with a thermal conductivity detector (TCD), a 1.83 m molecular sieve packed 5A column and an argon carrier gas. CO₂ concentration was measured with a second GC (model 310; SRI Instruments) with a TCD, a 0.91 m silica gel column, and using helium as the carrier gas. The concentrations of propionate, formate, and acetate were analyzed by high-performance liquid chromatograph (HPLC) (Thermo Scientific, Accela, country) equipped with a photo-diode array (210 nm) and an ultraviolet detector. An Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) was used to separate the
VFAs. Sulfuric acid (5 mM) was used as the mobile phase at a flow rate of 650 µL/min, and the pressure was maintained at 9650 kPa. The total elution time was 30 min, and each sample was measured in triplicate, and the average concentrations were reported. The performance of the MECs was evaluated by the current density of the reactor, \( I (\text{A/m}^2) \); coulombic efficiency, \( \text{CE} (%) \); propionate and acetate removal (%) as previously described.

4.2.4 16S rRNA gene sequencing

Over the course of the experiments, samples for microbial community analysis were periodically collected at different time periods (PL/AL: 0, 10, 30, 50, 70 and 100 days; PH/ AH: 0, 15 or 20, 35, 70 and 100 days) from the anode and suspension of each reactor. Day 0 for the anode samples represents the MFC anode that was transferred to individual MECs after three cycles of reproducible voltage (500 mV, over a 1 KΩ external resistor). The anode samples were collected by cutting ~ 0.2 g (wet weight) of the anode fibers using flame sterilized scissors. Genomic DNA was extracted using the PowerSoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. The quality (A260/A280) and quantity (A260) of the extracted genomic DNA was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Triplicate PCR reactions were performed for each sample in a 25 µL reaction volume using the HotStarTaqPlus Master Mix (Qiagen, Valencia, CA), 0.5 µM of each primer, and 100-200 ng of template DNA. Bacterial and archaeal 16S rRNA genes were amplified using domain specific primer sets: 341F (5’-Lib-L/A-Key-Barcode-CA Linker-CCTACGGGNGGCWGCAG-3’) and 785R (5’-Lib-L/A-Key-TC Linker-
GACTACHVGGGTATCTAATCC-3’) for bacteria; and 519F (5’-Lib-L/A-Key-Barcode-CA Linker- CAGCMGCCGCGGTAA--3’) and 1041R (5’-Lib-L/A-Key-TC Linker-GGCCATGCACCWCCTCTC-3’) for Archaea. A unique 8–bp error–correcting barcode was used to tag each PCR product. PCR was performed using a C1000 Thermal Cycler (Bio- Rad, Hercules, CA). For bacteria, the PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 27 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min. For archaea, the PCR conditions were as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min.

The triplicate PCR products from each sample were pooled and then loaded on agarose gel and purified using the Qiaquick gel extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration of the PCR products was measured with a Qubit® 2.0 Fluorometer using the PicoGreen® dsDNA quantitation assay (Invitrogen, Carlsbad, CA). The purified barcoded amplicons from each sample were pooled in equimolar concentration and sequenced on the Roche 454 FLX Titanium genome sequencer (Roche, Indianapolis, IN) according to manufacturer's instructions.

The bacterial and archaeal 16S rRNA sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME v 1.9.0) pipeline. Raw reads were first demultiplexed, trimmed and filtered for quality. The minimum acceptable length was set to 200 bp. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the uclust algorithm. A representative sequence from each OTU was aligned using PyNAST and these were phylogenetically assigned to a
taxonomic identity using the RDP Naive Bayesian rRNA classifier at a confidence threshold of 80% \(^{32}\). Chimeric sequences were identified and removed from the aligned sequences using Chimera Slayer as implemented in QIIME. Rarified OTU tables were used to generate alpha and beta diversity metrics by normalizing to 1100 sequences per sample. Rarified OTU tables were used to generate alpha and beta diversity metrics by normalizing to the lowest sequence read (1100 sequences) between the samples. For alpha diversity measurements, both non-phylogenetic based metrics (observed OTUs, Shannon diversity index (H) and Chao 1 richness estimator) and phylogenetic based metric (phylogenetic diversity (PD_whole)) were calculated with QIIME Temporal variation of bacterial community was analyzed by nonmetric multidimensional scale (NMDS) using PRIMER 6 software (version 6.1.13) and PERMANOVA+ add-on (version 1.0.3). NMDS ordination was generated based on Bray-Curtis matrix (beta diversity) in QIIME. Phylogenetic diversity of abundant bacterial taxa was visualized in a heatmap using PRIMER 7 software.

4.3 Results

4.3.1 Performance of MECs at low and high concentrations of acetate and propionate

A-reactors showed a higher current density (4.25±0.23 A/m\(^2\)) than P-reactors (2.7±0.28 A/m\(^2\)) \((P= <0.05\), Student’s \(t\)-test for all comparisons) (Figure 4.1) with a short lag time of 1-5 days (Figure 4.1 A and B), whereas, P-reactors showed a delayed startup of 15–20 days to reach maximum current density (Figure 4.1 C and D). After 5 days of operation, the electrical current reached stable value of 4-4.5 A/m\(^2\) in A-reactors, whereas it was dynamic in the P-reactors (1.5-3 A/m\(^2\)) (Figure 4.1C and D). For instance, the maximum
current density increased from 1 A/m² on day 2 to 2.3 A/m² on day 10 in the PL-reactors followed by a decrease in the maximum current density of 1.5 A/m² on day 22. Then maximum current density of 3.3 A/m² was reached on day 23 and remained steady until day 35 of operation (Figure 4.1C). Furthermore, it relatively decreased to 2.2 A/m² in the next four days and eventually reached a stable electrical current of 2.5 A/m² until the termination of the experiment on day 70. The PH-reactors showed maximum current density of 2.5 A/m² by day 15 and showed a stable electrical current trend till the end of the experiment (Figure 4.1D). In addition, the reactors fed with low concentration of acetate or propionate produced a relatively higher current density (AL: 4.4±0.17 A/m²; PL: 2.4±0.7 A/m²) (Figure 4.1A and C) than reactors fed with high concentration of acetate or propionate (AH: 3.9±0.13 A/m²; PH: 2.2±0.15 A/m²) (Figure 4.1B and D). It should be noted that cutting a portion of the of anode fibers at each sampling event affected the performance of the reactors irrespective of the substrate and concentrations tested (Figure 4.1). However, A-reactors showed a faster recovery than the P-reactors in relation to attaining maximum reproducible electrical current generation (Figure 4.1).
Figure 4.1. Current density profile in duplicate MECs fed with low and high concentrations of acetate and propionate. (A) MECs fed with low acetate concentration (AL); (B) MECs fed with high acetate concentration (AH); (C) MECs fed with low propionate concentration (PL); (D) MECs fed with high propionate concentration (PH). Arrows indicate anode biofilm sampling for DNA extraction.

The average CE (%) for the whole period (i.e. 70 days) of operation of the MECs were: AL (99±11 %), AH (93±5 %), PL (73±9 %) and PH (63±10 %) reactors (Figure 4.2). The A-reactors yielded higher CE (96±8 %) (Figure 4.2 A and B) than P-reactors (68±9.5 %) (P= < 0.05) (Figure 4.2C and D). Particularly, the CE of few batches of A-reactors were > 100%. Furthermore, the CE in the A-reactors was more stable in comparison to the P-reactors (Figure 4.2). Irrespective of the substrate tested, low concentration reactors (AL/PL: 86±10 %) produced a relatively higher CE than the high concentration reactors (AH/PH: 78±7.5 %) (P= < 0.05) (Figure 4.2).
Substrate removal was nearly complete in A-reactors (98.8±1.2 %) with no significant difference between AL and AH-reactors ($P > 0.2$) (Figure 4.3 A and B), whereas, variable percentage of substrate removal was noticed in P-reactors (PL: 93± 8.6; PH: 75±14) (Figure 4.3 C and D). The substrate removal rates (g COD)/L/Day) were, for
AL (0.33±0.05), AH (0.90±0.14), PL (0.27±0.06) and PH (0.46±0.05) reactors. Also, the pH of the medium was 7.6±0.5 in A- and P-reactors at the end of fed-batch cycle.

Figure 4.3. Substrate removal trend in duplicate MECs fed with low and high concentrations of acetate and propionate. (A) MECs fed with low acetate concentration (AL); (B) MECs fed with high acetate concentration (AH); (C) MECs fed with low propionate concentration (PL); (D) MECs fed with high propionate high (PH). The values correspond to the average of the duplicate reactors.

As stated above, cutting a portion of the anode fibers at each sampling event affected the performance of the reactors irrespective of the substrate and concentrations tested. This explains the drop in propionate removal efficiency after cycles 23 (PL) and 4 (PH).
During the anode biofilm sampling (day 10, 20, 30, 50 and 70), the P-reactors showed a relatively stable performance in terms of current density (A/m²), coulombic efficiency (%), and propionate removal (%) (Figure 4.4).

![Figure 4.4](image)

**Figure 4.4.** Performance of P-reactors in terms of current density (A and D), coulombic efficiency (C and E), and propionate removal (E and F) during anode biofilm sampling; MECs fed with low propionate concentration (A, B, and C); MECs fed with high propionate concentration (D, E, and F).

### 4.3.2 Microbial community analysis

16S rRNA gene sequencing was used to characterize the bacterial and archaeal communities of anode and suspension samples from duplicate MEC reactors (AL, AH, PL, and PH). A total of 1,066,983 (bacteria) and 503,327 (archaea) high quality reads (average length of ~400 bp) were obtained after denoising, quality filtering and removal of chimeric sequences. For downstream analysis, OTUs with 97% sequence identity threshold were used.
4.3.2.1. Bacterial community diversity

For alpha diversity measures, we subsampled the dataset (normalized abundance values) to an even depth of 1100 sequences across the samples to remove inherent heterogeneity of sampling depth. This number was chosen, as it corresponds to the lowest number of sequence reads detected. The diversity values across the anode and suspension samples of A- and P-reactors ranged as follows: observed operational taxonomic units (OTUs: 74-1225), Chao 1 (216-3465), Shannon diversity index (H; 2.2-6.9), and phylogenetic diversity (PD; 12-79) (Tables 4.1 and 4.2). The bacterial diversity was higher in the anode and suspension of P-reactors than A-reactors based on observed OTUs, Chao 1, H, and PD (Table 4.1 and 4.2). Also, the high concentration-fed reactors (AH/PH) revealed a higher diversity than low concentration-fed reactors (AL/PL) (Table 4.1 and 4.2). Time series analysis of A-reactors indicated that bacterial diversity of anode and suspension samples was higher on day 0, and considerably reduced at the end of the experiment (i.e. day 70) (Table 4.1). Likewise, the P-reactors, particularly, PH-reactors showed a similar trend on day 0 (anode and suspension), and relatively decreased at the end of the experiment (Table 4.2). In contrast, PL-reactors (anode and suspension) revealed that the bacterial diversity was higher on day 0 and significantly reduced with time until day 50 ($P < 0.05$), followed by an increase in diversity on day 70 (Table 4.1).

Table 4.1. Measures of alpha diversity of bacterial phylotypes in acetate reactors

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<tr>
<th>Sample</th>
<th>Chao1</th>
<th>Observed Otus</th>
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<th>Shannon diversity index</th>
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<tr>
<td>A_AL_0</td>
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A anode, S suspension

Table 4.2. Measures of alpha diversity of bacterial phylotypes in propionate reactors

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<td>641±10</td>
<td>51±2</td>
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</tr>
<tr>
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<td>38±0.7</td>
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<td>44±0.05</td>
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<td>62±0.03</td>
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<td>625±15</td>
<td>41±1</td>
<td>4.42±0.02</td>
</tr>
</tbody>
</table>

A anode, S suspension
4.3.2.2. Bacterial community structure

Non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis revealed that all the samples (anode and suspension) had a gradual succession away from the initial conditions and a relatively similar pattern of succession was observed between low and high concentration-fed reactors (Figure 4.5). However, the development and succession paths of anodic bacterial communities were different between A- and P-reactors (Figure 4.5). For example, A-reactors showed that all the anodes (AL: 10, 30, 50 and 70 days; AH: 15, 35 and 70 days) were clustered together and distantly away from the initial anode samples (0 days) (Figure 4.5A and B). In contrast, P-reactors exhibited that the anode samples (PL: 10, 30 and 50; PH: 20 and 35 days) were clustered together and successive sampling of the anode at day 70 was a distance away from the other anode samples (Figure 4.5 C and D). Also, NMDS results showed that the bacterial community structure of the suspensions (A- and P- reactors) were more dynamic than the anode as can be seen by their wider distribution in the ordination plot (Figure 4.5).
Figure 4.5. Nonmetric multidimensional scaling plots based on Bray-Curtis distance for the (A) AL, (B) AH, (C) PL and (D) PH reactors. Each symbol represents a specific sampling day (AL/PL: 0, 10, 30, 50 and 70; AH/PH: 0.15, 20, 35, and 70). A, anode and S, suspension.

4.3.2.3. Bacterial community composition and dynamics

A heat map was generated to represent the various phylotypes (down to the lowest classification level possible) identified from the A- and P-reactors (Figure 4.6 and 4.7). Highly abundant phylotypes belonging to the different detected bacterial classes are discussed below:

*Deltaproteobacteria*

Four phylotypes belong to the class *Deltaproteobacteria* were relatively abundant in the A- and P-reactors (Figure 4.6 and 7). Among those *Geobacter* was highly dominant over time (10 to 70 days) in the anode of A- and P-reactors (Figure 4.6A and 4.7A). *Geobacter* was detected in very low fraction (< 1%) in the A-reactors at day 0. However, it became highly dominant (AL: 52±13%; AH: 49±4%) over time (10 to 70 days). The relative abundance of *Geobacter* in the anode of P-reactors was different between low and high concentration-fed reactors (Figure 4.7A). For example, the relative abundance of *Geobacter* at day 0 of PL-reactors was 1.4%, and it significantly increased to reach 70±3.4% between days 10 and 50 days followed by a significant decrease to 40±2.7% ($P < 0.05$) on day 70 (Figure 4.7A). In contrast, PH-reactors revealed that the relative abundance of *Geobacter* was dynamic over time (Figure 4.7A). For instance, the relative abundance of *Geobacter* was 12±1.7% on day 0, and increased to 49±2.3% on day 20 (Figure 4.7A). Following operation at day 50, the relative abundance of *Geobacter* was
drastically reduced to 23±2.4%. But it increased again to reach 42±3% on day 70. The suspensions of A- and P-reactors revealed that relative abundance of *Geobacter* was dynamic over time (3-45%) (Figure 4.6 B and 4.7 B). *Desulfovibrio* was relatively identified in all the samples of A- and P-reactors (1-14%) (Figure 4.6 and 4.7). *Pelobacteraceae* was relatively abundant in anode of A-reactors (1-23%) than P-reactors (2-11%). *Desulfuromonadales* was present during the early stage of A- and P-reactors (1-5%) (Figure 4.6 and 4.7).
Figure 4.6. Heat map distribution of bacterial phylotypes of A-reactors: anode of A-reactors (A) and suspension of A-reactors (B). A, anode and S, suspension.
Bacterial phyla representing less than 1% of the total sequences reads are classified as others.
**Figure 4.7.** Heat map distribution of bacterial phylotypes of P-reactors: anode of P-reactors (A) and suspension of P-reactors (B). A, anode and S, suspension. Bacterial phyla representing less than 1% of the total sequences reads are classified as others.

**Clostridia**

Seven phylotypes belonging to the class *Clostridia* were frequently observed in all the samples of A- and P-reactors (Figure 4.6 and 4.7). *Copothermobacter* was highly abundant in A-reactors than P-reactors (Figure 4.6 and 4.7). Specifically, it was abundant during earlier stages of reactor operation (between day 0 and 20) (A-reactors: 45±11%; P-reactors: 22±3.5%) and was significantly reduced during later stages of reactor operation (day 50 and 70) (A-MECs: 7.7±5%; P-MECs: 2.5±1.7%) ($P < 0.05$) (Figure 4.6 and 4.7). *Sedimentibacter* was relatively abundant over time in the P-reactors (1-14%) than A-reactors (2-7%). Particularly, it was more prevalent in the suspension than the anode of A- and P-reactors. *Sohengenia* was present in the anode of A-reactors over time (1-6%) and it was present only during the early stages of operation in the P-reactors (1-9%). *Syntrophomonas* was present only in the anode of PH-reactors (2%). *Desulfoспоромуса* was observed only in the suspension, specifically, it was more prevalent in the A-reactors (1-8%) than P-reactors (1-4%) (Figure 4.6 and 4.7).

**Synergistia**

Two different phylotypes (*Dethiosulfovibrionaceae* and *Synergistaceae*) belonging to the class *Synergistia* were consistently observed in the A- and P-reactors over time (Figure 4.6 and 4.7). Specifically, *Dethiosulfovibrionaceae* was dominant throughout the operation of the P-reactors (anode and suspension) than A-reactors (Figure 4.6 and 4.7).
However, *Synergistaceae* was found to be dominant in the suspension than the anode of A- and P-reactors (Figure 4.6 and 4.7).

**Betaproteobacteria**

Four different phylotypes belonging to the class *Betaproteobacteria* were observed in the samples of A- and P-reactors (Figure 4.6 and 4.7). *Dechloromonas* was considerably abundant in the suspension of P-reactors over time (2-41%). *Rhodocyclaceae* was relatively abundant (3-19%) in the suspension of AH-reactors over time (Figure 4.6 B).

**Gammaproteobacteria**

Three different phylotypes (*Pseudomonas*, *Pseudomonadaceae*, and *Shewanella*) belonging to the class *Gammaproteobacteria* were observed in the A- and P-reactors (Figure 4.6 and 4.7). Specifically, *Pseudomonas* and *Pseudomonadaceae* were relatively dominant over time in the A-reactors than the P-reactors ((Figure 4.6A and B). *Shewanella* was noticed as a minor fraction in the A- and P-reactors (Figure 4.6 and 4.7). However, it was more prevalent (29±2.5%) in the suspension of PL-reactors on day 70 (Figure 4.6 and 4.7B).

**Bacteroidia**

Three different phylotypes of the class *Bacteroidia* (*Dysgonomonas*, *Bacteroidales*, *Porphyromonadaceae*, and *Parabacteroidetes*) were noticed in the A- and P-reactors (Figure 4.6 and 4.7). *Porphyromonadaceae* and *Bacteroidales* were relatively more dominant in the P-reactors (7±4%) than the A-reactors (3±0.4%) (Figure 4.7), particularly it was more prevalent in the suspension than the anode of the P-reactors (Figure 4.7B). These results suggest that *Porphyromonadaceae* and *Bacteroidales* preferentially may
have contributed for propionate fermentation. *Parabacteroidetes* was present only in the suspension of A-reactors (Figure 4.6 B).

*Anaerolineae*

Only one phylotype of the class *Anaerolineae* namely *Anaerolinaceae* was observed in the A- and P-reactors. It was more abundant (3-16%) in the P-reactors than the A-reactors. Specifically, it was more abundant in the anode than the suspension of P-reactors (Figure 4.7).

4.3.2.4. **Archaeal community composition and dynamics**

Although methanogenesis was not an important electron sink in this study, archaeal 16S rRNA gene sequences of A- and P-reactors revealed the dominance of *Methanobactericeae* (74.5±13%) (hydrogenotrophic methanogens) in all the samples (anode and suspension) of A- and P-reactors (Figure 4.8). Particularly, the most abundant genera was *Methanobacterium* in all the samples of A-reactors (65±13%) (Figure 4.8A and B) and anode of P-reactors (57±15%) (Figure 4.8C). Whereas, *Methanobrevibacter* was more abundant in the suspension of P-reactors (61±12%) (Figure 4.8C and D).
Figure 4.8. Relative abundance of archaeal phylotypes for the anode of A-reactors (A), suspension of A-reactors (B), anode of P-reactors (C) and suspension of P-reactors (D). A, anode and S, suspension. Archaeal phylotypes representing less than 1% of the total sequences reads are classified as others. Samples A (AL-0 and 10) and S (AL-0 and 10) failed to amplify.

Temporal analysis of archaeal 16S rRNA gene sequences of A-reactors revealed the predominance of *Methanobacterium* (65±13 %) over time followed by *Methanobrevibacter* (10±6 %), *Thermoplasmata* (WCHD3-02) and *Crenarchaeota* (MCG) (excluding day 0 and 10 of AL-reactor samples which were failed to amplify) (Figure 4.8A and B). Also, A-reactors contain a minor fraction of unclassified *Methanobacteriaceae*, *Methanospirillum*, *Methanosarcina*, and *Methanoseta*. In contrast, temporal analysis of the archaeal community of P-reactors displayed that the anodes were dominated by the hydrogenotrophic methanogens, *Methanobacterium* (PL: 51±12%; PH: 64±18%) followed by acetoclastic methanogens, *Methanoseta* (PL: 10±6%; PH: 14±4 %) (Figure 8C). In addition, other sub-dominant communities were
observed namely unclassified *Methanobacteriaceae*, *Methanobrevibacter*, *Methanospirillum*, *Methanosarcina*, and *Thermoplasma* (WCHD3-02). *Methanobrevibacter* (PL: 64±15 %; PH: 60±14 %) was dominant in the suspension samples of P-reactors followed by *Methanobacterium* (PL: 12±8 %; PH: 25±15 %) (Figure 8 C and D).

### 4.4. Discussion

An examination of microbial community dynamics in MECs fed with fermentable or non-fermentable substrates with different concentrations showed that A-reactors revealed stable performance in terms of current density, CE, and substrate removal efficiency regardless of the concentrations tested (Figure 4.1, 4.2 and 4.3). Acetate can be directly utilized by exoelectrogens enhambling them to outcompete other acetate consuming organisms  resulting in a better performance than P-reactors. In addition, A-reactors produced CEs greater than 100% in some of the batches (Figure 4.2 A and B) possibly due to H$_2$ recycling (H$_2$ production either during fermentation or via cathodic H$_2$ evolution process that cross through membrane), as stated previously in other MEC studies 27,34. Also, CEs greater than 100% is possibly due to the oxidation of intracellular biopolymers such as polyhydroxyalkanoates 17,35 or utilization of stored energy in the cells 34. P-reactors showed a longer lag phase and relatively lower reactor performance than A-reactors (Figure 4.1, 4.2 and 4.3). Microbial partnership between exoelectrogens and fermenters are required for propionate degradation in the P-reactors 22,35. Also, loss of electrons to various other competing electron sinks (biomass synthesis and production of soluble microbial products (SMPs) might have occured as previously described 22,36-38.
In general, high concentration-fed reactors (AH/PH) exhibited a lower reactor performance in term of maximum current density and CE (Figure 4.1, 4.2). than the low concentration-fed reactors (AL/PL), which may be due to further losses of electrons to other competing electron sinks as described above (Hari et al. 2016; Sleutels et al. 2011) (Figure 4.2 B and D). In addition, all the reactors (AL, AH, and PL) showed a nearly complete removal of substrate (97±2 %) excluding PH-reactors, which showed 75±14% (Figure 4.3D) substrate removal. Methanogenesis was not an important electron sink in this study.

Anaerobic digester sludge at most domestic wastewater treatment plants, which was the original inoculum for this study, predominantly contains acetoclastic methanogens. Recently, the presence of hydrogenotrophic methanogens either in the inoculum or during MEC operation showed increase in VFAs (propionate, and acetate) removal efficiency, and enhanced reactor performance. Also, our experience from other propionate fed MEC studies (4g COD/L) inoculated with a different inoculum source (effluent from anaerobic membrane bioreactors operated with synthetic municipal wastewater medium containing starch, milk powder, and yeast) showed higher enrichment of hydrogenotrophic methanogens (1-11%) and complete removal of propionate (unpublished data). Therefore, this suggests that the presence of hydrogenotrophic methanogens in MECs is essential for the complete removal of propionate at higher concentrations. Interestingly, sampling of the anode biomass affected the performance of P-reactors (i.e. slower recovery of reproducible current generation) (Figure 4.1C) likely due to the loss of fermentative bacterial biomass. However, anode sampling of A-reactors did not affect the reproducible reactor
performance (Figure 4.1, 4.2 and 4.3). Similar to previous studies on acetate-fed MFCs\textsuperscript{18,39}. These results indicate that retaining the undisturbed biomass in the anode of P-reactors (PL/PH) is needed for stable and reproducible reactor performance.

A relatively similar anodic bacterial community structure was observed in the A-reactors over time, whereas P-reactors had a varying microbial community structure over time (Figure 4.5). The succession observed in the anode of A- and P-reactors (from \textit{Copothermobacter}, \textit{Anaerolinaceae} to \textit{Geobacter} (day 0 to 10) and eventual dominance of \textit{Geobacter} over time (day 10 to 70) (Figure 4.5, 4.6 and 4.7) suggest that the selective pressure imposed by MEC operation not only influenced the dominance of \textit{Geobacter} but also decreased the bacterial diversity over time (10-70 days) (Table 4.1 and 4.2), which are in line with previous MET studies\textsuperscript{40,41}. The relatively stable current density observed in this study (except PL-reactors) and the abundance of \textit{Geobacter} on the anode are consistent with previous MET studies with acetate and propionate, as \textit{Geobacter} is essential for achieving high electrochemical performance\textsuperscript{18,36,41,42}. Recently, Ishii et al. 2014\textsuperscript{36} showed the dominance of many strains and species of \textit{Geobacter} in MFCs fed with different substrates (sucrose, acetate, and a mixture of butyrate and propionate). They suggested that different phylotypes of \textit{Geobacter} can play a role in the oxidation of more complex substrates together with fermenting organisms. This signifies the robustness of MXC systems by exhibiting higher abundance of \textit{Geobacter} regardless of the substrates tested, and this likely may result in stable performance under real scale wastewater treatment.

At the end of MECs operation (70 days), all reactors (except PH) showed a decrease in the abundance of \textit{Geobacter} accompanied by an increase in the abundance of diverse
phytotypes (Clostridia, Synergistia, Anaerolineae and Gammaproteobacteria) (Figure 4.6A and 4.7A), however, the current density remained stable (Figure 4.1). These results suggest that functional stability was maintained despite changes in community structure. Previous studies reported that the microbial communities in MFCs are flexible and are able to self-select and self-optimize to maintain functional stability\textsuperscript{15,17}.

In contrast to AL results, PL-reactors showed a dynamic trend in terms of current density over a time (Figure 4.1C). After sampling of an anodic biofilm, a dynamic trend of current density was seen (Figure 4.1C). However, the current density again reached to a steady state after a few batches (Figure 4.1C). These results suggest that anode sampling might influenced the cooperation between microbial partners which affected propionate oxidation. Such a cooperation between fermenters and exoelectrogens likely needs a longer duration for a stable reactor performance. It is of interest that a relatively stable performance was observed during the sampling period in PL-reactors (i.e. day 10, 30, 50 and 70) (Figure 4.1) perhaps due to the presence of a relatively stable microbial community structure (except day 70). These findings suggest that an undisturbed anodic biofilm is required to maintain a stable operation of the reactors fed with propionate.

In the case of the PH-reactors, a relatively stable trend in the maximum current density with each cycle was observed with varying microbial community structure over a time (day 20-70). The dominance of diverse bacterial communities such as Geobacter, Clostridia, Synergistia, and Anaerolineae was observed in PH-reactors (Figure 4.7A). The members of these phylotypes are well-known fermenters, exoelectrogens and secondary metabolites (acetate, formate, and \( \text{H}_2 \)) consumers\textsuperscript{41,43-47}. During the end of operation of both P-reactors (PL: day 70; and PH: day 50), a decrease in the abundance of
*Geobacter* accompanied by an increase in the abundance of *Syneristia* and *Anaerolineae* was observed, however the current density remained unchanged (Figure 4.1 C and D). This result suggests the presence of functional redundancy within the anodic microbial communities, and which likely resulted in a stable reactor performance. An earlier study on pilot scale MEC treating domestic wastewater showed the dominance of the hydrolytic microorganism *Syneristia (Dethiosulfovibrionaceae)*, which likely resulted in more positive impact on the reactor performance. *Anaerolineae* was found as a predominant group in the electrode of integrated MEC-AD system and was also detected as a dominant organism in the anode of MFCs fed with the root exudates of rice field soil, suggesting that it likely played a role as an exoelectrogen and/or fermenter; however, its role in this study is unclear.

The bacterial community structure in the suspension of A and P-reactors were highly dynamic and diverse as revealed by NMDS and bacterial diversity measures (Figure 4.5, Table 4.1 and 4.2). In particular, P-reactors showed a higher phylogenetic diversity than the A-reactors (Table 4.1 and 4.2), which likely due to the fermentable nature of propionate that requires fermenters and secondary metabolite consumers. However, acetate can be directly utilized by exoelectrogens, resulting in a lower phylogenetic diversity in the A-reactors. The suspension of P-reactors were dominated by diverse phylotypes belonging to *Betaproteobacteria, Deltaproteobacteria, Clostridia, Syneristia*, and *Anaerolineae* (Figure 4.6B and 4.7B). Members of these classes were reported to be propionate oxidizers and were abundant in propionate and acetate fed MECs, propionate enriched soils, and anaerobic digester sludge. The sub-class of *Betaproteobacteria, Dechloromonas* and *Thaurea* were abundant in the suspension of
A and P-reactors (Figure 4.6B and 4.7B). These are mainly denitrifiers and previously been detected in the anode and cathode of MFCs and \textit{Thauera} was reported to utilize propionate and acetate.

During the startup of A and P-reactors, the dominance of \textit{Coprothermobacter} was seen, however it was significantly reduced over time (Figure 4.6 and 4.7). \textit{Coprothermobacter} has been observed in thermophilic MFCs fed with acetate suggesting that their presence was likely to consume diverse electron donors rather than current production. Between day 0 and 70, the A- and P-reactors exhibited high abundance of \textit{Desulfovibrio}, and \textit{Pseudomonadaceae}. It has been reported that members of \textit{Desulfovibrio} can play a role in electric current generation in MFCs. Also, members of \textit{Desulfovibrio} are known as H$_2$ scavengers. \textit{Pseudomonadaceae} likely functioned as exoelectrogens as they are known to produce current by forming electroactive biofilm and also produce electron shuttles like phenazines and flavins in the anode.

### 4.5. Conclusions

Our findings indicated that MECs performance are affected based on the carbon source (acetate and propionate) and concentrations (0.5g COD/L and 4g COD/L) tested. \textit{Geobacter} was the dominant genus in the anode of all the tested conditions. In particular, A-reactors exhibited a greater and stable reactor performance (i.e. current production, CE, and substrate removal) and less dynamics of microbial community than the P-reactors. P-reactors showed a dynamic performance (i.e. current production, CE, and substrate removal), that was likely due to the fermentable nature of substrate and the incremental anode biofilm sampling that might caused a disturbance to the syntrophic
populations involved in propionate degradation. These P-reactors also exhibited a diverse and varying microbial community structure over a time. Regardless of two different substrates tested, a reduction in the reactor performance was observed at high substrate concentration. The fundamental insights obtained in this study on the dynamics of microbial communities in MECs and linking it to reactor performance is essential for successful integration of MEC with AD system for the treatment of VFAs.

4.6. References


**CHAPTER 5 Summary and Conclusions**

The work presented in this dissertation focuses on fundamental insights into the propionate oxidation in the anode of MECs. Acquiring such fundamental knowledge could aid in improving MECs as an efficient wastewater treatment process either as an independent systems or integrated systems. To address this, several studies were conducted to evaluate 1) the paths of electron flow in the anode of propionate fed MECs during propionate oxidation 2) the effect of different set anode potentials on the performance, electron fluxes and microbial communities of propionate fed MECs (SAPs) (–0.25, 0 and 0.25 V vs. standard hydrogen electrode, SHE) and 3) temporal dynamics of microbial communities in MECs fed with fermentable (low (0.5 g COD/L) or high concentration (4 g COD/L) of propionate) and non-fermentable substrates (low (0.5 g COD/L) or high concentration (4 g COD/L) of acetate).

Propionate is a model fermentable substrate to study microbial partnerships in natural and engineered methanogenic systems and an important intermediate in the anaerobic decomposition of organic matter in AD processes. Accumulation of propionate (>20 to 37 mM) at high organic loading rates is detrimental to anaerobic digestion processes.¹ ² ³ Thus, propionate removal is necessary for the stable operation of
anaerobic digestion systems. In such systems, acetogenic bacteria oxidize propionate to acetate and \(\text{H}_2\) (or formate), which are then utilized by acetoclastic (acetate) and hydrogenotrophic methanogens (formate and \(\text{H}_2\)) to generate \(\text{CH}_4\) and \(\text{CO}_2\) \(^4\). In the anode of MECs, little is known about the paths of electron flow during propionate degradation. The work presented in the chapter 2 determined the paths of electron flow in the anode of MECs fed with low and high concentrations of propionate.

The results showed that multiple paths of electron flow to electrical current could occur simultaneously during propionate oxidation in the anode of MECs despite of the tested propionate concentrations. At both concentrations tested, current was a major sink of electrons, whereas \(\text{CH}_4\) was a minor sink of electrons. In methanogenic systems, processing of substrates through multiple routes in parallel is essential for maintaining functional stability in response to a perturbation \(^5,6\). In a similar fashion, we hypothesized that multiple paths of electron flow from the substrate in the anode of MECs should result in a higher functional stability of the system. However, this should be tested in the anode of MECs in future studies. In general, accumulation of high concentrations of propionate (>20 to 37 mM) is detrimental to anaerobic digestion processes \(^1-3\). Regardless of the high concentration (36 mM) of propionate used in this study, the removal of propionate was still high (78 %) in the PH-MEC reactors. Therefore, the anode of MECs could potentially be integrated with existing AD processes to improve propionate degradation. In addition, further studies are needed to elucidate the conditions that favor multiple paths of electron flow and the ones that favor the dominance of one pathway over another.
In MECs, setting the anode potentials can impact the electrochemical performance, microbial community structure, and theoretical maximum energy gain ($\Delta G_0'$) by exoelectrogens for its growth and maintenance \(^7\). Such studies have been examined using non-fermentable substrates in MECs including acetate, however the effect of these SAPs on fermentable substrates is still unknown, for instance, propionate. One new and important application of MECs is the addition of electrodes directly into an AD, in order to improve performance and increase the methane concentration in the product gas. However, the impact of a set anode potential (SAP) on MEC performance has not been well examined for such environments where there can be high concentrations of fermentable substrates, especially for the case of propionate which is slowly degraded, as most studies on SAPs in MECs have mainly focused on acetate. Therefore, the work presented in the chapter 3 examined the effect of three different SAPs (–0.25, 0 and 0.25 V vs. standard hydrogen electrode, SHE) MEC performance using propionate (36 mM) in terms of its degradation rate, electron fluxes to various sinks (current, CH4 and undefined sinks), and microbial community structure. It was shown that: (i) SAPs affected the electron fluxes to various electron sinks, where current was a significant electrons sink followed by methane in all the SAPs tested; however, current was relatively higher in the positive SAPs (0 and 0.25 V). In contrast, methane was higher in the negative SAP (–0.25 V); and (ii) SAPs affected the anodic microbial community structure and diversity where higher microbial diversity was detected at SAP of 0.25 V than SAP of 0 and –0.25 V, and the relative abundance of the most dominant members (Geobacter, Smithella and Syntrophobacter) at the anode varied among the tested SAPs. Microbial community structure in the biofilm anode and suspension imply that the degradation of propionate in
all the tested SAPs was facilitated by syntrophic interaction between fermenters (*Smithella* and *Syntrophobacter*) and *Geobacter* at the anode and fermenters (*Smithella* and *Syntrophobacter*) and methanogens (mainly hydrogeonotrophic methanogens) in suspension.

MECs has been proved as an efficient technology to rectify the technical issues oriented with AD systems such as VFAs accumulations. So far, many attempts were made to integrate MECs and AD with the focus on engineering aspects, reactor design and materials optimization. However, microbial communities involved in the anode are a key component of MECs and these were not studied in detail. Yet, only few studies have been reported on understanding the microbial ecology of MECs towards practical evaluations as a wastewater treatment technology. Also, such microbial studies on MECs focused on a single sampling event (typically at the end of the MECs operation). Single sampling event does not provide a clear understanding about the temporal dynamics of microbial communities and its related system performances. Therefore, a deeper insights into the dynamics of microbial communities over a time (multiple sampling events) in relation to the system performance are needed for a practical application of wastewater treatment. The work presented in chapter 4 examined the temporal dynamics of microbial communities in MECs fed with low (0.5 g COD/L) or high concentration (4 g COD/L) of acetate or propionate. This is the first study investigating the temporal microbial community dynamics in MECs fed with different concentrations of fermentable and non-fermentable substrates, which are usually present in real wastewater. Our findings indicated that MECs are functionally stable systems regardless of the carbon source (acetate and propionate) and concentrations (0.5g COD/L and 4g COD/L) tested. Also,
Geobacter was the dominant genus in the anode of all the tested conditions. In particular, A-reactors exhibited a greater and stable reactor performance (i.e. current production, CE, and substrate removal) and less dynamics of microbial community than the P-reactors. Whereas, P-reactors showed a dynamic performance (i.e. current production, CE and substrate removal) which likely due to the fermentable nature of substrate and the incremental anode biofilm sampling that might cause a disturbance to the syntrophic populations involved in propionate degradation. These P-reactors also exhibited a diverse and varying microbial community structure over a time. Despite of the type of substrate tested, a reduction in the reactor performance was observed at high substrate concentration. These fundamental insights obtained in this study on the dynamics of microbial communities in MECs and linking it to reactor performance is essential for successful integration of MEC with AD system for the treatment of VFAs.

I believe that outcome of this dissertation provides fundamental knowledge of propionate oxidation in the anode of MECs. Such knowledge likely helps in improving MECs as a robust and efficient anaerobic wastewater treatment system to recover energy and to treat high strength wastewater. However, further studies in a pilot scale that evaluate the performance of MECs with actual wastewater contain higher organic concentration are needed to further develop this technology toward implementation. Also, MECs could be integrated with AD system for enhancing the energy recovery while maintaining the process stability of the systems under propionate accumulated conditions still needs to be investigated. Such integrated systems have the potential to become an energy positive, sustainable wastewater treatment system in near future.
5.1. References


10. Feng, Y., Liu, Y. & Zhang, Y. Enhancement of sludge decomposition and hydrogen production from waste activated sludge in a microbial electrolysis cell with cheap
