Building Microbial Communities and Managing Fermentation

In Microbial Electrolysis Cells

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved June 2015 by the
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ARIZONA STATE UNIVERSITY

August 2015

ABSTRACT

Microbial electrochemical cells (MXCs) offer an alternative to methane production in anaerobic water treatment and the recapture of energy in waste waters. MXCs use anode respiring bacteria (ARB) to oxidize organic compounds and generate electrical current. In both anaerobic digestion and MXCs, an anaerobic food web connects the metabolisms of different microorganisms, using hydrolysis, fermentation and either methanogenesis or anode respiration to break down organic compounds, convert them to acetate and hydrogen, and then convert those intermediates into either methane or current. In this dissertation, understanding and managing the interactions among fermenters, methanogens, and ARB were critical to making developments in MXCs. Deep sequencing technologies were used in order to identify key community members, understand their role in the community, and identify selective pressures that drove the structure of microbial communities. This work goes from developing ARB communities by finding and using the best partners to managing ARB communities with undesirable partners. First, the foundation of MXCs, namely the ARB they rely on, was expanded by identifying novel ARB, the genus *Geoalkalibacter*, and demonstrating the presence of ARB in 7 out of 13 different environmental samples. Second, a new microbial community which converted butyrate to electricity at ~70% Coulombic efficiency was assembled and demonstrated that mixed communities can be used to assemble efficient ARB communities. Third, varying the concentrations of sugars and ethanol fed to methanogenic communities showed how increasing ED concentration drove decreases in methane production and increases in both fatty acids and the propionate producing genera *Bacteroides* and *Clostridium*. Finally, methanogenic batch cultures, fed glucose and sucrose, and exposed to 0.15 – 6 g $N\text{-}NH_4^+$ L^{-1} showed that increased NH_4^+ inhibited methane production, drove fatty acid and lactate production, and enriched Lactobacillales (up to 40% abundance) above 4 g $N\text{-}NH_4^+$ L^{-1}. Further, 4 g $N\text{-}NH_4^+$ L^{-1} improved Coulombic efficiencies in MXCs fed with glucose and sucrose, and showed that MXC communities, especially the biofilm, are more resilient to high NH_4^+ than comparable methanogenic communities. These developments offer new opportunities for MXC applications, guidance for efficient operation of MXCs, and insights into fermentative microbial communities.

DEDICATION

For my family, I owe you more than I can ever express or repay. My mother, my dad, and my father, separately and together, raised me to be curious, to believe in myself, and to persevere in difficult times. Your support and unconditional love has made all the difference. My sister Christina, who is always there for me and has always shared her wisdom with me (being four years older). My brother, Adolfo, and sisters, Nicole and Daniella, who help me laugh and put things in perspective. My incredible huge step-family, who I love and would not trade for the world. My parents-in-law, who love me like a son, and my brother-in-law, who is just as into comics and video games as I am.

For my wife, Allison Kenney Haynes Miceli. You moved across the country to be with me, stood by my side in good times and held me up in bad times. You are my best friend and better half; I don't even know who I would be without you. This is as much yours as mine.

ACKNOWLEDGEMENTS

Simultaneously, I must thank Dr. Rosa Krajmalnik-Brown and Dr. César Torres for their constant support and guidance in my Ph.D. studies. Unfortunately, someone's name must always go first when I list my two co-advisors, and so I had to place one of their names first here as well. Dr. Rosy wins the honor, as she spent an entire semester asking me if I had decided to join her and Dr. César to work on MXCs. When I agreed to work with them as my co-advisors, I was very worried about having the dreaded "two bosses." This worry has never come to pass, thankfully, and I have nothing but great things to say about how my two co-advisors have worked together in my time as a Ph.D. student with them. Each of them has taught me many things and encouraged me through the years. I can only thank you both for all of the hard work you have put in, the effort you've made in being available when I need your help, and the encouragement which helped me push through the ups and downs of research.

I thank Dr. Bruce Rittmann for his lessons over the years, in committee meetings, group meetings, and especially his class on Environmental Biotechnology. I greatly appreciate the insightful questions Dr. Rittmann brings to my research and I am very grateful for the support he provided during my studies which enabled me to focus on my research. I hope that I have learned to "think like the microbes" in my time at ASU.

I also wish to thank my friends in EB (or SCEB as it is now), who have helped me along the way: Dr. Prathap Parameswaran, who knows where everything is and how every lab protocol is done and managed to get work done despite everyone needing him so badly we may have placed a locator beacon on him. Dr. Sudeep Popat, who has given me guidance at the bench and sat through many discussions over coffee, helping me with all the worries a grad student has. Dr. Anca Delgado, thank you for helping me get oriented in the lab and to not worry so much when things go wrong. Finally, thanks to Dr. Jon Badalamenti, who convinced me that Dr. Rosy and Dr. César would be able to work together and not make my life a nightmare trying to answer to two advisors. The four of you helped me grow as a scientist and kept me sane while doing so.

Thank you to two of my students, who I had the great pleasure to mentor: Jonah Rucker and Suyana Lozada. Jonah helped me learn how to be a better mentor, and to never let my

students give up on looking for a chemical in the cabinet. Suyana took countless samples over weekends, showing great dedication and enthusiasm for our research.

My work received generous support from Science Foundation Arizona, the Swette Center for Environmental Biotechnology at Arizona State University, the Institute for Maximizing Student Development at Arizona State University (NIH Award R25GM099650), the National Science Foundation (CAREER Award 1053939), and the Office of Naval Research (Grant N00014-10-M-0231).

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LIST OF ABBREVIATIONS

ADS	Anaerobic digested sludge
ARB	Anode-respiring bacteria
BLAST	Basic localized alignment search tool
CCA	Canonical Correspondence Analysis
CE	Coulombic efficiency
CV	Cyclic voltammetry
EET	Extracellular electron transfer
GC	Gas chromatography
HPLC	High pressure liquid chromatography
HRT	Hydraulic retention time
LAB	Lactic acid bacteria
MEC	Microbial electrolysis cell
MHV	Marine hydrothermal vent
MFC	Microbial fuel cell
MXC	Microbial electrochemical cell
NH_4^+	Ammonium
OTU	Operational Taxonomic Unit
PC	Principal Component
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PICRSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
qPCR	Quantitative real-time polymerase chain reaction
SRB	Sulfate reducing bacteria
VSS	Volatile suspended solids

CHAPTER 1

INTRODUCTION

1.1 Limitations to Sustainable Development

Water and energy are two pillars of modern society and they are highly interdependent on each other (Palaniappan et al. 2010). Fresh water is necessary for daily survival and energy extraction, while access to clean water reduces the spread of preventable diseases. Energy enables the manufacturing and movement of goods, including clean water, and the use of modern tools required to grow economically. Both are needed for the intensive agricultural that is required in order to feed the world's growing population. Despite all this, access to clean water and energy continue to be major challenges for many in the world, the UN's Conference on Sustainable Development identifying them as two of the seven critical problems facing the world and society's sustainable development (UN, 2012, 2011).

Due to growing populations, the availability of fresh, clean water remains insufficient to meet the world's needs (USAID, 2013; WHO and UNICEF, 2014, 2013; WWAP, 2014) and demand for water is predicted to increase by 55% worldwide between 2000 and 2050 (OECD, 2012). Human activities such as agriculture, industry, and disposal of human waste introduce organics, nutrients, and hazardous chemicals into freshwater sources (Palaniappan et al. 2010). All too often, these contaminated waters are returned to the environment without treatment, especially in developing countries. The input of additional nutrients into rivers and bays can cause excessive growth of vascular plants and algae which make the water anaerobic, killing off fish and other aerobic organisms (WWAP, 2009). Human waste, whether through open defecation or insufficient treatment, can introduce pathogenic organisms into water supplies which can affect both local and downstream communities (Carr and Neary, 2008; UNEP GEMS, 2007; WHO and UNICEF, 2008). Toxic contaminants can poison wildlife and people, both directly and through bioaccumulation (Carr and Neary, 2008; Palaniappan et al., 2010). While technology exists to desalinate abundant salt waters, this process does not prevent the ecological and health impacts of releasing untreated water, in addition to being expensive and energy intensive (Ghaffour et al. 2013). We must therefore treat these waste waters to remove the

contaminants prior to release in order to preserve the environment at large and fresh water sources in particular.

Although worldwide concern for depleting fossil fuel stores continues to increase (Owen et al. 2010; Aleklett et al. 2010; Höök et al. 2009), the effects resulting from the use of fossil fuels are more pressing (SDSN and IDDRI, 2014). Global energy use reached 12,700 million tons of oil equivalents (or 17.4 TW on average) in 2013, with fossil fuels (oil, coal, and natural gas) accounting for 86.7% of it (BP, 2014). Utilization of currently available stores of fossil fuels will produce more CO_2 than many predictions indicate can be released without terrible consequences for the environment (SDSN and IDDRI, 2014). Observations in multiple locations report CO_2 concentrations in the atmosphere above 400 ppm (WMO, 2014), a symbolic mark, but also a signpost of the great likelihood that temperatures will increase beyond 2 ℃ on average worldwide (SDSN and IDDRI, 2014). If the world is to stay below the 4℃ increase generally considered to be catastrophic, changes must be made and new innovations fostered. Two paths of research must be pursued in order to address this concern. First, we must continue to increasingly leverage renewable energy sources and decrease our reliance on volatile, dwindling energy sources. Second, we must improve the efficiency of current processes and practices in order to maximize the use of energy sources we already utilize. As scientist/engineers, we can work on possible solutions that address these paths.

1.2 Wastewater Treatment

Wastewater treatment lies at one of the intersecting points of these two issues. Biological waste water treatment uses bacteria to break down undesirable compounds in waste streams and converts them into less harmful substances. Treatment technologies include both aerobic and anaerobic processes. Aerobic treatment requires the pumping of oxygen into the system, a process which accounts for up to 50% of the energy cost of aerobic treatment (US EPA, 2008), and the disposal of large amounts of biomass generated, called biosolids (Low and Chase, 1999; Wei et al., 2003). Anaerobic treatment avoids the need to pump oxygen into the system, and results in less biomass production (Pfeffer et al. 1967). Often, these two are used in concert to offset their individual downsides. During anaerobic digestion, the main form of

anaerobic waste water and biosolids treatment, methane is the major chemical product. Although methane can be used productively as a source of heat or fuel for a generator, this is not often pursued with only 1% of daily flow in the US going through digesters with gas utilization as of 2000 (US EPA, 2008). Instead, the methane produced is flared and converted to CO_2. Methane is a greenhouse gas, currently estimated to be twenty-eight times more potent than CO_2 (Myhre et al., 2013); therefore, its release into the atmosphere is highly regulated.

Microbial electrochemical cells (MXCs) are a developing form of anaerobic treatment that may hold part of the answer to the water and energy problems facing our society. MXCs utilize bacteria as catalysts for converting between chemical and electrical forms of energy (Lovley, 2008). These organisms are called anode respiring bacteria (ARB), due to their use of an anode at the end of their respiratory chain (Torres et al., 2009). By using waste and polluted waters, we can produce electricity (or energy bearing chemical compounds), decrease the required energy input for treatment of these waters, and produce clean water (Oh et al., 2010).

Applications in wastewater treatment and bioremediation often involve fairly complex organic substrates, or electron donors (ED) (Erable et al., 2010). The known ARB capable of highly efficient anode respiration typically consume relatively simple EDs such as acetate, a two carbon short chain fatty acid (Bond and Lovley, 2003). In order to obtain energy from more complex compounds, we must find novel ARB or ARB must work in tandem with other organisms capable of breaking down the more complex compounds in their environment and providing appropriate intermediate compounds for the ARB to consume (Torres et al. 2007; Parameswaran et al. 2009; Ren et al. 2007).

Several different groups of microorganisms are often found in MXCs including: fermenters, homoacetogens, and methanogens. Figure 1.1 depicts their interactions in the anaerobic food web as arrows between intermediate compounds. Fermenters convert sugars to alcohols, fatty acids (including acetate), CO_2, and hydrogen (H_2) (Madigan and Martinko, 2003). The majority of acetate in anaerobic systems is produced from fermentation. Some of it can come from homoacetogens, bacteria that consume H_2 and produce acetate (Hugenholtz and Ljungdahl, 1991; Ljungdahl, 1986; Ragsdale and Pierce, 2008). There are two types of

organisms who produce methane: acetoclastic methanogens produce methane from acetate and hydrogenotrophic methanogens produce methane from H_2 (Wolfe, 1992).

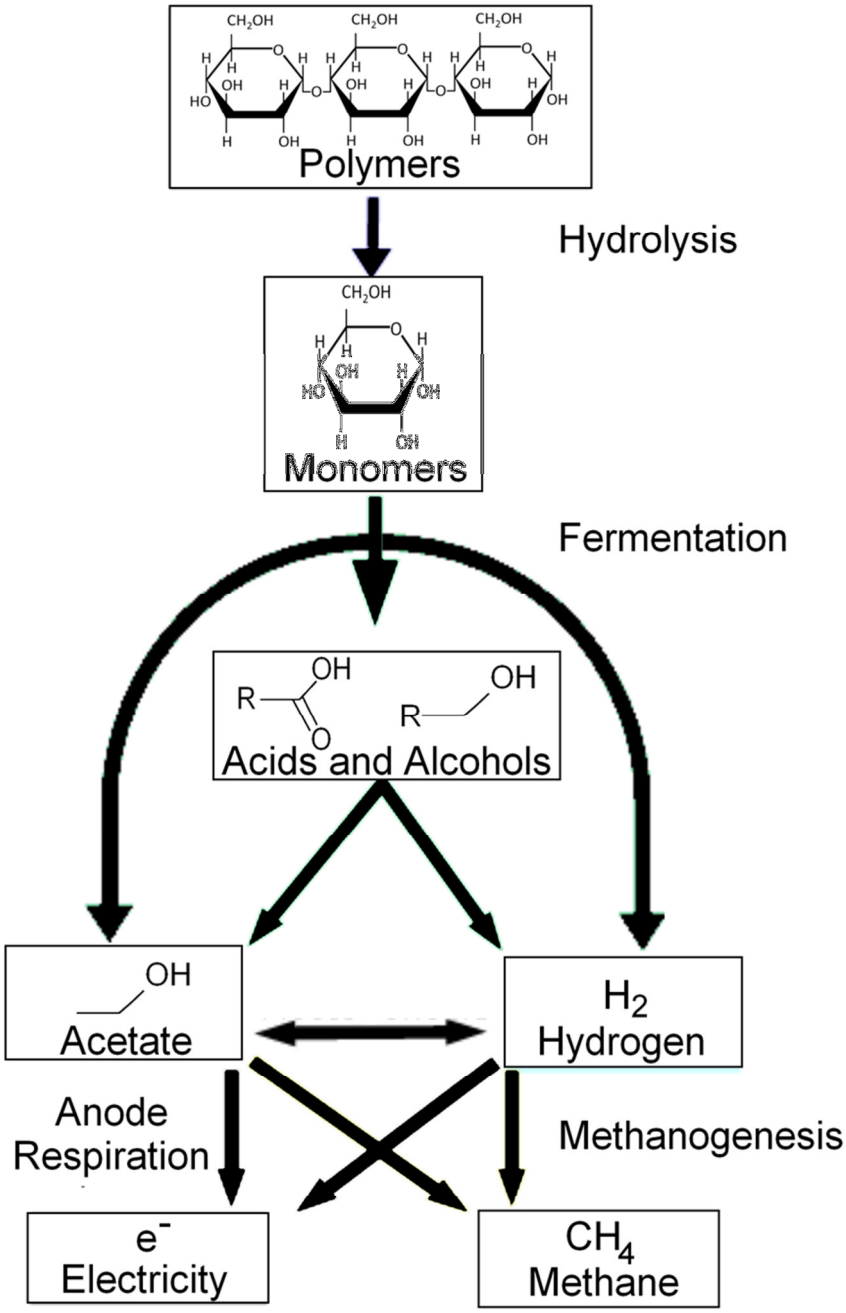


Figure 1.1. Flow of electrons between intermediates in MXCs. Bacteria mediate the transformations, depicted as arrows, between different intermediates, example compounds are illustrated.

The interactions among all of these organisms play a critical role in the operation of anaerobic systems and specifically in MXCs. The loss of electrons from the initial ED to biomass (of any group of microorganisms) or to methane results in a decrease in the efficiency of the system, as these electrons do not end up at the electrode to be turned into current or electrical power (Parameswaran et al., 2009). Coulombic Efficiency (CE) in an MXC measures the fraction of electrons converted to current by the ARB from the net amount of electrons removed from initial COD in the MXC. It can be represented by the equation:

$$CE = \frac{Q_{output}\,(C)}{96485\left(\frac{C}{mol\,e^-}\right) * (COD_{in} - COD_{out})\left(\frac{mg}{L}\right) * \frac{mol\,O_2}{16000\,mg\,O_2} * 4\left(\frac{mol\,e^-}{mol\,O_2}\right) * V(L)} \quad (1)$$

Where Q_{output} is the total charge passed as current, chemical oxygen demand (*COD)* is a measure of the organic compounds present, and V is the volume of the reactor. In order to maximize CE from EDs which ARB cannot directly utilize, we need at least three conditions to be met in regards to the microbial community present: 1) it must be able to efficiently transfer electrons from some organic compound to the anode, 2) it must be able to convert the provided ED to intermediate organic compounds which the ARB can utilize, and 3) it must minimize the diversion of electrons to alternative processes.

First, the community must contain ARB capable of performing efficient anode respiration. When I began my studies, researchers had already identified several ARB; however, the options as to their salt, pH, and temperature optima were limited and wide ED specificity was not combined with high current production. One organism, *Shewanella oneidensis*, directly utilizes a wide variety of EDs (Venkateswaran et al., 1999) but produces low current densities (Rosenbaum et al. 2010; Torres et al. 2010; Borole et al. 2011; Clauwaert et al. 2008). *Geobacter sulfurreducens*, one of the most common ARB, has been shown to convert acetate to current with high efficiency and current density, but does not utilize a wide variety of EDs (Bond and Lovley, 2003). One of my goals starting out was to identify novel ARB with broader ED specificities and wider salt, pH, and temperature tolerances, a development that would open up future applications for MXCs.

Second, the community must be able to convert the ED provided into intermediate compounds capable of being utilized by the ARB present. An MXC utilizing the bacteria *G. sulfurreducens* as the ARB requires a mixed community that produces acetate in order to produce current from complex EDs. It is unclear if amending an MXC community by simply adding complementing microbial members, which have the metabolic pathways missing in the MXC community, will result in positive microbial interactions and enable the production of current from a given ED. As my studies progressed, I became interested in the microbial community interactions in MXCs and decided to see if assembling MXC communities in this manner would result in efficient operation.

Third, the community must be able to minimize losses of electrons to processes that do not result in current. Methane production has been reported as a major electron sink in many MXC systems. Preventing methane production has previously increased CE in MXCs fed fermentable EDs (Parameswaran et al. 2009; Zhu et al. 2015). Cost-effective strategies for inhibiting methanogenesis are still needed to improve the process. Considering these challenges, I became further interested in how we might manage microbial communities in MXCs in order channel electrons towards current and away from methane and looked into alternatives to previously described methods.

1.3 Dissertation Objectives and Outline

Analyzing the microbial communities in MXCs that develop under different operating conditions will provide the knowledge to manage microbial communities during and attain the conditions required for efficient MXC operation. I therefore pose several questions to be answered in my research. First, how widespread is the capacity for anode respiration in the natural environment? Second, can the mixed microbial communities in MXCs be combined with non ARB communities in order to perform multi-step processes? Third, can I craft strategies such as controlling organic loading or providing certain inhibitors to help manage the microbial community by selectively inhibiting competition by methanogenesis? In preparation to answering these questions, Chapter 2 will provide background information on MXCs, the microbial organisms that form communities in them, and techniques used to study them.

A number of different environmental and wastewater treatment derived samples have been used to inoculate MXCs with varying results. It is unknown whether these differences are due to the presence or absence of ARB in the starting inoculum or if differences in laboratory setup play a more significant role. Some locations around the world may host conditions more amenable for ARB to thrive or conditions where ARB with novel capabilities have evolved. Therefore, I enriched a variety of environmental samples from around the world and demonstrated the prevalence of ARB in multiple environments which led to the identification of a novel ARB, *Geoalkalibacter*. I present these already published data (in a modified format) in Chapter 3.

While most ARB prefer to use acetate as their electron donor, most waste streams are composed of much more complex organic compounds. Real world applications of MXC technologies therefore require the use of mixed cultures. In the event that a MXC community is incapable of utilizing a given electron donor, it should be possible to amend the community with organisms that can bridge the metabolic gap between substrate and acetate. A commonly used mixed ARB culture in the Krajmalnik-Brown and Torres labs, which produces high current density on acetate, did not contain the biological pathways required to form acetate from butyrate. By introducing a microbial community capable of fermenting butyrate to acetate, but incapable of using an anode as the terminal electron acceptor, I developed a microbial community capable of consuming butyrate for the production of electricity. CEs with this community rival the best natural communities reported so far, and indeed surpass the majority of them. I present these already published data (in a modified format) in Chapter 4.

Under anaerobic conditions, fermenting bacteria produce acetate and H_2 as two of their main end products; the proportions depend on the electron donors and microbial organisms present, as well as prevailing environmental parameters. Unfortunately, methanogens use acetate and H_2 to produce methane, an undesirable electron sink with respect to maximizing current production because it is a compound unusable by ARB. By adjusting the initial ED concentration in batch fed fermentation cultures, the balance of products from fermentation can be shifted to produce more VFAs and less hydrogen, leading to lower methane production. I

show how this shift in fermentation affects the microbial communities involved, using several different fermentable EDs. These data are presented in Chapter 5.

Previous work has demonstrated that inhibiting methanogens can improve the CE of MECs. Several waste streams contain high concentrations of ammonium, a known inhibitor of methanogens. I showed that increasing ammonium (NH_4^+) in fermenting, methanogenic batch cultures can shift the balance of products during fermentation away from methane and towards VFAs, and, in some cases, lactate. In MECs, NH_4^+ improved efficiency with glucose as the ED, but did not show improvement with sucrose as ED. NH_4^+ showed a marked effect on the microbial community structure of fermenting, methanogenic cultures; however, the presence of ARB and another electron acceptor (the anode) in MECs appeared to greatly diminish the effects of NH_4^+ on microbial community structures. These data are presented in Chapter 6.

Finally, in Chapter 7 I present my conclusions and in Chapter 8 I provide guidance for future work in identifying novel ARB, crafting more efficient microbial communities, and managing communities for specific outcomes. Altogether, my work expands the tool set for designing and managing the microbial communities used in MXC systems by providing options for the choice of starting communities, demonstrating the capacity to tailor microbial communities involving ARB to society's needs, and developing methods for controlling electron losses during the utilization of fermentable EDs.

CHAPTER 2

BACKGROUND

2.1. Waste waters

2.2.1 Overview

Human activities, including urban sewage production, animal husbandry, agriculture, and food processing, lead to the production of a variety of contaminated waste waters (Palaniappan et al., 2010). If these waters are released untreated, not only can they cause great ecological harm, they can also spread disease (UNICEF and Who/Unicef, 2008; WHO and UNICEF, 2014). These particular waste streams tend to have organic contaminants and nutrients that make them amenable to biological treatment using aerobic and anaerobic bacteria (Rittmann and McCarty, 2001).

2.1.1 Aerobic water treatment

During aerobic treatment of waste water, pumps force air into the water in order to provide oxygen to aerobic microorganisms (Rittmann and McCarty, 2001). The microorganisms consume organic contaminants in the waste water, forming CO_2 and biomass. Due to the highly favorable energetics of aerobic respiration, these microorganisms form large amounts of (Russell and Cook, 1995). This biomass, also called biosolids, continues to accumulate during treatment, and operators must remove the excess biosolids in order to maintain process efficiency and prevent overflow of biosolids into effluent waters. While these biosolids were once routinely landfilled, increasingly strict regulations require the use of methods of stabilization and encourage energy and nutrient recapture (Wang et al. 2008; Oleszkiewicz and Mavinic 2001). The addition of oxygen to aerobic waste water treatment systems accounts for up to 50% of the electricity used by waste water treatment plants (US EPA, 2008). In order to minimize the environmental effects and energy costs of aerobic waste water treatment, anaerobic technologies should be employed.

2.1.2 Anaerobic digestion

Anaerobic digestion is used to treat biosolids, the biomass generated during aerobic treatment. The use of anaerobic processes not only decreases the amount of biosolids but also

decreases energy use by decreasing the amount of air needed to treat waste streams (Mccarty, 1964). Unfortunately, only 19% of the estimated 16,029 publicly owned wastewater treatment plants in the United States use anaerobic digestion, and only 1% utilize the gas produced (US EPA, 2008). In spite of its low adoption, anaerobic digestion is a mature technology that continues to develop, with growing opportunities to harvest both energy and nutrients from waste streams (McCarty et al. 2011; Oleszkiewicz and Mavinic 2001; Rulkens et al.1998; Rajeshwari et al. 2000; Mata-Alvarez et al. 2000).

The main goal of anaerobic digestion is the removal of biodegradable matter in order to stabilize biosolids (Mccarty, 1964; Parkin et al., 1986). During anaerobic digestion, waste waters with organic compounds are fed into an anaerobic tank. Microorganisms in the tank break down the organics and convert them to methane. Anaerobic digestion of waste waters requires different microorganisms interacting with each other through intermediate metabolite compounds, where one microbe's waste products are the ED for another in a microbial food web, as illustrated in Figure 1.1. Fermenting bacteria convert sugars, amino acids, and alcohols into acetate and H_2 which methanogenic archaea convert into methane (CH_4), the end product of anaerobic digestion.

2.2. Fermentation

2.2.1 Overview

Fermentation is an anaerobic process that bacteria perform in order to extract energy from organic compounds where the electron acceptor is an intermediate metabolite generated from the initial ED. The majority of energy extracted during fermentation is obtained through substrate level phosphorylation. In some cases, when fermentation reactions are unable to drive phosphorylation directly, chemical gradients can be produced and used to drive ATP synthase, similar to more conventional electron transport chains with terminal electron acceptors (Hilpert and Dimroth, 1982). Essentially, fermentation rearranges a substrate molecule into two, or more, product molecules, one of which is more oxidized and one of which is more reduced (Jin and Bethke, 2007).

2.2.2 Thermodynamics of Fermentations

During fermentation, microorganisms generate H_2 in addition to short chain fatty acids (SCFAs), alcohols, and other carbon compounds (Rodríguez et al. 2006; Thauer et al. 1977). Consider the fermentation of glucose, an abundant sugar in the environment and many waste streams. Theoretically, the fermentation of glucose could produce the following reaction, with the production of acetate and 4 moles of H_2 per mole of glucose (Jungermann et al. 1973; Thauer et al. 1977):

$$C_6H_{12}O_6 + 4\ H_2O \rightarrow 2\ CH_3COO^- + 4\ H_2 + 2\ HCO_3^- + 4\ H^+ \qquad \Delta G°_{rxn} = -206.1\ kJ/mol \quad (2)$$

The theoretical, complete conversion of glucose to CO_2 would proceed according to this equation:

$$C_6H_{12}O_6 + 12\ H_2O \rightarrow 12\ H_2 + 6\ HCO_3^- + 6\ H^+ \qquad \Delta G°_{rxn} = 3.0\ kJ/mol \quad (3)$$

Therefore, the formation of 12 moles of H_2 from 1 mole of glucose would require an additional input of energy in order to be thermodynamically favorable. This illustrates the importance of considering the thermodynamics of the reactions being used to provide energy in biological systems. The theoretical value of 4 moles of H_2 per mole of glucose fermented with 2 moles of acetate, however, has not yet been produced. The control of H_2 formation in glucose fermentation was demonstrated by varying pH (and thus the availability of protons) by Lee et al.(2008).

Figure 2.1 shows some of the pathways involved with the fermentative breakdown of glucose which begins with glycolysis. During glycolysis, NAD^+ is reduced to NADH and pyruvate is formed, reviewed in Bodner et al. (1986). The NADH must be re-oxidized in order to replenish the NAD^+ pool for further glycolysis to proceed. Under fermentative conditions, the electrons from oxidizing NADH can be sent to reduce protons for H_2 production (Rodríguez et al., 2006). Pyruvate can be sent to the TCA cycle for acetate formation, which involves the reduction of the protein ferredoxin. Similar to NADH, ferredoxin can be re-oxidized with the evolution of H_2 (Mortenson et al. 1962; Tagawa and Arnon 1962; Mortenson et al. 1963). In both cases, the production of H_2 faces certain thermodynamic limitations, reviewed by Schink (1997).

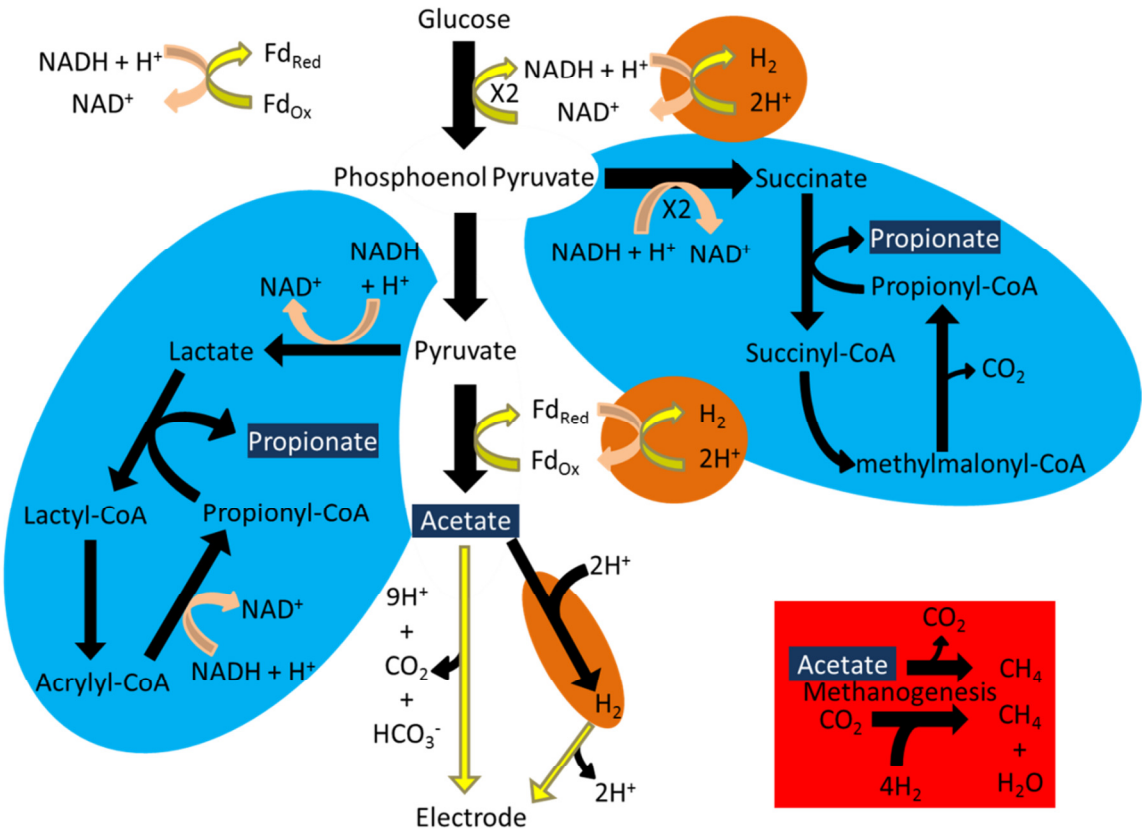


Figure 2.1. Selected pathways involved in the fermentation of glucose. This includes the production of acetate, propionate, and H_2, as well as the subsequent transference of electrons from acetate to an electrode (anode respiration) and the use of either acetate or H_2 for methane production (methanogenesis).

Briefly, the pathways that produce H_2 during fermentation are favored only at low H_2 partial pressures due to the lower Gibbs free energy for the reactions (Tanisho et al. 1989; Angenent et al. 2004; Schink 1997). According to Angenent et al. (2004), the partial pressure for H_2 at which further H_2 production becomes thermodynamically unfavorable can be calculated using equation 4, assuming the oxidized and reduced forms of the electron donor are at equal concentrations and a pH of 7:

$$P_{H_2,max} \leq \exp\left\{\frac{2F\left(E_{H_2}^{O'} - E_X^{O'}\right)}{RT}\right\} \quad (4)$$

where F is Faraday's constant (9.6485 x 10^4 C mol^{-1}), R is the ideal gas constant (8.3144 J mol^{-1} K^{-1}), T is the temperature in Kelvin, $E_{H_2}^{O'}$ is the standard redox potential of H_2 formation, and $E_X^{O'}$ is the standard redox potential for the electron donor for proton reduction, either NADH or reduced

ferredoxin. The reduction of protons ($E^{O'}_{H_2}$ = -414 mV) using electrons from NADH ($E^{O'}_{NADH}$= -320 mV) during fermentation is inhibited at H_2 partial pressures above 0.0006 atm, or 0.47 µM in the solution, at equilibrium (Angenent et al., 2004). Due to the more negative redox potential of ferredoxin ($E^{O'}_{fer.}$= -400mV), compared to NADH, the production of H_2 through this pathway can be sustained up to H_2 partial pressures of 0.3 atm, or 237 µM in solution (Angenent et al., 2004). During most fermentation reactions, a greater number of molar equivalents of NADH are produced than ferredoxin, which limits the amount of electrons which pass through ferredoxin to H_2. The redox potentials for these compounds also shift with the ratio of their reduced to oxidized forms and with the pH of the environment (Tanisho et al. 1989). Since NADH based formation of H_2 shuts down at lower H_2 partial pressures, high NADH concentrations can drive ferredoxin reduction (Petitdemange et al., 1976). When the concentration of H_2 increases, the change in Gibbs free energy for each of the associated reactions also increases until it becomes positive and the reaction is no longer thermodynamically favorable. Increasing H_2 eventually inhibit H_2 formation through NADH and ferredoxin.

In anoxic environments such as sediments, wetlands, landfills, and sludge digesters, hydrogenotrophic organisms relieve the buildup of H_2 by consuming it, which encourages faster fermentation rates (McInerney et al., 2008; Schink, 1997). Hydrogenotrophic methanogens, with the production of methane, decrease H_2 concentrations low enough that they keep the production of H_2 during fermentation thermodynamically favorable (Lee and Zinder 1988). Homoacetogens also consume H_2, but produce acetate instead (Ragsdale and Pierce, 2008). When the thermodynamic threshold is reached, due to H_2 production outpacing H_2 consumption, the fermenting organisms must slow down production of H_2.

Fermenters do not cease consuming the available high energy EDs when H_2 production slows down. Instead, when other fermentation pathways are present, there is a shift in the metabolites produced (Angenent et al., 2004). This occurs because the biochemistry of fermentation requires available NAD^+ and oxidized ferredoxin molecules. In order to replenish the NAD^+ and oxidized ferredoxin pools, the microorganisms begin to produce other reduced

compounds such as ethanol, propionate, and butyrate. During propionate formation, bacteria use electrons from NADH in either of two pathways for the production of propionyl-CoA (Hetzel et al., 2003; Swick and Wood, 1960) followed by the release of propionate (shown in the blue ovals in Figure 2.1). These reactions re-oxidize the NADH pool, making NAD^+ available to continue fermentation. These, and similar reactions, enable the continued fermentation of more complex, higher energy carrying compounds through the production of simpler compounds without necessarily producing H_2. These pathways have been further demonstrated in the absence of higher organic compounds for the production of C-6 and C-8 carbon fatty acids using acetate (a two carbon fatty acid) as the starting carbon source with H_2 as the electron source, thus building up fatty acids with longer carbon chains from a shorter one (Steinbusch et al., 2011). When H_2 formation from ferredoxin becomes unfavorable, the electrons in ferredoxin can be transferred to either NAD^+, and used for product formation as described above, or to $NADP^+$, and used for biosynthesis (Jungermann et al., 1973).

2.3. Methanogenesis

In most environments where fermentation occurs, methanogens assist the fermenters by consuming H_2 and acetate. By converting H_2 and acetate to methane, methanogens remove the end products of fermentation and enable the continued production of H_2 by fermenters in a symbiotic process called syntrophy. Syntrophic H_2 consumption by methanogens also enables the oxidation of SCFAs like propionate and butyrate, by making the oxidation of propionate and butyrate to H_2 and acetate thermodynamically favorable.

Using either H_2 or acetate as ED and methane as end product provides methanogens only a small amount of energy available for their metabolism, as equations 5 and 6 show (Agler et al., 2011):

$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$ $\Delta G^{\circ\prime}$= -39.06 kJ/mol CH_4 (at 37 °C) (5)

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ $\Delta G^{\circ\prime}$= -125.84 kJ/mol CH_4 (at 37 °C) (6)

Acetoclastic methanogens (Eq. 5) receive all the energy available from their metabolic reaction. Hydrogenotrophic methanogens (Eq. 6), however, share their energy with fermenters by maintaining the low concentration of H_2 required for keeping H_2 production thermodynamically

favorable for the fermenters (Dolfing, 2014; McInerney et al., 2008; Schink, 1997). Additionally, hydrogenotrophic methanogens must reduce their own CO_2, which requires more energy than making use of already reduced carbon. In both cases, the energy budgets of methanogens translate into very slow growth rates.

Anaerobic digesters rely on methanogens to produce methane (Mccarty, 1964; Parkin et al., 1986). Unfortunately, due to their slow growth rate, methanogens are much slower growing than fermenting bacteria (Rittmann and McCarty, 2001). This leads to the long treatment times observed in anaerobic digesters operated as continuously stirred tank reactors. Upsets in anaerobic digesters often take long periods of time to correct. While fermenters can quickly reproduce to make use of additional incoming ED or simply bounce back after toxic compounds are diluted out, methanogens take longer to respond. Without the removal of acetate by acetoclastic methanogens and syntrophic oxidation of other SCFAs, due to the lack of hydrogenotrophic methanogens, acids build up in the systems leading to a drop in pH. This creates a cascading effect where methanogens are inhibited by the low pH and the digester ceases to produce methane (Mccarty, 1964).

2.4. Microbial Electrochemical Cells

2.4.1 Overview

MXCs offer an alternative to methane production during anaerobic water treatment in addition to other uses currently being researched which include: energy production at large and small scales, chemical production, and use in sensors. Using biomass from waste streams such as sewage systems (Ahn and Logan, 2010; Kan et al., 2011; Min and Logan, 2004), agriculture (Nimje et al. 2012; Deng et al. 2012), animal husbandry (Fornero et al., 2010; Jung et al., 2008; Li et al., 2013; Min et al., 2005; Yokoyama et al., 2006), or food processing (Mansoorian et al., 2013; Oh and Logan, 2005) as the substrate for MXCs potentially enables energy recapture from these streams as opposed to further energy input during treatment (Fornero et al., 2010; Lovley, 2006; McCarty et al., 2011; Nimje et al., 2012; Oh et al., 2010). Development of small MXCs for powering remote sensors is currently underway with testing in river sediments showing promise (Bond et al., 2002; Donovan et al., 2013; Holmes et al., 2004; Lee et al., 2015; Tender et al.,

2002). Given the high sensitivity of microbes to their environment, MXCs are also being tested for their capacity to act as sensors themselves (Deng et al., 2015). Recently, work has expanded to using MXCs to drive the production of H_2, formate, acetate, and peroxide at the cathode (Aulenta et al., 2012; He and Angenent, 2006; Jeremiasse et al., 2010; Lovley and Nevin, 2013; Rozendal et al., 2009).

MXCs use bacteria as catalysts on at least one of the electrodes of an electrochemical cell. Just as in a normal electrochemical cell, an MXC has two electrodes: an anode, where electrons enter the circuit donated by an electrochemical reaction, and a cathode, where electrons exit the circuit by another electrochemical reaction. The difference between the changes in free energy (ΔG) of each of those chemical reactions defines what mode the MXC is operating in. When ΔG is negative, the MXC is called a microbial fuel cell (MFC), and energy is available for use in the electrical circuit. If ΔG is positive, the MXC is called a microbial electrolysis cell (MEC) and energy, in the form of additional voltage, must be applied through the electrical circuit in order to drive the reaction at the cathode. (Logan et al., 2006; Torres, 2014)

The general schematic of an H-type MXC is shown in Figure 2.2. In the anode chamber, the ARB can grow as a biofilm directly on the anode, consume EDs, and deliver electrons to the anode. Additional processes transform the starting substrates into intermediate EDs which the ARB can utilize and alternative processes can direct electrons away from current production. In this schematic, a membrane is used to separate the two chambers, preventing complete mixing but allowing ions to pass as the ionic current which must match electrical current in order to attain electroneutrality. In the cathode chamber, an electron acceptor is reduced by electrons delivered from the circuit.

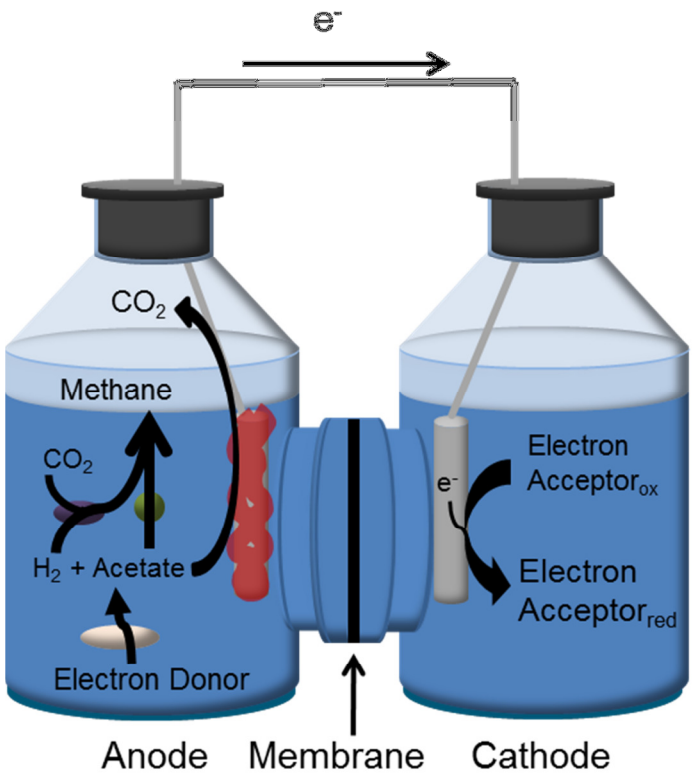


Figure 2.2. Schematic of an H-Type MXC setup.

2.4.2 Anode Respiring Bacteria

ARB enable MXC applications by performing a unique form of respiration. During respiration, organisms extract electrons from an ED in a chemical half reaction with a given ΔG, pass the electrons through a series of steps that transform the energy in the electrons into forms more suitable for use inside the cells of the organism, and then pass the electrons to a terminal electron acceptor in a chemical half reaction with a more positive ΔG. This terminal electron acceptor is normally a compound that is water soluble and can diffuse or be pumped in and out of the microbial cell. In contrast, ARB are heterotrophic organisms that can use solid, extracellular terminal electron acceptors (Bond and Lovley, 2003; Kim et al., 1999; Snider et al., 2012; Torres et al., 2010). In their natural environments, ARB often reduce metals and minerals such as Fe (III), U(VI), and Mn(IV) which are insoluble either before or after electron transfer, thus requiring the need for the capacity to perform extracellular electron transfer (EET) (Caccavo et al., 1994a; Frankel and Bazylinski, 2003; Lovley et al., 2011; Venkateswaran et al., 1999; Weber et al.,

2006). Interestingly, the ability to reduce an extracellular electron acceptor does not guarantee the capacity to interface with an electrode (Richter et al., 2007). In the case of the anode, ARB transfer electrons to, or respire, the anode. In order to do so, electrons from inside the bacteria, where metabolism takes place, must cross the cell membrane and make their way to the anode. These steps are performed by chemical compounds, from small molecules to proteins, that can undergo repeatable oxidation and reduction reactions (Bond et al., 2012; Hernandez and Newman, 2001; Torres et al., 2010).

Three methods of anode respiration are known to exist: mediator based, direct contact, and indirect contact, reviewed and compared in Torres et al. (2010). In mediator-based anode respiration, electron shuttles reversible accept electrons from the bacteria and donate them to the anode. Natural shuttles are produced by the bacteria (Rabaey et al., 2005; Von Canstein et al., 2008), but bacteria are known to use synthetic electron shuttles as well (Park and Zeikus, 2000; Sund et al., 2007). Mediator based anode respiration limits the rate of current production to the diffusion rate of the shuttles between the bacteria and the electrodes. In direct contact anode respiration, redox enzymes in the outer cell membrane of the bacteria directly contact and donate electrons to the anode (Magnuson et al., 2001; Myers and Myers, 1992). Current production with direct contact anode respiration is limited by the need for each cell to be in contact with the anode, limiting the bacteria to a single cell's thickness on the anode. In indirect-contact anode respiration, bacteria form a network of extracellular proteins that passes electrons to the anode (Bond et al., 2012; Reguera et al., 2006; Torres et al., 2008b). This network enables the bacteria to grow into a biofilm up to 10s of μm thick and vastly increases the current which can be produced for a given anode surface area. Different microorganisms exhibit combinations of these mechanisms for anode respiration.

2.4.3 Known ARB

The two most well studied ARB are *Shewanella oneidensis* (Venkateswaran et al., 1999) and *Geobacter sulfurreducens* (Caccavo et al., 1994a). Both organisms are freshwater, neutrophilic, mesophilic, gram-negative bacteria. *S. oneidensis* consumes a variety of EDs and forms biofilms on an anode; however, it makes use of mostly shuttle based anode respiration,

limiting its capacity for high current densities. *Gb. sulfurreducens* makes use of indirect contact anode respiration, enabling high current densities; however, it prefers acetate as its main electron donor. Prior to the work presented in Chapter 3, studies of ARB were mostly limited to these two organisms. Since then, thermophilic ARB (Fu et al., 2013; Parameswaran et al., 2013), gram positive ARB (Parameswaran et al., 2013; Wrighton et al., 2011), and halophilic/alkaliphilic ARB (Badalamenti et al. 2013) have been identified and characterized. In Chapter 3, I demonstrate the presence of ARB in a variety of environmental samples and identify *Geoalkalibacter,* a genus containing halophilic and alkaliphilic organisms, as a candidate ARB, later confirmed and characterized by my colleague Dr. Jon Badalamenti (Badalamenti et al. 2013).

Similar to the overall electrochemical cell, the amount of energy available to ARB depends on the thermodynamic potential difference between their electron donor and their electron acceptor, in this case an anode (Jin and Bethke, 2007; Rittmann and McCarty, 2001). When ARB oxidize simple electron donors with relatively positive electrochemical potentials and they are exposed to an anode with a relatively negative electrochemical potential, they can only obtain a small amount of energy for their metabolic functions. As the difference between the two increases, the ARB can harvest more energy, which supports faster growth of biomass.

2.5. Mixed Communities in MXCs

2.5.1 Development of Mixed Communities

Mixed ARB communities fed with EDs of increasing complexity tend to form more diverse communities (Finkelstein et al., 2006b; Torres et al., 2009). Outside of pure culture experiments, anode respiring biofilms are rarely comprised solely of ARB. Although ARB perform anode respiration very efficiently, they are typically limited by either ED choices or anode surface area availability. In the case of very efficient ARB who can mainly consume acetate, they must rely on other organisms to break down and reduce other compounds available in the environment into acetate for their use (Torres et al. 2007; Parameswaran et al. 2009). In the case of ARB that can consume a wider variety of electron donors, their biomass, and therefore their capacity for electron-donor utilization, is limited by the amount of surface area available, thus they often must compete with other organisms capable of utilizing the available EDs. Both conditions lead to the

development of fermenting and methanogenic organisms being able to thrive in the environment of MXCs (Lee et al. 2008; Parameswaran et al. 2010).

2.5.2 Flow of electrons in Mixed Communities in MXCs.

In MXCs, methanogens lead to decreases in CE (Parameswaran et al. 2009; Parameswaran et al. 2010; Lee et al. 2008). ARB do not use methane as an ED, and methane quickly leaves the liquid phase, taking away the electrons it carries. The majority of losses to methane in MXCs appears to occur through H_2 production (Parameswaran et al., 2011, 2009). ARB have a lower K_s, the substrate concentration at which the rate of substrate utilization is half of its maximum value, for acetate than acetoclastic methanogens; thus, they are expected to outcompete methanogens for the acetate present (Parameswaran et al., 2009). ARB rely on acetogens to convert H_2 to acetate, and acetogens are typically outcompeted by methanogens (Parameswaran et al., 2012). Additionally, a large hydraulic retention time, paired with a small anode and high ED concentrations will provide opportunity for acetoclastic methanogens to build up biomass and take a significant share of the electron flow (Logan, 2012; Logan et al., 2007; Rittmann and McCarty, 2001).

2.5.3 Building Mixed Communities

Knowing that microbial communities form networks of interacting organisms, many microbial communities have been assembled for specific functions such as chemical production and bioremediation. Binary cultures have been assembled to convert starting EDs into desirable chemical products (Agler et al., 2011; Banerjee et al., 2003; Wen et al., 2014). The field of bioaugmentation relies on adding organisms with a specific function to an environment in order to transform hazardous chemicals into non-hazardous or non-soluble chemicals (Morasch and Meckenstock, 2005). Microorganisms are chosen for their ability to perform specific, complementing functions and placed in an environment where they can thrive, carrying out the processes we want as part of their metabolism. The capacity to add in needed metabolic pathways offers an opportunity to improve MXC communities (Ren et al., 2007; Rosenbaum et al., 2011; Speers and Reguera, 2012). Although implementing this piece-by-piece assembly of communities often works, it has failed for unknown reasons (Read et al., 2010). It is therefore of

interest to see how well such strategies work in MXC systems, starting with simple EDs. In Chapter 4, I assemble a community capable of converting butyrate, a four carbon fatty acid, into electricity with high efficiency.

2.6. Effects of NH_4^+ in Mixed Communities

2.6.1 Overview

NH_4^+ is a key building block of life on earth (Fowler et al., 2013). It forms a component of amino acids, the building blocks for proteins which are in turn the workhorses of cellular life. It enters the biosphere through the fixation of atmospheric N_2 gas by bacteria in the soil. Industrially, about 1% of world energy is spent producing ammonia through the Haber-Bosch Process every year (Rafiqul et al., 2005). In order to remove it microbiologically from waste streams, NH_4^+ is usually first oxidized to nitrite and nitrate, and then reduced to N_2 gas which escapes to the atmosphere.

NH_4^+ is present in a variety of waste streams including: agriculture, food processing, and sewage treatment (Chen et al., 2008). In agriculture, nitrogen in the form of NH_4^+ or nitrate is applied to fields to supply plants with this needed nutrient; however, rain and irrigation often wash large amounts of both into the watershed where nitrates can be reduced to NH_4^+ in anoxic zones (Palaniappan et al., 2010). Food processing wastes and sewage naturally contain high concentrations of NH_4^+ (Fornero et al., 2010). Although runoff from agriculture is difficult to collect, food processing and sewage wastewaters, which are already collected, must be treated prior to returning them to the environment, providing an opportunity to use them and exploit their benefits during treatment.

2.6.2 NH_4^+ Effects in Engineered Systems

Previous work on the effects of NH_4^+ on microbial communities has focused on its disruptive effects on: anaerobic digesters (Yenigün and Demirel, 2013), fermentative H_2 production (Pan et al., 2013; Salerno et al., 2006), and acidogenesis (Lü et al., 2008). In anaerobic digestion, a rise in NH_4^+ levels inhibits methanogens and can lead to halts in methanogenesis as described above. After a period of acclimation, some digesters have shown the capacity to function under high NH_4^+ levels (Borja et al., 1996; Schnürer et al., 1999; Sung

and Liu, 2003; Van Velsen, 1979; Werner et al., 2014; Westerholm et al., 2012). This ability to function under higher NH_4^+ concentrations has been attributed to a shift in the methanogen population to hydrogenotrophic methanogens, which are more resilient to NH_4^+ toxicity than acetoclastic methanogens (Sprott and Patel 1986). In fermentative biohydrogen production, an optimum NH_4^+ concentration increases H_2 production, above which the toxicity of NH_4^+ takes over and H_2 production decreases and stops (Pan et al., 2013; Salerno et al., 2006). In acidogenesis of food wastes, NH_4^+ altered the distribution of products away from SCFAs and encouraged the production of lactate and alcohols (Lü et al., 2008). Whether or not increased NH_4^+ results in a disruption of an engineered system depends on the desired outcome of the system and what organisms are required to perform the necessary functions.

2.6.3 NH4+ Inhibition Mechanisms of Action

NH_4^+ has several inhibitory effects, some of which affect a broader range of organisms and some of which are specific to methanogens. NH_4^+ enters cells by diffusing across the cell membrane in its un-protonated form, NH_3. The protonated form, NH_4^+, cannot diffuse freely due to its positive charge, and the pK_a of NH_4^+/NH_3 is 9.24, so only a small amount of NH_3 is typically free to diffuse across the cell membrane (Kleiner, 1985). When the pH inside of the cell is the same as or higher than the pH of the environment, NH_4^+/NH_3 concentrations equilibrate between the inside and outside of the cell. High intracellular NH_4^+/NH_3 has been reported to inhibit acetyl-CoA synthetase in yeast during fermentation (Zheng et al., 2012), and acetyl-CoA synthetase is required for acetoclastic methanogenesis (Jetten et al. 1990). When the pH inside the cell is lower than the outside, NH_3 entering the cell gets protonated, decreasing the concentration of NH_3 inside the cell and producing a gradient that allows more NH_3 to diffuse into the cell and causing the cell to accumulate NH_4^+ above the environmental levels (Kadam and Boone, 1996). Intracellular NH_4^+ formation, driven by either a high extracellular concentration or in tandem with an intracellular pH below the environmental pH, raises the intracellular pH because free protons are lost in the formation of NH_4^+. In order to fix the pH imbalance, a K^+/H^+ antiporter exports K^+ and imports H^+. The loss of intracellular K^+ could be deleterious in its own right as K^+ is required for a variety of bacteria (Lester, 1958) and helps with osmoregulation (Laimins et al. 1981). This

proton exchange further disrupts the proton motive force that drives ATP production in methanogens (Sprott et al. 1984), whereas fermenters mainly have to contend with the effects on pH homeostasis as they rely mostly on substrate level phosphorylation. The fact that methanogens live on a smaller energy budget than most organisms may make them more susceptible to disruptions in their metabolisms.

In methanogens, NH_4^+ has been shown to have an additional method of inhibition. When subjected to high concentrations of NH_4^+, *Methanospirillum hungatei* cells ceased producing methane and became depleted of intracellular K^+. Upon addition of Ca^{2+}, Mg^{2+}, or Mn^{2+}, methanogenesis was restored although intracellular pH and K^+ were not. It is unclear what causes this sensitivity (Sprott et al., 1985). Inhibitory concentrations of NH_4^+ in methanogens are further discussed in Chapter 6.

2.6.4 Ammonia in MXCs

Recently, investigations into the use of MXCs for treatment of high NH_4^+ waste streams have been published testing NH_4^+ toxicity on ARB (Clauwaert et al., 2008b) and the feeding of high NH_4^+ waste streams to MXCs (Damiano et al., 2014; Ganesh and Jambeck, 2013; Jung et al., 2008; Li et al., 2013; Mansoorian et al., 2013; Min et al., 2005; Nimje et al., 2012; Oh and Logan, 2005; Puig et al., 2011; Yokoyama et al., 2006). Acetate fed MXCs showed that ARB could operate at up to 5g N-NH_4^+ L^{-1} without inhibition (Clauwaert et al., 2008b). Feasibility tests of various wastes indicate that MXCs could offer treatment options capable of decreasing organic and nutrient levels in animal wastes (Jung et al., 2008; Li et al., 2013; Min et al., 2005; Yokoyama et al., 2006), landfill leachates (Damiano et al., 2014; Ganesh and Jambeck, 2013; Puig et al., 2011), and food wastes (Mansoorian et al., 2013; Nimje et al., 2012; Oh and Logan, 2005). These studies leave a gap, however, in our understanding of how NH_4^+ affects the microbial communities and distribution of electrons during fermentation in MXCs, which my work in Chapter 6 seeks to fill.

2.7. Studying Mixed Microbial Communities

2.7.1 Overview

Understanding mixed microbial communities and how their members interact requires the capabilities to identify which organisms are present and what processes they can and are performing (Rittmann et al., 2008). A recent mainstay of microbial community analysis, sequencing the 16S rRNA gene from genomic DNA extracted from a community provides information on what organisms are present (Bartram et al., 2011). Chemical analysis of liquid and gaseous products describes the function of microorganisms, i.e. what processes are occurring based on the consumption and production of different metabolites. Combining these data with the literature enables the interpretation of what processes are occurring and what organisms are responsible for those processes in experiments. In MXC systems, electrochemical data and current production are also highly useful (Logan et al., 2006; Torres, 2014).

2.7.2 Tools for community analysis

In microbial community analysis, an estimate is made of the abundance of different organisms in a community. Sequencing of the 16S rRNA gene has been very popular, due to the combination of variable and conserved regions in the gene which enable amplification of the gene from genomic DNA from a wide variety of organisms and determination of the phylogenetic identity of the organism from which the DNA derived (Olsen et al., 1986). Recent developments in sequencing technology have enabled the amplification of DNA from multiple different templates at the same time (Margulies et al., 2005), the combination of multiple samples in sequencing runs (Hamady et al., 2008), and huge increases in the number and length of reads obtained (Bartram et al., 2011), leading to a greatly increased capacity to analyze microbial communities and improved resolution of the phylogenetic identity and abundance of the organisms present in a sample.

Once sequence reads of the 16S rRNA genes are obtained, they are screened for quality, and sequences with very high similarity are collected into operational taxonomic units (OTUs), based on the assumption that sequences of sufficiently high similarity (usually >97% similarity for genera) come from the same taxonomic group (Edgar, 2010). A representative

sequences is pulled out of the OTU and used to assign taxonomy to the OTU based on that representative sequence's similarity to sequences in a database of identified sequences (Cole et al., 2013). The number of sequences assigned to a given OTU is summed and used as the abundance for that OTU. Most often, the relative abundance of an OTU, among all the sequences obtained, is used for analysis. The taxonomic identity of an OTU is used to consult the literature on what is known about the given taxonomic group and when combined with its abundance in the community, inferences are made regarding what function the OTU Is performing in a community and how important it is to the community.

Another method used to understand the capabilities of a microbial community, PICRUSt is a software tool to predict the presence of functional genes from the abundance of 16S rRNA gene sequences (Langille et al., 2013). Sequences are binned into phylotypes by similarity to identified 16S rRNA gene sequences from a database of complete microbial genomes. Sequences that do not match closely enough to a sequenced strain are removed. The abundances of phylotypes are then normalized against the copy number of 16S rRNA genes in the genomes assigned to the phylotypes, which corrects the abundance of each phylotype for those organisms with multiple copies of 16S rRNA genes. The abundances of each phylotype are then used to calculate the abundances of genes contained in the assigned genomes, forming a collection of genes from all of the assigned phylotypes and their abundances, or a metagenome, for the microbial community as a whole. The metagenome can then be queried for the presence and abundance of specific functional genes that encode enzymes for pathways of interest. The abundances and identities of phylotypes used to generate the prediction of a particular gene can also be extracted for analysis. These are predictions based on DNA extracted from the community and thus represent the abundances of genes present in the community and may not indicate that a given gene is in fact active in the community. Transcriptomic, proteomic, or metabolomics data are required to verify activity of specific functional genes.

Quantitative polymerase chain reaction, qPCR, complements broader community analysis by quantifying the abundance of specific target genes (Smith and Osborn, 2009). These

can be either functional, such as FTHFS targeting homoacetogens (Xu et al., 2009), or phylogenetic, such as using specific versions of the 16S rRNA gene to target subclades of the methanogenic archaea (Luton et al., 2002). In qPCR, template DNA is amplified using targeted primers and a method of producing fluorescent signal that is proportional to the amount of double stranded DNA present. The signal in qPCR is produced by different chemistries and approaches, two common ones include TaqMan and Sybr green chemistries. In TaqMan probe qPCR, this is a probe that binds between the two PCR primers and is covalently bound to a molecule which fluoresces once it is cleaved from the probe. The fluorescent molecule is cleaved from the probe once the probe is incorporated into the growing strand by DNA polymerase (Stults et al., 2001). In Sybr green qPCR, a fluorescent molecule intercalates into double stranded DNA and only fluoresces when bound, generating signal as amplification occurs regardless of whether the amplified sequence is the target or not (Wittwer et al., 2013). The increase in fluorescence in both cases is tracked during amplification and compared against a standard curve of target DNA in order to quantify the target in the sample. When used to target functional genes, qPCR can show the presence of particular pathways in a microbial community, regardless of whether it belongs to one phylogenetic clade or is redundantly produced across several. In qPCR, the template used can be either genomic DNA, providing information on the presence of the target in the sample, or complementary DNA, reverse transcribed from mRNA and thus providing information on the presence of genes which are actually transcribed and thus active to some extent in the community.

2.8. Interpreting Trends in Community Data

2.8.1 Principal Coordinate Analysis

The increasing amount of data generated by microbial community analysis requires increasingly sophisticated tools to understand. Looking at a variety of similar microbial communities, it can be quite difficult to pick out important trends by eye. A frequently used method, principal coordinate analysis (PCoA) (Gower, 1966; Legendre and Andersson, 1999), relies on the use of an underlying calculation to determine how closely related different microbial communities are to each other. UNIFRAC (Hamady et al., 2010), a method based on how much

phylogenetic trees of different microbial communities match each other, provides a distance metric which can either include weighting of taxons by abundance or leave this information out.

Once UNIFRAC distances are calculated between each of the microbial communities to be considered, an ordination space is constructed. First, one community is picked as the origin point. The next community is plotted its (UNIFRAC calculated) distance away, creating the first axis. A third community is plotted at a distance from both the first and second communities equal to its UNFIRAC distance from each. This generally involves creation of a second axis. More communities tend to introduce more axes, often ending with n-1 axes for the ordination space, where n equals the number of communities compared.

Once the communities are plotted in this ordination space, principal component analysis (PCA, not to be confused with PCoA, a technique which makes use of PCA) (Hotelling, 1933) is performed, generating new axes on which the data is plotted. These new axes and the coordinates of the communities on them represent a transformation of the data. The first axis, called principal component 1 (PC 1), is created such that it explains the greatest amount of variability between samples across the ordination space. The second axis, which is created orthogonal to the first and so does not correlate with the first axis, then accounts for the next greatest portion of the variability possible and is called principal component 2 (PC 2). This continues with additional axes, typically equal to n-1 as well; however, most of these explain so little of the variability that they are ignored during further analysis. Clusters of microbial communities in the ordination space can then be analyzed in order to attempt to identify what parameters from other, related data drive the development of changes in the microbial communities. The abundance of individual OTUs in each community can be used as weights along with the location in the ordination space of each community in order to plot the location of OTUs in the ordination space as a way of comparing which OTUs are most closely associated with which communities.

PCoA can best be illustrated with an example. Figure 2.3 shows distances, such as UNIFRAC calculated distances, between hypothetical microbial communities A, B, and C. The communities can be plotted according to their distances from each other, as in Figure 2.4. First,

community A is plotted five units from community B, creating the first axis (Figure 2.4, A). In order to plot community C both 4.24 units from A and 3.6 units from B, another axis must be generated (Figure 2.4, B). This creates n-1 axes, or an (n-1)-dimensional space, where n is the number of microbial communities analyzed. Here, the space is only two dimensions but it can be much greater in real cases. The number of dimensions is then reduced, in this case from two axes to only one, by translating the location of cultures in (n-1)-dimensional space to the desired number of dimensions (Figure 2.4, C). This does cause the loss of some information contained in the data.

By using PCA to reduce the number of dimensions, new axes are generated to account for the greatest amount of variability among the sample data. By finding these axes, the loss of information during the reduction of dimensions is minimized. An example of PCA on two dimensional data is presented in Figure 2.5. A simple example will help illustrate the principle behind PCA. Data is first graphed according to known variables, such as pH and temperature (Figure 2.5, A). Then algorithms are applied which generate new axes through the data that correspond with the variability in the data (Figure 2.5, B). These new axes (PC 1, PC 2, etc.) can then be used to examine the data and draw conclusions (Figure 2.5, C). In Figure 2.5, A, it is trivial to see a trend in the data between increasing pH and increasing temperature and the transformation of the data on to the new axes is unnecessary to observe the trend. When data is encoded in four or more dimensions, however, visualization becomes much more difficult. Observing trends across four or more dimensions, visualized in only two dimensions, is incredible difficult. PCA allows visualization of high dimensional data by shifting perspective to the area where the greatest amount of variation is encoded, as each principal component (PC) is correlated to the greatest amount of variability in the data not explained by previous PCs. Usually only the first two or three PCs are visualized. The use of this method allows high dimensional data to be examined in a space where the greatest variability occurs, enabling trends to be pulled out and examined.

	A	B	C
A	0		
B	5	0	
C	4.24	3.60	0

Figure 2.3. Distance matrix showing example distances between hypothetical communities.

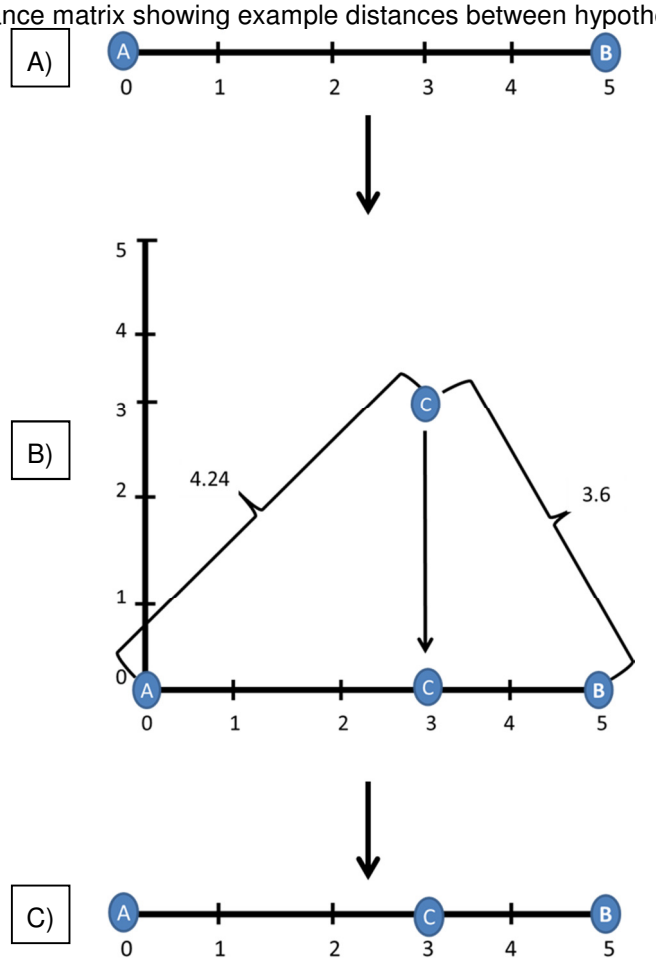


Figure 2.4. Example PCoA construction using distances between hypothetical communities in Figure 2.3. (A) Using community A as the origin, community B is plotted its characteristic distance away, generating an axis. (B) In order to plot C its characteristic distances from both A and B, a new axis must be generated. (C) In order reduce the number of dimensions, community C's location can be translated onto the first axis.

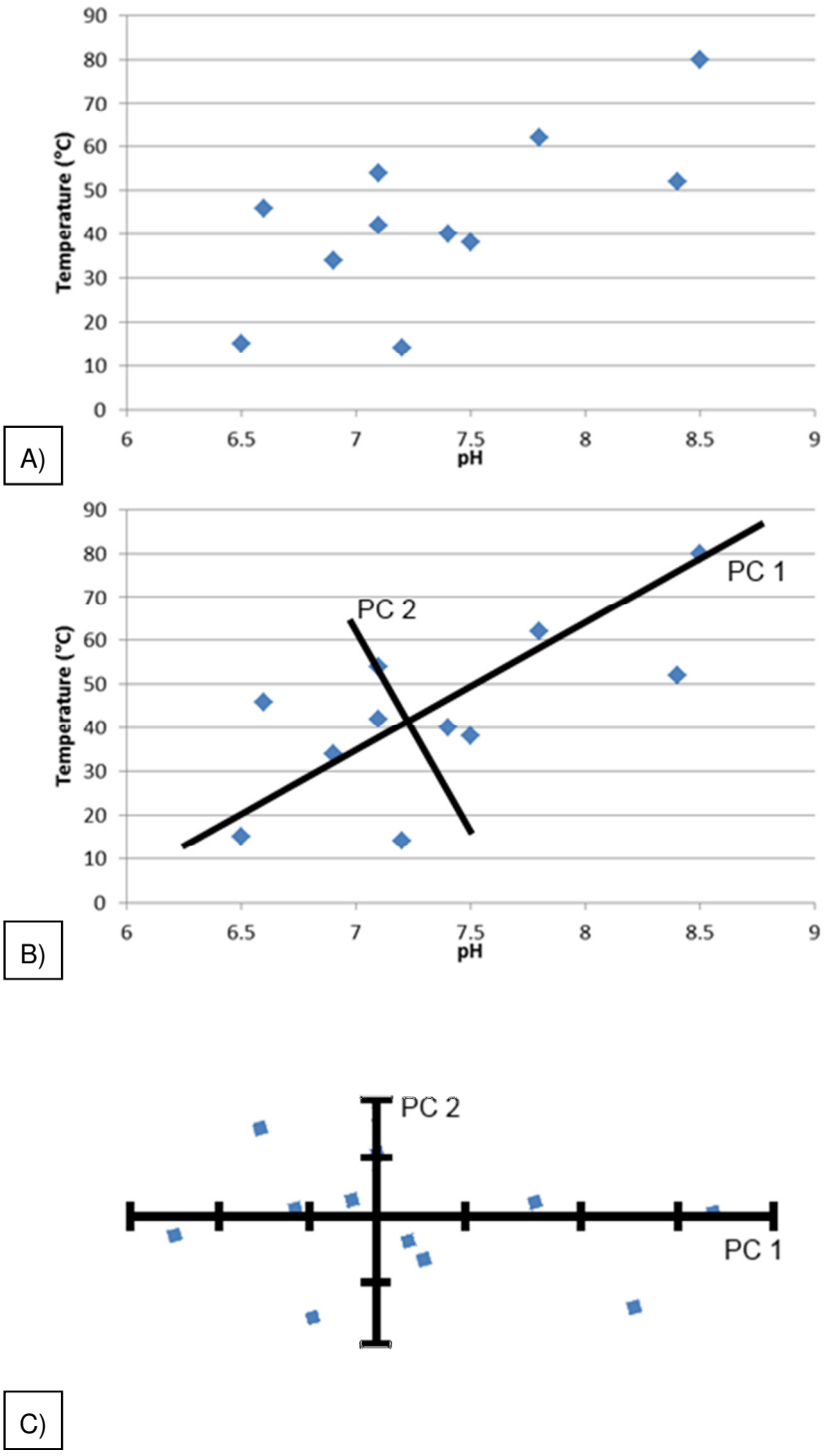


Figure 2.5. Example of PCA using hypothetical pH and temperature data. (A) Samples are plotted according to pH and temperature. (B) Axes are defined along which the variability of the data is greatest, with each added axis being orthogonal to all previous. (C) The samples are plotted on the new axes and used to examine the data for trends.

In order to illustrate how PCoA graphs are interpreted, a hypothetical example is useful. An experiment was conducted where microbial cultures were fed one of three different EDs (ethanol, glucose, and sucrose) at three different concentrations (concentrations 1, 2, and 3), for a total of nine different experimental cultures. The microbial communities of the nine different cultures were analyzed using 16S rRNA gene sequencing and PCoA as described above. In Figure 2.6, two different possible outcomes of this analysis are shown. Microbial communities are represented by colored spheres with numbers over laid on them. The color of the spheres indicates the ED fed, and the numbers represent the concentration of ED fed. In the first example result, Figure 2.6 A, the communities cluster together according to the ED fed, regardless of the concentration which was fed to them. This would indicate that the ED is the determining factor for the structure of the communities. In the second example, Figure 2.6 B, the communities cluster according to the concentration fed to them, with the cultures fed different EDs intermingled among each other. This would indicate that the concentration of ED determined the microbial community structure rather than the concentration of ED. In this manner, examining how communities cluster together in the PCoA is used to identify clusters of communities with high similarity segregated away from communities which are dissimilar and knowledge about the communities is used in order to explain what causes these communities to be similar.

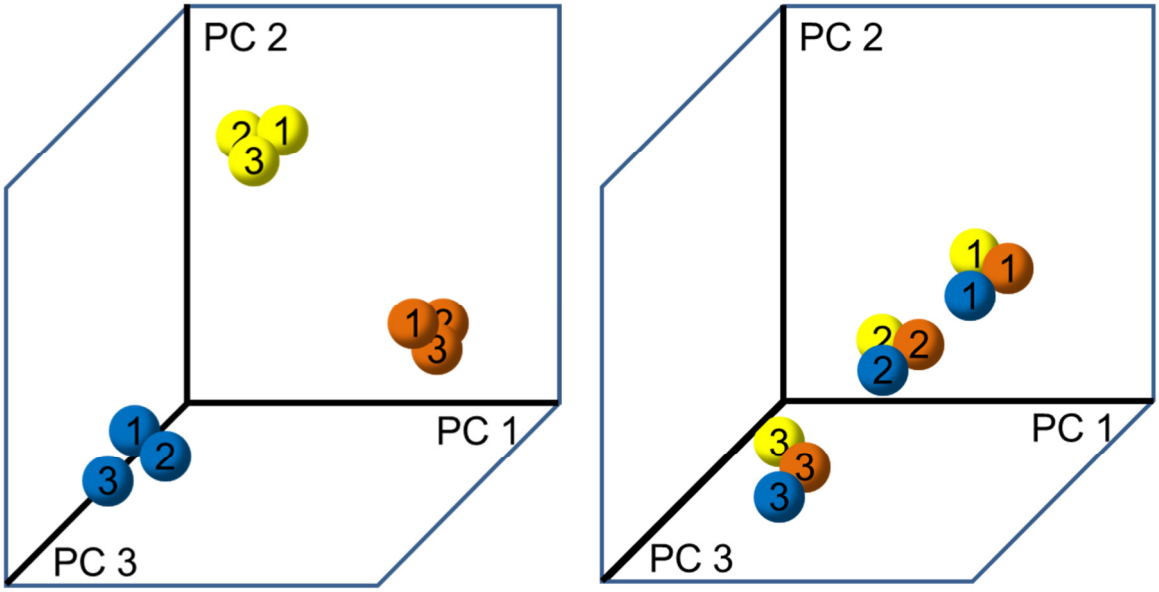


Figure 2.6. Example outcomes of PCoA analysis of microbial communities in a hypothetical experiment. Spheres represent microbial communities, color represents what ED was fed, and number indicates the concentration of ED provided. A) Communities cluster by ED B) Communities cluster by concentration

2.8.2 Canonical correspondence analysis (CCA)

CCA is a statistical method originally designed to interpret the abundances of plant species in different geographical locations with varying environmental conditions (ter Braak, 1986). It correlates environmental data with abundance data of different taxa in order to identify variables that explain microbial community composition and has been adapted for use in microbial ecology. First, each community is arbitrarily assigned a unique "site score", forming an axis (called CCA 1 on graphs) on which the different communities fall. A "species score" is then calculated for each taxa, which is an average of the abundance of the taxa across all communities, weighted by the site scores. Next, a new site score is calculated for each community. This site score is an average of the abundance of all the taxa at the site, weighted by species scores. Regression coefficients are then calculated using a weighted multiple regression of the site scores on the environmental variables. Using these regression coefficients, site scores are recalculated, generating site scores fitted to the environmental data. The process is then repeated until convergence between cycles is reached. Additional axes (CCA 2, etc.) are further calculated, each orthogonal to all previous axes. Each axis thus accounts for a decreasing amount of the variability in the data as each axis is uncorrelated to all previous axes. In this way,

axes are correlated to environmental data, creating constraints on how the axes are generated. CCA graphs look similar to PCoA graphs, except that gradients, for continuous variables, can be drawn as arrows showing how the variable changes across communities in the graph and centroids, for both continuous and categorical variables, show the weighted center of subsets of the communities. Rather than requiring a researcher to infer relationships between community structure and environmental parameters, CCA will show if community structure changes correlate with changes in the environmental data provided.

2.9. Using MXCs to Study ARB Communities.

Development at the laboratory scale takes place in MECs and MFCs. In comparison to MFCs, MECs enable experiments that insulate the microbial community at the anode (or cathode) from limitations in the design of the system being used such as O_2 penetration and changing anode potential (Harnisch and Rabaey, 2012; Logan et al., 2006). Limitations in the system can hamper the ARB as the ARB take advantage of the anode to take up electrons from their metabolic machinery (Bond and Lovley, 2003). In an MFC, the electrons must go through the electrical circuit and be removed by the reaction at the cathode. If the reaction at the cathode cannot take up electrons produced by the bacteria at the anode, because the cathodic reaction is thermodynamically unfavorable, then the potential of the anode decreases. When the potential of the anode decreases, the transfer of electrons from ARB to the anode slows down and can halt if the potential goes too low, the particular inhibitory value depending on the given ARB and the proteins or other chemical compounds they use to interact with the electrode (Torres et al., 2008b). In the case of *Gb. sulfurreducens*, for example, anode respiration ceases between -0.25 and -0.30 V vs SHE (Torres et al., 2010).

In an MEC, where external voltage is applied to the circuit, the potential of the working electrode is held constant, regardless of what electrochemical reaction occurs at the counter electrode. For the production of energy bearing chemicals, this provides the extra energy needed to drive the cathodic reaction, which is otherwise unfavorable. For the study of ARB, an anode poised at the correct potential provides a non-limiting electron acceptor for the ARB. When the ARB deliver electrons to the anode, the applied voltage drives the cathode reaction and maintains

the potential at the anode. This has the added benefit of excluding O_2 from the cathode side of the MEC by driving oxygen reduction despite its low kinetics. O_2 present in the cathode can leak into the anode, poison anaerobic organisms, and provide an alternative electron acceptor. Previous work has shown that the use of -0.03V vs SHE as the potential for the anode encourages ARB colonization of anodes (Torres et al., 2009).

In MEC experiments, the three electrode setup in an H-type reactor with a potentiostat is commonly used to poise the potential of the anode. The three electrodes are a working electrode (the anode), a counter electrode (the cathode), and a reference electrode, situated next to the working electrode, like that used by Torres et al. (2009). The potentiostat monitors the potential difference between the anode and the reference electrode and regulates the potential difference by increasing and decreasing resistance between the anode and cathode. A minute amount of current must pass through the reference electrode in order to measure the potential drop from the anode; this is allowed through a large resistance between anode and reference. The additional current generated by the anode is circuited to the cathode, thus preventing the reference electrode from corroding. This enables a set potential to be maintained on the anode throughout experiments. In addition, the potentiostat records the current produced and allows the performance of electrochemical techniques such as cyclic voltammetry (Bard and Faulkner, 2001).

Cyclic voltammetry (CV) is an electrochemical method that has been used to study ARB, both isolates and in mixed culture, to provide information regarding the mechanisms the ARB use to deliver electrons to the anode (Liu et al., 2008; Richter et al., 2009; Torres et al., 2010, 2007). In CV, the potential of the chosen electrode, the anode in the case of ARB, is changed at a constant rate across a chosen range of potentials and back to the starting potential. During this sweep, the current generated by the ARB biofilm is measured. Characteristic shapes appear in the current response which can indicate whether the biofilm uses shuttles, contact based electron transfer, or both. The generated curves can help establish appropriate conceptual electron transfer models. For example, contact based electron transfer can be modelled in relation to the potential of the anode using the Nernst-Monod equation (Marcus et al., 2007):

$$j = j_{max}\left(\frac{1}{1+\exp\left[-\frac{F}{RT}(E-E_{KA})\right]}\right) \quad (7)$$

where E_{KA} is the potential at which the current is half of its maximum value ($j = \frac{1}{2} j_{max}$, called the midpoint potential), and E is the potential of the anode. The midpoint potential observed during the CV is indicative of the rate limiting step in the metabolic machinery of the cells performing electron transfer in direct contact and indirect contact based electron transfer. The midpoint potential is different for different organisms and can provide indication, although not confirmation, of the presence of particular ARB.

The ability to establish and maintain a set potential on the anode enables the selection of ARB using an anode as the main electron acceptor in a microbial community (Torres et al., 2009). When an ED, such as acetate, is available in liquid media containing an anode with a set potential and other electron acceptors are minimized, ARB will form a biofilm on the anode. In this manner, a microbial community from an environmental sample can be enriched with ARB.

CHAPTER 3

ENRICHMENT AND ANALYSIS OF ANODE-RESPIRING BACTERIA FROM DIVERSE ANAEROBIC INOCULA

This Chapter was published, in a modified format, in *Environmental Science & Technology* (Miceli et al., 2012).

3.1. Introduction

3.1.1 Environmental repositories of ARB

Although practical applications will require mixed communities of microorganisms, understanding the building blocks of such complex systems helps to enhance the desired functions. In this chapter, I focus on enriched biofilm communities that contain the key organisms that make MXCs possible: the ARB. In order to fully realize the potential of MXC technologies, it is important to study a variety of organisms and mixed communities capable of anode respiration so that a wider array of metabolic processes might be known, understood, and leveraged. This can only be accomplished if we expand our search for novel ARB beyond the environments so far tested.

In the natural environment, known ARB make use of a variety of extracellular electron acceptors including manganese and iron (Lovley, 1993; Nealson and Saffarini, 1994; Weber et al., 2006). Previous work has confirmed the presence of ARB in a number of environments including freshwater sediments (Chae et al., 2009; Holmes et al., 2004), marine sediments (Bond et al., 2002; Chae et al., 2009; Holmes et al., 2004; Tender et al., 2002), salt marshes (Holmes et al., 2004; Logan et al., 2005), anaerobic sludge from potato processing (Rabaey et al., 2004), waste water treatment plants (Borole et al., 2009; Harnisch et al., 2011; Jung and Regan, 2007; Kan et al., 2011; Kim et al., 2004; Lee et al., 2003; Lefebvre et al., 2010; Torres et al., 2007), and recently in mangrove swamp sediments (Salvin et al., 2012). From setting up sediment MXCs (Holmes et al., 2004) and encouraging colonization by treating the electrodes (Liu et al., 2007), to using high shear stress (Boon et al., 2008) and serial dilutions (Liu et al., 2008; Wang et al., 2010), many different methods have been devised to obtain cultures containing ARB. The great

variability in the performance of the biofilm communities from these studies (e.g., current densities ranging from 4.5 μA/m^2 to 5 A/m^2) underscores the need to use a reliable enrichment technique for obtaining efficient ARB biofilm communities, which I define as those capable of generating greater than 1.5 A/m^2, based on calculations of the maximum current density capable of being produced by mediator based electron transport (Torres et al., 2010).

3.1.2 Enriching ARB

Researchers have already used MXCs to enrich ARB biofilm communities from wastewater samples (Torres et al., 2009, 2007). Previous work shows that enriching ARB from waste water at low anode potentials (< -0.09 V vs SHE) resulted in faster biofilm growth, higher maximum current densities, and biofilms highly enriched for *Gb. sulfurreducens* (Finkelstein et al., 2006a; Torres et al., 2009). Given that many previous enrichment strategies were unsuccessful in achieving high current densities, I selected this enrichment strategy and improved upon it.

The results of experiments feeding different electron donors to MXCs with identical inocula established that MXC community structure responds to and is highly dependent on the ED supplied (Chae et al., 2009; Jung and Regan, 2007; Kan et al., 2011; Lee et al., 2003). Relatively simple electron donors, such as acetate, have a more positive thermodynamic potential and provide the microbial community with a relatively small amount of free energy (Lee et al., 2003). More reduced compounds make more energy available to the community (Chae et al., 2009; Jung and Regan, 2007; Kan et al., 2011). In such cases, not all the members of the community are responsible for anode respiration, since fermentation and other respiratory processes allow different organisms to proliferate (Parameswaran et al., 2011, 2010, 2009). Limiting the electron donor to acetate has previously resulted in enrichments containing: 49% *Azospira* and 11% *Acidovorax* (Borole et al., 2009); almost entirely *Geobacter* (Harnisch et al., 2011; Jung and Regan, 2007); and 21% Deltaproteobacteria (Lee et al., 2003). With the objective to selectively enrich ARB, I applied selective pressure on my microbial cultures by performing a biofilm transfer step and providing acetate as the electron donor. Biofilm transfers have proven effective previously (Liu et al., 2008; Wang et al., 2010) and work by moving a portion of biofilm from a working MXC into a new MXC where natural selection should increase

the population of ARB. Acetate was the only electron donor provided in abundance in order to control the available energy in the system.

In the environment, ARB could be present in anaerobic sediments and soils where they have access to reduced compounds for use as electron donors and insoluble electron acceptors. Many locations around the world should easily fit these requirements while providing variations in other environmental parameters to which organisms will have adapted themselves. I therefore hypothesized that a greater variety of organisms capable of living as highly efficient ARB abound in the natural environment than have previously been discovered. In order to demonstrate their presence and find novel ARB, I used an ARB enrichment method previously developed in Torres et al. (2009). I obtained 13 samples from locations around the world, placed them in MXCs with electrodes poised at -0.30 V vs Ag/AgCl provided as the sole electron acceptor, and provided acetate as an ED. Cultures were examined for current production, electrochemical responses, and changes in their microbial community from before and after enrichment based on pyrosequencing analysis.

3.2. Materials and Methods

3.2.1 Environmental samples

I obtained samples from: 1) an anaerobic soil from Superior, AZ, USA; 2) a saline microbial mat from Cabo Rojo, PR, USA; 3) a saltwater sediment from a mangrove swamp near Cabo Rojo, PR, USA; 4) a soil from a pine forest near Cabo Rojo, PR, USA; 5) sediment from a mangrove swamp in Carolina, PR, USA; 6) an iron rich soil from Mayaguez, PR, USA; 7) an anaerobic soil from Cuzdrioara, Romania; 8) a sediment from the Brazi Reservoir in Rosia Montana, Romania; 9) an anaerobic soil from Crow's Rock in Rosia Montana, Romania; 10) an anaerobic soil from a pine tree forest in Rosia Montana, Romania; 11) an anaerobic soil from woods in Yokohama City, Kanagawa Prefecture, Japan; 12) an anaerobic sediment from a beach in Kochin, India; and 13) a river sediment from Kerala, India. Samples were obtained using 50 mL Falcon tubes that were capped to minimize O_2 intrusion and stored at 4ºC prior to use. Two samples, Carolina Mangrove and Cabo Rojo Salt Flat, were collected from bodies of saltwater, and thus the media used for the related experiments contained an increased salt concentration.

Samples were collected from between 2 and 20 cm of depth below the surface. Samples from abroad were sealed in zip lock bags, packed in boxes and shipped to the laboratory facilities.

3.2.2 Enrichment strategy

I enriched environmental samples in single chamber MECs. The MECs contained 300 mL of culture media composed of 50 mM bicarbonate buffer (pH ~7.4), 2mM K_2HPO_4, 7mM NH_4Cl, 25μM $FeCl_2$, 0.1 mM H_2S, with trace minerals (Torres et al., 2008c) added, and 25 mM acetate as the electron donor for freshwater samples. Saltwater samples were enriched with the same medium composition amended with 0.342 mM NaCl and 14.8 mM $MgCl_2$. Media was sparged with 80:20 N_2:CO_2 for 30 minutes to remove O_2, then autoclaved at 120 ºC for 30 minutes, and capped immediately after autoclaving to prevent oxygen intrusion. The media was only opened afterward, immediately prior to use, in an anaerobic glove box (Coy Laboratory, Michigan, USA) containing an atmosphere of ~97% N_2: 3% H_2. Both the assembly of MXCs and the transfer of enrichment cultures were performed inside the anaerobic glove box. I used single cylindrical graphite rods (graphitestore.com) for the working and counter electrodes, (6.56 cm x 0.3 cm, surface area of 6.25 cm^2). A 250 mL bottle (VWR Cat. No. 89000236) housed the media and electrodes. The graphite electrodes and bottle were autoclaved prior to use while the Ag/AgCl reference (Bioanalytical Systems Inc.) was sterilized with 70% isopropanol (VWR International Cat. BDH11334-LG). The working electrode was poised at -0.30 V vs Ag/AgCl using a multi-potentiostat (VMP3, Biologic, USA). I monitored the current continuously through the multi-potentiostat and recorded measurements every two minutes automatically. For the regular MXC media, the applied potential was measured to be -0.19 vs SHE, for the saltwater media the potential was -0.13 V vs SHE, after correction. I controlled the temperature at 32 ºC and chambers were mixed constantly with a magnetic stir bar at 150 rpm.

The cultures were subjected to two sequential enrichments. For the first enrichment, 5 g of each environmental sample was added to a clean, autoclaved MEC inside a sterile hood (SterilchemGARD III Advance Model SG403TX, Baker Company, Sanford, ME). The reactors were monitored until approximately 70% of the electron equivalents contained in the electron donor had been recovered as current, current increased above 1mA and plateaued for more than

24 hours, or 21 days had passed without current going above 1 mA, whichever occurred first. Samples which produced greater than 1 mA (1.59 A/m^2) of current during the time allotted were utilized for secondary enrichments.

For the second enrichment, I scraped approximately 1 cm^2 of biofilm from the anode surface and, along with approximately 5 mL of suspension, used this to inoculate the second enrichment MEC. These samples were provided fresh media. Once current production increased above 1mA and stabilized for a period of 24 hours, cyclic voltammetry was performed on the biofilm and a DNA sample was taken directly afterwards as described below.

3.2.3 CV

CVs were performed as described in Torres et al. (Torres et al., 2008b) The potential was swept from the open cell potential up to -0.10 V and down to -0.60 V vs Ag/AgCl, then back again for a second full cycle at a scan rate of 1mV/s. In the results, I only present my measurements from the second sweep. The midpoint potentials for the biofilms were calculated from these CVs.

3.2.4 Pyrosequencing Analysis

DNA from sediment samples and biofilm samples was obtained using the MoBio Laboratories Power Soil DNA kit (MoBio Laboratories, Carlsbad, CA, Cat #12888) as per the manufacturer's directions. DNA samples were sent to Research and Testing Laboratory (Lubbock, TX) for 454 pyrosequencing as described previously in Garcia-Pena et al. (2011). The primers used were their “Blue” primers (104F, 530R), targeting the V2 and V3 region of the bacterial 16S rRNA gene. Sequences were analyzed using the mothur software suite (Schloss et al., 2009) as described by Garcia-Pena et al. (2011). Sequences containing mismatched barcodes, with quality scores below 25, with any uncalled nucleotides, shorter than 200bp, longer than 450bp, containing homopolymers greater than 8bp long, or labeled chimeric sequences by the chimera checker in mothur were discarded. Primers were trimmed off and clustering was performed to generate OTU's with a distance cutoff of 0.03 to provide representative sequences for taxonomic identification down to the genus level. I used a 50% confidence threshold for taxonomic classification using the RDP Classifier (Cole et al., 2009). The sequences of the most

abundant OTUs from each dominant genus were submitted to the Basic Local Alignment Search Tool (BLAST) supported by the National Center for Biotechnology Information in order to obtain their similarity to the most closely related genbank database sequence.

3.3. Results

3.3.1 Current Production by Enriched ARB Biofilms

A total of 13 environmental samples were tested for the presence of ARB using MXC based enrichments monitored for current production. Table 3.1 summarizes the originating locations of the environmental samples, the maximum current density produced during the first and second enrichments, and the abbreviations used in figures for each culture. Cultures were not tested further in a second enrichment if they did not produce sufficient current (>1.59 A/m^2) by the end of 21 days.

Table 3.1: List of environmental samples used for enrichment experiments. Included are the origin location, the maximum current densities produced at each enrichment step, and the names used in the accompanying figures.

Number	Sample Name	Location	Current Density: 1st Enrichment	Current Density: 2nd Enrichment
1	Superior	Superior, Arizona, USA	6.99 A/m^2	5.59 A/m^2
2	Salt Flat (Saltwater)	Cabo Rojo, Puerto Rico, USA	2.46 A/m^2	4.45 A/m^2
3	Playa Sucia Mangrove (Saltwater)	Cabo Rojo, Puerto Rico USA	3.87 A/m^2	4.23 A/m^2
4	Pine Forest	Cabo Rojo, Puerto Rico, USA	0.016 A/m^2	NA
5	Carolina Mangrove	Carolina, Puerto Rico, USA	7.62 A/m^2	10.77 A/m^2
6	Mayaguez Soil	Mayaguez, Puerto Rico, USA	4.13 A/m^2	2.33 A/m^2
7	Cuzdrioara Soil	Cuzdrioara, Romania	9.85 A/m^2	9.15 A/m^2
8	Brazi Reservoir	Rosia Montana, Romania	0.66 A/m^2	NA
9	Crow's Rock	Rosia Montana, Romania	0.0024 A/m^2	NA
10	Pine Soil	Rosia Montana, Romania	0.002 A/m^2	NA
11	Forest Soil	Yokohama City, Japan	1.2 A/m^2	NA
12	Kochin Shoreline	Kochin, Kerala, India	8.73 A/m^2	8.92 A/m^2
13	River Sediment	Kerala, India	0.24 A/m^2	NA

NA - Not applicable, these cultures were not transferred for a second enrichment, due to low current density during the first enrichment.

After the second enrichment and once cultures reached a steady value of current production, I performed a CV on each culture. The results of the CVs are displayed in Figure 3.1: Carolina Mangrove was producing ~71% of its maximum current at that time, Cuzdrioara ~81%, Kochin Shoreline ~98%, Mayaguez ~93%, Playa Sucia ~83%, Salt Flat ~84%, and Superior only ~61%. The majority of cultures were operating near their maximum current densities given the potential at which they were being grown (the black line). Increasing the potential of the electrode would not significantly increase the current production of the community, except in the case of the Superior, AZ culture. This indicates that the cultures were limited by transport processes within the biofilm, not by the electron transfer occurring at the electrode surface. In the case of the Carolina Mangrove biofilm, DNA sampling and CV were performed after current production had declined significantly. Following the initial peak of current (up to 10.77A/m^2), the culture did not stabilize again. In light of the high current density generated, I performed a CV and analyzed the microbial community in spite of this event.

Using the CV results, I calculated the midpoint potential of the ARB biofilms. The midpoint potential is the potential at which half the maximum current is produced when sweeping the potential and depends on the electron-transfer mechanism used by ARB. Figure 3.2 shows the maximum current density and the calculated midpoint potential for each enriched culture. Given that the CVs were performed at the end of the experiment, the maximum current observed is in some cases higher than the maximum current at the time of the CV. Most of the CVs closely, but not perfectly, follow the Nernst-Monod equation (Marcus et al., 2011; Torres et al., 2008b), with some samples showing higher potential losses than expected through Nernst-Monod. These deviations could be the result of either an electron transfer limitation or limitation by the transport of protons out of the biofilm in accordance with the PCBIOFILM model (Torres et al., 2010). Alternatively, they may be the result of more than one redox reaction controlling the current produced. Due to the variations in microbial communities and the possible presence of ARB not yet characterized, the latter explanation is likely. Results from a previously reported culture developed from the Mesa Northwest Water Reclamation Plant in Mesa, AZ (Mesa WWTP)

are included in Figure 3.2 for comparison. A large proportion of this culture (92% when enriched at -0.35 V vs Ag/AgCl) was found to be *Geobacter* in a clone library (Torres et al., 2009).

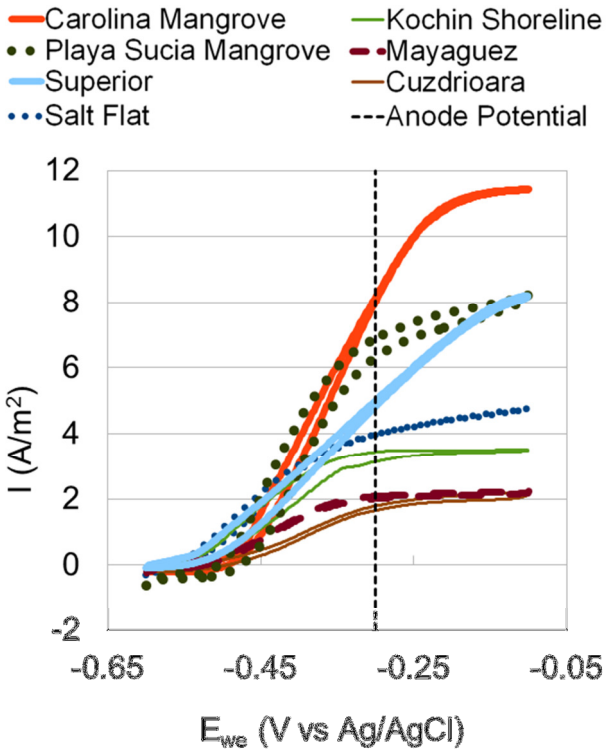


Figure 3.1. Cyclic voltammograms of enriched biofilms from current producing cultures.

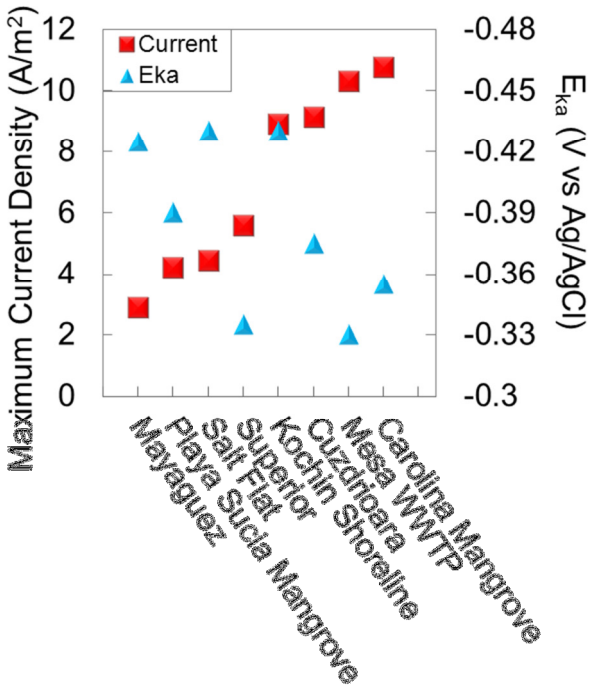


Figure 3.2. Maximum current density and midpoint potential measured for each biofilm. Samples are organized by maximum current density. The Mesa WWTP sample, which consisted of 92% *Geobacter*, is included for comparison as a previously studied sample.

3.3.2 Enrichment of Community Members in ARB Biofilms

In my community analysis, I assigned taxonomy to OTUs at the genus level. In Figures 3.3-3.9, each graph shows the enriched community from a single biofilm after the second enrichment and the current production during both enrichment periods. Five out of the seven cultures that went through both enrichment steps showed enrichment for particular groups of organisms as compared to their original environmental communities. Current densities were not correlated with the period of time in each enrichment phase for the samples tested. For those interested, a comparison between enrichment cultures and their corresponding environmental sample as well as details on the closest Genbank matches to the sequences of the most abundant OTU in each culture are provided in Appendix A.

In five samples, the communities were clearly enriched for particular OTUs. Two samples, Cuzdrioara Soil (Figure 3.3) and Superior Soil (Figure 3.4), contained a high abundance of sequences related to the genus *Geobacter*. Kochin Shoreline (Figure 3.5) and Playa Sucia Mangrove (Figure 3.6) were predominantly *Geoalkalibacter* sequences. *Geoalkalibacter* are closely related to *Geobacter*, gram negative, and do not form spores. They can be either alkaliphilic or alkalitolerant, use Fe(III) and sulfur as electron acceptors, and can make use of a variety of simple organic acids (including acetate) and ethanol as electron donor (Greene et al., 2009; Zavarzina et al., 2006). The Mayaguez Soil (Figure 3.7) resulted in a biofilm highly enriched with an OTU related to the genus *Desulfonispora*. *Desulfonispora* are known to ferment taurine, resulting in acetate, ammonia, and thiosulfate. They are not known to reduce sulfate, sulfite, or nitrate (Denger et al., 1999).

The two biofilms that did not appear to become enriched, Salt Flat (Figure 3.8) and Carolina Mangrove (Figure 3.9), were also distinct from each other. Carolina Mangrove's biofilm was the only biofilm that showed a community structure very similar to its originating soil and its most abundant sequence was classified as the *Fusibacter* genus. *Fusibacter* are gram positive, ferment carbohydrates, and use thiosulfate and sulfur as electron acceptors (Ravot et al., 1999). The Salt Flat biofilm contained two dominant OTUs, each contributing approximately one-third of the sequences identified: one from the genus *Proteiniphilum* and one from the genus

Geosporobacter. *Proteiniphilum* are proteolytic, gram negative organisms not known to consume carbohydrates or alcohols but capable of producing acetate and propionate (Chen and Dong, 2005). A previous report found *Proteiniphilum* associated with an MXC; however, it was not a major component of the community there either (Ishii et al., 2012). Members of the genus *Geosporobacter* are strictly anaerobic, gram positive, spore formers that ferment carbohydrates, but they have not previously been demonstrated to consume acetate nor use metals as electron acceptors (Klouche et al., 2007).

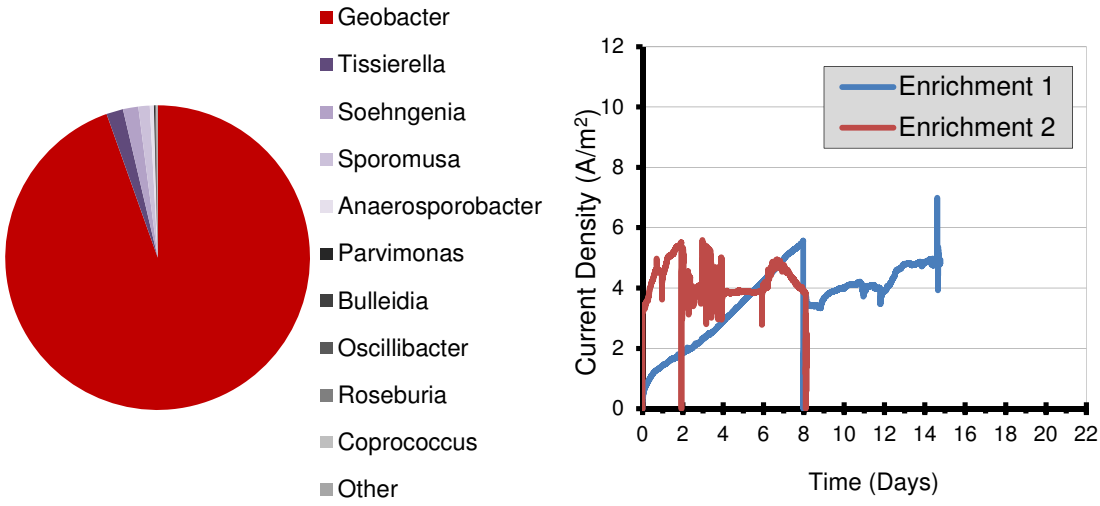


Figure 3.3. Superior, AZ biofilm microbial community analysis (genera classification) and current production.

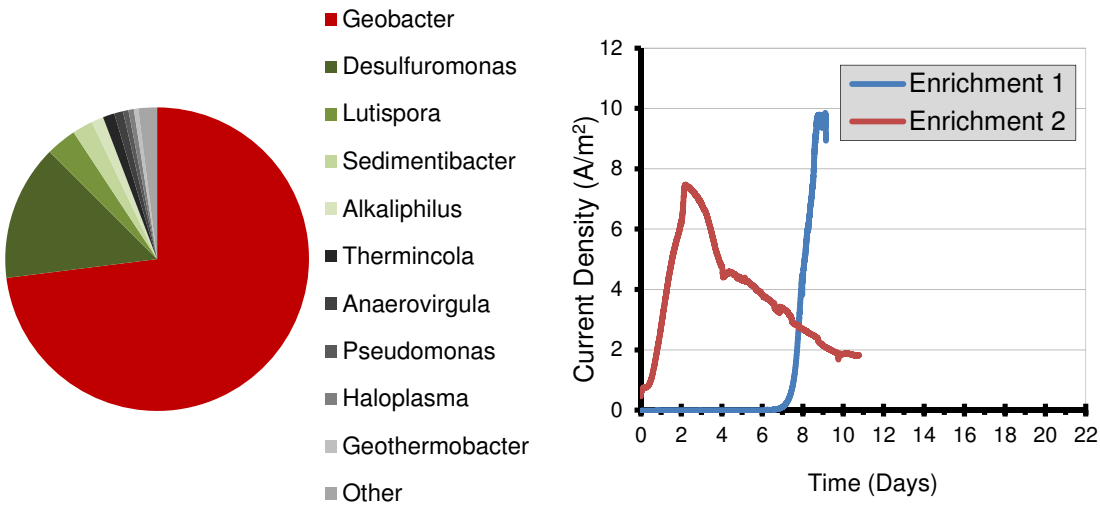


Figure 3.4. Cuzdrioara biofilm microbial community analysis (genera classification) and current production.

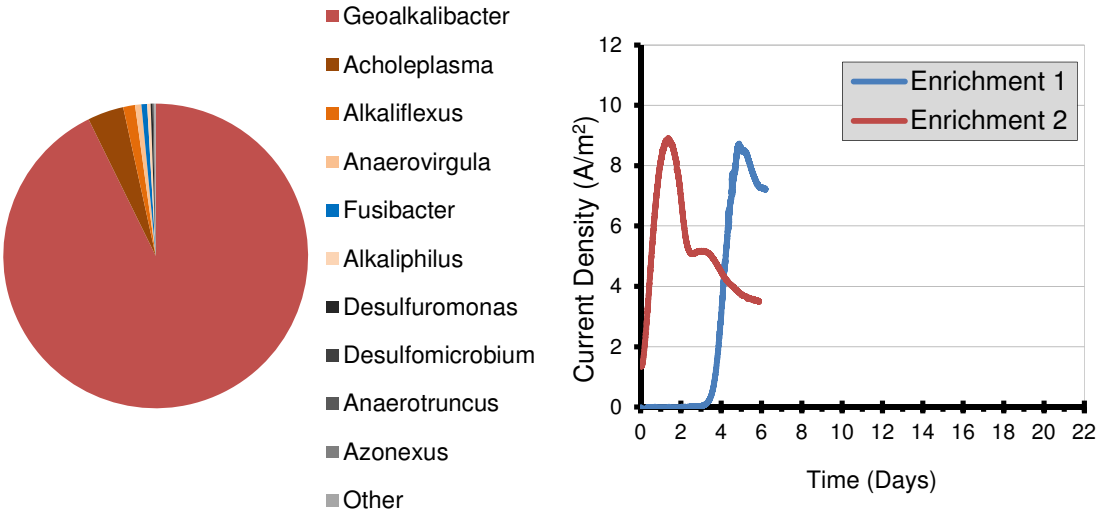


Figure 3.5. Kochin Shoreline biofilm microbial community analysis (genera classification) and current production.

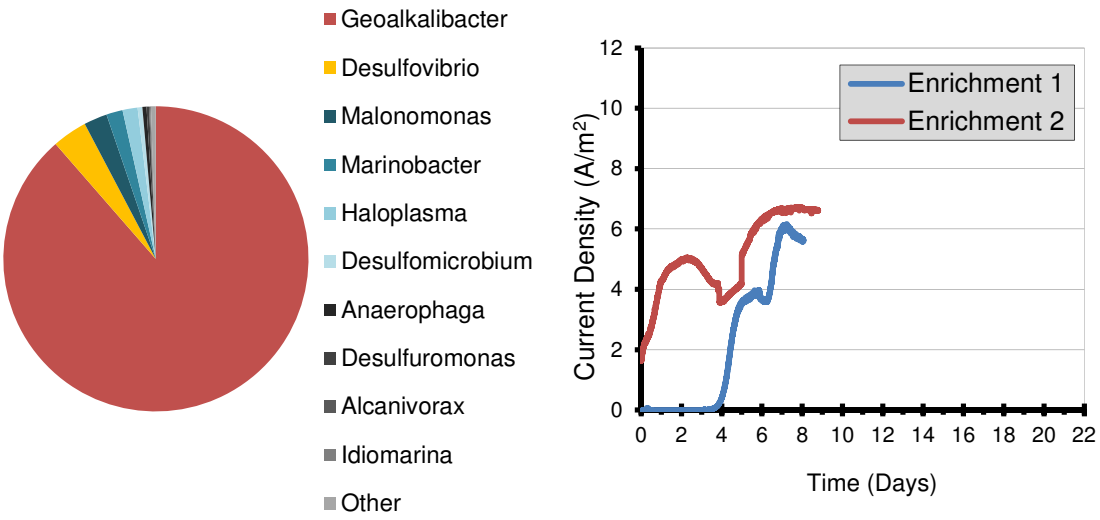


Figure 3.6. Playa Sucia Mangrove biofilm microbial community analysis (genera classification) and current production

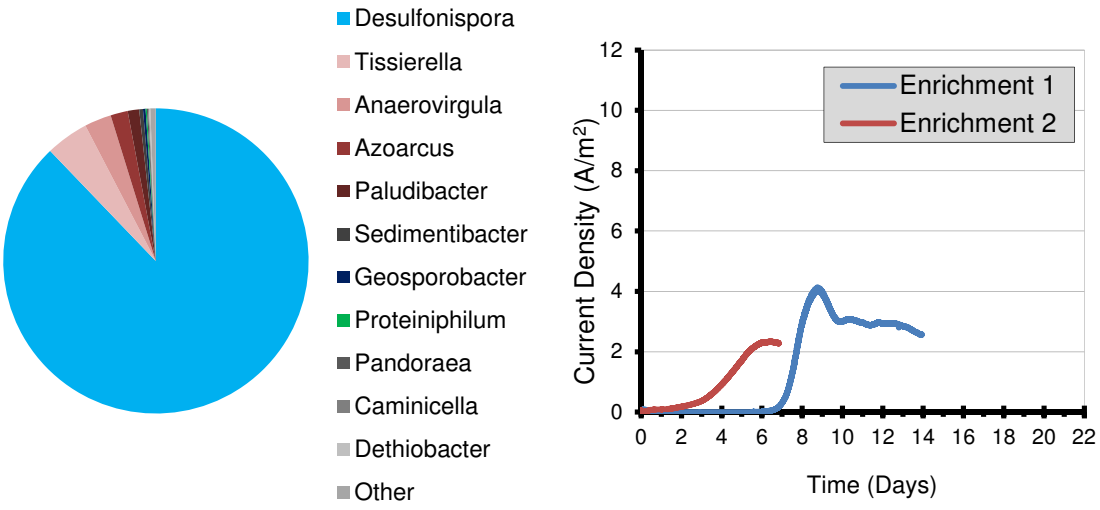


Figure 3.7. Mayaguez biofilm microbial community analysis (genera classification) and current production

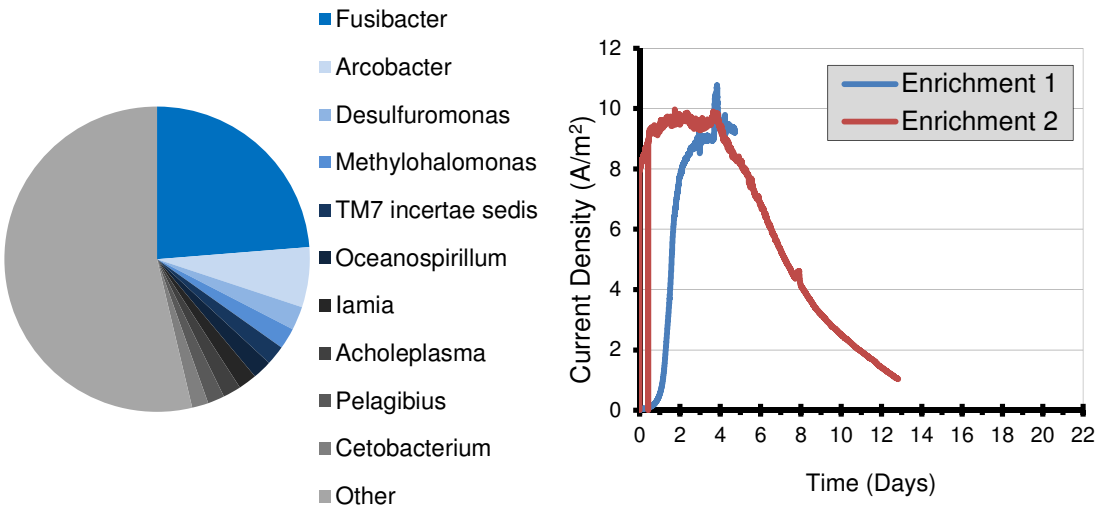


Figure 3.8. Carolina Mangrove biofilm microbial community analysis (genera classification) and current production

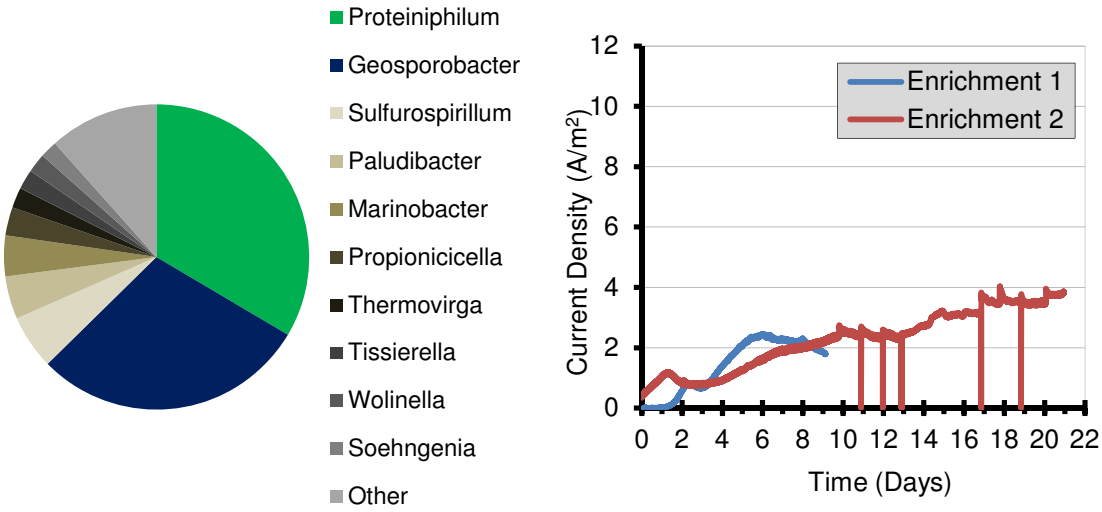


Figure 3.9. Salt Flat biofilm microbial community analysis (genera classification) and current production

3.4. Discussion

These experiments demonstrate the successful enrichment of environmental samples for ARB capable of producing high current densities (> 1.5 A/m^2) in 7 out of 13 environmental samples using MECs fed with acetate and with an anode poised at -0.30 V vs Ag/AgCl. Among these samples, several identified organisms are likely candidates for novel ARB deserving of further scrutiny. Clearly, the capacity for anode respiration is not a rare trait, but rather a widespread mechanism to conserve energy, likely involved in metal oxide respiration in natural environments as these can exist as insoluble electron acceptors similar to electrodes (Weber et al., 2006).

Identifying organisms related to *Geobacter* in the two samples, Superior, AZ and Cuzdrioara, demonstrates that my enrichment strategy selects for known ARB. In these samples, the current densities of 5.59 A/m^2 and 9.25 A/m^2 correlate well with known values for *Geobacter*. The midpoint potentials for Superior, AZ (-0.335 V vs Ag/AgCl) and Cuzdrioara (-0.375 V vs Ag/AgCl) biofilms are slightly more positive than literature values for *Geobacter* (-0.42V vs Ag/AgCl). It is unclear what causes this difference.

I report here the first finding of organisms related to *Geoalkalibacter* dominating the population of MEC biofilms in the Kochin Shoreline and Playa Sucia samples. Both cultures produced significant current densities (8.92 A/m^2 and 4.23 A/m^2), and also produced different midpoint potentials (-0.39 V and -0.43 V vs Ag/AgCl). This may be due to the differences in media composition between the two MXCs, as Playa Sucia was fed media with a higher salt concentration. The use of Playa Sucia Mangrove sediments represents only the second time mangrove sediments have been used as inocula for ARB biofilm communities (Salvin et al., 2012), where they also reported greater than 10 A/m^2 of current, and the first time the associated community has been analyzed.

In the Mayaguez culture, this is the first report of a highly enriched OTU related to the genus *Desulfonispora* in a biofilm community producing high current densities (4.13 A/m^2). Notably, organisms from the class Clostridia, of which *Desulfonispora* is a member, have been detected in MXCs previously; however, they typically occur in lower current density producing

systems such as that reported by Zhu et al. (which produced a maximum of 1.5A/m^2) (2011). The genus *Desulfonispora* performs at least one fermentation reaction, which opens up the possibility that it is scavenging cell products from the rest of the biofilm and not producing current itself. The observations that 88% of the sequences recovered were related to *Desulfonispora* and that the biofilm was steadily producing greater than 3.75 A/m^2 at the time of sampling encourages the interpretation that this organism is performing anode respiration or has a stronger role than just scavenging cell products. The midpoint potential for Mayaguez (-0.425 V vs Ag/AgCl) was similar to the Kochin Shoreline culture; however, given the differences in their microbial populations, it is difficult to make definitive conclusions regarding the nature of the EET pathway(s) taking place in these biofilm communities.

OTUs related to the class Clostridia also appeared in considerable numbers in my Salt Flat and Carolina Mangrove samples. The Salt Flat sample exhibited a similar midpoint potential (-0.43 V vs Ag/AgCl) to that of the Mayaguez culture. In Salt Flat, however, the OTU that is most closely related to Clostridia represented only one-third of the sequences. Although it is possible that the other dominant OTU, related to the class Bacteroides, generates the current being observed, the data gathered here shows multiple biofilm ARB communities with large populations of Clostridia related phylotypes. These observations indicate that organisms from the class Clostridia may perform anode respiration in these cultures.

The Carolina Mangrove biofilm's midpoint was more positive than the other two Clostridia containing samples at -0.36 V vs Ag/AgCl, a value closer to that found for pure or highly enriched *Geobacter* biofilms. It is noted that I sampled the Carolina Mangrove biofilm after current production had declined significantly. It appears that due to the high current density generated (10.77 A/m^2) the culture quickly exhausted its electron donor and quickly declined in activity. Given this and the fact that organisms in the genus *Fusibacter* (the organism most closely related to the dominant OTU) are known to perform fermentation, it is possible that the related organisms detected are not producing current in the system but instead consuming material from dead cells. While none of the major OTUs were matched to known ARB, considering that the biofilm

continued to produce significant current density at the time of sampling (~1A/m^2), it is reasonable to conclude that an efficient ARB must still be present.

High current densities were not correlated with the presence of any single micro-organism across the different samples; however, they did show a minor inverse correlation to E_{ka} values. My community analysis indicates that some of the ARB enriched in my cultures may represent as yet unknown ARB capable organisms including organisms closely related to known ARB, such as the *Geoalkalibacter*, as well as more distantly related, such as the *Geosporobacter* and *Desulfonispora*. Finding relatives of *Geoalkalibacter* as potential ARB is rather exciting, given recent reports of the importance of controlling pH in MXCs (Torres et al., 2008a, 2008c). The production of large electrical currents may require high concentrations of alkalinity to prevent acidity buildup in MXCs which would in turn require organisms like *Geoalkalibacter*, capable of surviving in highly alkaline environments (Greene et al., 2009; Zavarzina et al., 2006).

Given that acetate is not fermented and the lack of alternative electron acceptors in the enrichment MECs, the majority of organisms capable of surviving must efficiently transfer electrons to the electrode to survive. My community analysis supports this finding in the lack of cultures dominated by fermenting organisms, with the minor exception of the Carolina Mangrove and Mayaguez samples as discussed above. The lower current densities observed in the second enrichments of the Superior, Mayaguez, and Cuzdrioara samples compared to the first enrichments may be due to the dilution of electron donors in the second enrichment stage which were added along with the inocula during the first enrichment stage. Using a potentiostatically controlled anode in a single chamber MXC, H_2 is generated at the cathode, making it possible for ARB to survive hydrogenotrophically (Bond and Lovley, 2003; Lee et al., 2009; Ra et al., 2008; Torres et al., 2007). Previously, the observed current density generated by *Geobacter* containing biofilms fed with H_2 was much lower than that generated with acetate (Parameswaran et al., 2011). Additionally, H_2 will only be generated once current has begun to flow in the circuit. These factors combined with the availability of acetate make it unlikely, although still possible, that current generated comes from hydrogenotrophic ARB and not acetate consuming ARB. In either

case, this work focuses on the search for high current density producing ARB, regardless of the electron donor they consume.

Two of the cultures tested here originated from locations near bodies of salt water: Cabo Rojo Salt Flat and Playa Sucia Mangrove. These cultures were enriched in media with increased levels of salt (NaCl and $MgCl_2$) in order to more closely mimic their natural environment. Although these two cultures did not produce the highest current densities, they did show significant current densities (~4.2 - 4.5 A m^{-2}) and their tolerance for higher salt concentrations make them prime candidates for future studies in MXC systems containing higher concentrations of salts than normally utilized.

The small amount of free energy available between acetate, or even hydrogen, and the anode in conjunction with the current density cutoff I used ensured that my cultures were enriched for organisms capable of highly efficient EET, as demonstrated by the high current densities produced. The high current densities achieved indicate that the ARB most likely reside in the biofilm and not in the suspension. Biofilms are better suited for high current densities according to the analysis by Torres et al. (2010). My results also provide further support for the importance of low anode potential MECs as a viable method for enriching ARB from diverse environmental samples, in agreement with previous observations with only one starting inoculum (Torres et al., 2009).

In these experiments only two different media compositions and one anode potential were used for enrichment of ARB capable organisms. These conditions may have prevented the growth and detection of ARB more suited to different environments. In addition to the 7 samples which produced high current densities, two additional samples produced current densities between 0.1 and 1.5 A m^{-2} during the first enrichment stage; however, this was deemed insufficient to continue working with these samples in my search for efficient ARB. The failure of these and other samples to produce high current density generating biofilms may result from factors such as the suitability of the media used for the growth of the organisms present, varying capacities for electrode respiration or differing methods of biofilm formation between different

organisms. These factors may also explain the variations in current density generated by the enriched biofilms.

My results show that ARB other than *Geobacter* related organisms can be enriched using a simple electron donor by expanding the search for ARB. The presence of highly diverse biofilms producing electricity may result from either functional redundancy among organisms or some unknown synergistic factors. It is therefore important to study a multitude of organisms from a large variety of locations in order to enrich and further study those with the greatest capacity to perform under the conditions in which they will be used. Towards this end it may be necessary to establish favorable media conditions for as yet uncharacterized ARB. I expect that further research using these enriched ARB cultures will produce novel isolated ARB strains or mixed ARB communities which will be instrumental for further ARB and EET characterization and will be used as building blocks for microbial cultures in new applications.

CHAPTER 4

COMBINING MICROBIAL CULTURES FOR EFFICIENT PRODUCTION OF ELECTRICITY FROM BUTYRATE IN A MICROBIAL ELECTROCHEMICAL CELL

This Chapter was published, in a modified format, in *Bioresource Technology* (Miceli et al., 2014).

4.1. Introduction

4.1.1 Partners for ARB.

MXC technology has been proposed as a complementing process for current wastewater treatment techniques (Oh et al., 2010). In this setting, MXCs reclaim a small amount of energy from wastewaters while simultaneously decreasing energy input into the treatment process. In order to accomplish this, ARB either produce current directly from the EDs supplied (Kim et al., 1999) or rely on other organisms to hydrolyze and ferment complex EDs prior to current production (Kiely et al., 2011; Parameswaran et al., 2009; Torres et al., 2007). In this chapter, I examine an option available when the ARB community cannot make use of the ED provided: amendment of the community with organisms capable of transforming the given ED to an intermediate which ARB can consume. I specifically examine the use of butyrate, a simple, four carbon SCFA.

Following fermentation of complex compounds and wastewater streams, significant amounts of butyrate often remain (Agler et al., 2011), containing up to 45% of the remaining electrons (Fang et al., 2002). Unlike acetate, butyrate is not used as an electron donor by *Geobacter sulfurreducens* (Caccavo et al., 1994b), one of the ARB found most often in MXC systems and linked to high current densities. Under low partial pressures of hydrogen, such as in the presence of methanogens, butyrate fermentation to acetate and H_2 becomes thermodynamically favorable (Kleerebezem and Stams, 2000). This is also important because high concentrations of SCFAs, including butyrate, inhibit the complete anaerobic degradation of more complex compounds (Siegert and Banks, 2005). MXCs that consume SCFAs beyond just

acetate, therefore, stand to prosper by additionally increasing the degradation of more complex EDs.

Previous studies have demonstrated that mixed cultures from the environment and domestic wastewaters are capable of producing electricity from butyrate in MXCs (Freguia et al., 2010; Liu et al., 2005; Min and Logan, 2004; Teng et al., 2010; Torres et al., 2007; Zhang et al., 2011). The CE with butyrate as electron donor ranges from 8% to 67%, while current densities varied from 0.16 A/m^2 to 0.77 A/m^2 (Freguia et al., 2010; Liu et al., 2005; Torres et al., 2007). It is important to note that these current densities generated are often well below those obtained with ARB fed acetate as the sole electron donor (~10 A/m^2). In many cases, variations in current density can be related to the design of the reactors used, including factors such as cathodic potential losses and ohmic resistance, or how the system is operated (fixed potential vs floating potential); however, the microbial community used to convert EDs to current remains a key factor regardless of the reactor design. It appears that simply relying on naturally derived inocula or even mixed communities developed for other purposes is not the ideal method for obtaining efficient microbial cultures for converting complex compounds to electricity in MXCs.

4.1.2 Co-cultures in MXCs

The development of co-cultures with the intent of generating increased current from particular EDs is an area that has been raising attention recently; so far research has focused on combining pure cultures with defined activities. Known ARB have been paired with different organisms in order to generate current from glucose (Rosenbaum et al., 2011), cellulose (Ren et al., 2007), and corn stover (Speers and Reguera, 2012). Read et al. (2010) also demonstrated that, when known ARB were paired with *Clostridium acetobutylicum* and fed a mixture of acetate and lactate, current production decreased compared to the ARB alone, although a detailed explanation was not provided. It is quite clear that further work is needed in order to understand the possibilities and limits of generating novel microbial cultures for electricity and H_2 production in MXCs. Such work should focus on the mechanisms which enable syntrophic interactions to form between microbial populations and improve CE. Given the complexity of wastewaters, we

not only require efficient pure cultures, but also robust, mixed microbial communities that are suitable for real world, large scale systems.

In this work, I inoculated butyrate fed H-type MECs with two different, but complementary mixed microbial cultures. One inoculum came from a marine hydrothermal vent (MHV) and had the capacity to ferment butyrate to acetate during batch culturing. The other inoculum was from a wastewater treatment plant, but had been previously enriched in an MEC resulting in a large proportion of *Geobacter* in the population and was able to generate current through the consumption of acetate. I hypothesized that, by combining these complementing cultures, I could produce an efficient co-mixed culture able to ferment butyrate and produce high current densities in an MEC.

4.2. Materials and Methods

4.2.1 Microbial Cultures for Inoculating MECs

For these experiments, marine hydrothermal vent sediment samples were obtained from shallow hydrothermal vents (depth 10 m) located in Punta Mita, Nayarit, Mexico (Mexican Pacific Ocean coast) and registering an average temperature of 85 °C (Guerrero-Barajas and García-Peña, 2010). Pyrite (FeS) is present at this location, suggesting the biological formation of sulfide produced by the reduction of sea water sulfate and its combination with the Fe^{2+} derived from iron oxides and the corresponding reactions with organic matter. Hydrothermal vents are a natural source of microbial diversity responsible for biogeochemical cycling in regular and extreme conditions depending on the geology of the sites. The conditions established in these sites favor the presence of sulfate reducing bacteria (SRB), which promote the production of sulfides commonly found in the deposits of these hydrothermal regions. Previous work documented that these sediments harbored a microbial consortium capable of fermenting butyrate to acetate under sulfate reducing conditions (Guerrero-Barajas and García-Peña, 2010). The samples were stored in the dark and at 4 °C prior to use and contained a volatile suspended solids (VSS) content of 0.037 gVSS/g wet sediment as reported previously (Guerrero-Barajas and García-Peña, 2010). The pH of the sample was 8.91. The ARB inoculum was effluent from an acetate-fed MEC seed reactor inoculated with anaerobic sludge from the Mesa Northwest Water

Reclamation Plant, Mesa, AZ. The ARB biofilms of previous MECs started with samples from this waste water treatment plant have routinely contained significant populations of *G. sulfurreducens* (Parameswaran et al., 2009; Torres et al., 2009). The MEC used for inoculum was producing steady current (~9 A/m^2) under a poised anode (-0.30 V vs Ag/AgCl) at the time of inoculum collection.

4.2.2 Mineral media

Batch cultures for the enrichment of sediments and MEC systems contained mineral media prepared as reported by Parameswaran et al. (2009). The media contained 21.3 mM Na_2HPO_4, 3.7 mM KH_2PO_4, 7.7 mM NH_4Cl, 34 mM NaCl, 10 mL of a trace mineral solution, 25 µM $FeCl_2$ solution and 0.1 mM H_2S. The composition of the trace mineral solution was also previously reported by Parameswaran et al. (2009). Sodium acetate and sodium butyrate (at 14 mM) were used as EDs for the different experiments. The enrichment cultures also contained 15 mM sulfate as the electron acceptor.

4.2.3 Enrichment of Sulfate Reducing Fatty Acid Consumers

Separate batch experiments verified the capacity of the MHV microbial population to use acetate as electron donor with sulfate as the electron acceptor and to ferment butyrate to acetate. Batch experiments were performed in serum bottles with 160 mL total volume; 5 g of wet sediments and 100 mL of medium at an initial pH of 7.6 was added to each bottle. After the bottles were sealed with Teflon lined rubber septum and aluminum crimps, N_2 was sparged through the headspace for 15 minutes, ensuring an anaerobic atmosphere. Incubation of the bottles took place in a shaker at 37 °C. Liquid and gas phase samples were taken periodically for analysis.

4.2.4 MEC Setup

MXCs were operated in MEC mode in order to better retain anaerobic conditions inside and to keep the anode constantly polarized. A multi-potentiostat (Bio-Logic VMP3) monitored current production and poised the anode at -0.30 V vs Ag/AgCl using an Ag/AgCl reference electrode (BASi Instruments). This potential was chosen to avoid any limitations to already characterized, efficient ARB (*Geobacter*) for growth on the anode, while encouraging the

selection of any other novel ARB from the sediments by providing a non-limiting anode potential. The MECs were H-types, similar to the schematic shown in Figure 2.2, consisting of two graphite rod electrodes (electrode 1: 6.55 cm x 0.3 cm; electrode 2: 6.05 cm x 0.4 cm; total surface area ~14.1 cm^2; graphitestore.com) for the anodes and two rods (both 6.55 cm x 0.3 cm; total surface area ~12.6 cm^2) for the cathodes. The two chambers were separated by an anion exchange membrane (AMI-7001, Membranes International). The cathode chamber contained distilled water adjusted to pH 12 with NaOH and the anode chamber contained the same medium described above (~360 mL).

After initial set up, the anode chamber was sparged with pure N_2 gas in order to obtain anaerobic conditions. The anode and cathode chambers were stirred with magnetic stir bars at 150 rpm. Four MECs were setup in this manner and kept in an incubator at 37 °C. The control experiment (MEC 1) consisted of 25 mM butyrate in the media and 5 mL of ARB inoculum. Two MECs were inoculated with 20 g of sediment from the marine hydrothermal vent, MEC 2 and MEC 3. 25 mM butyrate as electron donor was provided to MEC 2 and 25 mM acetate to MEC 3. Both MEC 2 and 3 were performed in duplicate. Finally, MEC 4 contained 25 mM butyrate, 20 g of sediment, and 5 mL of ARB inoculum. Like MECs 1-3, MEC 4 was initially run in batch mode; however, it was later switched to continuous flow at a hydraulic retention time of 1 day.

4.2.5 ED and Metabolite Analysis

Liquid samples (1 mL) from the suspension of the MECs and the batch microbial cultures were filtered with 0.22 µm syringe filters (Pall Life Sciences Cat 4455) and analyzed using high performance liquid chromatography (performed on a HPLC Model LC-20AT, Shimadzu) to quantify volatile fatty acids and alcohols, including acetate and butyrate. The samples were analyzed on a HPX-87H (Bio-Rad Laboratories) column at 50 °C with sulfuric acid (2.5 mM) as the eluent at 0.6 mL min^{-1} in order to separate the components and the effluent was analyzed using a photo-diode array (at 210 nm) over 35 minutes. Gas samples (200 µL volume) from the cultures and MECs were taken using a gas-tight syringe (SGE 500 µL, Switzerland) and analyzed by gas chromatography (GC) for gaseous products of microbial activity, specifically CO_2, CH_4 and H_2. The samples were analyzed on a Shimadzu GC 2010, using a packed column (ShinCarbon

ST 100/120 mesh, Restek Corporation, Bellfonte, PA) for separation and a thermal conductivity detector. The carrier gas was N_2 fed at 10 mL min^{-1} at 5.4 atm. The injection loop was set to 110 ℃, the column to 140 ℃, and the detector to 160 ℃. A standard curve was made for calibration using analytical grade H_2, CH_4, and CO_2.

4.2.6 Community Analyses.

Biomass samples from the marine hydrothermal vent sediment, as well as the biofilm and suspension portions of MECs 1 and 4 at the end of each experiment were used for community analysis. DNA was extracted from the biomass using the MoBio Powersoil DNA Extraction kit according to the manufacturer's directions. A clone library was created from the DNA extracted from MEC 1 using primers targeted to the 16S rRNA gene as previously described (Torres et al., 2009). 52 clones were picked and sequenced from the sediment, 43 from the biofilm, and 23 clones from the suspension.

DNA extracted from the MHV sediment and from the biofilm and suspension of MEC 4 was sent to Research and Testing Laboratory (Lubbock, TX) for 454 pyrosequencing as described in Garcia-Peña et al. (2011). The gene target was the 16S rRNA gene of general bacteria using the commercially available Blue primers (104F-530R) targeting the V2 and V3 regions of the 16s rDNA. Sequences were analyzed using the mothur software (www.mothur.org) as described in Garcia-Peña et al. (2011). Single sequences were removed and the number of sequences was normalized in all sets to that of the smallest set: 5973 sequences. For classification, a bootstrap cutoff of 50 % was used as recommended by RDP when using sequences below 250 bp in length.

Quantitative real-time PCR was used to quantify methanogens in DNA extracted from the MHV sediment as well as the biofilm and suspension from MEC 4 using TaqMan probes. The probes targeted general Archaea (primers Arc787F, Arc1059R, TaqMan probe Arc915F), the orders Methanobacteriales (primers MBT857F, MBT1196R, TaqMan probe MBT929F) and Methanomicrobiales (primers MMB282F, MMB832R, TaqMan probe MMB749F), and the families Methanosarcinaceae (primers Msc 38F, Msc828R, TaqMan probe Msc492F) and Methanosaetaceae (primers Mst702F, Mst862R, TaqMan probe Mst753F) as previously

described (Yu et al., 2005). Reactions were performed in 10 µL volumes on 96 well plates. Each reaction mixture contained 4.5 µL of Real Master Mix (5' Prime, Cat #2900237), 0.03 µL of TaqMan Probe (final concentration 300 nM), 0.5 µL each of forward and reverse primers (final concentration 5 µM) and 4 µL of template DNA at ~10 ng μL^{-1}. TaqMan probes were labeled with FAM and the reactions were performed and monitored using a Realplex Mastercycler ep gradient S (Eppendorf). Archaea, Methanobacteriales, Methanomicrobiales, and Methanosaetaceae assays were initiated at 93 °C for 30 seconds followed by 45 cycles of melting (93 °C for 10 seconds) and binding/elongation (63 °C for 30 seconds). For Methanosarcinaceae, binding/elongation was performed at 60 °C.

4.2.7 ED Spike Experiments.

In order to investigate if butyrate was directly consumed by the biofilm or if initial fermentation of butyrate to acetate by the co-mixed culture was needed prior to utilization by ARB, spike experiments were performed on MEC 4 in batch mode after it had a well-developed biofilm, as determined by steady current production under continuous flow operation. The current in an ED-depleted MEC was first allowed to decrease below < 0.5 A m^{-2}. Then, either butyrate or acetate was introduced to the MEC using a syringe. Butyrate was introduced at 1 M (5 mL) to a final concentration of ~14 mM. Acetate was introduced at 1 M (5 mL) to a final concentration of ~14.2 mM. The instantaneous change in current density after each ED spike was recorded.

4.3. Results and Discussion

4.3.1 Acetate and butyrate consumption in batch experiments

In order to enrich the microbial population of the MHV sediments for either acetate or butyrate consumers, I inoculated sediments in two batch cultures using either acetate or butyrate as electron donor and sulfate as electron acceptor. Electron balances for these cultures are shown in Figure 4.1. Acetate (Figure 4.1, A) was used as a control to show that the sediments were viable and that the culture technique was appropriate to grow anaerobic sulfate reducers, which include relatives of *Geobacter*, regardless of successful butyrate fermentation. In the acetate fed cultures, around 70% of the total acetate supplied was consumed during the first four days of cultivation; complete acetate degradation occurred after 11 days. In the butyrate fed

cultures (Figure 4.1, B), butyrate was initially transformed to acetate by the microbial population starting at day 4, and the complete removal of both EDs was observed after 11 days. Detection of CO_2 gas in the headspace indicates that some mineralization of acetate and butyrate occurred. These data suggested the possible use of the MHV sediments in MEC systems using either acetate or butyrate as electron donors.

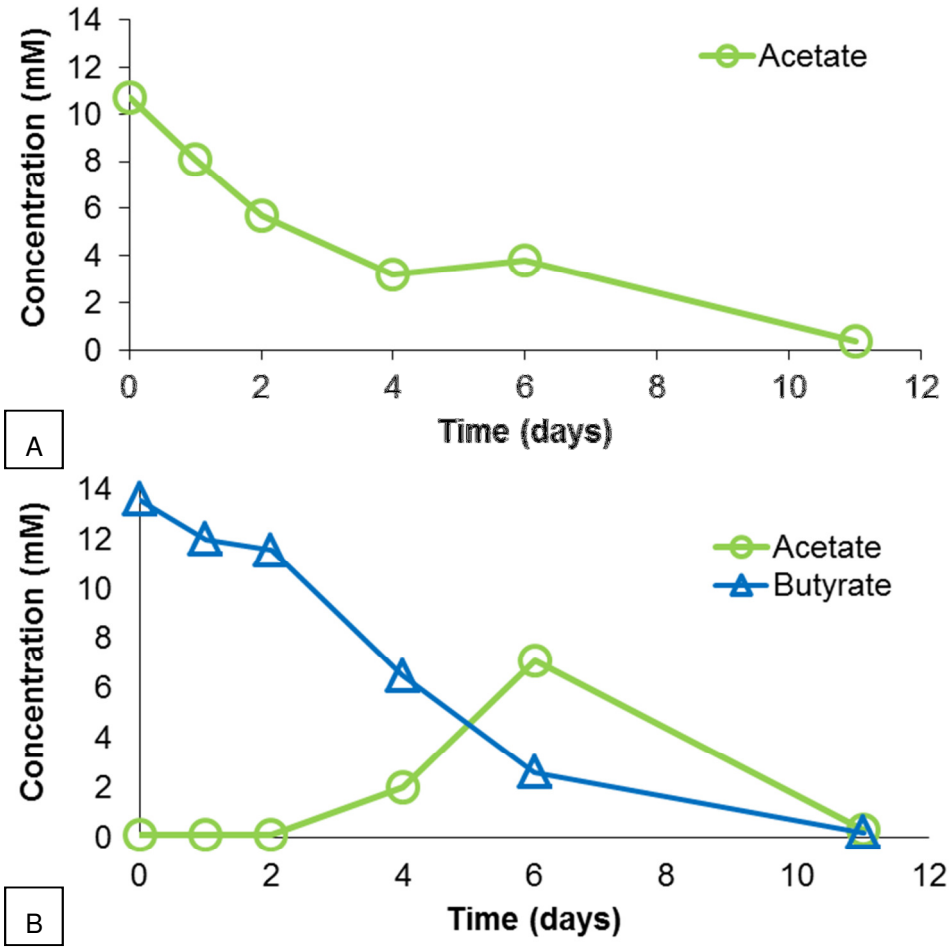


Figure 4.1. Electron balances of fermentation by serum bottle cultures of MHV bacteria performing: (A) degradation of acetate and (B) production of acetate from butyrate followed by degradation of acetate.

4.3.2 Current generation by individual microbial cultures

Two separate mixed-culture MECs (MECs 1 and 2) proved unable to produce high current densities from butyrate. When I placed the inocula from a highly enriched, current producing, acetate fed MEC in a new MEC with butyrate as the sole electron donor (MEC 1), very little current was produced (<0.1 A m^{-2}, see Figure 4.2, panel A). The inoculum from the acetate-fed MEC consisted mostly of *G. sulfurreducens* (Torres et al., 2009), an ARB known to consume acetate but not butyrate (Caccavo et al., 1994b). Without organisms to ferment butyrate to acetate, the *Geobacter* rich culture could not produce current from butyrate. Both MHV sediment culture only MECs (MECs 2 and 3) also showed only minor electrogenic activity, producing current densities of ~0.2 A m^{-2} (Butyrate fed MEC 2 shown in Figure 4.2, panel B; Acetate fed MEC 3 data not shown). Figure 4.3 shows the electron balance of MEC 2 where butyrate was clearly fermented to acetate. In spite of this, only a very small amount of the electrons provided as butyrate were ultimately channeled into current, indicating either a lack of acetate consuming ARB or unfavorable conditions for current production. MEC 3's failure to generate more than ~0.2 A m^{-2} further confirmed that the mixed culture from the MHV sediments lacked acetate consuming ARB.

I analyzed the microbial community of the MHV sediment as well as the suspension and the small biofilm attached to the electrode of MEC 2 using a clone library (Figure 4.4). The organisms detected were mainly fermenters along with some organisms known for only weak interactions with the anode. No *G. Sulfurreducens* sequences were detected.

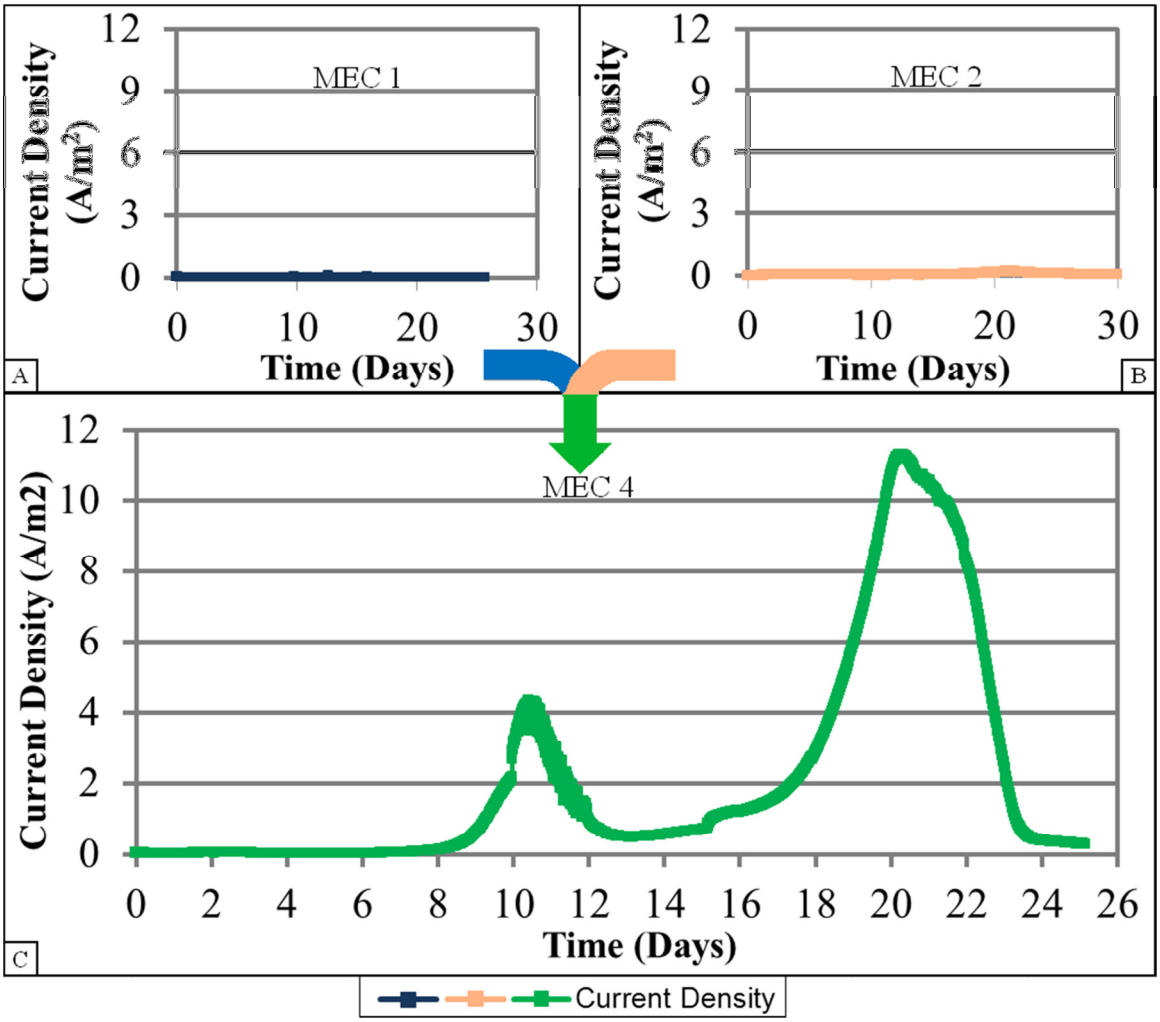


Figure 4.2. Current density from three MECs: (A) Butyrate fed *Geobacter* culture (MEC 1) (B) Butyrate fed MHV culture (MEC 2), and (C) Butyrate fed, MHV-*Geobacter* co-mixed culture (MEC 4).

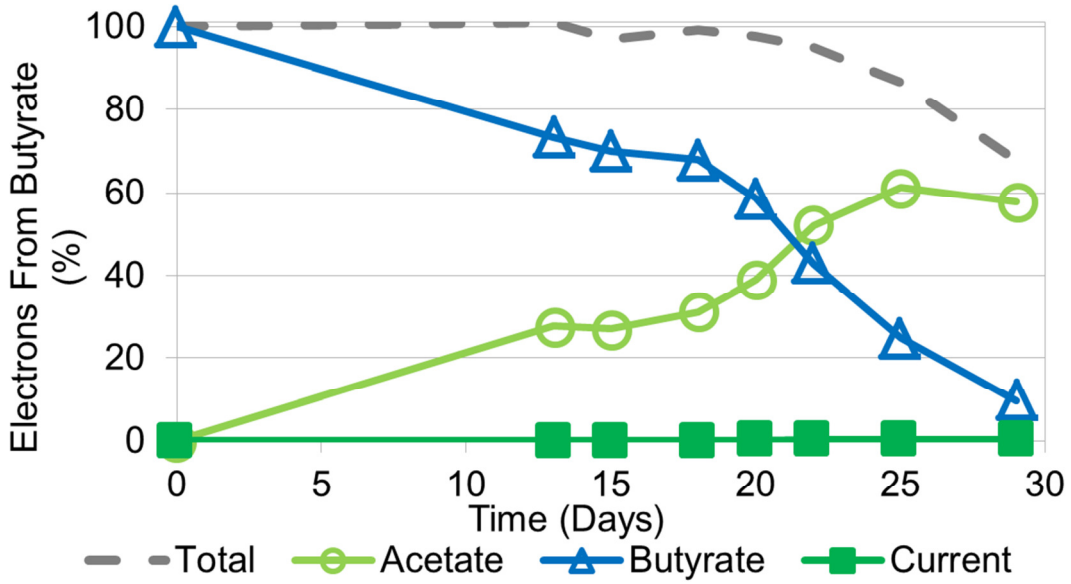


Figure 4.3. Electron balance for MHV sediment culture in an MEC, fed with butyrate. MEC 2.

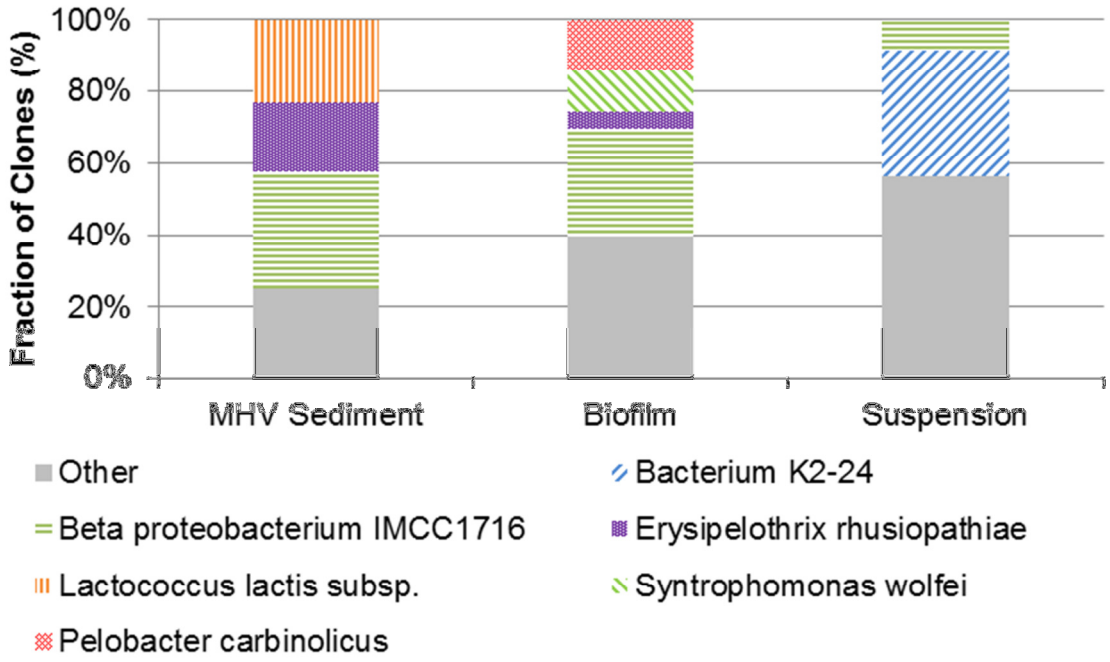


Figure 4.4. Clone library results from butyrate fermenting, low current MHV culture, species level.

4.3.4 Current generation by combining the MHV and *Geobacter* rich ARB cultures in a butyrate fed MEC

Next, I amended the MHV sediment culture with the *Geobacter* containing culture in an MEC fed with butyrate (MEC 4). Once combined together, this co-mixed culture fermented butyrate and successfully produced electricity. Current densities (Figure 4.2, panel C) were similar to those found previously with *Geobacter* cultures fed on only acetate (~10 A m^{-2}) in other fixed potential systems. Data from volatile fatty acid analysis indicates that the electricity was produced through intermediate acetate production and consumption (Figure 4.5). CE for this culture was ~70 % by the end of the experiment. Methane was detected by GC and hydrogenotrophic methanogens (Methanobacteriales) were detected using qPCR (Figure 4.6). The missing electrons in Figure 4.5 were likely diverted to methane and biomass, although neither sink was quantified. The high recovery of electrons in the MHV culture alone as products (Figure 4.3) was likely due to greater biomass being present, resulting in a very small loss of electrons to biomass production.

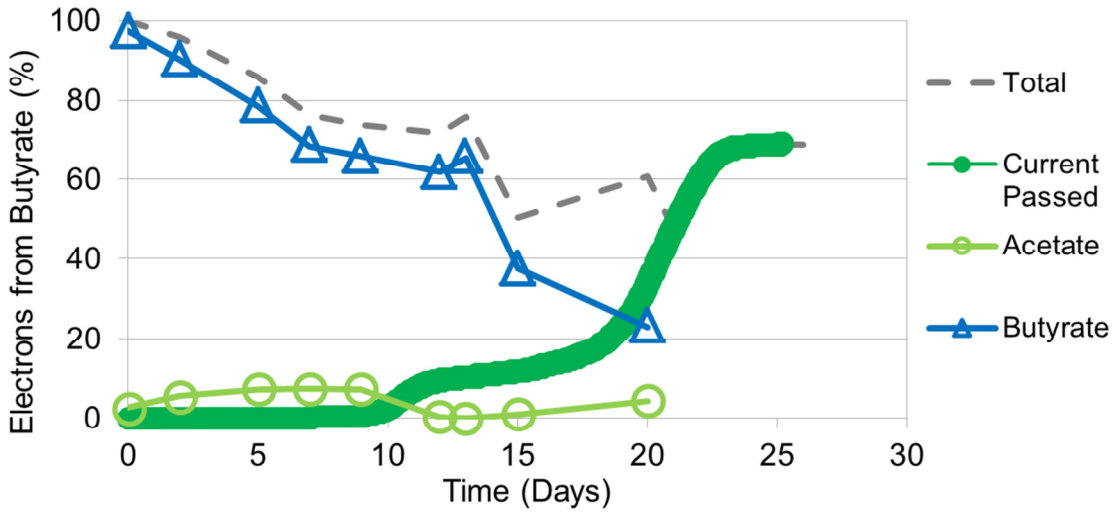


Figure 4.5. Electron balance for a butyrate fed MEC inoculated with a culture from a marine hydrothermal vent, capable of butyrate fermentation, and a *Geobacter* rich culture enriched from anaerobic digested sludge (MEC 4).

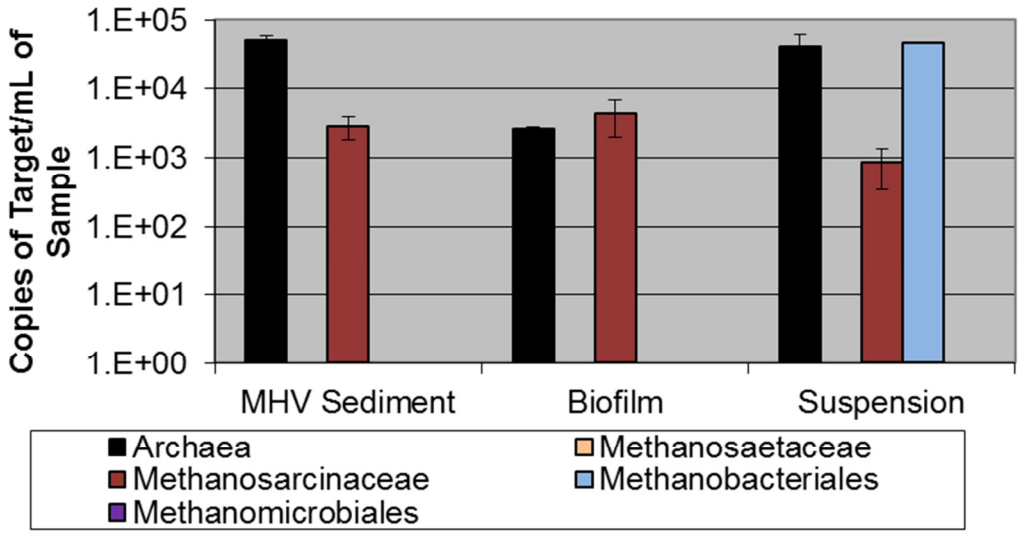


Figure 4.6. Methanogen groups detected in MHV sediment and MEC 4.

The ARB and butyrate fermenting organisms appear to be largely physically separated in MEC 4, as observed using pyrosequencing. A summary of the community analysis results at the class level are shown in Figure 4.7, along with community data from the ARB inoculum, published previously (Torres et al., 2009). Deltaproteobacteria (the class that includes the known ARB *Geobacter*) dominated the microbial culture in the current-producing biofilm, similar to the current producing biofilm community used as ARB inoculum. Evaluating these data together with the high current density obtained and the presence of acetate in the system, I therefore conclude that *Geobacter* were the main ARB generating current.

The suspension appears to demonstrate influences from the sediment and the biofilm communities: a high proportion of Gammaproteobacteria, similar to the sediment, and some Clostridia, Betaproteobacteria, and Epsilonproteobacteria, which are seen in the biofilm and suspension, but not the original sediment. Clostridia have been recorded previously in a MXC fed with butyrate and inoculated with anaerobic sludge (Chae et al., 2009), while Gammaproteobacteria are known fermenters and have been linked to the production of current from glucose (Chung and Okabe, 2009) and acetate (Pham et al., 2003). Butyrate was fermented to acetate by a mixture of mainly Gammaproteobacteria and Clostridia. The large

proportion of Deltaproteobacteria sequences found in the suspension likely resulted from anode biofilm detachment.

This physical separation between communities performing different functions has important implications, both positive and negative, for the design of MXC systems. Suspended and biofilm communities can be subjected to different retention times for the control of biomass production. Unfortunately, suspended communities will also be more susceptible than biofilm communities to washout at higher flow rates.

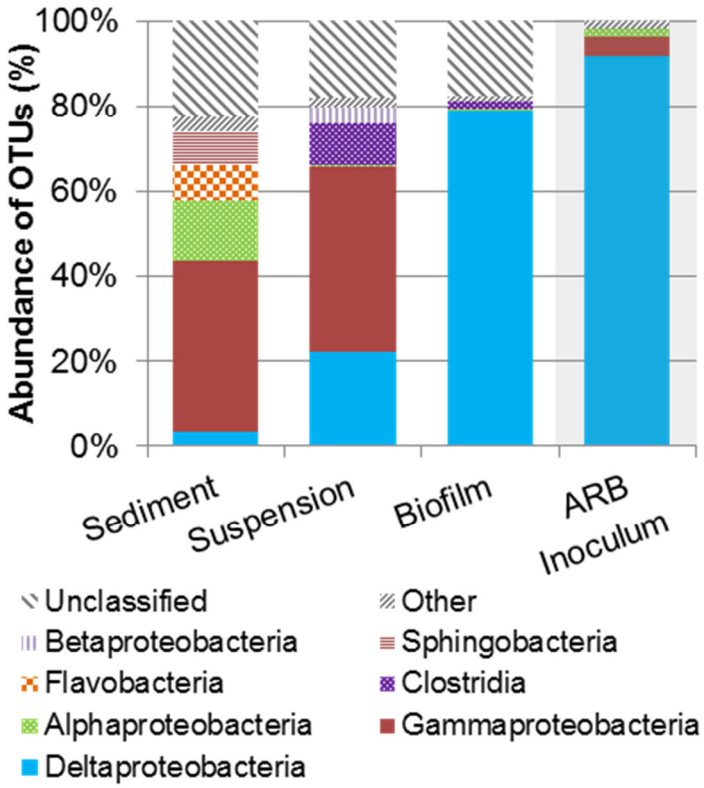


Figure 4.7. Microbial community analysis at the class level for the hydrothermal vent sediment, the butyrate fed MHV-Geobacter co-mixed culture MEC 4 suspension, the same MEC's biofilm, and the ARB inoculum used (previously published in Torres et al. (2009)). ARB Inoculum is highlighted in grey to emphasize it was analyzed at an earlier time point.

4.3.5 Current Response to Acetate and Butyrate Spikes in MECs.

I performed spike experiments on MEC 4 to better understand whether current production came from direct oxidation of butyrate or through intermediate acetate fermentation in the butyrate fed MECs. Organic acid analysis indicates that the consortium fermented butyrate to acetate when butyrate was used as an ED for electricity production by the ARB. Figure 4.8, panel A, shows the lagging response of the anode biofilm upon addition of butyrate, on the order of hours, in comparison to a much quicker response to the addition of acetate, on the order of minutes. Figure 4.8, panel B, shows the decrease in butyrate observed over time, with only a minor and temporary increase in acetate concentration. Acetate levels in the bulk liquid (~7 mM) do not reach those often associated with the high levels of current density found here. Given that acetate is a known ED for ARB biofilms(Caccavo et al., 1994b; Freguia et al., 2010; Kiely et al., 2011; Torres et al., 2009), the lag in current response to ED addition and the presence of acetate in the media indicate that the consortium fermented butyrate to acetate prior to acetate oxidation at the anode for the production of current.

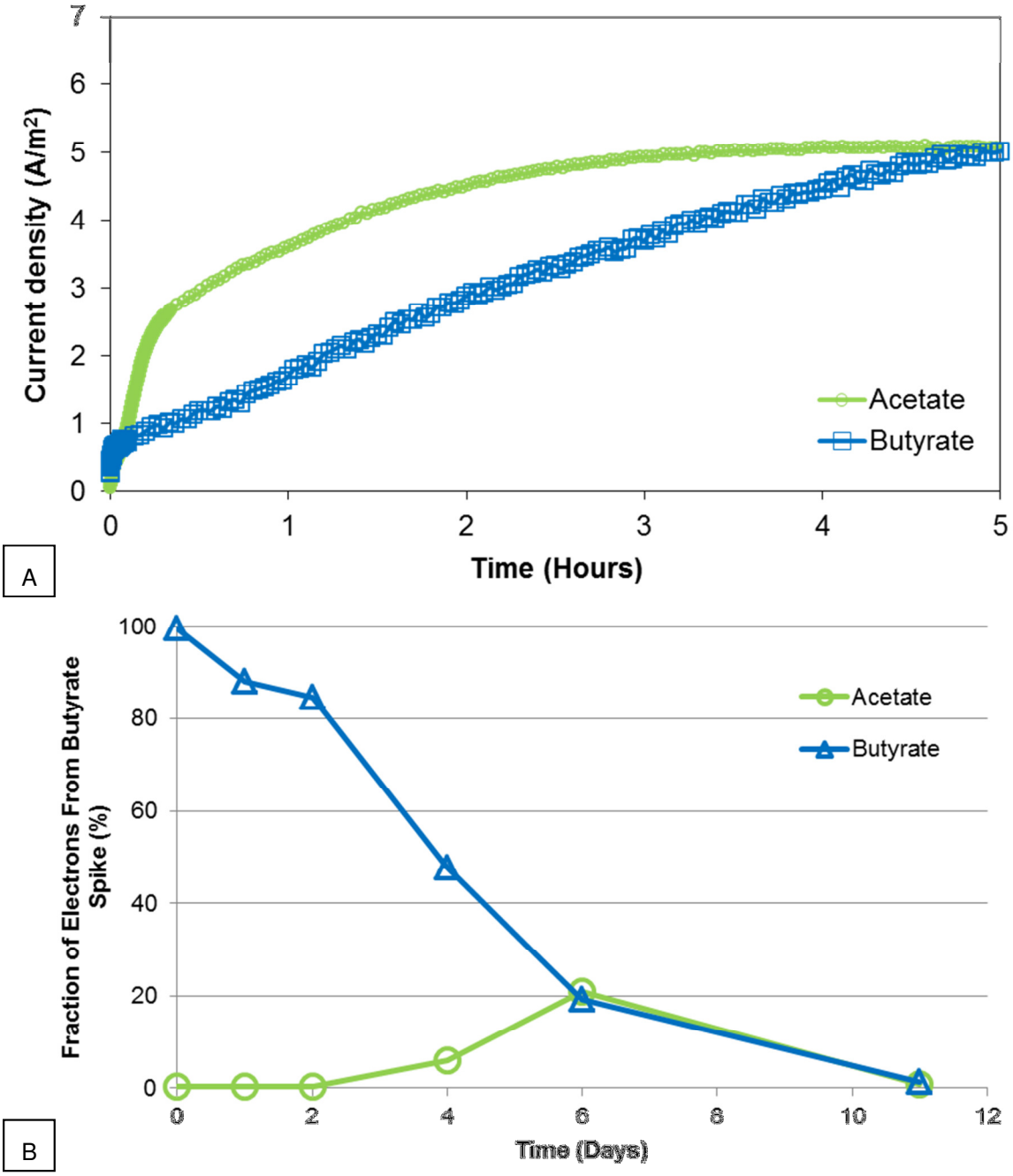


Figure 4.8. Co-mixed culture: A) current response to butyrate and acetate spikes, B) butyrate and acetate concentrations during butyrate spike

4.4. Conclusions

This work demonstrates the capacity to amend highly efficient ARB cultures with other microbial cultures displaying interesting metabolic capabilities in order to build metabolic pathways for the utilization of complex feeds in MXCs. This enhanced co-culture rivals and in some cases surpasses those derived naturally or through enrichment, by producing high current densities (~10 A m^{-2}) and high CE (~70 %). By assembling microbial communities containing the best organisms for each desired metabolic step I can quickly, easily, and reliably produce mixed communities to perform desired applications.

CHAPTER 5

THE EFFECTS OF SHIFTING THE BALANCE OF FERMENTATION PRODUCTS BETWEEN HYDROGEN AND VOLATILE FATTY ACIDS ON MICROBIAL COMMUNITIES

5.1. Introduction

5.1.1 Overview

After searching for novel ARB and building mixed microbial communities in Chapters 3 and 4, I became interested in the question of how these systems could be managed more efficiently. During initial work on the project that would become Chapter 6, I encountered issues in controlling methane production during fermentation. My control cultures, fed with fermentable EDs and provided anaerobic digested sludge as inoculum, repeatedly failed to produce methane. This led me to the research discussed here, in Chapter 5, where I examine fermentation and associated microbial communities.

Fermentation is a process of great importance in many industrial and environmental biotechnologies as well as in the natural environment. It is used industrially to produce beverages, foods, and chemical compounds as well as to clean wastewaters and to enhance bioremediation processes in groundwater (Agler et al., 2011; Parkin et al., 1986; Ziv-El et al., 2012a, 2012b). In human intestines, it improves digestion, and recent work indicates that fermentation is a key element for host-microbe interactions and may contribute to some diseases (Krajmalnik-Brown et al., 2012; Zhang et al., 2009). Fermentation is a metabolic process, often performed by microorganisms, where energy is extracted from carbon compounds without the use of an external electron acceptor, such as oxygen or sulfate (Hoelzle et al., 2014).

5.1.2 Fermentative H_2

During fermentation, microorganisms generate H_2, SCFAs, and alcohols (Rodríguez et al., 2006; Thauer et al., 1977). Figure 5.1 illustrates some of the metabolic pathways involved in the fermentative breakdown of glucose. During glycolysis, NAD^+ is reduced to NADH and pyruvate is formed (reviewed in Bodner, 1986). The NADH must be re-oxidized in order to replenish the NAD^+ pool for further glycolysis. Under fermentative conditions, the electrons from oxidizing NADH can be sent to reduce protons for H_2 production (Rodríguez et al., 2006).

Pyruvate can be channeled to the TCA cycle for acetate formation, which involves the reduction of the protein ferredoxin. Similar to NADH, ferredoxin can be re-oxidized with the evolution of H_2 (Mortenson et al., 1963, 1962; Tagawa and Arnon, 1962). In both cases, the production of H_2 faces thermodynamic limitations, reviewed by Schink (1997).

Briefly, the pathways that produce H_2 during fermentation are favored only at low H_2 partial pressures due to the lower Gibbs free energy of the chemical reactions (Angenent et al., 2004; Reichardt et al., 2014; Schink, 1997; Tanisho et al., 1989). According to Angenent et al. (2004), generating H_2 with electrons from NADH during fermentation is inhibited at H_2 partial pressures above 0.0006 atm, or 0.47 µM in the solution (Angenent et al., 2004). Due to the more negative redox potential of ferredoxin ($E^{O'}_{fer.}$= -400 mV) compared to NADH ($E^{O'}_{NADH}$= -320 mV), the production of H_2 through ferredoxin can be sustained up to H_2 partial pressures of 0.3 atm, or 237 µM in solution (Angenent et al., 2004). As the concentration of H_2 increases, the change in free energy caused by the reaction also increases until it becomes positive and the reaction is no longer thermodynamically favorable.

In anoxic environments such as sediments, wetlands, landfills, sewage digesters, and the human intestines, hydrogenotrophic methanogens, which produce methane, and homoacetogens, which produce acetate, keep H_2 concentrations low enough that the production of H_2 during fermentation remains thermodynamically favorable (Lee and Zinder, 1988; Ragsdale and Pierce, 2008). This leads to faster fermentation rates (Krajmalnik-Brown et al., 2012; McInerney et al., 2008; Schink, 1997; Zhang et al., 2009).

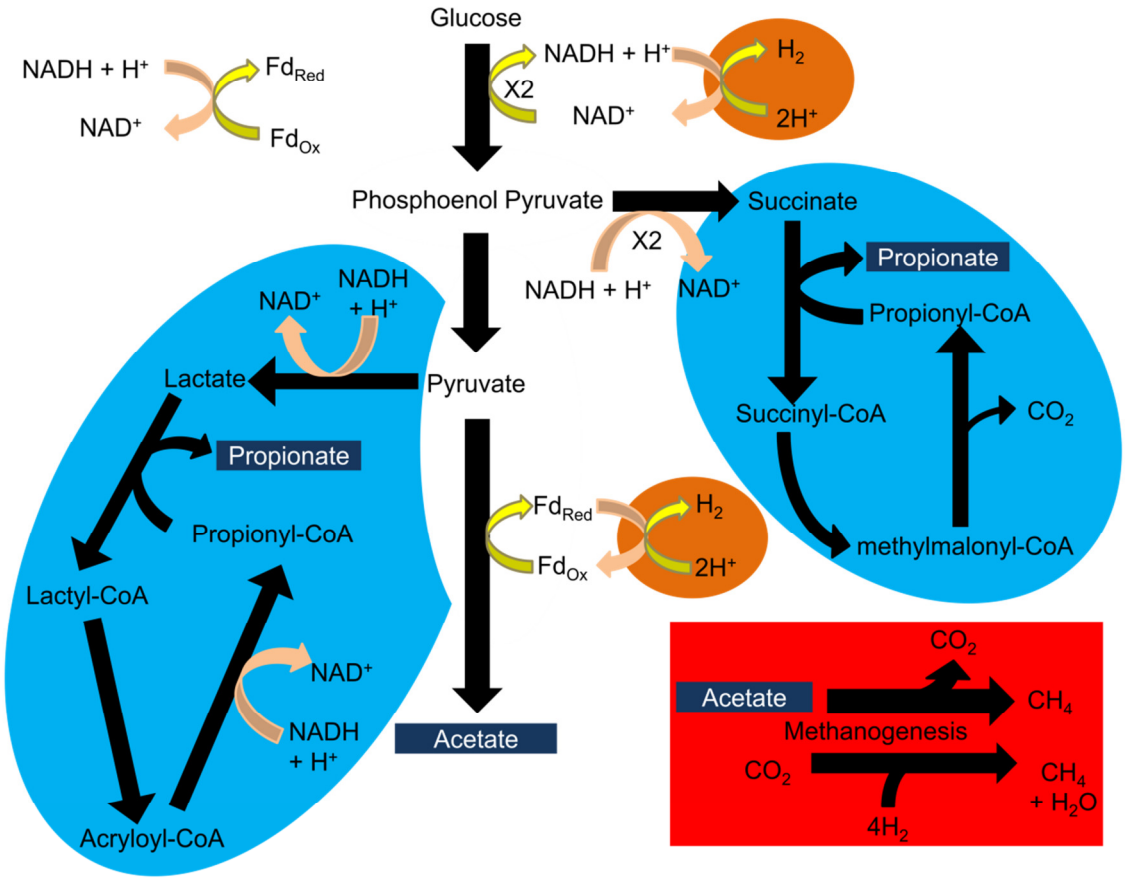


Figure 5.1. Selected pathways involved in the fermentation of glucose with the production of acetate, propionate, and H2, as well as the use of either acetate or H_2 for methanogenesis. A (+) indicates gene was predicted by PICRUSt, (NA) indicates gene was not predicted.

5.1.3 Production of Propionate

When the thermodynamic threshold is reached, due to H_2 production outpacing H_2 consumption, the fermenting organisms must slow down H_2 production. Under these conditions, fermenters do not cease consuming available EDs. Instead, there is a shift in the metabolites produced as discussed by Ruzicka (1996), Angenent et al.(2004) and Hoelzle et al.(2014) In order to replenish the pools of NAD^+ and oxidized ferredoxin, the microorganisms produce compounds such as ethanol, propionate, and lactate (Hoelzle et al., 2014). During propionate formation, bacteria use electrons from NADH in either of two pathways for the production of propionyl-CoA (Hetzel et al., 2003; Swick and Wood, 1960), followed by the release of propionate (highlighted by blue ovals in Figure 5.1). These, and similar reactions, enable the continued fermentation of more complex, higher energy-carrying compounds, such as glucose, through the

production of simpler compounds, such as propionate and butyrate, without necessarily producing more H_2. These pathways have been further demonstrated in the absence of higher organic compounds for the production of C-6 and C-8 carbon fatty acids using acetate (a two carbon fatty acid) as the starting point with H_2 as the electron source, thus building up fatty acids with longer carbon chains from a shorter fatty acid (Steinbusch et al., 2011). When forming H_2 from ferredoxin becomes unfavorable, the electrons in ferredoxin can be transferred to either NAD^+, and used to form more reduced products as described above, or the electrons can be transferred to $NADP^+$ and used for biosynthesis (Jungermann et al., 1973).

The thermodynamic inhibition of H_2 production and its resultant shift in fermentation product spectrum has important implications in different environments such as anaerobic digestion, the human gut, and biohydrogen production. In anaerobic digesters, the increased production of propionic and butyric acids that occurs when H_2 builds up can decrease the pH so much that methanogenesis shuts down (McCarty et al., 1963; Parkin et al., 1986). In the human gut, the production of SCFAs, through fermentation, leads to an increase in calories available from foods for absorption (Krajmalnik-Brown et al., 2012; Zhang et al., 2009). In biohydrogen production, it is important to maintain low H_2 concentrations in order to obtain the greatest yield of H_2 from EDs (Logan et al., 2002; Van Ginkel and Logan, 2005).

Controlling the balance of fermentation products also has applications in emerging biotechnologies such as dechlorination and microbial electrochemical cells. In dechlorination, H_2 is the preferred ED for anaerobic organisms which break down chlorinated ethenes (Ziv-El et al., 2012a, 2012b). In microbial electrochemical cells, anaerobic organisms convert SCFAs to electricity and methanogenesis can take up electrons through H_2 (Parameswaran et al., 2010, 2009). Diverting electrons to H_2 thus improves some systems and is undesirable in others.

5.1.4 Previous work

Although the shift of fermentation products due to H_2 production has been described in the literature (Logan et al., 2002; Ruzicka, 1996; Van Ginkel and Logan, 2005), the major focus has been on glucose fermentation as a representative compound, the process of methanogenesis is not usually included, nor have the associated microbial communities and their

responses to these changes been examined. Waste streams are not only made of glucose, or compounds which decompose strictly into glucose; therefore it is important to include other common fermentable EDs when studying fermentation. Given the difficulty in preventing methanogenesis in systems which have a continuous influx of microbial biomass, it is important to include methanogens when studying the effect of ED loading on the distribution of fermentation products. Finally, whether or not microbial communities respond to changes in ED concentrations, as opposed to responding only to ED type, by altering their structure or function can have consequences in bioreactor stability and efficiency. For the first time, I report changes in the microbial community structure related to changes in the balance of fermentation products produced from various fermentable EDs fed at different concentrations.

In order to study the shift in the balance between H_2 and SCFAs production during fermentation and its effect on microbial communities, I set up fermentation cultures in batch serum bottles fed ethanol, lactate, glucose, sucrose, and molasses as ED. I varied the initial concentrations of EDs and monitored SCFAs, methane, and H_2 as fermentation products and the associated microbial community structures and predicted functions that developed as a result of these ED variations.

5.2 Materials and Methods

5.2.1 Fermentation Only Batch Experiments

I performed fermentation batch experiments in triplicate in 150 mL serum bottles. The serum bottles contained 100 mL of enrichment media: 1.5 mM KH_2PO_4, 9.3 mM NH_4Cl, 17.1 mM NaCl, 4.2 mM $MgCl_2$, 6.7 mM KCl, 1.4 mM $CaCl_2$, 100 mM $NaHCO_3$, 0.1 mM H_2S, 25 µM $FeCl_2$, and 2 mL L^{-1} of trace minerals solution. The trace minerals solution contained 1.7 mM EDTA, 345 µM $CoCl_2$, 1 mM $CaCl_2$, 162 µM H_3BO_3, 97 µM Na_2MoO_4, 5.8 µM Na_2SeO_3, 39 µM Na_2WO_4, 154 µM $NiCl_2$, 12.2 mM $MgCl_2$, 4.7 mM $MnCl_2$, 367 µM $ZnCl_2$, 400 µM $CuSO_4$, and 23 µM $AlK(SO_4)_2$. I sparged 80% N_2:20% CO_2 gas through the media to make it anaerobic, prior to and during the addition of the reagents, adjusted the initial pH of the media to ~7.6 with 1M HCl, and sterilized all culture bottles. I performed fermentation experiments with ethanol, lactate, glucose, sucrose, and molasses at starting concentrations of 100, 200, and 400 me^- eq. L^{-1} (or 800 – 3200

mg L^{-1} COD). The starting concentrations were normalized to electron equivalents for comparison across EDs. The corresponding ethanol and lactate concentrations are approximately 8.3, 16.6, and 33.3 mM. For glucose, the concentrations are approximately 4.2, 8.3, and 16.6 mM. The equivalent concentrations for sucrose are 2.1, 4.2 and 8.3 mM. As will be explained later, I also performed lactate experiments at 0.83, 1.66, and 3.33 mM. I inoculated each culture with 1mL of anaerobic digested sludge (ADS) obtained from the Mesa Northwest Water Reclamation Plant (Mesa, AZ). The cultures were incubated at 37 ºC and 150 rpm. Both liquid and gas samples were taken daily for the first four days, and then periodically to quantify SCFAs, H_2 and methane.

5.2.2 Microbial community analysis.

In order to perform community analysis, I collected biomass samples and extracted DNA. At the beginning of each experiment, I took 1 mL of the anaerobic digested sludge, placed it in a clean sterile 1.5 mL falcon tube previously cross linked in a SpectroLinker for 5 minutes, and froze it for future DNA extractions. I also obtained 1 mL of each culture when the original ED was depleted (day 4 for ethanol, glucose, sucrose, and molasses and day 3 for lactate). The samples were placed into -80 ºC storage. DNA was extracted from the samples using the MoBio Power Soil DNA kit (MoBio Laboratories, Carlsbad, CA, cat. 12888) and quality and quantity were checked on a Nanodrop ND-1000 spectrophotometer. DNA samples were sent to the University of Minnesota Genomics Center's Core facility for sequencing on an Illumina MiSeq sequencer using 3rd generation chemistry. Primers targeting the V4 –V6 region (V4F: GTGCCAGCMGCCGCGGTAA and V6R: CGACRRCCATGCANCACCT) 515F – 1064R were used for paired end sequencing and approximately 280 bp were sequenced from each end.

Paired ends were combined using Pandaseq (Masella et al., 2012), the assembly algorithm from the RDP paper (Cole et al., 2013), elimination of all sequences with uncalled nucleotides, and a threshold of 0.95. Sequences were analyzed using the QIIME software package as described in Ruiz et al. (2014), except as noted below. I used QIIME version 1.8 (Caporaso et al., 2010) and a sequence length cutoff of 500bp. I assigned taxonomy using the 97% similarity set of OTUs from the Greengenes 13_5 database (McDonald et al., 2012). I

rarefied the samples to 6242 OTUs, the number of OTUs present in the smallest sample as described previously (Delgado et al., 2014). I used the QIIME beta diversity through plots script to generate a PCoA of the communities based on the weighted UNIFRAC distance metric (Lozupone et al., 2005) and the plots were visualized using Emperor (Vázquez-Baeza et al., 2013). I used the QIIME make distance boxplots script to compare the distances, also based on the weighted UNIFRAC distances, between samples based on concentration and ED provided, including a two sample t-test to test the significance of the differences in the distances between sets of samples.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) was used to predict the metagenome of the microbial communities observed (through the Galaxy web application available at http://huttenhower.sph.harvard.edu/galaxy/). Phylotypes were picked using Greengenes 13_5 database (McDonald et al., 2012) as a closed reference set, with 0.97 similarity used as the cutoff, and assigned taxonomy. Phylotypes were normalized by 16S rRNA gene copy number and a metagenome was constructed based on the taxonomic assignments of the phylotypes. This metagenome was used to identify and quantify the presence of genes related to propionate production. These genes were identified from the KEGG database and the work of Reichardt et al. (2014) . The metagenome was used in the metagenome contributions script to determine which phylotypes generated the prediction of propionate production related genes.

5.2.3 Analysis of Fermentation Products.

I analyzed liquid samples from the cultures using HPLC (Model LC-20AT, Shimadzu, Columbia, MD) and gas samples from the headspace of the cultures using GC as previously described (Miceli et al., 2014). For sugars, I analyzed the liquid samples on the same HPLC system, at 30 ℃ with 18 MΩ reverse osmosis water as eluent and a refractive index detector (Waters, RID 2414).

5.3. Results and Discussion

5.3.1 Increasing ED Concentration Decreases Methane Production

Figure 5.2 shows the distribution of electrons from the initial EDs to acetate, propionate, butyrate, and methane when the original ED was depleted (day 4 for ethanol, glucose, sucrose, and molasses and day 3 for lactate). The days shown were selected based on the initial ED having been consumed and acetoclastic methanogenesis not yet having clearly taken over. Across all fermentation cultures, as the starting substrate concentration increased, the fraction of electrons going to methane decreased (averaged across substrates, from 34% to 6%) while the fraction of electrons going to acetate, propionate and butyrate increased (from 52% to 82%). Both increasing ED concentration and complexity of the ED lead to increasing ratios of propionate:acetate and butyrate:acetate. For breakdowns of the products over time for individual experimental conditions, see Appendix A.

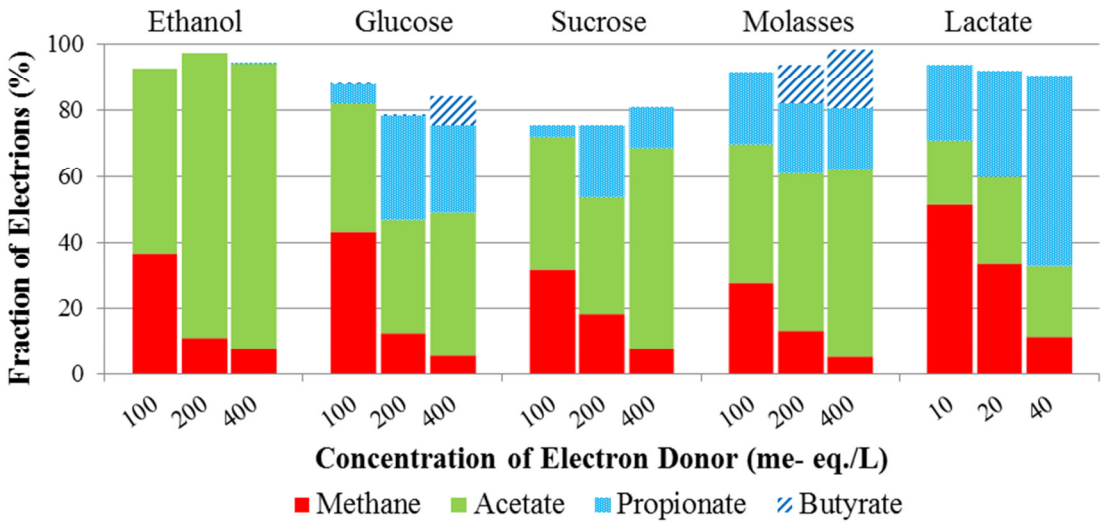


Figure 5.2. Distribution of electrons from initial ED to methane, acetate, propionate, and butyrate. The data shown are from day 4 of the ethanol, glucose, sucrose, and molasses experiments and from day 3 of the lactate experiment.

Experiments performed with lactate at 100, 200, and 400 me^- eq. L^{-1}, corresponding to 8.3, 16.6, and 33.3 mM, did not show the above mentioned pattern. The electron balance for lactate fed cultures at these concentrations, shown in Figure 5.3, shows low production of methane with coincident production of acetate and propionate, including a high ratio of propionate to acetate, across the experimental conditions. Additional tests with lactate concentrations at

$1/10^{th}$ the concentration of the previous experiments successfully replicated the trend of high methane production at low concentrations of initial ED; an electron balance for these experiments is shown in Figure 5.4. This variation appears to be due to the metabolic pathways involved in lactate utilization. Lactate is an intermediate in the pathways of propionate formation, as seen on the left hand side of Figure 5.1. In order to utilize lactate as ED, the step from pyruvate to lactate, removing reducing equivalents, must work in reverse of the direction shown. In this manner, pyruvate would be generated from lactate while also generating reducing equivalents. In order to get rid of these reducing equivalents in an environment with a high H_2 partial pressure, propionate must be formed from additional molecules of lactate, removing ED from the environment. I therefore attribute lactate's lower threshold for hydrogen, and thus the lower initial concentration required for the shift in products, to lactate's direct use as an acceptor of reducing equivalents.

Additionally, I observed that longer SCFAs than acetate (i.e. propionate and butyrate) were produced in increasing quantities as I increased initial ED concentrations. This agrees with my expectations that by producing sufficient H_2, and thus overloading the thermodynamics of H_2 production and the rate of consumption, fermentation cultures were forced to route electrons to more reduced carbon compounds.

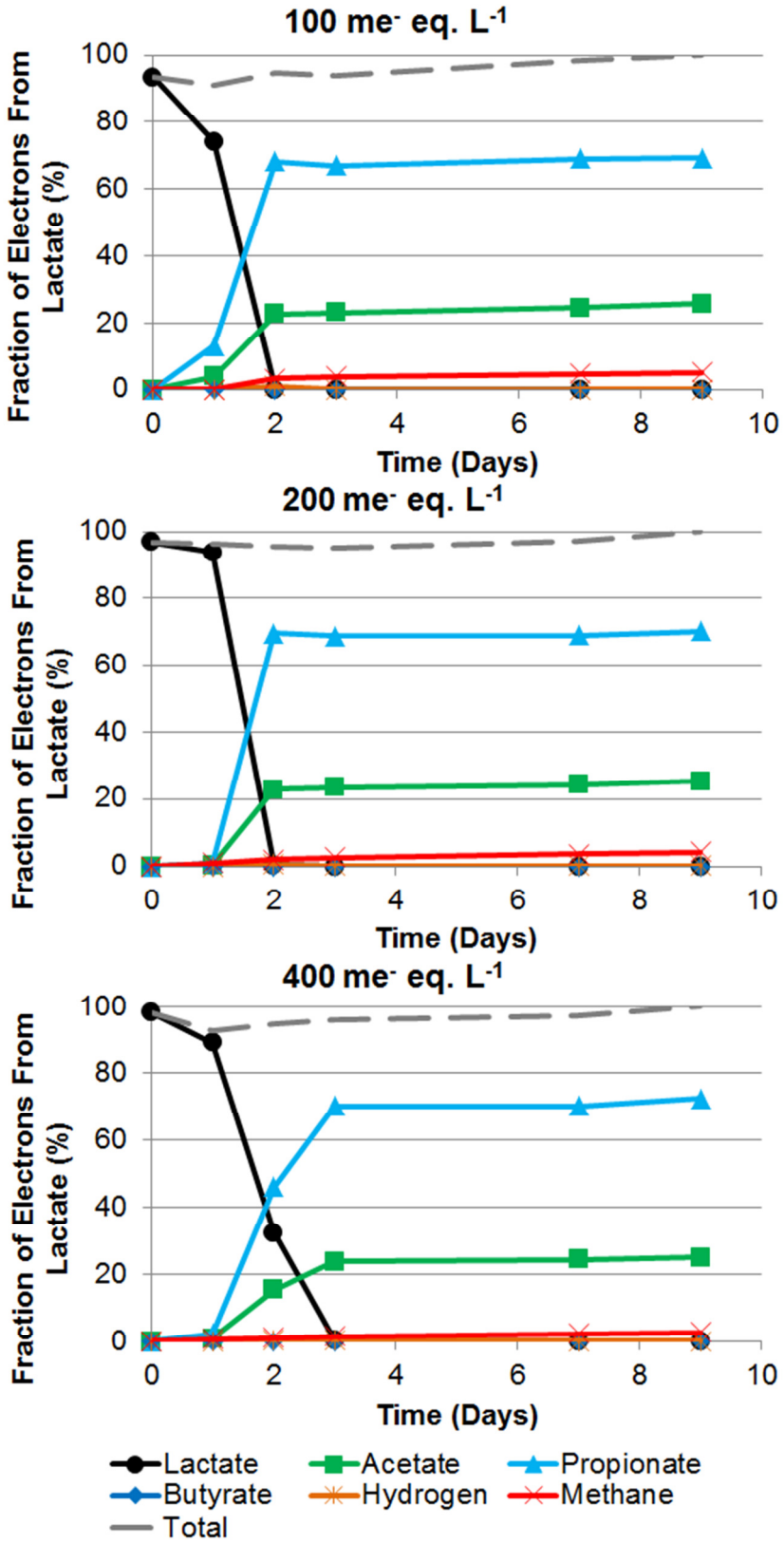


Figure 5.3. Electron balance for lactate fed fermentation cultures, at high concentrations.

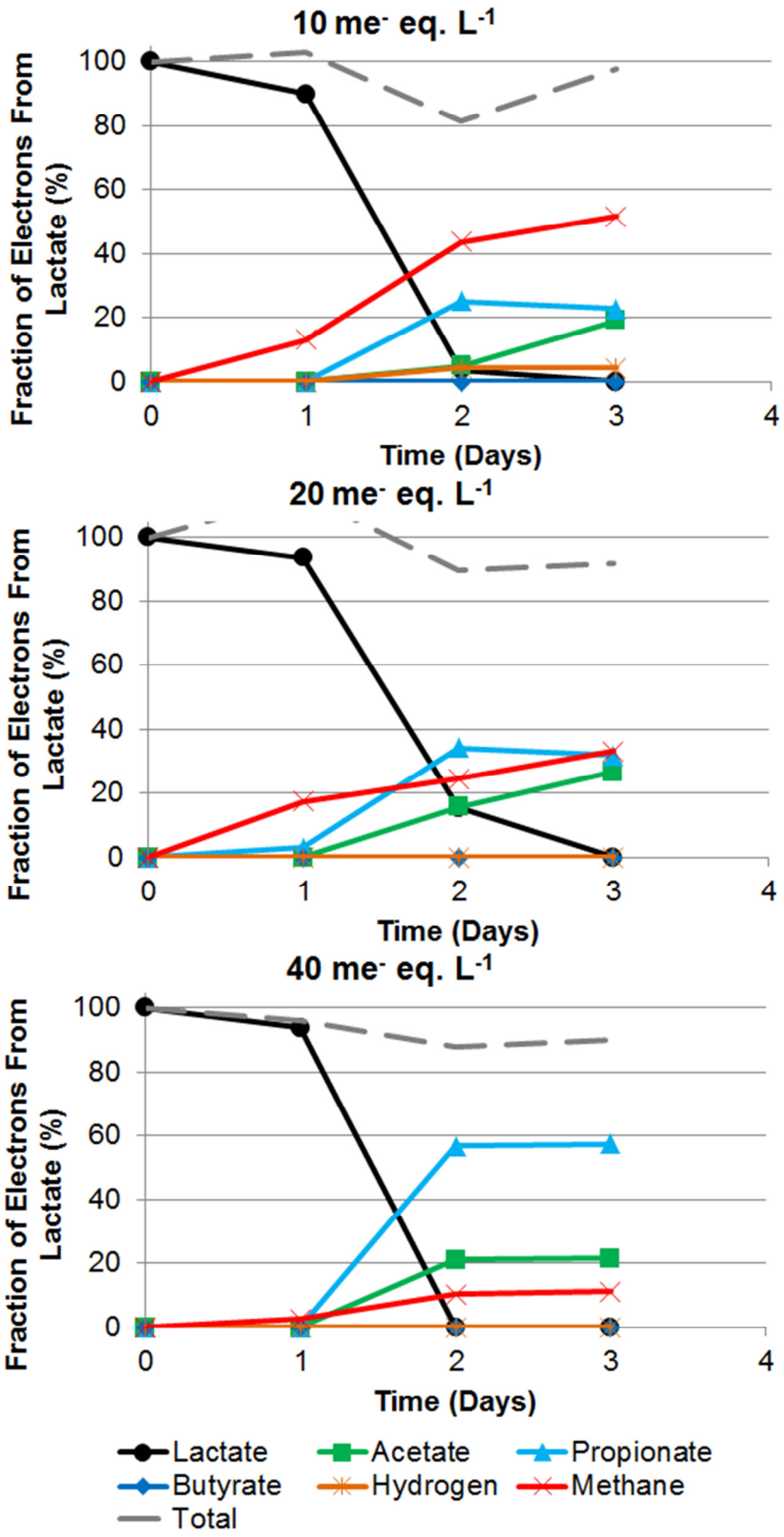


Figure 5.4. Electron balance for lactate fed fermentation cultures, at low concentrations.

5.3.2 ED Type is a Major Determinant of the Overall Microbial Community Structure

Figure 5.5 shows the microbial community structures plotted on PCs 1, 2, and 3 from a PCoA of the distances, measured by weighted UNIFRAC, between the microbial communities. The inoculum communities were grouped very tightly, indicating that the starting inoculums, although collected at different time points from the Mesa Northwest Water Reclamation Plant, were not likely to affect the outcome of the experiments. The experimental communities cluster more by type of ED fed to them rather than by ED concentration.

Figure 5.6 shows the distribution of phylogenetic distances measured by weighted UNIFRAC, between different sets of microbial communities. The left side shows the distribution of distances between cultures fed the same initial ED concentration and the distribution of distances between cultures fed different initial ED concentrations. The distribution of distances are the same among both sets, indicating that cultures fed the same initial ED concentration are no more or less similar to each other than they are to cultures fed different initial ED concentrations. The right side shows the distribution of distances between cultures fed the same ED and the distribution of distances between cultures fed different EDs. The average distance between cultures fed the same ED is smaller than the average distance between cultures fed different EDs. Therefore, the cultures fed the same ED are, on average, more similar to each other than they are to cultures fed different EDs. This difference is statistically significant (p-value < 0.001) according to my two sample t-test.

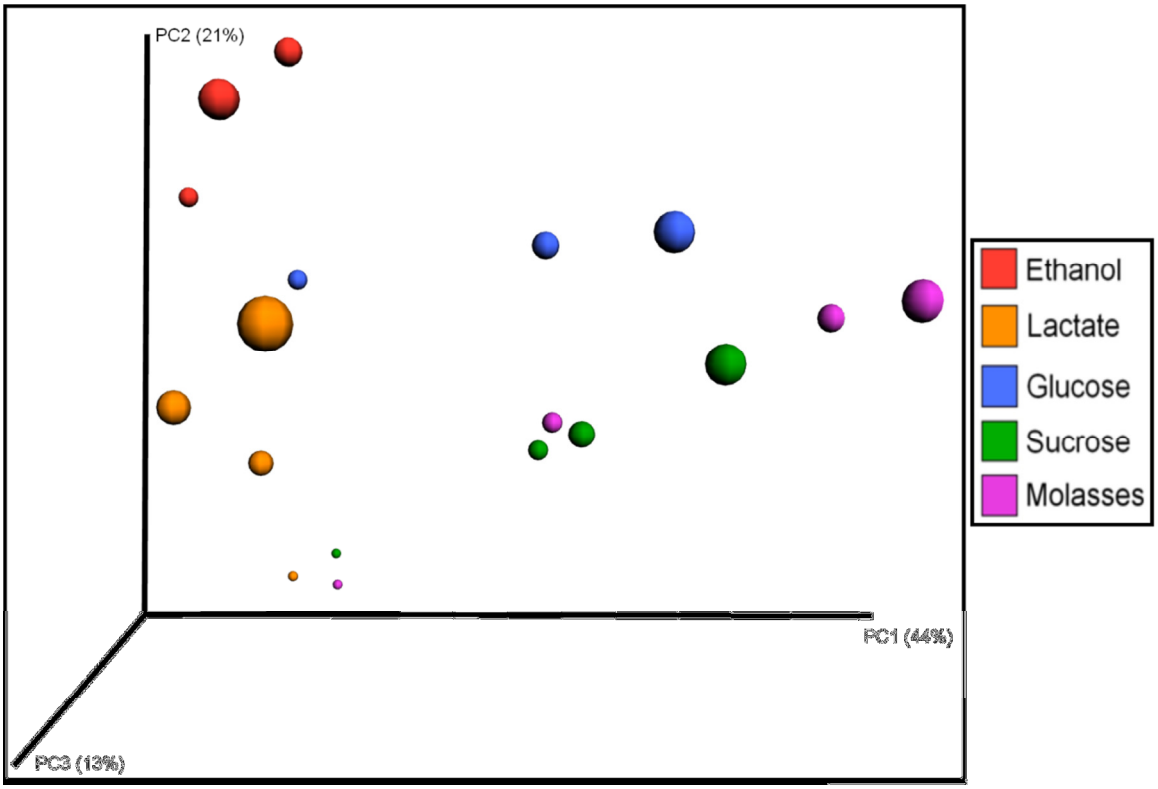


Figure 5.5. PCoA of microbial communities based on weighted UNIFRAC distances. Size of spheres correlates to inoculum (smallest) and increasing starting ED concentration: 100 me^- eq. L^{-1}, 200 me^- eq. L^{-1}, and 400 me^- eq. L^{-1}. 10, 20, and 40 me^- eq. L^{-1} for lactate.

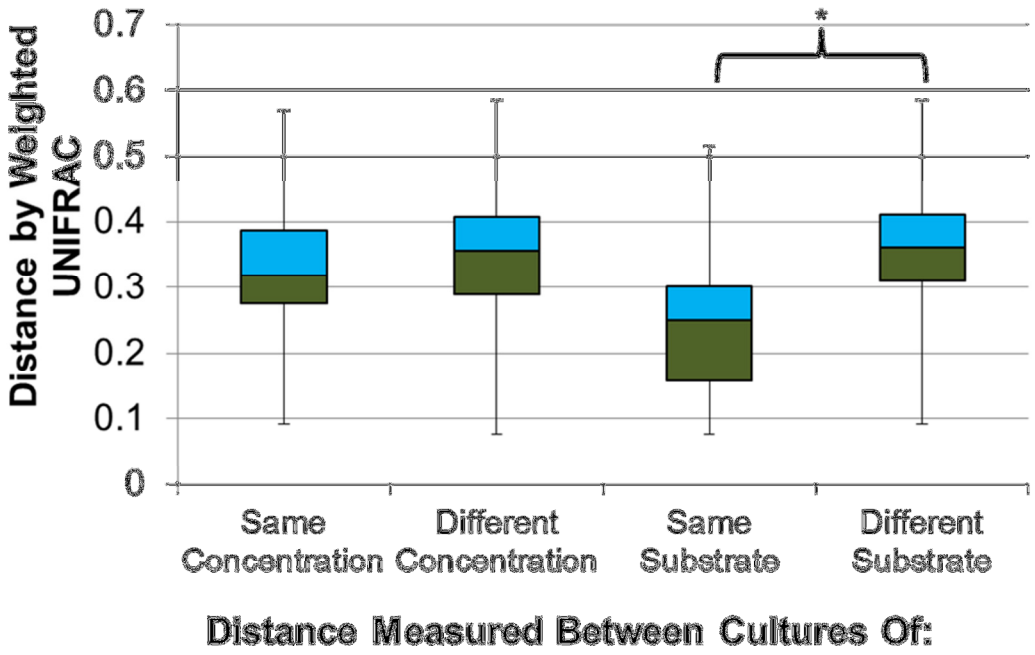


Figure 5.6. Distribution of weighted UNIFRAC distances between microbial communities. Subsets of communities correspond to communities: fed the same concentration of ED, fed different concentrations of ED, fed the same ED, and fed different EDs

5.3.4 Initial ED Concentration is a Secondary Determinant of Overall Microbial Community Structure

In Figure 5.5, clustering of the microbial communities related to varying the initial concentration of ED does show up as a trend, especially across the more complex EDs, spreading out across PC 1. Looking at the microbial communities in more detail, Figure 5.7 shows the abundance of OTUs, identified at the genus level, of known propionate producing microorganisms. OTUs related to the genus *Bacteroides* (15-35%) (Macy et al., 1978; Wallnöfer and Baldwin, 1967) increased in abundance as initial ED concentration increased for the EDs glucose, sucrose, and molasses. The lactate cultures have a high abundance of OTUs related to the genus *Clostridium* (30-62%) (Grupe and Gottschalk, 1992; Leaver et al., 1955; Vasconcelos et al., 1994). In the inoculum, OTUs identified as known propionate producing organisms represented only a small fraction of the communities. All OTUs which increased in abundance with increasing ED concentration in at least one set of experiments and accounted for at least 10% of one microbial community are shown in Figure 5.8 at the family level. These organisms may contribute to propionate production or may play some alternative role such as biomass degradation.

The initial concentration of ED drives microbial communities by determining what fermentation products can be produced. In cultures with higher ED concentrations, H_2 production leads to thermodynamically unfavorable conditions for further production of H_2 and acetate. Instead of producing acetate and hydrogen, the communities shift towards organisms capable of excreting propionate. The decrease in thermodynamic energy available from H_2 production acts as a selective pressure on the community, and the capacity to produce propionate thus becomes advantageous under these conditions by allowing additional ED to be consumed efficiently.

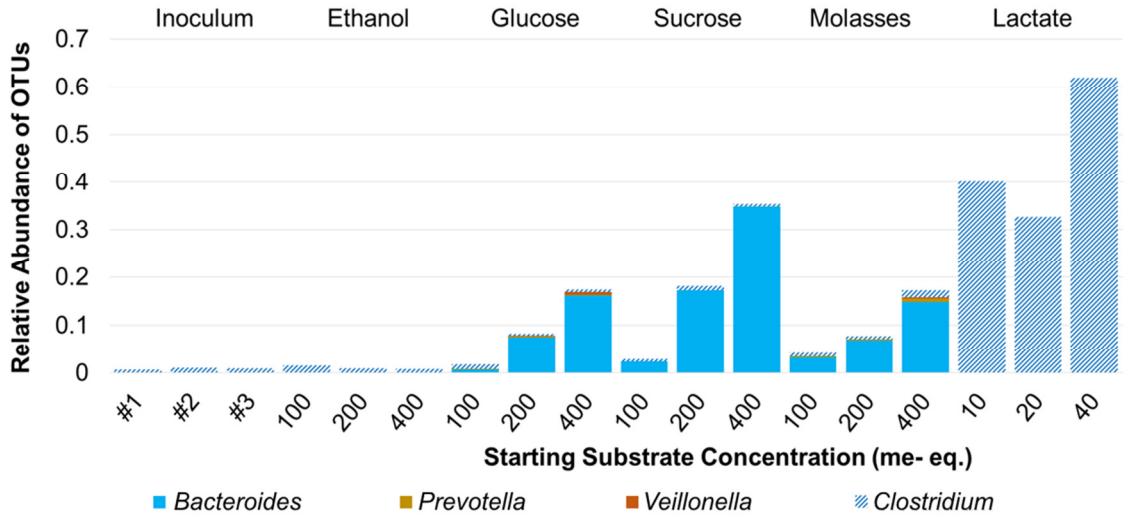


Figure 5.7. Relative abundances of known propionate producing microorganisms in ADS inoculum and fermentation only batch cultures fed ethanol, glucose, sucrose, molasses, or lactate at varying concentrations. Organisms identified at the genus level.

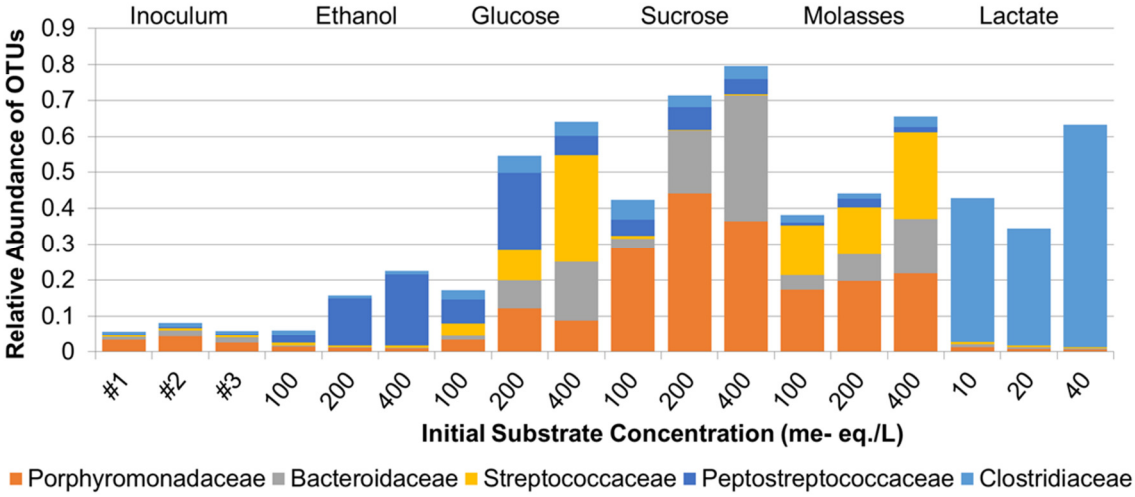


Figure 5.8. Relative abundances of OTUs which increased in abundance with increasing ED concentration in at least one set of experimental cultures and accounted for greater than 10 % of an experimental community. Identified at the family level.

5.3.5 Increased Propionate Production is defined by Metabolic Redundancy

I used PICRUSt to predict metagenomes for the microbial communities. Figure 5.9 shows the related metabolic steps for genes that encode enzymes that catalyze propionate production along two metabolic pathways: through succinate and through acryloyl-CoA. Table 5.1 shows the summed relative abundance of all phylotypes which have at least one copy for each of the genes considered. The lactate cultures were not included in this analysis due to the difference in ED concentration used. The ethanol cultures were not included because they did not produce propionate.

The PICRUSt analysis did not show clear trends for abundances of the genes for propionate production related to increasing ED concentration. Propionyl-CoA transferase is utilized in both pathways (K01026 [EC: 2.8.3.1]), where it transfers CoA from propionyl-CoA to either lactate or succinate. The gene encoding propionyl-CoA transferase [EC: 2.8.3.1] increased across increasing initial ED concentrations only for glucose fed cultures. In sucrose and molasses, the abundance of propionyl-CoA transferase was predicted to remain low across the varied concentrations tested. In the succinate pathway, the genes encoding methylmalonyl-CoA decarboxylase (K11264 [EC: 4.1.1.41]) and succinyl-CoA synthetase decreased with increasing ED. Methylmalonyl-CoA mutase (K01847 [EC: 5.4.99.2]) increased with increasing ED. Methylmalonyl-CoA epimerase (K05606 [EC: 5.1.99.1]) was low across all substrates. In the acryloyl-CoA pathway, the gene that encodes a medium chain acyl-CoA dehydrogenase (K00249 [EC: 1.3.99.3]), specific to propionate production, decreased as ED increased. The gene for L-lactate dehydrogenase (K00016 [EC: 1.1.1.27]) was abundant in glucose and molasses cultures with no change across ED concentration, and decreased in abundance as ED concentration increased in sucrose cultures. Given these results, the PICRUSt analysis does not clearly support a community shift that can be traced to specific genes in the pathway..

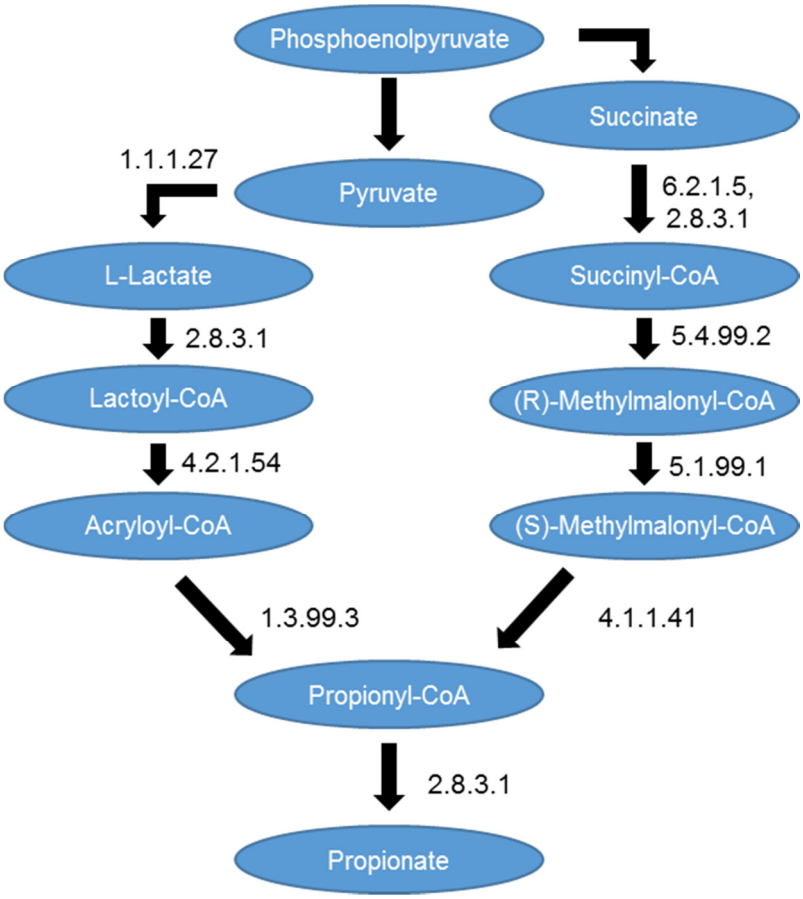
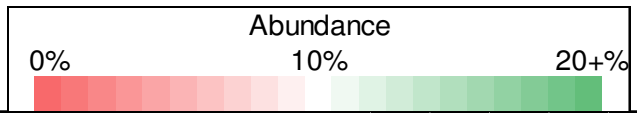


Figure 5.9. Metabolic steps in two propionate production pathways. Genes identified by EC number.

Table 5.1. Relative abundance of phylotypes with genes related to propionate production. Predicted by PICRUSt.

Substrate		Glucose			Sucrose			Molasses		
Concentration (me^- eq./L)		100	200	400	100	200	400	100	200	400
EC	Enzyme									
Common										
Propionyl-CoA + Lactate/Succinate ➔ Propionate + Lactate-CoA/Succinate-CoA										
2.8.3.1	propionate CoA-transferase K01026	8.1	7.4	19.2	2.9	1.9	1.9	3.0	2.0	3.0
Succinate Pathway										
(S)-Methylmalonyl-CoA ➔ Propionyl-CoA										
4.1.1.41	methylmalonyl-CoA decarboxylase K11264	39.9	22.9	17.6	20.8	7.8	3.3	19.8	17.5	11.6
(R)-Methylmalonyl-CoA ➔ (S)-Methylmalonyl-CoA										
5.1.99.1	methylmalonyl-CoA epimerase K05606	6.4	1.1	0.3	5.5	1.2	0.3	2.8	1.1	1.3
Succinyl-CoA ➔ (R)-Methylmalonly-CoA										
5.4.99.2	methylmalonyl-CoA mutase K01847	27.3	31.3	39.0	48.8	68.6	78.9	44.0	47.2	51.6
5.4.99.2	methylmalonyl-CoA mutase α, K01848	48.6	35.9	9.4	34.5	16.3	9.3	31.4	34.8	22.3
5.4.99.2	methylmalonyl-CoA mutase β, K01849	49.8	36.1	9.0	36.4	16.9	9.5	33.7	35.8	22.9
5.4.99.2	methylmalonyl-CoA mutase K11942	1.3	0.6	0.2	0.5	0.3	0.2	0.8	0.4	0.3
Succinate ➔ Succinyl-CoA										
6.2.1.5	succinyl-CoA synthetase alpha subunit K01902	68.3	53.5	29.8	62.8	31.7	14.7	55.4	49.2	33.1
6.2.1.5	succinyl-CoA synthetase beta subunit K01903	68.0	53.5	29.8	62.5	31.6	14.6	55.2	49.0	33.0
2.8.3.1	propionate CoA-transferase K01026	8.1	7.4	19.2	2.9	1.9	1.9	3.0	2.0	3.0
Acryloyl Pathway										
Acryloyl-CoA ➔ Propionyl-CoA										
1.3.99.3	medium chain acyl-CoA dehydrogenase K00249	43.5	31.5	4.5	26.0	10.2	4.2	25.6	26.0	14.2
Lactoyl-CoA ➔ Acryloyl-CoA										
4.2.1.54	lactoyl-CoA dehydratase (No KO)	0	0	0	0	0	0	0	0	0
L-Lactate ➔ Lactoyl-CoA										
2.8.3.1	propionate CoA-transferase K01026	8.1	7.4	19.2	2.9	1.9	1.9	3.0	2.0	3.0
Pyruvate ➔ L-lactate										
1.1.1.27	L-lactate dehydrogenase K00016	53.3	38.9	63.9	34.1	19.7	13.8	38.0	36.3	40.8



In order to determine if PICRUSt was simply failing to predict the metagenome correctly, I examined the abundance of individual phylotypes that contributed to the predicted gene abundances for two genes: phylotypes generating predictions for the propionyl-CoA transferase gene [EC: 2.8.3.1] are shown in Figure 5.10 and for the methylmalonyl-CoA decarboxylase gene [EC: 4.1.1.41] phylotypes are shown in Figure 5.11, at the class level. Propionyl-CoA transferase [EC: 2.8.3.1] transfers CoA from propionyl-CoA to either lactate or succinate. Methylmalonyl-CoA decarboxylase [EC: 4.1.1.41] converts methylmalonyl-CoA to propionyl-CoA by removing a carboxyl group. Methylmalonyl-CoA decarboxylase allows the bacteria to capture energy from the decarboxylation of methylmalonyl-CoA in the form of Na^+ gradients, which can be used to generate ATP, and H^+ gradients, used to maintain homeostasis (Dimroth and Schink, 1998; Hilpert and Dimroth, 1982; Hoffmann et al., 1989). Multiple organisms were predicted to contribute to the abundance of both of these genes in the metagenome; however, the abundant phylotypes do not coincide with known propionate producers.

Given that OTUs closely related to known propionate producers (*Bacteroides*, Figure 5.7) were observed in the community analysis above but corresponding phylotypes are not contributing to the predictions of these genes (Figures 5.10 and 5.11), it seems that the community shift is not being entirely captured by the PICRUSt analysis. Test predictions using a mock community containing all of the *Bacteroides* genera with sequenced genomes confirmed that PICRUSt does not generate a prediction of the methylmalonyl-CoA decarboxylase gene (EC: 4.1.1.41), a gene demonstrated to be used by *Bacteroides* during propionate formation (Reichardt et al., 2014). Additionally, the genera *Clostridium* is known to make use of the acryloyl-CoA pathway and OTUs related to *Clostridium* were identified in the lactate fed cultures; however, the gene for lactoyl-CoA dehydratase (No KO assigned [EC: 4.2.1.54]) is not predicted using PICRUSt. Although the PICRUSt analysis is incomplete, it clearly shows that some of the genes involved with propionate production were not predicted from individual phylotypes, but rather from multiple phylotypes, indicating redundancy in the microbial communities for propionate production.

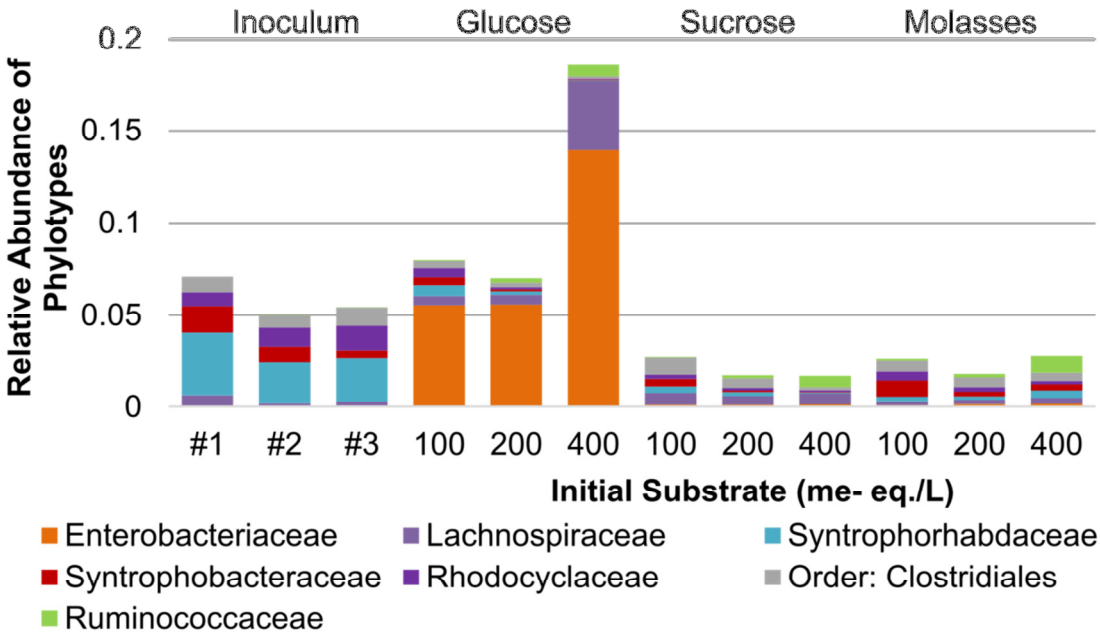


Figure 5.9. Relative abundances of phylotypes which contributed to the PICRUSt prediction of the propionyl-CoA transferase gene (K01026 [EC: 2.8.3.1]). Identified at the family level. Lactate and ethanol cultures are not included as discussed in the text.

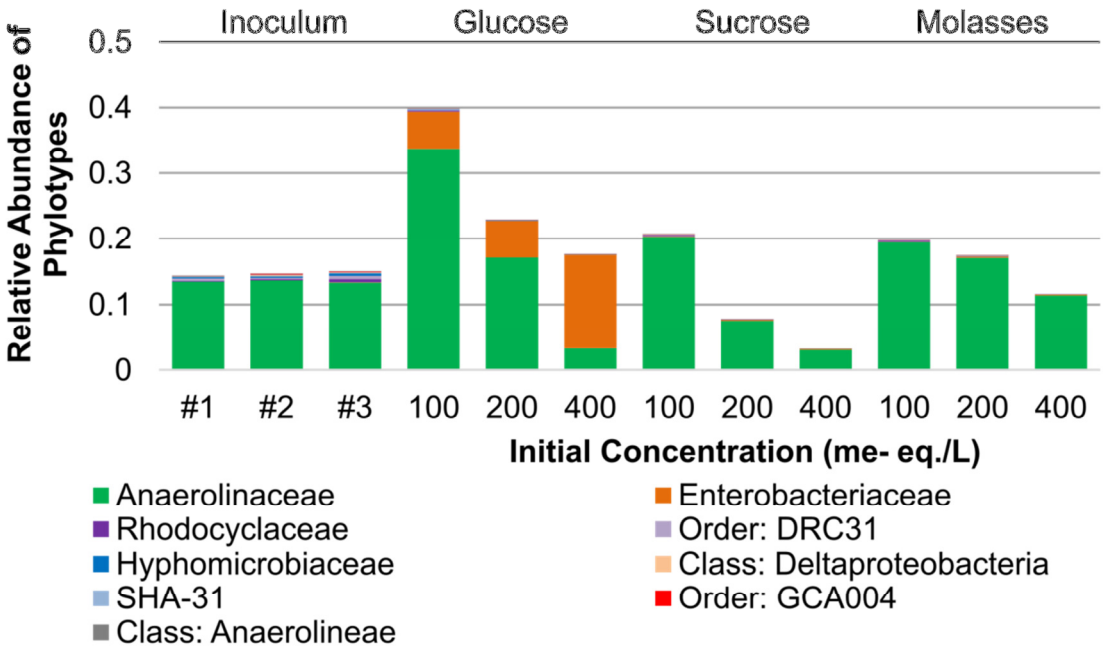


Figure 5.10. Relative abundances of phylotypes which contributed to the PICRUSt prediction of the methylmalonyl-CoA decarboxylase gene (K11264 [EC: 4.1.1.41]). Identified at the family level. Lactate and ethanol cultures are not included as discussed in the text.

5.4. Conclusions

Regardless of ED provided, when initial ED concentration was higher, the fermentation cultures shifted from producing acetate and methane to producing SCFAs. The occurred for most of the EDs tested between 100 and 200 me^- eq. L^{-1}. Lactate, due to its use as an acceptor of reducing equivalents, required even lower concentrations of ED for this shift to occur (between 10 and 20 me^- eq. L^{-1}). The microbial community analysis indicated that the type of ED was the major driver in the development of the microbial community structures, but that the initial concentration of ED fed was also an important factor that crafted communities. Known propionate producers were detected: *Bacteroides* dominated glucose, sucrose, and molasses fed cultures, and *Clostridium* dominated lactate fed cultures. The analysis of the predicted metagenomes showed that redundancy in genes related to propionate production existed in the communities, and the succinate pathway played an important role in these systems. Using ED loading to shift the balance of the products of fermentation not only deserves greater exploitation in industrial settings such as biohydrogen production, dechlorination, and microbial electrochemical cells, it also offers insights in to managing microbial communities in the human gut, where the distribution of fermentation products has been linked to obesity.

CHAPTER 6

AMMONIA INHIBITION OF METHANOGENESIS IN MICROBIAL ELECTROCHEMICAL CELLS FED WITH FERMENTABLE SUBSTRATES

6.1. Introduction

6.1.1 Overview

In Chapter 5, I established the conditions required to operate batch cultures which would result in particular distributions of the electrons from fermentable EDs. I made use of that knowledge in the following experiments, in order to establish control conditions which would result in ~30% of the electrons from a fermentable ED ending up in methane, so that I could assess an alternative method to inhibit methanogenesis and re-channel these electrons. In this chapter, I present my work in using NH_4^+ to inhibit methanogenesis in MECs.

MXCs convert energy stored in chemical compounds into electrical energy through the metabolic action of anode respiration (Logan et al., 2006; Rittmann, 2008). ARB, the bacterial catalysts in MXCs, prefer acetate as their ED, a two carbon carboxylic acid (Pant et al., 2010). Given that most applications for MXCs involve the utilization of feed stocks of organic compounds other than acetate, understanding and controlling the processes of fermentation becomes an important area of MXC operation (Freguia et al., 2010, 2008; Torres et al., 2007).

Anaerobic bacteria ferment complex organic compounds into smaller organic compounds, including acetate and propionate, in the absence of electron acceptors. Fermentation often produces H_2 in addition to organic compounds (Lee et al., 2008b). Methanogens consume the H_2, along with CO_2, or acetate while generating methane (Schink, 1997). Methane is an insoluble gas and has not been shown to be oxidized by ARB; therefore, the production of methane in MXCs represents an electron sink which diverts electrons away from current production (Freguia et al., 2008, 2007; Parameswaran et al., 2009). In a process called homoacetogenesis, H_2 and CO_2 can also be utilized by homoacetogenic bacteria to produce acetate (Ljungdahl, 1986). Under most circumstances, hydrogenotrophic methanogenesis takes the majority of H_2 (Le Van et al., 1998).

CE measures the ratio of electrons converted to current to the total number of electrons

removed from the provided electron donor in MXCs; thus CE represents an important benchmark for MXC performance. MXCs yield the highest CEs with acetate, which contains 8 e^- eq. per mol (Pant et al., 2010). This occurs because the majority of electrons from acetate go to respiration rather than biomass production. When other electron donors are used, however, methanogenesis results in the loss of up to 33% CE in MXC systems. The use of inhibitors such as 2-bromo ethanosulfate or alamethicin to suppress methanogenesis in MXC systems improves CE (Parameswaran et al., 2009; Zhu et al., 2015). The inhibition of methanogenesis also fosters the formation of a positive syntrophy where homoacetogenic organisms consume H_2 and produce acetate, which the ARB then utilize for current production (Parameswaran et al., 2012, 2010). In order to maximize CE in MXCs, methanogenesis must be prevented. Therefore, I am interested in alternative methods of managing the microbial communities associated with MXCs.

6.1.2 Using Ammonia to control methanogens

One possible method of controlling methanogenesis is by selective inhibition of the methanogens using increased NH_4^+ concentrations in the waste stream to be treated. NH_4^+ toxicity for methanogens has been examined in the past for its effects on anaerobic digestion, where its presence in high quantities can be detrimental (Angelidaki and Ahring, 1994; Borja et al., 1996; Chen et al., 2008; De Baere et al., 1984; Koster and Lettinga, 1988). High NH_4^+ content waste streams, ranging from 0.1 to 8g NH_4^+/L, include animal waste, run off from agricultural fields where fertilizer has been applied, and food wastes (Borja et al., 1996; De la Rubia et al., 2010; Desloover et al., 2012; Walker et al., 2011; Yabu et al., 2011). Given its availability in certain waste streams, NH_4^+ may represent a method of inhibiting methanogenesis while providing a beneficial effect on the treatment of these waste streams with MXCs.

NH_4^+ inhibits methanogens at concentrations higher than those typically supplied in previous MXC experiments, ~0.1 - 0.3 g $N\text{-}NH_4^+ L^{-1}$ (Lee et al., 2008a; Parameswaran et al., 2009). Increasing NH_4^+ has several effects which may inhibit the growth of different microorganisms such as: disturbance of the intracellular pH (Bakker, 1990; Sprott et al., 1984), a shift in intracellular cation concentrations (Sprott et al., 1985, 1984), and the direct inhibition of enzymes by increased $N\text{-}NH_4^+ L^{-1}$ (O'Sullivan and Ettlinger, 1976; Zheng et al., 2012). Reported

values for initiation of methanogen inhibition range from 0.18 to 7.85 g $N\text{-}NH_4^+$ L^{-1} (De Baere et al., 1984; Koster and Lettinga, 1988). The large variability reported stems from multiple sources such as: testing cultures acclimated over time to high concentrations of ammonium chloride (NH_4Cl), pH of the system, and the groups of methanogens present (Kadam and Boone, 1996). The pk_a of NH_4^+ is ~9.24, therefore, a greater proportion of the NH_4^+ begins to be un-protonated as the pH of a system approaches this value. Typical pH values in MXC systems range from 6.5 to 8 (Bullen et al., 2006). De Baere et al.(1984) stated that 100 mg L^{-1} of free ammonia (approximately 3.4 g $N\text{-}NH_4^+$ L^{-1} at pH 7.6) is the inhibitory concentration for methanogens as the un-protonated form crosses cell membranes more easily (Kleiner, 1985). Sprott and Patel (1986) demonstrated with pure cultures that acetoclastic methanogens are inhibited typically when $N\text{-}NH_4^+$ is present at concentrations around 1.4 g $N\text{-}NH_4^+$ L^{-1} while hydrogenotrophic methanogens function at up to 9.6 g $N\text{-}NH_4^+$ L^{-1}.

6.2.3 Previous work with MECs

Previous work has explored NH_4^+ tolerance by ARB in MECs fed with acetate (Clauwaert et al., 2008b; Kim et al., 2011; Nam et al., 2010). Clauwaert et al. (2008b) demonstrated that an ARB biofilm fed acetate tolerated NH_4^+ up to 5g $N\text{-}NH_4^+$ L^{-1} with coincident increases in both CE and current densities, from 20-30% to 40-45% CE and 2 to 5.3 A m^{-2} respectively, as NH_4^+ increased from 0 to 5 g $N\text{-}NH_4^+$ L^{-1} at a pH of ~6.7. The high NH_4^+ (5 g $N\text{-}NH_4^+$ L^{-1}), at this pH, should produce an extracellular environment of only 0.012-0.019 g $N\text{-}NH_3$ L^{-1}, which would be well below the inhibitory levels reported for methanogens. Work by Nam et al. demonstrated an MEC without loss of efficiency at 0.038g $N\text{-}NH_3$ L^{-1} (Kim et al., 2011). These reported levels for the inhibition of ARB overlap with inhibitory levels for methanogens; therefore, a compromise between inhibition of methanogens and loss of current production may represent an ideal middle ground in practical applications.

I hypothesized that high concentrations of NH_4^+ applied to MECs will improve the CE obtained using fermentable EDs by inhibiting methanogens and directing more electrons from the electron donor to ARB through soluble intermediates and thus to electricity. To test this hypothesis, I performed experiments to measure the effects of varying NH_4^+ on different microbial

members of the mixed communities involved with electricity production from fermentable EDs. First, I varied NH_4^+ concentrations in fermenting, methanogenic batch cultures fed glucose and sucrose as model fermentable EDs. Second, I tested high NH_4^+ conditions in MECs fed glucose and sucrose and compared them against MECs with control conditions. I also analyzed the microbial communities developed in response to the varying conditions in each of these experiments in order to understand what effects NH_4^+ has on fermenting and anode respiring bacterial communities.

6.2. Materials and Methods

6.2.1 Hydrogenotroph Enriched Microbial Culture

I established a microbial culture enriched for hydrogenotrophs using anaerobic digested sludge (ADS) from the Mesa Northwest Water Reclamation Plant, Mesa, AZ. I prepared anaerobic bottles for the cultures by first filling 160 mL bottles with 50 mL of enrichment media made similar to Chapter 5, except the phosphate buffer was 25mM, prepared with Na_2HPO_4 and KH_2PO_4. Prepared culture bottles were inoculated with 5 mL of ADS and the headspace was sparged with 80% H_2:20% CO_2 gas mixture in order to provide H_2 as the electron donor for the hydrogenotrophs. I placed the cultures into incubators set to 37º C and 150 rpm. The cultures were fed by filling a 100 mL frictionless syringe with 80% H_2:20% CO_2 gas mixture and attaching the syringe to the culture bottle with a needle, allowing the headspace in the culture bottle to equilibrate with atmospheric pressure. Once the culture consumed 80 mL of gas for two days in a row, the culture was diluted 1:10 into a new culture bottle with fresh media. The culture was enriched in this manner twice prior to use in experiments. This hydrogenotrophic inoculum is referred as Inoc-H_2 in microbial community analyses.

6.2.2 NH_4^+ Inhibition of Fermentation Cultures

I performed the inhibition of fermentation cultures in batch culture experiments. I used the media described above and varied the NH_4^+ by replacing the control media, which included 0.15 g N-NH_4^+/L, with a high concentration NH_4Cl stock solution, prepared anaerobically, bringing the NH_4^+ up to the desired value. Cultures were set up with 0.15 g, 2 g, 4 g, or 6 g N-NH_4^+ L^{-1}. I filled each of the 150 mL bottles with 100 mL of media and performed the experiments in

triplicate. I inoculated the cultures with 1 mL each of ADS and Inoc-H_2, described above. I tested glucose and sucrose as fermentable electron donors, and added each at an initial concentration of 100 me^- eq. L^{-1}. I prepared a 100x stock solution of each ED with the enrichment media and used this to supply 100 me^- eq. L^{-1} to the cultures. I placed the cultures in an incubator at 37 ºC at 150 rpm and took both liquid and gas samples to measure SCFAs, sugars, alcohols, H_2 and methane as described below.

6.2.3 Analysis of Fermentation Products

I analyzed liquid samples using HPLC (Model LC-20AT, Shimadzu, Columbia, MD) and gas samples from the headspace of the cultures using GC as previously described (Miceli et al., 2014). Sugars were analyzed as described in Chapter 5.

6.2.4 Microbial Ecology

For community analysis, I took biomass samples for DNA extraction as described in Chapter 5. At the beginning of each experiment, I took 1 mL of ADS and 5 mL of the Inoc-H_2 culture. From the batch bottle experiments, I took 5 mL of the culture on day 4. The batch bottle samples and the Inoc-H_2 culture samples were then centrifuged for 5 minutes at 13,000 rpm, and 4 mL of the media were removed in order to concentrate the samples in 1 mL of media. For the MEC experiments, samples of the pre-grown biofilm and the biofilm on day 4, each scraped from 8 cm^2 of the anode, were taken along with 5 mL of the suspension on day 4 for analysis. Biofilm samples were suspended in 1mL of blank media and the suspension samples were concentrated as above. I stored the samples at -80 ºC until extraction.

The V4-V6 region of the 16S rRNA gene was targeted for sequencing on a MiSeq Illumina sequencer, and sequences were analyzed as described in Chapter 5. The sequences from the batch fermentation cultures were rarefied to 9926 sequences and those from the MEC cultures were rarefied to 10,000 sequences, in order to normalize the number of sequences per sample.

In order to see how the microbial communities correlate with ED and NH_4^+ conditions, I performed canonical correspondence analysis CCA (ter Braak, 1986). This is done by calculating eigenvectors, which are linear combinations of the environmental/chemical data provided, that

best correlate with the taxa data observed, in this relative abundance obtained through sequencing. The comparison of microbial communities performed is thus constrained by the given environmental/chemical data provided. The eigenvectors can be plotted as axes for graphs, and the samples and taxa can be plotted on these axes. Each eigenvector, or axis, is uncorrelated to all other eigenvectors in order to best explain the variability seen in the data. In CCA, samples (in this case the different cultures) form clusters on the graph based on how similar their microbial community structure and environmental parameters are to each other. Arrows (called gradients) can be drawn based on how well the microbial communities correlate to continuous environmental parameters. The gradients form additional axes on the graph along which the variation of the variable which they represent is greatest. Although the gradients can align with the CCA axes, they do not have to do so. The CCA axes are statistical abstractions which correlate the changes in the microbial community structure with changes in the environmental data. In the case of environmental parameters which are not continuous, only have a small number of unique values by design of the experiment, or are categorical, centroids are useful for analysis. The locations of cultures in a given group can be averaged together to calculate the centroid, which is the center of the group in the space created by the axes generated during CCA and can give indications on how different values of environmental variables affect groups of cultures.

Interpreting a CCA is similar to interpreting a PCoA. Microbial communities of similar structure cluster together. The difference, however, is that with the addition of environmental/chemical data, specific environmental parameters can be directly connected to the clustering, rather than being left up to the interpretation of the viewer. Due to the inclusion of the environmental data, interpretations of driving forces in communities are more reliable when using CCA. For further readings, ter Braak's work is recommended (ter Braak and Braak, 1987; ter Braak, 1994, 1990; ter Braak et al., 1995).

I performed CCA (ter Braak, 1986) using the R software package (RCT, 2015) with the vegan package (Oksanen et al., 2015), using abundance at the taxonomic level of order for OTUs which represented greater than 10% of at least one sample. For the fermentation only cultures

two constraining variables were used. The first constraining variable was the type of culture, grouping the cultures as inoculum (for the anaerobic digested sludge and hydrogenotrophic culture samples) and either sucrose or glucose (for experimental cultures fed the given ED). The second constraining variable I used was the concentration of NH_4^+, setting the inoculum to 0 for the purposes of analysis. Values used for the constraining variables in the fermentation only cultures are shown in Table 6.1. For the MEC cultures, the constraining variables used were the conditions the culture was grown in, the ED fed to the culture, and the location of the culture. These values for the MEC cultures are summarized in Table 6.2.

Table 6.1: Constraints used for CCA of fermentation only cultures.

Sample ID	Set	Condition
Glucose Control	Glucose	0.15
Glucose 2g	Glucose	2
Glucose 4g	Glucose	4
Glucose 6g	Glucose	6
Sucrose Control	Sucrose	0.15
Sucrose 2g	Sucrose	2
Sucrose 4g	Sucrose	4
Sucrose 6g	Sucrose	6
ADS-1	Inoculum	0
ADS-2	Inoculum	0
H2-1	Inoculum	0
H2-2	Inoculum	0

Table 6.2: Values used as constraints for CCA of MEC communities.

Culture	Location	ED	Condition
ADS 1	ADS	ADS	ADS
ADS 2	ADS	ADS	ADS
H2 1	H2	H2	H2
H2 2	H2	H2	H2
Glucose Control 1 - Biofilm Start	Biofilm	Acetate	InoculumBF
Glucose Control 1 - Biofilm End	Biofilm	Glucose	Control
Glucose Control 1 - Suspension End	Suspension	Glucose	Control
Glucose Control 2 - Biofilm Start	Biofilm	Acetate	InoculumBF
Glucose Control 2- Biofilm End	Biofilm	Glucose	Control
Glucose Control 2- Suspension End	Suspension	Glucose	Control
Glucose 4g 1 - Biofilm Start	Biofilm	Acetate	InoculumBF
Glucose 4g 1 - Biofilm End	Biofilm	Glucose	4g
Glucose 4g 1 - Suspension End	Suspension	Glucose	4g
Glucose 4g 2 - Biofilm Start	Biofilm	Acetate	InoculumBF
Glucose 4g 2 - Biofilm End	Biofilm	Glucose	4g
Glucose 4g 2 - Suspension End	Suspension	Glucose	4g
Sucrose Control 1 - Biofilm Start	Biofilm	Acetate	InoculumBF
Sucrose Control 1 - Biofilm End	Biofilm	Sucrose	Control
Sucrose Control 1 - Suspension End	Suspension	Sucrose	Control
Sucrose Control 2 - Biofilm Start	Biofilm	Acetate	InoculumBF
Sucrose Control 2- Biofilm End	Biofilm	Sucrose	Control
Sucrose Control 2- Suspension End	Suspension	Sucrose	Control
Sucrose 4g 1 - Biofilm Start	Biofilm	Acetate	InoculumBF
Sucrose 4g 1 - Biofilm End	Biofilm	Sucrose	4g
Sucrose 4g 1 - Suspension End	Suspension	Sucrose	4g
Sucrose 4g 2 - Biofilm Start	Biofilm	Acetate	InoculumBF
Sucrose 4g 2 - Biofilm End	Biofilm	Sucrose	4g
Sucrose 4g 2 - Suspension End	Suspension	Sucrose	4g

6.3. Results and Discussion

6.3.1 Increasing NH_4^+ Shifts Product Distribution during Fermentation

Fermentation only batch cultures were set up in order to understand how fermentation and methanogenesis are affected by increasing NH_4^+, to assess how NH_4^+ affects microbial community structure, and to identify target levels of NH_4^+ suitable for use in MEC experiments. Figure 6.1 summarizes the electron balances from the fermentation of glucose and sucrose in batch fermentation cultures (without electrodes). Two time points are shown for each ED, days 3 and 10 for glucose cultures and days 2 and 9 for sucrose cultures. Days 3 (glucose) and 2 (sucrose) show the results of the early portion of fermentation while days 10 (glucose) and 9 (sucrose) show the results of longer fermentation. For more detailed breakdowns of the electron balances for the fermentation cultures over time, see Appendix D.

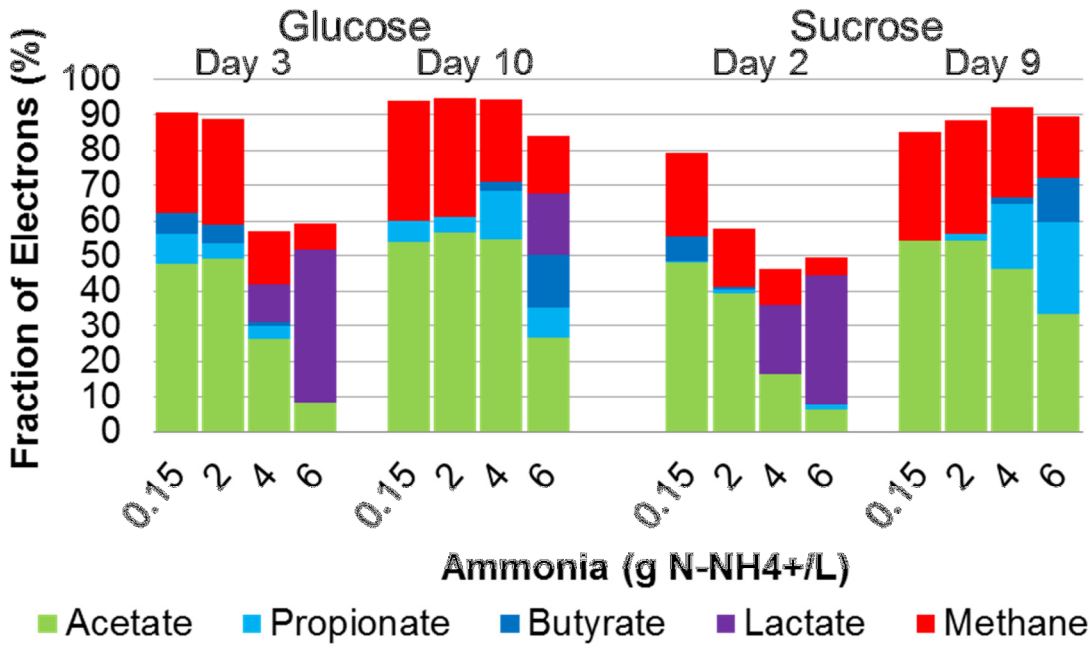


Figure 6.1. Electron balances for fermentation only batch cultures, fed either glucose or sucrose, at two selected time points. Balance calculated from total initial ED provided.

Overall, the fraction of electrons from the initial ED which end up in SCFAs by the end of fermentation increased with increasing NH_4^+, from ~60% to ~70%. The fraction of electrons which end up as methane decreased, as NH_4^+ increased, from ~30% to ~16% of the electrons from the initial ED. Methanogenesis may be inhibited directly or the fermenting organisms may be affected by the presence of increased NH_4^+. Given that NH_4^+ can disrupt pH homeostasis in cells, it is possible that fermenters must divert energy away from cell synthesis and towards ATP production when exposed to high NH_4^+, thus sending more electrons from their metabolism towards excreted SCFAs, alcohols, and other compounds. In addition, the early time points show that, as NH_4^+ increased, lactate was formed during the early time periods and later fermented to SCFAs, and likely hydrogen. The diversion of electrons away from methane and towards soluble carbon compounds during fermentation is promising for applications in MXCs.

6.3.2 NH_4^+ at High Concentrations Alters Microbial Communities

I analyzed the microbial communities of the fermentation only batch cultures by targeting the 16S rDNA. Figure 6.2 shows these communities identified down to the genus level where possible. OTUs from the family Clostridiaceae, known fermenting bacteria, dominate communities (50-77%) at low NH_4^+ concentrations (0.15 g and 2 g NH_4^+ L^{-1}) along with some Pelobacteraceae (3.5-16%), and in the glucose control cultures some *Arcobacter* (21%). A drastic shift occurs between 2 g and 4 g N-NH_4^+ L^{-1}, where Lactobacillales takes over the high NH_4^+ cultures (42-47%). Clostridiaceae OTUs decrease drastically (< 0.001% at 6 g NH_4^+/L) at high NH_4^+ levels. In sucrose, a switch between Porphyromonadaceae, a family in the order Bacteriales, and an unknown member of the order Bacteriales switch, with Porphyormonadaceae increasing and the unknown Bacteriales decreasing as NH_4^+ increases.

The presence of Lactobacillales, the order which contains lactic acid bacteria (LAB), corresponds with lactate production in the higher NH_4^+ cultures and has been observed in high NH_4^+ conditions during anaerobic digestion and co-digestion of vegetable and fruit residues (Garcia-Peña et al., 2011). While LAB are known to acidify their environment, making it inhospitable to other organisms, pH in these experiments was maintained with a high buffer concentration, ruling out this as a likely explanation for their high abundance. It is possible that

the adaptations which make LAB successful during fermentation at low pH may also provide resistance to NH_4^+. Clear experimental evidence of the effects of NH_4^+ on LAB is lacking in the literature; however, there are reports of LAB maintaining intracellular pH above environmental pH (Kasjet, 1987), which would confer some resistance against NH_4^+, and LAB are known to ferment meat, a high NH_4^+ environment (Fontana et al., 2011). Further, the maintenance of pH homeostasis in LAB, which proves so useful during low pH fermentation, relies on pumping protons out and K^+ in to the cell (Hutkins and Nannen, 1993). Given that their regular metabolism allows them to expend energy on this process in preparation of the pH dropping, the addition of NH_4^+ and its effect of removing intracellular protons may not be as detrimental to LAB as it is to other fermenting bacteria.

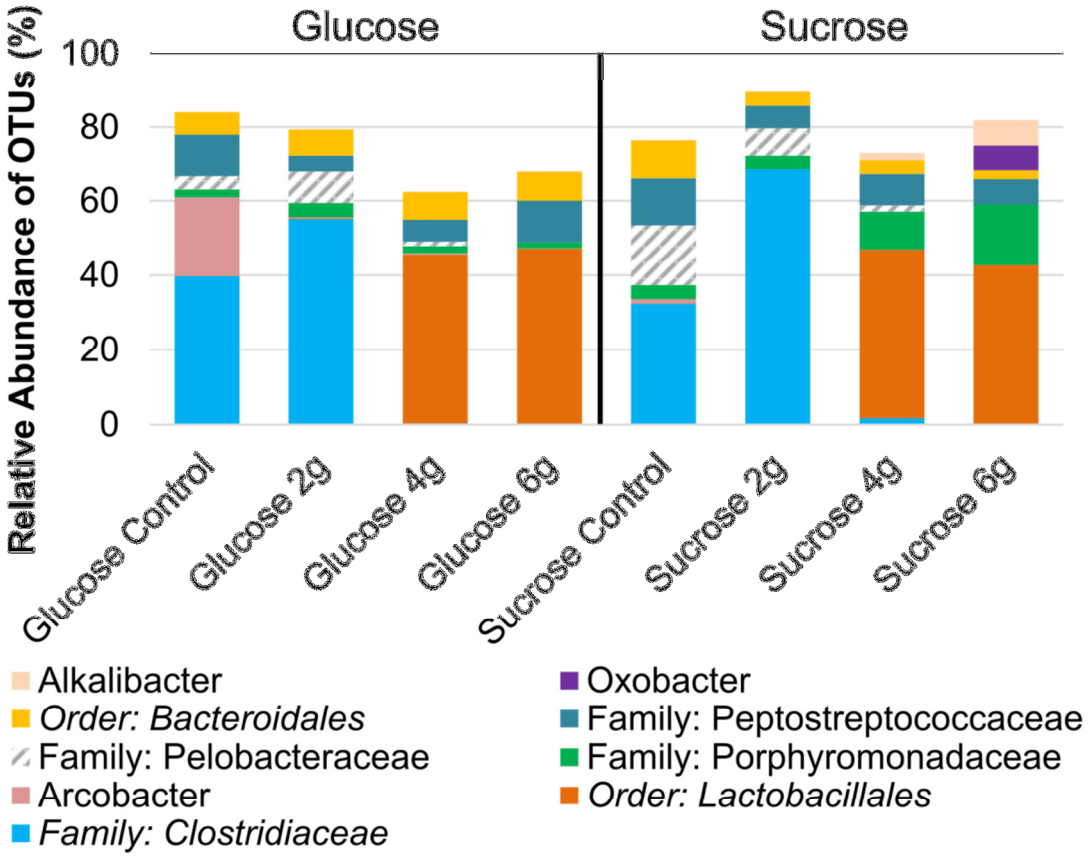


Figure 6.2. Relative abundances of OTUs in fermentation only batch cultures. Data from day 4, identified at the genus taxonomic level where possible and at family and order where necessary.

For the fermentation only cultures, CCA shows that the cultures (shown as squares and triangles in Figure 6.3) cluster according to NH_4^+ concentration (denoted by color) and ED they were fed (denoted by shape). The NH_4^+ effect accounts for more of the variations in the overall community structures of the cultures than ED type, as seen by how the cultures form two clusters: low (control and 2 g N- NH_4^+/L) and high (4 g and 6 g N- NH_4^+/L) ammonia. With ED, there is some separation between the clusters of glucose and sucrose cultures; however, one glucose culture (2 g N- NH_4^+/L) clusters more with the low NH_4^+ sucrose cultures than with its matching low NH_4^+ glucose culture (the glucose control). Only OTUs which stood out in the analysis are graphed, shown as X's on Figure 6.3. OTUs related to Lactobacillales lie nearly perfectly on the axis defined by the NH_4^+ gradient (shown as an arrow, pointing in the direction of increasing NH_4^+), indicating that its abundance is almost entirely determined by the NH_4^+ concentration, regardless of ED. The five other phylotypes shown each have a particular association described in the CCA. Porphyromonadaceae are partially associated with increaseing NH_4^+ and sucrose. Bacteroidales overall shows almost no effect due to NH_4^+ and a minor ED connection. Clostridiaceae show an inverse relation with NH_4^+, given its location in relation to the direction of the NH_4^+ gradient, but little ED dependant change. Pelobacteraceae are associated with low NH_4^+ and (slightly) sucrose, given its proximity to the sucrose centroid. *Arcobacter* are associated with glucose and low NH_4^+. These associations indicate that the abundance of a given phyltype is associated with a condition, not that it is exclusively found in the given condition.

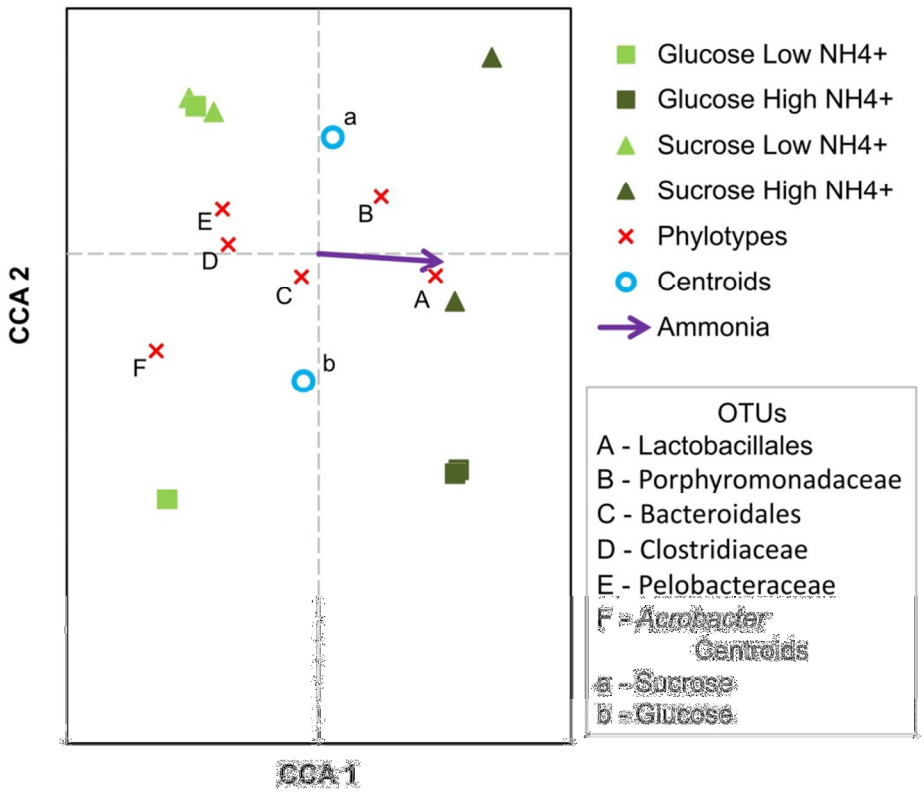


Figure 6.3. Canonical correspondence analysis (CCA) of fermentation only batch cultures. Communities (Glucose Fed – triangles, Sucrose Fed - squares) form clusters of low NH_4^+ (Control and 2 g N-NH_4^+/L, shown in light green) and high NH_4^+ (4 g and 6 g N-NH_4^+/L shown in dark green) with some clustering by ED. X's represent the location of OTUs, identified by uppercase letters. Hollow circles represent the locations of centroids, weighted average location of cultures of a given subset, identified by lowercase letters.

6.3.3 Higher NH_4^+ Improves CE in Glucose MECs

Given that 5 g $N\text{-}NH_4^+ L^{-1}$ is the upper limit of NH_4^+ that ARB can withstand and our results in the fermentation only batch experiments indicate that higher NH_4^+ would not divert more electrons to soluble EDs, I tested 4 g $N\text{-}NH_4^+/L$ in glucose and sucrose fed MECs as the high NH_4^+ concentration. Figures 6.4 and 6.5 show the CE for glucose and sucrose fed MECs, at control and high NH_4^+ conditions. As seen in Figure 6.4, in glucose fed MECs, the 4 g $N\text{-}NH_4^+ L^{-1}$ condition increased CE from 75% to 90%, with coincident decreases in methane production. In the sucrose fed MECs, Figure 6.5, CEs in the 4 g $N\text{-}NH_4^+ L^{-1}$ MECs were similar to those in control conditions (Figure 6.5). Closer inspection of the sucrose fed, 4 g $N\text{-}NH_4^+ L^{-1}$ MEC 1 shows that it produced current at a slower rate than replicate 2 in the first days and there was more lactate and acetate accumulation than in other 4 g $N\text{-}NH_4^+ L^{-1}$ MECs. This could be the result of excessive disturbance to the biomass of the anode during initial sampling, leading to a decreased capacity for acetate uptake in the early stages of operation. Given that additional methane was not observed, the fate of these electrons is unclear. While lactate built up in the 4 g $N\text{-}NH_4^+ L^{-1}$ treated MECs, it was removed from the solution more quickly than in the fermentation only cultures.

The presence of the ARB biofilm enabled the rapid degradation of lactate in the MECs, perhaps by removing acetate, one of the end products of lactate fermentation, more efficiently than could be accomplished in fermentation only cultures. Given that acetate and propionate are produced as lactate disappears, it is likely that acetate is the intermediate between lactate and current production. The results with glucose are very promising, and the lack of detrimental effects seen in the sucrose MEC indicate that at the least, NH_4^+ concentrations around 4 g $N\text{-}NH_4^+ L^{-1}$ are not detrimental to CE in more complex waste streams which rely on fermentation as part of the treatment process. It is unclear why sucrose fed MECs failed to respond positively to NH_4^+ treatment.

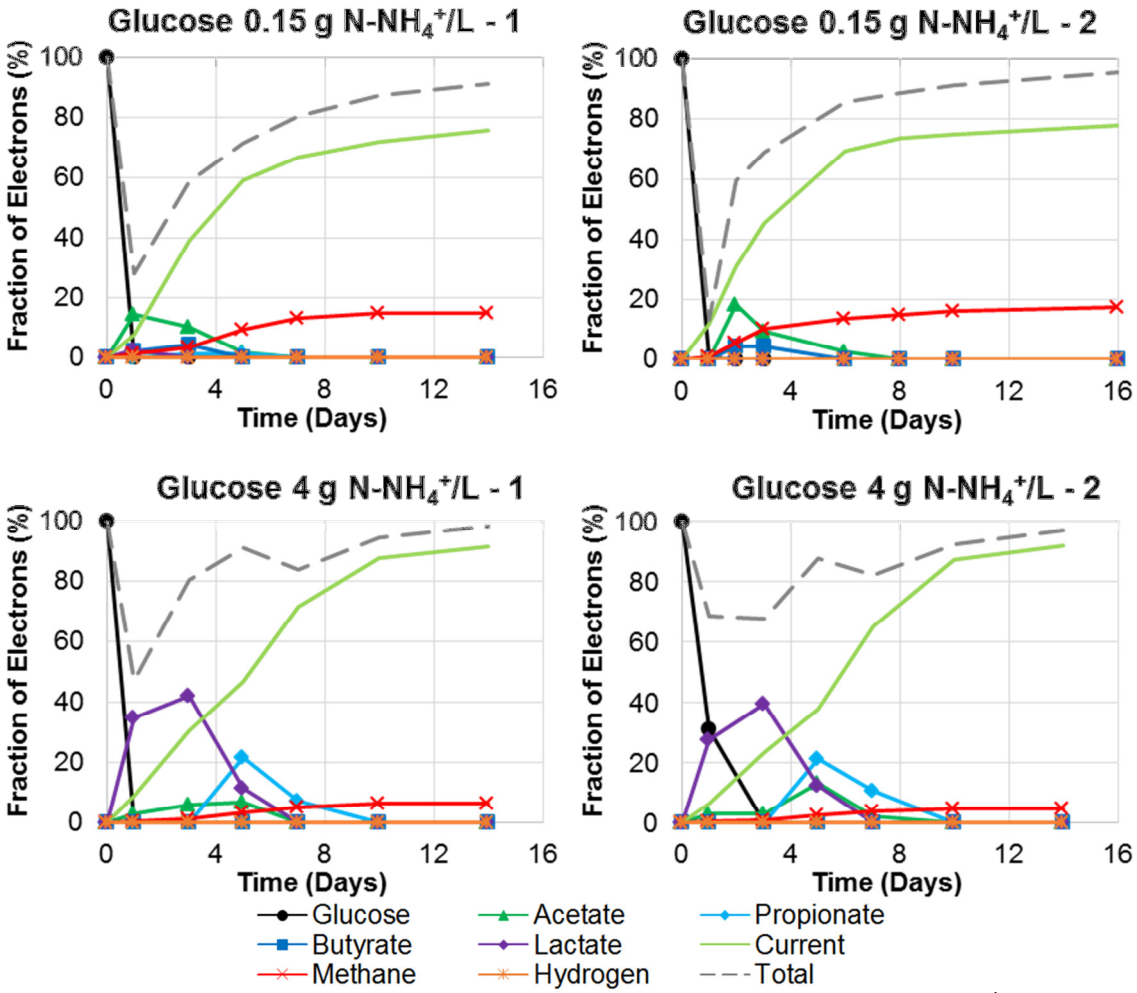


Figure 6.4. Electron balances for the glucose fed MECs at control (0.15 g $N\text{-}NH_4^+$ L^{-1}) and high (4 g $N\text{-}NH_4^+$ L^{-1}) NH_4^+ conditions. Performed in duplicate.

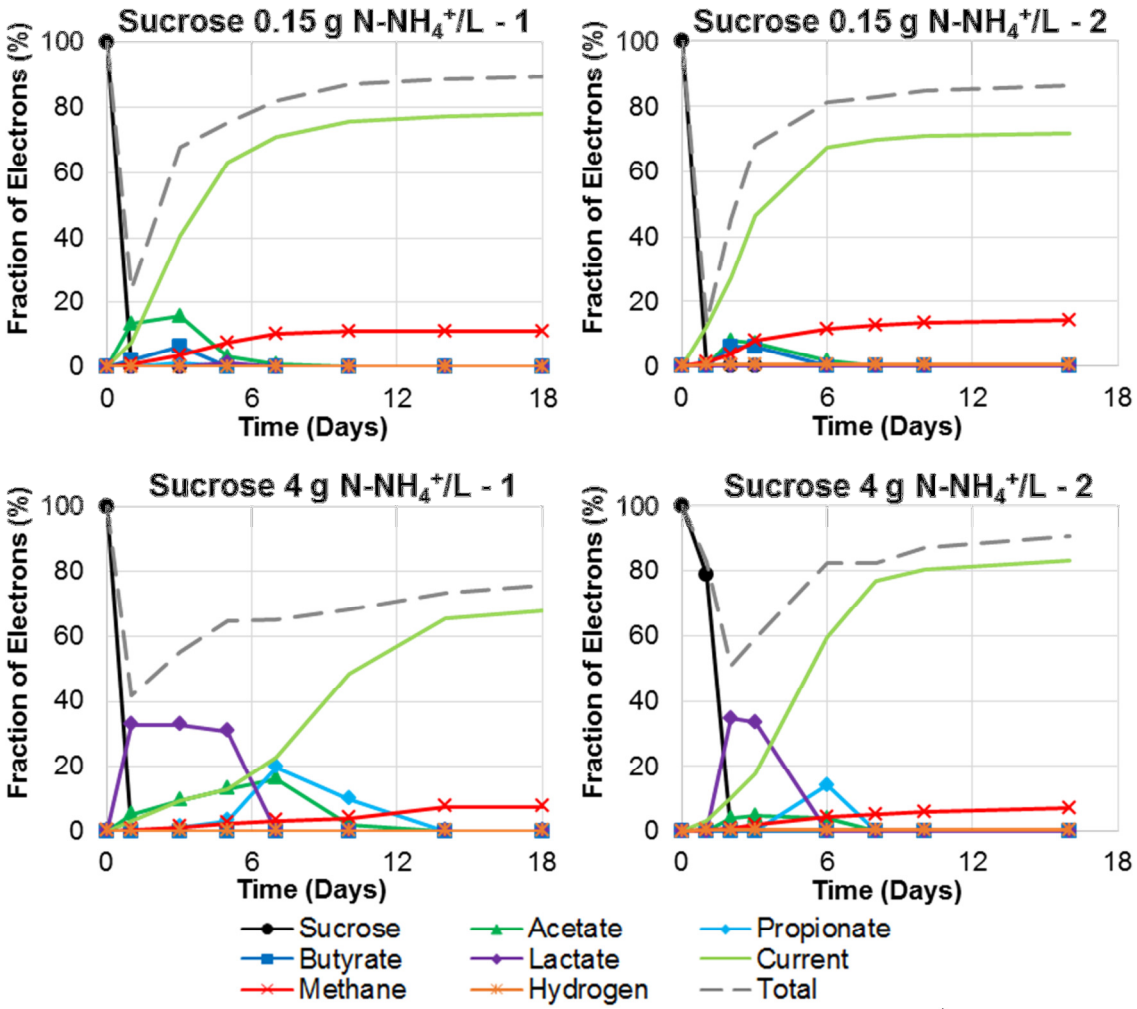


Figure 6.5. Electron balance for sucrose fed MECs at control (0.15 g $N\text{-}NH_4^+$ L^{-1}) and high (4 g $N\text{-}NH_4^+$ L^{-1}) NH_4^+ conditions. Performed in duplicate.

6.3.4 Higher NH_4^+ Has Only Minor Effects on Overall Community Structure in MECs

The microbial communities sampled for community analysis in the MECs were the added hydrogenotrophic enrichment cultures, the added ADS inoculum, initial starting biofilms, ending biofilms, and ending suspension communities. The shifts in microbial communities due to NH_4^+ treatment were less drastic in the MECs than in the batch cultures. In these communities, OTUs were identified down to genus where possible, and down to family and order where necessary. Only OTUs which accounted for more than 5% of one of the communities are shown in each figure. Across all figures, the same colors were used to represent the same OTUs, while OTUs which clustered separately at the genus level but which could not be identified as separate taxa at the given level are shown with the same color but different patterns.

The effect of NH_4^+ on MEC communities was most pronounced in the suspension communities, shown in Figure 6.6. Although members of the order Clostridiales dominated the suspension communities of glucose fed MECs in both conditions and suspension communities in sucrose fed control MECs (34-66% abundance), the dominant OTUs did not cluster into the same OTU at the family and genus levels. The major Clostridiales OTU in MEC suspension communities (40-62% abundance) under control conditions was assigned to the family Clostridiaceae and was the same OUT observed in the low NH_4^+ fermentation only batch cultures. The major Clostridiales OTU in the MEC suspension communities under high NH_4^+ conditions formed a separate OTU for which family could not be determined, indicating a shift occurred among Clostridiales members. This OTU accounted for a large amount of the population in glucose fed, high NH_4^+ MEC suspension communities (34-66%), but was less abundant in sucrose fed, high NH_4^+ MEC suspension communities (10-27%). The application of NH_4^+ treatment thus drove a shift between members of the order Clostridiales in MECs.

Both sucrose and glucose high NH_4^+ suspension cultures showed enrichment in Lactobacillales related OTUs, however the enrichment was neither as consistent across replicates nor to the same abundance as in fermentation only communities. Lactobacillales reached 27.5% in only one community, the suspension of glucose fed 4 g N-NH_4^+ L^{-1} MEC 2, and accounted for less than 10% in all other suspension communities. I conclude that NH_4^+ did shift

the communities; however, it did not provide as great of a selective pressure in the MEC suspension communities as I observed in the fermentation only batch cultures.

NH_4^+ had even less effect in the biofilm communities, shown in Figures 6.7 (glucose fed MECs) and 6.8 (sucrose fed MECs). Most of the biofilm communities were dominated by the genera *Geobacter,* with the minimum being 20% abundance in one community. *S. oneidensis*, the only known mesophilic, lactate consuming ARB, was below 1% abundance in all communities. None of the OTUs showed an increase or decrease which correlated with NH_4^+ treatment in the biofilms across replicates. The biofilm communities appear particularly resilient to increased NH_4^+ treatment.

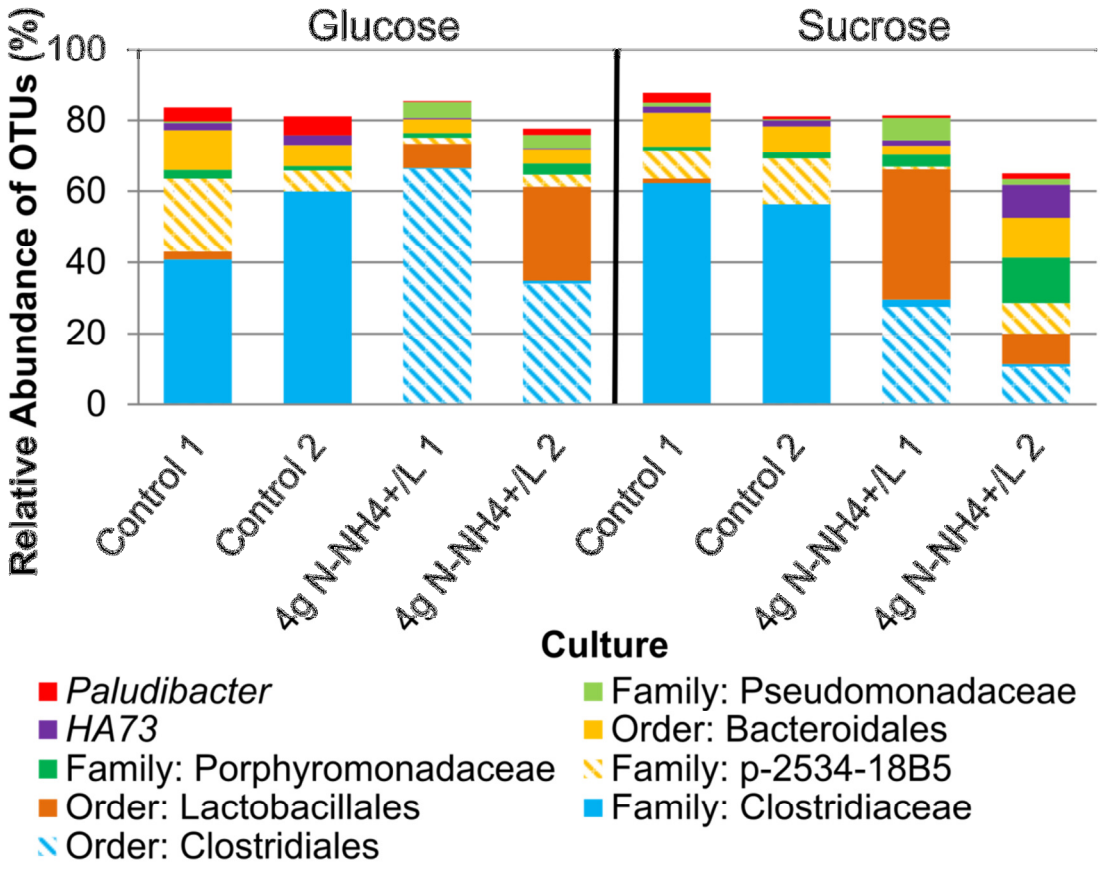


Figure 6.6. Relative abundance of OTUs in suspension communities from duplicate glucose and sucrose fed MECs, with either control (0.15 g N-NH_4^+/L) or high (4 g N-NH_4^+/L) NH_4^+ conditions. DNA collected at the end of the experiments. OTUs presented at the genera level, family and order identified when necessary.

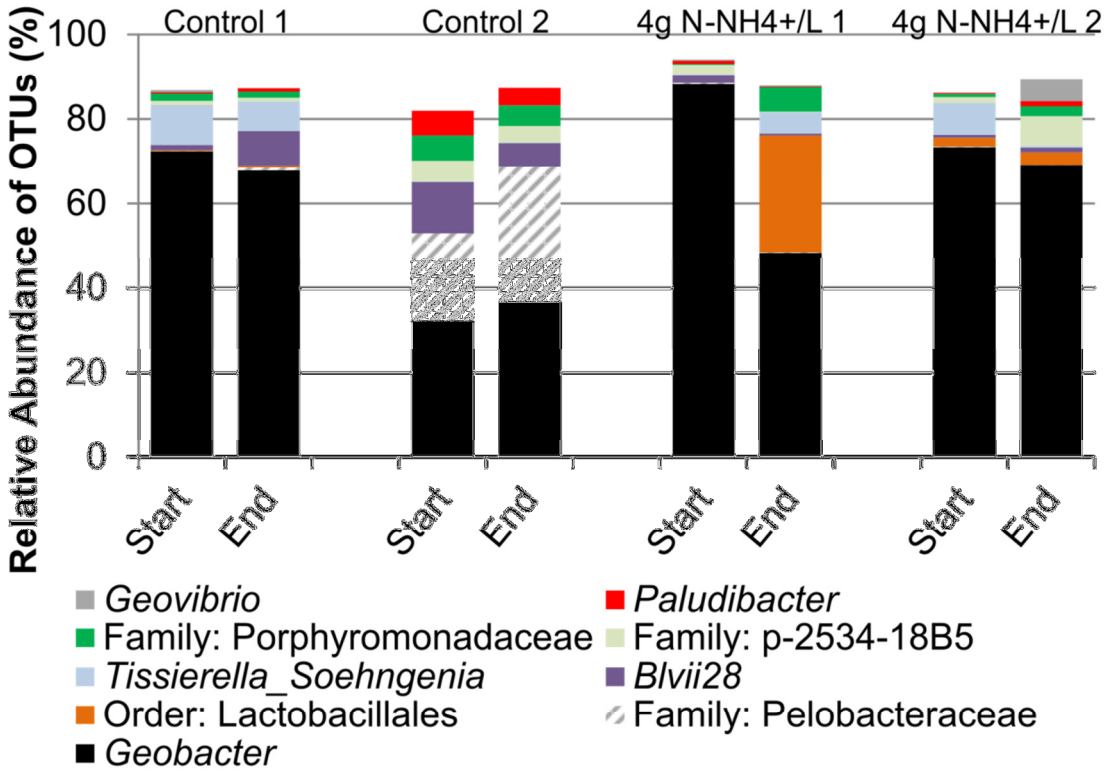


Figure 6.7. Relative abundance of OTUs of communities from starting and ending biofilms of glucose fed MECs, with either control (0.15 g $N\text{-}NH_4^+/L$) or high (4 g $N\text{-}NH_4^+/L$) NH_4^+ conditions. DNA collected at the end of the experiments. OTUs presented at the genera level, family and order identified when necessary.

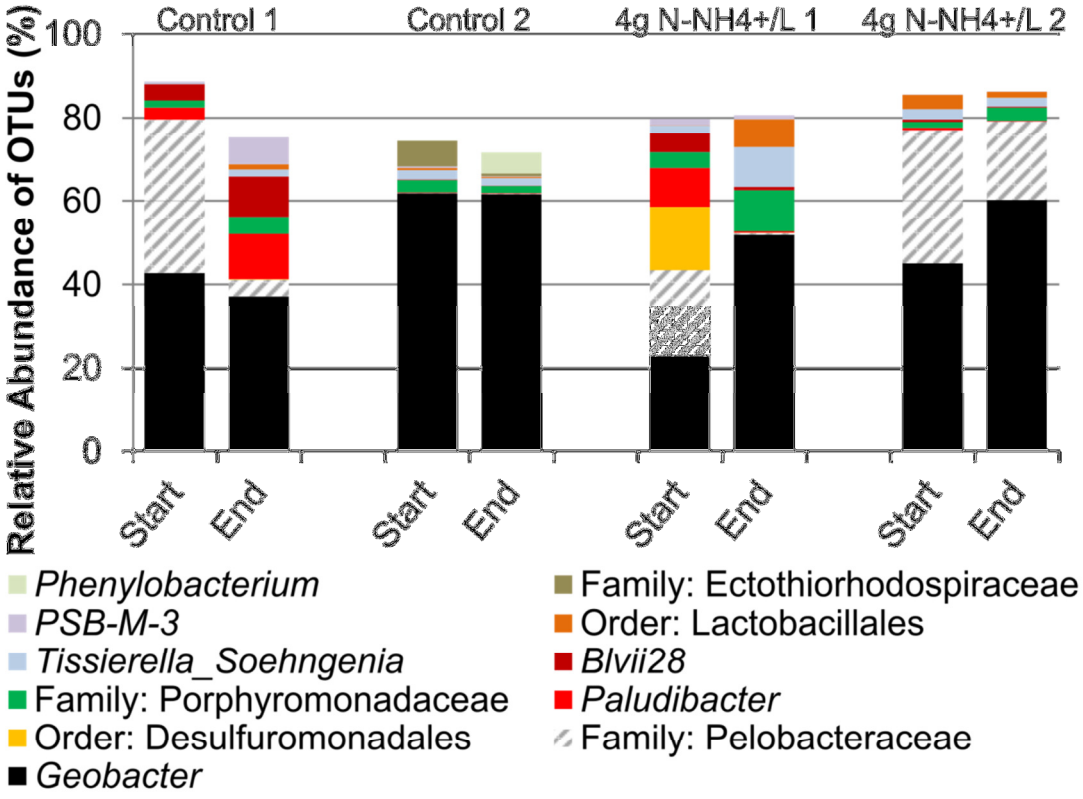


Figure 6.8. Relative abundance of OTUs of communities from starting and ending biofilms of sucrose fed MECs, with either control (0.15 g $N\text{-}NH_4^+$/L) or high (4 g $N\text{-}NH_4^+$/L) NH_4^+ conditions. DNA collected at the end of the experiments. OTUs presented at the genera level, family and order identified when necessary.

6.3.5 CCA Supports NH^{4+} as Only a Minor Factor in MEC Community Structure

CCA correlates taxa and environmental data, making it easier to interpret trends in the data caused by experimental conditions. Figure 6.9 shows a CCA plot with microbial communities from the Inoc-H_2 cultures, ADS inoculums, MEC starting biofilms, MEC ending biofilms, and MEC ending suspensions. Here we can see that, as expected, the hydrogenotrophic cultures formed their own cluster to the top left, indicating that they are very dissimilar to all other communities. Suspension communities and biofilm communities form two separate clusters, and the ADS inoculum samples clustered with the suspension communities. This is further illustrated by examination of the centroids, which represent sets of the communities which have a common environmental variable, such as ED or location in the system. The greatest distance, and thus difference in community structure, lies between centroids for communities from the biofilm and communities from the suspension. Suspended or biofilm growth in the MEC is thus a greater determinant than what ED was fed or whether NH_4^+ was elevated in determining community structure. In contrast, the centroids for glucose and sucrose communities and the centroids for control and 4 g N-NH_4^+ L^{-1} communities are much closer together, indicating they are not as large a driving force for the structures of the communities. In this CCA, the constraints used accounted for 80.3% of the variability in the data, indicating that the chosen constraints largely explained the changes observed in the community.

To further understand the driving forces of the microbial communities, the biofilm and suspension communities were analyzed with CCA, without the inoculum communities. In the results from analyzing only the suspension communities, shown in Figure 6.10, control communities form a cluster while the NH_4^+ suspension communities are separated from both the control communities and each other. The variability explained in the suspension communities only CCA is only 42.6%, thus the constraints used only partially explain the driving forces in the communities. In the CCA of only the biofilm communities, shown in Figure 6.11, the only major clustering evident is in samples from the starting biofilms. In this CCA, the variability explained was only 30.1%, indicating that the constraints were very poor at explaining the microbial community structures. These results indicate that NH_4^+ plays a role in driving the suspension

communities but not the biofilm communities; however, in these MEC experiments NH_4^+ was not the major driving force in shaping the microbial communities, as evidenced by the low variability explained in the biofilm and suspension CCAs. For greater detail on the microbial communities in these experiments, see Appendix D.

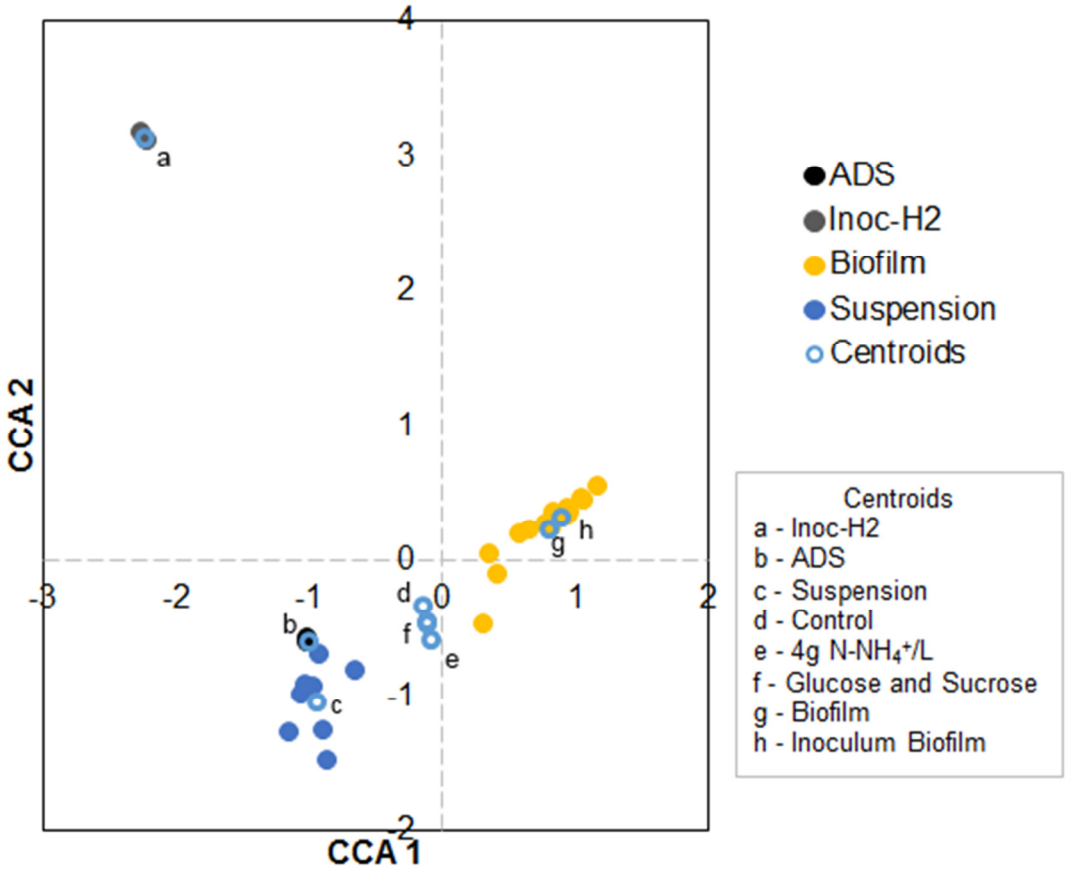


Figure 6.9. CCA of inoculum and communities from control and high NH_4^+ MECs fed glucose or sucrose. Circles indicate microbial communities and hollow circles indicate centroids for subsets of the communities. Centroids are identified by lowercase letters.

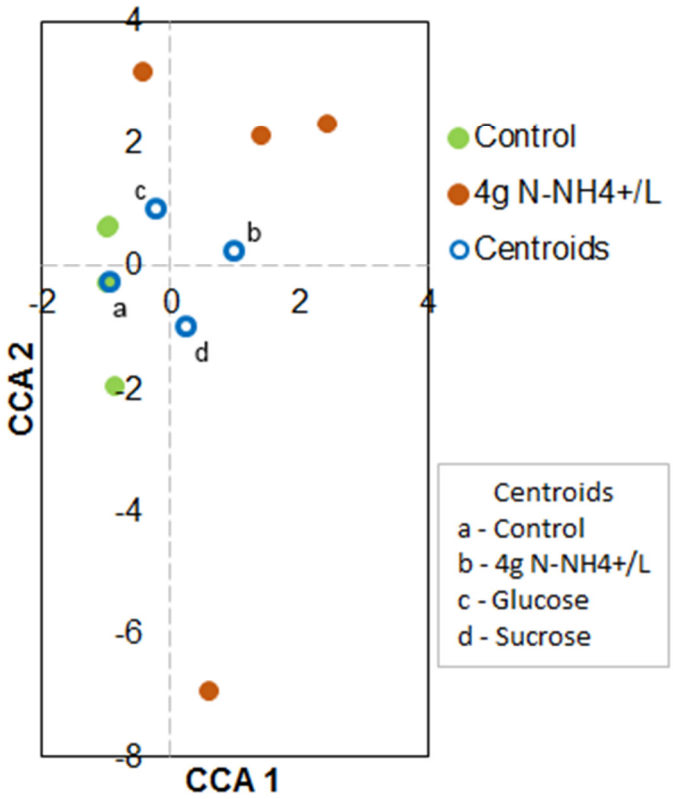


Figure 6.10. CCA of only suspension communities from control and high NH_4^+ MECs fed glucose or sucrose. Full circles indicate microbial communities; hollow circles indicate the centroids for subsets of the communities. Centroids are labeled by lowercase letters.

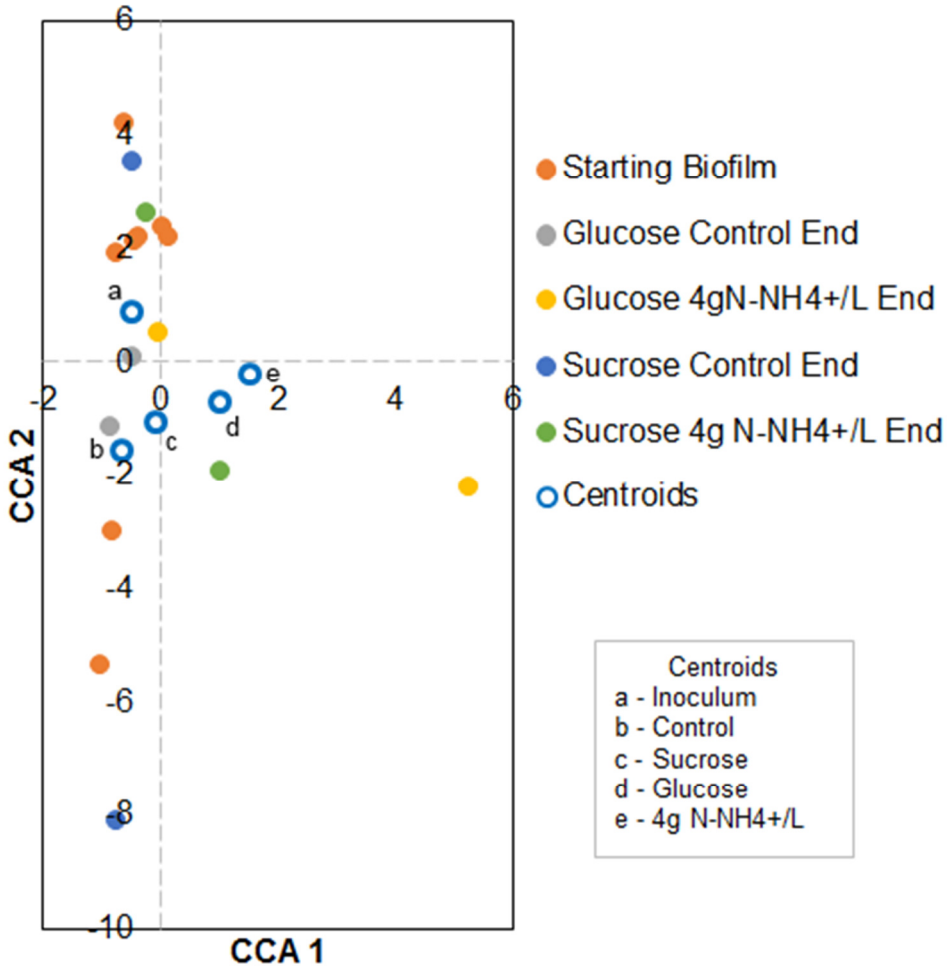


Figure 6.11. CCA of only biofilm communities from control and high NH_4^+ MECs fed glucose or sucrose. Full circles indicate microbial communities; hollow circles indicate centroids of subsets of the microbial communities. Centroids are labeled with lowercase letters.

6.4. Conclusions

This work encourages the use of MECs to treat high NH_4^+ waste streams. Increased NH_4^+ shifted the products of fermentation of glucose and sucrose away from methane and towards SCFAs and lactate in fermentation only cultures, with a shift in the microbial communities towards the family Lactobacillales at NH_4^+ concentrations greater than 4g N- NH_4^+ L^{-1}. While CE in glucose fed MECs improved from 75 to 90% with high NH_4^+, only one of the two replicate sucrose MECs demonstrated improvement. NH_4^+ did not cause major changes in the microbial communities of MECs, indicating a stabilizing effect of anode respiration, and its removal of end products from fermentation, that helps the microbial communities resist NH_4^+ toxicity. Clearly, the use of 4g N- NH_4^+ L^{-1} in MXC streams is acceptable, and I would suggest up to 5g N- NH_4^+ L^{-1} might prove beneficial given ARB tolerance. This work fills an important gap in fundamental knowledge, showing how NH_4^+ affects fermenting microbial communities in MECs using simple sugars and will inform future work with real world waste streams.

CHAPTER 7

SUMMARY

MXCs offer an answer to the questions of how do we maintain and improve the cleanliness and safety of environmental water sources. Their development will help improve the energy efficiency of wastewater treatment, making it more affordable for developing nations to implement and in doing so avoid the contamination of their already stretched water supplies. My work in MECs, expanding our repertoire of ARB to choose from, building microbial communities, and managing the fermenting microbial communities associated with MECs also applies to MFCs, as the same communities are involved, and thus will help advance MXC technologies.

When I began my research, the variety of known ARB were limited in their capacity to work in different environments, which in turn limited the types of wastes which MXCs could utilize and the variety of applications where MXCs could be implemented. The abundance of locations which fit the known requirements for ARB indicated that a wider variety of ARB should exist and were merely waiting to be found. In Chapter 3, I demonstrated the enrichment of ARB capable of generating > 1 A m^{-2} in 7 out of 13 environmental samples tested. I used MECs to provide an anode as electron acceptor and limited the available ED to acetate, thus placing a selective pressure on the microbial communities introduced from the environmental samples which enabled ARB to thrive and take over the microbial communities present. I used microbial community analysis based on sequencing the 16S rRNA gene in order to characterize the microbial communities and identify the genera which were enriched. From these experiments, I identified a candidate novel ARB genus, *Geoalkalibacter*, which has proven to contain the first identified halophilic and alkaliphilic ARB species, as characterized by Badalamenti et al (2013). Furthermore, the presence of highly diverse microbial communities in the anode respiring biofilms in my experiments after two stages of enrichment indicates that greater redundancy in the capacity for anode respiration may exist than previously expected. These results open up the development of MXCs with high salinity and high alkalinity wastes in the anode compartment.

While I started my research at the base of the MXC food chain with ARB, I was very interested in exploring how MXC systems could be improved through the optimization of microbial

interactions in complex microbial communities. Additionally, the problem of some ARB having a limited repertoire for ED utilization came to my attention. Previous research demonstrated the simple concept of combining isolated strains of bacteria with complementing metabolisms in order to build a desired network of metabolic reactions, including work in MECs. In Chapter 4, I expanded upon the concept of developing co-cultures to perform multi-step processes for electricity production by introducing the use of mixed cultures in place of isolates. The co-mixed culture I constructed successfully converted butyrate to current, through acetate as an intermediate, at high CE (~70 %). Its CE rivaled the best natural microbial communities and outperformed the majority of natural communities reported in the literature, demonstrating the utility of assembly over relying on the natural presence of needed organisms. The majority of work in combining chosen microbial partners comes in the form of co-cultures with isolates. In the natural environment, however, microorganisms work together among a large number of competing organisms and thus keep each other in check and provide redundancy of function in the face of shifting environmental conditions. Assembling communities may therefore prove even more successful given the self-correcting nature of diverse communities. Furthermore, wastewater treatment naturally involves the use of mixed microbial communities, eliminating the possibility of using binary co-cultures that may work well in laboratory experiments.

After my work assembling microbial communities in MECs, it became a natural progression to consider how to manage microbial communities in order to obtain desired outcomes. Initial work on the topic of Chapter 6, using ammonia to manage fermentation, gave me great trouble. Every time I ran a batch culture to test the inhibition of methane by NH_4^+, the batch cultures directed only 5-10% of their electrons to methane. While investigating the failure of my cultures to produce methane, I came to understand that the concentration of EDs which I provided to the batch cultures caused inhibition in the pathways for producing H_2. Once I understood what was causing this from a thermodynamic standpoint, it opened up the opportunity to make use of this as a method for controlling fermentation to prevent the production of methane and improve CE in MXCs. Previous research along these lines mostly examined glucose as a model ED, excluded methanogenesis, and failed to examine how the microbial communities are

affected under these conditions. In Chapter 5, I identified shifts in the microbial communities of cultures fermenting the simple EDs ethanol, lactate, glucose, sucrose, and molasses. I set up different fermenting batch cultures for each ED and varied initial ED concentrations, resulting in a decrease in the fraction of electrons going to methane production and an increase in the fraction of electrons going to acetate, and in some cases propionate and butyrate. The thermodynamic requirements of fermentation, altered by varying the ED concentration and the resultant H_2 partial pressure, determined the microbial community structure in these experiments. I was able to link these together using chemical and community data in a study which included methanogenesis across a variety of model compounds for the first time. The microbial communities showed a shift towards the known propionate producers *Bacteroides* and *Clostridium*, depending on ED. The metagenome, predicted by PICRUSt, indicated that the communities contained redundancy in taxa able to produce propionate. My results here offer improvements not only to CE in MXCs fed fermentable EDs but also help inform research on other fermenting communities in bioremediation and the human microbiome.

Once I understood how ED concentration could be used to control the products of fermentation, I applied this information to my experiments using NH_4^+. Fermentation in MXCs often leads to a loss of electrons to methane and methanogens are known to have a lower threshold for NH_4^+ toxicity than fermenters. The majority of literature examined the effects of ammonia in anaerobic digestion and how methanogenesis inhibition causes digester upset and failure. Review of the literature also indicated a favorable comparison with NH_4^+ toxicity in ARB. This brought me to test the effects of NH_4^+ on the fermentation of simple sugars in MECs. If NH_4^+ inhibits methanogenesis at concentrations where fermentation and anode respiration continue unaffected, then CE will improve in the presence of NH_4^+ concentrations which inhibit methanogens. In Chapter 6, I compared the effects of NH_4^+ on fermentation with methanogenesis, to the effects of NH_4^+ on fermentation in MECs and the associated microbial communities. Increasing NH_4^+ increased production of SCFAs and, at higher NH_4^+, lactate in fermentation only cultures fed glucose and sucrose while CE in MECs improved only with glucose as ED. The fermentation only cultures showed a marked change in community structure with a

high abundance of OTUs related to the order *Lactobacillales* above 4g $N\text{-}NH_4^+$ L^{-1} while MEC communities appeared less affected. It appears that the combination of anode respiration with fermentation at high NH_4^+ provides stability to the community, possible by drawing off fermentation products more quickly than methanogenesis. At 4g $N\text{-}NH_4^+$ L^{-1}, MECs fed with fermentable ED exhibit improved CE (from 75 to 91%) or no change, depending on ED, providing further support for their use in treating high NH_4^+ streams. Further work may demonstrate improvements in CE with sucrose as well, if damage to the biofilm during sampling was responsible for the inconsistent results. This work builds upon the fundamental knowledge of NH_4^+ treatment in MECs by focusing on what occurs during fermentation, filling in the gaps between treating real waste streams and testing NH_4^+ toxicity on only ARB, and indicates that MXC technologies are less susceptible to upsets caused by high NH_4^+ waste streams than methanogenic systems . Finally, my work comparing fermentation only and MEC communities indicates that results in one area do not always translate into the other; anode biofilm communities do not simply replace acetoclastic methanogenesis but apply their own selective pressures on fermenting communities.

In closing, my work has both broadened and deepened our knowledge of microbial communities in MECs and how efforts to control fermentation affect them. Novel ARB remain undiscovered in the environment with unique properties, such as salt and pH tolerance, and searches for them should be tailored towards desired characteristics of their biology. Novel communities assembled using mixed microbial communities can be just as efficient as naturally derived communities at performing useful functions and likely result in more resilient communities than binary co-cultures. In fermenting systems, the maintenance of fermentable EDs above 400 me^- eq. L^{-1} should divert >80 % of electrons to SCFAs production, contingent on the size of methanogen populations. The use of 4g $N\text{-}NH_4^+$ L^{-1} in MXC influent streams should be considered as it can result in improvements in CE of 10-15% with some fermentable EDs and MXC communities appear to be resilient to NH_4^+ driven community changes. In the following section, I present directions for future research related to the work I have done.

CHAPTER 8

Suggestions for Future Research

8.1. Continuing the Search for Novel ARB

It is likely that the natural environment continues to harbor unidentified ARB with novel characteristics. In my experiments in chapter 3, the selective pressures chosen were tailored to known ARB: low salinity, neutral pH, mesophilic temperature, and acetate as ED. While this ensured my experiments would enrich some ARB, those already known, it could not guarantee the enrichment of novel ARB. Future bioprospecting should therefore continue by tailoring the enrichment strategies towards organisms which thrive in different environments. This has already proven successful with regards to temperature with the discovery of thermophilic ARB. In addition, consideration of the characteristics of the environmental sample should be included, for example using a low pH enrichment strategy for samples which come from a low pH environment. Specifically, efficient low pH ARB remain undiscovered. The discovery of ARB which thrive in low pH environments could offer improvements in power production by MXCs, given the limitations imposed by acid production during fermentation, proton production in biofilms performing anode respiration, and the limitations of cathode reactions at neutral pH (Popat et al., 2012).

Throughout my work and that of others, organisms in the order Clostridiales appear in anode respiring biofilms on a regular basis. Clostridiales are known fermenters and even contain known ARB: *Thermincola potens* and *Thermincola ferriacetica* (Parameswaran et al., 2013; Wrighton et al., 2011). These two known ARB are thermophilic; however, the repeated presence of Clostridia at relatively high abundance (>10%), in cultures which have been enriched on acetate with an anode as electron acceptor, at mesophilic temperatures, indicates they may be contributing to anode respiration at lower temperatures as well. I propose further examination of the capacity of members of Clostridia for anode respiration. Specifically, I suggest looking at members of the genera *Desulfonispora*, *Fusibacter*, and *Geosporobacter* due to their abundance in my enrichment experiments in Chapter 3. See Appendix A for genera level breakdown of enriched communities.

Given the expense of *in vivo* experiments and the increasing accumulation of sequenced genomes and bioinformatics tools, it may be efficient to increase the search for ARB through database analysis. Sequenced genomes can be screened for the presence of c-type cytochromes with high homology to those found to be related to extracellular electron transfer in *Gb. sulfurreducens* and *S. oneidensis* (Franks et al., 2012; Inoue et al., 2010; Myers and Myers, 2001) *,* muli-heme cytochromes (Butler et al., 2009), and the presence of large numbers of outer membrane c-type cytochromes, a trait of both *Gb. sulfurreducens* and *S. oneidensis* (Methé et al., 2003; Meyer et al., 2004). Previous work compared the genomes across the family Geobacteraceae in order to explain the adaptation of a common ancestor into respiring organisms, such as *Geobacter* and *Geoalkalibacter*, and fermenting only organisms, such as *Pelobacter* (Richter et al., 2007). This work should help target the search for genes necessary for *Geobacter*-like anode respiration and further comparisons with related organisms should enable screening for novel ARB. Organisms known to be incapable of anode respiration can help rule out candidate genes while unknown organisms can be sorted based on the presence of candidate genes and then tested for the capacity perform anode respiration.

8.2. Applications Managing Microbial Communities

In anaerobic digestion, two phase systems have been used to separate fermentation from methanogenesis so that each process can be managed and optimized separately (Demirel and Yenigün, 2002; Muha et al., 2013). Given the results I obtained in Chapter 5, this may be a viable route for MXCs to take in wastewater treatment. A fermenting bioreactor can be operated at low pH, inhibiting the methanogens and driving the production of SCFAs. The fermenting bioreactor could also be optimized for its particular feedstock with less concern for the effects on the MXC. The effluent can then be sent to an MXC in place of a methanogenic digester. This would allow the optimization of the MXC, especially given that MXCs operate most efficiently on SCFAs as opposed to when they must include fermentation in their design. The combination of operating an MXC at a relatively short hydraulic retention time (HRT) and the growth of ARB at the surface of the anode, inhibiting methanogen retention on the anode's surface, would inhibit

methanogenesis in the system. This two stage system mainly requires optimization of the size of the fermenter to the MXC, in order to match output and input optimally.

In addition, such a system could be used in order to first produce a spectrum of fermentation products, in the fermenter, and then remove the lower SCFAs in the MXC with a relatively short HRT. The short HRT in the MXC would leave the medium chain fatty acids, which require longer HRTs to be oxidized, in the effluent. In this manner, a stream of medium chain fatty acids might be produced for collection and use as a chemical product of the process. In this scenario, the key is to have an undersized MXC for the fermenter's output. Optimization studies would need to focus on maximizing acetate consumption and minimizing higher carbon SCFA consumption.

Preliminary experiments, based on Chapter 5, have already proven fruitful in implementing ED loading based control of CE directly in fermentable ED fed MECs. In these experiments, batch MECs fed with sufficiently high concentrations of fermentable EDs shifted the products of fermentation from methane to current, compared to control MECs. Given that many planned implementations of MXCs operate in continuous mode, it would be useful to determine how well this strategy works in continuous operation. Previous work with continuous flow MECs provide high input concentrations of ED (Chung and Okabe, 2009; Freguia et al., 2008; Parameswaran et al., 2010, 2009; Torres et al., 2007); however, due to the kinetics of bacterial growth, the concentration of ED inside the reactor is much lower than the influent (Rittmann and McCarty, 2001). Further, the retention of biomass often enables methanogens to grow and consume (or utilize) part of the electrons supplied (Freguia et al., 2008; Parameswaran et al., 2010, 2009; Torres et al., 2007). Therefore, experiments with continuous operation should focus on the concentration of EDs inside the MXC in order to determine whether ED is sufficiently high to decrease H_2 production and hinder methanogenesis. The higher growth rate of fermenters should enable the maintenance of higher ED concentrations through the use of lower HRT, without washing out the fermenters. Low HRT will also assist with managing methanogens, through washout due to their low growth rates. So long as the anode surface is well matched to the output of the fermenting community, the removal of acetate should assist with decreasing

effluent concentrations of organics. The effect of high acetate removal, by ARB, on H_2 production during high rate fermentation should also be considered. Although some reports in the literature appear similar to this concept (Chookaew et al., 2014; Chung and Okabe, 2009; Min and Logan, 2004), the combination of using high ED loading and short HRT to drive acidogenesis with an anode matched to the output of fermentation productivity do not seem to have been implemented fully and tested yet.

8.3. Other Implementations of MXC Principles

Biogeobatteries are natural MFCs where a conductor crosses from an anaerobic environment with high organics to an aerobic zone and enables the transfer of electrons across the boundary (Hubbard et al., 2011; Revil et al., 2010). Biogeobatteries can increase the breakdown of environmental contaminants by acting as the electron acceptor in a reduced environment (Revil et al., 2010). It should be a relatively simple matter to insert conductors into contaminant plumes and artificially generate a biogeobattery, thus stimulating bioremediation. Related concepts exist at the laboratory scale, such as placing contaminated soil into MFCs, however, these would likely incur greater cost due to construction expenses and so the costs and benefits must be weighed. Given that much bioremediation is done at the lowest possible cost, the construction of artificial biogeobatteries may be more cost effective. First, laboratory scale experiments should be used to determine the rates at which such remediation can occur, which will likely be constrained by surface area of the electrodes and diffusion of ionic current between anode and cathode. Similar to MXCs, the design of the electrodes is likely to have large effects on the efficiency of the system, however, if the goal is bioremediation at low cost and not power production, then the answer could be as simple as sticking iron or graphite coated rods across the oxic/anoxic boundary.

The idea of biogeobatteries can also be inverted. Instead of using the inserted electrode as an anode, a polarizing potential could be applied in order to introduce reducing power to contaminant plumes, making them more amenable to reduction. Nitrate reduction has been demonstrated at the cathode (Virdis et al., 2009); however other contaminants remain to be exploited in such a manner. Recent work with biocathodes has shown the uptake of electrons by

hydrogenotrophic organisms though unproven mechanisms. If the mechanism of electron transfer in these biocathodes is H_2 transfer, then dechlorination of trichloroethenes could be possible using a poised cathode in the environment as dechlorinating bacteria, such as *Dehalococcoides*, prefer H_2 as their ED. The presence of hydrogenotrophic organisms near the cathode should maintain a low H_2 partial pressure, making H_2 more thermodynamically favorable.

8.4. Fundamental Questions

In my work, several fundamental questions arose which were not answered. Although these questions have less immediate practical implications, they are of interest and may open up new methods of managing fermentation. Is the production of propionate (and other higher fatty acids) solely due to changes in the microbial community structure or can the metabolism of key propionate producing fermenters shift between propionate and acetate plus H_2 production? What other organisms, other than those identified in the literature, are responsible for propionate production in anaerobic environments? Do MXC communities offer better process stability than methanogenic communities in anaerobic digestion?

During my research presented in Chapter 5, I originally expected to find little change in the microbial communities related to the change in the balance of fermentation products. Given that many bacteria can adjust to changing environmental conditions, I expected to see the microbial communities remain unchanged with increasing ED concentration, indicating a change in the metabolisms of the organisms already present. Instead, I saw shifts in the microbial community that were connected to changes in the products of fermentation. Further, the genes I identified as being related to propionate production were not predicted to be present in the majority of community members, indicating that only some of the community members were capable of using propionate production to get rid of excess reducing equivalents. I think it would be interesting to know whether or not fermenters capable of propionate production can shift their fermentation products based on prevailing conditions. In order to test this, I propose the use of pure cultures of known propionate producing organisms, such as *Clostridium propionicum* (Hetzel et al., 2003; Leaver et al., 1955) and *Bacteroides fragilis* (Macy et al., 1978), in experiments measuring their fermentation products at varied ED concentrations and H_2 partial pressures.

These experiments would help to determine whether propionate producers can alter their metabolisms to generate different products based on prevailing conditions or if changes in the products of fermentation are solely the result of competition between different organisms. While the former seems likely, my results in Chapter 5 indicate the latter occurs more readily.

Also in Chapter 5, a number of organisms increased in abundance, according to 16S rRNA gene analysis, with increasing ED concentration and propionate production; these organisms have not been identified as propionate producers. In addition, a second set of organisms were predicted by PICRUSt to carry the methylmalonyl-CoA decarboxylase gene (Hoffmann et al., 1989), key to the production of propionate through the succinate pathway. It is unclear whether either of these groups include actual propionate producers or if they play some other important role in the fermentation of the provided EDs. I therefore propose experiments to test the identified clades further, using simple fermentable EDs and pure cultures, in order to establish whether they are capable of producing propionate during fermentation or if they halt at some intermediate compound when isolated. Classifying organisms as propionate producers will aid in future microbial community studies by providing insight into the role of these organisms in mixed microbial communities.

In Chapter 6, the resiliency of anode respiration and the associated microbial communities at high NH_4^+ concentrations indicates that MXC communities may be more resilient than methanogenic communities to drastic changes in environmental conditions. The MXC communities maintained operation in the presence of 4g $N\text{-}NH_4^+$ L^{-1}, concentrations that inhibited methanogenic communities. Perhaps, MXCs might prove more stable to changes in influent waste streams than conventional anaerobic digestion. I propose a direct comparison between the two technologies, but rather than relying only on efficiency with a given waste stream, waste streams should be varied in concentration and make up, including the presence of toxic compounds, and efficiency as a measure of resilience to these changes be measured. The ability of such systems to correct after major inhibitory events, such as those that would normally lead to a failed anaerobic digester, would be very interesting. If MXCs do indeed offer greater resiliency

in the face of drastic changes in waste streams, this would add another mark on the scorecard for their development and future implementation.

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APPENDIX A

COMPARISION OF ENVIRONMENTAL COMMUNITIES WITH ENRICHMENT COMMUNITIES AND BLAST RESULTS FROM CHAPTER 3

Provided here is an overview of how well enrichment worked in the ARB enrichment MECs of chapter 3 and details regarding the most abundant OTUs observed. Figure A.1 compares the microbial communities of the environmental samples to the corresponding biofilm communities enriched in MECs, identified at the class level. As discussed in detail in chapter 3, five communities became highly enriched for particular OTUs. Two communities, Carolina Mangrove and Salt Flat, were not highly enriched for single OTUs, an observation more common with fermentable EDs than acetate. Further, the Carolina Mangrove community, a high current producing biofilm community, appears very similar to its originating environmental sample.

Table A.1 shows the most abundant genera in each enriched biofilm community from chapter 3, the closest genbank match obtained by BLAST to the corresponding sequence, and the similarity between the obtained sequence and the genbank match.

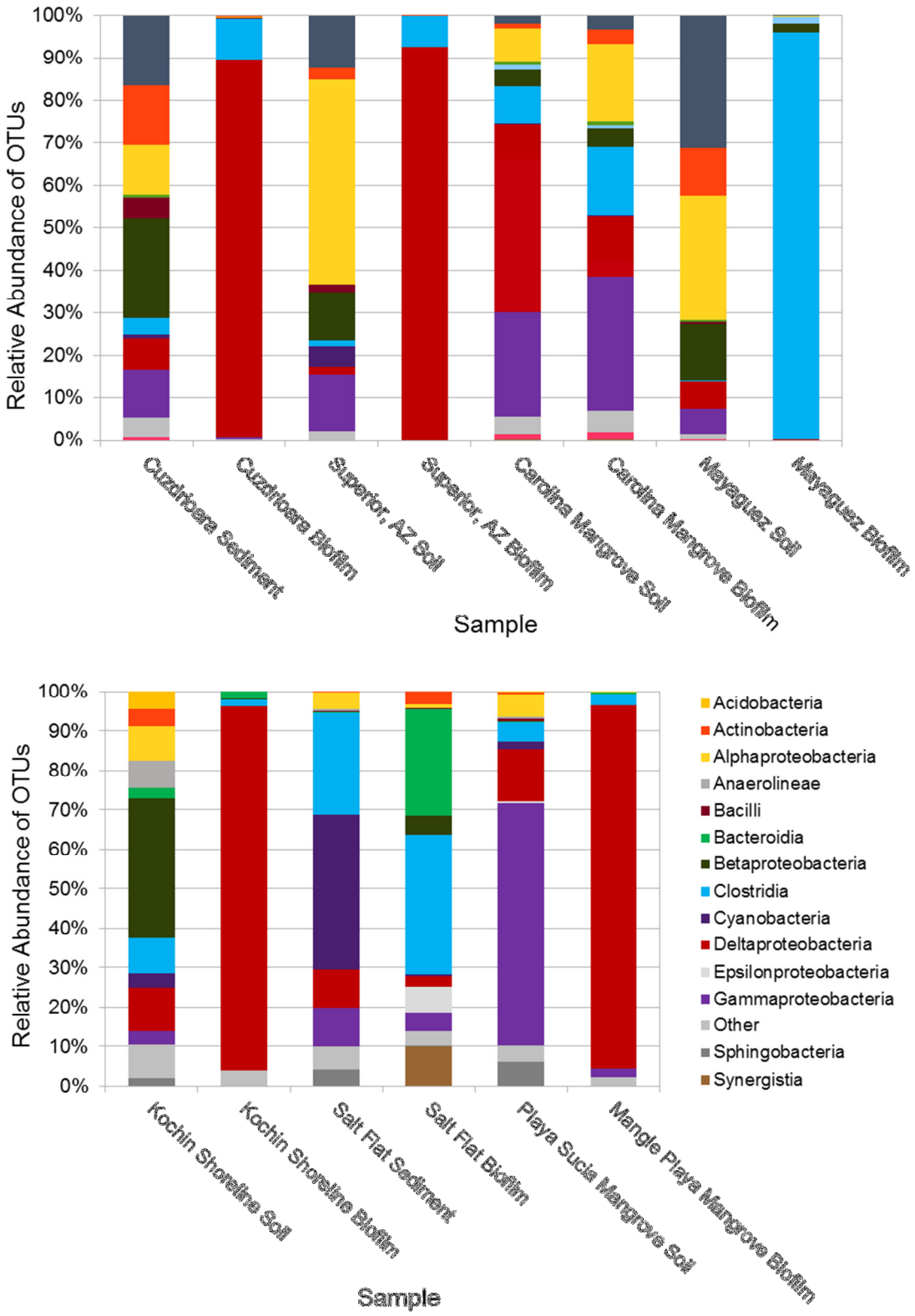

Figure A.1. Community analysis by class for environmental samples and anode enriched biofilms.

Table A.1. Dominant genera and closest genbank matches of abundant OTUs.

Sample	Dominant Genus	Abundance (% of sequences)	Closest Genbank match (as of July 24, 2012)	Percent Identity
Mayaguez	*Desulfonispora*	88%	*Desulfonispora thiosulfatigenes strain GKNTAUT 16S* (NR 026497.1)	92%
Playa Sucia Mangrove	*Geoalkalibacter*	89%	*Geoalkalibacter subterraneus strain Red1 16S* (NR 044429.1)	99%
Salt Flat	*Geosporobacter*	34%	*Geosporobacter subterraneus strain VNs68 16S* (DQ643978.1)	93-94%
	Proteiniphilum	29%	*Proteiniphilum acetatigenes strain TB107 16S* (NR 043154.1)	97%
Superior	*Geobacter*	92%	*Geobacter metallireducens GS-15* (CP000148.1)	95-96%
Kochin Shoreline	*Geoalkalibacter*	93%	*Geoalkalibacter subterraneus strain Red1 16S* (NR 044429.1)	93%
Cuzdrioara	*Geobacter*	73%	*Geobacter pelophilus strain Dfr2* (NR 026077.1)	94-95%
Carolina Mangrove	*Fusibacter*	24%	*Fusibacter sp. BELH1 partial 16S* (FR851323.1)	96-97%

APPENDIX B

ELECTRON BALANCES FROM FERMENTATION ONLY CULTURES IN CHAPTER 5

Figures B.1-B.4 in this appendix show the electron balances from fermentation cultures fed the EDs ethanol, glucose, sucrose, and molasses, performed in chapter 5. The figures show the distribution of electrons throughout the course of the experiments, with the figures organized by ED. Each figure includes three graphs, one for each concentration of ED tested, and each graph shows averaged data from triplicate cultures. The figures show in detail the shift away from methane production and towards SCFA production as initial ED increases in greater detail than provided in the main text of chapter 5. Furthermore, the consumption of acetate, indicating the presence of acetoclastic methanogenesis, can be observed.

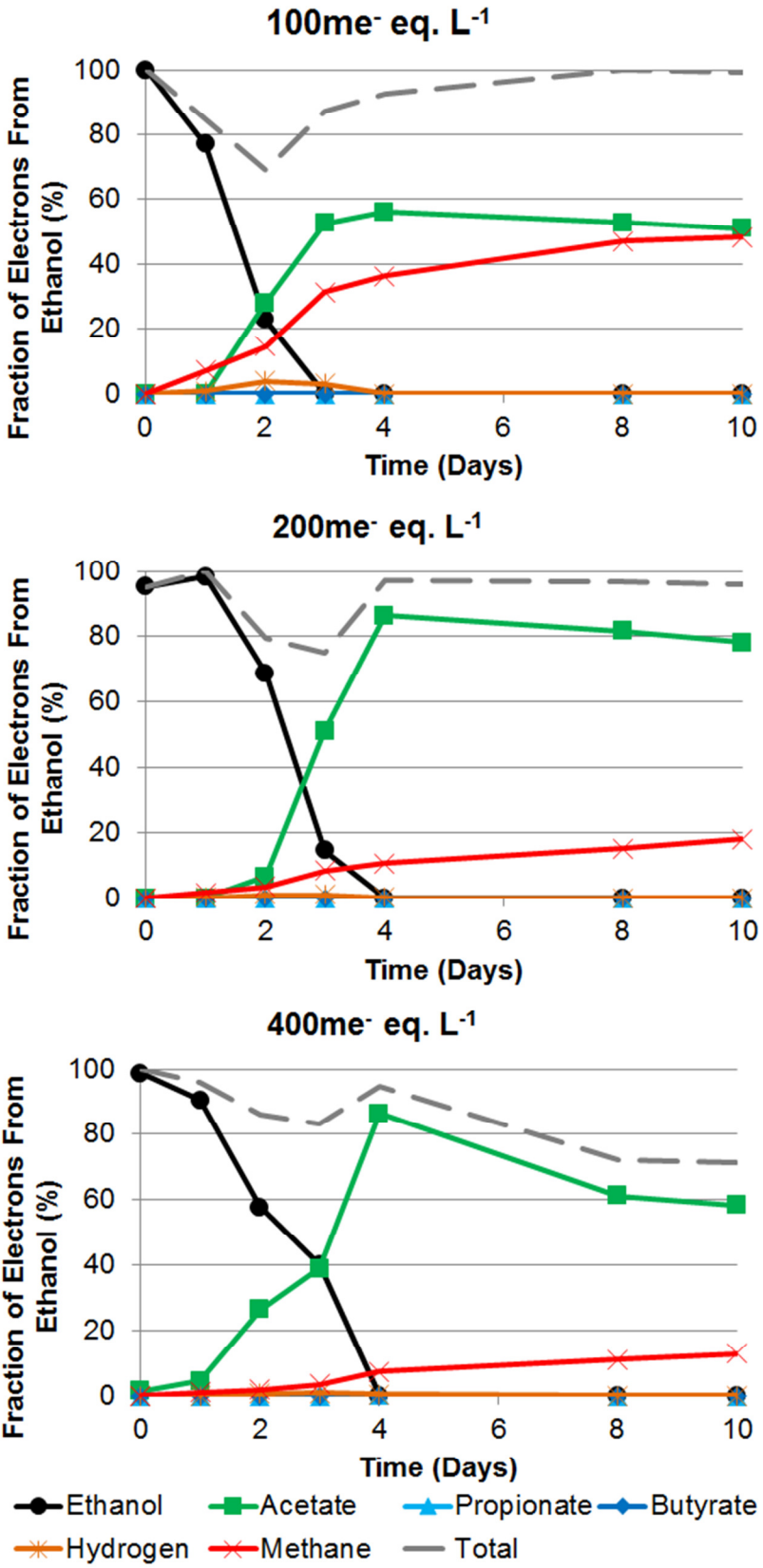

Figure B.1. Electron balances of ethanol fed fermentation cultures.

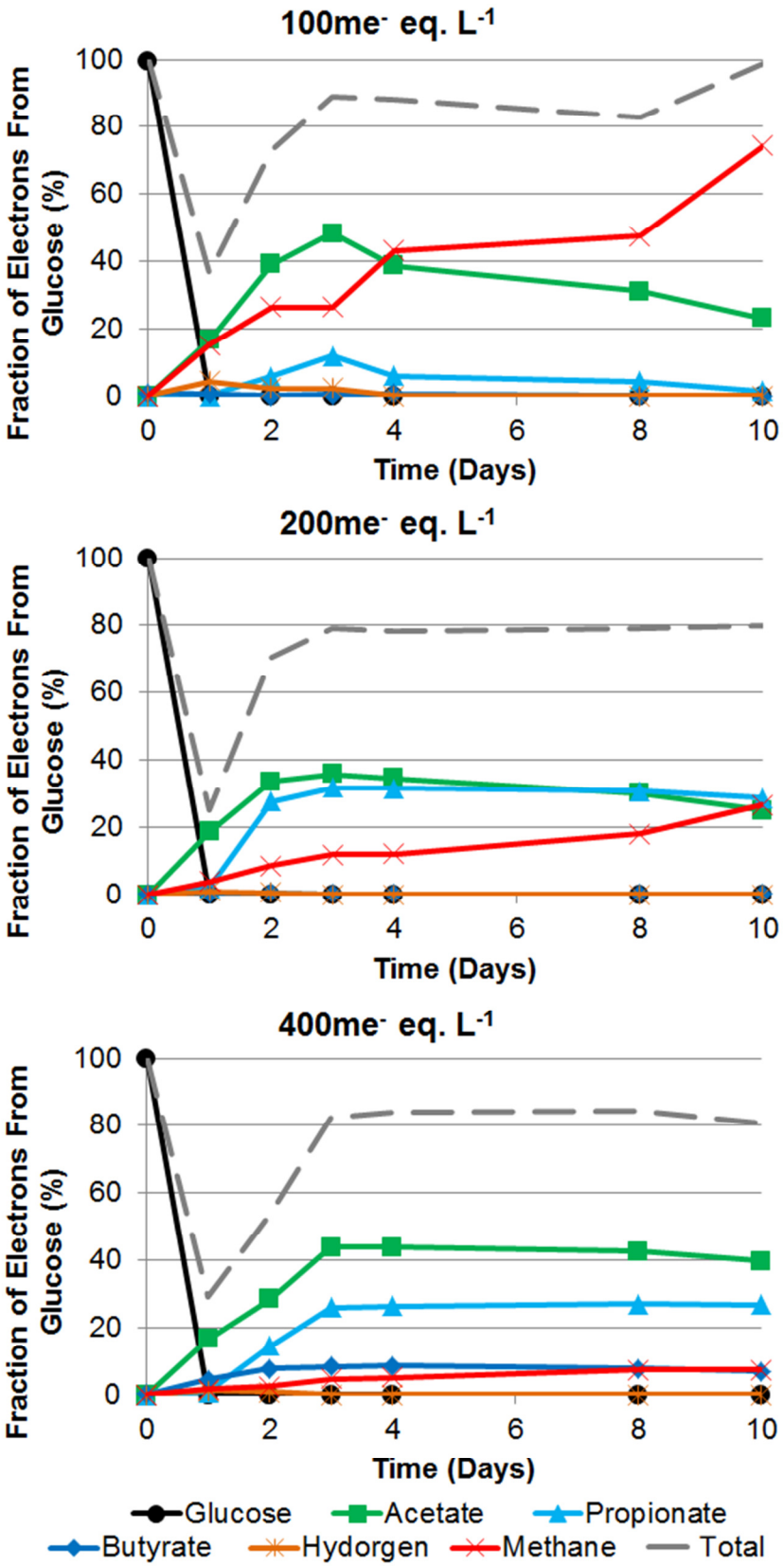

Figure B.2. Electron balances of glucose fed fermentation cultures.

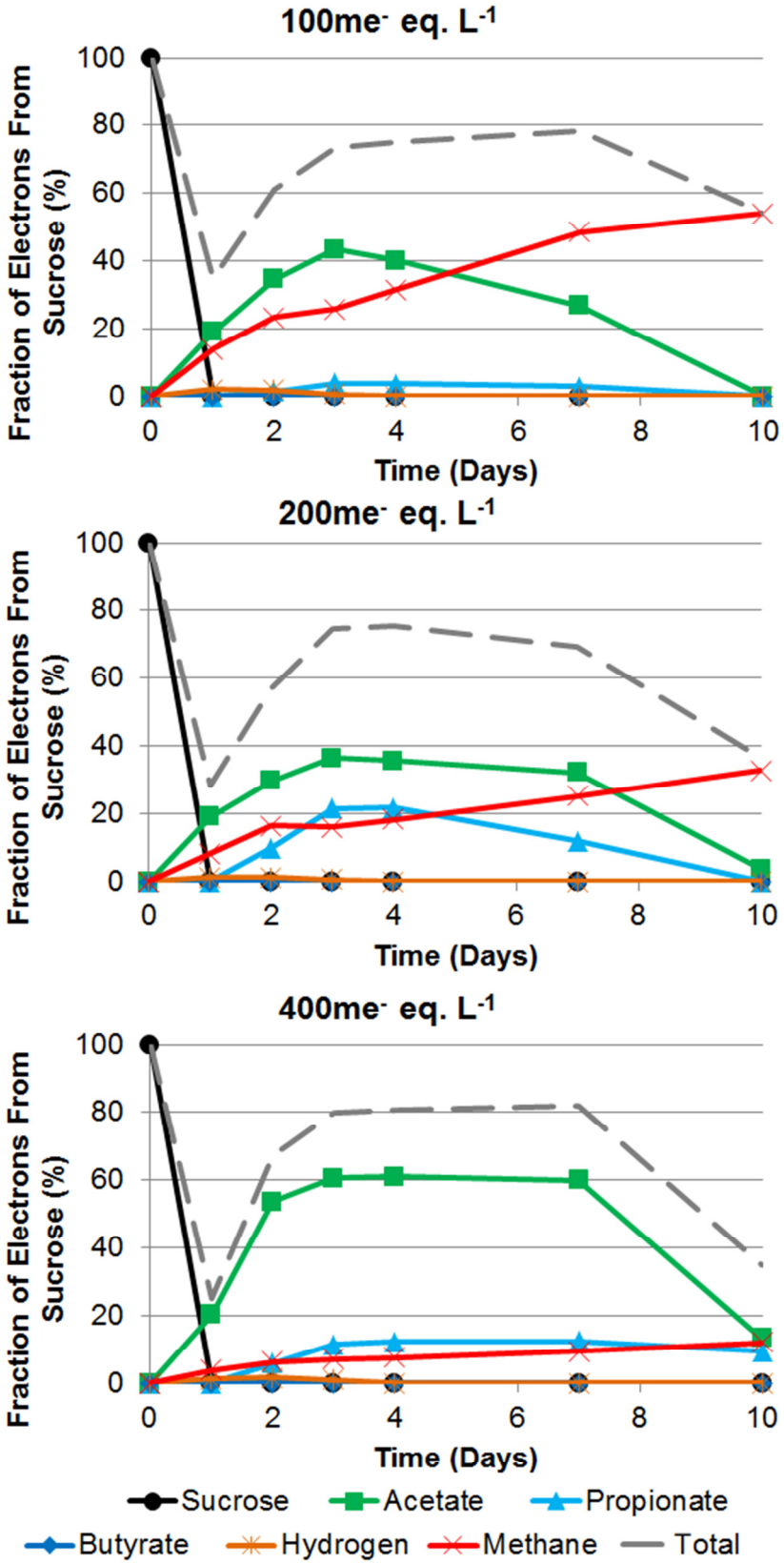

Figure B.3: Electron balances of sucrose fed fermentation cultures.

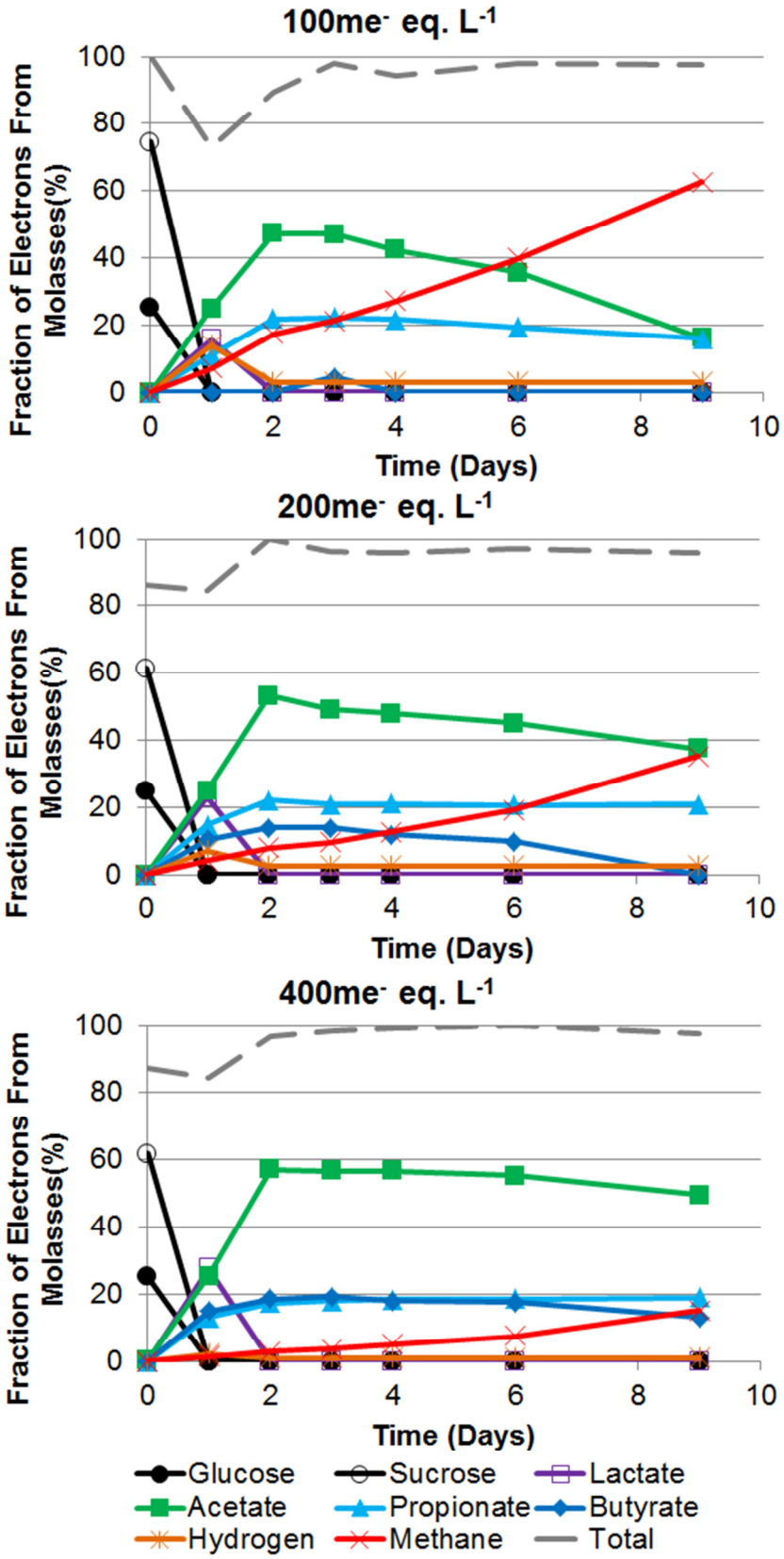

Figure B.4. Electron balances of molasses fed fermentation cultures.

APPENDIX C

DETAILED BREAKDOWN OF ELECTRON BALANCES FROM FERMENTATION ONLY CULTURES IN CHAPTER 6

In chapter 6, a summary was provided of the electron balances from the fermentation only cultures. Given the dynamic nature of microbial communities, I present here a detailed breakdown of the electron balances from the fermentation only cultures treated with increasing NH_4^+. The electron balances are separated by ED fed and each graph represents the average of triplicate experimental cultures.

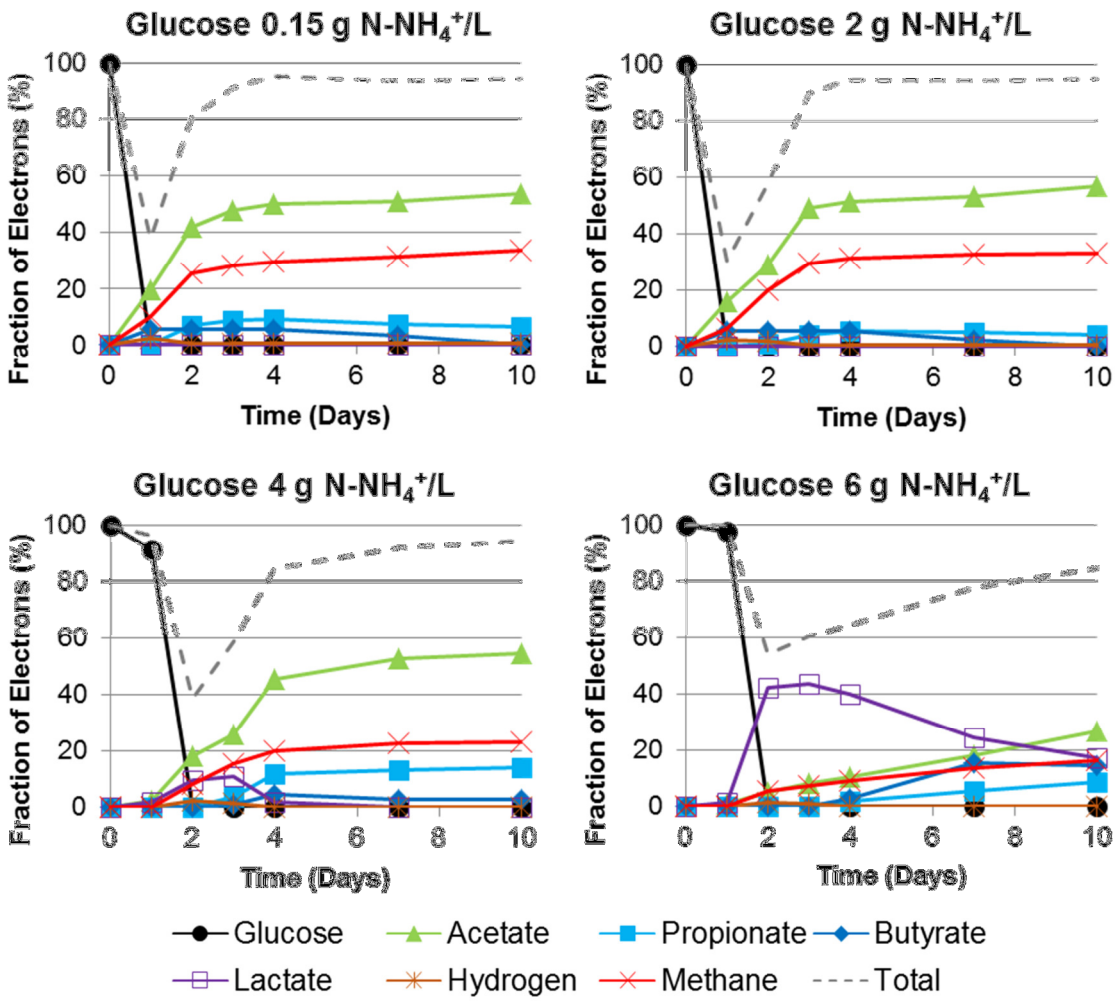

Figure C.1. Electron balances for glucose fed fermentation only cultures.

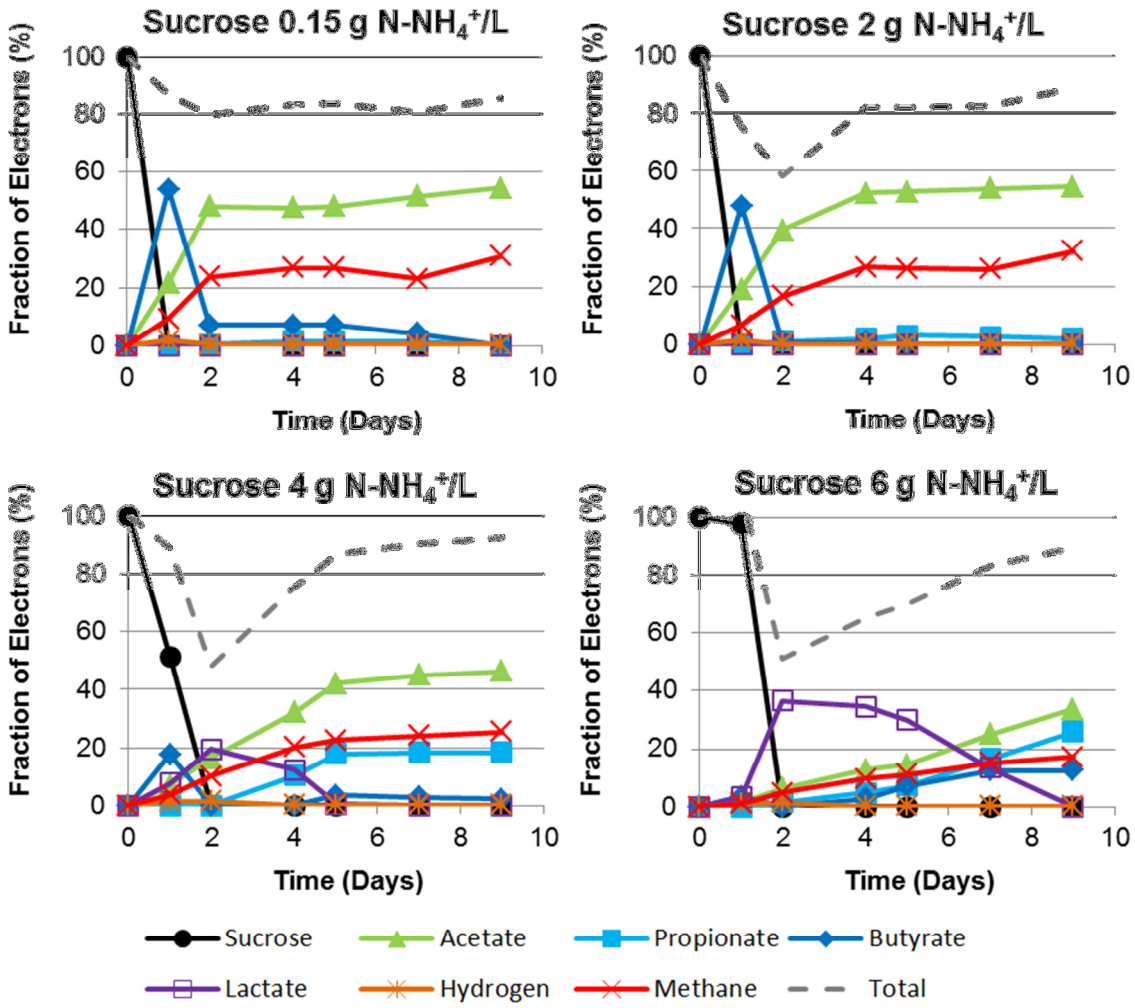

Figure C.2. Electron balances for sucrose fed fermentation only batch cultures.