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METHANE PRODUCTION AND METHANOGENIC COMMUNITIES IN MICROBIAL ELECTROLYSIS CELLS, ANODIC POTENTIAL INFLUENCE ON MICROBIAL FUEL CELLS, AND A METHOD TO ENTRAP MICROBES ON AN ELECTRODE

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Rachel Cain Wagner

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The dissertation of Rachel Cain Wagner was reviewed and approved* by the following:

Bruce E. Logan
Professor of Environmental Engineering
Dissertation Adviser
Chair of Committee

John M. Regan
Associate Professor of Environmental Engineering

Jennifer Macalady
Associate Professor of Geosciences

Christopher House
Professor of Geosciences

Peggy Johnson
Professor of Civil and Environmental Engineering
Head of the Department Civil and Environmental Engineering

*Signatures are on file in the Graduate School.

ABSTRACT

Microbial fuel cells (MFCs) and related technologies (bioelectrochemical systems, BESs) use microbes as catalysts for reactions that donate electrons to or use electrons from a circuit. At the anode, exoelectrogens oxidize organic matter and donate electrons to the electrode. At the cathode, electrotrophs consume electrons for their metabolic needs. BESs have potential for a number of practical purposes, from generating useful fuels such as hydrogen and methane to generating electricity while treating wastewater. The work described here addresses some of the issues in understanding microbial communities in these systems and in improving the functionality of BESs.

Hydrogen and Methane from Swine Wastewater. The production of a useful and valuable product during swine wastewater treatment, such as hydrogen gas, could help to lower treatment costs. Hydrogen can theoretically be produced from wastewater by electrohydrogenesis in a microbial electrolysis cell (MEC) or by fermentation. Using a single-chamber MEC with a graphite-fiber brush anode, hydrogen gas was generated at 0.9-1.0 m^3-H_2 m^{-3} day^{-1} using full-strength or diluted swine wastewater. COD removals ranged from 8-29% in 20-h tests, and from 69-75% in longer tests (184 hours) using full-strength wastewater. The gas produced was up to 77 ± 11% hydrogen, with overall recoveries of up to 28 ± 6% of the COD in the wastewater as hydrogen gas. Methane was also produced at a maximum of 13 ± 4% of total gas volume. The efficiency of hydrogen production, based on the electrical energy needed (but excluding the energy in the wastewater) compared to the energy of the hydrogen gas produced, was as high as 190 ± 39% in 42-h batch tests with

undiluted wastewater, but was lower in longer batch tests of 184 hours (91 ± 6%). Hydrogen gas could not be recovered in fermentation tests using wastewater with a heat-treated inoculum. Hydrogen production was shown to be possible by fermentation when the wastewater was sterilized, but this process would not be practical or energy efficient. We therefore conclude from these tests that MECs are an effective method for hydrogen recovery from swine wastewater treatment, although the process needs to be further evaluated for reducing methane production, increasing the efficiency of converting the organic matter into current, and increasing recovery of hydrogen gas produced at the cathode.

Optimal Set Anode Potentials Vary in Bioelectrochemical Systems. In BESs, the anode potential can be set to a fixed voltage using a potentiostat but there is no accepted method for defining an optimal potential. Microbes can theoretically gain more energy by reducing a terminal electron acceptor with a more positive potential, for example oxygen compared to nitrate. Therefore, more positive anode potentials should allow microbes to gain more energy per electron transferred than a lower potential, but this can only occur if the microbe has metabolic pathways capable of capturing the available energy. This review of the literature shows that there is a general trend of improved performance using more positive potentials, but there are several notable cases where biofilm growth and current generation improved or only occurred at more negative potentials. This suggests that even with diverse microbial communities, it is primarily the potential of the terminal respiratory proteins used by certain exoelectrogenic bacteria, and to a lesser extent the anode potential, that determines the optimal growth conditions in the reactor. Our analysis suggests that

additional bioelectrochemical investigations of both pure and mixed cultures, over a wide range of potentials, are needed to better understand how to set and evaluate optimal anode potentials for improving BES performance.

Immobilization of anode-attached microbes in a microbial fuel cell. Current-generating (exoelectrogenic) bacteria in BESs may not be culturable using standard in vitro agar-plating techniques, making isolation of new microbes a challenge. More *in vivo* like conditions are needed where bacteria can be grown and directly isolated on an electrode. While colonies can be developed from single cells on an electrode, the cells must be immobilized after being placed on the surface. Here we present a proof-of-concept immobilization approach that allows exoelectrogenic activity of cells on an electrode based on applying a layer of latex to hold bacteria on surfaces. The effectiveness of this procedure to immobilize particles was first demonstrated using fluorescent microspheres as bacterial analogs. The latex coating was then shown to not substantially affect the exoelectrogenic activity of well-developed anode biofilms in two different systems. A single layer of airbrushed coating did not reduce the voltage produced by a biofilm in an MFC, and more easily applied dip-and-blot coating reduced voltage by only 11% in a MEC. This latex immobilization procedure will enable future testing of single cells for exoelectrogenic activity on electrodes in BESs.

Characterization of anodic and cathodic communities in a pilot-scale bio-electrochemical system. MECs can be used to produce hydrogen or methane gas, but there is little information on the archaeal communities in these systems. Methane production in these systems decreases hydrogen production, but the methane

produced may be higher than that possible by anaerobic digestion alone, and methane itself is a valuable fuel with a readily available infrastructure. Communities from biofilms on the anode and cathode and from the suspension of a 1000-L pilot-scale MEC reactor were analyzed for archaea and bacteria. Hydrogen gas was initially produced by the reactor, but it was completely replaced by methane production within ~6 weeks that continued through the life of the experiments (~14 weeks). Archaea identified by rRNA analysis were mainly unidentified clones. Among the organisms that were successfully identified as methanogens, those at the cathode were more likely than the methanogens at the anode to consume hydrogen and carbon dioxide. Visual fluorescent *in situ* hybridization image analysis confirmed the presence of archaea, but the great majority of microbes were bacteria (~90%). Based on 16S rRNA gene clone library analysis, *Pelobacter*, *Geobacter*, and *Clostridium* were present in large numbers in both anodic and cathodic communities, while they were not dominant in the suspension, suggesting that they are involved in substrate transformation processes that resulted in electron transfer to and/or from electrodes. Microbes most similar to *Clostridium* were a higher percentage of microbes in bacterial communities at the cathode than the anode or suspension, indicating their importance in the cathodic community metabolism. High-throughput pyrosequencing analysis showed *Geobacter* dominating both anodic and cathodic communities, with *Clostridium* again having a more significant presence on the cathode.

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Chapter 1

Introduction

1.1. Water and Energy: Grand Challenges

Water and energy are key components to civilization, and the issues and potential solutions are linked in a critical connection termed the water-energy nexus [1]. Energy is required to clean and distribute water for direct human consumption, agriculture, and industry; energy production requires abundant and reliable water. Among the Grand Challenges faced by engineers and civilization in the coming decades are the challenges of providing clean water and producing energy sustainably, without compromising the Earth systems on which humans rely [2].

Energy availability and the consequences of energy production are tremendous issues confronting humanity. Fossil fuels are non-renewable resources, and though the predictions of timing for their peak and inevitable decline vary greatly, these resources are neither infinite nor renewable. Carbon dioxide additions to the atmosphere are an additional problem with burning fossil fuel. This greenhouse gas is inextricably linked to global warming and climate change [3, 4]; with an atmospheric lifetime of several decades, carbon dioxide added to the atmosphere becomes a long-term problem. Current energy transformations, such as from fossil fuels to electricity, are highly water-intensive, because many of these systems use steam generation to power turbines. Future work in energy development must consider water use and treatment.

Water and wastewater treatment and distribution uses 4-5% of electricity in the United States [5, 6]. In both treatment and distribution, a significant amount of energy is wasted in both water and wastewater. For example, water treated to the high standards necessary for drinking, which is energy intensive, is also used to transport wastes through drains and toilets; the water quality – and therefore energy requirement – needed for waste transport is much lower than what is actually used, wasting energy. In addition, water often leaves buildings at a warmer temperature than it enters, and the energy lost in that heat is as much as ten times higher than the energy used to treat the water and wastewater combined [7]. Cooling water for power plants generating electricity is also a source of lost heat. One innovative use of this wasted heat is forward osmosis using ammonia-carbon dioxide for seawater desalination [8]. Wastewater itself contains an estimated 1.9 kWh/m^3 of energy, while a typical wastewater treatment plant uses about 0.6 kWh/m^3 to treat the wastewater [9, 10], leaving a tremendous amount of potential for the wastewater itself to provide the energy necessary for treatment. The energy in the wastewater must first, however, be converted into a more useful form of energy. Anaerobic digestion of wastewater sludge successfully converts some of that energy into methane, which is a useful fuel; however, the energy in the dissolved organic portion of the wastewater is not captured by anaerobic digestion [9]. Instead, aerobic treatment processes, which are energy intensive, are the standard.

1.2. Bioelectrochemical Systems

Bioelectrochemical systems (BESs) harness the energy from organic matter in wastewater and convert it into usable forms. BES technology is based on microbial transfer of electrons to and from solid electrodes. Microbes in these systems are referred to as exoelectrogens or electrotrophs, given their ability to generate ("gen") current by transfer of electrons to electrodes or their ability to consume ("troph") electrons from electrodes. Microbial fuel cells (MFCs) are the most thoroughly studied form of BESs. In these systems, microbes consume organic matter and transfer electrons to an anode as their terminal electron acceptor. The electrons travel through an electrical circuit to the cathode, where a reduction reaction occurs. In an MFC, the current produced by the electrons moving from the anode to the cathode are harvested as electrical energy. At the anode, the microbes can consume complex substrates such as wasted organic matter in wastewater streams such as domestic or industrial wastewaters, creating a highly sustainable source of electrical energy. Other types of BESs can use the electrons available at the cathode for reactions that synthesize valuable products (such as hydrogen gas in a microbial electrolysis cell (MEC), [11-13]) or can use the electrochemical potential between the electrodes to drive desalination of salt water (in a microbial desalination cell (MDC), [14-16]).

1.3. Components of BESs

1.3.1. Anode

The anode of a BES consists of microbes, a substrate, an electrolytic solution, and an electrode. Exoelectrogenic microbes capable of using the electrode as a

terminal electron acceptor are the catalyst for anodic reactions. Other microbes may be necessary to support the exoelectrogens. The substrate can be a simple chemical, such as acetic acid or glucose, or complex mixture of organic matter, such as domestic or industrial wastewater. Acetic acid or other compounds that are readily oxidized by microbes allow for precise laboratory studies. For practical application of BESs, however, wastewater provides the substrate, rich in electrons that are essentially free in organic matter. The organic matter which is very costly to reduce in traditional aerobic wastewater treatment is the fuel for a BES: an energy source for the microbes. If the substrate is complex like most wastewaters, a mixed community of microbes may be required to break down the organic matter and achieve substantial wastewater treatment. The microbes capable of metabolizing more complex molecules then provide simpler substrates in the form of break-down products that can be used by exoelectrogens. Transfer of electrons from microbes to a solid electrode can happen indirectly, through soluble mediators, or through direct contact between the microbes and the electrode. A variety of exoelectrogens have been identified, and the most prevalent microbes found in BESs are *Geobacter* spp. [17]. In addition to the substrate, the microbes require an electrolytic solution with a near-neutral pH, and conductivity and micro-nutrients suitable to their growth requirements. The electrode must also support microbial growth while retaining conductivity without corroding. Most electrodes in BESs are made of carbon, in a variety of forms from carbon paper to graphite fiber brushes [18].

1.3.2. Cathode

The most typical cathodic reaction in an MFC is the oxygen reduction reaction, $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$. Other oxidizing agents such as ferricyanide can be used, but oxygen from air is the most sustainable. In addition to harvesting the electrons as electricity in an MFC, the electrons at the cathode of BESs can be used to generate valuable products as well. One of the first uses of these electrons was in a MEC, in which oxygen was excluded from the reactor so that the electrons were combined with protons to form hydrogen gas, $2H^+ + 2e^- \rightarrow H_2$. While the oxygen reduction reaction is spontaneous in MFCs, occurring at a theoretical potential of +0.8 V, the potential at which hydrogen will evolve from protons and electrons is –0.414 V under standard conditions (25^{o}C) at neutral pH. An MFC produces an anode potential of approximately –0.3 V. With no overpotentials, the theoretical voltage that must be applied to generate hydrogen is therefore the difference: (–0.414 – –0.3) = –0.114 V. However, due to overpotentials and inefficiencies, most MECs require –0.4 to –0.9 V applied to the cathode to achieve reasonable rates of hydrogen production, supplied by a power supply [6]. The energy value of the hydrogen gas in most MECs is greater than the energy required to boost the voltage, due to the voltage provided by the anodic reactions [19].

Other valuable products, such as hydrogen peroxide [20], caustic [21], acetate [22], or methane [23, 24], can be produced using the cathodic electrons, creating additional value from BESs. In addition to the product value, cathodic reactions can consume CO_2, making the system carbon neutral if the electrons come from a bio-

anode (which produces CO_2 as the microbes respire) or carbon negative if the electrons are supplied by non-carbon-based electricity such as solar or wind.

1.3.3. Membranes

The cathode can be separated from the anode by a membrane that allows for proton transport, or the BES can exist as a single chamber. In a dual-chambered system, proton transport occurs directly through a proton exchange membrane such as Nafion (Ion Power) or indirectly through an anion exchange membrane, with protons bound to negatively charged phosphate ions [25]. A membrane allows separation of the anolyte and catholyte as well as separation of microbes and some reaction products. However, internal resistance usually increases in the presence of a membrane, lowering the power output of a BES [26-28]. A single-chamber design not only increases mass transfer between the electrodes, but also decreases the cost of the BES while increasing power output [29-32].

1.4. Exoelectrogenic and Electrotrophic Microbes

The microbes capable of transferring electrons to an anode or from a cathode are of great interest. One strategy for improving BESs is to target environmental conditions to encourage growth of the best-performing microbes. These improvements require a comprehensive understanding both of the microbes that are most capable of extracellular electron transfer to and from electrodes and of the communities that most successfully support these microbes in a mixed community with a complex substrate [17]. For example, *Geobacter* spp. have been dominant in numerous community analyses of anodic communities [33-37], and *Geobacter* spp. are

successful exoelectrogens in pure culture studies as well [38, 39]. In one pilot-scale reactor, environmental amendments such as the addition of acetic acid and fumarate were included to enhance growth of these specific microbes, and current increased in part due to these enrichments [40]. Cathodic communities can also be selected to target specific microbial reactions. For example, methanogens must be excluded from or limited in mixed culture, single chamber MECs in which hydrogen is the desired product [41, 42], or they will convert a large fraction of the hydrogen into methane. In a denitrifying biocathode, the community structure appears to affect performance. Specifically, an increase in both richness [number of species or other OTUs (operational taxonomic units)] and evenness (distribution of OTUs), was seen in the system with the highest current and nitrate reduction [43].

In addition to improving conditions for exoelectrogens or electrotrophs in mixed communities, BES performance can be improved by engineering or evolving certain microbes to improve their performance, or by creating pure cultures of the most electrically active microbes. In this case, isolation of novel exoelectrogens or electrotrophs is a valuable tool for finding the best microbe for a given environmental condition or for engineering. Two strategies have been used for isolation of exoelectrogens from BESs. In the first strategy, microbes from anodic communities are isolated on iron-reducing agar plates. This method is not ideal because iron-reduction capability does not always also confer anode-reduction capability [44]. The second method is dilution-to-extinction in an MFC. In this case, an anodophilic consortium is diluted in series until current generation ceases. DGGE or another type of community analysis is performed to determine if the most dilute reactor that

generated current is comprised of a pure isolate. While this method directly isolates anode-reducers, it is time, material, and labor-intensive [44, 45]. An ideal system would involve depositing an array of single cells onto a flat electrode. Such an array is possible using printing technology [46]. In a BES, the array must be immobilized to allow for colony development of successful exoelectrogens.

In most cases, the electrical potential of the anode is determined by the microbes present and their reactions at the anode. If the anodic potential is set externally, rather than allowed to vary, the ability of microbes to use the anode as a terminal electron acceptor can be affected; in addition, the type of microbe using the anode, and the microbial exoelectrogenic activity, can be influenced by different potentials [47]. The anode interacts with microbial cytochromes, which have potential ranges in which they typically operate, influenced by the terminal electron acceptors that a given microbe is capable of using. If the cytochromes are not able to donate electrons to an anode at a given potential, current generation is not possible.

1.5. Cathodic Methane Generation

The cathodic potential determines, in part, the reactions that occur at the cathode. The microbial community present and the environmental conditions are also important in determining whether or not a desired reaction will occur. In MECs used for hydrogen production, methane generation is often problematic. In hydrogenotrophic methanogenesis ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$), four moles of hydrogen gas are consumed for every mole of methane produced, significantly reducing the total energy produced, since methane is not as energy dense as hydrogen

(approximately 55 MJ/kg for CH_4 vs. 143 MJ/kg for H_2). Although both methane and hydrogen are potentially valuable fuels, current controls on whether an MEC produces methane or hydrogen are weak, and production of a pure gas would be preferable to the current systems which produce mixed gases when wastewaters are used.

Methods to reduce methane production and promote hydrogen production during fermentation have been tried by several research groups [48, 49], but the only successful methods have been addition of large amounts of sugars [50] or high-heat sterilization of inoculum to kill non-spore forming microbes such as methanogens [51, 52]. Heat treatment of the inoculum is ineffective, however, with a substrate such as swine wastewater that also contains methanogens [48]. Hydrogen production in MECs is more successful than standard fermentation experiments, but the methane generation is still problematic. Environmental efforts to control methane in MECs, such as lowering pH or temperature or exposing biofilms to oxygen, are not effective. Chemicals such as 2-bromoethanesulfonate are successful at inhibiting methanogens and subsequent increases in hydrogen capture from MECs are seen when this chemical is used [41]. However, adding this compound would not be useful in practice.

Although methane production is problematic when hydrogen is desired, methane is itself a valuable fuel. Because methane is the main component of natural gas, the infrastructure for methane uses as a fuel is already in place. Biogenic methane production, rather than fossil fuel methane, provides carbon neutral energy. Methane production at a BES cathode is produced either indirectly, with hydrogen as

an intermediate, or directly, from electrons at the cathode [23, 24]. The potential at which CO_2 converts to CH_4 is less negative than that for hydrogen production from protons (–0.24 V for CH_4 compared to –0.414 V for hydrogen). Theoretically, the potential of –0.24 V can be reached by a BES anode, but overpotentials require that some additional voltage is applied. Several studies have now shown evidence that cathodic biofilms are directly catalyzing methane production from CO_2 and cathodic electrons [23, 24]. If the source of the electrons is anodic oxidation, the system is carbon neutral; if non-carbon based electricity such as solar or wind is used to provide the cathodic electrons, this system can theoretically be a carbon negative source of methane.

1.6. Conclusions

Reducing the use of fossil-based fuels will require a number of different alternatives. Available resources such as wind and solar energy will determine the best technology to implement in a given location. Converting currently wasted energy, including the wasted energy in wastewaters, into usable forms of energy will also be a valuable alternative to fossil fuels. Conversion of wasted energy could turn wastewater into a net energy producer using tools such as MFCs and MECs, and other version of these technologies: bioelectrochemical systems. Energy can be converted in these systems into a variety of forms including electricity, hydrogen, and methane.

Research in all aspects of BESs is necessary to improve their efficiency to enable their practical use. Among the critical components that require investigation

are the microbes functioning in these systems, exoelectrogens and electrotrophs, and their use as catalysts for both anodic and cathodic reactions. BESs and the microbes that are key to their function have great potential for converting a wasted resource into useful energy.

1.7. Description and Objectives of this Dissertation

A study of hydrogen and methane gas production from swine wastewater is described in Chapter 2. I was the primary author of this research. My work focused on hydrogen production from swine wastewater using membrane-less MEC technology. My co-authors generated the research ideas for obtaining hydrogen from swine wastewater, and they performed the data collection and analysis of the non-MEC experiments for this work. My focus was the use of swine wastewater in MECs, analysis and interpretation of this data, and why this method showed improvements over the other methods.

A review of studies with set anodic potentials is given in Chapter 3. I wrote the manuscript, except for the section on methods to set electrode potentials which was written by Douglas Call. All of the co-authors participated in editing the manuscript.

A method for trapping microbes on an anode using a latex overlay is described in Chapter 4. I generated the idea for this research from work in other labs developing successful latex-microbe films. The need for microbe entrapment arose with the development of an isolation technique for exoelectrogens. Isolating microbes in an array on a single electrode in an MFC was the original goal, but this requires

that the microbes be trapped in a single location on the electrode. I performed the experiments along with Sikandar Porter-Gill (undergraduate), and I wrote the original manuscript.

A community analysis of a pilot-scale MEC built to treat wastewater from a winery is presented in Chapter 5. My co-authors and other members of Dr. Bruce Logan's laboratory group designed, built, and maintained the reactor as previously described [40], and gathered the DNA samples. I processed the DNA and performed the clone library analysis. Researchers at Yale University (Hamid Rismani-Yadzi, Kyle Bibby, and Jordan Peccia) performed the pyrosequencing and assembled the raw data from the sequencer. I also performed the FISH analysis with advice from Douglas Call and I interpreted the results from the different analyses and wrote Chapter 5.

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Chapter 2

Hydrogen and Methane Production from Swine Wastewater Using Microbial Electrolysis Cells

Abstract

The production of a useful and valuable product during swine wastewater treatment, such as hydrogen gas, could help to lower treatment costs. Hydrogen can theoretically be produced from wastewater by electrohydrogenesis in a microbial electrolysis cell (MEC) or by fermentation. Using a single-chamber MEC with a graphite-fiber brush anode, hydrogen gas was generated at 0.9-1.0 m^3 m^{-3} day^{-1} H2 using full-strength or diluted swine wastewater. COD removals ranged from 8 to 29% in 20-h tests, and from 69 to 75% in longer tests (184 hours) using full-strength wastewater. The gas produced was up to 77 ± 11% hydrogen, with overall recoveries of up to 28 ± 6% of the COD in the wastewater as hydrogen gas. Methane was also produced at a maximum of 13 ± 4% of total gas volume. The efficiency of hydrogen production, based on the electrical energy needed (but excluding the energy in the wastewater) compared to the energy of the hydrogen gas produced, was as high as 190 ± 39% in 42-h batch tests with undiluted wastewater, but was lower in longer batch tests of 184 hours (91 ± 6%). Hydrogen gas could not be recovered in fermentation tests using wastewater with a heat-treated inoculum. Hydrogen production was shown to be possible by fermentation when the wastewater was sterilized, but this process would not be practical or energy efficient. We therefore conclude from these tests that MECs are an effective method for hydrogen recovery from swine wastewater treatment,

although the process needs to be further evaluated for reducing methane production, increasing the efficiency of converting the organic matter into current, and increasing recovery of hydrogen gas produced at the cathode.

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2.1. Introduction

Considerable amounts of animal wastewater are generated each year that require extensive treatment. In the US alone there are 64 million hogs and the amount of animals being used in food production is increasing [1]. Conventional methods of treating animal wastewaters include anaerobic lagoons, constructed wetlands, and storage with landspreading [2]. Common environmental problems associated with these strategies include surface runoff of nutrients, organics, and pathogens [3]; odors; emissions of methane, nitrous oxide, and ammonia [4]; and deteriorated system performance due to excessive nitrogen accumulation [5].

Energy can be extracted from wastewater during treatment, providing products that can help offset treatment costs. Microbial fuel cells (MFCs) have been examined as a method for generating electricity while simultaneously treating wastewater [6]. In these systems, bacteria oxidize organic matter and release electrons to an anode, which then flow to the cathode and combine with oxygen and protons to form water. Swine wastewater was successfully treated using MFCs [7], and it was

recently shown that MFCs could also be used to remove odors [8]. A more conventional approach to swine wastewater treatment is anaerobic digestion, in which organic matter is broken down by bacteria, releasing volatile fatty acids and hydrogen gas. These intermediate products are used by methanogens to produce methane. Hydrogen gas, however, contains more energy (on a mass basis) and is therefore more valuable than methane. Hydrogen has been successfully produced by fermentation using food processing wastewaters, municipal wastewater sludge filtrate, and paper hydrolysates [9, 10], as well as from solids such as wheat starch, bean curd waste, wheat and rice bran, and municipal solid wastes [11-14]. Recently, hydrogen from domestic sewage sludge fermentation was reported [15], but both enzymatic and heat pre-treatments were necessary for hydrodgen production. While recovering hydrogen gas from swine wastewater may allow for a more cost-effective treatment process, high hydrogen gas yields have not yet been achieved using swine wastewater by a fermentation process.

Hydrogen gas can also be produced from biomass using electrohydrogenesis [16], in a device called a microbial electrolysis cell (MEC) [17, 18]. The MEC is a modified MFC in which the cathode is completely anoxic, and a voltage is added to that produced by the bacteria to allow for hydrogen evolution. At the cathode, electrons combine with protons to form hydrogen via the hydrogen evolution reaction (HER): $2H^+ + 2e^- \rightarrow H_2$ (g). Bacteria at the anode consume organic matter and produce a voltage of approximately -0.3 V, while the HER requires -0.41 V, requiring a theoretical input of 0.11 V. In practice, a higher voltage input of 0.25-0.8 V is required for the HER to occur in an MEC [19]. It has been shown that three times as

much energy can be recovered in the hydrogen gas than is added as electrical energy using the process with acetate and several other volatile fatty acids [16].

The purpose of this study was to examine the feasibility of MECs or fermentation for producing hydrogen gas from swine wastewater. In both methods, the main barrier to hydrogen recovery is hydrogen consumption by methanogens. There are very few studies on using MECs for hydrogen generation, and only one using wastewater. Current was produced in an MEC using domestic wastewater, but the low strength of the wastewater required the use of relatively high added voltages, and the reactor had high internal resistance leading to low hydrogen recoveries [20]. A new single-chamber MEC reactor was recently designed [21] that has a lower internal resistance than the MEC used by Ditzig et al. [20], and it produced much higher hydrogen gas flow rates from acetate than in other MEC studies [22, 23]. We therefore examined hydrogen production using this MEC reactor with swine wastewater to see if we could achieve reasonable hydrogen recoveries. We compared this approach to a more conventional fermentation-based approach using a heat-treated inoculum to select for non-methanogenic microorganisms, and determined the upper limit for the efficiency of a fermentation-based approach by completely sterilizing the wastewater. We demonstrate here that while fermentation of swine wastewater does not produce hydrogen without energy-intensive pretreatments, electrohydrogenesis in an MEC can easily achieve high hydrogen recoveries even in a single-chamber reactor.

2.2. Materials and Methods

2.2.1. Swine wastewater

Swine wastewater was collected from the slurry pits of the swine farm located at the Pennsylvania State University in University Park, PA and stored at 4^{o}C for <30 days. The wastewater had a chemical oxygen demand (COD) of approximately 12,000 – 17,000 mg/L.

2.2.2. Microbial Electrolysis Cell

In preparation for MEC operation, MFCs were constructed of lexan, with an air cathode with a platinum catalyst [24]. Graphite-fiber brush anodes were enriched with exoelectrogenic bacteria using diluted swine wastewater (COD = 2,000 mg/L) in duplicate, single-chamber MFCs having a cylindrical chamber 4-cm long by 3-cm in diameter (empty volume = 28 mL). After acclimation, the MFCs consistently produced a maximum voltage of approximately 550 mV. After at least three consistent cycles at this maximum voltage, the reactors were converted to operate as MECs as previously described [21] by covering the air cathode with a plate to exclude air and eliminating the oxygen reduction at the cathode. Gas was collected from an anaerobic culture tube glued to fit on top of a 1.6-cm opening on the top of the reactor. MECs were exposed to air for 30-60 minutes after each cycle to reduce methane production by oxygen-sensitive methanogens that could be present in the biofilm. Gas production from the reactor was measured using a respirometer (AER-200; Challenge Environmental). A power source (3645A; Circuit Specialists, Inc.) was connected to the circuit to add voltage, and a multimeter (2700; Keithley

Instruments, Inc.) was used to monitor the voltage across an external resistor (R_{ex}=10 Ω) to calculate current. For all experiments, 0.5 V was applied from the external power source to the reactor. All tests were run at 30°C in a constant temperature room. All tests used non-diluted (ND) wastewater without amendments, except (as noted) in some tests the wastewater was diluted (D) to a COD of 2,000 mg/L and buffered (50mM phosphate buffer, pH = 7) with trace minerals (per liter: 15mg NTA, 30mg $MgSO_4 \cdot 7H_2O$, 5mg $MnSO_4 \cdot H_2O$, 10mg NaCl, 1mg $FeSO_4 \cdot 7H_2O$, 1mg $CoCl_2 \cdot 6H_2O$, 1mg $CaCl_2$, 1mg $ZnSO_4 \cdot 7H_2O$, 0.1mg $CuSO_4 \cdot 5H_2O$, 0.1mg $AlK(SO_4)_2 \cdot 12H_2O$, 0.1mg H_3BO_3, 0.1mg $Na_2MoO_4 \cdot 2H_2O$) and trace vitamins (per liter: 2 mg Biotin, 2.0 mg Folic acid, 10 mg Pyridoxine hydrochloride, 5 mg Thiamine HCl, 5.0 mg Riboflavin, 5.0 mg Nicotinic acid, 5.0 mg Calcium D-(+)-pantothenate, 0.1 mg Vitamin B12, 5 mg p-Aminobenzoic acid, 5.0 mg Thioctic acid) [25]. The non-diluted wastewater had a pH of around 6.8; ammonia was not measured for this experiment, but wastewater from the Penn State swine farm typically has ammonia concentrations of approximately 2,000 mg-N/L.

Reactors were operated in fed-batch mode for two different lengths of time, referred to as “long” (L) or “short” (S) batch cycles. A long batch cycle was conducted until the complete gas production cycle ended, as indicated by zero gas production rate for one hour or more. For short batch cycles, the test was discontinued once the gas production rate had reached a peak.

2.2.3. Measurements and Chemical Analyses

Total COD analysis of the solution was performed at the beginning and end of each batch cycle (method 8000; HACH COD system, HACH Company, Loveland, CO) [26]. Sugar was analyzed using the phenol-sulfuric acid method for reducing sugars [27], with sample filtration through a 0.2- μm pore diameter membrane filter for dissolved sugar. Total ammonia (ammonia and ammonium ion) was analyzed using an ATI Orion Model 720A Benchtop pH/ISE meter and an ammonia probe (ATI Orion, Boston, MA). A pH probe and meter (Fisher Scientific accumet® model 10 and VWR SympHony) were used for pH measurements. Redox potential in the continuous flow fermentation experiments was measured with a redox probe (Combination Red/Ox probe Pt4805-DXK-S8/225, Mettler-Toledo, Columbus OH). In the fermentation tests, the concentrations of solvents, alcohols, and organic acids (acetone, ethanol, propanol, butanol, acetate, propionate, and butyrate) in the liquid phase were measured by gas chromatography (Varian Star 3400) with injector and flame ionization detector temperatures of 250^{o}C. After each batch cycle, gas from a gas bag and the reactor headspace was sampled using a gas-tight syringe (100 or 200 μL injection volume) and analyzed by gas chromatography for hydrogen, methane, carbon dioxide, and nitrogen gas (GC; Models 310 & 8610B, SRI Instruments, Torrence, CA) [21].

2.2.4. Calculations

The cathodic hydrogen recovery efficiency, defined as the fraction of electrons reaching the cathode that are recovered as hydrogen gas, is calculated as

$$r_{H2,\,cat} = \frac{n_{H2}}{n_{H2,\,cat}} \tag{1}$$

where n_{H2} is the number of moles of hydrogen recovered ($n_{H2} = 4.02\times10^{-5}\ v_{H2}$), and $n_{H2,cat}$ is the number of moles of hydrogen that can theoretically be produced from the current, calculated as

$$n_{H2,\,cat} = \frac{\sum_{1}^{n} I\Delta t}{2F} \tag{2}$$

where 2 is used to convert moles of electrons to moles of hydrogen gas, F is Faraday's constant (96,485 C/mol e^-), and $I = E/R_{ex}$ is the current (A) calculated from the measured voltage (E, V) across the resistor ($R_{ex} = 10\Omega$).

The overall hydrogen recovery, $r_{H2,\,COD}$, which is defined as the ratio of the hydrogen recovered to the maximum possible hydrogen recovery based on the organic matter oxidized in the wastewater on the basis of COD, is

$$r_{H2,\,COD} = \frac{n_{H2}}{n_{H2,COD}} \tag{3}$$

where $n_{H2,COD} = \Delta COD v_L b_{H2/S}$, ΔCOD is the COD (mg/L) consumed during the batch cycle, v_L is the volume of the liquid, and $b_{H2/S}$ is a conversion factor based on stoichiometric conversion of electrons in COD to hydrogen gas equaling 1 mol H_2 per 16 g O_2.

The Coulombic efficiency (CE), or the fraction of electrons obtained from the consumption of COD that are available for hydrogen production at the cathode, is

$$CE = \frac{n_{H2,cat}}{n_{H2,COD}} = \frac{r_{H2,COD}}{r_{H2,\,cat}}. \tag{4}$$

The energy recovery, η_W, is the ratio of the energy content of the hydrogen recovered compared to the electrical energy input required for the cathodic reaction. In terms of the number of moles of hydrogen recovered compared to the energy consumed by the power source converted into equivalent moles of hydrogen, energy recovery is calculated as

$$\eta_W = \frac{n_{H2}}{n_{H2,PS}}. \tag{5}$$

A small external resistor (10Ω) is used to measure current, so the energy added by the power source, W_{PS}, is corrected for losses across the external resistor as previously described [16, 21, 28] by the following equation:

$$W_{PS} = \sum_{1}^{n} IE_{PS}\Delta t - \sum_{1}^{n} I^2 R_{ex}\Delta t. \tag{6}$$

The equivalent number of moles of hydrogen is then calculated as

$$n_{H2,PS} = \frac{W_{PS}}{\Delta H_{H2}}, \tag{7}$$

where E_{PS} =0.5 V is the voltage applied using the power source, Δt (s) is the time increment for n data points measured during a batch cycle, and ΔH_{H2} is the upper heating value of the heat of combustion of hydrogen gas (285.83 kJ/mol).

Energy added by the power source is normalized by COD removed as $W_{PS}/(v_L \Delta COD)$. Note that this result is different from a calculation using the applied voltage of the power source (E_{app}) and assuming all of the electrons from the COD removed are harvested as current, or $E_{app} F\, b_{COD}$, where the constant b_{COD} is used to convert COD removed to electrons (b_{COD} = 1 mol e^-/8 g COD). For example, at E_{app}

=0.5 V, the maximum energy requirement based on the applied voltage and assuming all COD removed was recovered as current would be 1.67 kWh/kg COD."

The recovery of methane from COD ($r_{CH4,\,COD}$), defined as the ratio of methane produced to the maximum possible methane recovery based on organic matter oxidized, is

$$r_{CH4,\,COD} = \frac{n_{CH4}}{n_{CH4,COD}} \tag{8}$$

where $n_{CH4,COD} = \Delta COD v_L b_{CH4/S}$ and $b_{CH4/S}$ = 1 mol CH_4 per 64 g O_2.

2.2.5. Fermentation Experiments

2.2.5.1. Hydrogen production testing

Batch tests were conducted in glass bottles (299 mL capacity; Wheaton Scientific) using diluted wastewater volumes (200 mL) as previously described [29]. Swine wastewater was diluted either 2× or 8× using distilled water and tested at initial pH values of 7.2 (buffered with a 0.02 M phosphate buffer) and 5.8 (buffered with 0.05 M 2-(N-morpholino)ethanesulfonic acid monohydrate [MES; J.T. Baker]), with initial pH adjustments made using HCl or NaOH. Bottles were sparged with nitrogen to remove oxygen and were capped with a rubber stopper and an aluminum seal. All tests were run in duplicate at 30°C. Biogas production in each bottle was continuously measured using a respirometric system as previously described [30].

Various inocula and sample pretreatment methods were evaluated for their effectiveness in increasing hydrogen production from fermentation. Tests were conducted using: (1) no inoculum; (2) a sludge inoculum consisting of 6.6 g of dewatered sludge collected from the Pennsylvania State University Wastewater

Treatment Plant in State College, PA; or (3) a heat-treated (HT) inoculum consisting of 1.0 g dewatered anaerobic sludge (0.2-cm thick) baked for two hours at 104^{o}C. To assess the maximum hydrogen production possible from the organic matter in the sludge under conditions that theoretically eliminate methanogenic losses of hydrogen, a swine wastewater sample was autoclaved (121^{o}C, 15 psi) and combined with an HT sludge inoculum at a pH of 5.8. To assess the amount of hydrogen production possible in the presence of indigenous methanogens and other hydrogen-consuming microbes, glucose (1 g) was added to diluted swine wastewater with an HT inoculum (pH = 5.8).

Since methanogens are slow growing and can often be washed out in continuous flow reactors with short hydraulic retention times (HRTs) [31], the possibility of fermentative hydrogen production from swine wastewater was further investigated in continuous culture under optimal conditions to assess the maximum potential for hydrogen production. Wastewater was pumped into a fermentor (New Brunswick BioFlo 110) with an operating volume of 1 L, an HRT of 24 h, and an HT agricultural soil inoculum [30]. While the microbial composition of this inoculum differs from that used above in the batch tests, previous work has demonstrated equivalent hydrogen production using either inoculum [29]. The reactor was initially filled with an equal mixture of swine wastewater and distilled water. L-Cysteine (0.5 g/L) was initially added to consume oxygen in the medium, thereby reducing the redox potential, and the solution pH was adjusted to 5.5 with 1 M KOH. The reactor was sparged with nitrogen gas for 1 hr and inoculated with the HT agricultural soil (5 g). In an additional test, the wastewater was first sterilized by autoclaving before

being introduced to the reactor. In a third test, glucose (5g/L) was initially added to the reactor to ensure the germination of hydrogen-producing bacteria.

In each case, the reactor was operated in batch mode until the redox potential was reduced to below -200 mV (~3 days, measured against the Argenthal system), and then switched from batch to continuous flow. In continuous flow operation, the reactor was only fed with swine wastewater diluted 2× with distilled water with no additional L-cysteine or glucose. The feed bottle was continuously sparged with nitrogen gas to maintain anaerobic conditions, and the reactor pH was maintained at 5.5 by automated addition of KOH. Gas production was measured daily using a water exclusion apparatus, and hydrogen concentrations in the headspace were measured every 24 hours.

2.2.5.2. Hydrogen consumption testing

To measure the potential for acetogenesis from hydrogen gas, we conducted hydrogen consumption tests using the HT sludge inoculum at four different pHs (5, 6, 7, and 8). In previous tests using glucose, methanogenesis was successfully eliminated by a combination of low pH and heat treatment of the inoculum [30]. Serum bottles were inoculated with HT sludge (0.5 g), filled with 200 mL of sulfate-free nutrient mineral solution (0.5 M phosphate buffer, 1 g sodium bicarbonate), and the initial pH adjusted with HCl or NaOH. Bottles were flushed with hydrogen gas and capped with a rubber stopper and an aluminum seal. CO_2 (50 mL) was added to the bottles using a syringe, and the pressure was then released using a needle. Abiotic (no inoculum) and biotic (inoculum with nitrogen headspace) controls were also prepared. Hydrogen in the headspace was measured over time.

2.3. Results

2.3.1. Hydrogen Production and Wastewater Treatment in Microbial Electrolysis Cells

Hydrogen gas was produced in all MEC tests. In the short-cycle (S) batch tests, 17 ± 7% of the COD removed was recovered in hydrogen gas (14 ± 5 mL) using non-diluted (ND) wastewater (20 h cycle time), while 22 ± 4% of the COD was recovered in hydrogen gas (9 ± 3 mL) using the diluted wastewater (16 h cycle time; Figure 1, Table 1). Using a long (L) cycle time required for completion of hydrogen production for the diluted (D) sample (42 h) slightly increased conversion of COD removed to hydrogen gas to 28 ± 6%, and the volume of hydrogen gas produced to 15 ± 2 mL. A complete cycle time of the non-diluted sample required 184 h and resulted in a similar conversion efficiency of COD removed to hydrogen of 20 ± 1%, but the volume of hydrogen gas increased to 77 ± 11 mL due to combined high COD concentration and duration of the test.

The energy yield was 190 ± 39% based on the energy content of the hydrogen gas recovered in the tests with the non-diluted wastewater in the short batch cycle (ND-S; Figure 1). COD was reduced by 19 ± 15% in the ND-S test (Figure 1), with the best COD reduction of 72 ± 4% achieved in the longer test with non-diluted wastewater (ND-L; Figure 1). However, the ND-L experiment was the least efficient with respect to energy recovery as hydrogen versus required energy input (ηW = 91 ± 6%), in large part due to the long time of the test (184 hours), which resulted in current generation, and therefore power input, approximately 10 times greater than

the D-L test (42 h), but a volume of hydrogen only approximately 5.5 times greater. A larger portion of the current was recovered as methane in the longer test (ND-L), which produced nearly 13 times as much methane as the shorter test (D-L). The other tests had energy efficiencies of 179 ± 4% and 165 ± 21% for the D-L and D-S tests respectively, based on the electrical energy input and the hydrogen recovered. Energy input from the power source ranged from 0.47 to 1.09 kWh/kg COD, with an average of 0.8 kWh/kg COD.

There was a large variation in the recovery (as current) of electrons from the COD removed, with CEs ranging from 29 ± 17% to 70 ± 2% (Table 1). In general, long batch cycle times resulted in higher CEs based on comparisons made for tests with the same initial COD. The highest CE was achieved in the long batch test, but the long-cycle time (184 h) resulted in a low recovery of hydrogen produced at the cathode (29 ± 2%). The other tests with cycle times of 16-42 hours achieved cathodic hydrogen recoveries of 53- 61%.

The hydrogen production rate (Q) was similar in all tests. It was approximately 1.0 m^3 m^{-3} day^{-1} H_2 (total reactor volume) for the long batch cycle time tests, and 0.9 m^3 m^{-3} day^{-1} H_2 for the short batches (Table 1).

The MECs produced methane in addition to hydrogen (Table 1). Hydrogen was 77 ± 5% of the total gas volume for the D-L test, and 74 ± 4% for the D-S test. For the tests using non-diluted wastewater, the percentage of hydrogen in the gas was slightly smaller, with 64 ± 1% for the ND-L test and 58 ± 1% for the ND-S test. The balance in each case was methane and carbon dioxide. Methane recovery from COD

($r_{CH4,\ COD}$) was slightly less than hydrogen recovery from COD for all tests, between 5 ± 2% to 21 ± 7% for $r_{CH4,\ COD}$ and from 17 ± 7 to 28 ± 6% for $r_{H2,\ COD}$ (Figure 2).

2.3.2. Hydrogen Production and Consumption in Fermentation Tests

Fermentation tests were conducted under various conditions in order to evaluate the potential for hydrogen production. However, even with the addition of an HT inoculum and conditions that limit hydrogen-consuming reactions (the wastewater was autoclaved and the solution buffered to pH 5.8), hydrogen gas only reached a concentration of 6% in the headspace after 75 hours (Figure 3). After 200 hours, the hydrogen gas was completely re-consumed by the culture. The amount of energy needed to autoclave the wastewater (approximately 1-2 kWh/kg COD) would not justify the small amount of hydrogen produced (equivalent to <0.001 kWh/kg COD, data not shown), but this procedure does demonstrate that the upper limit for hydrogen production under optimal conditions by fermentation is quite small. In tests with wastewater (no inoculum amendment, buffered to either pH 5.8 or 7.2), no net hydrogen was produced. There was biogas production (with or without a sludge inoculum), but the gas contained methane and carbon dioxide, and no hydrogen. When the pH was lowered to inhibit methanogenesis (adjusted to 5.8 and to 5.0), gas production decreased but still no hydrogen was produced.

Additional tests were conducted to see if other factors were interfering with hydrogen production. Glucose was added to wastewater to ensure germination of hydrogen-producing bacteria, the wastewater was diluted either 2× or 8× to reduce the concentration of methanogens and ammonia, and a low pH was used to restrict

methanogenesis (pH = 5.8). In tests with 2× or 8× diluted and autoclaved wastewater, HT inoculum, and glucose, the biogas produced was approximately 50% hydrogen (Figure 4). Without the HT inoculum or autoclaving, the 2× diluted wastewater with glucose produced biogas with approximately 23% hydrogen gas over 70 h. When 2× diluted and autoclaved wastewater with HT inoculum was tested without any glucose addition, the hydrogen in the biogas was <10%. The molar hydrogen yields were 1-1.2 mol H_2/mol glucose for the HT sludge alone (not plotted on Figure 4), 1.89 for the 2× diluted wastewater with HT sludge, and 0.59 for the 8× diluted wastewater with HT sludge.

2.3.2.1. Hydrogen consumption tests

The lack of net volume of hydrogen gas evolved in fermentation tests could be due to an equivalent rate of hydrogen consumption via acetogenesis. When hydrogen was added to the headspace of wastewater with an HT sludge inoculum at a concentration of ~60%, the hydrogen was rapidly consumed when the initial pH was 6, 7, or 8 (Figure 5), and acetate concentrations increased (data not shown). The abiotic control bottles without inoculum did not consume hydrogen in the headspace, and the biotic control bottles with nitrogen in the headspace did not produce hydrogen (data not shown). At a pH of 5, the rate of hydrogen consumption was substantially reduced, but as previously stated, tests at pH 5 showed little potential for hydrogen production. Thus, it appears that there is a great ability for the sample to consume hydrogen gas, making it difficult to recover small amounts of hydrogen gas under fermentation conditions.

2.3.2.2. Continuous flow reactor tests

Hydrogen production by fermentation was also examined in continuous culture to test the hypothesis that hydrogen production might be achieved under conditions that would wash out hydrogen-consuming microorganisms. There was no sustained hydrogen production using either raw or autoclaved swine wastewater (diluted 2×) in a reactor operated under continuous culture conditions (data not shown). There was hydrogen initially produced when glucose was added during the reactor start up, but when the reactor was operated in continuous flow mode there was no hydrogen production under steady state conditions (data not shown).

2.4. Discussion

High COD wastewater such as swine wastewater can be used to produce hydrogen gas using an MEC, but not by fermentation. In fermentation tests, no hydrogen gas was recovered unless the wastewater was autoclaved, and even under those conditions the small amount of hydrogen gas that was evolved was consumed by the sample. In an MEC, hydrogen gas was evolved at a fast rate, and thus appreciable amounts of hydrogen gas could be recovered before it could be used by microorganisms in the wastewater. The rates of hydrogen gas production measured here were similar to those found in recent studies using acetate [16, 21]. Treatment efficiencies in MEC tests with swine wastewater ranged from 19 ± 15 to 72 ± 4% based on COD reduction. While this is a large range, it is similar to the range of treatment seen in more conventional digesters, which produce COD reductions from 28 to 87% [32].

This suggests that treating swine wastewater in an MEC may be a viable option for both producing hydrogen gas and reducing the COD.

Coulombic efficiencies are used to evaluate the percent of electrons from the COD that are transferred into current. The CEs in the short-cycle tests were 29 ± 17% and 43 ± 2%, and 48 ± 9% and 70 ± 2% in the long-cycle tests. The lower CEs in all experiments except for the long test with the undiluted waste (ND-L) indicated that a large percentage of electrons were not successfully transferred into current. A large percentage of the COD removed was therefore transferred to electron acceptors such as CO_2, stored in cells, or used for cell growth, as shown in Figure 2. Sludge production may be problematic if electrons are shuttled into cell synthesis rather than current. Methane was produced in all tests, with 5 ± 2% to 21 ± 7% of the COD removed converted to methane. Less methane was recovered in tests with undiluted waste than with the diluted waste.

The use of the single-chamber design, in which the hydrogen is evolved into the wastewater, allows for a simple reactor design (compared to reactors with a membrane) and can achieve high hydrogen production rates. However, the lack of a membrane can reduce hydrogen recoveries due to hydrogen consumption by microorganisms in the wastewater. In the longest test using non-dilute wastewater, 70% of the electrons were recovered as current (CE) but only 29% of these electrons were successfully recovered as hydrogen gas ($r_{H2,\ CAT}$). The increase in CE compared to the other tests may be due to reoxidation of hydrogen at the anode, which supplies electrons to the circuit without a concurrent COD reduction. In addition, acetogenesis may be occurring, as seen in the hydrogen consumption tests from the fermentation

experiments. Acetate could either be oxidized or converted into methane. In contrast, tests conducted using only a single substrate (acetate) in a defined medium resulted in nearly 80% of the electrons recovered as hydrogen gas (same applied voltage of 0.5 V, and a comparable conductivity of 7.5 mS; [21]). Hydrogen production rates here were 0.9 or 1.0 m^3 m^{-3} day^{-1} with the swine wastewater, compared to 1.5 m^3 m^{-3} day^{-1} for tests using acetate. The current densities using swine wastewater were only slightly lower (92 A m^{-3} for dilute wastewater, 109 A m^{-3} for non-dilute wastewater; Table 1) compared to 145 A m^{-3} for the Call and Logan [21] study. Thus, it appears that using swine wastewater achieves good current densities but that hydrogen recovery is much more challenging with a wastewater containing a high number of hydrogen-consuming microorganisms. Despite these biological hydrogen losses, overall recoveries ($r_{H2,\ COD}$) were still between 17% ± 7% and 28% ± 6%, a range that is much higher than a previous MEC study with wastewater using a two-chamber system with a membrane separating the anode chamber from the cathode [20].

The observation that hydrogen was only minimally produced during fermentation using the autoclaved sample demonstrates that hydrogen production is intrinsically possible by this method, but that other factors limit hydrogen gas recovery. The lack of hydrogen recovery is concluded to be due to utilization of hydrogen by microorganisms in the wastewater and low hydrogen production rates. This conclusion is based on tests showing little hydrogen evolution from even autoclaved wastewater, and tests showing that hydrogen added to the gas phase is rapidly consumed by microorganisms in the wastewater. There are several factors that could have contributed to low hydrogen production. For example, hydrogen

evolution could simply be due to a lack of suitable sugars in the wastewater, as most hydrogen evolved in high-rate hydrogen fermentation tests is a result of sugar in the sample. Only 4% of the nearly 7,000 mg/L of total reducing sugar (data not shown) in the raw wastewater was soluble (evaluated using the phenol-sulfuric acid method for reducing sugars, [27]). The addition of glucose to any wastewater sample stimulated hydrogen evolution (Figure 4), but there was no hydrogen production without adding glucose or sterilizing the wastewater. Thus, slow kinetics of combined sugar hydrolysis could have limited hydrogen fermentation rates. It is also possible that ammonia inhibited hydrogen-producing bacteria from germinating or growing rapidly. However, the ammonia concentration in batch tests using full-strength wastewater was ~2000 mg-N/L, and using 2× diluted wastewater was ~1000 mg-N/L. These ammonia concentrations are below concentrations reported to inhibit either hydrogen production rates or yields (at pH of 5.2 or 6.2) [33], and below inhibitory levels reported for methanogenesis at the two pH values used in these tests [34]. Ammonia concentrations were the same in the autoclaved and untreated wastewater tests. As a result of all these factors, it appears that hydrogen recovery from swine wastewater will not be feasible by fermentation processes unless some breakthrough is made in changing the nature of the wastewater or the conditions for microbial growth that inhibit the utilization of the hydrogen by microorganisms in the wastewater.

2.4.1. Outlook for swine wastewater treatment using MECs

The use of MECs for wastewater treatment will depend on many factors including the cost of the materials, and the amount of energy needed. The cost of

materials for large scale treatment is not yet known. The energy efficiencies based on the energy value of the hydrogen gas produced compared to the electrical energy input were high here using the single-chamber MEC and wastewater, and thus from an energy standpoint MECs may be a promising method of treatment. Although MECs are unlikely to become cheaper than anaerobic digestion for high-strength wastewater applications due to capital costs for the electrodes, MECs can produce a high value product and may have a role in wastewater treatment in special circumstances where the hydrogen produced has a value that can offset the capital costs. Rozendal et al. [35] offer a similar argument for electricity produced by MFCs. The efficiency of the short-cycle time reactor with non-diluted wastewater was 190 ± 39%. Energy efficiencies in other tests were similarly high when the total cycle time was short, primarily as a result of the low energy input from the power source over this shorter time. Note that the energy content of the organic matter is not included in this energy balance since it is "free" compared to the electrical energy needed, and thus the efficiency can be over 100%. The amount of energy provided by the power source per mass of COD removed was approximately 0.8 kWh/kg-COD. If all the electrons available in the removed COD (1 mol e-/8 g COD) had been successfully harnessed as current, 1.67 kWh/kg-COD would be required by the power source applying 0.5 V. However, since approximately 50% of the electrons from the COD did not produce current, the energy requirement in these experiments was about half that expected by an applied voltage of 0.5 V. These values for energy requirements for this process are within the 0.7-2 kWh/kg-COD range [36] needed for activated sludge treatment. However, in some of the MEC tests almost twice as much energy

was created here in the form of hydrogen gas. Using the energy in the gas to power the system with a fuel cell, for example, could make the process nearly self-sustaining. Assuming 55% efficiency in a hydrogen fuel cell (for electricity production), using the hydrogen from the best-performing MECs in this study would provide ~10% more energy than the energy from combustion of both the hydrogen and methane (assuming 40% combustion efficiency). This might require clean up of the gas for use in a hydrogen fuel cell, a process which would need to be further examined. In the best scenario from this study, approximately 1 kWh/kg-COD removed could be achieved by combusting the hydrogen and methane. This is similar to the energy output achieved from combusting the methane produced by anaerobic digestion. The options for energy recovery should be further explored.

2.5. Conclusions

Swine wastewater was successfully treated while producing hydrogen gas using an MEC. In contrast, little hydrogen gas could be recovered from fermentation of the wastewater unless it was autoclaved, a procedure that would not be practical on a larger scale. In an MEC, the COD removals ranged from 19 ± 15 to 72 ± 4%, with hydrogen recoveries of 17 ± 7 to 28 ± 6% based on COD removed. The advantage of producing hydrogen gas in an MEC compared to other methods is the high energy content and market value of the hydrogen. However, appreciable amounts of methane gas were also produced in this process, resulting in a biogas with several components. The use of this product gas for other purposes will need further evaluation to

determine the overall practical nature of using an MEC for swine wastewater treatment.

2.6. References

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2.7. Tables

Table 2.1. Summary of microbial electrolysis cell experimental results. D = dilute, ND = not dilute wastewater; S=short-cycle time; L = long-cycle time.

Conditions	Batch cycle time[a] (hr)	Initial COD (mg/L)	CE (%)	$r_{H2,\ cathode}$ (%)	$r_{H2,\ COD}$ (%)	$r_{CH4,\ COD}$ (%)	Q[b] ($m^3\ m^{-3}\ day^{-1}\ H_2$)	Current densities (A/m^3)	H_2 (%)
D-S	16	2,000	43 ± 2	53 ± 6	23 ± 4	21 ± 2	0.8 ± 0.2	93 ± 22	74 ± 4
ND-S	20	12,825	29 ± 17	61± 12	17 ± 7	5 ± 2	0.9 ± 0.2	106 ± 6	58 ± 1
D-L	42	2,000	48 ± 9	58 ± 1	28 ± 6	21 ± 7	1 ± 0.1	92 ± 13	77 ± 5
ND-L	184	12,825	70 ± 2	29 ± 2	20 ± 1	14 ± 5	1 ± 0.1	112 ± 25	64 ± 1

[a]Short-cycle = batch ended immediately after peak gas production rate; long-cycle = batch ended when gas production ceased.
[b]Maximum rate sustained for ≥ 2 hours

2.8. Figures

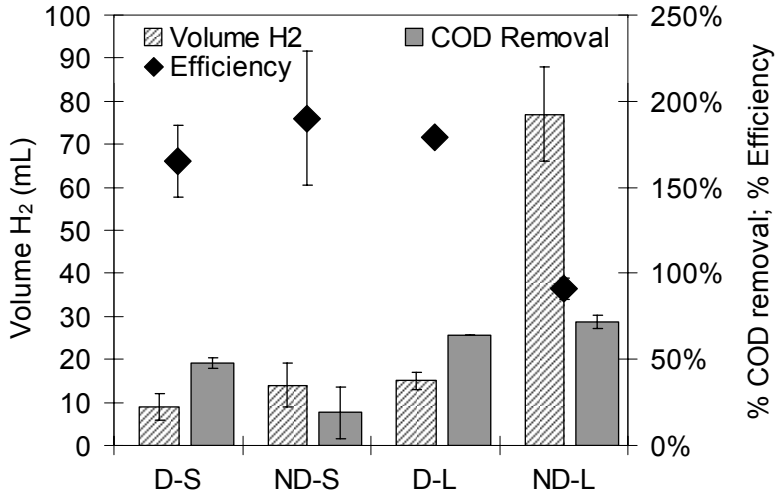

Figure 2.1. Hydrogen production (volume), wastewater treatment (COD removal), and hydrogen gas production efficiency, based on the electrical energy needed compared to the energy of the hydrogen gas produced (η_W). Error bars ± SD based on duplicate measurements for each experiment.

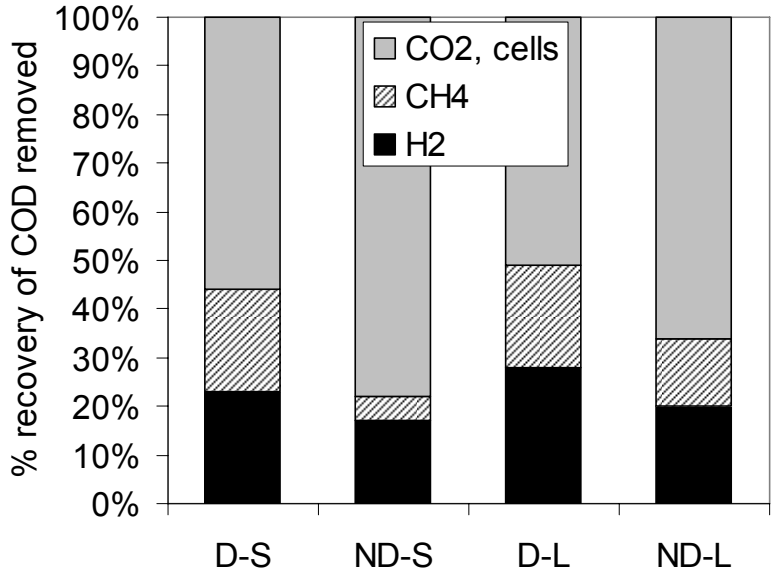

Figure 2.2. The fate of the COD removed in MEC experiments, based on $r_{H2,\ COD}$ and $r_{CH4,\ COD}$. Variation across duplicates is not shown.

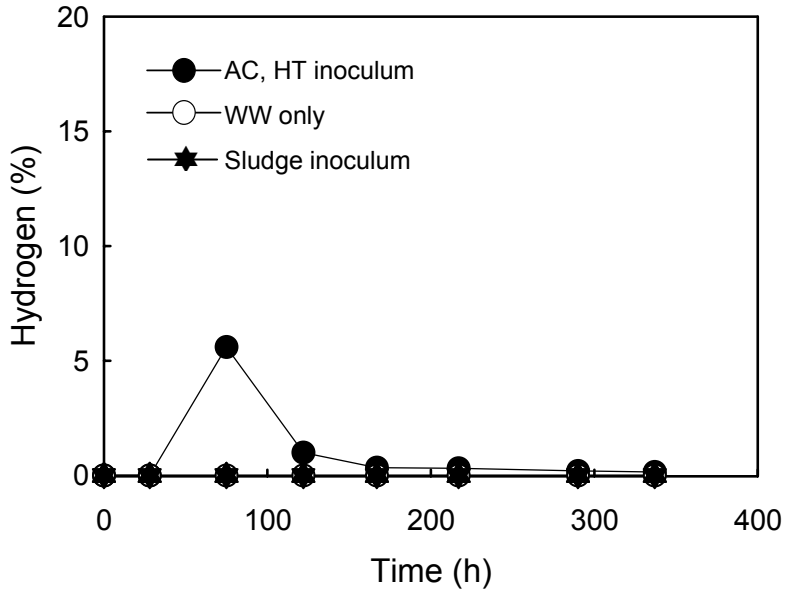

Figure 2.3. Hydrogen production from swine wastewater by fermentation (pH 5.8). AC = autoclaved; HT = heat-treated inoculum.

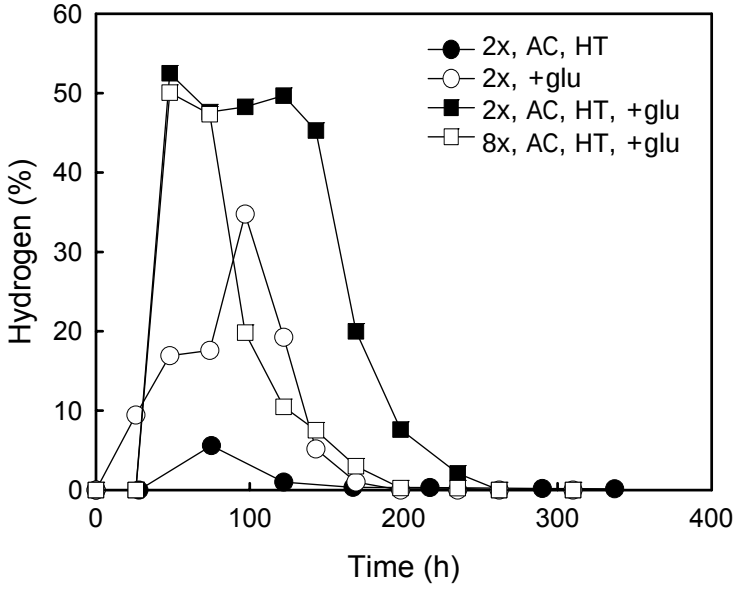

Figure 2.4. Hydrogen production by fermentation with adjustments to dilution (2x or 8x) and bacterial community. Some samples included an external inoculum (HT sludge); some were amended with glucose (+glu). AC = autoclaved; HT = heat-treated inoculum.

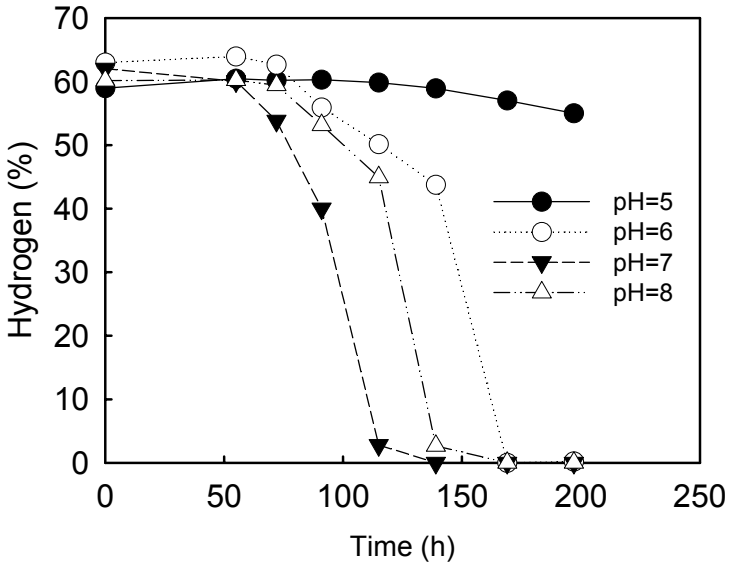

Figure 2.5. Hydrogen consumption by HT sludge inoculum in swine wastewater at variable pH.

Chapter 3

Optimal set anode potentials vary in bioelectrochemical systems

Abstract

In bioelectrochemical systems (BESs), the anode potential can be set to a fixed voltage using a potentiostat but there is no accepted method for defining an optimal potential. Microbes can theoretically gain more energy by reducing a terminal electron acceptor with a more positive potential, for example oxygen compared to nitrate. Therefore, more positive anode potentials should allow microbes to gain more energy per electron transferred than a lower potential, but this can only occur if the microbe has metabolic pathways capable of capturing the available energy. Our review of the literature shows that there is a general trend of improved performance using more positive potentials, but there are several notable cases where biofilm growth and current generation improved or only occurred at more negative potentials. This suggests that even with diverse microbial communities, it is primarily the potential of the terminal respiratory proteins used by certain exoelectrogenic bacteria, and to a lesser extent the anode potential, that determines the optimal growth conditions in the reactor. Our analysis suggests that additional bioelectrochemical investigations of both pure and mixed cultures, over a wide range of potentials, are needed to better understand how to set and evaluate optimal anode potentials for improving BES performance.

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3.1. Introduction

Microorganisms respire and capture energy for the production of ATP through the oxidation of organic and inorganic matter and reduction of a terminal electron acceptor. ATP is generated by the proton motive force, which results from protons that are pumped outside the inner cell membrane as electrons are transferred from a reduced to an oxidized compound via the electron transport chain during respiration. Thus, there is the possibility of generating more energy using electrons acceptors with higher potentials, but only if the microorganism can utilize the additional energy through the pumping of additional protons across the membrane.

The maximum amount of energy that can be captured from the oxidation and reduction of two chemical species can be calculated from the Gibbs free energy as

$$\Delta G^{0'} = -nF\Delta E_0^{'} \quad (1)$$

where $\Delta G^{0'}$ is the Gibbs free energy at standard biological conditions (T = 25^{o}C, pH = 7), n the number of electrons transferred, F Faradays constant (96,485 C/mol e^{-}), and $\Delta E_0^{'}$ the difference in the potentials between the electron donor and the electron acceptor for a particular chemical reaction. Larger values of $\Delta E_0^{'}$, therefore, could provide more energy for the cell. For example, using hydrogen as an electron donor and assuming equimolar concentrations of the terminal electron acceptor, *Paracoccus denitrificans* can obtain more energy by reducing oxygen than by reducing nitrate, due to the greater energy available from reduction of oxygen. Capture of this

additional energy could result in increased biomass yields, depending on the metabolic efficiency of this microbe under these conditions. Thermodynamic potentials can not be used to predict growth rates of microbes, as this is determined by kinetics, but in general aerobes usually grow faster than anaerobic microorganisms. In addition to the different redox potentials of electron acceptors, the energetic requirements of a cell vary depending upon whether an internal electron acceptor (such as fumarate) or an external electron acceptor (such as insoluble Fe(III) or an electrode) is available [1]. With an internal electron acceptor, protons are consumed within the cell during reduction of the electron acceptor, whereas with an external electron acceptor, protons produced from substrate oxidation must be transported out of the cytoplasm to avoid acidifying the cytoplasm. This proton transport, required by use of an external electron acceptor, is an energetically intensive process that Mahadevan et al. suggested [2] could have significantly decreased the growth yield of *G. sulfurreducens* on the external electron acceptor.

In a bioelectrochemical system (BES), the anode can be used as a terminal electron acceptor for microorganisms. Microbes must have a mechanism, provided through direct contact or a chemical mediator, for transferring electrons outside of the cell to the anode [3]. In order for the reaction to be thermodynamically favorable, the anode must have a higher (more positive) potential than either the terminal protein in the cell's electron transport chain or the mediator that is used. In theory (Eq. 1), a more positive anode potential will allow the cell to capture more energy, but only if the cell is capable of capturing this energy by pumping additional protons across its inner membrane. Thus, the microbe must possess terminal respiratory proteins that

can use this additional potential provided by the anode. For example, if the anode of an acetate fed ($E_{0\,\text{donor}}^{'}$ = –0.3 V) BES is set to a highly positive value (e.g. $E_{0\,\text{anode}}^{'}$ = +0.4 V) and a microbe can only adjust its respiratory enzymes to a lower potential (e.g. $E_{0\,\text{enzyme}}^{'}$ = –0.05 V, as suggested by [4]), then the microbe will only be able to capture part of the total available free energy. The additional free energy between the terminal respiratory enzyme and anode potential is wasted.

Setting the anode potential in a BES has allowed researchers to study the electrochemical capabilities of microbes that can transfer electrons to an anode (referred to as exoelectrogens or anode-reducing bacteria [3]). Optimal anode potentials, defined here as those producing high current densities and more rapid start-up times, have not been established. Known exoelectrogens are widely dispersed among many different genera, and factors that can affect an optimal anode potential are diverse, for example: differing redox potentials of the various cytochromes; different mechanisms for transferring electrons from the cell to the anode; and variability in the effectiveness of electron transfer from cells to different anodes materials (for example carbon versus stainless steel). A review of 28 studies (Table 1) reveals that in 50% of these studies, a single potential was used to set the anode for experimentation. When different anode potentials are compared, 71% (10) of these 14 comparison studies show improved performance (i.e. faster start-up or higher current density) at higher potentials, 14% (2) showed mixed results, and 14% (2) show improved performance at lower potentials.

We review here the literature in terms of the effects of anode potentials on start-up time, maximum current densities, and biomass production. Our results show

that optimal electrode conditions for mixed communities cannot be set *a priori*, and at present must be individually determined; for pure cultures, electrochemical techniques can assist in determining the optimal anode potential.

3.1.1. Setting Anode Potentials

Accurately setting an anode potential requires the use of a potentiostat in conjunction with a reference electrode [5]. Commonly used reference electrodes include silver/silver chloride (Ag/AgCl) and saturated calomel electrodes (SCE). The standard value of the saturated Ag/AgCl reference is +0.197 V (T = 25^{o}C) versus the standard hydrogen electrode (SHE) [6]. Under unsaturated conditions, the potentials are slightly different (e.g. +0.209 V in 3M KCl). The SCE reference has a standard value of +0.244 V (T = 25^{o}C) under saturated conditions, but this value is also dependent on the electrolyte concentration. The actual potential of a reference electrode depends on experimental conditions, such as temperature and solution chemistry, and it can be determined by calibrating the reference electrode using Zobell's solution [7]. At the end of an experiment, the reference electrode should be checked for accuracy, as chemicals in solution, such as ammonia and sulfide, can form precipitates on the electrode, causing variability relative to the standard values reported above [8]. The reference electrode should be placed as close as possible to the working electrode, with the location fixed throughout the experiment, to minimize potential loss through the solution and limit variability. Placing a reference electrode 0.6 cm from the anode, for example, resulted in a drift of 0.042 V between the reference potential and the anode potential [9].

Set anode potentials in BESs typically range from −0.2 V to +0.8 V vs. SHE at pH 7, with the anode potential more positive than the calculated potential of the substrate. Anode potentials are often set to mimic typical terminal electron acceptors found in natural environments, such as insoluble iron (ferrihydrite/Fe^{2+}, −0.100 to +0.100 V) [10] and oxygen (O_2/H_2O, +0.818 V) [11]. Hydrogen can theoretically form at potentials more negative than −0.414 V and oxygen can be produced at potentials more positive than +0.818 V; however, all electrode materials have overpotentials and the actual potentials at which these reactions occur can be determined using cyclic voltammetry on abiotic controls. Electrochemical hydrogen or oxygen production should be avoided as it may lead to the growth of non-exoelectrogenic bacteria and interfere with interpretation of results. In addition, hydrogen peroxide can be produced at a theoretical potential of +0.28 V when oxygen is present, which has been shown at the cathode of several BES [12-14]. Although potentiostats provide the most accurate method for setting anode potentials, they can be a cost-prohibitive lab item and an alternative method for setting an anode potential using a power supply unit is described by Bond [8].

All voltages are given here with respect to the SHE, pH = 7. In cases where the voltage was not reported vs. SHE, Ag/AgCl voltages were approximated by adding +0.2 V and SCE voltages by adding +0.24 V to the reported voltage. We recommend that researchers working on BESs maintain consistency across the discipline by always reporting voltages adjusted to SHE, and ensure accuracy of given values by always checking their reference electrodes as mentioned above using Zobell's solution. Differences in potential can be described as "more negative" and

"more positive" than one another or as "lower" and "higher," with higher always indicating a more positive potential.

3.2. Results supporting the use of higher anode potentials

Studies in which better performance is seen at higher potentials suggest that more positive potentials provided more free energy to the microorganisms, although in many cases the range of potentials examined was limited. In one study, for example, *G. sulfurreducens* produced a higher current, and more quickly, at +0.8 V than at the lower potential of +0.3 V [15], but no other potentials were examined. The anode with the more positive potential also produced a thicker biofilm. Dumas et al. [16] also examined the effect of different potentials on current production by *G. sulfurreducens*, but they used stainless steel electrodes rather than graphite, which prevents a direct comparison. Current was only generated at the highest set potential of +0.51 V, but similar to the Busalmen study [15] all the other potentials examined were fairly high (+0.31, +0.21, and +0.11 V).

Examining a wider range of potentials, Wei et al. [17] compared current and biomass production of *G. sulfurreducens* growing on anodes set to −0.16 V, 0 V, and +0.4 V. They observed faster start-up times, higher current generation, and greater biomass production for the anode poised at 0 V versus −0.16 V; the anode set to +0.4 V showed similar current and biomass production to the one poised at 0 V, suggesting an upper limit to the amount of energy that could be captured by the bacteria, regardless of an increase in available potential. They concluded that the key to high

current production was faster and thicker biofilm development, which could be achieved by using higher anode potentials (0 and +0.4 V).

In addition to the pure culture studies mentioned above, Finkelstein et al. [11] showed improved performance with a mixed culture at higher set potentials than lower potentials. Three different potentials (+0.142 V, +0.303 V, and +0.818 V) were applied to anodes submerged in marine sediment. The highest current and fastest substrate oxidation rate was produced by the anode with the most positive set potential. A mixed culture from garden compost also produced higher current at higher set anode potential (+0.94 V vs. +0.74 V and +0.64 V, in separate reactors) [18].

Setting the anode potential at more positive voltages has a clear rationale based on providing the possibility for more energy for the microorganisms, but it creates conditions which are quite different than those that develop in most BESs. When microbial fuel cells are operated, the anode potentials measured during peak voltage generation are often quite negative, with typical values around –0.20 V to –0.28 V for mixed cultures oxidizing acetate [19]. The exact value is dependent on the substrate used, the cathodic reaction, the external resistor, and the current density. The potential of a non-poised anode becomes more negative over time (until it reaches a steady-state potential) as an exoelectrogenic biofilm develops [5], whereas a poised anode is controlled externally (e.g. by a potentiostat) at the same potential throughout the duration of the experiment, regardless of biofilm activity. There are few studies comparing current densities achieved using a non-poised (and therefore variable potential) anode to those produced by a set (and therefore stable) potential. In

one such study, Wang et al. [20] used a mixed culture (domestic wastewater and anaerobic sludge). The anode of one reactor was set at +0.4 V, while the potential in the anode of the second reactor was not fixed. The non-poised anode potential decreased from approximately +0.49 to –0.16 V over 11 batch cycles (52 days). The final cell voltage of the non-poised reactor (measured across a 1000 Ω resistor) was the same as that achieved with the poised-anode reactor (disconnected and measured across a 1000 Ω resistor). However, the poised-anode reactor produced a reproducible current in fewer cycles (6 batch cycles, 30 days), showing that setting the anode potential can result in more rapid reactor acclimation for maximum power. The experiments were not continued past day 60, so longer-term stability of the non-poised anode was not tested. Faster start-up time using an anode poised at +0.4 V versus a non-poised anode was also shown with *G. sulfurreducens* (2 and 3.5 days, respectively, to reach the same current) [17]. Reducing the length of time it takes an anode to reach a stable maximum voltage is important when considering the use of BESs for practical applications. Wang et al. [20] showed that poising an anode reduced start-up time, and in a study using *S. oneidensis*, Cho and Ellington [21] showed that the most positive potential had the shortest start-up time (+0.5 V vs. +0.35 V, +0.2 V and 0 V).

In the studies mentioned in this section (10 of the 28 studies reviewed here), there appears to be a clear trend of improved performance with higher potentials. These observations of improved performance at higher potentials do not consider many studies where relatively low potentials were used. Thus, it is clear that more positive potentials can be used to study microorganisms on the anodes in BESs, but

comparisons with a broader range of potentials – including those much closer to the potential of the substrate – are necessary for a complete understanding of BES performance.

3.3. Results supporting the use of lower anode potentials

The low anode potential developed by a non-poised MFC is typically only slightly more positive than the E_0' for the half cell reaction for oxidation of typical substrates, such as –0.30 V for acetate and –0.43 V for glucose [22]. This suggests that setting a more negative potential will create conditions most similar to those in a BES, but using a single potential does not allow us to probe the capabilities of the pure or mixed culture at other electrode potentials.

In order to see what potentials might produce the most optimum conditions for growth in BESs, Torres et al. [23] examined biofilm growth and current generation in a two-chamber BES containing four anodes set at different potentials using a single reference electrode. The anode chamber was inoculated with a mixture of return activated sludge and anaerobic digester sludge. The two anodes set at negative potentials (–0.15 V and –0.09 V) generated current sooner, reached higher current densities, and developed visually thicker biofilms than the anodes set at positive potentials (+0.02 V and +0.37 V). These results suggest that in a single competitive environment for acetate, exoelectrogenic microorganisms were most successful (relative to growth and current production) using relatively low anode potentials. The biofilm that developed at –0.15 V was dominated by *Geobacter* (97% similarity to *G. sulfurreducens*), a known exoelectrogen that produces high current densities in BESs

[24-27]. In contrast, the community at the anode set at +0.37 V was much more diverse and generated very little current. The authors surmised that non-exoelectrogens, and/or mediator-producing exoelectrogenic microbes, may have colonized the +0.37 V anode first, interfering with the ability of *Geobacter* to make direct contact with this anode.

Setting multiple electrodes to different potentials within the same chamber creates conditions that may not exist when only one anode is used per reactor. For example, electrodes placed at different distances from the reference electrode in the same chamber may create small but significant variation from the intended applied potential. Biofilms growing on one electrode could produce mediators at one potential that affect growth and current production of biofilms on electrodes at other potentials. The use of electrodes at different potentials in the same chamber produces surfaces with different charges, promoting negatively-charged particle (e.g. bacterial) migration to the more positively charged electrodes. Only one study has examined BESs using both single and multiple electrodes per anode chamber [18]. Three dimensionally stable anode (DSA) electrodes were placed into a single reactor, set at three different (positive) potentials of +0.54, +0.74, and +0.94 V, and inoculated with garden compost. In direct contrast to their anodes in separate reactors, average current densities in the single reactor/multiple anodes experiment were highest (129 mA m^{-2}) for the electrodes set at the lowest applied potential (+0.54 V), and current densities decreased at the higher potentials. In an experiment with three electrodes placed in a single chamber, all at +0.74 V, each electrode produced the same current. However, the current from an electrode in this reactor, with all three electrodes at +0.74 V, was

lower than that obtained at the same potential in the presence of two electrodes set at different potentials. The different results for anodes placed in separate reactors compared to a single reactor suggest that the presence of other electrodes set at different potentials within a reactor affected the results. Electrode materials and potentials used by Torres et al. [23] and Parot et al. [18] were also different, and for these reasons further studies are necessary to determine if it is acceptable to use multiple electrodes with different set potentials in the same chamber in BES tests.

Other studies comparing high and low anode potentials show mixed results, in terms of power or current, with different set potentials. For example, Aelterman et al. [28] concluded that the optimal anode potential was 0 V, compared to –0.2 V and +0.2 V, for a mixed culture obtained from a long-running MFC anode. The conclusion that 0 V was optimal was based on maximum attainable power as well as the highest current production at maximum power, although the lowest set potential (–0.2 V) reached its maximum power most quickly. The lowest amount of biomass was produced on the electrode at the lowest potential (–0.2 V). However, even though there was less biomass on the electrode set to –0.2 V, this biofilm was the most active, producing considerably more current per unit of biomass than the biofilms on the electrodes set at higher potentials. This suggests that different potentials may be necessary to encourage different biofilm characteristics: first a potential to encourage biofilm growth on the electrode, and then a potential to increase the current production.

Setting a highly negative anode potential over long periods has been shown to result in modification of a pure culture by increasing the amount of current that can

be produced. Yi et al. [29] set an anode at −0.2 V and obtained a strain of *G. sulfurreducens* capable of current densities over five times larger than the wild type strain, showing that the negative anode potential acted as a selective pressure for the evolution of a more efficient strain. Identical experiments at positive anode potentials were not performed, and it is unknown if different anode potentials can also be used to apply this selective pressure.

Although microbes can theoretically gain more energy from a higher potential terminal electron acceptor, the studies described in this section suggest a more complex picture. Four out of the 28 studies reviewed here suggest that microbes can produce higher current densities and thicker biofilms at low potentials, and reinforce that further study is necessary to understand microbial activity across a broad range of applied potentials.

3.4. Electrochemical tests to examine the conditions of exoelectrogenic behavior

The use of electrochemical techniques, such as cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), may help to identify the reasons for these various findings with different set potentials and microorganisms. For example, there is now evidence using CV and EIS techniques that *G. sulfurreducens* can discharge electrons at more than one redox potential, allowing this microbe to function well at more than one set potential. CV scans of *G. sulfurreducens* biofilms set at two different potentials (+0.8 V and +0.3 V) showed two different sets of redox peaks [15]. When set at +0.3 V, there was an oxidation peak at +0.22 V and a reduction peak at –0.16 V. However, biofilms set at +0.8 V showed a different

response, with an oxidation peak at +0.85 V and a reduction peak at +0.43 V. The locations of these peaks were dependent on acclimation to a specific potential and were not permanent. Switching the biofilm from the high set potential to low potential produced peaks similar to those in the biofilm originally acclimated at the lower potential. Conversely, Wei et al. [17] obtained identical redox peaks for *G. sulfurreducens* using CV for anodes set to −0.16, 0, and +0.4 V as well as a non-poised anode. The peaks for the electrodes set to +0.4 and 0 V were slightly larger in magnitude than the other anodes, which was consistent with the higher potentials producing more biomass than the lower potential. Marsili et al. [4] found very similar CV results between a *G. sulfurreducens* biofilm grown at −0.16 V and +0.24 V, but using differential pulse voltammetry to compare the two potentials, two differences in the peaks were evident.

EIS of a *G. sulfurreducens* biofilm showed that electron transfer could be altered by the choice of set potential [30]. Charge transfer resistance decreased by over 100-fold, resulting in an increase in the electron transfer rate, using an electrode set at –0.16 V, compared to one set at +0.042 V. Setting the potential even lower, at –0.26 V, also increased the charge transfer resistance by more than 10 times that at –0.16 V. A lower charge transfer resistance improves the kinetics of electron transfer, allowing for improved power densities in an MFC. A first-derivative analysis of a CV scan of this microorganism (set potential conditions during growth at +0.24 V; Figure 1) showed a large inflection point at –0.16 V, characteristic of a single rate-limiting reaction indicative of the redox potential of the electron transfer protein. There were also secondary (but substantially smaller) peaks at –0.02 V and –0.22 V, indicating

the presence of additional electron transfer proteins. These results suggest the presence of multiple different redox pathways, with the more optimal one at –0.16 V.

This optimal potential is consistent with other studies that show an optimal potential for an anodic biofilm in the range of –0.15 V to –0.2 V. For example, Torres et al. [23] showed better performance of a mixed culture dominated by *G. sulfurreducens* at –0.15 V, compared to three more positive potentials. A potential of –0.16 V was reached in the non-poised electrode experiment by Wang et al. [20], and –0.2 V was the potential with the most active biomass obtained by Aelterman et al. [28]. Other microbes such as *S. oneidensis* show different responses to CV [31] compared to *G. sulfurreducens*. This likely results from the various types of mechanisms used for electron transfer, which can include for *Shewanella* electron shuttles [32], direct contact [33], and nanowires [34], while *Geobacter* employs direct contact [24], different types of nanowires [35], and recently it has been shown that cytochromes can be localized on these nanowires [36]. Thus, they use different terminal respiratory proteins and can have different pathways for electron transfer, resulting in much different performance in terms of power production in MFCs [3]. Electrochemical techniques can help to determine the optimal potential for an anodic biofilm with these and other microbes. Furthermore, insight into the reasons for the optimal set potentials could be obtained by using CV and EIS to examine biofilms for midpoint potentials and redox peaks.

3.5. Outlook

Desirable characteristics for a microbial fuel cell, such as high current density, high power, and fast start-up, may require setting the anode potential to grow a biofilm most capable of achieving these traits. Our survey of the literature shows that no one set potential will always yield the best results, suggesting that the outcome of a set potential experiment is dependent on culture conditions, electrode materials, and inoculum. The potential to best promote bacterial activity could be set relatively high, which would encourage electron pathways that allow for fast growth and a high energy gain for the cells. Alternatively, a more negative potential may be most useful to produce a high current density, improving the desired output for a functioning BES. Whether a single set potential should be used, or the potential may need to be changed after the biofilm grows during start-up, is not known and should continue to be examined. Furthermore, the effect of setting an anode potential, compared to allowing a mixed community to evolve a potential at a fixed resistance, has not been well studied. Additional comparisons are needed to understand if an initially poised anode will result in improved current production once the anode potential is no longer set. Electrochemical tests, community analyses, and further study of the response of both pure and mixed cultures to set potentials and different resistances will improve our understanding of the behavior of microbial communities in various redox environments and different types of BESs.

3.6. References

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3.7. Tables

Table 3.1. Applied potential studies comparing set anode potentials.[a]

Set Potentials (V vs. SHE)	Inoculum	Reference Electrode[b]	Working Electrode	Results[c]	Reference
-0.2, 0, +0.2	MFC suspension	Ag/AgCl	graphite granule	Most active biomass at -0.2 V; highest current at maximum power at 0 V; maximum power at 0 V. Similar start-up at all voltages.	[28]
-0.36, -0.16, +0.04, +0.44, +0.64	*D. desulfuricans*	SCE	graphite plate; stainless steel	Current obtained only at -0.16 V	[37]
-0.26, -0.16, -0.06, +0.04, +0.14, +0.24	*G. sulfurreducens*	SCE	polished graphite blocks	At constant biomass, -0.16 V and -0.26 V had lower current; similar CVs between biofilms grown at -0.16 V and +0.24 V suggesting limited ability to adjust terminal reductase to different voltages.	[4]
-0.15, -0.09, +0.02, +0.37	Domestic WW	Ag/AgCl (0.27 V vs. SHE)	graphite rods (multiple)	At -0.15 V and -0.09 V, obtained higher current, and faster start-up, than at other voltages; lower potentials produced a thicker biofilm dominated by *G. sulfurreducens*	[23]
-0.16, 0, +0.4, floating potential	*G. sulfurreducens*	SCE (0.242 V vs. SHE)	carbon paper	Higher current, faster start-up, greater biomass for 0 V and +0.4 V; lower use of possible metabolic energy gain at +0.4 V suggesting an upper limit of the terminal reductase for G. sulfurreducens between 0 V and +0.4 V.	[17]
+0.4, floating potential	Domestic WW	Ag/AgCl (0.197 V vs. SHE)	graphite plate	Faster startup using poised vs. non-poised anode potentials.	[20]

0, +0.2, +0.35, +0.5	*S. oneidensis*	Ag/AgCl	graphite plate	Higher current at +0.5 V	[21]
+0.11, +0.21, +0.31, +0.51	*G. sulfurreducens*	Ag/AgCl (0.31 V vs. SHE)	stainless steel	Current obtained only at +0.51 V	[16]
+0.54, +0.74, +0.94	Garden compost	SCE	dimensionally stable anodes (DSA)	Higher current, faster biofilm development at +0.54 V within one chamber; higher current at +0.94 V when in separate reactors, but this result was inconsistent across replicates; current at +0.74 V > +0.34 V > +0.64 V in one experiment; +0.74 V selected as best potential	[18]
+0.3, +0.8	*G. sulfurreducens*	Ag/AgCl (0.197 V vs. SHE)	graphite plate	Higher current, and faster start-up, at +0.8 V; +0.8 produced a thicker biofilm	[15]
+0.14, +0.30, +0.82	Marine sediment	Ag/AgCl	graphite rod	Higher current, faster substrate oxidation at +0.82 V than other voltages.	[11]

[a]Many studies used a single applied potential and did not compare different potentials: [24, 35, 38-48]. Three additional studies not reviewed here suggest improved performance at more positive potentials: [49-51]. [b]The value in parentheses is the value reported in the article. No value means that the reference electrode value compared to the SHE was not reported. [c]Results in which a lower anode potential improved performance are underlined.

3.8. Figures

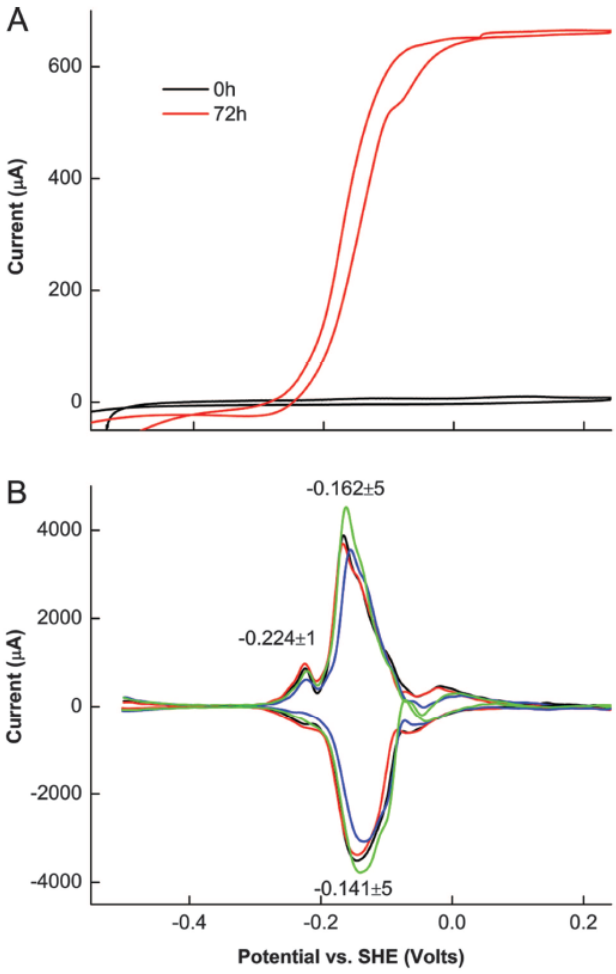

Figure 3.1. From [30]. CV (A) and first derivative of CV (B) of *G. sulfurreducens*. Source: American Society for Microbiology.

Chapter 4

Immobilization of anode-attached microbes in a microbial fuel cell

Abstract

Current-generating (exoelectrogenic) bacteria in bioelectrochemical systems (BESs) may not be culturable using standard in vitro agar-plating techniques, making isolation of new microbes a challenge. More *in vivo* like conditions are needed where bacteria can be grown and directly isolated on an electrode. While colonies can be developed from single cells on an electrode, the cells must be immobilized after being placed on the surface. Here we present a proof-of-concept immobilization approach that allows exoelectrogenic activity of cells on an electrode based on applying a layer of latex to hold bacteria on surfaces. The effectiveness of this procedure to immobilize particles was first demonstrated using fluorescent microspheres as bacterial analogs. The latex coating was then shown to not substantially affect the exoelectrogenic activity of well-developed anode biofilms in two different systems. A single layer of airbrushed coating did not reduce the voltage produced by a biofilm in a microbial fuel cell (MFC), and more easily applied dip-and-blot coating reduced voltage by only 11% in a microbial electrolysis cell (MEC). This latex immobilization procedure will enable future testing of single cells for exoelectrogenic activity on electrodes in BESs.

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4.1. Introduction

Bioelectrochemical systems (BESs) are based on electron transfer between microbes and an electrode surface. Most investigations into the mechanisms of electron transfer from a microbe to an anode have focused on two microorganisms, *Geobacter sulfurreducens* [1-6] and *Shewanella oneidensis* [7, 8], where it has been shown that specific genes and proteins are involved in exogenous electron transfer. Further study of current-generating (exoelectrogenic) bacteria and biofilms will benefit from isolating and identifying other microorganisms that are capable of electron transfer to an electrode.

Isolation techniques to identify novel exoelectrogens have typically involved dilution-to-extinction in BESs, or isolation on ferric iron agar plates. A U-tube reactor was developed [9] that would allow a single microbe, obtained by serial dilutions, to deposit by sedimentation onto a flat anode surface. This technique was used to identify novel exoelectrogens *Ochrobactrum anthropi* YZ-1 [9] and *Enterobacter cloacae* FR [10]. However, the cumbersome process required many serial transfers to obtain these isolates. A microbe related to *Clostridium butyricum* was isolated from a microbial fuel cell (MFC) using ferric iron agar plates [11], but this method of isolation does not target all exoelectrogens as some microbes have been isolated that can generate current but not reduce iron [9, 12].

In addition to spread-plating techniques, screening of arrays of microorganisms on ferric iron agar plates is possible through printer technology [13]. This approach can be used to print very small droplets of a cell suspension diluted to contain single microbes. To take advantage of this technology, for example by printing single cells in a grid pattern onto an electrode for isolation, a robust immobilization layer is required to bind the cells to the electrode so that they do not move after application to the electrode surface. This layer should not interfere with the ability of microbes to transfer electrons to an electrode surface, or with the diffusion of substrate to the cells. Latex films were evaluated here to see if they could be used to fulfill these requirements. Latex films have previously been used to entrap microbes on non-conducting surfaces, producing a high density of organisms in a thin film that survived freezing and drying [14-16]. We show here effective entrapment of bacteria-sized particles using fluorescent microspheres, and demonstrate that latex entrapped anode biofilms allow exoelectrogenic activity.

4.2. Materials and Methods

Latex was applied to two different types of anodes, carbon paper (without wet proofing; E-Tek) or graphite blocks (Grade GM-10; GraphiteStore.com Inc.), in two different types of BESs in order to evaluate the immobilization method under different conditions. Carbon paper was used as the anode in a single-chamber 28-mL microbial fuel cell (MFC) reactor with a platinum-catalyzed air cathode [17, 18] (both electrodes with projected surface area of 7 cm^2). Graphite blocks (projected surface area of 4.6 cm^2) were used as anodes for a single-chamber 5-mL microbial

electrolysis cells (MECs) with a 1.0 × 1.5 cm^2 304 stainless steel 90 × 90 mesh cathode [19]. Carbon paper (projected surface area of 3.0 cm^2) was also used as anode material in some 5-mL MECs. All reactors were inoculated using cell suspensions from pre-acclimated MFCs that were originally inoculated with domestic wastewater and acetate. A multimeter (2700, Keithley Instruments, Inc.) was used to monitor the voltage across an external resistor (R_{ex} = 10 Ω, MEC; 1000 Ω, MFC). A power source (3645A, Circuit Specialists, Inc.) was connected to the MEC circuit to add –0.7 V to the cathode. All BESs were maintained at 30°C.

MFC medium was 100 mM phosphate buffer with 17 mM acetate as the substrate (per L: 0.62 g NH_4Cl, 4.9 g $NaH_2PO_4 \cdot H_2O$, 9.15 g Na_2HPO_4, 0.26 g KCl, 1.4 g sodium acetate, and Wolfe's vitamins and minerals) [20]. MEC medium was 30-mM bicarbonate buffer with 10-mM acetate as the substrate, based on the ATCC recipe for *G. sulfurreducens*, #1957 (per L: 1.5 g NH_4Cl, 0.6 g NaH_2PO_4, 0.1 g KCl, 2.5 g $NaHCO_3$, 0.82 g sodium acetate, and Wolfe's vitamins and minerals), without the addition of the electron acceptor. MFC and MEC reactors were operated in fed-batch mode until they successively produced at least 3 equivalent batch cycles, indicating a well-established anodic biofilm.

A monodisperse latex emulsion (SF-091; Rohm & Haas) was amended with 5% glycerol to optimize the degree of coalescence and subsequent diffusivity of the film to the substrate [14, 19]. This solution was applied in two different ways to well-established biofilms in the different BESs by removing the anodes temporarily from the reactors. Glycerol-amended latex (referred to simply as "latex") was applied to the carbon paper biofilm from the MFC using an air brush (Paache, BearAir, S.

Easton, MA; 4.5 L/min of airflow). One, three, or five layers were applied, allowing 15 minutes between each layer, and one hour after the final layer, for drying at room temperature. For the graphite blocks and carbon paper anodes from the MEC a simpler application procedure was used, where the latex was applied by dipping the blocks or paper into the latex, and excess solution was drawn off the anode with a laboratory wipe. In other experiments, the glycerol-amended latex was diluted in water to 30% to see if performance improved with a thinner layer of latex.

The effectiveness of the latex to immobilize bacteria on the anode materials was examined using several different techniques. Direct observation of individual bacteria on an electrode, when bacteria were stained using acridine orange, was not possible due to high levels of background fluorescence. Therefore, application of individual microbes on an electrode was simulated by applying droplets of fluorescent microspheres (Fluoresbrite spheres, 4.1-μm diameter, Invitrogen) to graphite electrodes. Latex was applied by the dipping method described above. After drying, the latex-coated electrode was immersed in MFC medium to simulate the electrode in a BES. The droplets were observed with fluorescence microscopy before and after latex application and MFC simulation.

For SEM visualization, small sections of carbon paper anodes with exoelectrogenic biofilms with and without latex coating were mounted in cryo-matrix and frozen. Thin slices were removed from the cross-section with a cryotome until a smooth surface was obtained. The surface was etched with the cryo-SEM electron beam to remove ice crystals before viewing.

4.3. Results

4.3.1. Latex preparation

Application of glycerol-amended latex with the airbrush resulted in ~2.1 mg dry weight of latex per cm^2 anode area per layer. Application by dipping and blotting of the glycerol-amended latex onto graphite block resulted in ~5.3 mg/cm2/layer for 100% latex-glycerol, and 0.67 mg/cm^2/layer for 30% latex-glycerol. On carbon paper, ~8.1 mg/cm^2/layer was applied for 100% latex-glycerol, and 2.5 mg/cm^2/layer for 30% latex-glycerol.

4.3.2. Immobilization of microspheres and microbes

Fluorescent microspheres are often used as analogs for microorganisms [22, 23]. The location and shape of a droplet of microspheres (4.1-μm diameter) on an electrode were retained after latex application and drying, and after submersion in standard MFC media.

The latex film applied with an airbrush to an exoelectrogenic biofilm on a carbon paper anode remained completely intact, without dissolving or cracking, after 6 cycles in an MFC (Figure 1). The layers of latex coalesced into one continuous overlay. The biofilm was not visible in SEM images due to preparation requirements for the latex; however, the presence of the biofilm was confirmed by the exoelectrogenic activity through current production in the MFC. The latex layer applied to the MEC carbon paper anode using the dip-and-blot method also remained visibly intact throughout the experiment. The latex layer applied to the graphite block with the dip-and-blot method had variable performance. The layer made using the 30% dilution remained intact. However, at full strength, the latex layer did not

consistently remain adhered to the block, and in some reactors, the latex began to peel off after ten days.

4.3.3. Latex coatings on anode biofilms

When one layer of glycerol-amended latex was applied with the airbrush to a biofilm on carbon paper in an MFC, the reactor recovered immediately to its pre-latex voltage. When three layers were applied, the reactor returned to its original performance in 6 cycles. However, when five layers were applied, the MFC only reached 45% of its original voltage even after 6 cycles (Figure 2).

Using undiluted glycerol-amended latex for immobilization of microbes on a graphite block, the MEC with graphite block anode returned to 42% (±8%) of its original current within three cycles of latex application by dipping and blotting. However, after three cycles, which took approximately 10 days, the overlay had started to delaminate from the graphite block, so testing was discontinued. Using a 30% dilution of the latex-glycerol, the current recovery in the MECs improved, reaching 85% (±9%) of the original current within 3 cycles of latex application with consistent results over 3 additional cycles (Figure 3). In addition, the latex remained adhered to the anode.

When the undiluted overlay was applied to carbon paper anodes in the MECs, current returned to 43% of the original level within 3 cycles and was maintained in further batches. With the thinner, 30% diluted layer, current returned to 89% (±6%) of the original current within 3 cycles of application and remained consistent in subsequent batches (Figure 4).

4.4. Discussion

Latex films were shown to be effective in holding individual particles (fluorescent microspheres) or active biofilms on electrically conductive surfaces. Microbes trapped on two different surfaces (carbon paper and graphite block) using different application methods (airbrushing and dip-and-blot) retained most of their exoelectrogenic capability. On both surfaces, and in both MFC and MEC reactors, increasing the amount of latex applied onto the biofilm adversely affected the ability of the anode to recover exoelectrogenic activity to pre-application current levels. Lyngberg et al. [21] found that effective diffusivity through the latex was highly dependent on layer thickness. Therefore, this decrease in activity was likely due to a reduction in mass transfer to (substrate) and from (protons) the biofilm with thicker layers of latex.

The latex coating thickness, measured by dry weight, on the graphite block was less than that of the graphite paper, and the full strength latex coating did not stick well to the block. The coating on the carbon paper when applied by the air brush to the MFC anode or the dip-and-blot method (at 30% strength) to the MEC anode was similar (slightly more than 2 mg/cm^2/layer). While the MFC regained 100% of its pre-application performance, the MEC was limited to about 89% of its pre-application performance. It is unlikely that there was any decrease in the performance of the MEC in these experiments due to exposure of the biofilm to oxygen during the latex application, as MEC biofilms are routinely exposed to air when it they are refilled (often intentionally to reduce methanogenesis) without adverse affects to current production [24]. In addition, the biofilm in an MFC is routinely exposed to

oxygen in air due to oxygen diffusion through the cathode and into the anode chamber without apparent adverse effects. If desired, the latex film could be applied under strictly anoxic conditions in an anaerobic glove box. Previous work with bio-catalytic films used for hydrogen gas production has shown that the coating itself is not adversely affected by the presence or absence of air, nor is the performance of that biofilm [14]. However, it is possible that some strict anaerobes might be affected by oxygen during this procedure, so anaerobic application of the latex biofilm may be of interest in future studies.

The ability to immobilize microbes on an electrode using a latex film has two valuable applications for BESs, but for successful application in BESs, immobilization of microbes on electrodes must not interfere with the ability of cells to transfer electrons. Bioelectrochemical features seen in cyclic voltammograms of pectin-entrapped *Geobacter* biofilms have been shown to be similar to naturally-grown *Geobacter* biofilms [6]. This suggests that entrapment by itself is not changing the electrical capability of the cells, although they found current was somewhat decreased as observed here as well. One application of an immobilization layer for cells on a BES electrode is isolation of microbes directly on an electrode. This requires immobilization of an array of single cells, without greatly compromising current generation, which our latex overlay achieves. In addition, a biofilm of specific microbes can be developed on an electrode in a controlled setting, immobilized and protected under a latex coating, and then introduced to a more complex, non-sterile environment. Under the coating, these organisms would not have to compete with other microbes for the electron-accepting surface. Exoelectrogenic biofilm activity

under a glycerol-amended latex film can be restored to nearly the same levels as pre-application activity, making it a suitable immobilization layer for these applications.

4.5. References

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4.6. Figures

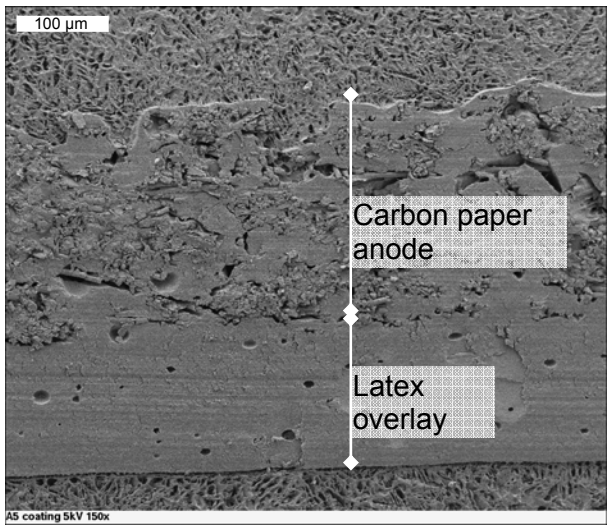

Figure 4.1. SEM image of 3 layers of latex ("latex overlay"; approximately 165 µm thick) on a carbon paper anode with exoelectrogenic biofilm after 6 cycles in an MFC. The biofilm is not visible due to SEM preparation techniques necessary to maintain the latex layer.

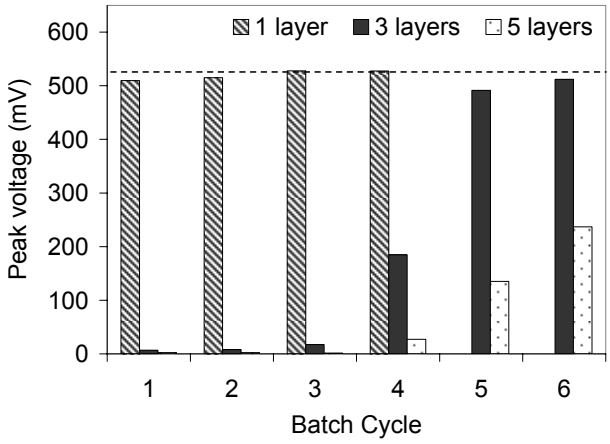

Figure 4.2. An exoelectrogenic biofilm on a carbon paper anode in an MFC with 1, 3, or 5 layers of latex applied to a carbon paper anode using an airbrush, compared to a reactor with no latex (dashed line). Representative reactors are shown.

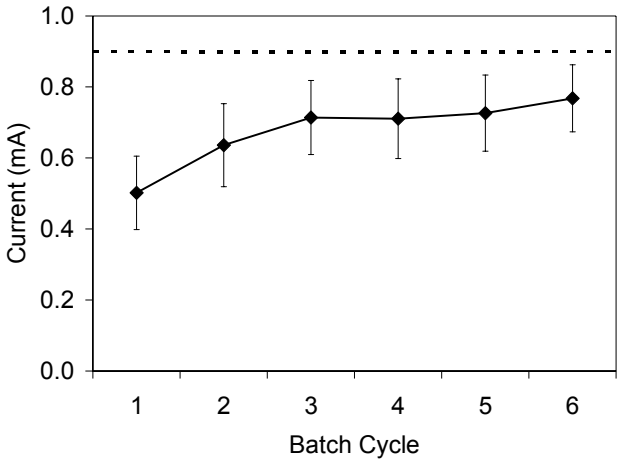

Figure 4.3. An exoelectrogenic biofilm on a graphite block anode in an MEC immobilized with glycerol-amended latex diluted to 30% strength, compared to the biofilm with no overlay (dashed line).

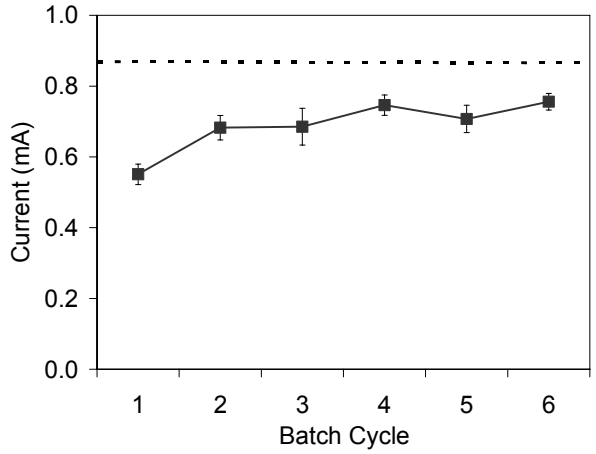

Figure 4.4. An exoelectrogenic biofilm on a carbon paper anode in an MEC immobilized with glycerol-amended latex diluted to 30% strength, compared to the biofilm with no overlay (dashed line).

Chapter 5

Characterization of anodic and cathodic communities in a pilot-scale bio-electrochemical system

Abstract

Microbial electrolysis cells (MECs) can be used to produce hydrogen or methane gas, but there is little information on the archaeal communities in these systems. Methane production in these systems decreases hydrogen production, but the methane produced may be higher than that possible by anaerobic digestion alone, and methane itself is a valuable fuel with a readily available infrastructure. Communities from biofilms on the anode and cathode and from the solution of a 1000-L pilot-scale MEC reactor were analyzed for archaea and bacteria. Hydrogen gas was initially produced by the reactor, but it was completely replaced by methane production within ~6 weeks that continued through the life of the experiments (~14 weeks). Archaea identified by rRNA analysis were mainly unidentified clones. Among the organisms with sequences similar to known methanogens, those at the cathode were more likely than the methanogens at the anode to consume hydrogen and carbon dioxide. Visual fluorescent *in situ* hybridization image analysis of anodic brush fibers confirmed the presence of archaea, but the great majority of microbes were bacteria (~90%). Based on 16S rRNA gene clone library analysis, *Pelobacter*, *Geobacter*, and *Clostridium* were present in large numbers in both anodic and cathodic communities, while they

are not dominant in the solution, suggesting that they are involved in substrate transformation processes that resulted in electron transfer to and/or from electrodes. Microbes most similar to *Clostridium* were a higher percentage of microbes in bacterial communities at the cathode than the anode or solution, indicating their importance in the cathodic community metabolism. High-throughput pyrosequencing analysis showed *Geobacter* dominating both anodic and cathodic communities, with *Clostridium* again having a more significant presence on the cathode.

5.1. Introduction

Microbial electron transfer to and from electrodes is the key process in bioelectrochemical systems (BES). A mixed community of anodic microbes allows a complex substrate to be consumed, producing intermediary products for further consumption and allowing electron transfer both within the community and from microbes to the electrode. Electrons captured by the solid electrode can be harnessed as electrical current, such as in a microbial fuel cell (MFC), or can be used to produce a desired product at the cathode, such as hydrogen gas [1, 2] acetate [3], hydrogen peroxide [4], sodium hydroxide [5], and methane [6]. In addition to donating electrons to an anode, *Geobacter* and other microbes are also able to use the solid cathode, at an acceptable potential, as the sole reductant [7, 8]. These cathodic reactions can therefore be engineered to occur either electrochemically or via microbial electrosynthesis.

Anodic communities consume organic material and ultimately transfer electrons to an anode. These well-studied communities [9], largely regardless of

substrate type or complexity, are typically dominated by *Geobacter* spp. [10-13], in particular *G. sulfurreducens*. *Pelobacter propionicus*, a close relative of the *Geobacter* (which arguably should be a member of the *Geobacteracea* family rather than *Desulfuromonaceae* [14, 15]), has also been found to be predominant in several mixed exoelectrogenic communities [16]. However, neither the type strain of *P. propionicus* nor an isolate with a high similarity to this microbe have produced current. Other dominant organisms in anodic biofilms include *Clostridium* spp. (especially in systems with cellulosic substrates) [17], *Thauera* spp., *Azoarcus* spp., *Desulfuromonas* spp., and *Bacillus* spp. [9]. Syntrophic interactions and diverse communities appear to be critical for substantial transfer of the energy in complex organic substrates into current [18, 19].

The communities that develop in cathodic biofilms can be more varied than their anodic counterparts, depending on the type of reaction occurring at the cathode [8, 20-24], and have not been as well studied. Methanogenic archaea are particularly in need of study in MEC communities, since these organisms are solely responsible for methanogenesis. Cathodic communities may consume acetate from solution and hydrogen either from the cathode or from the solution. Electrotrophic organisms use electrons directly from the cathode and compounds such as carbon dioxide as a carbon source. In MECs, microbes can reduce protons using cathodic electrons, producing hydrogen [25-27] or methane [6, 28]. A hydrogen-producing biocathode was dominated by bacteria most similar to *Desulfovibrio vulgaris* [27], and a methane-producing biocathode was dominated by an organism most similar to the archaeon *Methanobacterium palustre* [6]. These single studies, however, do not give

a comprehensive picture of the communities expected in these types of BES. Biocathode communities may need a variety of microorganisms present to complete the metabolic pathways for certain desired compounds. While the key organism for functionality of a biocathode is one capable of using an electrode as a source of electrons. However, it may be necessary or beneficial for that microbe to transfer electrons, either directly or indirectly, to other organisms in the community that are capable of synthesizing the product desired as the output from the BES.

Methane is produced biologically exclusively by members of the domain Archaea, and methanogens can be found in numerous diverse environments, both extreme and temperate. The biosynthetic pathways for methanogenesis are complex. In most anaerobic digesters, methanogens are a small percentage of the microbes compared to bacteria [29-31]. In contrast, methanogens were found in substantial numbers relative to bacteria in one methane-producing biocathode in a BES [6]. Methanogens have long been known function syntrophically with bacteria [32-34], forming aggregates to facilitate interspecies hydrogen transfer [35]. The ability of the methanogens to keep hydrogen concentrations very low allows the bacteria to gain energy from reactions that would not be energetically favorable under standard conditions having higher hydrogen concentrations. The bacterial degradation of organics to hydrogen is not thermodynamically favorable unless the hydrogen gas produced by the bacteria is rapidly consumed [36]. Hydrogenotrophic methanogens will readily consume the hydrogen, producing a low partial pressure of hydrogen allowing the bacteria to gain energy from hydrogen production. The consumption of hydrogen by methanogenesis utilizes carbon dioxide reduction, and is expressed by

the equation $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (Equation 1). This reaction is exergonic ($\Delta G^{0'} = -131$ kJ/mol-CH_4) and therefore is energetically beneficial for methanogens, and requires no applied potential (theoretically) for the electrochemical reaction in a BES. The hydrogen in this reaction can be in the form of hydrogen gas (H_2) or disassociated into protons and electrons, which would be the case in a methanogenic BES that is directly consuming electrons from the cathode. Acetoclastic methanogenesis, $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$ is also exergonic ($\Delta G^{0'} = -31$ kG/mol-CH_4). Although there are few known acetotrophic methanogens, this pathway can dominate certain environments in which these microbes are highly active [37].

Biocathodic methanogenesis has been examined under several different conditions, including set-potential electrodes and elemental iron. Cheng et al. [6] developed a methanogenic biocathode that produced methane at ~ –0.9 V (vs. SHE; and lower) applied potential, with evidence strongly suggesting that the methane was produced by direct electron transfer from the cathode to the microorganism. Daniels et al. [38] demonstrated that elemental iron could be the sole electron source for microbial methanogenesis. Iron reduction was separated physically from microbial growth in several tests, implying that several different archaeal methanogens used hydrogen gas produced via cathodic depolarization of the elemental iron rather than direct electron transfer from the iron to the microbes. Iron oxidation with hydrogen gas generation, $4Fe^0 + 8H^+ \rightarrow 4Fe_2^+ + 4H_2$, (Equation 2) is energetically unfavorable ($\Delta G^{0'} = +3.2$ kJ/reaction) at standard conditions and pH = 7. However, if the hydrogen gas concentration is kept low, as is the case with hydrogenotrophic methanogenesis, then the reaction can become thermodynamically favorable. For

example, at 1 mM Fe^{+2} and 10 Pa H_2, the reaction is exergonic [38]. In the iron-reduction study, unlike the MEC, hydrogen gas is most likely the electron carrier between the elemental iron and the microbes. In contrast to the earlier iron-reduction study by Daniels et al. [39], a methanogen isolated from marine sediments produced methane while growing on elemental iron at a faster rate than a pure culture of a known hydrogenotrophic methanogen, suggesting that the electrons from the iron are accessible to the isolate more readily than by way of a hydrogen intermediary. This new isolate also produced methane faster than a *Methanococcus thermolithotrophicus* type strain used in the first study (e.g. ~1 mM in 100 hours for the isolate vs. ~20 uM for *M. thermolithotrophicus*). The mechanism for the electron transfer in these instances was not fully elucidated.

A pilot-scale MEC was built to treat wastewater from wine production while producing hydrogen gas. The single chamber reactor, with applied voltage (E_{ap}) of 0.9 V, operated at approximately 30°C. For the two weeks prior to biological sampling, the reactor was producing a current of ~5.5-6.0 A/m^3, with gas containing ~85% CH_4 (~0.2 L/L-d) [40]. After initial inoculation with winery wastewater and wastewater from a local domestic wastewater treatment plant, the reactor was enriched (on days 52-65) from a separate tank used to promote the growth of exoelectrogens such as *Geobacter* by supplementing wastewater with acetic acid and fumarate [40]. After a 3 week start-up phase, the reactor produced H_2 in the biogas. By week 6 and through the remaining experimental phase (week 14), only CH_4 was produced. These results that showed the evolution of the gas composition from

hydrogen to methane suggests that we need a better understanding of type and growth of methanogens, and their presence and location throughout the MEC.

The objectives of this study were to identify and compare the organisms present in the communities found on the anode and cathode of this pilot-scale, methane-producing MEC, and to analyze the proportion of archaea and bacteria in these communities. The communities found on the anode, cathode, and in solution of the reactor were analyzed by 16S rRNA clone library and pyrosequencing analyses. In addition, the anodic brush fibers were stained with fluorescent *in situ* hybridization to ascertain the relative density of each domain in the community.

5.2. Materials and Methods

5.2.1. DNA extraction

Biofilm was scraped from a graphite fiber brush anode (Figure 1) and stainless steel cathode of a 1000-L pilot MEC that had been operating for approximately 94 days. Genomic DNA was extracted from the anode and cathode biofilms and from the planktonic microbes in the reactor solution as described previously [41] using a Power-Soil DNA isolation kit (Mo Bio Laboratories).

5.2.2. Clone Library Community Analysis

Archaeal primers Arch-21F (5'–TTC CGG TTG ATC CYG CCG GA–3') and Arch-958R (5'–YCCGGCGTTGAMTCCAATT–3') [42] were used to amplify the 16S rRNA gene fragments of the extracted genomic DNA, and to construct a clone library for archaeal 16S rRNA gene fragments. Bacterial 16S rRNA gene fragments

were amplified by PCR using universal bacterial primers 530F (5'–GTCCCAGCMGCCGCGG–3') and 1490R (5'–GGTTACCTTGTTACGACTT–3') [43]. Both archaeal and bacterial PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and then ligated and cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Colonies were plated in a 96-well grid on LB-ampicillin plates. Plasmid extractions were carried out according to manufacturer's instructions on these colonies using the E-Z 96 Fastfilter Plasmid Kit (Omega Biotek). Plasmids were sequenced with the T7 primer using an ABI 3730XL DNA sequencer (Applied Biosystems; Penn State Genomics Core Facility – University Park, PA). BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the sequences from the clone library using the "Nucleotide collection (nr/nt)" database. In both cases the blastn algorithm was used with a word size of 11. For archaeal and bacterial clones, a percent similarity of 97% or greater was used to identify sequence matches. If an isolate or known organism was found within the range of 97-100%, this match was chosen rather than an uncultured clone. Approximately 48 sequences were examined for each community and primer set.

5.2.3. High Throughput Sequencing Community Analysis

Bacterial phylogenetic libraries were produced from the anode and cathode samples by pyrosequencing utilizing the GS FLX sequencer and Titanium series chemistry (454 Life Sciences, 146 Branford, CT). The primers used were universal bacterial PCR primers 343F (5'-TACGGRAGGCAGCAG-3') and 926R (5'-CCGTCAATTYYTTTRAGTTT-3') [44, 45] including pyrosequencing adaptors,

keys, and multiplex identifiers. PCR was conducted in 50 μL volumes containing 1 X PCR master mix (Roche Applied Science, Indianapolis, IN), 0.4 μM of each primer, and 2 μL of DNA template (5 ng/μL). PCR was performed at the following cycling conditions: initial denaturation at 94^{o}C for 5 min, and 25 cycles of 94^{o}C denaturation for 15 s, annealing at 56^{o}C for 45 s, 152 and a 1 min extension at 72^{o}C, followed by a final extension at 72^{o}C for 8 min. Four reactions were conducted for each sample and amplicons were combined before removing salts and unincorporated primers using a Qiagen MinElute PCR purification kit (Qiagen Inc., Valencia, CA, USA) [46]. Amplicons were visualized on a 1.2% agarose gel and smearing was observed then the amplicons were extracted with Qiagen's MinElute gel extraction kit (Qiagen Inc.). Each purified DNA amplicon was pooled at equal concentrations for sequencing. DNA concentration and purity were confirmed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Sequencing was performed using an emPCR Lib-A Kit (Roche Applied Science, Indianapolis, IN) at the Yale Center for Genome Analysis. Reads were subjected to quality control at the machine based on run-produced Phred scores. Keypass, dots, and mixed filters assessed the quality of the whole read while the quality of read ends was checked by signal intensity and primer filters.

Quantitative sequence analysis was performed using programs in the Quantitative Insights Into Microbial Ecology (QIIME) tool box [47]. Sequences were sorted, and primer and multiplex identifier sequences were trimmed. Sequences were denoised with Titanium Pyronoise software [47-49]. Sequences were clustered into

operational taxonomic units based on 97% identity and aligned with the Greengenes core set [50]. The RDP classifier [51] was used to assign taxonomy.

5.2.4. Fluorescent In Situ Hybridization (FISH) of Bacteria and Archaea

Samples for FISH analysis were obtained by adding fibers from the anode to phosphate buffered saline (PBS) solution. Cells were fixed in 4% paraformaldehyde and stored at 4^{o}C. Samples were then rinsed in PBS and stored in 50% ethanol/PBS at –20^{o}C. FISH analysis was carried out on the fibers as described previously [52]. Domain-specific probes were used to target bacteria (EUB338 [53]) and archaea (ARCH915 [54]). Fibers were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Samples were examined on an Olympus BX61 epifluorescent microscope equipped with DAPI, FITC, and Texas Red filter sets as described previously. Images were recorded using a DP72 digital camera and analyzed using the DP2-BSW software. The proportion of archaeal cells to bacterial cells was approximated by visually counting the cells on the FISH-stained images.

5.3. Results

5.3.1. Archaea in the MEC

In clone libraries from the cathode, anode, and solution, the majority of the archaeal sequences do not match any known organisms (Figure 2). Organisms with sequences similar to the archaeal clones (≥97%) from the anode include *Methanosaeta spp.* (9%), *Methanosarcina spp.* (9%), and *Methanobacterium palustre* (2%). Organisms with sequences similar to the archaeal clones from the cathode

include *Methanosarcina spp.* (4%), *Methanosaeta concilii* (2%), and *Methanobacterium curvum* (4%). In solution, the organisms matched by sequences were *Methanosaeta thermophila* (2%) and *Methanothermobacter thermautotrophicus* (2%).

5.3.2. Bacteria in the MEC

Bacteria with similarity (≥87%) to *Pelobacter propionicus* were the most dominant organisms represented by sequences from the clone libraries of the anodic (40%) and cathodic (20%) communities (Figure 3). *Geobacter* spp., *Tolumonas auensis*, and *Clostridium* spp. were all represented by about 7% of matches in the anodic community. *Clostridium* spp. (11%) and *Tolumonas auensis* (10%) were prevalent on the cathode, as well as *Geobacter* spp. (7%) and *Veillonella parvula* (6%, a member of the Clostridiaceae family).

The clones from the solution phase community within the reactor were dominated by bacteria with the most similarity to *Tolumonas auensis* (24%). *Veillonella parvula* comprised 11% and *Pelobacter propionicus* and *Enterobacter cloacae* each comprised 7% of the solution clones. *Escherichia coli* and *Klebsiella* spp. were also present in the solution.

5.3.3. High Throughput Sequencing Community Analysis for Bacteria

Geobacter was identified as the predominant genus in both anode (30%) and cathode (35%) samples, based on pyrosequencing through the RDP classifier (Figure 4). Approximately 15% of each sample was comprised of *Paulidibacter*.

Clostridiaceae were also well represented in this analysis, consistent with the clone library analysis, consisting of about 10% of the anode and cathode samples sequences. *Pelobacter* was not identified as a significant representative in either community, comprising less than 5% of total sequences in each sample.

5.3.4. Fluorescent In Situ Hybridization (FISH) of Bacteria and Archaea

Based on FISH analysis, archaea were present in much smaller numbers than bacteria. Approximately 8% of the cells in images of anodic fibers were archaea, and the remaining 92% were bacterial cells (Figure 5). Little to no auto-fluorescence was observed by the cells. Since the combination of the two FISH probes target all the microbes also seen by the DAPI stain (not pictured), it is unlikely that either probe missed any targets.

5.4. Discussion

Most sequences in the archaeal clone libraries from the anode, cathode, and solution matched most closely to uncultured clones. All the sequences with similarity to known organisms identified as methanogens in the archaeal clone libraries. Analyses of the biofilm community using FISH suggest that the number of archaea at the anode was approximately 8% compared to bacteria. This was lower than the percentage of archaea found in the cathodic biofilm in another methanogenic MEC [6], but higher than studies of anaerobic digestion [29, 30]. The considerable proportion of methane in the biogas [40] was attributable to the methanogens.

Of the methanogens identified using the clone analysis with a similarity of ≥97%, we know that *Methanosaeta* are obligate acetoclasts, *Methanobacterium* and *Methanobrevibacter* require hydrogen and carbon dioxide, and *Methanosarcina* are capable of consuming either hydrogen or acetate. At the cathode, obligate acetoclasts (*Methanosaeta*) were approximately 2% of the community, while methanogens capable of using hydrogen (or either hydrogen or acetate) comprised 8% of the community. Obligate acetoclasts were more prevalent at the anode than the cathode, comprising 9%; other methanogens also comprised 11% at the anode. Since the hydrogenotrophic pathway (Equation 1) consumes hydrogen and electrons as well as CO_2, this suggests that the most likely mechanism for direct electron transfer from the electrode to methanogens was through hydrogenotrophic pathways. Hydrogenotrophs at the cathode may have rapidly and completely consumed hydrogen gas produced electrochemically by the electrode as well (Figure 6). Given the large proportion of methane in the gas collected from the reactor, and the corresponding decline in CO_2 as CH_4 increased, hydrogenotrophic methanogens in the cathodic biofilm appear to be the primary source of the methane. This methanogenesis may be direct electrosynthesis, using cathodic electrons directly, or may be indirect, with cathodic hydrogen as an intermediary that is completely consumed by the methanogens.

The archaea present on the anode and in solution may have several possible metabolic roles the reactor. They may be acetoclastic methanogens, consuming acetate and therefore reducing substrate available for current generation by exoelectrogens. These archaea may also be methane-oxidizing exoelectrogens, consuming methane and producing current [55] in a similar manner to hydrogen

cycling seen in some MECs [41]. Methanotrophs in the order Methanosarcinales include organisms related to those found in the MEC, including *Methanosarcina barkeri* and *Methanosaeta thermophila*. The majority of the archaea in the reactor were not identifiable, and their role in the MEC is yet unknown.

Geobacter was present in both community analysis results – the clone library and the pyrosequencing – on both the cathode and the anode. *Geobacter sulfurreducens* are able to both donate and accept electrons from solid electrodes in BES (Figure 6). In the clone library, *Pelobacter propionicus* was dominant, consisting of 20% of the cathode and 40% of the anode clones. *P. propionicus* is a fermentative microbe with the ability to reduce ferric iron or elemental sulfur. It can also oxidize ethanol, an operation that may have been useful for the anodic biofilm, breaking down the wastes from the winery and generating acetic acid for exoelectrogens to use as substrate. Although *P. propionicus* is not known to have exoelectrogenic activity, its dominance in this BES and in other anodic biofilms has several possible explanations, in addition to its role upstream in the metabolic pathway of the reactor. It is very closely related to *Geobacter* and may have obtained the necessary suite of genes for exoelectrogenic activity through horizontal gene transfer from neighboring *Geobacter* in the biofilm. *P. propionicus* may also directly transfer electrons to *Geobacter* [56]. It is also possible that although laboratory conditions have not induced current production from *Pelobacter* isolates, it is in fact capable of producing current under environmental conditions (perhaps in syntrophy with other microbes in the community) found in the pilot reactor. Finally, given their close taxonomic relationship, clone library methods of community analysis may be

misidentifying *Pelobacter*, and it should in fact be *Geobacter*, a known exoelectrogen.

Tolumonas auensis, another dominant organism in the reactor according to the clone library, is, like *P. propionicus*, capable of producing potential substrates for exoelectrogens such as acetate and formate from sugars [57]. This organism is dominant in the solution community, indicating that it is less likely to be exoelectrogenic, but rather breaking down organic matter that is then available for exoelectrogens.

Clostridiaceae, which were present in both the clone library and the pyrosequencing results, are ubiquitous and diverse anaerobes that are capable of producing, among other compounds, molecular hydrogen and organics, which would be useful to anodic exoelectrogens. Clostridia have been found in other biocathodes that produced hydrogen [27] or methane [6]. An isolate closely related to *C. butyricum* has been shown to have exoelectrogenic activity [58], indicating that these organisms may be contributing to the current. Their presence in biocathodes, including the biocathode in this study, also suggests that they may be capable of using an electrode as a source of electrons (Figure 6).

5.5. Outlook

Archaeal activity at the anode may have either a positive or a detrimental effect on current generation, so understanding the activities of these organisms will help facilitate control for improved current production. Understanding the microbes involved with biocathodic methane production and the pathways through which

cathodic electrons reduce CO_2 to CH_4 will allow enhancement of anaerobic digestion. Improvement of this technology will increase the likelihood that it will be used to decrease COD in wastes while sustainably producing a valuable energy source in the form of methane gas.

5.6. References

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5.7. Figures

Figure 5.1. Biofilm on the graphite brush anodes.

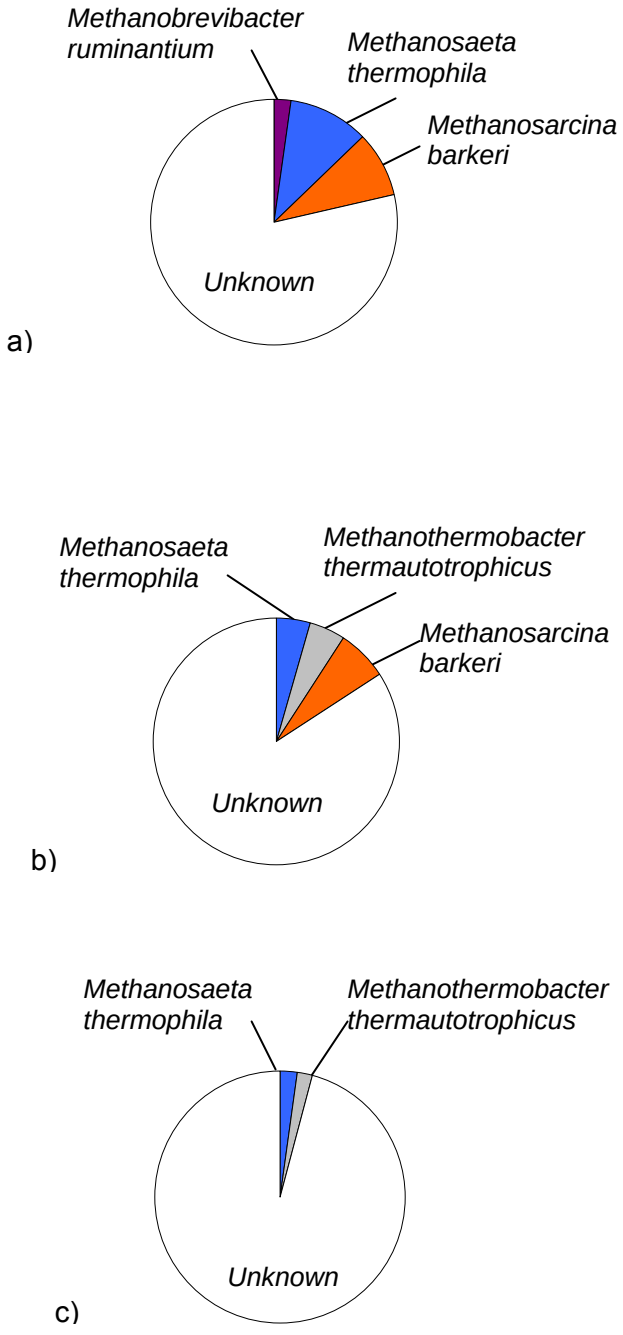
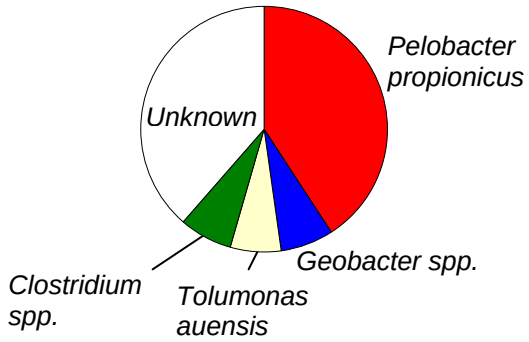
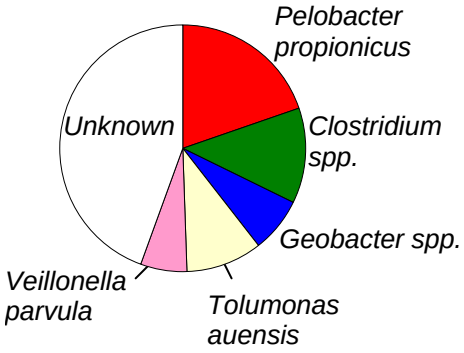


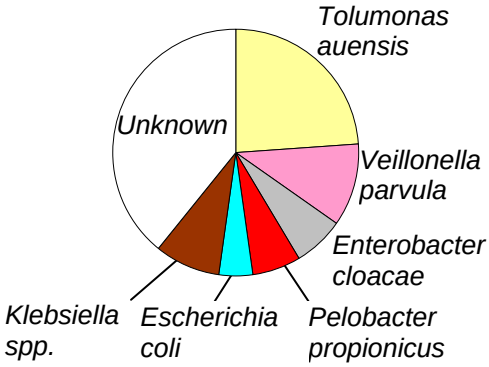
Figure 5.2. Clone library analysis of 16s rRNA sequences generated with archaeal primers from a) graphite brush anode, b) stainless steel cathode, and c) solution from the pilot-scale BES fed wastewater from a winery.



a)



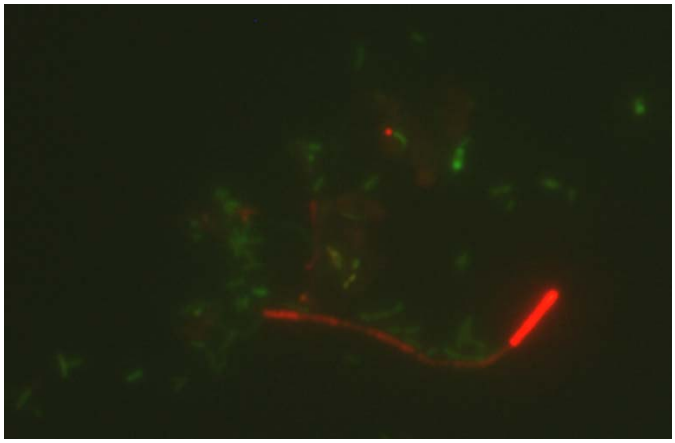
b)



c)

Figure 5.3. Clone library analysis of 16s rRNA sequences generated with bacterial primers from a) graphite brush anode, b) stainless steel cathode, and c) solution from the pilot-scale BES fed wastewater from a winery.

a)



b)

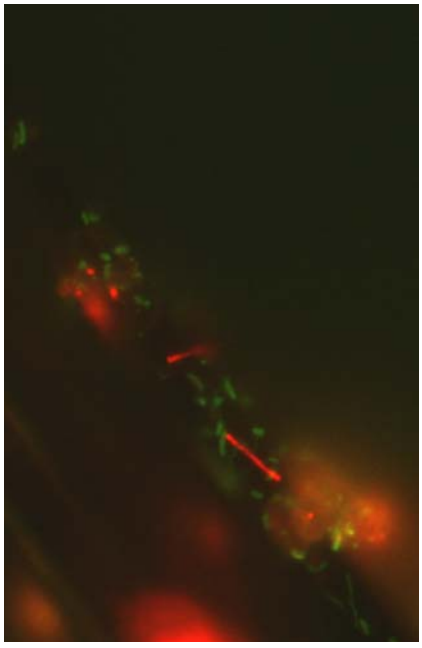


Figure 5.4 (a & b). Representative fluorescent *in situ* hybridization (FISH) of anodic fibers. Green cells are targeted by the bacterial probe EUB338 and red cells by the archaeal probe ARCH915.

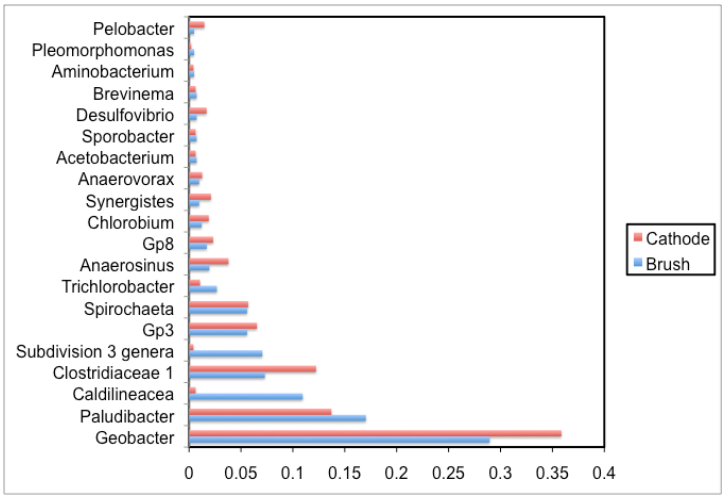


Figure 5.5. Breakdown by genus of pilot-scale BES cathode and brush samples, determined by 454 pyrosequencing.

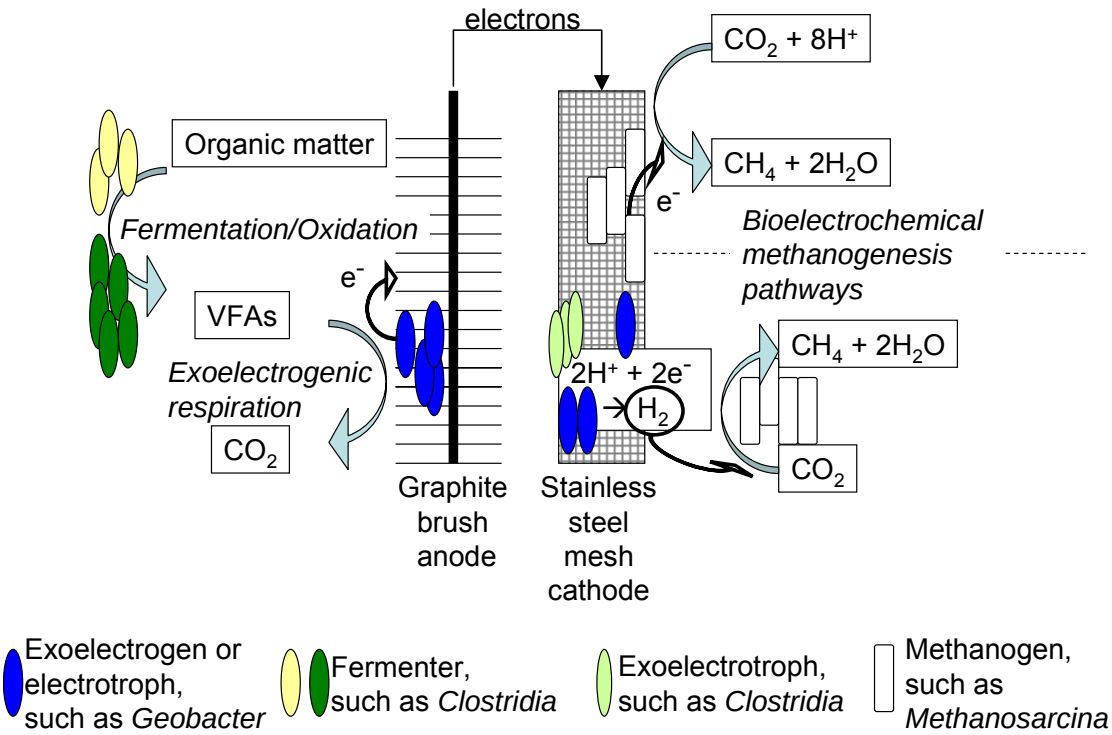


Figure 5.6. Possible reaction pathways at the anode and cathode in a methanogenic bioelectrochemical system.

RACHEL CAIN WAGNER

2072 Mary Ellen Lane, State College, PA 16803, 814-482-0623,
rcw186@psu.edu, rcw186@gmail.com

EDUCATION

Ph.D. Environmental Engineering, May 2012
Penn State University, University Park, PA
Advisor: Bruce E. Logan, Kappe Professor of Environmental Engineering
M.S. Biological Systems Engineering, May 2004
Virginia Tech, Blacksburg, VA
Advisor: Theo A. Dillaha, Professor of Biological Systems Engineering
B.S. Biology, May 2000
Virginia Tech, Blacksburg, VA

FELLOWSHIPS, HONORS, AND AWARDS

National Science Foundation (NSF) Graduate Research Fellowship, 2007-2010
Poster Award Winner - Association of Environmental Engineering and Science Professors Tampa Conference, 2011
Cecil M. Pepperman Memorial Graduate Fellowship, Penn State University, Spring 2011
PEO Scholar Award, Philanthropic Education Organization International, 2010-2011
University Graduate Fellowship, Penn State University, 2006-2007
General Electric's First-Year Faculty for the Future, Penn State University, 2006-2008
Air and Waste Management Association Scholarship, Spring 2007
Community Baboon Sanctuary Lifetime Membership, Spring 2006
William Steele Memorial Scholarship, Virginia Tech, Fall 2003
Waste Policy Institute Summer Fellowship, Summer 2003
Gamma Sigma Delta (Agriculture Honor Society), inducted 2003
Alpha Epsilon (Agricultural and Biological Engineering Honor Society), inducted 2003
Phi Beta Kappa National Honor Society, inducted Spring 2000
Virginia Tech Honors Program, 1999-2000

RESEARCH AND RELEVANT EXPERIENCE

Graduate Research Fellow & Assistant, Environmental Engineering, Penn State, 2006-present
Peace Corps Volunteer, Community Baboon Sanctuary, Belize, 2004-2006
Graduate Research Assistant, Biological Systems Engineering, Virginia Tech, 2001-2004
Associate Biologist, CropTech Corporation, 2000-2001