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CONVERGENT DEVELOPMENT OF MICROBIAL COMMUNITIES IN MICROBIAL FUEL CELLS

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ABSTRACT

Microbial fuel cells (MFCs) are often inoculated with rich sources of microorganisms from a single wastewater source, but it is not known to what extent this inoculum choice affects community development or power production. The temporal development of microbial communities in MFCs was examined using inocula from three different sources: a local wastewater treatment plant known to produce consistent power densities; a second nearby wastewater treatment plant; and an anaerobic bog sediment. The bog inoculum MFCs initially produced much higher power densities than the wastewater samples, but after 60 d all MFCs on average converged to similar voltages (0.47 ± 0.02 V, 1000 Ω external resistor) and maximum power densities (590 ± 170 mW/m^2). While voltages produced by MFCs inoculated with bog sediment were not significantly different among triplicate reactors, one of the MFCs inoculated with wastewater produced substantially less voltage. Denaturing gel gradient electrophoresis (DGGE) profiling showed the development of a stable exoelectrogenic biofilm in all samples after 30 d, consistent with an analysis using 16S rRNA clone libraries. After 60 days, 58 ± 10% of clones had a high similarity to *Geobacter sulfurreducens*. Analysis using a FISH probe for *G. sulfurreducens* confirmed the predominance of this microbe in the anode communities (63 ± 6% of all microbes). One of the wastewater inoculated MFCs that produced less voltage than other MFCs (UAJA3) had a significantly lower percentage of 16S clones similar to *G. sulfurreducens* (36%) with an increased number of Bacteroidetes (36%). This suggested that a high predominance of *G. sulfurreducens* was needed for convergent power densities and that an overabundance of Bacteroidetes could be harmful, although a lower percentage of this microbe in the UAJA3 MFC could not be verified by FISH analysis. These results suggests that MFC replicates need not be sampled and analyzed for their individual microbial communities when exhibiting similar performance, but sub-optimal performance can arise from a lack of

predominance of specific microorganisms in the anode communities. The extent to which this finding can be generalized for MFCs needs to be further examined for other substrates, inocula, and reactor types.

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Chapter 1

Introduction

1.1 The need for sustainable energy

Over the last 2 decades, there has been increasing concern over the degradation of the environment due to the burning of fossil fuels. Experts have also expressed concern that the supplies of fossil fuels are steadily running out. The US DOE reports that the amount of energy consumption worldwide will more than double by 2035 (739 quadrillion Btu) as compared to 1990 (355 quadrillion Btu) (1). Despite only having ~5% of the world's population, the United States accounts for 21% of the total energy consumption worldwide (1). More than 85% of this energy consumed in the United States is derived from fossil fuels (2). This trend of increasing consumption of fossil fuels cannot continue as it is a non-renewable resource. In addition, the burning of fossil fuels releases carbon dioxide, which is a known greenhouse gas and is responsible for global temperature changes (3). For this reason sustainable energy has become an increasingly important field of study in recent years.

There are many different areas of research to meet the diminishing fuel supply and increasing of CO_2 concentration in the atmosphere. In 2009, 8% of the energy consumed in the United States came from sustainable sources. Sustainable energy technologies include energy sources such as wind, solar, nuclear, hydroenergy, and biofuels. Energy sources such as wind, solar, and hydroenergy are only suitable in certain areas due to the need for specific climates or conditions, which limits their viability as a long-term, widespread solution. Biofuels is another area of research in development that attempts to break down cellulosic materials to it base sugars

that can then be fermented to ethanol which can be used for fuel. There are, however challenges associated with trying to develop biomass conversion to a large-scale sustainable technology. Much of the challenge lies in the cost of producing the fuel (4). A major obstruction to using cellulosic material to convert to renewable fuels is that this material is difficult to break down so a large amount of energy needs to be input into the system to recover the cellulose. Nuclear energy has been previously shown to work, but is not a widespread source of energy due to the danger arising from the use of radioactive materials to generate energy. Another difficulty with nuclear energy is the amount of available uranium would only be able to generate power for approximately one decade (5). This does not present a long-term, sustainable option. An additional obstacle to these technologies is that the energy content of gasoline is higher than many of these other fuels (6). This would necessitate an increase in production to meet consumer demands and could increase costs more. Without government tax incentives there would be little motivation for investors to spend capital on these technologies when fossil fuels are still the cheapest alternative.

1.2 Waste and wastewater as an energy source

Another resource that has yet to be utilized is wastewater streams from both industrial and municipal sources. Over 126 billion liters of wastewater are treated per day with an annual cost of greater than 25 billion dollars (7). These waste streams can have an influent chemical oxygen demand ranging from 200-1500 mg/L (8). The COD is derived from organics present in the wastewater, which need to be removed to meet certain regulatory standards before the water can be discharged into a natural body of water. Bacteria are used in various wastewater treatment processes to consume organics present to treat the wastewater and simultaneously use them for energy to grow and multiply. The bacteria that are enriched for in these processes are aerobic

heterotrophs because of their consumption of organics as an energy source along with their characteristic high growth and substrate utilization rates as compared to other types of bacteria (9). As a result, oxygen must be pumped into the system through aerators, which accounts for 45 to 60 % of the total energy to treat the wastewater (10). Many countries do not have the capital to take advantage of these large-scale, aerobic methods of water treatment. Therefore, anaerobic methods of treating water would provide a more easily accessible method of wastewater treatment.

1.3 Microbial fuel cells for bio-electricity production

One emerging anaerobic treatment technology is microbial fuel cells (MFCs). MFCs use exoelectrogenic bacteria that grow under anaerobic conditions and have the ability to transfer electrons externally to the anode of the reactor. The electrons then pass through an external circuit to the cathode of the system. At the cathode, the electrons combine with protons and an electron acceptor. In single chamber reactors, the electron acceptor used is oxygen. The substrate used for this system can be the wastewater stream of a wastewater treatment plant. This would allow the simultaneous treatment of the wastewater and production of electricity which can be collected and used to power other operations in the treatment plant.

1.4 Objectives

Determining the types of bacteria that are involved in the production of electricity in MFCs is important to designing systems with optimal operating conditions. Wastewater plants in different areas experience different loads and service areas with different types of waste. It is important to study the convergence of the anodic bacterial communities that arise from different

inoculum sources to ensure that these exoelectrogenic bacteria can be enriched from different influent wastewater streams and are not found in specific areas, which would limit their applicability. Furthermore, it is necessary to explore the convergence of these bacterial communities to determine the number of replicate reactors that should be used in order to accurately characterize the community for a given system and inoculum source without spending excess time and money analyzing replicates that do not yield any additional information.

Chapter 2

Literature Review

2.1 Voltage and power production of single-chamber MFCs

The first MFCs that were developed consisted of two-chamber reactors that needed to have an aerated cathodic chamber (11-14). This system was not viable for large-scale applications because of the need to aerate the cathode chamber. Breakthroughs such as removing the proton-exchange memebrane (PEM) (15) and the development of an air cathode (16) eliminated this need for aeration and allowed for higher power production. Liu et al. (17) compared a two-chambered and one-chambered MFC and found that the single chamber MFC produced 5.2 times more voltage (146 ± 8 mW/m^2) than a two-chambered MFC (28 ± 3 mW/m^2). These single chamber MFCs have since advanced to use different reactor configurations and electrode materials. Cheng et al.(16) used a brush anode because of its high surface area (9600 m^2/m^3) for bacterial growth and were able to produce 2400 mW/m^2 of power. In addition, a new cathode configuration was developed to further increase power from 538 to 776 mW/m^2. Since then, new configurations such as stacked electrode assemblies have been shown to produce high powers (627 W/m^3) with a higher coulombic efficiency (71% versus 35%) than previous MFCs (18).

2.2 Anodic community analysis methods

Anodic communities are the current producers of MFCs. Thus, it is important to study these communities to identify species that play key roles in power generation. Three major techniques used to characterize the anodic communities are denaturing gradient gel

electrophoresis (DGGE), 16S rDNA clone libraries, and fluorescent in-situ hybridization (FISH). DGGE and 16S rDNA both amplify the 16S gene of the bacteria using polymerase chain reaction (PCR), while FISH uses fluorescently labeled probes that bind to the ribosome of the bacterial cell.

Using DGGE, amplified 16S fragments are electrophoretically run in a denaturing gel and sequences are separated into bands based on the GC content of the sequence (19). These bands can then be excised and identified. It can be difficult to detect community shifts using this method by visually inspecting two gel images because of the large amount of bands that can be present on each gel. Images of the gels are therefore compared in a statistical manner to see an overall shift in the anodic community by generating plots using principle component analysis (PCA) (20, 21). The method of obtaining the PCA plots using DGGE varies, but it provides a method to find overall differences in microbial communities (19, 21, 22). DGGE bands can also contain more than one species per band due to co-migration (19). This can skew what is thought to be a more prominent band by making it darker than it would be if there was only one species in that band.

A 16S clone library, analysis, unlike DGGE, allows for the quantification of individual members of a microbial population (23). By inserting DNA fragments into a vector and transforming the vector into competent *E. coli*, the plasmid is multiplied to a high enough concentration for direct sequencing after it has been extracted from the host *E. coli*. The sequences can then be identified and a quantitative measure of the community is obtained. The disadvantage of this technique is that many vectors must be transformed and extracted to obtain a large enough number of clones to statistically capture the majority of the microbes within the community.

FISH is a useful tool to identify specific types of bacteria in a sample. Because it uses probes that bind to ribosomes, the sample does not need to undergo PCR and is therefore not

subjected to any bias associated with PCR (24). It also visually verifies the presence of target bacteria in the community. Cells can be counted using a microscope to quantify the presence of targeted bacteria using FISH probes in conjunction with stains such as DAPI that stain all bacteria present in the sample. FISH is, however, limited in its application because a specific bacterium must be first targeted before probes can be designed and FISH can be carried out. The length of the probes used to identify the target bacteria is much shorter than the PCR fragments used in 16S and DGGE analysis so the specificity of the probes must be tested before they can be used.

2.3 MFC communities using acetate as a substrate

Acetate is a commonly used substrate in MFC studies not only because it is a well defined chemical substrate, but also because it is known to be able to be used as an electron donor in respiration by many anaerobic bacteria (25). It has also been shown to be directly oxidized by *Geobaceter sulfurreducens*, which is a known dissimilatory metal-reducing bacterium, through the TCA cycle (26). This ability to reduce metals is thought to confer exoelectrogenic capabilities to *Geobacter* and other genera of dissimilatory metal-reducing bacteria (27).

Community analysis of the anodes of different types of acetate fed reactors using 16S clone library analysis has produced a wide spectrum of results (Table 2.1). Anodes were developed in a single chamber continuous upflow reactor in the study conducted by Lee et al. (28). Nearly equal percentages of Alphaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria were found after 17 days. The study by Holmes et al (29) was conducted using sediment MFCs where the anode was placed directly in the anoxic sediment. A clear predominance of *G. sulfurreducens* (70%) was found after the reactors had run for at least three months. A single chamber, batch mode bottle MFC was used by Xing et al. (21). After ~6 days, the community was dominated by Gammaproteobacteria and contained approximately equal

percentages of Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria. Anodic communities had predominance of Betaproteobacteria (49%) and Deltaproteobacteria (32%) after acclimation for ~11 days in a two-chambered batch system used by Chae et al. (30) before the community was analyzed. Single-chambered, batch-fed cubic reactors were acclimated to the system for ~1 year before the anode was sampled and the community analyzed in the study by Kiely et al (31) The communities of reactors that were acclimated for more than two months give a similar percentage of Deltaproteobacteria, but other taxonomic groups are present in different amounts. Reactors that were acclimated for less than a period of months show very little similarity in the community, even though they are being fed the same substrate. This suggests that for reactors fed the same substrate, the initial community is mainly a function of the inoculum source and that the community shifts over time.

Table 2.1 Community analysis results of acetate-fed reactors (% representation of each group).

Study	Proteobacteria				Firmicutes	Bacteroidetes	Others	Acclimation time	Ref
	α	β	γ	δ					
Lee et al. 2003	24	7	21	21	7	0	20	17 days	(28)
Holmes et al. 2004	8	0	2	70	12	0	8	> 90 days	(29)
Xing et al. 2009	19	18	36	15	5	7	0	6 days	(21)
Chae et al. 2009	2	49	0	32	0	0	17	11 days	(30)
Keily et al. 2011	7	30	0	63	0	0	0	> 365 days	(31)

2.4 Comparison of different inoculum sources in MFCs

There are two main categories of inoculum sources for MFCs: mixed and pure culture. Mixed culture inocula are usually sampled from various environmental locations such as marine,

freshwater, and soil sediments (32-34) or wastewater treatment plant sources such as activated sludge or primary clarifier effluent (35, 36). These inoculum sources all contain rich and diverse microbial communities (37, 38) that contain exoelectrogenic microorganisms. Pure culture studies have also been done to try to verify that key microorganisms present in the biofilm community are exoelectrogenic (39). These studies are done with organisms isolated from a mixed culture MFC or with known dissimilatory metal reducing bacteria such as *Shewanella oneidensis* and *Geobacter sulfurreducens* (20, 40, 41).

Different inoculum sources have been compared in several studies to attempt to elucidate the inoculum source with the most effective microbial community. In a study conducted by Holmes et al. (29) marine, freshwater, and salt-marsh sediments were used as inocula in sediment MFCs, where the anodes were placed directly into the anaerobic sediment and cathodes were placed in the overlying water containing dissolved oxygen. All of the anodes in that study were found to be dominated by Deltaproteobacteria. The marine sediments produced a significant portion of members of the *Geobacteraceae* class and also had the largest steady current production (~20 mA/m^2). Inoculation with freshwater sediments produced a lower steady current of 9 mA/m^2 and yielded a significant percentage of *Geothrix fermentans* in the anodic community. The anode inoculated with estuarine sediments was dominated by *Desulfobulbaceae* and produced a steady current of 7 mA/m^2. This suggested that the capacity for higher power production was associated with communities dominated by members of the *Geobacteraceae* class. Others have compared the performance of mixed and pure cultures. *Shewanella oneidensis* was compared to a mixed culture from a primary clarifier effluent in various types of MFCs by Watson et al (42). *S. oneidensis* was always produced less power than the mixed culture. In one case using a single chamber, air cathode MFC, the mixed culture reactor (858 ± 9 mW/m^2) produced 5 times more power than the pure culture reactor (148 ± 20 mW/m^2. *Geobacter sulfurreducens* has also previously been compared to wastewater and environmental samples.

Reactors inoculated with pure *G. sulfurreducens* were shown to produce a similar maximum power to reactors inoculated with river water and aerobic wastewater effluent in a continuous flow system (43). In a batch study that compared *G. sulfurreducens*, soil, and wastewater treatment plant influent as inocula, Jiang et al found that MFCs inoculated with wastewater produced the highest maximum open circuit voltage (785 mV), followed by soil sediments (684 mV) and pure *G. sulfurreducens* (625 mV) (44). These results were not based on the working potentials, but because the reactor conditions are the same, the difference should be caused by the anode potential assuming unchanged performance of the cathodes. The lowest anode potential of the wastewater inoculated reactor should have yielded the highest working potential. The results of these studies imply that the source of a mixed inoculum may be unimportant as long as there is a diverse community that initially contains exoelectrogenic bacteria. However, these studies did not directly investigate the effect of different inocula on the development of the anodic community.

2.5 Replicates in MFC studies

In MFC studies there seems to be no generally accepted amount of replicate reactors that are needed to make statistically sound findings. A single reactor was used in many studies (27, 45, 46). This prevents a statistical analysis of reactor. Other studies have used duplicate (29, 47-49) or triplicate reactors (34, 50, 51). There was only one study that addressed the number of replicates that are needed to maximize information in MFC studies. Eight reactors were run in parallel and a statistical analysis was used to compare changes in COD removal, voltage production, and VFA content in order to recommend an optimal number of replicates. However, it is not clear from the results why four replicates were chosen as the minimum number of replicates needed. The conclusion of this study was that 4 replicates were needed to generate

sufficient data per unit time rather than running less reactors for more cycles, at the expense of repeatability (52). However, if repeatability was sacrificed to accumulate data then the reliability of the data accumulated could be called into question. In summary, these studies suggest that at least duplicate reactors are needed, but the amount of variability there is among replicates has not been investigated.

Chapter 3

Convergent development of bacterial communities in MFCs

3.1 Introduction

Microbial fuel cells (MFCs) have received much interest in recent years because these systems can produce energy while concurrently removing organics from waste streams. In an MFC, a biofilm of exoelectrogenic bacteria on the anode degrades organic matter and produces electrical current. Exoelectrogenic bacteria occur widely in nature (39), and MFCs have been successfully inoculated using aerobic and anaerobic wastewater streams (35, 36) as well as environmental samples such as seawater, rivers, and soil (27, 43, 44). In many of these studies when acetate is the carbon source, particularly freshwater-sediment type MFCs and those inoculated with wastewater, it has been found that Deltaproteobacteria are predominant in the community. For example, Bond et al found that 71.3% of clones from an acetate-fed sediment MFC reactor were similar to Deltaproteobacteria and that ~45% of these clones were from the family *Geobacteraceae* (27). However, this dominance of *Geobacteraceae* is not always observed as *Pelobacter* and Betaproteobacteria have also been found to dominate the anodic community of acetate fed reactors (30, 53). The communities of acetate-fed reactors have been shown to contain a very diverse consortium of microorganisms despite being fed a single and simple substrate. Lee et al found an array of different taxonomic groups including 24% Alpha-, 7% Beta-, 21% Gamma- , 21% Delta-proteobacteria, 7%Firmicutes, and 21% other (28).

The role of the inoculum source on the differences that develop in reactors that are fed a similar substrate has not been well studied. There have been several studies of the communities that evolve in MFCs with different inocula, but there have been few studies that examine the use

of different inocula in the same system to try to find the most efficient microbial consortium. In one study by Holmes et al (29), samples were obtained from salt-water marshes, freshwater, and marine sediments. The anodic communities and reactor performances were then compared in sediment-type MFCs. They found that all anodic communities were dominated by different orders of Deltaproteobacteria. The resulting community from marine inoculum produced the highest steady power (~20 mA/m^2) and was dominated by *Geobacteraceae*, followed by resultant freshwater community (9 mA/m^2), which contained large amounts of *Geothrix fermentans*, and salt-marsh sediment community (7 mA/m^2), which predominantly contained *Desulfobulbaceae*. Mixed inocula have been examined in other studies using wastewater (aerobic and anaerobic effluent), environmental samples (river water and soil), and pure inocula in either batch or continuous-flow reactor systems (42-44). Mixed inocula sources usually produced a higher maximum power than pure cultures. *Shewanella oneidensis* produced less power than a mixed inoculum in several different types of reactors (single, two and three chamber MFCs) (42). *G. sulfurreducens* has produced less current than a mixed inoculum in one case (54) and the same amount in another study (55). There have been a limited number of comparisons of different mixed culture inocula in MFCs. Previous studies have shown that wastewater-inoculated reactors produced more power in some instances than those seeded with environmental inocula, such as soils and sediments, and lower voltage and power in others. Anaerobic and aerobic wastewater effluent inocula were compared to a soil inoculum in a continuous flow MFC (43). On average, at low flow (1.25 mL/hr) the aerobic effluent (1.16 mW/m^2) and river water (1.14 mW/m^2) inoculated reactors produced a higher maximum power than the anaerobic effluent inoculated reactor (0.89 mW/m^2). In another study, the influent to a wastewater plant was compared to a soil sample as an MFC inoculum source in a batch-mode MFC (44). The wastewater inoculated reactor (785 mV) produced a higher maximum open circuit potential than the soil inoculated

reactor (684 mV). The anodic communities were not analyzed in these studies to see if there were differences in the microbes present.

There seems to be no general convention on the number of replicates used in MFC studies. There are many studies that use only one reactor for their experiments (27, 45-47). Fewer studies use reactors in duplicate (29, 48, 49) or triplicate (34, 50, 51). However, the reproducibility of replicates has not been well investigated. There has also been little attempt to analyze the system to identify a potential cause for this difference in performance among replicates.

Many times only one molecular technique is used to analyze the anodic community. Because the 16S rRNA gene is a highly conserved region of the genome, and is ubiquitous among prokaryotic genomes, this is often used to distinguish between and identify different members of the community. One community analysis technique that can use the 16S gene and is used in many studies is denaturing gel gradient electrophoresis (DGGE) (33, 50, 56, 57). Another commonly used technique in MFC community analysis is 16S rRNA clone library analysis (31, 32, 35, 53, 58). Both of these techniques are PCR based. Fluorescent in-situ hybridization (FISH) is non-PCR based and can be used to corroborate the findings of other molecular techniques (59). Most of these studies use one molecular technique to describe the anodic community, but often times do not use a non-PCR based technique, such as FISH, to verify the results.

To determine the effect the inoculum source has on the bacterial community and to better understand the extent of reproducibility of replicate reactors, three different inocula were obtained and run in triplicate with one source being the “control” inoculum (PSU wastewater, which produces consistent and stable voltages). All reactors were fed the same medium containing sodium acetate, which has been used in many studies and produces power densities equal to or higher than other substrates (60). By monitoring the reactor performance and the community shift over time it can be determined if the performance of the reactors is attributable

to the inoculum source. Three different community analysis techniques (DGGE, 16S clone libraries, and FISH) were all used in conjunction to obtain a comprehensive portrayal of the community.

3.2 Materials and methods

3.2.1 Reactor Configuration and Data Collection

Single-chamber air-cathode MFCs were constructed from Lexan cubes to form a single 4 cm cylindrical chamber 3 cm in diameter as previously described (61). Reactors were 28 mL in total volume. Anodes were made from a graphite fiber brush 2.5 cm in outside diameter and 2.5 cm long (62) (PANEX33 160K, ZOLTEK) and cathodes from carbon cloth (type B-1B, E-TEK). Cathodes contained 0.5 mg/cm^2 Pt catalyst as previously described (16). Reactors were kept in a constant 30°C chamber (63, 64) and were fed a medium consisting of 1 g/L sodium acetate with Wolfe's vitamins (5 mL/L) and minerals (12.5 mL/L) (65) in 50 mM phosphate buffer saline (PBS) (Na_2HPO_4, 4.56 g/L; NaH_2PO_4 monohydrate, 2.45 g/L; NH_4Cl, 0.31 g/L; KCl, 0.13 g/L)(31, 66).

The voltage was measured across an external 1 kΩ resistor using a Kiethley data collection system (Kiethley, Cleveland, OH, United States). Power was calculated as $P = E^2/R$ and current was calculated using Ohm's Law (E=IR). Power and current densities were normalized by the cathode projected surface area (7.1 cm^2).Polarization curves were generated using the single-cycle method (67) by varying external resistances every 20 minutes from 10 kΩ to 50 Ω. All statistical calculations using a t-test are based on $\alpha = 0.05$.

3.2.2 Inoculum Sources and DNA Extraction

Reactors (triplicate) were inoculated using samples from three different sources: the Pennsylvania State University Wastewater Treatment Plant (PSU WW); University Area Joint Authority Wastewater Treatment Plant (UAJA WW); and sediment from a stagnant bog in the Black Moshannon Lake State Park, PA (Bog). These two wastewater treatment plants were selected because the UAJA WWTP is much larger and serves rural communities, while the PSU WWTP is smaller and treats only city wastewater. The wastewater inocula were taken from the primary clarifier effluent from both plants. Wastewater reactors were inoculated in a 1:1 ratio of wastewater to PBS solution. The Bog reactors were inoculated using a 1:3 ratio of sediment to PBS solution to obtain a similar viscosity to the wastewater inoculation procedure. All reactors were fed only a PBS plus acetate solution after the first cycle and were fed every three days.

Anode samples were taken by carefully cutting approximately 2 g (wet weight) of the brush using flame-sterilized scissors 7 times throughout the experiment. The anode samples were taken in the middle of a cycle during peak electrochemical activity. Recent tests with identical reactor configuration have shown that up to 75% of the anode brush can be removed without adversely affecting voltage or power generation (68). DNA was extracted using the PowerSoil DNA Isolation Kit (MO-BIO Laboratories) according to the manufacturer's instructions. An additional anode sample was taken at each sampling time for fluorescent in-situ hybridization (FISH) analysis (sample preparation described below). All samples were stored at –20 °C for analysis of the microbial communities.

3.2.3 16S rRNA Clone Libraries

16S rRNA clone libraries were generated as previously described (31), but using different primers. Extracted DNA was amplified via PCR with an annealing temperature of 55ºC to obtain fragment lengths of 960 base pairs. The bacterial primers used were 530F (5'- GTCCCAGCMGCCGCGG -3') and 1490R (5'- GGTTACCTTGTTACGACTT -3') (69). PCR products were purified using the Qiagen PCR Purification Kit (Qiagen) and then ligated and amplified using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Colonies were blue/white screened and appropriate colonies were plated in a 96-well grid format on LB plates (Amp^r 50 µL/mL). The plasmids were extracted using an EZ Fastfilter Plasmid Kit (Omega-Biotek) according to the manufacturer's procedure, and inserts were sequenced using the M13R primer using a DNA sequencer (ABI 3730XL, Applied Biosystems). The chromosomal NCBI BLAST algorithm was then used to analyze the DNA sequences (70).

Shannon diversity indices, Good's coverage values, and rarefaction curves were calculated using the Mothur computer software (71). Sequences were first aligned in MEGA 4 (72). Rarefaction curves are an average composed of 1000 randomizations. Approximately 95 clones were analyzed for each sample and deposited into GenBank under accession numbers JF817397 - JF818119.

3.2.4 DGGE Analysis

A 16S rDNA fragment was amplified via PCR on the extracted DNA using a total reaction volume of 20 µL containing 9 µL of 2x Go-Taq Green Master Mix (Promega, Madison, WI, United States), 8 µL of sterile ddH_20, 25 ng of DNA, and 1 µL of each primer. The universal primers used for amplification were: 968F (5'- AACGCGAAGAACCTTAC-3') with a GC clamp

(5'-CGCCCGCCGCGCCCCGCGCCCGGCCCG CCGCCCCCGCCCC-3') attached and 1401R (5'-CGGTG TGTACAAGACCC-3')(73). The DNA samples were amplified with a G-Storm 1 Thermocycler (GRI Laboratories, UK) using the following temperature profile: 95°C for 5 min, then 20 cycles of 95°C for 30 s, 60°C for 30 s, and 1 min at 72°C with the annealing temperature being decreased by 0.1°C each cycle to 58°C. Then 15 cycles were run with 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C. A ten minute period was used for a final extension. All samples were stored at 4°C until use. DGGE gels were then prepared as previously described (74) and run using a DCode universal mutation detection system (Bio-Rad Laboratories, United States). The denaturing gradient in the gel ranged from 30% to 60%, where 100% denaturation corresponds to 40% (v/v) formamide and 7 M urea. PCR products were dispensed into a gel consisting of 7% (w/v) polyacrylamide (37.5: 1 acrylamide/bisacrylamide) in a 1× TAE buffer solution. The gel was cast with a gradient delivery system (Model 475, Bio-Rad, USA). Electrophoresis was then conducted for 12 hours at 60°C and 70 V in a 0.5× TAE buffer solution to achieve effective band separation. Gels were then silver stained as previously described (75).

The most prominent bands from each gel were excised using a flame and ethanol sterilized razor blade, crushed in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and allowed to equilibrate overnight at 4°C. The buffer containing DNA was then used as the template for PCR as described above excluding the GC clamp. The PCR product was then purified using a PCR cleanup kit (Qiagen) followed by ligating the DNA fragments into a TOPO 2.1 vector (Invitrogen, US) according to manufacturer's instructions, then inserted into chemically competent *E. coli* cells. Eight random colonies were then taken and sequenced by first extracting the plasmid from the *E. coli* using a 96 well Plasmid Extraction Kit (Omega Bio-Tek), and sequencing the DNA (ABI 3730XL, Applied Biosystems).

The information used for Principal Component Analysis (PCA) of the DGGE gels was obtained using two methods in order to determine the shift of the microbial community over time.

For the first method a computer software program (GelCompar II, Applied-Maths, Austin, TX, USA) was used to identify the presence or absence of bands and the relative densities of bands for PCA plots. For the second method, gel images were manually scored based on the presence/absence of specified bands, and a scale from 0-5 based on the relative density of the bands. PCA plots were generated in Minitab 16 (Minitab, USA) to determine the similarity of the replicates at different times and to evaluate temporal community shifts in the reactors.

3.2.5 FISH

Samples for FISH analysis were taken at peak voltage at the same time as samples used for the 16S clone libraries/ DGGE analysis. These samples were immediately fixed using 4% paraformaldehyde for 6 hours total at 4° C and stored at –20° C in 50% ethanol/PBS mixture. A *Geobacter sulfurreducens* probe (100 ng/uL) with a red fluorescent tag (Alexa Fluor 594) (GAAGACAGGAGGCCCGAAA) (Invitrogen) along with 2 helper probes at 100 ng/uL (CTAATGGTACGCGGACTCATCC and GTCCCCCCCTTTTCCCGCAAG) were hybridized to the rRNA for 1.5 hours at 46° C (76). Samples were then stained using Slow Fade Gold® with DAPI (Invitrogen, Carlsbad, CA) to label all bacterial cells. Visualization was then performed on an Olympus FV100 confocal laser scanning microscope. Images were taken using different filters to show the fluorescence of the *Geobacter* probe in the sample versus the total number of bacteria present. The total amount of *Geobacter w*as calculated by comparing the amount of red and blue fluorescence of the same image using different filters. The percentage of *G. sulfurreducens* in each reactor sample was compared by counting at least 1000 total cells from 10 different locations of the biofilm for each sample.

3.3 Results

3.3.1 Voltage and Power

Initially, the Bog reactors produced higher voltages than the wastewater inoculated reactors. After 60 days, the reactors from each inoculum source converged to a similar voltage output of 465 ± 6 (PSU), 453 ± 25 (UAJA), and 480 ± 2 mV (Bog) (Fig. 3.1). The maximum voltages generated by the two wastewater inocula were very similar to each other throughout the entire study. UAJA 3 gave significantly (t-test, P = 0.005) lower power and voltage reading throughout the course of the experiment.

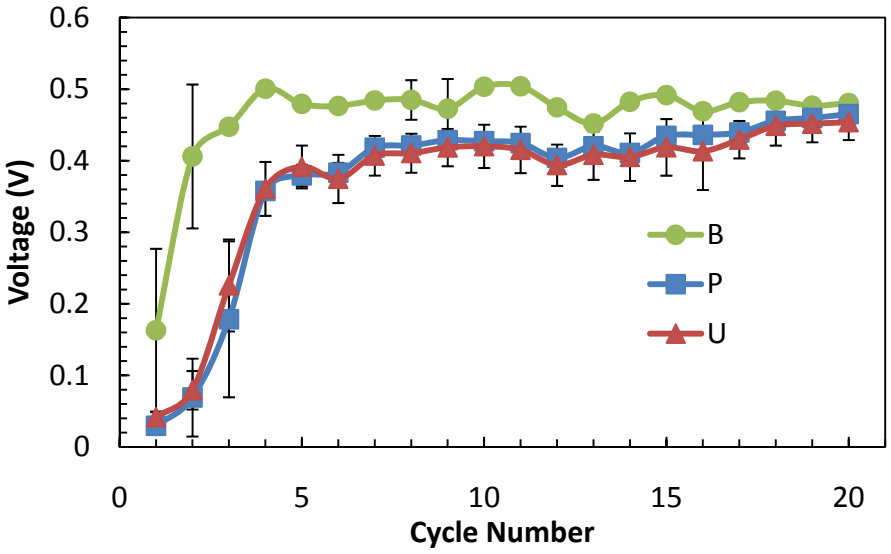

Figure 3.1. Plot of average maximum voltage for each cycle. Initally Bog-inoculated reactors (B) produce more voltage than wastewater inoculated reactors (P, PSU) (U, UAJA). By day 60 all reactors produce similar voltages. Wastewater reactors produce the similar voltages throughout the length of the experiment.

Based on polarization tests, maximum power densities on day 48 were not significantly different for eight reactors (t-test, P > 0.05), with the UAJA 3 sample producing significantly less power (354 mW/m^2) than the other samples (t-test, P = 0.005) (Fig. 3.2). The maximum power

density was 590 ± 110 mW/m^2 based on all 9 reactors, and 620 ± 60 mW/m^2 excluding the UAJA 3 replicate (n = 8). These results demonstrate that based on power densities the inoculum was not important for achieving maximum power if tests were run in triplicate. However, if only a single reactor had been used the UAJA sample could have produced different results than the other inocula.

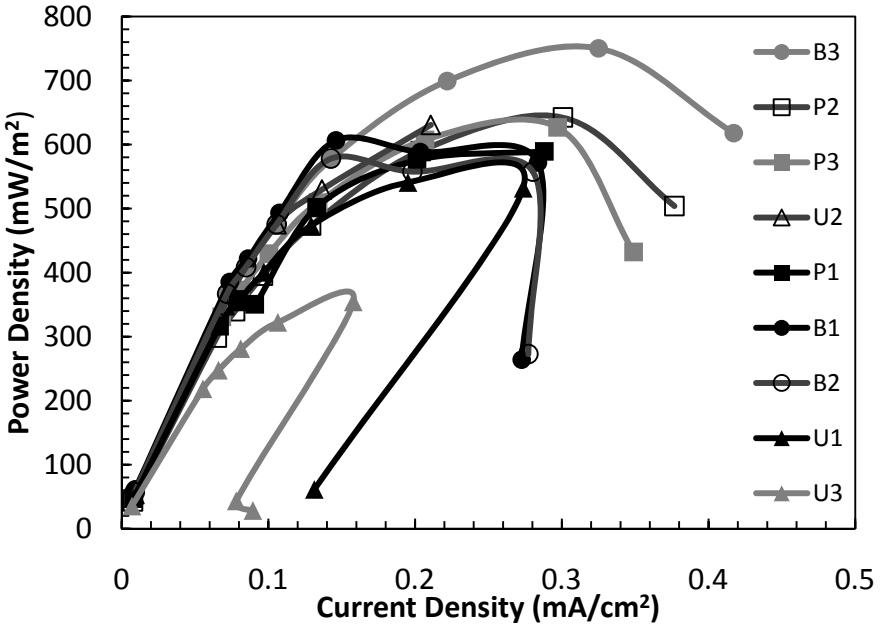

Figure 3.2. Power density curves generated from cycle 16 (day 48) showing that UAJA replicate 3 produced significantly (t-test, $\alpha = 0.05$, $P = 0.005$) less power than all other reactors, including other UAJA replicates.

Additional analysis of the results suggests that duplicate reactors would have been sufficient for comparison of the different inocula. The average voltages and maximum power calculated using only the two most disparate replicates from the UAJA inoculated reactors produced 450 ± 34 mV and 490 ± 190 mW/m^2, compared to 453 ± 25 mV and 510 ± 140 mW/m^2 based on triplicate reactors. These values for voltage ($P = 0.90$) and power ($P = 0.92$) are not significantly different when either duplicates or triplicates are used. This similarity shows that,

while replicate reactors are needed, using triplicate reactors yields little more information than duplicate reactors.

3.3.2 16S Clone analysis

All reactors were found to be predominated by clones most similar (>95%) to *Geobacter sulfurreducens* based on 16S clone libraries. All of the reactors had clones similar to *G. sulfurreducens* present in the range from 67 to 51% (n=8) with an average of 61 ± 6 %, except for replicate UAJA 3, which only had 36%. Additionally, clones distantly related to Bacteriodetes (< 89% similarity) were present as 36% of the community of replicate UAJA 3. These clones related to Bacteriodetes were the second most abundant in all of the anode communities, with an average of 11 ± 6 % (n=8). Other clones identified in the clone analysis were <95% similar to members of Betaproteobacteria (5 ± 3 %, n = 9) and Alphaprotoeobacteria (2 ± 2 %, n = 9) (Fig. 3.3).

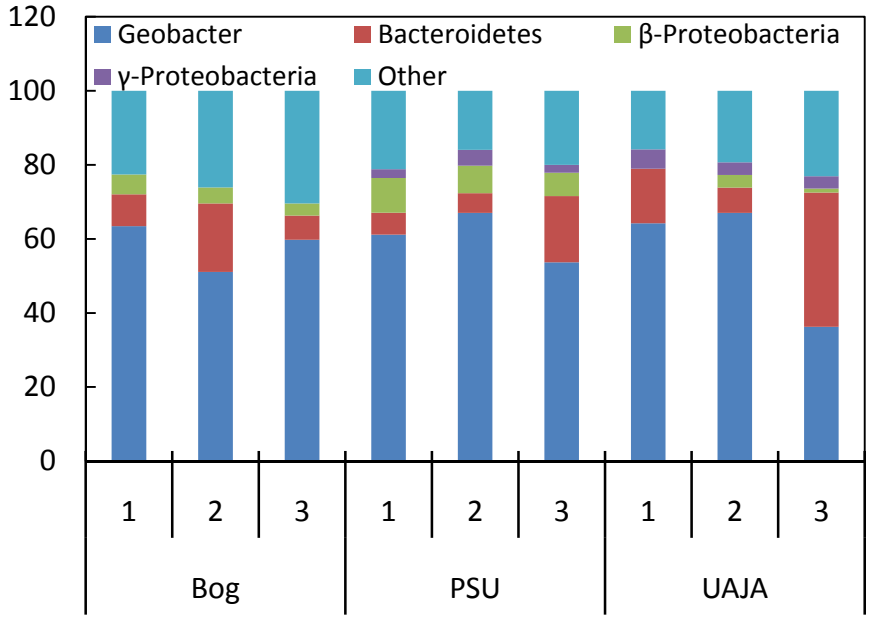

Figure 3.3. Abundance of major phylogenetic groups in each reactor found using 16S rDNA clone libraries of the sample taken at cycle 16. UAJA 3 has the least amount of G. sulfurreducens, possibly leading to decrease in voltage and maximum power.

There was no difference in diversity among the different inocula based on the Shannon Diversity Indices of each reactor (1.78 ± 0.22, n=9) indicating that there was convergence among the anodic communities of the different reactors. The UAJA3 replicate, however, was the only replicate found to have a diversity index that was more than one standard deviation higher than the average (2.07). This added diversity could account for the underperformance of this reactor. Good's Coverage values indicated that on average 82% of the total species richness was accounted for in each community (77) (Table 3.1). Rarefaction curves generated using Mothur (Fig. 3.4) show that there is little difference between the diversity of each of the reactors. All rarefaction curves seem to be approaching a plateau, but one was never reached. This suggests that the diversity of the bacterial population, beyond the dominance of the Geobacter and Bacteriodetes, may not have been fully captured in the clone analysis. However, it is unlikely that obtaining additional information on the least abundant members of the bacterial community would have improved our understanding of the system.

Table 2.1. Shannon Diversity Indices and Good's Coverage Values calculated from 16S clone library data.

	# of clones	Shannon Diversity	Goods Coverage (%)
Bog 1	93	1.57	86
Bog 2	92	1.94	80
Bog 3	92	1.45	89
Bog-Average	277	1.65	85
PSU 1	86	1.86	81
PSU 2	94	1.53	84
PSU 3	95	1.99	82
PSU-Average	275	1.79	82
UAJA 1	95	1.90	79
UAJA 2	88	1.72	79
UAJA 3	91	2.07	81
UAJA - Average	274	1.90	80

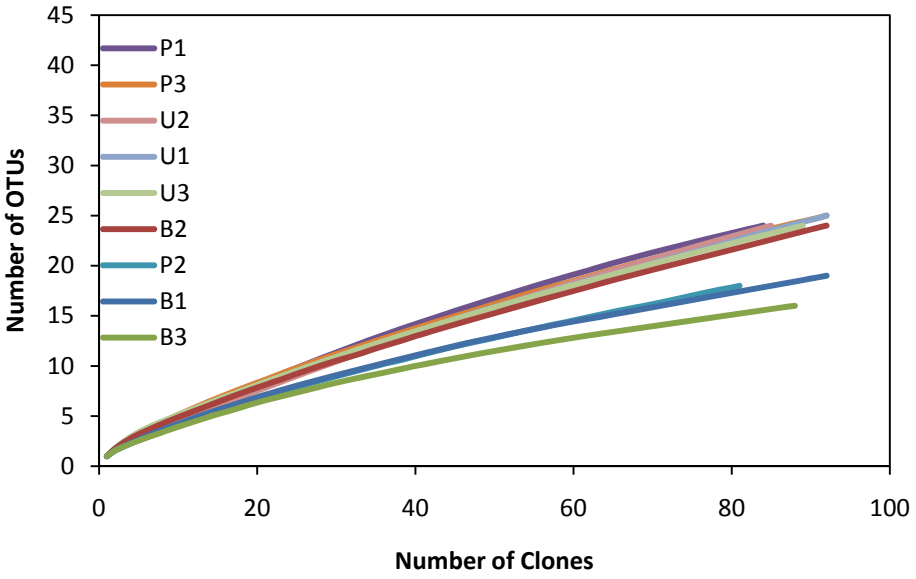

Figure 3.4. Rarefaction curves generated using Mothur computer software. These curves show that the diversity between the replicates is visually very similar. A distinct plateau was not reached implying that the full diversity was not captured by the 16S clone libraries. However, more analysis to gain information on less abundant members of the community is unlikely to aid in understanding the system.

3.3.3 Community Shift with DGGE

Analysis using DGGE was able to better represent the diversity of the microbial community, although it did not capture as effectively the predominance of the community by *G. sulfurreducens*. Initially, gels had numerous bands from each source (>75). After three days, more than two-thirds of the original operational taxonomic units (OTUs) were eliminated from each inoculum source and only 8 distinct OTUs were identified from the Bog inoculated anode, 17 OTUs from the PSU WW anode, and 15 OTUs from the UAJA WW anode. These numbers decreased throughout the experiment. After 16 cycles, there were 7 OTUs from the Bog anodes, 9 OTUs from the PSU WW anodes, 7 OTUs from the UAJA WW anodes.

Analysis of the DGGE band patterns using PCA indicated a shift in communities over time. Initially, using the dominant bands from each gel, the Bog inoculated reactors cluster in a different part of the graph (quadrant 1) than the two wastewater inoculum sources (quadrant 4) (Fig. 3.5). By the end of the study, all of the data converged and clustered in quadrant 2. A PCA analysis using automated software (GelComparII) produced the same results as manual scoring, and thus there was no effect of the method used to score the bands on the conclusions reached with a PCA analysis.

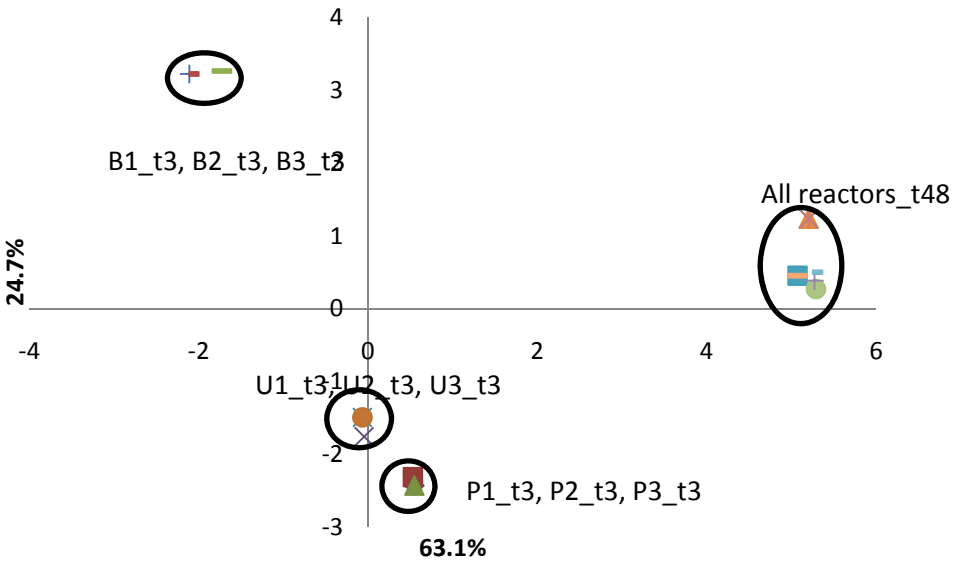

Figure 3.5. PCA plot of the dominant (darkest) bands from the gels representing the communities at cycle 1 and cycle 16. This plot contrasts the initial community of wastewater inoculated reactors with the bog inoculated reactors. Over the duration of the experiment all communities converge irrespective of inoculum source.

The darkest bands in the DGGE gels (bands 2, 3, 4, and 5) were excised and sequenced, assuming that these bands would represent the predominant species in the community. However, none of the dark bands initially analyzed (Fig. 3.6) were related to *G. sulfurreducens*, which was shown to be dominant by the 16S clone library analysis. Analysis of additional bands (1 and 6) revealed the presence of bacteria that were most closely related to the known isolates G.

sulfurreducens (95% similarity) and *Candidatus* Protochlamydia amoebophila (89%). Other bands sequenced contained members of the Colstridiaceae family, *Clostridium sticklandii* (89%), *Alkaliphilus oremlandii* (89%), *and Alkaliphilus metalliregedens* (88%), and the Gammaproteobacteium *Pseudomonas flourescens* (99%). All of these identified species were also found in the 16S rDNA clone analysis (Table 3.2).

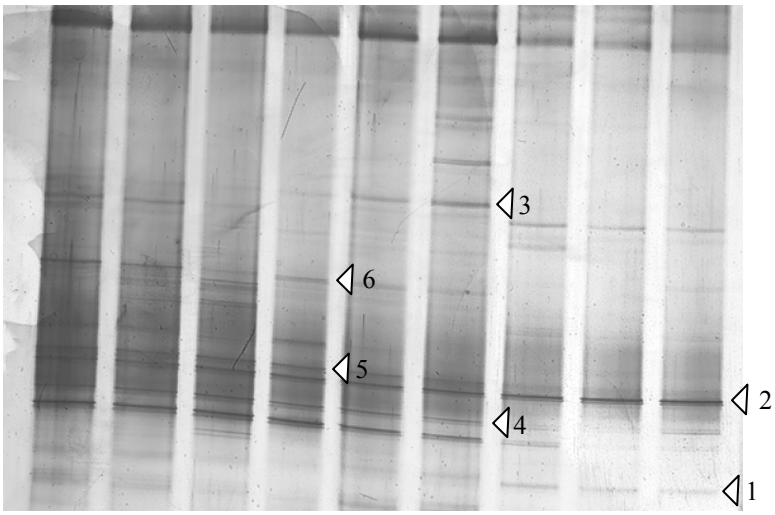

Fig. 3.6. DDGE gels showing bands that were excised and identified. Gels were used in the PCA analysis to monitor shifts in overall microbial community throughout the experiment.

Table 3.2. Identification of excised bands from DGGE gels.

	Closest known isolate	Similarity (%)	Found in 16S clone library
Band 1	*Geobacter sulfurreducens*	99	Yes
Band 2	*Alkaliphilus oremlandii*	89	Yes
Band 3	*Pseudomonas fluorescens*	99	Yes
Band 4	*Clostridium sticklandii*	89	Yes
Band 5	*Alkaliphilus metalliregidens*	88	Yes

Band 6	*Candidatus* Protochlamydia amoebophila	89	Yes

3.3.4 FISH

FISH was used to confirm that a large portion of the biofilm was dominated by *G. sulfurreducens.* The red cells shown in Fig. 7 represent the *G. sulfurreducens* while blue cells indicate all bacteria. Bacterial cell counts were used to quantify the differences between each sample. These counts showed that all of the samples contained 70 to 57% (n=30 per sample) with an average of 63 ± 6 % *G. sulfurreducens* (Fig. 8). Analysis of the samples using FISH was not able to confirm the finding of less *G. sulfurreducens* in reactor UAJA 3 by 16S clone library analysis.

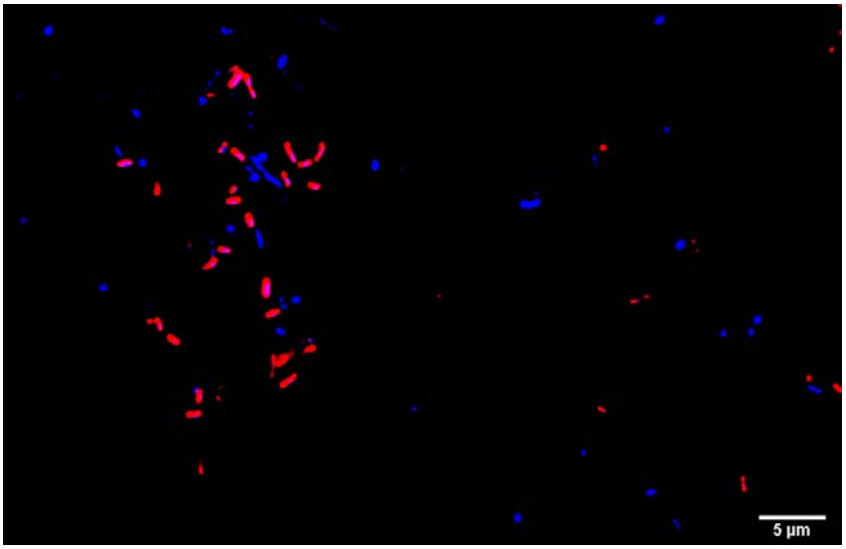

Figure 3.7. FISH image. Bacteria stained with DAPI (blue) and fluorescently labeled *G.sulfurreducens* (red).

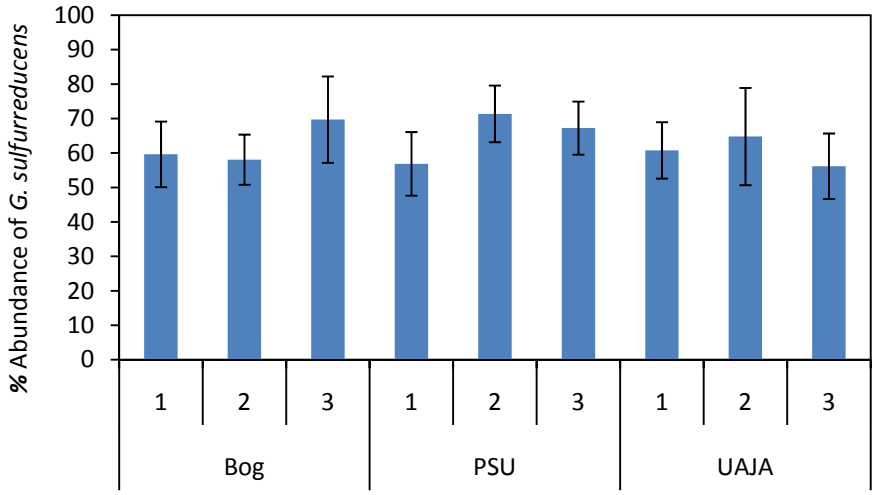

Figure 3.8 Percentage of *G. sulfurreducens* in each reactor determined by counting bacterial cells in FISH images that were captured during cycle 16. Ten images were taken from different parts of the sample and numbers were averaged to obtain the final result.

3.4 Discussion

3.4.1 Voltage and Power Comparison to Control Inoculum Source

After a long acclimation time (~60 days), the voltages of all the reactors converged to a similar voltage (470 ± 20 mV) and power density (590 ± 110 mW/m^2) regardless of inoculum source. Additional analysis showed that the same conclusions were obtained with either duplicate or triplicate reactors, suggesting that duplicate reactors are sufficient for MFC tests of different inocula. The Bog inoculated reactors initially produced a significantly higher voltage than the wastewater inoculated reactors. These MFCs reached their maximum voltage after only 3 cycles, and then this voltage remained relatively constant throughout the duration of the study. The bog inoculated reactors likely began producing higher voltages than the other inocula likely as a result of the long term acclimation to anaerobic conditions compared to the other wastewater samples obtained from the primary clarifier.

The PSU WW was used as the "control" inoculum source because it has previously been used as an inoculum source by our group (20, 31, 61, 62, 78). Average voltages produced by the different inocula here (470 ± 20 mV) were consistent with previous studies using the same 50 mM PBS and 1 g/L sodium acetate where the average maximum stable voltage was 500 ± 40 mV (n=5) (20, 31, 61, 62, 78). Maximum power densities obtained in previous studies were found to be larger in some cases (704 ± 30 mW/m^2, after the cathodic biofilm was removed) (31) and smaller in others (506 mW/m^2) (78) that obtained here (590 ± 110 mW/m^2) using the same buffer. In several studies, power densities were only reported at higher buffer concentrations (20, 61, 62). The differences in maximum power could be due to power overshoot, the use of different resistors or time before obtaining the voltage at each resistance, or biofilm on the cathode or better intrinsic performance of the cathodes made by others compared to those used here.. Some power density curves examined here exhibited Type D power overshoot, where the power density curve doubles back instead of reaching higher current densities. This overshoot arises from the inability of the bacterial community to transfer electrons fast enough to the anode when the external resistance is lowered (67). Power overshoot implies that the capacity for these reactors to function at higher current densities would have required additional acclimation to low resistances, which was not done here.

3.4.2 Comparison of the Community Composition using Different Molecular Techniques

The communities of the wastewater samples were shown to be similar throughout the study, but they were initially much different than those of the bog sample. However, after 48 days the communities from all inocula were similar based on an analysis using three different molecular techniques. Analysis of communities from the different inocula showed similar amounts of *G. sulfurreducens* based on 16S rRNA analysis (61 ± 6%) and FISH (63 ± 6%).

DGGE analysis was unable to determine predominance of particular species present in the community, but this technique did show a convergence to similar communities at the end of 16 cycles based on PCA of the band patterns. The similar responses of the wastewater inocula over time may be due to both inocula coming from a similar environment (primary clarifier) and subsequently being subjected to the same conditions in the MFCs. Both wastewater inocula had a rich microbial population allowing the communities to produce the same gradual shifts in voltages throughout the study. It is assumed that the selective pressures inside the MFCs favor the same types of bacteria that can flourish independent of the specific wastewater source.

Clone libraries and FISH identified that there was a clear predominance of *G. sulfurreducens,* while the multiple bands in DGGE gels implied a much more diverse community. This predominance of *Geobacter* in MFCs has been previously observed (27). However, the predominance of this genera or Deltaproteobacteria has not been found in all studies. Alphaproteobacteria (28) and Betaproteobacteria (30) have been found to be the predominant group in MFC anodic communities in other studies. One possible reason for this difference is that operating time of the MFCs in these previous studies prior to community analysis (6 to 17 days) was much shorter than that chosen here (60 days). This gives the anode community less time to develop and show the prominence of a specific group.

The use of DGGE to profile the communities clearly showed a shift in the community composition over time based on PCA plots. One disadvantage of this method, however, is that it was difficult to determine the predominance of a specific species of bacteria due to the large amount of prominent bands present in the gel. The prominence of *G. sulfurreducens* seen in 16S clone libraries could not be discerned using DGGE. Resolving individual species richness is difficult using DGGE due to co-migration of DNA. When the DNA co-migrates, a specific band appears to be prominent, which implies predominance of a single species, when it could be a mixture of more than one species. This is one possible reason the *G. sulfurreducens* band is

lighter than others. Co-migration in DGGE bands has been previously reported for other species of bacteria (79, 80). DGGE was more useful at showing the diversity of less abundant species because these species were more easily captured in the banding patterns.

Clone library analysis was a more useful quantitative method of comparing the abundances of specific bacteria within the anode community. This technique was the only one that provided an explanation that the reason for the underperforming replicate UAJA 3 could have been due to a reduced predominance of *G. sulfurreducens*. Alphaproteobacteria, betaproteobacteria and Bacteroidetes clones found in these samples have also been identified in variable amounts in other studies with acetate as the carbon source (28, 81). The analysis of all community structures revealed that a bacterial species related to Bacteroidetes (<89% similarity) was present in variable numbers (10 ± 7%) here. However, in sample UAJA 3, the community analysis showed an elevated number of clones (36%) similar to Bacteroidetes relative to other samples. This implies that a significant portion of the anode was dominated by Bacteroidetes and there was likely less *G. sulfurreducens* populating the anode, providing an explanation for the reduced performance of this MFC.

FISH was used as a non-PCR based molecular technique to independently verify the dominance of *G. sulfurreducens* found in the 16S rDNA analysis. Instead of targeting DNA or RNA, the FISH probes target the ribosomes of the bacteria of interest. The percentage of *G. sulfurreducens* (63 ± 6%) obtained from counting cells showed no deviation of the other inoculum sources from the control source. Each sample produced a statistically similar (t-test, $P > 0.05$) number of *G. sulfurreducens*. FISH gave very similar numbers of *G. sulfurreducens* compared to 16S rDNA values, except for UAJA 3. The FISH analysis did not show a decreased relative abundance of *G. sulfurreducens* in this reactor compared to the other reactors. However, a difference in community analysis is the most likely explanation for the reduced performance of the UAJA 3 MFC as the experimental conditions were otherwise identical.

These results suggest that more than one molecular technique should be used to verify any community analysis results. One technique can be used as the main source of community analysis, but another technique should be used for corroboration. Ideally, a non-PCR based technique such as FISH should be used to corroborate findings based on PCR analysis to eliminate any PCR bias.

Chapter 4

Conclusions

Three different inocula were used in triplicate reactors to understand the effect of the inoculum source on the community that arises after a long period of acclimation. It was determined that regardless of the inoculum source, the reactors performed similarly to the control reactors. In addition, the replicates all yielded similar results except for one under-performing reactor. Based on these findings, it seems to be unnecessary to run more than duplicate reactors along with one control reactor. In addition, if replicates are producing similar voltages and power and the control performs as expected, then analyzing the community structure of more than one replicate is unnecessary. Results of this study also suggest that at least one molecular technique, such as FISH, should be used to verify findings of initial community analysis to ensure that claims made are accurate and not due to biases inherent in a particular analysis.

Chapter 5

Future Work

There are other factors that need to be addressed to determine the effect of starting inocula on the resulting anodic community:

1. Additional inocula should be tested for convergence. Different environmental samples should be tested as inocula to determine if other environmental samples converge in the same manner as the wastewater samples. Also, anaerobic wastewater samples should also be tested to determine if they converge as well.
2. Different reactor designs should be tested to make sure that the community is not affected by factors such as electrode spacing or electrode materials. Future community analysis should involve switching the cathodes between reactors to eliminate that as a possibility for differences in performance.
3. Different molecular techniques such as qPCR and pyrosequencing could be used in conjunction with those described in this study to try to elucidate the optimal combination of techniques to most accurately depict the anodic community.

References

1. USEIA. International Energy Outlook 2010. [cited 2011 February 21]; Available from: http://www.eia.doe.gov/oiaf/ieo/world.html.

2. USDOE. Fossil Fuels. [cited 2011 February 21]; Available from: http://www.energy.gov/energysources/fossilfuels.htm.

3. EnergyQuest. Renewable Energy vs Fossil Fuels. [cited 2011 February 21]; Available from: http://www.energyquest.ca.gov/story/chapter17.html.

4. Wyman CE. BIOMASS ETHANOL: Technical Progress, Opportunities, and Commercial Challenges. Annual Review of Energy and the Environment. 1999;24(1):189-226.

5. Lewis NS, Nocera DG. Powering the planet: chemical challenges in solar energy utilization. Proceedings of the National Academy of Science. 2006;103(43):15729-35.

6. Ethanol. [cited 2011 February 21]; Available from: http://www.fueleconomy.gov/feg/ethanol.shtml.

7. Liu H, Ramnarayanan R, Logan BE. Production of electricity during wastewater treatment using a single chamber microbial fuel cell. Environmental Science & Technology. 2004;38(7):2281-5.

8. Henze M, Loosdrecht Mv, Ekama G, Brdjanovic D. Biological Wastewater Treatment: Principles, Modelling, and Design. London: IWA Publishing; 2008.

9. Rittmann BE, McCarty PL. Environmental Biotechnology: Principles and Applications. Boston: McGraw-Hill; 2001.

10. Bolles SA. Modeling Wastewater Aeration Systems to Discover Energy Savings Opportunities. Process Energy Services, LLC.

11. Bond DR, Lovley DR. Electricity production by *Geobacter sulfurreducens* attached to electrodes. Applied and Environmental Microbiology. 2003;69(3):1548-55.

12. Kim BH, Park DH, Shin PK, Chang IS, Kim HJ. Mediator-less biofuel cell. U.S. Patent 5976719. 1999.

13. Kim HJ, Park HS, Hyun MS, Chang IS, Kim M, Kim BH. A mediator-less microbial fuel cell using a metal reducing bacterium, *Shewanella putrefaciens*. Enzyme Microbial Technology. 2002;30(2):145-52.

14. Chaudhuri SK, Lovley DR. Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. Nature Biotechnology. 2003;21(10):1229-32.

15. Park DH, Zeikus JG. Improved fuel cell and electrode designs for producing electricity from microbial degradation. Biotechnology and Bioengineering. 2003;81(3):348-55.

16. Cheng S, Liu H, Logan BE. Increased performance of single-chamber microbial fuel cells using an improved cathode structure. Electrochemistry Communications. 2006;8:489-94.

17. Liu H, Logan BE. Electricity Generation Using an Air-Cathode Single Chamber Microbial Fuel Cell in the Presence and Absence of a Proton Exchange Membrane. Environmental Science & Technology. 2004;38(14):4040-6.

18. Fan Y, Hu H, Liu H. Enhanced coulombic efficiency and power density of air-cathode microbial fuel cells with an improved cell configuration. Journal of Power Sources. 2007;171(2):348-54.

19. Fromin N, Hamelin J, Tarnawski S, Roesti D, Jourdain-Miserez K, Forestier N, et al. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environmental Microbiology. 2002;4(11):634-43.

20. Xing D, Cheng S, Logan B, Regan J. Isolation of the exoelectrogenic denitrifying bacterium <i>Comamonas denitrificans</i> based on dilution to extinction. Applied Microbiology and Biotechnology. 2010;85(5):1575-87.

21. Xing D, Cheng S, Regan JM, Logan BE. Change in microbial communities in acetate- and glucose-fed microbial fuel cells in the presence of light. Biosensors and Bioelectronics. 2009;25(1):105-11.

22. Esseili MA, Kassem II, Sigler V. Optimization of DGGE community fingerprinting for characterizing Escherichia coli communities associated with fecal pollution. Water Research. 2008;42(17):4467-76.

23. Deng W, Xi D, Mao H, Wanapat M. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: a review. Molecular Biology Reports. 2008;35(2):265-74.

24. Lueders T, Friedrich MW. Evaluation of PCR Amplification Bias by Terminal Restriction Fragment Length Polymorphism Analysis of Small-Subunit rRNA and mcrA Genes by Using Defined Template Mixtures of Methanogenic Pure Cultures and Soil DNA Extracts. Appl Environ Microbiol. 2003;69(1):320-6.

25. Thauer RK, Zinkhan DM, Spormann AM. Biochemistry of Acetate Catabolism in Anaerobic Chemotrophic Bacteria. Annual Review of Microbiology. 1989;43(1):43-67.

26. Caccavo F, Jr, Lonergan DJ, Lovley DR, Davis M, Stolz JF, McInerney MJ. Geobacter sulfurreducens sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl Environ Microbiol. 1994;60(10):3752-9.

27. Bond DR, Holmes DE, Tender LM, Lovley DR. Electrode-reducing microorganisms that harvest energy from marine sediments. Science. 2002;295(5554):483-5.

28. Lee J, Phung NT, Chang IS, Kim BH, Sung HC. Use of acetate for enrichment of electrochemically active microorganisms and their 16S rDNA analyses. FEMS Microbiology Letters. 2003;223(2):185-91.

29. Holmes DE, Bond DR, O'Neil RA, Reimers CE, Tender LR, Lovley DR. Microbial Communities Associated with Electrodes Harvesting Electricity from a Variety of Aquatic Sediments. Microbial Ecology. 2004;48(2):178-90.

30. Chae K-J, Choi M-J, Lee J-W, Kim K-Y, Kim IS. Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells. Bioresource Technology. 2009;100(14):3518-25.

31. Kiely PD, Rader G, Regan JM, Logan BE. Long-term cathode performance and the microbial communities that develop in microbial fuel cells fed different fermentation endproducts. Bioresource Technology. 2011;102(1):361-6.

32. Ishii S, rsquo, ichi, Hotta Y, Watanabe K. Methanogenesis versus Electrogenesis: Morphological and Phylogenetic Comparisons of Microbial Communities. Bioscience, Biotechnology, and Biochemistry. 2008;72(2):286-94.

33. Erable B, Roncato M-A, Achouak W, Bergel A. Sampling Natural Biofilms: A New Route to Build Efficient Microbial Anodes. Environmental Science & Technology. 2009;43(9):3194-9.

34. Faimali M, Chelossi E, Pavanello G, Benedetti A, Vandecandelaere I, De Vos P, et al. Electrochemical activity and bacterial diversity of natural marine biofilm in laboratory closed-systems. Bioelectrochemistry. 2010;78(1):30-8.

35. Ki D, Park J, Lee J, Yoo K. Microbial diversity and population dynamics of activated sludge microbial communities participating in electricity generation in microbial fuel cells. Water Science & Technology. 2008;58(11):2195-201.

36. Min B, Cheng S, Logan BE. Electricity generation using membrane and salt bridge microbial fuel cells. Water Research. 2005;39(9):1675-86.

37. Llobet-Brossa E, Rossello-Mora R, Amann R. Microbial Community Composition of Wadden Sea Sediments as Revealed by Fluorescence In Situ Hybridization. Appl Environ Microbiol. 1998;64(7):2691-6.

38. Wagner M, Loy A, Nogueira R, Purkhold U, Lee N, Daims H. Microbial community composition and function in wastewater treatment plants. Antonie van Leeuwenhoek. 2002;81(1):665-80.

39. Logan BE. Exoelectrogenic bacteria that power microbial fuel cells. Nature Reviews Microbiology. 2009;7(5):375-81.

40. Fedorovich V, Knighton MC, Pagaling E, Ward FB, Free A, Goryanin I. Novel Electrochemically Active Bacterium Phylogenetically Related to Arcobacter butzleri, Isolated from a Microbial Fuel Cell. Appl Environ Microbiol. 2009;75(23):7326-34.

41. Yi H, Nevin KP, Kim B-C, Franks AE, Klimes A, Tender LM, et al. Selection of a variant of Geobacter sulfurreducens with enhanced capacity for current production in microbial fuel cells. Biosensors and Bioelectronics. 2009;24(12):3498-503.

42. Watson VJ, Logan BE. Power production in MFCs inoculated with *Shewanella oneidensis* MR-1 or mixed cultures. Biotechnology and Bioengineering. 2009:In press.

43. Ieropoulos I, Winfield J, Greenman J. Effects of flow-rate, inoculum and time on the internal resistance of microbial fuel cells. Bioresource Technology. 2010;101(10):3520-5.

44. Jiang D, Li B, Jia W, Lei Y. Effect of Inoculum Types on Bacterial Adhesion and Power Production in Microbial Fuel Cells. Applied Biochemistry and Biotechnology. 2010;160(1):182-96.

45. Jong BC, Kim BH, Chang IS, Liew PWY, Choo YF, Kang GS. Enrichment, performance and microbial diversity of a thermophilic mediatorless microbial fuel cell. Environmental Science & Technology. 2006;40(20):6449-54.

46. Reimers CE, Stecher HA, III, Westall JC, Alleau Y, Howell KA, Soule L, et al. Substrate Degradation Kinetics, Microbial Diversity, and Current Efficiency of Microbial Fuel Cells Supplied with Marine Plankton. Appl Environ Microbiol. 2007;73(21):7029-40.

47. Aelterman P, Rabaey K, Pham TH, Boon N, Verstraete W. Continuous electricity generation at high voltages and currents using stacked microbial fuel cells. Environmental Science & Technology. 2006;40:3388-94.

48. Rismani-Yazdi H, Christy AD, Carver SM, Yu Z, Dehority BA, Tuovinen OH. Effect of external resistance on bacterial diversity and metabolism in cellulose-fed microbial fuel cells. Bioresource Technology. 2011;102(1):278-83.

49. Sasaki K, Morita M, Sasaki D, Hirano S-i, Matsumoto N, Ohmura N, et al. Methanogenic communities on the electrodes of bioelectrochemical reactors without membranes. Journal of Bioscience and Bioengineering. 2011;111(1):47-9.

50. Jung S, Regan JM. External Resistance Influences Electrogenesis, Methanogenesis, and Anode Prokaryotic Communities in MFCs. Appl Environ Microbiol. 2010:AEM.01392-10.

51. Nelson KY, Razban B, McMartin DW, Cullimore DR, Ono T, Kiely PD. A rapid methodology using fatty acid methyl esters to profile bacterial community structures in microbial fuel cells. Bioelectrochemistry. 2010;78(1):80-6.

52. Larrosa A, Lozano LJ, Katuri KP, Head I, Scott K, Godinez C. On the repeatability and reproducibility of experimental two-chambered microbial fuel cells. Fuel. 2009;88(10):1852-7.

53. Kiely PD, Cusick R, Call DF, Selembo PA, Regan JM, Logan BE. Anode microbial communities produced by changing from microbial fuel cell to microbial electrolysis cell operation using two different wastewaters. Bioresource Technology. 2011;102(1):388-94.

54. Ishii S, Watanabe K, Yabuki S, Logan BE, Sekiguchi Y. Comparison of electrode reduction activities of *Geobacter sulfurreducens* and an enriched consortium in an air-cathode microbial fuel cell. . Applied and Environmental Microbiology. 2008;74(23):7348–55.

55. Nevin KP, Richter H, Covalla SF, Johnson JP, Woodard TL, Orloff AL, et al. Power output and columbic efficiencies from biofilms of *Geobacter sulfurreducens* comparable to mixed community microbial fuel cells. Environmental Microbiology. 2008;10(10):2505–14.

56. Zang G-L, Sheng G-P, Tong Z-H, Liu X-W, Teng S-X, Li W-W, et al. Direct Electricity Recovery from Canna indica by an Air-Cathode Microbial Fuel Cell Inoculated with Rumen Microorganisms. Environmental Science & Technology. 2010;44(7):2715-20.

57. Rismani-Yazdi H, Christy AD, Dehority BA, Morrison M, Yu Z, Tuovinen OH. Electricity generation from cellulose by rumen microorganisms in microbial fuel cells. Biotechnology and Bioengineering. 2007;97(6):1398-407.

58. Sun Y, Zuo J, Cui L, Deng Q, Dang Y. Diversity of microbes and potential exoelectrogenic bacteria on anode surface in microbial fuel cells. The Journal of General and Applied Microbiology. 2010;56(1):19-29.

59. Kiely PD, Regan JM, Logan BE. The electric picnic: synergistic requirements for exoelectrogenic microbial communities. Current Opinion in Biotechnology;In Press, Corrected Proof.

60. Pant D, Bogaert GV, Diels L, Vanbroekhoven K. A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. Bioresource Technology. 2009:doi:10.1016/j.biortech.2009.10.017.

61. Cheng S, Logan BE. Ammonia treatment of carbon cloth anodes to enhance power generation of microbial fuel cells. Electrochemistry Communications. 2007;9(3):492-6.

62. Logan BE, Cheng S, Watson V, Estadt G. Graphite fiber brush anodes for increased power production in air-cathode microbial fuel cells. Environmental Science & Technology. 2007;41(9):3341-6.

63. Liu H, Cheng S, Logan BE. Power generation in fed-batch microbial fuel cells as a function of ionic strength, temperature, and reactor configuration. Environmental Science & Technology. 2005;39(14):5488-93.

64. Logan BE, Aelterman P, Hamelers B, Rozendal R, Schröder U, Keller J, et al. Microbial fuel cells: methodology and technology. Environmental Science & Technology. 2006;40(17):5181-92.

65. Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS. Methanogens: reevaluation of a unique biological group. Microbiol Mol Biol Rev. 1979;43(2):260-96.

66. Xing D, S. Cheng, Regan JM, Logan BE. Change in microbial communities in acetate- and glucose-fed microbial fuel cells in the presence of light. Biosensors & Bioelectronics. 2009;25(1):105-11.

67. Watson VJ, Logan BE. Analysis of polarization methods for elimination of power overshoot in microbial fuel cells. Electrochemistry Communications. 2011;13(1):54-6.

68. Hutchinson A. Effect of Carbon Fiber Brush Anode Surface Area on Single Chamber MFC Start up Time and Internal Resistance. University Park, PA: Pennsylvania State University; 2011.

69. Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences of the United States of America. 1985;82(20):6955-9.

70. Altschul SF, Gish W, Miller E, Myers EW, Lipman DJ. Basic logic alignment search tool. J Molecular Biology. 1990;215:403-10.

71. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Appl Environ Microbiol. 2009;75(23):7537-41.

72. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution. 2007;24:1596-9.

73. Ren N, Xing D, Rittmann BE, Zhao L, Xie T, Zhao X. Microbial community structure of ethanol type fermentation in bio-hydrogen production. Environmental Microbiology. 2007;9(5):1112-25.

74. Xing D, Ren N, Rittmann BE. Genetic diversity of hydrogen-producing bacteria in an acidophilic ethanol-H2-coproducing system, analyzed using the [Fe]-hydrogenase gene. Applied and Environmental Microbiology. 2008;74:1232-9.

75. Bassam BJ, Caetano-Anollés G, Gresshoff PM. Fast and sensitive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry. 1991;196(1):80-3.

76. Kiely P, Call D, Yates M, Regan J, Logan B. Anodic biofilms in microbial fuel cells harbor low numbers of higher-power-producing bacteria than abundant genera. Applied Microbiology and Biotechnology. 2010;88(1):371-80.

77. Pommier T, CanbÄCk B, Riemann L, BostrÖM KH, Simu K, Lundberg P, et al. Global patterns of diversity and community structure in marine bacterioplankton. Molecular Ecology. 2007;16(4):867-80.

78. Liu H, Cheng S, Logan BE. Production of electricity from acetate or butyrate in a single chamber microbial fuel cell. Environmental Science & Technology. 2005;39(2):658-62.

79. Ercolini D, Moschetti G, Blaiotta G, Coppola S. Behavior of Variable V3 Region from 16S rDNA of Lactic Acid Bacteria in Denaturing Gradient Gel Electrophoresis. Current Microbiology. 2001;42(3):199-202.

80. Meroth CB, Walter J, Hertel C, Brandt MJ, Hammes WP. Monitoring the Bacterial Population Dynamics in Sourdough Fermentation Processes by Using PCR-Denaturing Gradient Gel Electrophoresis. Appl Environ Microbiol. 2003;69(1):475-82.

81. Jung S, Regan JM. Comparison of anode bacterial communities and performance in microbial fuel cells with different electron donors. Applied Microbiology and Biotechnology. 2007;77:393-402.

Appendix A

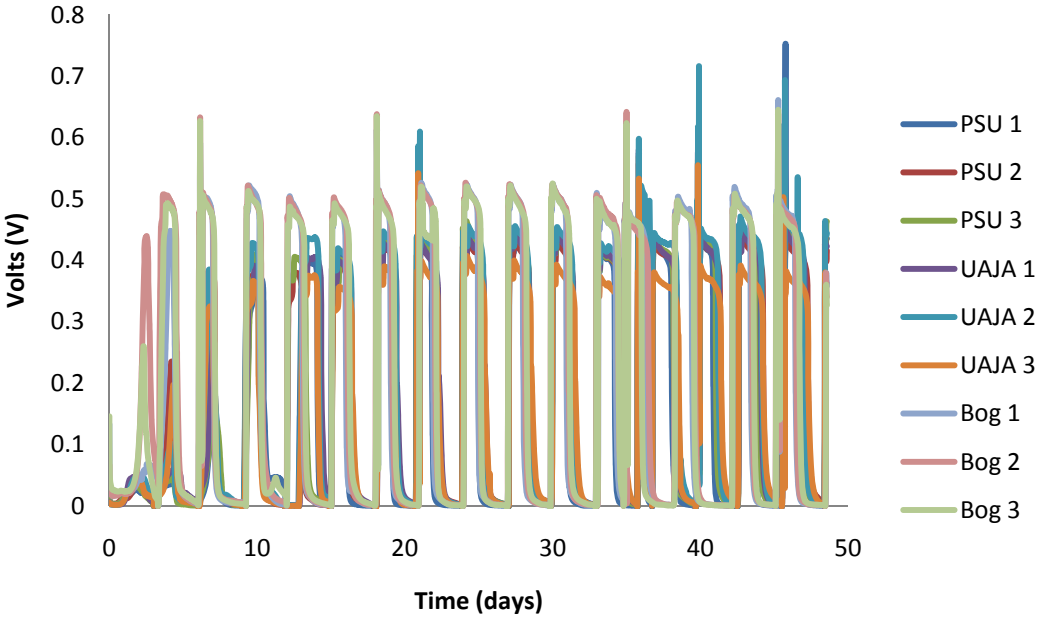

Figure A.1 Voltage versus time graph for all reactors. Spikes in voltage represent times where power density curves were taken.

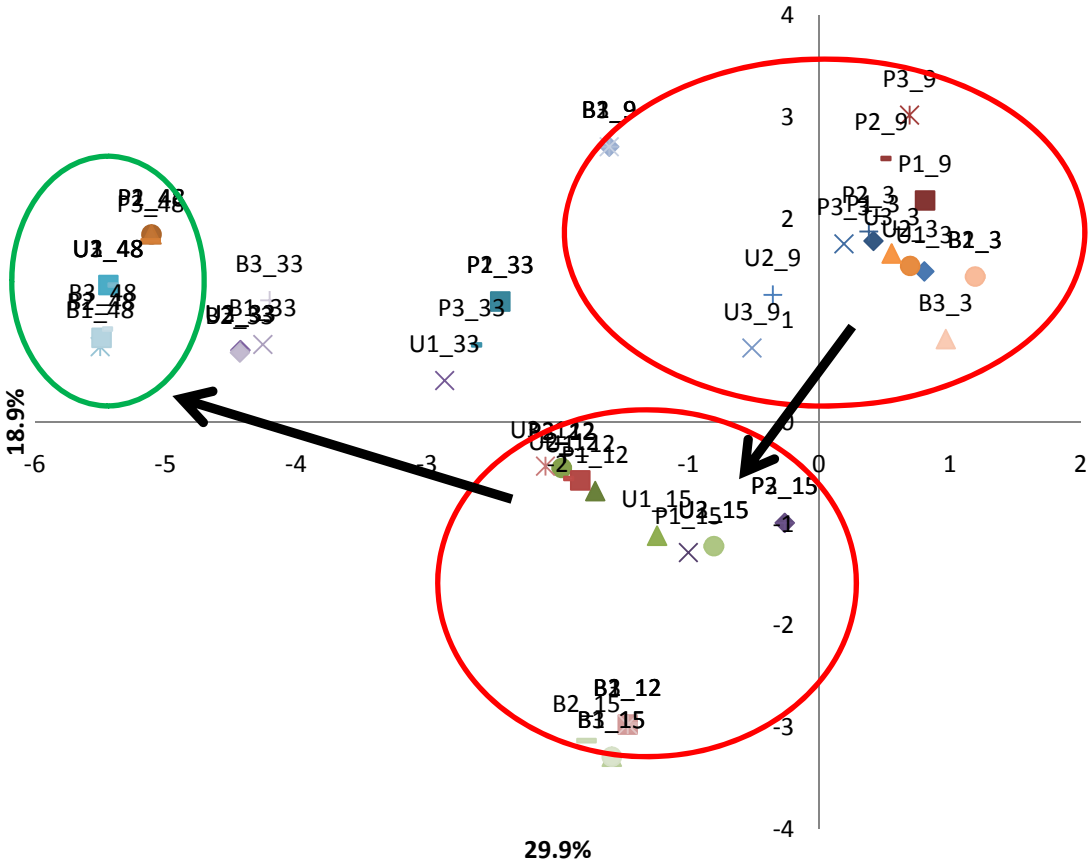

Figure A.2 PCA plot of all time points for all replicates of all inoculum sources. The plot shows a general trend from right to left with the inoculum sources converging at day 48. Between days 3 and 48, the Bog inoculated reactors are separated from the wastewater reactors, which are similar throughout the study. This plot was obtained using the "manual comparison" method.

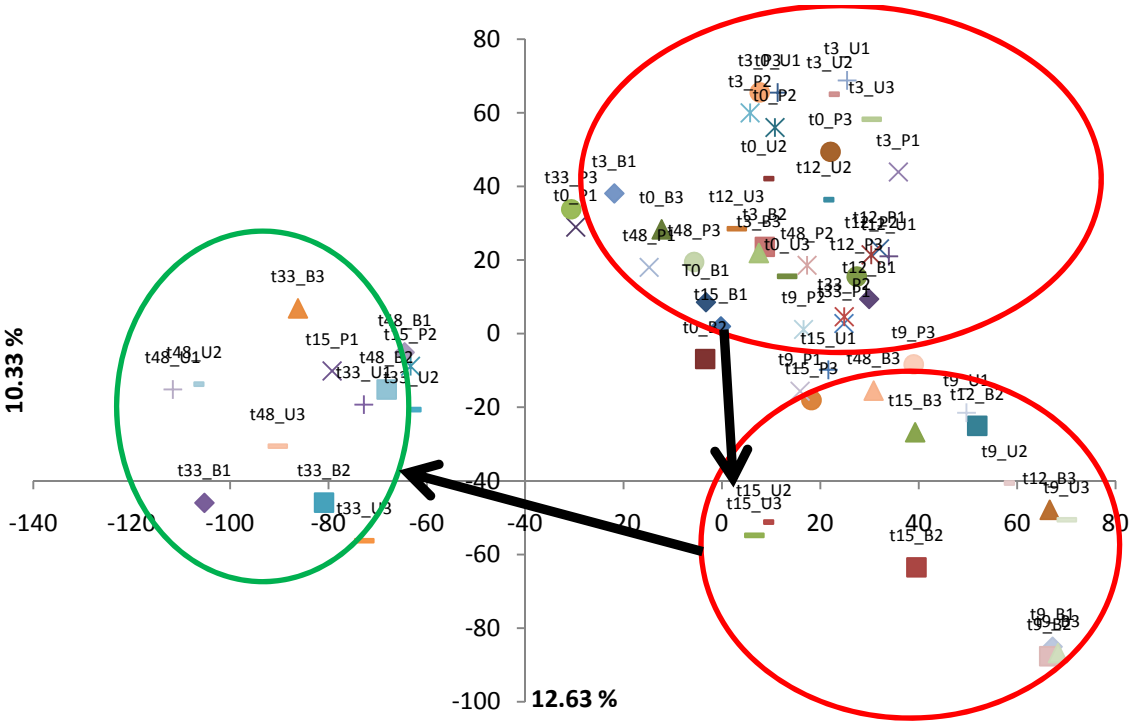

Figure A.3 PCA plot of all time points from all reactors using GelCompar II. This plot gives the same general trend as using the manual comparison method and shows a general convergence of all inocula after 16 cycles.