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**EFFECTS OF ACCLIMATION METHODS ON ANAEROBIC FLUIDIZED BED
MEMBRANE BIOREACTORS AND METHANOGENIC MICROBIAL
ELECTROLYSIS CELLS**

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by

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ABSTRACT

Microbial electrochemical technologies (METs) can help address energy needs by recovering energy from wastewater, decreasing energy input for wastewater treatment, and generating useful fuels. Microbial fuel cells (MFCs) can be used to produce electricity from organics, but they cannot treat the wastewater to levels needed to meet discharge limits while producing power. Anaerobic fluidized bed membrane bioreactors (AFMBRs) are an energy-efficient method for treating low strength wastewater, and thus they can be used to treat MFC effluent to meet discharge standards. Methanogenic microbial electrolysis cells (MECs) generate methane by fixing carbon dioxide. The purpose of these studies was to examine the effect of different acclimation methods on the performance and microbial communities of AFMBRs and MECs.

Different AFMBR startup methods were compared to determine effect of startup on reactor performance, and to assess microbial communities in AFMBRs. Similar (t-test, $\alpha = 0.05$, $P > 0.4$) COD removal was achieved for diluted wastewater using AFMBRs acclimated to methanol (M), wastewater (W), or anaerobic digester sludge (D), with an average COD removal of $63 \pm 12\%$. Acclimation to acetate (A) significantly increased COD removal to $84 \pm 6\%$. Improved removal efficiency by acetate acclimation was also seen after exposing reactor M to acetate feed for a week. When reactor M was subsequently fed diluted wastewater, the COD removal increased to $70 \pm 6\%$. Microbial communities on the GAC were significantly different ($P < 0.05$) from those in the influent and reactor fluid, and were distinguished by greater abundances of *Geobacter*, sulfur-reducing bacteria, *Syntrophaceae*, and *Chlorobiaceae*. Reactor

A demonstrated the highest relative abundance of *Geobacter*. These studies showed that acetate was a useful substrate for acclimation of AFMBR communities to achieve improved COD removal.

The cathode chamber of methanogenic MECs was inoculated with microbial communities pre-acclimated with GAC and chemical substrates to improve methane generation. Hydrogen-acclimated GAC had the highest relative abundance of *Methanobacterium*, which has been shown to dominate mixed-culture MEC archaeal communities. However, methane production from hydrogen-acclimated MECs was similar to communities acclimated to methanol and a VFA mix, which all averaged $23.5 \pm 9.7 \text{ nmol cm}^{-3} \text{ d}^{-1}$. Startup time was also insensitive to the substrate used in pre-acclimation. Pre-acclimation of communities with GAC promoted increased methane generation compared to MECs directly inoculated with GAC and an inoculum of bog sediment ($12.7 \pm 4.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$). Methane generation ceased after two cycles for MECs operated with bog sediment inoculum only. GAC could be a good growth support for enriching microbial communities associated with methanogenic MECs and exocellular electron transfer, as GAC communities had higher relative abundances of *Methanobacterium*, *Geobacter*, and sulfur-reducing bacteria which are known to be associated with microbial communities that develop in well-performing bioelectrochemical systems.

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Chapter 1

Introduction

Between 1950 and 2010, energy consumption in the United States has nearly tripled to 98 quadrillion BTU (EIA, 2012). Fossil fuel non-renewable sources comprise 83% of the 2010 U.S. energy consumption. Although the U.S. only makes up 4% of the world population, it uses 16% of the world's primary energy sources (IEA, 2015). Both the high energy consumption from nonrenewable sources and the high energy consumption of the U.S. specifically motivate efforts to promote alternative methods for energy production, reduce energy use, and use resources more effectively. Innovative wastewater treatment methods can address each of these objectives.

Increased treatment efficiency can decrease wastewater treatment energy requirements, which currently makes up 3% of U.S. electricity consumption (Water, 2006). Typical wastewater treatment, consisting of aerobic activated sludge and anaerobic sludge digestion, requires 0.6 kWh/m³ of wastewater treated. About half of this energy requirement is for aeration systems (McCarty, 2011). Instead of continuing to input this energy to treat wastewater as a waste, wastewater can be used as a resource. Energy potential in the organics alone can exceed the energy required to treat wastewater. One study estimated that a 500 mg COD/L wastewater stream can produce 1.93 kWh/m³ of energy (McCarty, 2011). In a study on waste streams in North Toronto, raw wastewater with 431 ± 8 mg COD/L was found to contain 1.76 kWh/m³ of energy (Shizas, 2004). When a freeze-drying method of sample preparation was used, less volatiles were lost prior to calorimetry, and an even higher energy content was measured: 2.1 ± 0.3 kWh/m³ for 427 mg COD/L domestic wastewater (Heidrich, 2011). The energy recovered

from organics in wastewater can be used to offset energy requirements to obtain safe effluent concentrations.

1.1 Energy recovery from wastewater using microbial electrochemical technologies

Microbial electrochemical technologies (METs) can treat wastewater while recovering energy from organics, using microbes that can transfer electrons exocellularly. In METs treating wastewater, biological oxidation of the organics occurs at the anode, treating the wastewater and sending electrons through the circuit (Logan, 2009). A wide variety of organics can be used as a substrate in METs (Pant, 2010).

In microbial fuel cells (MFCs) used for wastewater treatment, oxygen is reduced at the cathode in a spontaneous reaction, so energy in the wastewater is recovered as electricity. The cathode can operate through passive oxygen transfer to the cathode from air (Liu, 2004), which eliminates the need for energy-intensive aeration. In microbial electrolysis cells (MECs), an external voltage is applied to drive a non-spontaneous reaction, generating hydrogen or methane at the cathode. Hydrogen can be used as an energy source or in chemical synthesis. Methane generation occurs by biologically fixing carbon dioxide. If renewable sources of electricity are used, methanogenic MECs can be a carbon-neutral way of converting intermittent renewables into a fuel which is easy to store and transport, and can be used within the existing natural gas infrastructure.

METs treating wastewater require post-treatment in order to maximize energy or fuel generation, while still meeting discharge limits of 30 mg COD/L (EPA, 2010). MFCs exhibit a rapid decline in current production when wastewater is treated to less than 100-150 mg COD/L

(Zhang, 2015). MECs require high hydraulic retention times (HRTs) of over 30 h to produce high-quality effluent, as compared with 3-5 h for conventional secondary treatment (Metcalf & Eddy, 2003). A shorter time of 3–10 h is optimal for energy consumption and hydrogen production in MECs (Escapa, 2016).

1.2 Methanogenesis in MECs

Methane generation can occur through three main pathways. Acetoclastic methanogenesis converts acetate into methane and carbon dioxide. Hydrogenotrophic methanogenesis converts hydrogen molecules and carbon dioxide into methane. In this method, fermentative bacteria may ferment organics to produce acetate, protons, and electrons. The protons and electrons may be combined to form hydrogen molecules, which can be transported to hydrogenotrophic methanogens.

In a third mechanism, methanogens can directly uptake protons and electrons, combining with carbon dioxide to produce methane (Rotaru, 2014). Direct electron transfer may be of advantage to microbes, as the extra chemical step of producing hydrogen is no longer required, along with the enzymes required for the process biologically (Lovely, 2011). Direct electron transfer also has lower energy requirements than hydrogenotrophic methanogenesis. Hydrogen evolution theoretically occurs at -410 mV at pH 7, but if the microbe accepts electrons from the electrode, this step is skipped. Carbon dioxide would then be combined directly with protons and electrons, which theoretically occurs at -240 mV (Siegert, 2014). Mixed conclusions have been made about whether direct electron transfer is the primary mechanism of methanogenesis in

MECs (Cheng, 2009), with several papers concluding that hydrogenotrophic methanogenesis is the primary mechanism (Hara, 2013; Lohner, 2014).

1.3 Wastewater treatment using anaerobic fluidized bed membrane bioreactors

One effective post-treatment method for METs is an anaerobic fluidized bed membrane bioreactor (AFMBR). This reactor combines an anaerobic fluidized bed reactor with a membrane bioreactor, using granular activated carbon (GAC) to both provide a surface for biofilm growth and to scour the membranes for fouling-prevention (Kim, 2011). Systems combining energy recovery systems with AFMBRs have been shown to meet effluent requirements for organics when treating domestic wastewater (Kim, 2016; Ren, 2014; Shin, 2014; Yoo, 2012). These combined systems have also been shown to achieve energy production with low energy requirements. The studies using methane-generation as the method of energy recovery use an anaerobic fluidized bed reactor (AFBR) upstream of the AFMBR. In an AFBR-AFMBR setup, the energy requirements were about one tenth of those required for conventional treatment (Kim, 2011). In an MFC-AFMBR system, the energy consumed by the system was less than the electricity produced in the MFC (Ren, 2014). AFMBRs can also be used as a stand-alone reactor for low-strength (200 mg COD/L) wastewater treatment (Bae, 2014).

1.4 Research Objectives

AFMBR performance has been examined in terms of COD removal, but the impact of AFMBR inoculum and acclimation procedures on reactor performance has not been previously studied. In AFBR-AFMBR systems, the AFMBR was inoculated either from previously run

reactors, or from the upstream anaerobic reactor. In MFC-AFMBR systems, microbes were inoculated in a separate anaerobic reactor. No studies of microbial communities within an AFMBR have been conducted to date.

Direct electron transfer between microbes has been promoted through GAC addition (Liu, 2012), and pre-acclimation can impact reactor performance. In hydrogenotrophic MECs, pre-acclimation methods have been shown to impact subsequent COD removal efficiencies (Ivanov, 2013; Ren, 2013; Ullery, 2015). Methanogenic MECs have shown improved methane generation when using inocula with a greater abundance of hydrogenotrophic methanogens (Siegert, 2015a). Hydrogenotrophic methanogens, largely *Methanobacterium*, are also microbes that predominate in methanogenic MECs (Cheng, 2009; Siegert, 2015a; Siegert, 2015b; Zhen, 2015).

The objectives of this AFMBR study were to assess several different inoculation and startup methods to determine approaches effective in promoting COD removal (Siegert, 2015a; Ullery, 2015; Yates, 2012), and compare microbial communities with COD removals to infer which microbes are important for AFMBR function. The goal of this MEC study was to improve methane generation through the introduction of GAC in an inoculation step, with subsequent transfer of the GAC into MECs.

1.5 Research collaborations

I conducted the operation of reactors M, W, and D for AFMBR experiments, all MECs, and all GAC acclimation tests. I also conducted all measurements associated with their operation, including COD, gas analysis, and conductivity, and took all DNA samples and

performed DNA extraction. The data reported here for the AFMBRs treating MFC effluent were obtained by Dr. Kyoung-Yeol Kim, who previously published results for performance relative to COD removal, but did not analyze the microbial community (Kim, 2016). The data reported here for the AFMBR with acetate inoculation (A) were obtained by Yaoli Ye. DNA samples were taken part way through the operation of both Dr. Kim and Mr. Ye's reactors; therefore, COD data presented here only represents a portion of their full test results. DNA analysis (PCR, sequencing, and data processing) was performed by Dr. Peiying Hong at KAUST.

Chapter 2

Literature Review

Microbial electrochemical technologies (METs) and anaerobic fluidized bed membrane bioreactors (AFMBRs) are useful ways to treat wastewater efficiently, by reducing energy requirements and by recovering energy from the wastewater. AFMBRs treat low-strength wastewater efficiently through eliminating high energy costs for aeration by using anaerobic processes, and high costs needed to use biogas for membrane souring by instead scouring using fluidized GAC. Microbial electrolysis cells (MECs) can treat wastewater at the anode while producing fuel at the cathode with the input of an external voltage. Methanogenic MECs biologically fix carbon dioxide, and can be a carbon-neutral source of methane when coupled with renewable energy technologies. This chapter will review performance and operation of AFMBRs and methanogenic MECs, and microbial communities in MECs. Microbial communities in AFMBRs have not been previously examined.

2.1 Anaerobic Fluidized-bed Membrane Bioreactors (AFMBRs)

2.1.1 AFMBR operation

AFMBRs to date have been used as a polishing step for electricity-generating MECs (Kim, 2016; Ren, 2014), and as a polishing step for methane-producing anaerobic fluidized bed reactors (AFBRs) (Bae, 2014; Dutta, 2014; Kim, 2011; Shin, 2014; Yoo, 2012). In AFMBR studies using municipal wastewater, effluents have met BOD discharge limits based on COD measurements, assuming a 2:1 COD/BOD₅ ratio (Hays, 2011; Mara, 2003). In a lab-scale

AFBR-AFMBR setup treating primary clarifier effluent, the overall system achieved 84% COD removal, and the AFMBR achieved 64% COD removal at a short overall HRT of 2.3 h (Yoo, 2012). At this detention time, the effluent contained 25 ± 10 mg COD/L and 7 mg BOD₅/L, well below the EPA limit of 30 mg/L BOD₅ over 30 days (NPDES, 2010). In a pilot-scale AFBR-AFMBR run for 485 days, the AFMBR achieved 91% COD removal with 14 ± 8 mg/L COD in the effluent (3 mg/L BOD) in the summer, and 88% COD removal with 21 ± 4 mg/L COD in the effluent (9 mg/L BOD) in the winter (Shin, 2014). In a study feeding primary clarifier effluent to lab-scale MECs and using an AFMBR as the polishing step, the system achieved 93% overall COD removal, with 85% COD removal in the AFMBR, and 16 ± 3 mg/L of COD in the effluent (Ren, 2014). Another study on an MFC-AFMBR system demonstrated treatment of domestic wastewater (358 ± 89 mg COD/L) to below discharge standards (36 ± 6 mg COD/L) (Kim, 2016).

Constant COD removal efficiency has been observed over different hydraulic residence times (HRTs) for an AFMBR treating MFC effluent (Kim, 2016). The AFMBR used in that study achieved $89 \pm 3\%$ COD removal at HRTs of 1.2 to 3.8 h. However, a constant percentage removal of the COD resulted in an effluent concentration that was directly related to overall COD loading. At a COD loading of 2.8 ± 0.3 g COD/L-d, the reactor effluent contained 43 ± 6 mg COD/L, while at a lower COD loading of 0.8 ± 0.3 g/L-d, a decreased effluent concentration of 31.5 ± 5 mg/L was obtained. This showed that increasing the reactor HRT did not improve removal, so higher quality effluent would require greater COD removal in an upstream reactor. The effluent concentration obtained in this study of 36 ± 6 mg COD/L was substantially higher than the 16 ± 3 mg COD/L effluent obtained by the previous MFC-AFMBR study (Ren, 2014). This difference may be caused by a higher proportion of soluble COD (sCOD) in the higher-

effluent reactor (70 ± 6 mg sCOD/L, 108 ± 16 mg/L total) than in the lower-effluent reactor (57 ± 14 mg sCOD/L, 107 ± 10 mg/L total) or the different acclimation procedures.

Energy costs for AFBR-AFMBR or MFC-AFMBR systems treating primary clarifier effluent are significantly lower than conventional aerobic treatment processes, and their operation costs can potentially be offset by electricity generation through organics energy-recovery. For the lab-scale AFBR-AFMBR system fed wastewater, 0.047 kWh/m³ of electricity was required for operation (Yoo, 2012), which is an order of magnitude lower than typical aerobic treatment requirements (McCarty, 2011). Methane produced in the system could be used to cover this energy cost, with 0.082 kWh/m³ produced, assuming all methane was recovered and a conversion efficiency of 33%. For the pilot-scale AFBR-AFMBR study, methane production (0.14 kWh/m³) could offset system energy requirements by optimizing headloss in the recycle line (0.13 kWh/m³), assuming all methane produced can be recovered (Shin, 2014). In an MFC-AFMBR study, energy production by the MFC (0.0197 kWh/m³) was approximately equal to system electrical energy requirements (0.0186 kWh/m³).

Membrane fouling can be successfully controlled in AFMBRs by GAC scouring, membrane relaxation, and periodic solids removal. Fluidized GAC is a low-energy method of preventing membrane fouling through scouring (Aslam, 2014), with lower energy requirements than biogas sparging (Kim, 2011). These methods are even effective at short HRTs (2 – 4 h) and high AFMBR substrate concentration (>200 mg/L COD) (Bae, 2014). Membrane relaxation, accomplished by cycling the effluent pump on and off, is used to aid in maintaining low transmembrane pressure (TMP). The use of this technique resulted in TMP at 0.1 bar for 192 days in a lab-scale study fed wastewater (Yoo, 2012), below 0.24 bar for a 485-day pilot-scale study (Shin, 2014), and below 0.06 bar over 50 days in a lab-scale MEC-AFMBR system

treating wastewater (Ren, 2014). Chemical cleaning, membrane removal, or backwashing was not needed in any of those studies. In another MFC-AFMBR study, TMP remained between 0.04 and 0.07 bar for ~60 days, before increasing to 0.18 bar over ~20 days (Kim, 2016). Before this time, no solids had been removed from the reactor. Periodic solids removal maintained the TMP between 0.09 and 0.12 bar for the remaining ~40 days of operation, which demonstrated the importance of solids removal in maintaining low TMP. Solids removal has also been shown to be a more effective fouling mitigation method than chemical cleaning in an AFBR-AFMBR system treating synthetic wastewater (Kim, 2011). Before solids removal was conducted, a sudden TMP rise to 0.38 bar was addressed through chemical cleaning. While the chemical cleaning decreased TMP to 0.08 bar, TMP again rose to 0.25 bar. Solids removal by removing 4% of the reactor volume per day maintained the TMP at 0.25 bar for about 20 days. After a second chemical cleaning was performed, continued solids removal maintained TMP below 0.1 bar for nearly 40 more days until the end of the experiment.

Anaerobic processes produce less biological solids than aerobic processes, which decreases solids handling costs. AFBR-AFMBR systems have shown low biological solids production of 0.031 g VSS/g COD removed and 0.051 g VSS/g COD removed, which is an order of magnitude lower than for traditional aerobic treatment using activated sludge (Shin, 2014; Yoo, 2012). An AFBR-AFMBR system achieved superior removal of pharmaceuticals from wastewater compared to an aerobic treatment system (Dutta, 2014). Overall, AFMBRs are an energy-efficient method of wastewater treatment, are capable of low effluent COD concentrations, reliable membrane fouling control, and have other attractive operational benefits such as good removal of pharmaceuticals from a wastewater.

2.1.2 GAC acclimation methods in AFMBRs

In AFBR-AFMBR systems, microbial communities have been established in the AFMBR by adding fresh GAC to the AFMBR reactor, and installing downstream of a pre-acclimated AFBR (Bae, 2014; Dutta, 2014; Shin, 2014; Yoo, 2012). The upstream AFBRs have been inoculated in various ways: inoculation of fresh GAC with digester sludge and fed with wastewater (Shin, 2014) or simulated wastewater (Dutta, 2014), or transferring GAC from another AFBR used to treat simulated wastewater (Bae, 2014; Yoo, 2012). One study, which examined an AFMBR-only system, inoculated the AFMBR with GAC from another AFBR (Bae, 2014). An MFC-AFMBR system, however, does not have an upstream methane-producing reactor to serve as the seed or acclimation step for the AFMBR. In a study by Ren et al. (2014), GAC was acclimated in serum bottles, using anaerobic digester inoculum and primary clarifier effluent substrate before transferring into the AFMBR. In another study by Kim et al. (2016), GAC was acclimated in a separate AFBR fed domestic wastewater. Different communities may have been produced in the upstream systems or inoculation reactors. It is unknown how different acclimation methods could impact subsequent startup times and performance.

2.2 Methanogenic Microbial Electrolysis Cells (MECs)

2.2.1 MEC operation

MECs used for methane generation fix carbon dioxide at a biotic cathode to generate methane. Cathodic production of methane may occur at lower potentials than hydrogen generation. Conversion of electrons and protons into hydrogen gas theoretically occurs at -410

mV (vs SHE) at pH 7, while direct conversion of electrons, protons, and carbon dioxide to methane theoretically occurs at -240 mV (Siegert, 2014). The anode may be operated abiotically through water-splitting, with the cathode fixing carbon dioxide to form methane. When methane generation is coupled with an organic substrate at the anode, external voltage input may theoretically be eliminated. The cell potential of a reactor oxidizing acetate and generating methane is theoretically positive (71 mV), but electrode overpotentials may preclude spontaneous current generation. Hydrogen generation from acetate oxidation in an MEC has a negative theoretical cell potential of greater than ~ 0.2 V (Cheng, 2009).

Two-chamber MECs have been operated at a range of cathodic set potentials, from -500 mV to -850 mV vs SHE (Cheng, 2009; Lohner, 2014; Siegert, 2014; Siegert, 2015b; Villano, 2011; Zhen, 2015). Single chamber reactors produced methane at an applied external voltage of 1 V for a pure-culture MEC (Hara, 2013), and 0.7 V for a mixed-culture MEC (Siegert, 2015a). Most two-chamber MECs have been operated with an abiotic anode (Cheng, 2009; Lohner, 2014; Siegert, 2014; Siegert, 2015b; Zhen, 2015). A study conducted by Villano et al. (2011) using a two-chamber MEC with a biotic anode containing *Geobacter sulfurreducens* and a mixed-culture cathode, was operated at an applied cathodic potential of -850 mV. Acetate oxidation was found to be rate-limiting.

2.2.2 MEC acclimation and communities

Performance of hydrogenotrophic MECs have been improved by pre-acclimation of single-chamber MECs to domestic wastewater in multiple studies (Ivanov, 2013; Ren, 2013; Ullery, 2015). Acclimation methods of methanogenic MECs have also been shown to impact

MEC performance and startup. In one study, single chamber methanogenic MECs were inoculated with bog sediment containing hydrogenotrophic methanogens or anaerobic digester sludge containing no detectable presence of hydrogenotrophic methanogens (Siegert, 2015a). Bog-inoculated reactors produced more methane under high inoculum ratios than digester-inoculated reactors. Cathodic communities for both inoculation methods converged to *Methanobacterium*, which is a hydrogenotrophic methanogen. This suggests that the presence of hydrogenotrophic methanogens in the inoculum increases MEC performance, and that these specific archaea are important for improved rates of methane production.

Hydrogenotrophic methanogens have been shown to dominate methanogenic MECs, even for single-chamber reactors with acetate as the feed (Lee, 2009; Siegert, 2015a) and when acetoclastic methanogens dominated in the inoculum (Siegert, 2015a). *Methanobacterium*, a type of hydrogenotrophic methanogen, has been found to be dominant in the archaeal communities of many methanogenic MECs (Cheng, 2009; Zhen, 2015). This predominance was found in tests using different cathode materials, excluding platinum (Siegert, 2015b). *Methanobacterium* also dominated the archaeal communities of single-chamber MECs fed acetate, for both an inoculum containing hydrogenotrophic methanogens, and an inoculum with no detectable hydrogenotrophic methanogens (Siegert, 2015a).

2.2.3 Role of direct electron transfer

Studies have examined whether methane is generated by direct uptake of electrons in MECs. If this mechanism occurs, electrons would be directly accepted from the cathode, and biologically combined with protons and carbon dioxide to produce methane. Methane production

from carbon dioxide, electrons, and protons occurs at a less negative potential than hydrogenotrophic methanogenesis. Therefore, MECs could operate at lower energy consumption if microbes directly accept electrons from the cathode. Evidence of direct electron transfer between species has been observed in anaerobic brewery wastewater treatment by *Methanosaeta*, and confirmed in a co-culture of *Geobacter metallireducens* and *Methanosaeta harundinacea* (Rotaru, 2014). GAC has been added to anaerobic digesters to enhance biological stability and accelerate methanogenesis during startup, but GAC may also benefit anaerobic digestion as a conductive mediator for electron exchange between cells (Liu, 2012). Evidence of direct interspecies electron transfer was shown in co-culture tests of *G. metallireducens* and *Methanosarcina barkeri*. Addition of GAC increased methane metabolism, with cells attached to GAC without aggregating or formation of cell-to-cell connections.

Studies disagree on whether direct electron transfer is the dominant mechanism for methane generation in MECs. Cheng et al. (2009) concluded that direct electron transfer to the biofilm occurred, because abiotic hydrogen evolution was too low to account for methanogenesis rates at the cathode for both mixed culture tests and pure-culture tests of *Methanobacterium plaustris*. Coating the cathode with a hydrogen scavenger did not substantially decrease current densities, further supporting this conclusion. Siegert et al. (2014) stated that direct electron transfer was a likely mechanism for methanogenesis in MECs, as abiotic hydrogen production rates are not sufficient to explain biotic methane generation rates. They stated that hydrogen production rates could be enhanced by biofilm uptake of evolved hydrogen, but that other tests show little improvement by attempts to increase hydrogen mass transfer. Other pure-culture studies conclude that direct electron uptake is not the primary mechanism in methanogenic MECs, although the methanogens examined in these studies are not the same as those that

predominate in MECs. In one study, a pure-culture of *Methanothermobacter thermoautotrophicus*, which is in the same family as *Methanobacterium*, was used in single-chamber MECs (Hara, 2013). Identical LSVs were obtained for biotic and abiotic reactors, resulting in the conclusion that consumption of electrodes at the cathode did not depend on the presence of the methanogen. Abiotic molecular hydrogen production in these tests was equivalent to biotic methane production. In another pure-culture study using *Methanococcus maripaludis*, wild-type *M. maripaludis* was compared with a hydrogenase-deletion mutant strain (Lohner, 2014). The mutant strain carried deletions of all five catabolic hydrogenase genes, and one anabolic hydrogenase gene, so the mutant strain could only uptake electrons from formate or directly from the cathode. Wild-type tests produced more methane than the mutant strain at -600 mV vs SHE set cathode potential, indicating that methane is generated by consumption of abiotically developed hydrogen. This was further confirmed when a more negative cathodic potential of -700 mV was applied, and methane production increased for the wild-type tests only. Another study confirmed that H_2 formed by soluble enzymes released by the cell is sufficient to explain methane generation in MECs using pure-culture *M. paripaludis* (Deutzmann, 2015).

Chapter 3

Impacts of AFMBR Acclimation and Startup on COD Removal and Microbial Communities

3.1 Abstract

Anaerobic Fluidized Bed Membrane Bioreactors (AFMBRs) are useful for energy-efficient treatment of low strength wastewater. AFMBRs have been used primarily as a polishing step for microbial fuel cells (MFCs) or methane-generating reactors, which recover energy from the wastewater. In this study, different inoculation and startup methods were examined to determine whether they would affect AFMBR operation. AFMBR performance, as measured by chemical oxygen demand (COD) removal, was insensitive to acclimation and startup procedure for reactors acclimated using methanol and bog sediment inoculum (M), domestic wastewater (W), and anaerobic digester sludge (D). All showed $63 \pm 12\%$ COD removal using diluted-wastewater feed to simulate MFC effluent. This COD removal was slightly less than the 67% removal obtained using an AFMBR to treat the effluent of an MFC fed with non-diluted wastewater from the same treatment plant. An inoculation procedure based on acclimation of the GAC to acetate (A) significantly improved COD removal to $84 \pm 6\%$. After reactor M was switched to acetate feed for one week, COD removal significantly increased to $70 \pm 6\%$ when it was subsequently fed diluted domestic wastewater (M_A). DNA sequencing was performed on samples from four AFMBRs to infer which microbes contribute to COD removal in these systems. GAC microbial communities were differentiated from inlet and reactor fluid samples by greater abundances of *Geobacter*, sulfur-reducing bacteria, *Syntrophaceae*, and *Chlorobiaceae*, with reactor A having the highest relative abundance of *Geobacter*. These results showed that

acetate was the most useful substrate for either initial acclimation or to improve AFMBR performance of an operating reactor, and that different communities evolve on GAC compared to those in the suspended solution.

3.2 Introduction

Microbial fuel cells (MFCs) can be used for energy recovery from wastewater. MFCs produce electricity by promoting microbial oxidation of organic matter on the anode, with reduction to water at the cathode through passive oxygen transfer (Liu, 2004). Current production in MFCs rapidly declines when the COD decreases to less than 100–150 mg/L (Zhang, 2015). Therefore, to treat wastewater to below discharge limits while maintaining high current production, a post-treatment system is required.

One effective post-treatment method is an anaerobic fluidized bed membrane bioreactor (AFMBR), which has been developed for treating low-strength wastewater. In an AFMBR, granular activated carbon (GAC) is used as a surface for biofilm growth enabling long solid retention times, and when fluidized it can be used to scour the membranes and minimize fouling that increases transmembrane pressure. Systems combining an AFMBR with an upstream anaerobic fluidized bed reactor (AFBR) for methane-generation have already proved to be effective in treating domestic wastewater (Shin, 2014; Yoo, 2012). MFC-AFMBR systems have also been shown to achieve both energy production and low effluent concentrations (Kim, 2016; Ren, 2014).

No studies have been conducted to date on how startup procedure affects subsequent COD removal in AFMBRs. Previously, AFMBRs have been inoculated by the influent

microorganisms when the reactor was operated downstream of other methane-generating reactors (Bae, 2014; Dutta, 2014; Shin, 2014; Yoo, 2012), or by transferring GAC from a separate methane-generating reactor (Bae, 2014; Kim, 2016; Ren, 2014). In this study, different approaches to AFMBR inoculation and startup were tested to look for methods promoting improved COD removal (Siegert, 2015a; Ullery, 2015), and community analysis was conducted to determine microbes that are important for wastewater treatment using this system.

3.3 Materials and Methods

3.3.1 Reactor setup and operation

The AFMBR reactor body (65 mL) was made of a clear PVC tube (30 cm tall, 16 mm diameter; U.S. Plastic Corp.) as previously described (Ren, 2014) (Figure 3.1). The reactor contained a submerged membrane module consisting of eight, 24 cm-long polyvinylidene-fluoride (PVDF) hollow fiber membranes each with 2.0 mm outer diameter and 0.8 mm inner diameter (0.1 μm pore size, Kolon Inc., South Korea). For biogas collection, a Hungate tube (10 mL, Bellco Glass Inc., Vineland, NJ) with the bottom cut off was epoxied onto the top of the reactor and sealed with a butyl rubber stopper (20 mm diameter, Chemglass Inc., Vineland, NJ). Plastic mesh (27 \times 27, McMaster-Carr) was used at the bottom of the reactor body to retain the GAC (DARCO MRX, 10 \times 30 mesh, Norit Activated Carbon). The GAC used was manufactured from lignite coal, and contained 0.22 mL/g micropores (< 2 nm), 0.35 mL/g mesopores (2 – 50 nm), and 0.45 mL/g macropores (> 50 nm), according to the manufacturer.

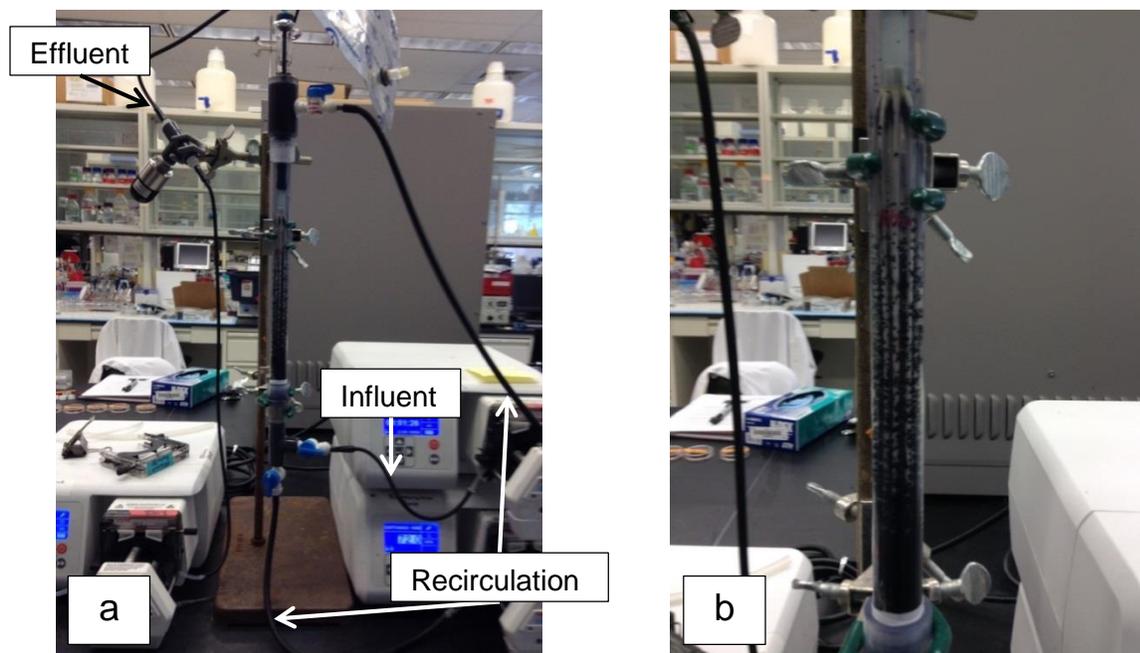


Figure 3.1. AFMBR with settling chamber (Reactor D), showing (a) the experimental setup and (b) a close-up view of the fluidized GAC.

The performance of the AFMBRs was assessed by measuring COD removal, to compare results to other studies which also used COD removal to assess AFMBR performance. COD was measured using standard methods (method 5220, HACH Company, Loveland, CO). Wastewater was diluted to 100 mg/L COD using distilled water for the feed. Sodium bicarbonate was added to increase the wastewater conductivity to 1.2 mS/cm, similar to that of a typical wastewater. If the pH approached 8.0 by sodium bicarbonate addition, sodium chloride was instead added to complete the conductivity increase. The feed reservoir was placed on ice, and the wastewater warmed to room temperature in the feed tube prior to entering the reactor.

Reactors were operated at an HRT of 1 h by cycling the pump for 10 minutes on at 1.2 mL/min, and then 1 minute off. This on/off cycling helps reduce fouling through membrane relaxation (Yoo, 2012). GAC was fluidized by recirculating liquid from inside the reactor at a

flowrate of 210 – 250 mL/min. Peristaltic pumps were used for influent, effluent, and recirculation lines (FH100M, Cole Parmer, Barrington, IL)

Methane was collected in a gas bag and measured by gas chromatography, (SRI 310C, SRI Instruments, Torrance, CA), and total gas volume was measured using a gastight syringe. Methane production in the gas phase was calculated by measuring the methane volume in the headspace over one to two days, with the reactor remaining sealed between measurements, as:

$$C_{m,g} = (V_2 - V_1) \times Q \times (t_2 - t_1)$$

where $C_{m,g}$ is the gaseous methane generation per volume wastewater treated, V_1 is the volume of methane in the headspace in the initial sample, V_2 is the volume of the final sample, Q is the wastewater flow rate, and t_1 and t_2 are the times of the initial and final samples of the measurement. To calculate the methane generation in the aqueous phase, the methane percentage in the headspace was averaged for the initial and final samples of each rate measurement. Saturated aqueous methane was calculated using Henry's law:

$$C_{m,l} = k_H y P_T V_m$$

where $C_{m,l}$ is the aqueous methane generation per volume of wastewater treated, k_H is the Henry constant for methane (0.014 mol/kg-bar), y is the percentage of methane in the headspace, P_T is the total pressure (1.101325 bar), and V_m is the molar volume of an ideal gas at 25 °C (24.465 L/mol) (Ren, 2014). An oversaturation of aqueous methane has been found in other anaerobic reactors of 124% (Smith, 2013, Ren, 2014). Therefore, the dissolved methane concentration was multiplied by 124% to obtain the final aqueous methane generation.

3.3.2 Acclimation and Startup methods

Different inoculum sources and startup procedures were used to assess their effect on reactor operation and performance. The inoculum sources each had different advantages. The bog sediment used is known to contain a diverse community of methanogens (Steinberg, 2008), while anaerobic digester sludge has very high concentration of methanogens but relatively low diversity, being composed mostly of acetoclastic *Methanosaeta* (Siegert, 2015a). Wastewater itself is also a rich source of microorganisms, with a relatively low solids content compared to the other samples. Three different startup methods were chosen for ease or to follow procedures used by others. Pre-acclimation of GAC in a fed batch reactor followed by transfer of the GAC to an AFMBR was examined based on a method previously used by Ren et al (2014). In a second method of inoculation, fresh GAC was added to an AFMBR operating without membranes, and membranes were later added. This method does not require a separate reactor for acclimation, and avoids membrane fouling while the biofilm is developing. The third method mimicked the startup of anaerobic membrane bioreactors and some AFBRs by using a long HRT of 11 h. The different inocula and procedures were identified here based on the distinguishing factors used in each startup method: methanol (M), wastewater (W), and anaerobic digester sludge (D).

For reactor M, GAC from a methanol-acclimated serum bottle (3.4 g dry weight) was added to the AFMBR reactor body and operated under 1 h HRT. Bog sediment was obtained from the Black Moshannon bog (40°54'20.6"N, 78°03'11.1"W) used in previous studies (Siegert, 2015a; Steinberg, 2008; Yates, 2012), and maintained in an anaerobic environment by flushing with nitrogen gas. Solids and fibers were removed from the bog sample by sieving under anaerobic conditions. The sieved sample was centrifuged for 5 min. at $7,650 \times g$ (Sorvall Evolution RC Centrifuge). Samples were decanted and mixed with bicarbonate-buffered

methanogen media (Siegert, 2015b) to create a 50/50 (v/v) slurry. Fed-batch serum bottles containing bog sediment slurry, GAC, and methanogen media were fed with methanol, a VFA mix (methanol, acetate, and propionate), hydrogen, acetate, or wastewater (without methanogen media). The methanol-acclimated GAC was selected from these different substrates to inoculate the AFMBR because methanol most quickly cultivated stable methane production, based on methane production from the bottles. The acetate-fed serum bottles inoculated in these tests failed to produce appreciable methane, with more detail available in Chapter 3.

To study the COD removal from only soluble COD, reactor M was exposed to an acetate feed for one week, followed by a filtered diluted wastewater feed for one week. Acetate feed was made by adding anhydrous sodium acetate to methanogen media, yielding 0.147 g/L sodium acetate. Filtered wastewater feed was made by diluting wastewater to 100 mg COD/L, adjusting conductivity to 1.2 mS/cm, and filtering to 0.45 μm with a nylon filter (Micron Separations, Inc, Westborough, MA). Reactor M was then switched back to dilute wastewater feed (M_A).

For reactor W, GAC was acclimated only to wastewater in the reactor in the absence of membranes. Fresh GAC (3.4 g) was washed and added into an AFMBR reactor body. The reactor was run without membranes at 1 h HRT for one month, with diluted wastewater as both the feed and inoculum source. Membranes were then added to the reactor and operated under normal 1 h HRT operation.

For reactor D, anaerobic digester sludge inoculum was added to the AFMBR, which was initially operated under long HRT. Secondary anaerobic digester sludge was obtained from the Penn State Wastewater Treatment Plant. Raw wastewater (340 mg/L COD), 3.8 g GAC, and 6.5 mL (10% v/v) of digester sludge were added to the AFMBR. The reactor was run without membranes under recirculation-only conditions (no influent or effluent flow) for one week to

emulate the AFBR startup used in another study (Shin, 2012). Membranes were then added, and the AFMBR was operated in continuous flow mode at an 11 h HRT with the feed of domestic wastewater (257 ± 60 mg COD/L) for three weeks, similar to previous startup methods used for both AFBRs (Shin, 2014) and anaerobic membrane bioreactors (Akram, 2008; Gao, 2010; Gao, 2011). Over one week, organic loading rates were gradually increased to the normal operational levels (1 h HRT, 2.5 g/L/d OLR) with dilute wastewater feed. To maintain anaerobic feed conditions, the feed was bubbled with N_2/CO_2 (20% CO_2 , 80% N_2), and gas bags containing N_2/CO_2 were attached to the closed feed bottle. A settling chamber was installed between the top of the reactor body and the Hungate tube to prevent overflow of GAC into the recirculation line.

GAC for reactor A was acclimated to acetate following acclimation in an AFBR fed with domestic wastewater. During acetate acclimation, GAC was transferred to a serum bottle and periodically fed a sodium acetate solution without media removal. GAC was later transferred to an AFMBR, which was operated at an HRT of 1.25 h.

The performance of reactors M, W, D, and A were compared to that of an AFMBR fed MFC effluent, as described in a previous study (Kim, 2016). The COD removals reported here were obtained from that study, but the microbial communities that developed in the AFMBR were not examined. GAC was acclimated in an AFBR fed with wastewater. The GAC from this reactor was then transferred to an AFMBR. The influent into the AFMBR was provided by the effluent of four MFCs, fed in two process trains (two reactors in series) with primary clarifier effluent (undiluted) obtained from the same source as the other AFMBRs listed here. The AFMBR in this study was operated at a slightly longer HRT of 1.2 h.

3.3.3 DNA testing and community analysis

Samples were taken from GAC, the reactor inlet, and reactor fluid for DNA extraction. DNA extraction of GAC was performed using 0.25 g samples. Four 13 mL influent samples were taken from the influent bottle where the tubing inlet was located. One 13 mL reactor fluid sample was taken from the main body of the AFMBR. All liquid samples were centrifuged at $4,500\times g$ for 1 h (Eppendorf 5804). Liquid samples were decanted, and cells were removed for DNA extraction by adding 750 μL of Power Soil kit bead solution and pipetting to mix. For inlet samples, the liquid was transferred to each centrifuge tube to incorporate the entire sample.

DNA isolation was performed using the MO Bio PowerSoil DNA extraction kit with the following modifications. Bead tubes with 0.1 mm glass beads were used instead of the garnet bead-beating tube included in the kit to prevent shearing of the DNA. The sample and 750 μL bead solution were added to the tube, and instead of vortexing for cell lysis, tubes were put to a bead mill (Bead Ruptor 12 Homogenizer, Kennesaw, GA) for 45 s on the medium setting. Tubes were centrifuged for 1 min rather than 0.5 min. Tubes were incubated at 4 °C for 10 min instead of 5 min.

PCR was performed on the isolated DNA using primer 515F-805R because of its coverage of both bacteria and archaea. Sequences were obtained using Illumina MiSeq amplicon sequencing and were classified using the Ribosomal Database Project (RDP) at a 95% confidence interval. To assess similarities among samples, Bray-Curtis similarities were computed based on square-root transformed relative abundances. Bootstrapped multidimensional scaling (MDS) plots were generated from these Bray-Curtis similarities using Primer-E software, version 7 (Harb, 2015).

3.4 Results and Discussion

3.4.1 COD removal efficiency

Pre-acclimation of GAC using acetate substrate (A) showed the highest COD removal of $84 \pm 6\%$ (Figure 3.3). This removal was significantly different from AFMBRs started up with methanol (M), wastewater (W), and anaerobic digester sludge (D) (t-test, $P < 5 \times 10^{-9}$). Reactors M, W, and D demonstrated similar COD removal of 62 – 64% ($P > 0.4$), despite differing inoculum sources and startup operation (Figure 3.2). The feed to reactors M and W were not significantly different ($P = 0.29$), with average influent CODs of 106 ± 25 mg/L for reactor M, and 112 ± 21 mg/L for W. Effluent concentrations for these reactors were also similar ($P = 0.29$), with 39 ± 14 mg/L for M and 43 ± 8 mg/L for W (Table 3.1). Reactor D had a slightly lower average influent COD of 92 ± 14 mg/L, with an effluent COD of 33 ± 13 mg/L. Constant percentage removal, with effluent COD directly proportional to organic loading was also observed by Kim, et al. (2016). The MFC-fed reactor achieved slightly higher removal ($P > 0.06$) than M, W, and D at $67 \pm 7\%$, with AFMBR feed averaging 114 ± 24 mg/L COD and effluent of 37 mg/L (Table 3.1).

After reactor M was fed with acetate and filtered dilute wastewater, COD removal by the AFMBR for diluted wastewater increased significantly ($P = 0.001$) to $70 \pm 6\%$ (M_A). During the period of acetate-feed, COD could not be measured due to interference of chloride and ammonia in the feed medium (Boyles, 1997; Kim, 1989). The reactor achieved $54 \pm 3\%$ sCOD removal during the following period with feeding of diluted wastewater.

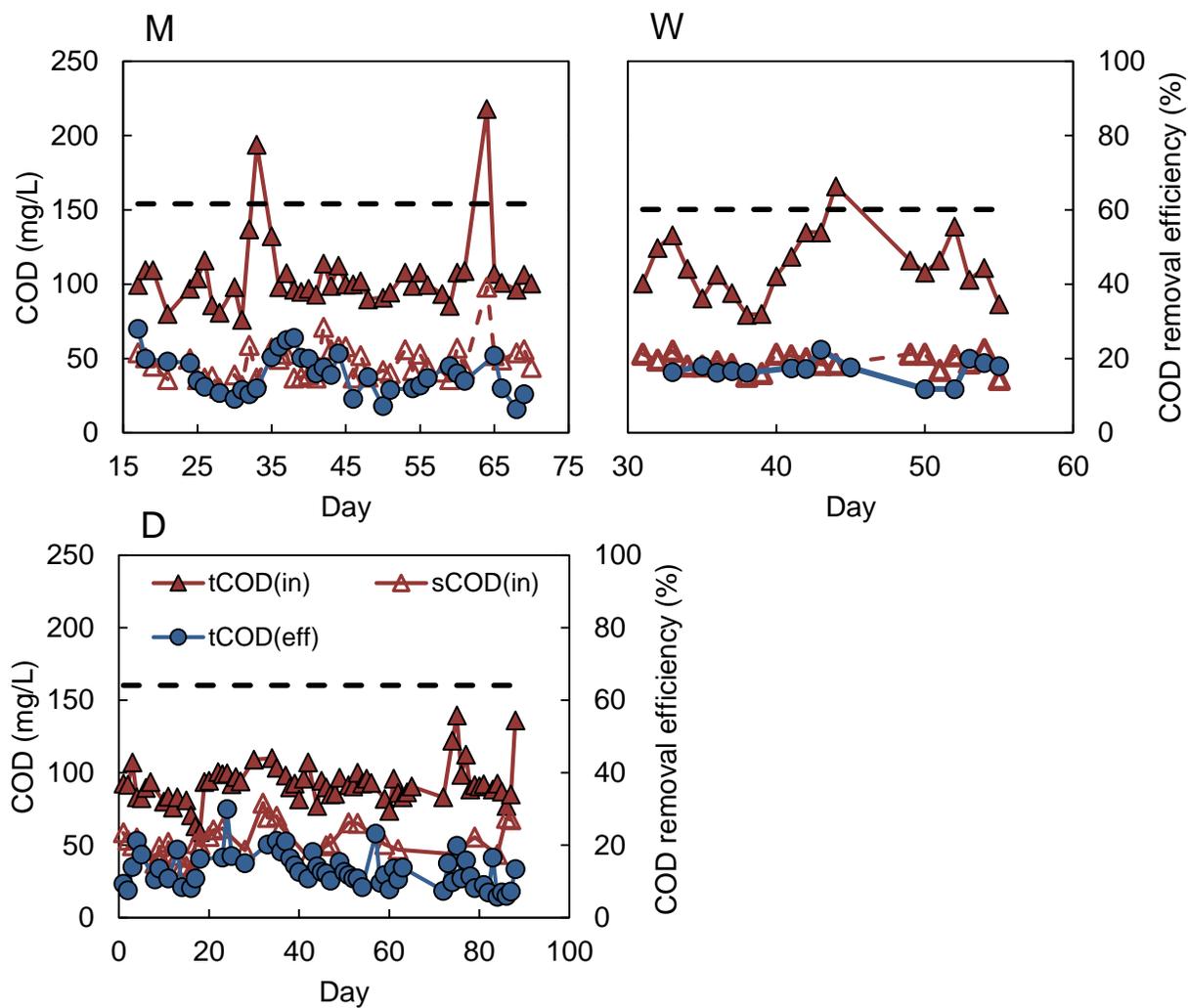


Figure 3.2. Influent total and soluble COD, effluent COD, and COD removal for the (M) methanol-startup AFMBR, (W) wastewater-startup AFMBR, and (D) digester sludge-inoculated AFMBR. Average COD removal was plotted as a dashed line.

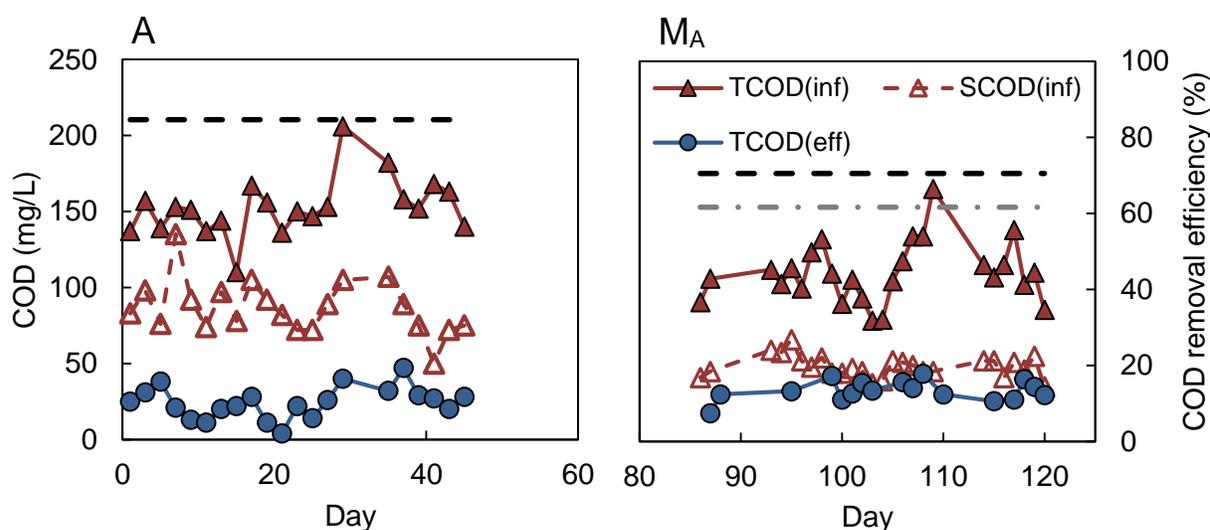


Figure 3.3. Influent total and soluble COD, effluent COD, and COD removal for operation of (A) AFMBR with fed-batch acetate feed included in startup, and (M_A) methanol-startup reactor after the soluble acetate feed and filtered wastewater feed. Averaged COD removal was plotted as a dashed line, while the average COD removal for reactor M is shown as a dashed and dotted line in plot M_A.

Table 3.1. COD removal for AFMBRs, where M is reactor M before the filtered feed test, M_A is reactor M after the acetate and filtered feed test, and MFC is the AFMBR fed with microbial fuel cell effluent.

	M	W	D	A	M _A	MFC
Influent tCOD (mg/L)	106 ± 25	112 ± 21	92 ± 14	153 ± 19	111 ± 20	114 ± 24
Influent sCOD (mg/L)	47 ± 11	48 ± 6	53 ± 11	87 ± 18	50 ± 7	72 ± 9
Effluent tCOD (mg/L)	42 ± 14	43 ± 8	33 ± 13	24 ± 10	33 ± 7	37 ± 5
COD removal (%)	62 ± 12%	63 ± 7%	64 ± 13%	84 ± 6%	70 ± 6%	67 ± 7%

Effluent COD has previously been observed to be directly proportional to COD loading (Kim, 2016). However, this relationship was not observed for the AFMBRs in this study (Figure 3.4). Reactor A received the highest COD loading, but shows the lowest effluent COD.

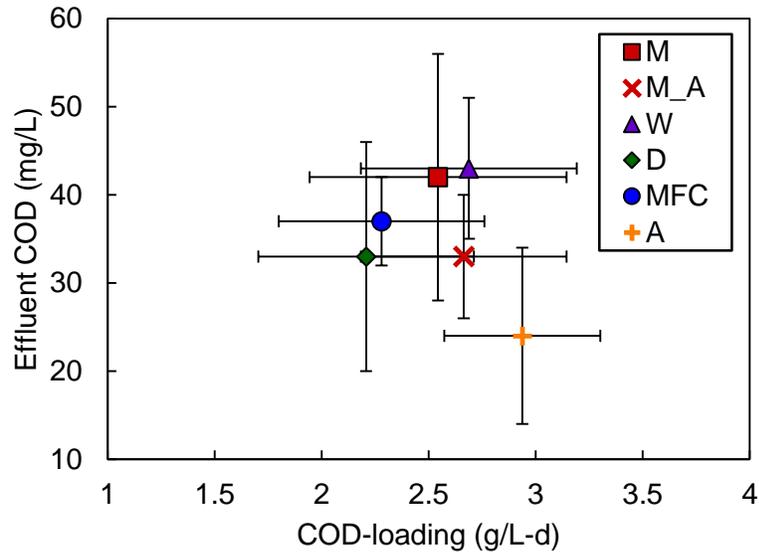


Figure 3.4. Relationship between COD loading rate on effluent COD for AFMBRs M, M_A, W, D, MFC-fed, and A.

All reactors reduced COD to levels permissible for discharge, assuming a typical COD:BOD₅ ratio of 2:1 (Mara, 2003), which has also been previously observed for wastewater samples from the same treatment plant used in this study (Hays, 2011). Effluent of lower-removal reactors M, W, and D averaged 36 ± 13 mg COD/L, or 18 ± 6 mg BOD₅/L, which is below the EPA secondary treatment standard of 30 mg BOD₅/L for a 30-day average (NPDES, 2010). Effluent COD concentrations for other reactors were even lower, with estimated effluent BOD concentrations of 18 ± 3 mg/L for the MFC-fed reactor, 17 ± 3 mg/L for reactor M_A, and 12 ± 5 mg/L for reactor A.

3.4.2 Methane generation

As COD removals were generally similar, methane generation was only monitored for reactor D. Gas samples were taken after day 10, when the AFMBR body was covered to prevent

light entry and visible algae growth. Two measurements were made of methane generation rate for days 16-18 and 62-63. For days 16-18, methane generation in the headspace was 0.54 mL methane/L wastewater treated, with a calculated 1.53 mL/L as dissolved methane, for a total of 2.1 mL/L. This volumetric production is in reasonable agreement with that previously obtained for the same reactor configuration by Ren et al. (2014) of 1.67 mL/L. The methane generation calculated for days 62-63 was an order of magnitude higher, at 13.5 mL/L total (1.15 mL/L gas, 12.4 mL/L aqueous). This single measurement may not represent methane generation for the entire reactor operation, but does indicate that increased methane generation may be obtained from this AFMBR configuration.

3.4.3 DNA sequencing results

DNA communities were examined for the GAC in reactors M_A, W, A, and the MFC-fed reactor, and compared those in suspension within the reactor body and in the feed. A comparison of biomass on the GAC can be seen in the appendix (Chapter 6), as estimated by DNA yield per gram of starting sample. Based on bootstrapping DNA sequencing results, microbial communities on all GAC particles were significantly different than those in the influent or reactor fluid ($\alpha < 0.05$) (Figure 3.5). Reactor fluid and influent samples were not significantly different from each other ($\alpha > 0.05$). The microbes that showed strong correlations among GAC samples (Pearson > 0.85), and that differentiated the GAC communities from the inlet and reactor fluid communities, were unclassified *Syntrophaceae*, sulfate-reducing bacteria (such as *Desulfocapsa*), unclassified *Bacterioidetes*, unclassified *Chlorobiaceae*, *Chlorobium*, and unclassified *Clostridiales* (Figure 3.6).

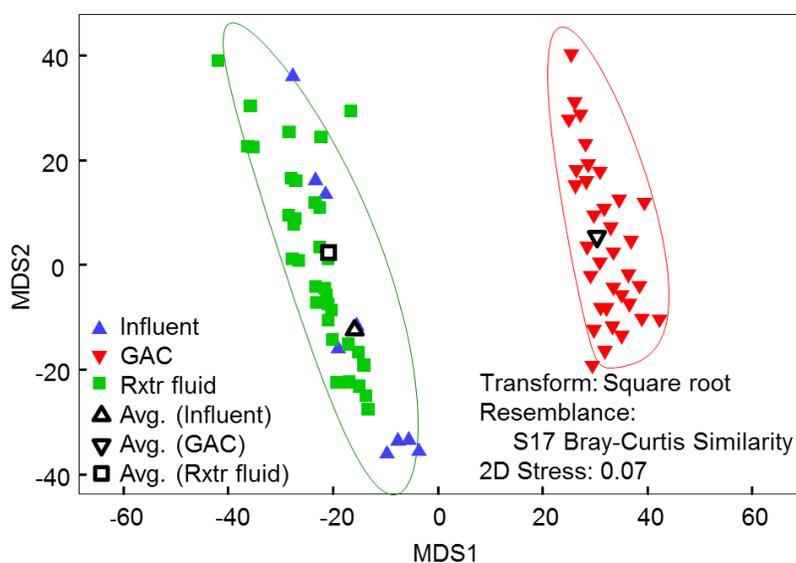


Figure 3.5. A multi-dimensional scaling plot based on bootstrapping results for influent, GAC, and reactor fluid samples from AFMBRs M_A, W, A, and the MFC-fed AFMBR.

Some of these distinguishing microbes on the GAC have connections to other systems exhibiting exocellular electron transfer. *Geobacter* and sulfate-reducing bacteria (*Desulfobulbus* and *Desulfomonas*) have been shown to transfer electrons exocellularly without mediators (Logan, 2009). *Desulfomicrobium* may play a role in microbiologically influenced corrosion (Duncan, 2014). Members of the family *Syntrophaceae* are strict anaerobes, and grow in conjunction with microbes that utilize H₂ or formate electron carriers. The family includes some sulfate-reducing bacteria (Kuever, 2014). Additionally, methanogenesis using a co-culture of *Geobacter metallireducens* and *Methanosarcina barkeri* has been enhanced by addition of GAC as a conductive surface for attached growth (Liu, 2012). While addition of GAC to co-cultures has been shown to promote direct exchange of electrons between species, its role in enriching *Geobacter* or SRB in mixed-culture studies has not been reported. GAC is more conductive than biologically produced

nanowires, so the greater conductivity of GAC may select for microbes capable of extracellular electron transfer. No methanogen enrichment was seen. The relative abundance of archaea measured, including methanogens, was lower than 1% of total reads for all GAC samples.

A high concentration of *Chlorobium* was found in the AFMBR treating MFC effluent. The genus *Chlorobium* and family *Chlorobiaceae* belong to the phylum *Chlorobia*, containing green-sulfur bacteria. Microbes within the genus *Chlorobium* are phototrophic obligate anaerobes. However, instead of undergoing oxygenic photosynthesis and producing oxygen, *Chlorobia* undergo anoxygenic phototrophy using hydrogen or sulfur as the electron donor (Eisen, 2002). *Chlorobium* can use sulfide and thiosulfate as electron donors, forming sulfate as an end product. Although this process does not release oxygen and compromise the anaerobic environment of the reactor, sulfate-reduction from the products of anoxic phototrophy can compete with methanogenesis, which is undesirable for methods relying on methane production for energy capture. Reactors M, W, and A were covered with aluminum foil during operation to prevent algae growth, but the MFC-fed reactor was operated without a cover, likely leading to the proliferation of phototrophic microbes.

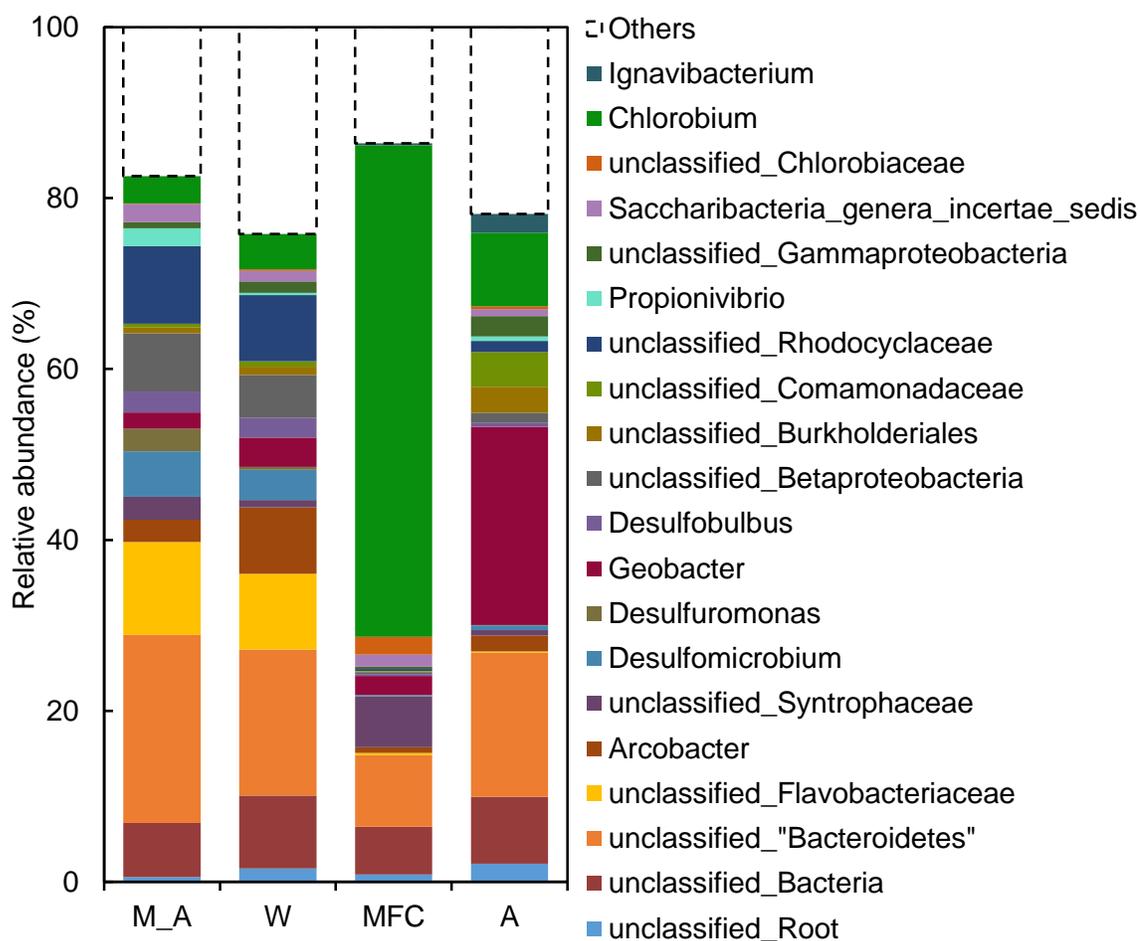


Figure 3.6. DNA sequencing results (>2% relative abundance) for GAC samples from M_A, W, MFC, and A AFMBRs.

Reactors M_A and W showed very similar communities (79% similarity, Bray Curtis, square root transformed). Reactor A still showed high similarity to M and W (62% for both), but showed slightly less SRBs and *Syntrophaceae*, and more *Geobacter* than M_A and W. The high abundance of *Chlorobium* in the MEC-fed reactor resulted in lower similarity among reactors M (54%), W (53%), and A (55%). Removing *Chlorobium* from the relative abundance plot for better visualization (Figure 3.7) shows that the MFC reactor also contains the distinguishing groups of *Syntrophaceae*, *Geobacter*, and SRBs.

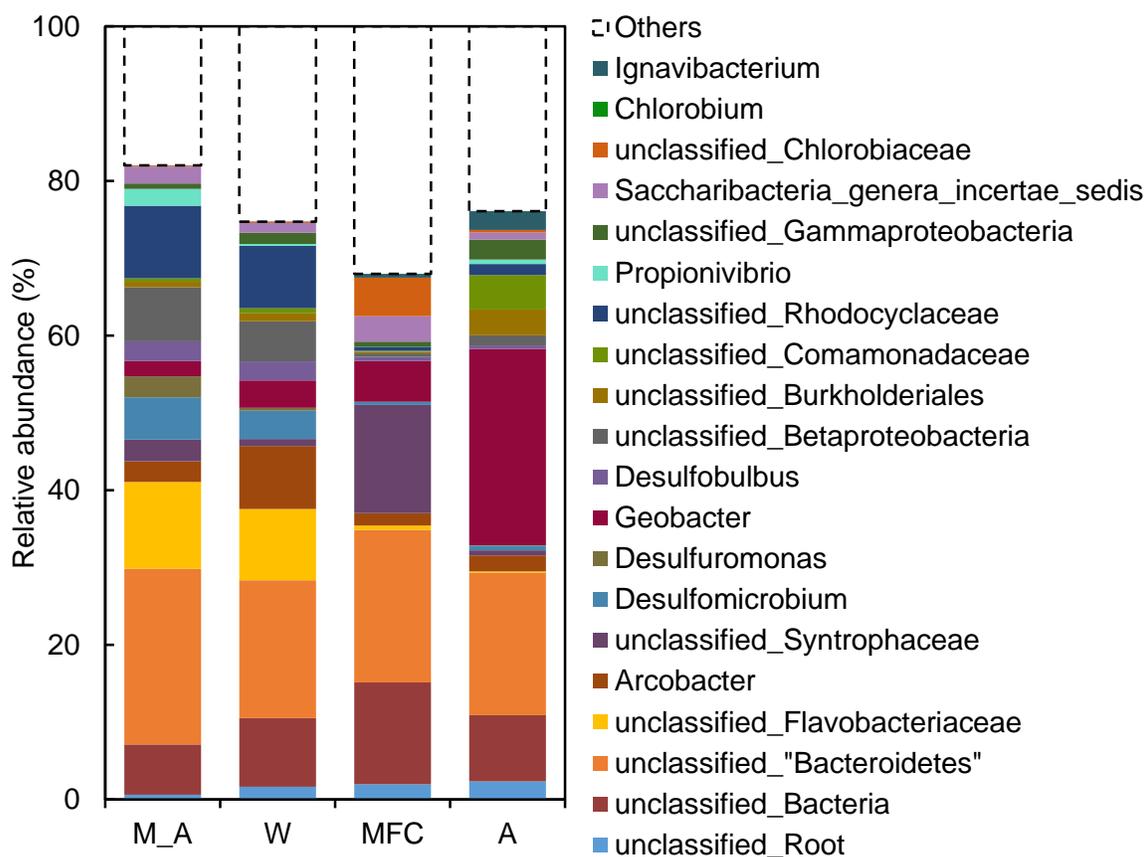


Figure 3.7. DNA sequencing results (>2% relative abundance) for GAC samples from M_A, W, MFC, and A AFMBRs, with *Chlorobium* removed and adjusting the total relative abundance to 100%.

There is less consistency of microbial populations among reactor fluid samples than among GAC samples (Figure 3.8). As reactors M_A and W received feed from the same bottle, they showed very similar microbial communities (77% similarity). Although reactor A also received a dilute wastewater feed from the same primary clarifier effluent, it showed low similarity to reactors M_A (35%) and W (31%). The AFMBR that received MFC effluent, rather than diluted wastewater, showed moderate similarity to reactors M_A (55%) and W (54%). Greater differences among reactor fluid for the four reactors tested suggest that the suspended environment is less selective for specific microbes.

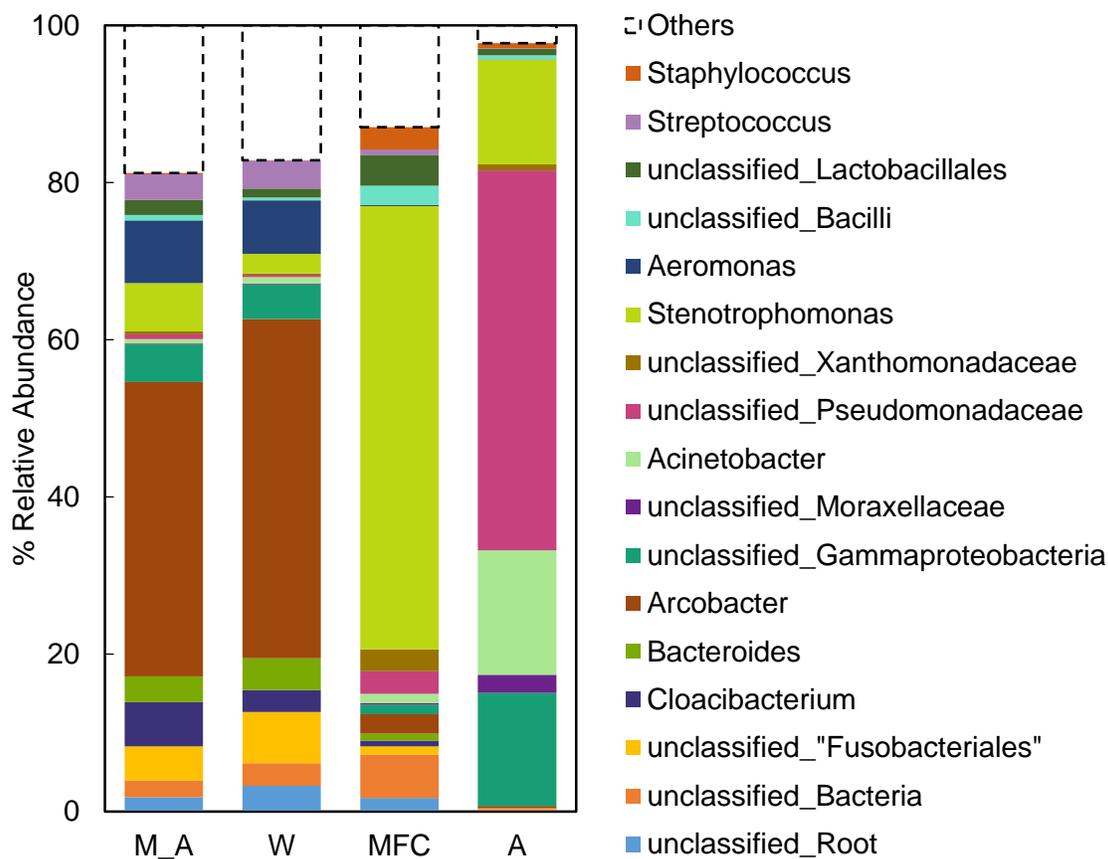


Figure 3.8. DNA sequencing results (>2% relative abundance) for reactor fluid samples from M, W, MFC, and A AFMBRs.

3.5 Conclusions

Acclimation of GAC to acetate significantly improved COD removal ($84 \pm 6\%$) compared to that obtained using other startup methods ($63 \pm 12\%$ COD removal) using a methanol feed and a bog sediment inoculum, wastewater as both feed and inoculum, or a wastewater feed with an anaerobic digester sludge inoculum. Exposing a methanol-acclimated reactor to acetate for one week also resulted in a subsequent increase in COD removal from 62% to 71%. *Geobacter* and sulfur-reducing bacteria have been previously associated with exocellular

electron transfer, and were two of the predominant microbial groups that distinguished microbial communities on GAC from those in the reactor fluid and influent. Activated carbon was a more selective environment for microbes (Pearson > 0.85) than the reactor fluid. These findings show that acclimation of GAC communities to an acetate substrate, or subsequent additional acclimation of a reactor using an acetate amendment, are useful methods for improving AFMBR performance.

Chapter 4

Effect of Pre-Acclimation of Granular Activated Carbon on Microbial Fuel Cell Startup and Performance

4.1 Abstract

Microbial electrolysis cells (MECs) can be used to generate methane for energy applications by fixing carbon dioxide, without requiring expensive catalysts. Methane production via direct electron transfer may decrease the applied voltage required to generate methane, as compared with evolving hydrogen for hydrogenotrophic methanogenesis. As GAC has been shown to stimulate direct transfer of electrons between species, microbial communities for MECs were pre-acclimated on GAC and fed different chemical substrates. GAC was then transferred into two-chamber MECs with carbon brush cathodes. MEC startup time and performance was insensitive to GAC acclimation substrate. Methane generation rates after the first three cycles were similar ($P > 0.4$) for GAC communities acclimated to hydrogen ($22 \pm 9.3 \text{ nmol cm}^{-3} \text{ d}^{-1}$), methanol ($25 \pm 9.7 \text{ nmol cm}^{-3} \text{ d}^{-1}$), and a volatile fatty acid (VFA) mix ($22 \pm 11 \text{ nmol cm}^{-3} \text{ d}^{-1}$), even though the use of a hydrogen feed selected for higher abundances of *Methanobacterium*. MECs started without pre-acclimation using GAC showed lower methane generation rates than pre-acclimated reactors ($13 \pm 4.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$), but performed better than MECs using the same inoculum without GAC. Compared to carbon brush cathodes, GAC selected for microbes which have previously been found in methanogenic MECs or have been associated with exocellular electron transfer. These results show that pre-acclimation of GAC to a chemical substrate before transferring to an MEC can improve startup time and methane generation as compared with no pre-acclimation.

4.2 Introduction

Microbial electrolysis cells (MECs) use microbes grown on electrodes to produce gaseous fuels with the addition of external electrical input (Siegert, 2015b). Certain microbes can be used to electrochemically produce hydrogen from the cathode via anodic biological reactions, or methane by the biological reduction of carbon dioxide at the cathode. When paired with a renewable source of electricity, methanogenic MECs can be a carbon-neutral way to generate methane. Although methane is less energy-dense than hydrogen, it is easier to transport and can be used with already available infrastructure.

At the anode of a methanogenic MEC, release of protons and electrons can be driven by biological oxidation of organics or by abiotic water splitting. Methane can be generated at the anode either through hydrogenotrophic methanogenesis, using hydrogen that is evolved at the cathode at -410 mV. Another method of methane generation is by direct uptake of electrons from the cathode, where electrons are combined protons and carbon dioxide at -240 mV (Siegert, 2014). Promotion of direct electron uptake in MECs is beneficial, as this mechanism consumes less energy than hydrogenotrophic methanogenesis. Some studies on mixed-culture MECs have concluded that direct electron uptake may be a primary mechanism for methanogenesis (Cheng, 2009; Siegert, 2014), and they found *Methanobacterium* as the dominant archaeal genus. Other studies of pure-culture MECs using different methane-generating archaea have concluded that hydrogenotrophic methanogenesis is instead the primary pathway for methane production in MECs (Hara, 2013; Lohner, 2014). Direct electron exchange between species has been enhanced in anaerobic digesters through the addition of granular activated carbon (GAC) (Liu, 2012).

In hydrogenotrophic MECs, pre-acclimation has been used to develop an electrochemically-active biofilm (Ivanov, 2013; Ren, 2013; Ullery, 2015). Performance of methanogenic MECs has also been increased by using an inoculum more similar to communities that were enriched in the MEC (Siegert, 2015a). Therefore, in this study, pre-acclimation of microbial communities on GAC with a chemical substrate before transferring to an MEC was tested to see if this acclimation method would improve subsequent methane generation rates, and decrease startup time.

4.3 Materials and Methods

4.3.1 Serum bottle acclimation

4.3.1.1 Setup

Bog sediment was chosen as an inoculum source for its microbial diversity, as it contains hydrogenotrophic methanogens (Steinberg, 2008), which have been shown to improve methane production in MECs (Siegert, 2015a). Sediment was obtained from the Black Moshannen bog (40°54'20.6"N, 78°03'11.1"W) and maintained under anaerobic conditions by flushing with nitrogen gas before storage. The sample was sieved to remove fibers in an anaerobic chamber. The sample was then centrifuged for 5 min at 7,650×g (Sorvall Evolution RC Centrifuge), decanted, and mixed with methanogen media to create a 50/50 (v/v) slurry.

An ammonium chloride electrolyte-based bicarbonate medium was used to support microbial growth (pH 7.0, containing 1.5 g/L NH₄Cl, 0.1 g/L KCl, and 0.6 g/L NaHPO₄). The medium base was sparged with nitrogen gas for 45 min, autoclaved, and cooled while flushing

with nitrogen gas to remove oxygen. Solutions of sodium bicarbonate, vitamins, and minerals were sterilized separately and added to the medium. Sodium bicarbonate (25 g/L) was added at 10% of the base medium volume. Vitamins and minerals each were added at 1% of the base volume (Vitamins: 2 mg/L biotin, 2 mg/L folic acid, 10 mg/L pyridoxin \times HCl, 5 mg/L thiamin \times HCl, 5 mg/L riboflavin, 5 mg/L nicotinic acid, 5 mg/L Ca-pantothenate, 5 mg/L B₁₂, 5 mg/L *p*-aminobenzoic acid, and 5 mg/L lipoic acid; Minerals: 1.5 g/L nitrilotracetic acid, 3 g MgSO₄ \times 7H₂O, 0.5 g/L MnSO₄ \times 2H₂O, 0.5 g/L NaCl, 0.1 g/L FeSO₄ \times 7H₂O, 0.1 g CoCl₂, 0.1 g/L CaCl₂ \times 2H₂O, 0.1 g/L ZnSO₄, 0.02 g/L NiCl₂ \times 6H₂O, 0.01 g/L AlK(SO₄)₂, 0.01 g/L H₃BO₃, 0.01 g/L Na₂MoO₄ \times 2H₂O, 0.01 g/L Na₂SeO₃, and 0.01 g/L Na₂WO₄).

4.3.1.2 Operation

GAC was acclimated in 120 mL glass serum bottles operated under fed batch mode, and run in duplicate. Serum bottles were prepared inside an anaerobic chamber, with each bottle containing 40 mL bicarbonate medium and 10 mL bog sediment slurry. GAC (3.4 g dry weight, DARCO MRX, 10 \times 30 mesh, Norit Activated Carbon) was added to each bottle. Substrates were added to the bottles by injection at the beginning of each cycle: methanol (M), a VFA mix (MAP), acetate (A), hydrogen (H), and wastewater (W) as substrates. For each reactor, control reactors were run in duplicate and operated identically, but they lacked substrate. Media was changed for a set of controls at the same time as their associated test duplicate. Methane generation by the controls was subtracted from maximum methane generation for the test bottles for each cycle.

M acclimation reactors were fed with 20 μL of methanol. MAP acclimation reactors were fed with 0.50 mL of a volatile fatty acid mixture containing 17 mL methanol/L, 37 g sodium acetate/L, and 10 g sodium propionate/L. Acetate and propionate were included to better simulate VFA diversity in wastewater, while methanol was added to promote rapid methane production (Aslam, 2014; Shin, 2012). To start cycle 1, H acclimation reactors were flushed with H_2/CO_2 (20% CO_2 , 80% H_2) for 15 minutes. For subsequent cycles, the headspace was flushed with H_2/CO_2 for 15 minutes, and then filled to 200 kPa. The A acclimation reactors were fed with 0.50 mL of a 100 g/L sodium acetate solution. Wastewater acclimation reactors (W) were fed with primary clarifier effluent (490 ± 90 mg COD/L) from the Penn State Wastewater Treatment Plant bubbled with nitrogen gas to remove oxygen. Wastewater (40 mL) was added to the reactor in the glove box instead of methanogen media.

Bottles were incubated on a shaker table at 31 °C and 80 RPM. Methane production was measured every 1–4 days and measured by gas chromatography (SRI 310C, SRI Instruments, Torrance, CA). The medium was changed after methane in the headspace plateaued. To change the medium, 40 mL of liquid was removed in the glove box after shaking. Fresh bicarbonate medium (40 mL) was added to the bottle. The bottles were flushed with N_2/CO_2 (20% CO_2 , 80% N_2) for 15 minutes to remove hydrogen from the headspace. Substrate was then added using a syringe.

4.3.2 MEC setup and operation

Two-chamber MECs were assembled from two glass bottles (VWR, Radnor, PA, U.S.A) with side-arms each 3.8 cm in length and 2.4 cm in inner diameter. Each half of the reactor held

100 mL of liquid, with 55 mL of headspace. The side arms were connected with a screw clamp (35/25, VWR, Radnor, PA, U.S.A), separated with a Nafion membrane (Fuel Cell Store, Boulder, CO, U.S.A), and sealed with an O-ring. Reactors were capped with PBT screw caps with a 3.5 cm hole (CG25, Corning, Corning, NY), and butyl rubber stoppers (20 mm, Chemglass, Vineland, NJ).

To prepare the electrodes, titanium wires (0.032 gauge, 12 cm in length) were cut and cleaned with coarse sand paper. For the anode, the wire was secured to a piece of ruthenium mixed metal oxide, and inserted through a black rubber stopper. For the cathode, the wire was attached to carbon brushes (23/311629, Millrose, Mentor, OH) by wrapping the wires around the brush stem. Brushes were baked at 450 °C for 1 h before use. The wire was inserted through black rubber stopper with a 4 mm hole for reference electrode. Reference electrodes used were Ag/AgCl electrodes (-200 ± 5 mV vs SHE; model RE-5B, BASi, West Lafayette, IN), filled with 3 M NaCl saturated with AgCl, and using molecular sieve frits (3A, 3-4 mm dia., Alfa Aesar, Lancashire, United Kingdom). Electrodes were refurbished by replacing the solution and frit.

Reactors were assembled in the glove box, with 100 mL methanogen media in the anode. Test reactors were assembled with 93 mL methanogen media and 7 mL (10 g wet weight) GAC from the serum bottle startup reactors in the cathode. Fresh GAC controls were made by adding 83 mL of bicarbonate medium, 7 mL GAC wetted with bicarbonate medium, and 10 mL bog sediment slurry to the cathode. For the bog-only controls, 90 mL of bicarbonate medium and 10 mL bog sediment slurry were added to the cathode. During the first three cycles, carbon brushes were not in contact with the lower GAC. During cycle 3 for all reactors, carbon brushes were

pushed into contact with the GAC. The headspace in the reactors was flushed with N₂/CO₂ for 15 min after assembly or media change.

Reactors were operated at a set cathode potential of –600 mV vs SHE (–800 mV vs Ag/AgCl electrode in 3 M sodium chloride). Methane volume in the headspace was measured weekly by gas chromatography. A cycle was ended when one of the duplicate reactors showed less than a 10% increase in headspace methane over a week, regardless of the performance of the other duplicate. Therefore, methane generation rate was chosen as a more representative metric for describing MEC reactor performance. To start a new cycle, 80 mL of liquid was removed from both the anolyte and catholyte, and 80 mL of fresh methanogen media was added to each in an anaerobic chamber. Catholyte with GAC or bog sediment was stirred by hand before removing liquid.

4.3.3 MEC calculations

Methane generation rate was used as the metric to assess MEC operation instead of total methane generated per cycle, as total methane produced was affected by the timing of media changes. Methane generation rate was calculated between each weekly headspace measurement. An overall methane generation rate for each cycle was found by averaging weekly rates, starting from the point of first measured methane in the headspace, and excluding the start of the stationary phase (less than 10% methane increase) (details in the Appendix).

Coulombic recoveries (*CR*) were used to assess how much of the charge transferred through the circuit was converted into methane. The *CR*s were calculated as:

$$CR = \frac{8 F V_m}{Q}$$

where CR is the coulombic recovery, F is the faraday constant, 8 refers to the number of electrons transferred in the reduction of CO_2 to methane, V_m is the volume of methane measured in the cathode headspace at STP, and Q is the total coulombs transferred through the circuit. Coulombs transferred was calculated as the integral current over time for the cycle.

4.3.4 DNA isolation

GAC samples were removed from the reactors at the end of reactor operation, with tests conducted using 0.25 g of GAC from each reactor. Carbon brush fibers were cut for DNA extraction, with fibers removed from the side of the brush facing the membrane. DNA extraction was performed on all of the brush fibers removed (~0.25 g). An MO Bio PowerSoil DNA extraction kit was used to isolate DNA from GAC and fiber samples. The same modifications were used as in Chapter 3, as well as DNA processing and sequencing methods.

4.4 Results and Discussion

4.4.1 GAC acclimation

Hydrogen-fed acclimation reactors (H) produced the greatest volume of methane, while methanol-fed acclimation reactors (M) most quickly promoted stable methane generation, as shown in Figure 4.1. The methane production of acclimation reactor M averaged 7.4 ± 1.2 mL (90% stoichiometric production), with a maximum of 9.1 mL (Table 4.1). Reactor H methane production was more variable, averaging 17.1 ± 15.5 mL per cycle (80% stoichiometric production), with a maximum of 39.1 mL. The VFA mix, consisting of methanol, acetate, and

propionate (MAP), produced approximately $\frac{1}{4}$ of the methane of the M reactors, averaging 1.9 ± 0.6 mg/L methane, with a maximum of 3.4 mg/L. The average production was only 18% of stoichiometric methane production, but is 53% of the stoichiometric production if only methanol was consumed. For the M, H, and MAP acclimation reactors, methane production increased with successive cycles. Both acetate-fed (A) and wastewater-fed (W) acclimation reactors did not demonstrate sustained methane production from VFAs or more complex organics.

Methane production for A and W acclimation reactors ceased on the third cycle. In cycles where there was detectable methane production, A reactors produced an average of 0.5 mL methane (4% of stoichiometric conversion), with a maximum of 1.2 mL. W reactors produced 0.4 mL on average (5% of stoichiometric conversion), with a maximum of 0.6 mL. The same outcome of minimal methane production was obtained for acetate feed sterilized by filtration, acetate feed sterilized by autoclaving, and acetate feed with five times the previous acetate concentration. The cause of this inability to generate methane from acetate was not determined, but reinforces the hypothesis that MAP acclimation reactors mainly produced methane from the methanol in the substrate.

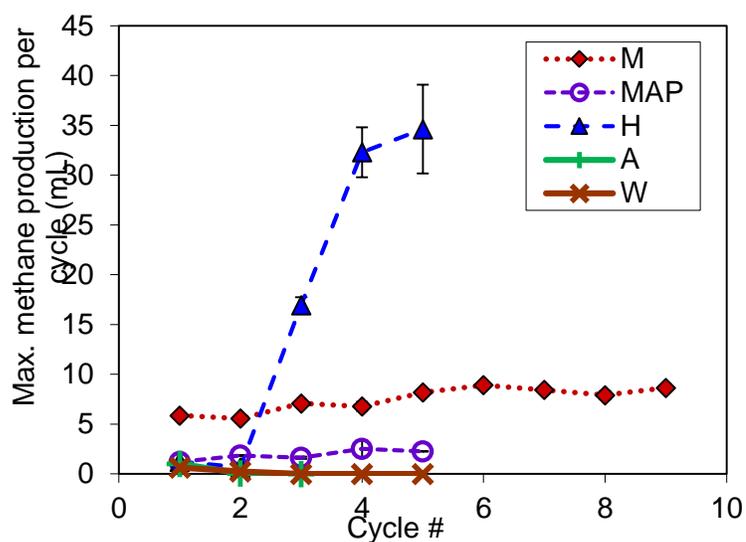


Figure 4.1. Maximum methane production per cycle for fed-batch serum bottle reactors with the following substrates: methanol (M); a methanol, acetate, and propionate mix (MAP); hydrogen (H); acetate (A); and wastewater (W). Error bars indicate the range for duplicates.

Table 4.1. Maximum methane production (mL at STP), and methane production based on stoichiometric conversion to methane (mL at STP) for GAC fed methane (M); a mixture of methanol, acetate, and propionate (MAP); and hydrogen (H).

Cycle	M		MAP		H	
	1	2	1	2	1	2
1	5.9	5.8	1.2	1.2	1.2	1.3
2	5.5	5.6	1.9	1.8	0.6	0.7
3	7.1	7.0	1.5	1.7	16.2	17.7
4	7.2	6.3	3.4	1.7	34.8	29.8
5	7.9	8.5	2.3	2.2	39.1	30.2
6	9.1	8.7				
7	8.6	8.3				
8	8.3	7.5				
9	8.8	8.4				
Average (all cycles)	7.4 ± 1.2		1.9 ± 0.6		17.1 ± 15.5	
Stoichiometric conversion	8.3		10.6		21.5	
% of stoichiometric conversion	90.0%		17.8%		79.7%	

4.4.2 MEC operation

GAC from M, H, and MAP acclimation reactors were used as the inoculum for M, H, and MAP MECs. As controls, fresh GAC with bog sediment inoculum (GAC+bog) was used, along with bog sediment only (bog) to see the effect of the GAC.

For MECs with pre-acclimated GAC, the cycle number had a greater effect on methane generation than acclimation substrate. ANOVA tests on the three pre-acclimated reactors (M, H, and MAP) showed that neither startup method nor cycle time significantly impacted the trend of methane generation rate (ANOVA, $P > 0.05$). However, this was likely due to poor replication by duplicates. By visual inspection of the data over time (Figure 4.2), it appears that methane generation rate increased with cycle number, but changes were not significantly different ($P > 0.05$) among pre-acclimated MECs when examining cycles 4-7 (excluding cycles without carbon brush/GAC contact). An average of $25.1 \pm 9.7 \text{ nmol cm}^{-3} \text{ d}^{-1}$ was achieved for M reactors, $22.3 \pm 10.8 \text{ nmol cm}^{-3} \text{ d}^{-1}$ for MAP reactors, and $22.1 \pm 9.3 \text{ nmol cm}^{-3} \text{ d}^{-1}$ for H reactors (Table 4.2). While there was no advantage of any specific method among the three different GAC acclimation methods, all three pre-acclimated MECs had statistically higher (t-test, $P < 0.04$) methane production rates than the fresh GAC+bog sediment control reactor, which produced methane at $12.7 \pm 4.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$. The GAC+bog sediment reactor showed significantly increased (regression, $P = 0.05$) methane production rates across subsequent cycles. Even if production rate would continue to increase, the MEC reactor was already operated for 441 days, over five times longer than hydrogen pre-acclimation (81 days) or MAP pre-acclimation (60 days). Such a long acclimation time would not be useful compared to the other methods. Methane production rate for the bog-only reactors decreased dramatically after the first cycle, with one duplicate producing no methane after the first cycle.

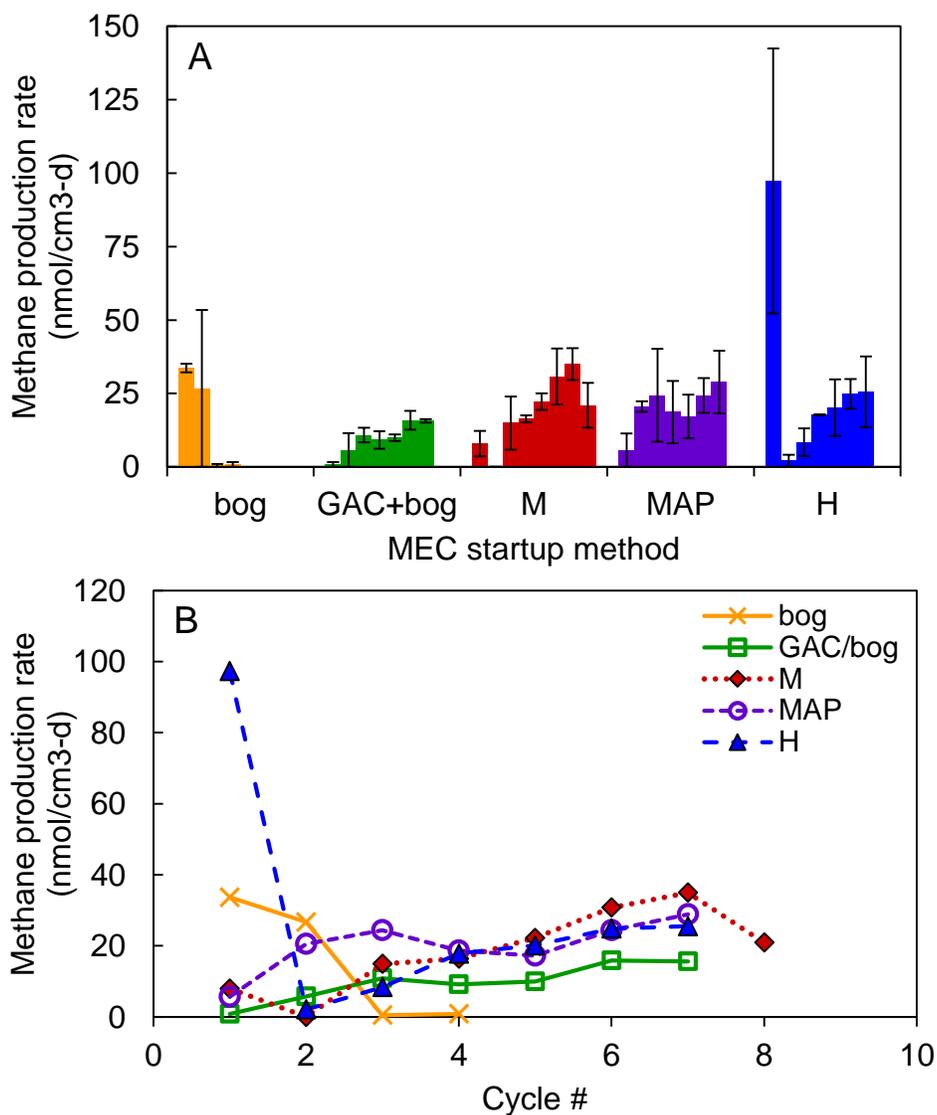


Figure 4.2. Both (A) and (B) show methane production rate across successive cycles in MECs (error bars show duplicate range). The MEC inoculum methods shown are: bog inoculum only (bog), bog inoculum with fresh GAC (GAC+bog), methanol-acclimated GAC (M), methanol/acetate/propionate acclimated GAC (MAP), and hydrogen-acclimated GAC (H).

Table 4.2. Methane generation rates ($\text{nmol cm}^{-3} \text{d}^{-1}$) for different MEC acclimations across successive cycles. Labeling is identical to that in Figure 4.2

Cycle	bog		GAC+bog		M		MAP		H	
	1	2	1	2	1	2	1	2	1	2
1	35.1	32.2	1.7	0.0	3.6	12.3	0.0	11.4	142.5	52.2
2	53.4	0.0	0.0	11.5	0.0	0.0	22.4	18.8	4.2	0.0
3	1.0	0.0	8.4	13.3	5.8	24.0	40.2	8.5	3.7	13.1
4	1.7	0.0	6.2	12.2	17.6	15.2	29.2	8.0	17.8	17.8
5			11.1	8.8	25.0	19.4	24.7	9.8	29.8	10.5
6			19.0	12.7	40.3	21.2	30.3	18.4	29.9	19.8
7			16.2	15.0	29.6	40.4	39.6	18.2	13.5	37.6
8					13.3	28.6				
Average (cycles 4-7)			12.7 \pm 4.1		25.1 \pm 9.7		22.3 \pm 10.8		22.1 \pm 9.3	

The reason for stable methane generation with the GAC+bog controls, while methane generation rate for the bog-only controls nearly ceased after cycle 2, is not clear. An inoculum ratio of 10% (v/v) was used for both types of control reactors, which is comparable to that used in some MEC studies (Siegert, 2015a; Villano, 2011), and ten times that of another two-chamber MEC study (Siegert, 2015b). The bog-only controls did not show effective consumption of electrons at the cathode, as the charge transferred for bog-only MECs was at least an order or magnitude lower than the other reactors (Table 4.3). Even during the initial cycles of high methane production rate (cycles 1 and 2), very little charge was transferred, with some duplicates exhibiting positive charge transfer. Thus, these results show that the GAC helped to facilitate charge transfer for microbes on the cathode.

Table 4.3. Coulombs transferred over each MEC cycle, including averages over cycles 1–4 and cycles 4–7. Labeling is identical to that in Figure 4.2

Cycle	bog		GAC+bog		M		MAP		H	
	1	2	1	2	1	2	1	2	1	2
1	-6.9E+0	4.2E+0	-1.6E+2	-1.2E+2	-1.2E+2	-1.7E+2	-6.9E+4	-1.3E+2	-4.9E+1	-1.7E+2
2	9.0E+0	-1.9E+0	-2.1E+1	-1.5E+2	-5.8E+1	-6.4E+0	-1.4E+2	-4.2E+1	-4.6E+1	-5.5E+1
3	-5.5E+0	-2.0E+1	-2.1E+2	-1.1E+2	-7.0E+1	-1.5E+2	-3.4E+2	-1.8E+2	-1.1E+2	-2.2E+2
4	-4.7E+0	-3.5E+0	-2.4E+2	-1.7E+2	-1.4E+2	-4.7E+1	-1.3E+2	-5.8E+1	-2.5E+2	-1.7E+2
5			-1.9E+2	-1.7E+2	-1.1E+2	-1.3E+2	-1.4E+2	-1.1E+2	-8.3E+1	-4.1E+1
6			-1.4E+3	-8.6E+2	-2.0E+2	-1.1E+2	-1.6E+2	-1.1E+2	-2.3E+2	-1.3E+2
7			-7.8E+2	-5.8E+2	-5.4E+1	-1.4E+2	-1.3E+2	-7.8E+1	-3.4E+2	-1.1E+3
8					-3.4E+1	-9.3E+1				
Avg. (1-4)	-3.7E+0 ± 8.6E+0		-1.5E+2 ± 6.7E+1		-9.5E+1 ± 5.8E+1		-8.7E+3 ± 2.4E+4		-1.3E+2 ± 8.0E+1	
Avg. (4-7)			-5.5E+2 ± 4.4E+2		-1.2E+2 ± 5.0E+1		-1.1E+2 ± 3.4E+1		-2.9E+2 ± 3.3E+2	

CRs based on recovery of methane for the measured current showed similar trends to methane production rate, with similar rates for reactors M, MAP, and H, and lower CRs for GAC+bog (Figure 4.3). The high initial CR for the bog-only MECs is an artifact of low current uptake by the microbes, suggesting that the methane produced was from organics in the inoculum. Methane measurements were only taken from the cathode chamber. Toward the end of the tests, methane was observed in the anode chamber. Therefore, the coulombic recoveries reported here do not reflect all of the current that was captured as methane, as some methane migrated into the anode chamber and was not measured.

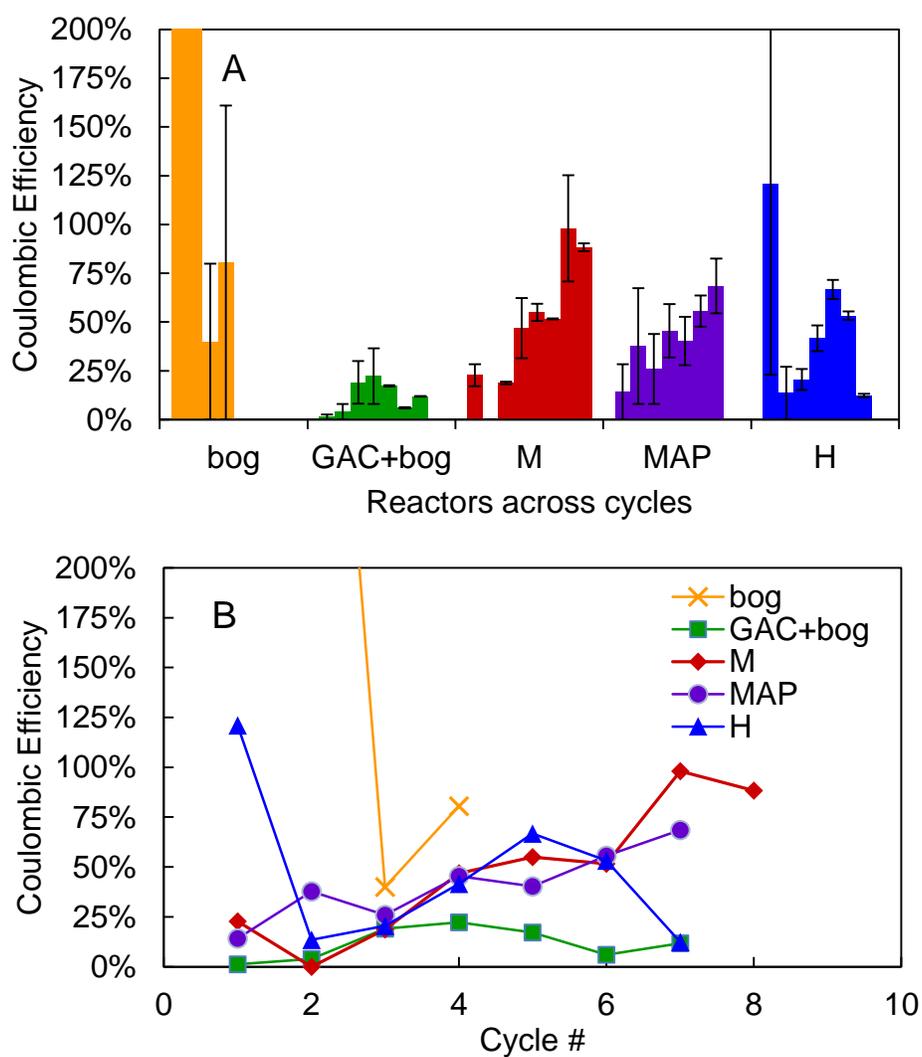


Figure 4.3. Both (A) and (B) show coulombic recoveries for different GAC pre-acclimation methods. Labeling is identical to that in Figure 4.2. Bog cycle 1 (2307%, 3635%) and bog cycle 2 (499%, 499%) are out of the range shown.

Table 4.4. Coulombic recoveries for duplicate MECs with different GAC pre-acclimation methods across successive cycles. Labeling is identical to that in Figure 4.2.

Cycle	bog		GAC+bog		M		MAP		H	
	1	2	1	2	1	2	1	2	1	2
1	2307%	3635%	3%	0%	17%	28%	0%	28%	219%	23%
2	499%	499%	0%	8%	0%	0%	8%	67%	27%	0%
3	80%	0%	8%	30%	19%	18%	44%	8%	15%	26%
4	161%	0%	8%	37%	31%	62%	59%	32%	35%	48%
5			18%	17%	59%	50%	53%	28%	62%	72%
6			6%	6%	51%	52%	64%	48%	51%	55%
7			12%	12%	125%	71%	83%	55%	13%	11%
8					90%	86%				

4.4.3 Microbial communities

GAC acclimation by substrate addition enriched the inoculum communities in archaea, *Geobacter*, and sulfur-reducing bacteria (SRB). Prior to acclimation, the bog sediment inocula for MAP and H reactors did not show any archaea above 2% relative abundance (Figure 4.4). *Geobacter* was the only bacterial group that was classified to the genus level, and only accounted for $3.0 \pm 0.05\%$ of DNA reads. Microbes capable of exocellular electron transfer grew favorably on the GAC, perhaps due to its high conductivity compared to biologically-produced electrical connections (Liu, 2012). Both bog sediment inocula showed high similarity of 80%, as expected for a microbial sample stored at 4 °C. An estimation of biomass in all samples can be seen in the appendix (Chapter 6), as the mass of DNA extracted per gram of starting sample.

The M and MAP acclimation reactors showed a high degree of similarity, averaging $74 \pm 8\%$ similarity among all M and MAP duplicates. This supported the hypothesis that only

methanol was consumed in the MAP reactors, as very similar communities would be expected through consumption of the same substrate. M and MAP acclimation reactors were enriched with SRB and *Geobacter*, which have both been associated with exocellular electron transfer in microbial electrochemical technologies (Logan, 2009). SRB compose $21 \pm 4\%$ of M reactor DNA reads and $31 \pm 2\%$ of MAP reactor reads (*Desulfomonile*, *Desulfovibrionales*, *Desulfovibrionaceae*, *Desulfovibrio*, *Desulfuromonadales*, and *Desulfobulbus*). In contrast, H reactors were only $5 \pm 3\%$ SRB. *Geobacter* comprised $50 \pm 17\%$ of M reactor DNA reads and $59 \pm 3\%$ of MAP reactor reads, while only comprising $16 \pm 7\%$ of H reads. *Geobacter* has been shown to engage in direct electron exchange with *Methanosarcina* (Liu, 2012) and *Methanosaeta* (Rotaru, 2014). *Geobacter* was also the most abundant bacterial genus in a single-chamber mixed-culture MEC fed acetate (Siegert, 2015a), but not in a two-chamber mixed-culture MEC without organic substrate addition (Siegert, 2015b). Observable levels of *Methanomethylovorans* were found in reactors M and MAP. *Methanomethylovorans* is in the family *Methansarcinaceae*, which are both aceto- and methylotrophic, and therefore able to consume methanol in the substrate (Demirel, 2008).

Methanobacterium dominated in H acclimation reactors, as it comprised $71 \pm 0.1\%$ of the total number of DNA reads for both bacteria and archaea (Figure 4.4). These hydrogenotrophic methanogens were the dominant archaeal genus found previously in many mixed-culture MECs (Cheng, 2009; Siegert, 2015a; Siegert, 2015b; Zhen, 2015). As a greater abundance of hydrogenotrophic methanogens in the inoculum has been shown to yield higher methane generation rates in MEC operation, GAC from H acclimation reactors were expected to show higher methane generation in MEC tests (Siegert, 2015a). The H acclimation reactors also

showed the presence of *Methanobrevibacter*, another hydrogenotrophic methanogen which is in the same family as *Methanobacterium* (Demirel, 2008).

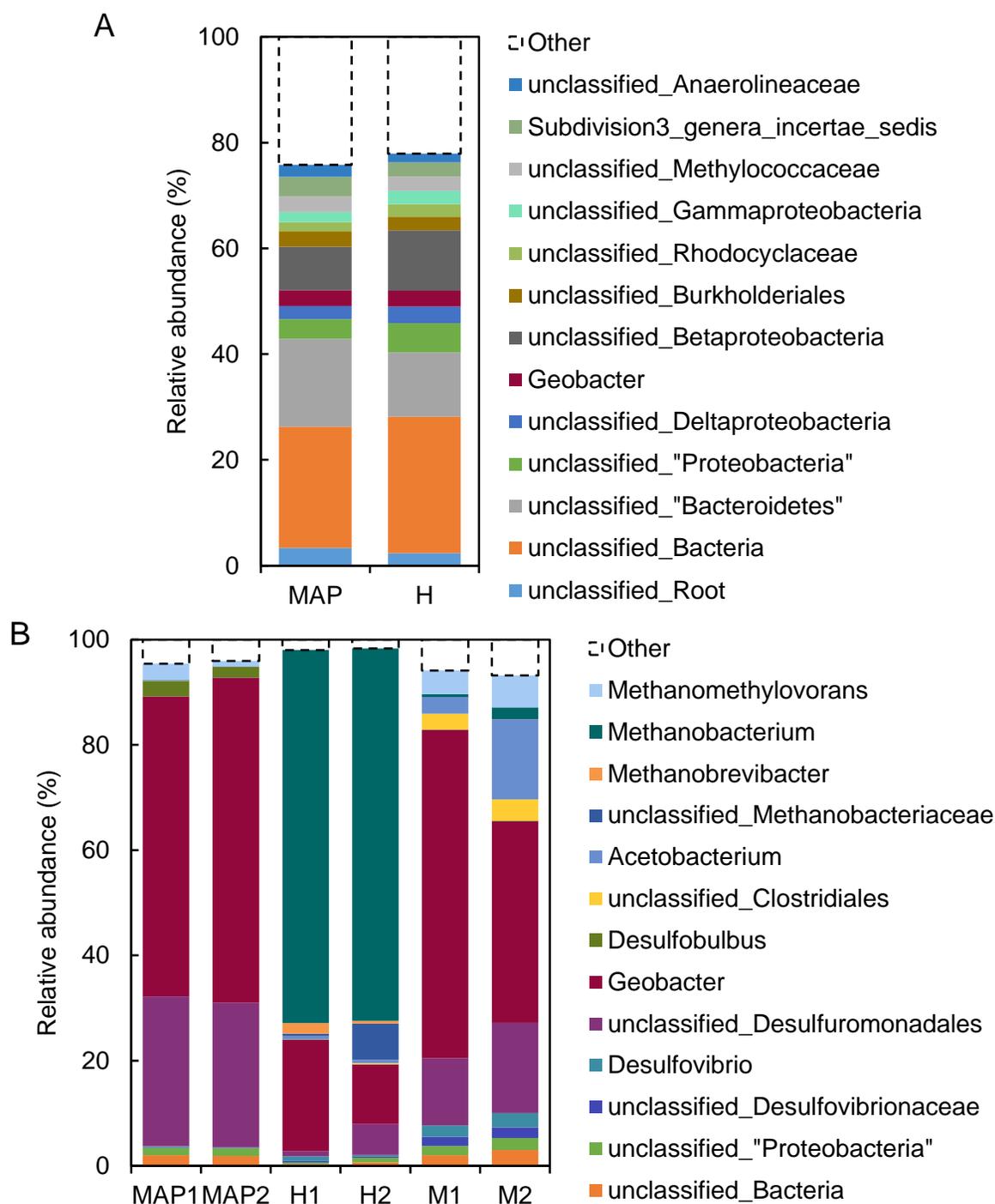


Figure 4.4. DNA results (>2% relative abundance) of (A) bog sediment inoculum used in MAP and H acclimation reactors, and (B) GAC samples from MAP, H, and M acclimation reactors.

Samples from M and MAP MECs showed similar communities ($67 \pm 8\%$), with greater similarity within GAC samples ($74 \pm 5\%$) and within brush fiber samples ($73 \pm 4\%$). Cathodic archaeal communities were dominated by *Methanobacterium*, which has also been found in many mixed-culture MECs (Cheng, 2009; Sievert, 2015a; Sievert, 2015b; Zhen, 2015). Many of the genera of microbes that were more highly abundant in GAC samples, as compared with carbon fiber brush samples, have previously been reported to be associated with methanogenic MECs or exocellular electron transfer. GAC samples showed greater relative abundance of *Methanobacterium* ($22 \pm 9\%$) compared to fiber samples ($8 \pm 1\%$). GAC samples also showed greater relative abundance of *Geobacter* ($15 \pm 6\%$) compared to fiber samples ($1 \pm 0.9\%$), and a greater abundance of SRB ($17 \pm 3\%$) compared to fiber samples ($8.0 \pm 2.9\%$). *Desulfuromonas* has been found to exist at high relative abundances on various cathode types in methanogenic MECs, including carbon brush cathodes (Sievert, 2015b). *Desulfuromonadales*, the order of *Desulfuromonas*, was more abundant in GAC samples ($7.1 \pm 3.4\%$) as compared to brush fiber samples ($0.13 \pm 0.17\%$). The reason GAC selected for *Geobacter*, SRB, and *Methanobacterium* over carbon brushes is not clear. Adsorption of natural organic matter onto the GAC may allow greater access to adsorbed substrate, or surface charge and electron transfer properties may select for certain microbes. The role of direct electron uptake in methanogenesis in the MECs could not be concluded, as the experiment did not include targeted mechanistic studies.

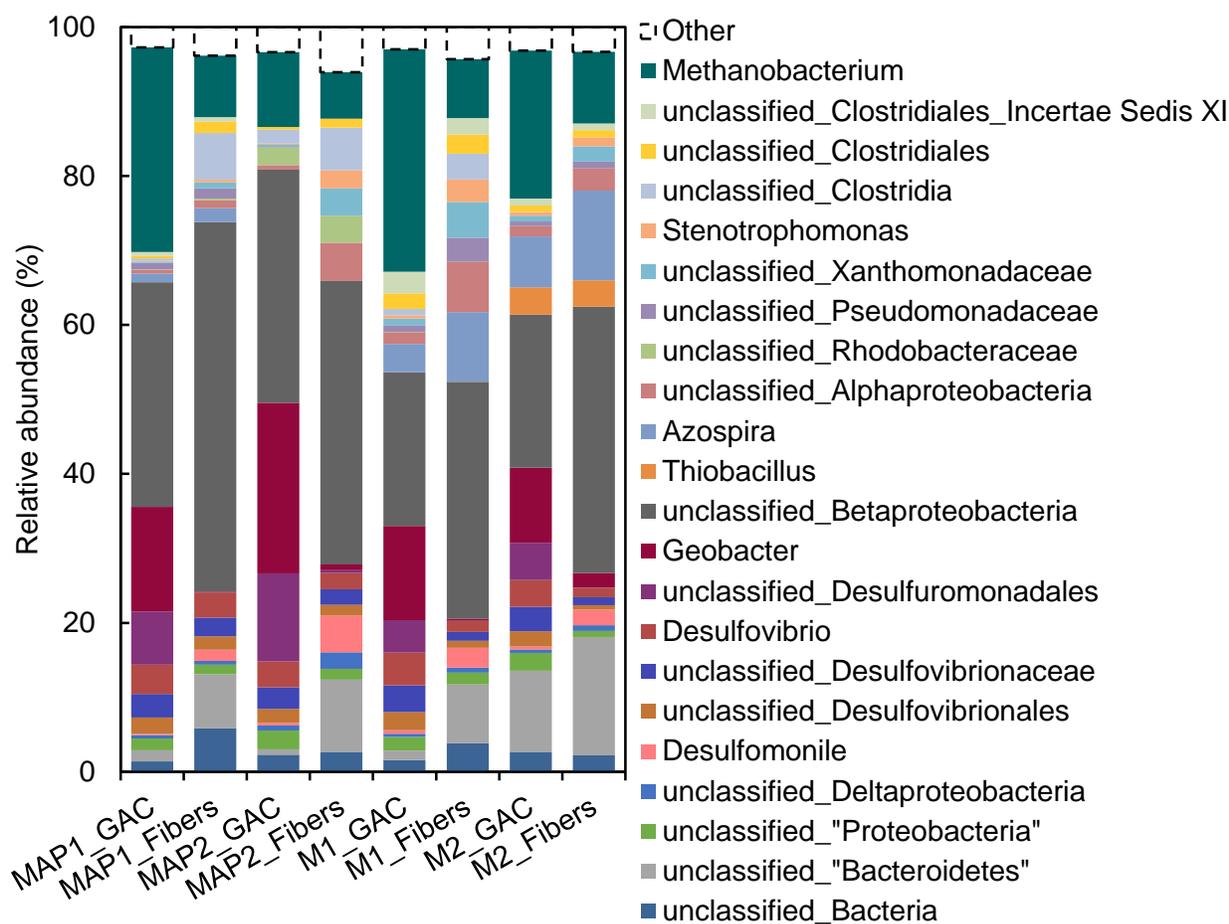


Figure 4.5. DNA sequencing results (>2% relative abundance) for MECs. Samples were taken from GAC and carbon brush cathode fibers taken from M and MAP reactors.

4.5 Conclusions

Hydrogen-fed, pre-acclimated GAC showed higher relative abundance of *Methanobacterium* ($71 \pm 0.05\%$) than methanol-fed and VFA-fed acclimation reactors ($0.7 \pm 1\%$). However, this high abundance of *Methanobacterium* at the beginning of MEC startup did not improve subsequent methane generation rate or reduce startup time. All pre-acclimated MECs showed similar methane generation rates and startup times ($23.5 \pm 9.7 \text{ nmol cm}^{-3} \text{ d}^{-1}$ for cycles 4–7). Pre-acclimation of GAC to a fed chemical substrate did show increased methane

generation rate during startup as compared to MEC startup with inoculum and fresh GAC ($12.7 \pm 4.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$). The MEC using bog sediment inoculum and fresh GAC may have eventually reached the methane generation rates obtained by pre-acclimated MECs after additional cycles, as the methane generation rate was increasing over subsequent cycles. However, total operation time of the MECs using fresh GAC was over five times longer than the pre-acclimation used for other MECs, making this approach impractical. The presence of GAC in MECs using bog sediment inoculum showed improved charge transfer and more consistent methane generation than MECs with only bog inoculum. MECs with pre-acclimation using methanol and a VFA mix had greater relative abundances of *Geobacter*, sulfate-reducing bacteria, and *Methanobacterium* on the GAC ($54 \pm 7\%$) as compared to the carbon fiber brush ($17 \pm 2\%$). GAC may be an effective growth support for enriching genera of microbes that have been shown to be associated with methanogenesis in MECs and exocellular electron transfer. Pre-acclimation of GAC to the organic chemicals used in this study suggest that this could be an effective way to achieve improved methane generation rates with a shorter startup time than using GAC without pre-acclimation.

Chapter 5

Future Work

5.1 AFMBR study

To confirm the finding that acetate acclimation improves AFMBR function, the following studies should be conducted:

- An acetate-fed, pre-acclimated AFMBR should be operated simultaneously with a control AFMBR to compare performance, and observe whether the acetate-acclimated AFMBR performs better than the control.
- A non-acetate acclimated AFMBR should be fed with acetate, to see whether COD removal improves after acetate amendment.
- DNA sequencing should be performed on samples from reactor D, to see whether communities from reactor D are similar to reactors M_A and W, which exhibited similar COD removal.
- Methane production and biomass production should be assessed throughout reactor operation, to observe how acclimation affects these parameters, and increase the sample size of methane generation measurements.

5.2 MEC study

- MEC carbon fiber brush cathodes should be pre-acclimated directly by feeding

with an organic substrate (ie. hydrogen, methanol), and compared with an MEC with no pre-acclimation to see whether pre-acclimation of the cathode to an organic substrate will improve performance.

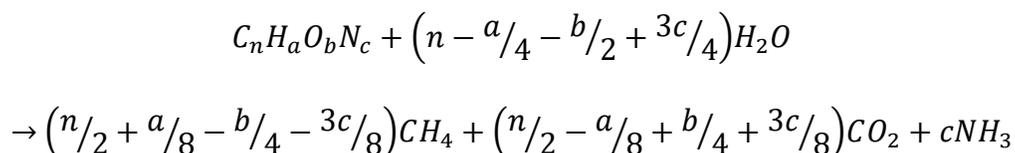
- MECs with carbon fiber brush cathodes should be compared to MECs using GAC as the cathode, similar to a study by Villano et al. (2011) which used graphite granules, to test whether GAC cathodes will have higher methane generation rates than carbon brush cathodes.

Chapter 6

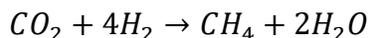
Appendix

6.1 Stoichiometric conversion of substrate to methane

For GAC pre-acclimation to organic substrates, volumetric methane production was compared with theoretical stoichiometric methane production. Moles of methane generated from methanol, acetate, and propionate were calculated using the following equation for anaerobic digestion:



Moles of methane generated from digestion of hydrogen molecule was calculated by the equation:



These results are summarized in Table 6.1.

Table 6.1. Theoretical methane generated per mole of substrate

Substrate	Chemical formula	mol CH ₄ / mol substrate
Methanol	CH ₃ OH	0.75
Acetate	CH ₃ COOH	1.0
Propionate	CH ₃ CH ₂ COOH	1.75
Hydrogen	H ₂	0.25

The theoretical volume of methane generated from substrates including methanol, acetate, and propionate was calculated as:

$$V_{methane} = \frac{V_s \rho_s n_s}{M_s V_m}$$

where V_s is the volume of substrate added, ρ_s is the density of substrate, n_s is the moles of methane generated per mole of substrate, M_s is the molar mass of substrate, and V_m is the molar volume of an ideal gas at standard temperature and pressure.

The theoretical volume of methane generated from hydrogen feed was calculated considering hydrogen in the headspace and dissolved in the medium. Methane generated from hydrogen in the headspace was calculated using:

$$V_{m,H} = \frac{f_s V_H P_s n_s V_m}{R T}$$

where $V_{m,H}$ is the theoretical methane generated from hydrogen in the headspace, f_s is the fraction of hydrogen gas in the feed, V_H is the headspace volume of the serum bottle, P_s is the pressure of hydrogen gas added, n_s is the moles of methane generated per mole of substrate, V_m is the molar volume of an ideal gas at standard temperature and pressure, R is the ideal gas constant, and T is room temperature. Theoretical methane generated by aqueous hydrogen was calculated using:

$$V_{m,L} = H_s V_L n_s V_m$$

where $V_{m,L}$ is the theoretical methane generated from hydrogen dissolved in the liquid, H_s is the solubility of hydrogen gas in water at 25 °C, V_L is the liquid volume in the serum bottle, n_s is the moles of methane generated per mole of substrate, and V_m is the molar volume of an ideal gas at standard temperature and pressure.

6.2 Methane generation rate

Methane generation rates were calculated as $\text{nmol cm}^{-3} \text{d}^{-1}$. Weekly methane generation rates were calculated between each GC measurement of the cathode headspace according to the equation:

$$R_m \left[\frac{\text{nmol}}{\text{cm}^3 \times \text{d}} \right] = \frac{\Delta V_m [\text{mL}] 10^9 [\text{nmol/mol}]}{\Delta t [\text{d}] 22.4 \left[\frac{\text{L}}{\text{mol}} \right] 10^3 \left[\frac{\text{mL}}{\text{L}} \right] V_h [\text{cm}^3]}$$

where R_m is the methane generation rate, ΔV_m is the change in methane volume between measurements, Δt is the time between measurements, and V_h is the cathode headspace volume. Weekly rates were averaged, starting after the lag phase (rates with a starting concentration of zero), and excluding the stationary phase (less than 10% increase in methane). An example of this calculation is shown in Figure 6.1, where the weekly production rates have been averaged to obtain overall methane production rates (slope of dotted line).

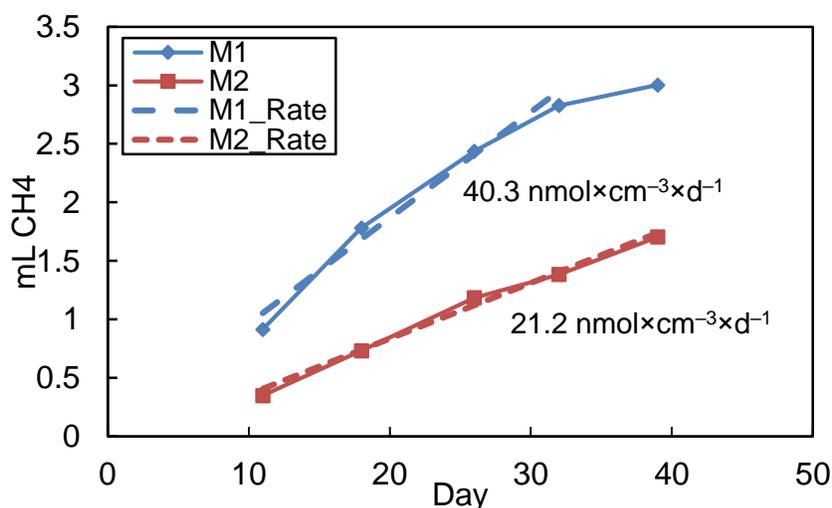


Figure 6.1. Methane in MEC cathode headspace for reactor M1 and M2, cycle 6. The figure shows averaged methane generation rates by the slopes of the fitted lines.

6.3 DNA extracted from samples

The mass of sample taken for DNA extraction, as well as the mass of extracted DNA in a 100 μL volume, is shown in Table 6.2. DNA concentration should only be considered an accurate measure of order of magnitude, as precision of Nanodrop readings was low.

Table 6.2. Mass of the sample used for DNA extraction, concentration of extracted DNA, and calculated DNA yield per gram of sample for the 100 μ L of final fluid containing the extracted DNA.

Reactor type	Reactor	Sample location	g sample	ng/ μ L DNA	ng(DNA) /g(sample)
Bog sediment inoculum	for H acclimation rxtr	bog sediment	0.475	17.1	3,603
	for MAP acclimation rxtr	bog sediment	0.317	34.9	11,013
Acclimation reactor	M1	GAC	0.446	2.9	650
	M2	GAC	0.396	4.1	1,036
	H1	GAC	0.327	5.8	1,772
	H2	GAC	0.390	9.9	2,537
	MAP1	GAC	0.514	3.5	681
	MAP2	GAC	0.406	4.0	984
MEC	M1	GAC	0.260	2.0	769
	M1	carbon fiber	0.235	4.0	1,701
	M2	GAC	0.314	4.8	1,531
	M2	carbon fiber	0.283	23.7	8,372
	H1	GAC	0.264	2.4	910
	H1	carbon fiber	0.164	3.1	1,891
	H2	GAC	0.259	2.4	928
	H2	carbon fiber	0.235	3.4	1,444
	MAP1	GAC	0.287	1.6	557
	MAP1	carbon fiber	0.241	33.0	13,687
	MAP2	GAC	0.301	6.0	1,991
	MAP2	carbon fiber	0.271	10.3	3,797
	GAC+bog1	GAC	0.302	1.4	464
	GAC+bog1	carbon fiber	0.329	1.1	334
	GAC+bog2	GAC	0.360	2.3	638
	GAC+bog2	carbon fiber	0.304	1.0	329
AFMBR	M_A	GAC	0.416	15.0	3,610
	M_A	Reactor fluid	N/A	7.7	N/A
	M_A	Influent	N/A	5.7	N/A
	W	GAC	0.419	14.4	3,438
	W	Reactor fluid	N/A	13.3	N/A
	W	Influent	N/A	5.7	N/A
	D	GAC	0.275	10.2	3,713
	D	Reactor fluid	N/A	34.3	N/A
	D	Influent	N/A	19.8	N/A
	A	GAC	0.322	11.0	3,413
	A	Reactor fluid	N/A	11.3	N/A
	A	Influent	N/A	38.0	N/A
	MFC	GAC	0.194	4.5	2,318
	MFC	Reactor fluid	N/A	7.4	N/A
	MFC	Influent	N/A	46.5	N/A

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