Exploring the extracellular electron transfer potential of biochar with soil microbes

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Summer 2017

Abstract

Biochar is an agriculture soil amendment that improves crop yields and soil health. One function that biochar may have is as a mediator of extracellular electron transfer, coupling microbial metabolism with distant electron acceptor reduction. This study aimed to enrich for soil bacteria capable of transferring electrons to biochar and to examine difference in oxygen profiles in biochar amended soils. Using acetate as an electron source in a microbial fuel cell, I enriched for a currently unidentified bacterium, however no strong electrical current was generated by these enrichment cultures. Spherical structures of possible bacterial origin were also found highly enriched on biochar from a microbial fuel cell using succinate as electron source. No differences were measured in oxygen profiles due to biochar in amended soils with either succinate or acetate as added electron sources. While this study is a first step, more thorough experiments are needed to confirm and describe extracellular electron transfer capability of biochar with soil microbes.

Introduction

Biochar is a soil amendment that, while used by indigenous people of South America and Africa for hundreds of years, has only recently been introduced into modern agriculture. Biochar is produced by the slow anoxic pyrolysis of organic matter such as wood chips or agriculture waste. The resulting material is highly enriched in pure carbon. Application of biochar to fields increases yields of various crops especially when used in conjunction with fertilizer and nutrient amendments. The growth promoting properties of biochar are not fully understood, but evidence points to its immense water holding and cation exchange capacities, as well as its ability to adsorb compounds from the soil such as metals, organic matter, and nutrients. Biochar can also be used as a carbon sequestration mechanism as it cannot be easily broken down by organisms and remains in soils for hundreds of years [1]. The pure carbon throughout biochar also makes the material able to conduct electricity similar to graphite used in basic microbial fuel cells.

Microbial fuel cells (MFC) harness microbial metabolism to generate an electric current. In a basic MFC, bacteria metabolize a given substrate using the anode, often graphite or carbon paper, as the electron acceptor. The electrons from this process then pass to a cathode where a final electron sink, such as oxygen, is reduced. The current produced by the electron movement between anode and cathode can be used for various applications such as powering environmental monitoring equipment. Electron transfer in an MFC requires either direct electron transfer between the bacteria and the electrode or by means of mediators such as humic acids, quinones, or Fe(II)/Fe(III) [2]. This extracellular electron transfer (EET) is also important in environmental systems where either electron donor and acceptor may not be easily accessible to organisms.

Microbes capable of EET can be found in many environments, all of which are good inoculum sources for MFCs. Soil has been shown to harbor many EET capable microbes such as members of the genres *Geobacter* [3] and *Rhizobiales* [4] and *Clostridium* [5]. *In situ* EET is believed to utilize electron acceptors such as iron oxides [5] and humic acids [6]. Due to its pure carbon chemical makeup, biochar has also been suggested as a possible electron conduit in amended soils [7]. Previous studies have found that it can be used effectively as an anode in MFCs inoculated with wastewater sludge [8]. Others have used biochar to connect *Shewanella oneidensis* metabolism, a known EET capable microbe, with Fe(III) reduction [9].

The goal of this study is to further examine the role of biochar as an electron conduit in soils. To do this I took both an MFC and microcosm approach to enrich for microbes capable of using biochar as sole electron acceptor and measure profiles of oxygen, the main environmental electron acceptor, in biochar amended soils.

Methods
Soil and biochar:

Soil was collected from a field at Coonamessett Farm in East Falmouth, MA (41.6762˚N, 70.5767˚W). The field site had not been planted since the Fall of the previous year and only contained a sparse covering of various weeds. Soil was taken from the top 10 cm and consisted primarily of the organic horizon. Soil was passed through a 2 mm sieve and incubated at room temperature with a depth of 3 cm for 5 days prior to succinate fuel cells, 6 days prior to succinate microcosms, 8 days prior to acetate fuel cells, and 11 days prior to acetate microcosms.

Two types of biochar, CC and SC, were used for this experiment, both of which were obtained from Brian von Herzen of the Climate Foundation. CC biochar is of cellulosic origin which underwent pyrolysis at around 500˚C. SC biochar is from sawdust feedstock pyrolyzed at a maximum of 480˚C and originally from ZHAW in Wadenswil, Switzerland.

Microbial Fuel Cells:

Biochar anodes were made by filling a 1 ml pipette tip with granulated biochar (Figure 1a). Holes were made in the side of the tip to allow better entry of anolyte media. A bare copper wire was inserted into wide end of the pipette tip into biochar and then sealed with silicone rubber.

Succinate MFCs were built as pictured in Figure 1b. The anaerobic and aerobic vessels consisted of a 250 mL and 500 mL Pyrex bottle respectively. Each chamber was sealed with a rubber stopper containing holes for electrodes and salt bridge. Succinate anolyte media contained per liter solution: 2.06 g sodium succinate dibasic hexahydrate, 0.31 g ammonium chloride, 0.13 g potassium chloride, 2.93 g sodium phosphate monobasic monohydrate, 4.09 g sodium phosphate dibasic anhydrous, 1 mL trace element solution, 5 mL MES buffer at pH 6.15, and 1 mL vitamin solution [8]. A 1% agarose in 3 M sodium chloride salt bridge spanning the anaerobic and aerobic chambers was made by injecting the molten solution into a 3 mm tube and solidifying. The counter electrode was a graphite rod (Alpha Aesar #14738) inserted into the aerobic chamber. The Ag/AgCl reference electrode (BASi MF-2052) was inserted into the anaerobic chamber. The experimental MFC used a biochar anode as the working electrode while the control MFC used an indium tin oxide (ITO) coated slide (Sigma Aldrich #703184)

attached to an alligator clip that was not submerged in the anolyte media. A constant nitrogen gas flow through the anaerobic chambers maintained an anoxic environment. Both MFCs were inoculated with 5 g soil. Chambers were filled with enough anolyte media to partially cover electrodes but maintain a small headspace for the nitrogen gas.

Acetate based fuel cells were single chambers consisting of 125 mL Pyrex bottles as shown in Figure 1c. Chambers were sealed with rubber stoppers with holes for working, counter and reference electrodes. Acetate anolyte media contained per liter solution 2.5 g sodium acetate anhydrous, 0.31 g ammonium chloride, 0.13 g potassium chloride, 2.93 g sodium phosphate monobasic monohydrate, 4.09 g sodium phosphate dibasic anhydrous, 1 mL trace element solution, 5 mL MES buffer at pH 6.15, and 1 mL vitamin solution [8]. Both MFCs used a graphite counter electrode and Ag/AgCl reference electrode. A constant nitrogen gas flow through the anaerobic chambers maintained an anoxic environment. The experimental MFC used a biochar anode as the working electrode while the control MFC used a second graphite rod. Both MFCs were inoculated with 5 g soil. Chambers were filled with enough anolyte media to partially cover electrodes but maintain a small headspace for the nitrogen gas. Acetate anolyte media was removed and replaced by fresh media after 4 days.

Figure 1: a) biochar anodes. b) succinate MFC with biochar anode using both anaerobic (small) and aerobic (big) bottles. c) Acetate MFC with biochar anode in one anaerobic bottle.

MFCs were hooked up to separate Gamry Instrument Interface 1000 potentiostats and controlled using Gamry Instrument Framework version 6.20 software. During run, MFCs were maintained using controlled potential coulometry at a potential of 200 mV, sampling time of 1 min, and maximum current of 1 mA. Prior to run and at the end of each run we performed cyclic voltammetry with a potential range from -500 to 400 mV, scan rate of 100 mV/s, step size of 2 mV, max current of 10 mA, and power factor correction of 50 ohm. Acetated MFCs were restarted 6 times in the first 4 days due to computer cutoffs. Cyclic voltammetry was performed at the beginning and end of each controlled potential coulometry run of all MFCs and before and after replacement of anolyte media in the acetate MFCs.

Microcosm set up:

All microcosms were set up in 20 x 150 mm Hungate tubes and sealed with Parafilm. There were eight microcosms to measure succinate use by soil microorganisms. Each succinate microcosm contained 10 mL lower soil slurry topped by 20 mL upper soil slurry. Succinate microcosm setup is described in table 1. Succinate solution contained 1.02 g sodium succinate dibasic hexahydrate in 500 mL water. Four microcosms were set up to measure acetate use. Acetate microcosm setup is described in table 2. Acetate solution contained 1.25 g sodium acetate anhydrous in 250 mL water. For both microcosms, after 24 hours of incubation, excess liquid on surface of soil was removed prior to microelectrode measurements.

Microcosm ID	Bottom 10 mL				Top 20 mL			
	Biochar added	g biochar / g Soil	Media added	ml media / g Soil	Biochar added	g biochar / g Soil	Media added	ml media / g Soil
-Biochar -Succinate	none	0	water	0.286	none	0	water	0.286
-Biochar +Succinate	none	0	7.6 mM sodium succinate	0.286	none	0	water	0.286
+CC -Succinate	CC	0.052	water	0.286	CC	0.052	water	0.286
+SC -Succinate	SC	0.013	water	0.286	SC	0.013	water	0.286
+CC in bottom only +Succinate	CC	0.052	7.6 mM sodium succinate	0.286	none	0	water	0.286
+SC in bottom only +Succinate	SC	0.013	7.6 mM sodium succinate	0.286	none	0	water	0.286
+CC +Succinate	CC	0.052	7.6 mM sodium succinate	0.286	CC	0.052	water	0.286
+SC +Succinate	SC	0.013	7.6 mM sodium succinate	0.286	SC	0.013	water	0.286

Table 1: Succinate microcosm designs.

Microcosm ID	Biochar added	g char / g soil	Media added	ml media / g soil
-Biochar -Acetate	none	0	water	0.4
-Biochar +Acetate	none	0	60 mM sodium acetate	0.4
+Biochar -Acetate	CC	0.1	water	0.4
+Biochar +Acetate	CC	0.1	60 mM sodium acetate	0.4

Table 2: Acetate microcosm designs.

Scanning Electron Microscopy:

Biochar anode, copper wire from biochar anode, and ITO slide from succinate MFCs were imaged using a Zeiss SUPRA 40VP scanning electron microscope. Samples were fixed in 4% paraformaldehyde at room temperature for 4 hours followed by 4˚C for 2 hours. They were then washed three times in 1x PBS. All samples were dehydrated by 25 minute submersions of 25%, 50%, 75% and 100% ethanol. Samples were stored in 100% ethanol until critical point drying and platinum coating.

HPLC:

Levels of acetate in samples of anolyte media from acetate MFCs and extracts from the acetate microcosms were measured using UV/vis detection on an HPLC (Shimadzu LC-2010C-HT with Bio-Rad Aminex HPX-87H column). Initial anolyte solution was sampled after 3 days and again from replenished anolyte solution after 7 days running in total. Fresh unused anolyte solution was measured for baseline acetate concentration. Soil extracts were made at the conclusion of the microcosm experiment, day 3. 25 mL pure water was added to each microcosm and the slurry was homogenized by shaking and vortexing tubes. 10 mL of slurry was then centrifuged at 4100 G for 10 minutes. Soil extract was purified by filtering 3 mL of slurry supernatant was filtered through 0.2 µm membrane filter.

Fluorescence in situ hybridization:

Anolyte solution with suspended cell aggregates was sampled from initial acetate MFCs after 4 days. Sample was fixed with 2% paraformaldehyde at 4˚C for 24 hours. After fixing,

sample was washed 3 times in 1x PBS and passed through 0.2 µm filter to capture cells. Two subsamples were hybridized for 2 hours at 46˚C in 30% formamide with 2 sets of probes, one set targeting *Gammaproteobacteria* (mix of Delta495a-CY3, Delta495b-CY3, and Delta495c-CY3) and the other targeting *Deltaproteobacteria* (GAM42a-CY3). Both probe sets also included a generic eubacterial probe (EUB338-FITC). After hybridization, samples were washed in standard wash buffer, PBS and milliQ water followed by dehydration in 96% ethanol and staining with DAPI. Samples were imaged using a Zeiss Axio-A2 fluorescent microscope.

Microelectrode oxygen measurements:

Oxygen profiles were measured in all microcosms using a 1.1 x 40 mm needle sensor oxygen probe (Unisense OX-N) coupled with a Unisense field microsensor multimeter. Sensors were controlled and data analyzed using Unisense SensorTrace software version 2.8.200.21688. Probes measured to a depth of 1 cm with a step size of 100 µm. Once all data was collected, gradients from a single sampling day were normalized to account for varying starting depths.

Results

MFC based enrichments:

No peak in current was observed in any MFC over the entire time course. There was a constant current at 6.25 µA in the succinate biochar MFC, 5.08 µA in the succinate ITO MFC, 4.71 µA in the acetate biochar MFC, and 4.53 µA in the acetate graphite MFC. Cyclic voltammograms for both succinate MFCs did not change between the start and end of run. Plots for the biochar anode in the succinate MFC and first measurement of the acetate MFC were mostly linear with no distinguishing peaks. The plot for the biochar anode in the acetate MFC changed between start of run and the pre- and post-replacement of anolyte media with peaks appearing at around 175 mV against the Ag/AgCl reference (Figure 2a). This peak went away in the final cyclic voltammogram when experiment was finished and the plot became relatively linear. The cyclic voltammogram for the graphite electrode in the acetate MFC also changed between time points with a peak at 260 mV initially, -200 mV pre-replacement of anolyte media, and 50 mV in both post-replacement of anolyte media and final reading, all against Ag/AgCl reference (Figure 2b). Samples of the anolyte media were taken from the acetate MFCs prior to replacement of media and at the end of the study for measurement by HPLC but no strong decrease in acetate concentration was observed when compared to fresh anolyte media.
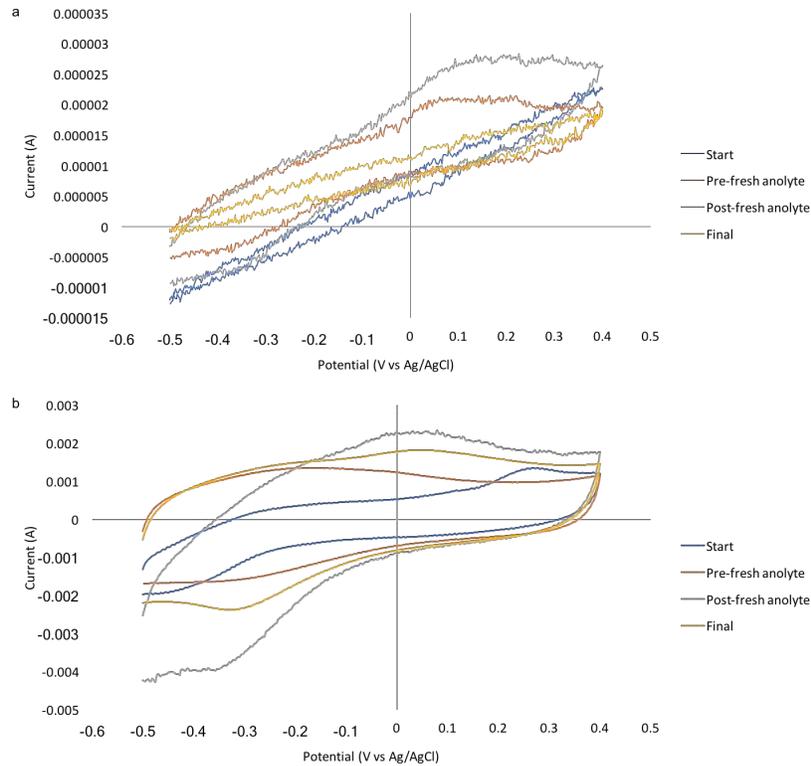
Figure 2: Cyclic voltammogram in acetate MFCs for start, pre-replacement of anolyte media, post-replacement of anolyte media, and end of experiment with the a) biochar anode and b) graphite anode.

While no clear bacteria were observed through SEM on the biochar anode from the succinate biochar MFC, spheres of fine filaments were seen throughout the biochar and coating the copper wire connector (Figures 3a-c). Numerous bacteria were found on the ITO slide anode from the succinate ITO MFC the majority of which were rod shaped bacteria with spiral markings running their length (Figure 3d). Also observed were cigar (Figure 3e), oval (Figure 3f), various other rod (Figures 3g-h) shaped bacteria and some other forms that may or may not be biological in origin. (Figure 3i). Various morphologies of bacteria were observed in the anolyte media of both succinate MFCs after runs but no clear enrichment was observed.
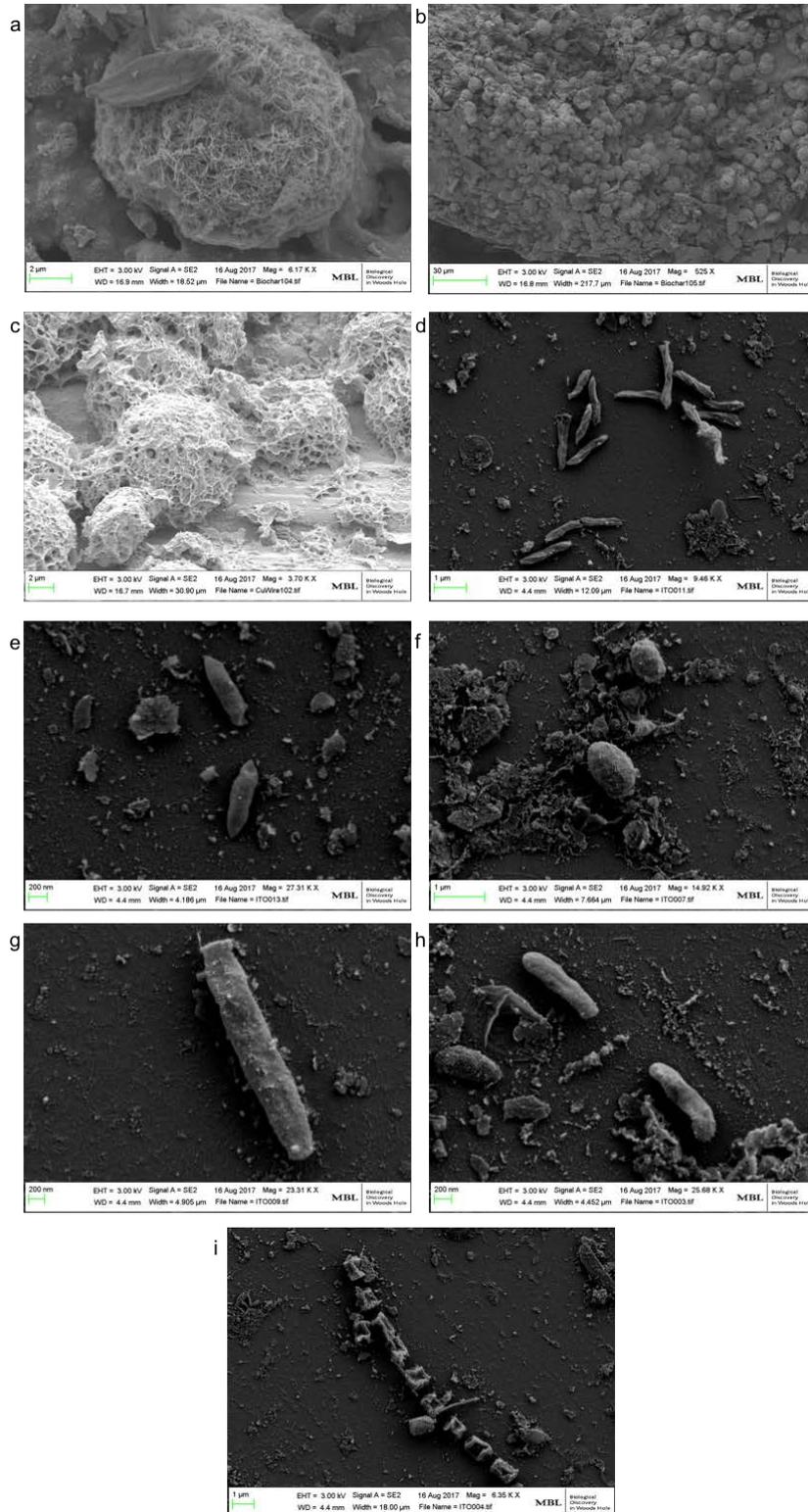
Figure 3: SEM images of a) fibrous sphere on biochar anode fragment from a succinate MFC, b) many fibrous spheres on biochar anode fragment from a succinate MFC, c) fibrous spheres on copper wire from biochar anode in a succinate MFC and bacteria of various morphologies found on the ITO slide anode from a succinate MFC including d) a rod with spiral pattern, e) cigar, f) oval, g) longer rod, h) rounded irregular rod, as well as i) other possible biological structures.

White wispy aggregates of bacteria were found floating and attached to surfaces in the acetate biochar MFC prior to refreshing of anolyte media (Figure 4a). The aggregates were formed by thick rod shaped bacteria containing numerous phase bright inclusions within the cells. Cells were arranged in clumps and twisted cords (Figure 4b). No fluorescence was observed with hybridization of either *Deltaproteobacteria* (Figure 5a) or *Gammaproteobacteria* (Figure 5b) FISH probes, however fluorescence was observed with the eubacteria probe and DAPI. No other bacteria showed clear enrichment. While aggregates continued to be seen, the enriched bacteria were not seen as widely in anolyte samples from the refreshed media biochar MFC. Aggregates imaged under phase contrast microscopy appeared to be made up of cellular debris from the previously enriched bacteria.
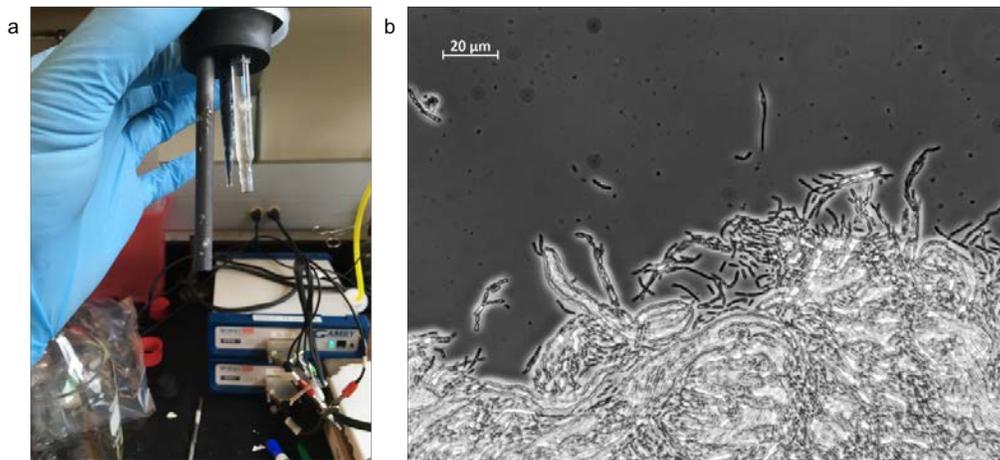
Figure 4: Enriched bacteria in the succinate MFC with biochar anode observed as a) white wispy aggregates floating and attached to MFC surfaces and as b) rod shaped bacteria forming twisted cord like structures in phase contrast microscopy
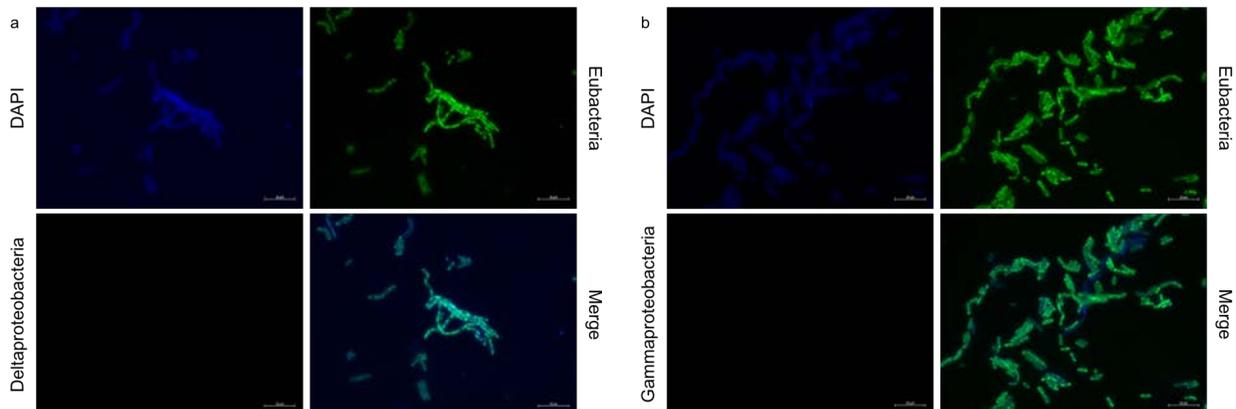

Figure 5: FISH image enriched bacteria from succinate MFC with biochar anode hybridized with probes for a) Deltaproteobacteria and b) Gammaproteobacteria along with Eubacteria and stained with DAPI. Note no fluorescence with either the Deltaproteobacteria or Gammaproteobacteria probes.

Microcosm based carbon utilization:

No difference was observed in the oxygen profiles of any succinate microcosms in any time point (Figure 6a-b). No difference was observed in the oxygen profiles of the acetate microcosms on day 1 (Figure 7a), but the microcosms containing acetate on day 3 showed a steeper decrease in oxygen with depth as compared to the no acetate controls. Biochar showed no influence on the microcosms containing acetate but seemed to increase the slope of the oxygen gradient in the no acetate controls (Figure 7b). Acetate concentration in soil extracts were determined at the conclusion of the experiment using HPLC resulting in 0.077 mM and 0.733 mM in the -biochar -acetate and +biochar -acetate microcosms respectively and 7.758 mM and 8.084 mM acetate in the -biochar +acetate and +biochar +acetate microcosms respectively.
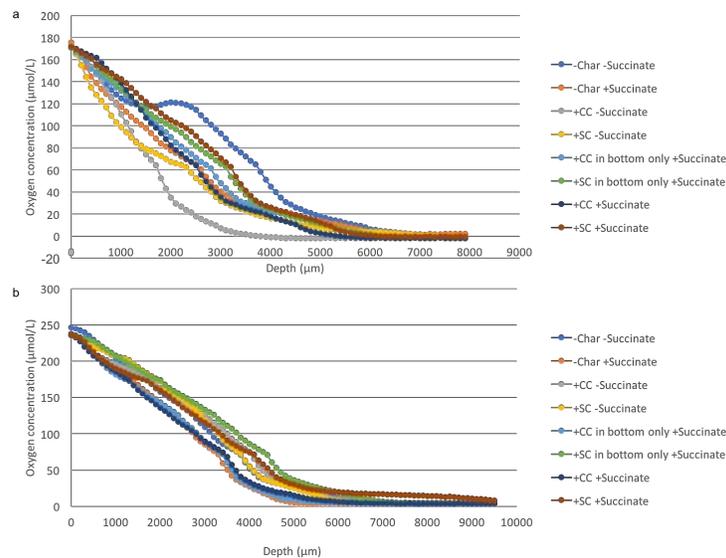

Figure 6: Oxygen profiles through first 1 cm of succinate microcosm soils, a) measured on day 1 and b) measured on day 3.
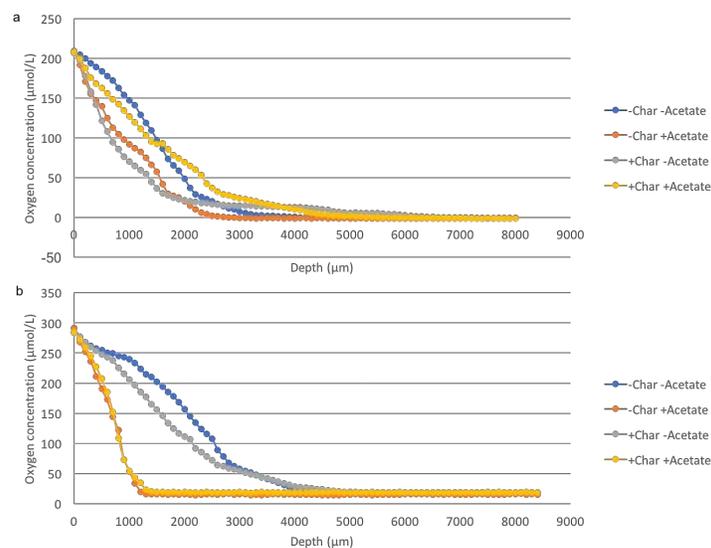

Figure 7: Oxygen profiles through first 1 cm of acetate microcosm soils, a) measured on day 1 and b) Measured on day 3.

Discussion

This study aimed to examine the ability of biochar to act as an electron conduit for soil microbes. While no increase in current was observed in the MFCs, the increased signal in the cyclic voltammogram over MFC run time suggest a change in electron transfer kinetics of the biochar electrode. Two possible causes for this could be change in anolyte solution or change to anode composition. Replacement of the anolyte solution resulted in no change to the cyclic voltammogram when compared to plot created just prior to replacement. This suggests that it is a modification to the anode itself that results in the electron transfer kinetics. Two possible anode modifications include complete saturation of anode by anolyte media or buildup of bacterial biofilm on the surfaces of the anodes. The high surface area and pore space of biochar could allow for more anolyte media to come into contact with the anode surface over time, increasing the interaction between anolyte ions and the anode [9]. This could also explain the higher constant current observed in the biochar anodes compared to graphite or ITO slides. When replacing the anolyte media in the acetate MFCs, the old media within the anode would largely transfer with the anode itself, keeping the increased conductivity when the potential was restarted. However, this shift in cyclic voltammetry was not seen in the succinate biochar MFC, where this saturation effect should also occur. Biofilm development on the anode is another possibility for the shift in electron transfer kinetics as enriched bacteria was observed in the anode during the run. Previous studies have shown biofilm development increasing cyclic voltammetry signal with carbon paper anodes [10]. The loss of the peaks in the cyclic voltammogram at the end of the experiment could then be due to the apparent loss of the enriched bacteria. Further studies should examine the extent of biofilm development on or bacterial interaction with biochar anodes to see if bacteria are indeed the cause of the shift cyclic voltammograms.

This study was able to enrich for a single bacterial type in the acetate biochar MFCs. I was unable to identify the bacteria but negative results from the FISH experiment suggest that it is not a member of either *Gammaproteobacteria* or *Deltaproteobacteria*, which contain known EET capable bacteria *Shewanella* and *Geobacter* respectively. More experimentation including isolation and sequencing will be needed to identify this bacterium. It is also unknown if this bacterium directly interacts with the biochar anode or conducts EET. While clusters of the bacteria were found attached to the anode, they were also found floating in the anolyte media as well as attached to the counter and reference electrodes. The twisted cord like structure of some of the bacterial aggregates is also intriguing and should be examined in more detail. The poor health of this enrichment after replacement of the anolyte media suggests a compound in the soil itself was required for bacterial growth. Understanding the micronutrients in soils will be important for further enrichment of soil microorganisms and in development of more efficient MFCs. SEM images of biochar and the copper wire from the succinate biochar MFC, while not producing clear signs of bacteria, do show interesting structures that may be of bacterial origin. These fibrous spheres are in high concentration on some of the biochar fragments as well as the copper wire, suggesting a close relationship to the electron potential of these surfaces in the working MFC. Proper identification and assessment of these structures is needed.

According to SEM images, rod-shaped bacteria also seemed to be enriched on the ITO slide anode of the succinate MFC. These bacteria had a spiral grooved structure running the

length of the cells. It is possible that these structures were caused by the dehydration and fixing processes however further imaging is needed to examine the nature of this cell morphology. Continued culturing and longer runs with this MFC are also needed to confirm the connection between the bacteria and the anode.

This study did not find any major difference in oxygen-depth profiles due to biochar, except possibly an increase in slope of the oxygen gradient in the biochar amended soil of the no-acetate microcosm as compared to the non-amended soil. Addition of acetate does show a sharp increase in oxygen gradient slope regardless of biochar addition. This is most likely due to increased microbial metabolism from excess carbon source. In order to fully test the electron transfer capability of biochar in soil it is important to measure conductivity throughout a soil profile with biochar amendments and to remove or reduce any other source of electron flow other than biochar. Use of biochar embedded membranes made of non-conductive material such as Nafion could accomplish this. Experiments with open air MFCs such as those used in Wolińska et al. [11] or Jiang et al. [5] or *in situ* MFCs could also be useful.

Conclusions

Biochar has the potential for being an electron conduit for microorganisms in an agricultural soil environment. Its use as an anode for MFCs suggests that microbes can use it as an electron acceptor and couple their metabolisms with reduction reactions away from the cell. More work needs to be done to show that this is occurring *in situ* and uncover the mechanisms behind the process. If EET is occurring with biochar, this process could explain one beneficial function this amendment has in treated soil.

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