Exploring the potential of sensing nutrient dynamics using soil-based microbial fuel cells

by

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B.S., Punjab Agricultural University, 2019

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Agronomy
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2022

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Abstract

A soil-based microbial fuel cell (MFC) is a bio-electric device that uses soil microorganisms to convert an organic substrate into electricity. The energy generation potential of MFCs may be exploited to 'sense' the nutrient status of agricultural soils, which would be faster than traditional soil sampling methods and analysis in laboratories. It could provide real-time data on available soil nutrients. Our studies focused on developing a soil-based MFC that tracks changes in nutrient availability and explores relationships between soil nutrient availability, microbial activity, and MFC performance. We hypothesized that 1) a change in the level of nutrients would produce a different microbial response; hence, a different electrical signal and 2) introducing a biofilm coating on the anode would enhance electrogenic microbial activity and its ability to capture changes in nitrogen dynamics and voltage. A total of five different studies with two soils were conducted sequentially to test these hypotheses. Soil-based MFCs were set up with natural, partially sterilized (a treatment in study 1), and sterilized (a treatment in study 2) soil at field capacity with nitrogen fertilizer (studies 1, 3, 4, and 5), organic carbon treatments (study 2), and *Geobacter* enriched inoculum (study 4) and pre-developed *Geobacter* anodic coating (study 5). Soil 1 was used for studies 1-3, while soil 2 was used for studies 4 and 5. The voltage generated was measured by a data logger and recorded every 15 minutes. Soil solution was analyzed to estimate NO_3^-, NO_2^- and NH_4^+, dissolved organic carbon, pH, and electrical conductivity. Soil gas samples (CO_2) were collected periodically as a proxy for soil microbial activity and soil organic carbon mineralization. The first study found that MFC performance was better in the control treatment than in higher nitrogen treatments. Voltage in higher nitrogen treatments was significantly low owing to possible nitrate reduction reactions using up the electrons, which could be used for voltage production, or the negative effect of N addition on organic matter

decomposition. The voltage of the sterilized treatment decreased significantly with increasing dissolved nitrogen levels. The second study showed that different organic carbon treatments did not differ significantly in voltage, most likely due to non-significant changes in dissolved organic carbon caused by the organic carbon treatments. However, the voltage of sterilized treatment was significantly low, suggesting that the voltages produced in the other treatments were a result of various biotic processes. Biofilm on anode with different nitrogen levels performed better than the control soil. Voltage generation (or MFC performance) was higher with higher nitrogen levels than the control in the second soil. Differences in voltage signals with nitrogen levels were observed, but they varied by soil type, nutrient content, and presence of biofilm. Future studies plan to use a hydrogel-based anode coating to protect the anodic biofilm from exogenous soil microorganisms and selective inoculum of microorganisms for the nutrient in question. Although results are encouraging, more work is needed to deconvolute other signals from the voltage signal corresponding to available soil N levels. If we can successfully model these relationships, this research could help improve crop production rates and ensure the Nation’s food security through 2050 demands.

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Acknowledgements

I would like to express my sincere gratitude to my advisor, Dr. Ganga Hettiarachchi for her excellent guidance, fullest support, and encouragement throughout my project. I would also like to thank her for giving me the wonderful opportunity to pursue research in soil science and for her encouragement with the various competitions I have been able to partake. I would like to extend my deepest thanks to my committee members; Dr. Charles Rice, Dr. Prathap Parameswaran, and Dr. Ryan Hansen for their guidance, valuable suggestions, and time for my research. I want to thank the soil chemistry group members, especially Ruwandi Kumarasinghe for helping me in this project, Sevendeep Kaur, Dr. Madhubhashini Galkaduwa, Dr. Zafer Alasmary, Kasuni Gamage, and Chandima Wekumbura for their constant support and motivation. I am extremely grateful to my friends, James Lin, and Kaila Carnley for helping me in every possible way and making my master's journey more fun and memorable.

I am incredibly thankful to the NSF EAGER Sits grant number 1841613 and in part to the USDA NIFA Multistate Project NC 1187 for providing the necessary funding and graduate assistantship. I am thankful to the Department of Agronomy, the Soil Testing Laboratory, and the staff for their assistance. I extend my thanks to the Soil Microbial Agroecology Lab, especially Carlos Pires, Victoria Dutra, Priscila Marchioro, and many others for their guidance and help with the lab equipment. I am especially thankful to Chinthaka Weerasekaramudiyanselage for helping me with the datalogger programming. I acknowledge the research laboratory of Dr. Prathap Parameswaran, especially Priyasha Fernando and Evan Heronemus for providing me with the biofilm.

Lastly, I am indebted to my family in India including my mother, sister, nephew, and relatives for their unconditional love and support.

Dedication

This thesis is dedicated to my late father Harkanwaljit Singh, my mother, Parminder Kaur, and my sister, Harleen Kaur for their immense sacrifices, hard work, and support which helped me reach where I am today.

Chapter 1 - Introduction

Improving the efficiency of crop production requires the continuous on-site monitoring of nutrients. Traditional soil sampling and lab analysis could be time-consuming, costly, and often not an accurate representation of the field. A microbial fuel cell is a technology that has received considerable attention in the last two decades for its energy production using microorganisms. The electrical energy produced is a renewable and sustainable technology that is considered efficient (Salgado, 2009; Sengodon & Hays, 2012) and carbon-neutral (Lovley, 2006). A microbial fuel cell (MFC) is a bioelectric device that can generate electricity as microorganisms consume organic substrates and release electrons (Huang et al., 2018; Barbato & Gronwald, 2018). This process occurs naturally in microorganisms as a part of anaerobic respiration, where microbes produce electrons and transfer them extracellularly to electron receptors (Lovley, 2008). One of the most popular and extensively studied microorganisms in the MFCs capable of producing high current densities is *Geobacter sulfurreducens* and is considered as the "gold standard" (Slate et al., 2019). *Geobacter sulfurreducens* is a gram-negative, rod-shaped, delta-proteobacterium and is a non-fermentative, obligate anaerobe with flagella and type IV pili production. It belongs to the group of dissimilatory metal-reducing microorganisms, which reduces metal oxides under anaerobic conditions in soils and sediments and produces biologically useful energy as ATP (Du et al., 2007). An important factor in the efficient transfer of electrons in an MFC is the bacterial attachment and formation of a biofilm on an anode surface (Franks et al., 2010). To date, microbial fuel cells have been researched for electricity generation (Allen & Benetto, 1990; Du et al., 2007), wastewater treatment (Frank & Nevin, 2010; Zhou et al., 2013 Oh et al., 2010), remediation of toxic compounds (Zhang et al., 2020;

Huang et al., 2011; Wang et al., 2012) and biosensing in wastewater (Kim et al., 2003; Cui et al., 2019). However, there is a lack of studies on the biosensing capability of soil-based microbial fuel cells. The focus of our research studies is based on exploring the energy generation potential of soil-based microbial fuel cells for development as a biosensor for estimating the nutrient status of soil in real-time.

Overapplication of fertilizers in agricultural fields is a matter of concern. It not only affects crop productivity but also causes serious environmental and health issues. Overapplied nutrients can wash into rivers, streams, and lakes, and can cause eutrophication leading to algal bloom and other problems like acid rain and global warming (Zhang et al., 2020b). To prevent the overapplication of nutrients, it is crucial to monitor overall soil health in the field in real-time, so farmers can make informed decisions for agricultural practices, nutrient applications, reduce costs, and increase crop productivity. In recent decades, MFC-based biosensors have drawn increasing attention because of their sustainability and low cost, with applications ranging from monitoring water quality to the detection of air quality (Cui et al., 2019). An MFC-based sensor was recently proposed for in-field early detection of eutrophication (Lorenzo et al.,2020). This sensor was found to be correlated to algal concentration and dissolved oxygen levels. In a study by Cristiani et al. (2008), cathodic polarization detected as electrical signals were correlated with microbial growth and biofilm development on stainless steel electrodes when the electrochemical probes were directly inserted into the soil. Several studies have found relationships between soil properties, microbial community, and electrical signals (Dunaj et al., 2012; Deng et al., 2015; Jiang et al., 2015).

In an attempt to understand the biosensing ability of MFCs, we conducted soil-based microbial fuel cell studies and a plant-based microbial fuel cell study to develop an MFC-based

biosensor that tracks changes in nutrient availability via changes in electrical signals. For the overall research, we hypothesized that a change in the level of nutrients such as nitrogen or organic carbon would give a different microbial response, generating a different electrical signal. For studies 4 and 5, our additional hypothesis was that treatments with pre-developed biofilm on the anode using *Geobacter* inoculum would enhance electrogenic microbial activity and its ability to capture changes in nitrogen dynamics. The objective of our first study was to develop relationships between nitrogen availability, microbial activity, and MFC performance. Our second study focused on different organic carbon levels to see a change in the electrical signal. The focus of our third study was to measure the differences in voltage produced in a plant microbial fuel cell with varying nitrogen levels. The fourth and fifth studies were conducted with a much simpler soil than before and a different setup with *Geobacter* inoculum on the anode (fourth study) with various nitrogen treatments. The fifth study exclusively used pre-developed *Geobacter* anodic coating to provide a stable biofilm with different nitrogen treatments. The objectives of the fourth and fifth studies were i) to understand the effects of pre-developed anodic biofilm on MFC performance and detection of nitrogen availability, and ii) to develop relationships between nitrogen availability, microbial activity, and MFC performance in the presence of biofilm.

References

Allen, R. M., & Benneto, H. P. (1993). Microbial Fuel Cells: Electricity Production from Carbohydrates. *Applied Biochemistry and Biotechnology*, *39*, 27-40. http://dx.doi.org/10.1007/BF02918975

Barbato, R., & Gronwald, F. (2018). A Study of Soil Based Microbial Fuel Cells. *International Journal of Scientific Research and Engineering Development*, *1*, 60-157.

Cristiani, P., Franzetti, A., & Bestetti, G. (2008). Monitoring of electro active biofilm in soil. *Electrochimica Acta*, *54*, 41–46.

Cui, Y., Lai, B., & Tang, X. (2019). Microbial Fuel Cell-Based Biosensors. *Biosensors*, *9*, 92. https://doi.org/10.3390/bios9030092

Deng, H., Jiang, Y. B., Zhou, Y. W., Shen, K., & Zhong, W. H. (2015). Using electrical signals of microbial fuel cells to detect copper stress on soil microorganisms. *European Journal of Soil Science*, *66*, 369–377.

Du, Z., Li, H., & Gu, T. (2007). A state of the art review on microbial fuel cells: A promising technology for wastewater treatment and bioenergy. *Biotechnology Advances*, 25, 464–482.

Dunaj S. J., Vallino J. J., Hines, M. E., Gay, M., Kobyljanec, C., & Rooney-Varga J. N. (2012). Relationships between soil organic matter, nutrients, bacterial community structure, and the performance of microbial fuel cells. *Environmental Science & Technology*, *46*, 1914–1922.

Franks, A. E., Malvankar, N., & Nevin, K. P. (2010). Bacterial biofilms: the powerhouse of a microbial fuel cell. *Biofuels*, *1(4)*, 589–604. https://doi.org/10.4155/bfs.10.25

Franks, A.E., & Nevin, K.P. (2010). Microbial Fuel Cells, A Current Review. *Energies*, *3*, 899-919.

Huang, D. Y., Zhou, S. G., Chen, Q., Zhao, B., Yuan, Y., & Zhuang, L. (2011). Enhanced anaerobic degradation of organic pollutants in a soil microbial fuel cell. *Chemical Engineering Journal*, *172*, 647–653. https://doi.org/10.1016/j.cej.2011.06.024

Huang, H., Cheng, C., Yang J., Li, C., Sun, Y., & Cen, K. (2018). Effect of nitrate on electricity generation in single-chamber air cathode microbial fuel cells. *Chemical Engineering Journal*, *337*, 661-670, ISSN 1385-8947. https://doi.org/10.1016/j.cej.2017.12.150

Jiang, Y. B., Deng, H., Sun, D. M., & Zhong, W. H. (2015). Electrical signals generated by soil microorganisms in microbial fuel cells respond linearly to soil Cd^{2+} pollution. *Geoderma*, 255-256: 35–41.

Kim, B.H., Chang, I.S., Gil, G.C., Park, H.S., & Kim, H.J. (2003). Novel BOD (biological oxygen demand) sensor using mediator-less microbial fuel cell. *Biotechnology Letters*, *25*, 541-545.

Lovley, D.R. (2006). Bug juice: harvesting electricity with microorganisms. *Nature Reviews Microbiology*, *4*, 497-508. https://doi.org/10.1038/nrmicro1442

Oh S., Kim J., Premier, G., Lee, T., Changwon, K., & Sloan, W. (2010). Sustainable wastewater treatment: how might microbial fuel cells contribute. *Biotechnology Advances*, *28*, 871–881. https://doi.org/10.1016/j.biotechadv.2010.07.008

Olias, L.G., Otero, A.R., Cameron, P.J., & Lorenzo, M.D. (2020). Ceramic Soil Microbial Fuel Cells Sensors for Early Detection of Eutrophication. *Proceedings*, *60*, 64. https://doi.org/10.3390/IECB2020-07039

Salgado, C.A. (2009). Microbial fuel cells powered by *Geobacter sulfurreducens*. *Basic Biotechnology* 5, 5:1.

Sengodon, P., Steen, E., & Hays, D.B. (2012). Future Fuel Technologies, National Petroleum Council (NPC) Study.

Slate, A.J., Whitehead K.A., Brownson, D.A.C., & Banks, C.E. (2019). Microbial fuel cells: An overview of current technology. *Renewable and Sustainable Energy Reviews*, 101, 60-81, ISSN 1364-0321. https://doi.org/10.1016/j.rser.2018.09.044

Wang, X., Cai, Z., Zhou, Q., Zhang, Z., & Chen, C. (2012). Bioelectrochemical stimulation of petroleum hydrocarbon degradation in saline soil using U-tube microbial fuel cells. *Biotechnology and Bioengineering*, *109*, 426–433. https://doi.org/10.1002/bit.23351

Zhang, J., Cao, X., Wang, H., Long, X., Li, X. (2020a). Simultaneous enhancement of heavy metal removal and electricity generation in soil microbial fuel cell. *Ecotoxicology and Environmental Safety*, *192*, 110314. https://doi.org/10.1016/j.ecoenv.2020.110314

Zhang, X., Davidson, E. A., Zou, T., Lassaletta, L., Quan, Z., Li, T., & Zhang, W. (2020b). Quantifying nutrient budgets for sustainable nutrient management. *Global Biogeochemical Cycles*, *34*, e2018GB006060. https://doi.org/10.1029/2018GB006060

Zhou, M., Wang, H., Hassett, J.D., & Gu, T. (2013). Recent advances in microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) for wastewater treatment, bioenergy and bioproducts. *Journal of Chemical Technology & Biotechnology*, *88*, 508–518. https://doi.org/10.1002/jctb.4004

Chapter 2 - Literature Review

2.1. Microbial fuel cell definition

Microbial fuel cells are bioelectric devices that use the inherent capability of microorganisms to generate electricity (Huang et al., 2018; Barbato & Gronwald, 2018). The chemical energy stored in the organic substrate, such as sugars, is converted to electrical energy by microorganisms. Microbial fuel cells or MFCs work similarly to a battery which uses the electrochemical processes to convert chemical energy into electricity (Barbato & Gronwald, 2018). A battery essentially consists of i. anode (the negative terminal) ii. cathode (the positive terminal), and iii. electrolyte, an ionic conduction medium that allows the movement of ions from the anode to the cathode (Barbato & Gronwald, 2018). Similarly, microbes oxidize organic matter in an MFC and produce electrons that are transferred to the anode and flow to the cathode through a conductive material (Logan et al., 2006). As a part of anaerobic respiration, some bacteria can perform extracellular respiration in which they can transfer the electrons produced to extracellular electron acceptors (Lovley, 2008). This extracellular respiration is commonly found in metal-reducing organisms such as *Geobacter spp.* and *Shewanella spp*. (Sure et al., 2016).

To be classified as an MFC, the device should have the substrate oxidized at the anode restored either continuously or intermittently; otherwise, it is considered as a biobattery (Logan et al., 2006). There are numerous advantages associated with this technology; it is inexpensive, has a low impact on the environment, and is sustained for prolonged periods. This chapter will review some uses of MFCs, their history, materials required to build MFCs, types of MFCs, evolution, prospects of soil-based MFCs, and some challenges to this technology.

2.2. History of MFCs

The first reported occurrence of electrical activity by microorganisms can be traced back to the 1900s when Porter discovered the production of electricity with bacteria (*E. coli*) and yeast (*Saccharomyces*) in a primitive MFC containing platinum electrode (Potter, 1911). Later, the design was improved, and the activity of bacteria was confirmed by Cohen (1931), who observed a voltage of 35V at a 0.2 mA current with stacked bacterial fuel cell systems [as cited by Barbato & Gronwald, 2018]. This advancement showed some interest. However, it was not until the 1980s when Bennetto and Allen developed a one-of-a-kind microbial fuel cell design, revolutionizing the original design through an understanding of the electron transport chain with considerable upgrades in technology. This design is still being used at the core of modern-day MFCs (Barbato & Gronwald, 2018; Bennetto, 1990).

In 1990, Habermann and Pommer reported a long-term MFC for the first time that was employed for 5 years continuously without malfunction or maintenance. This MFC used wastewater as substrate, becoming the first-ever MFC to treat domestic wastewater. This study also reported using bacteria for indirect electron transfer via soluble mediators (sulfate/sulfide in this study) (Slate et al., 2019). In 1999 when Kim developed a mediator-less MFC using natural electrochemically active bacteria to transport electrons (Kim et al., 1999; Logan et al., 2006). This design diminished the cost and prospects of otherwise potentially toxic mediators used in MFCs earlier (Barbato & Gronwald, 2018). Subsequently, there has been a tremendous interest within the field of electromicrobiology in terms of researchers and applications, and the reported electric current from MFCs has also significantly improved (Pant et al., 2010).

2.3. Working principle

A typical MFC consists of two chambers, an anode and a cathode separated by a proton exchange membrane in the case of a dual-chamber MFC. In the anode chamber, under anaerobic conditions, an organic substrate is oxidized by microorganisms, generating electrons and producing carbon dioxide (reduction reaction). The electrochemically active microorganisms can transfer these electrons to the anode, from where these travel through an external circuit to the cathode. The cathode is placed in an aerated environment (cathode chamber) where the terminal electron acceptor oxygen is abundant. Subsequently, electrons combine with protons and oxygen at the cathode to form water (oxidation reaction). A typical soil microbial fuel cell with oxidation and reduction reactions is shown in Figure 2.1. An example of the reactions occurring at the anode using acetate as a fuel source and at the cathode is as follows (Kumar, 2017; Du et al., 2007):

$$CH_3COOH + 2H_2O \rightarrow 2CO_2 + 8H^+ + 8e^-$$

$$2O_2 + 8H^+ + 8e^- \rightarrow 4H_2O$$

The microorganisms capable of transferring electrons are defined by Logan et al. (2006) as exoelectrogens. These exoelectrogens can transfer electrons to the anode by electron mediators (Sund et al., 2007; Turick et al., 2003; Chui et al., 2003), nanowires (Gorby, 2005), or by direct membrane-associated transfer (Bond & Lovely, 2003). In the cathodic chamber, oxygen is the prime electron acceptor due to its abundance and high reduction potential (Zhao et al., 2006; Rizmani-Yazdi et al., 2008). Oxygen can be provided in the cathode chamber by either bubbling the water or by using the cathode in air. Other electron acceptors commonly used in different MFC studies are ferricyanide, persulfate, nitrogen species, permanganate, mercury, iron, copper, chromium, hydrogen peroxide, etc. (summarized by Ucar et al., 2017).

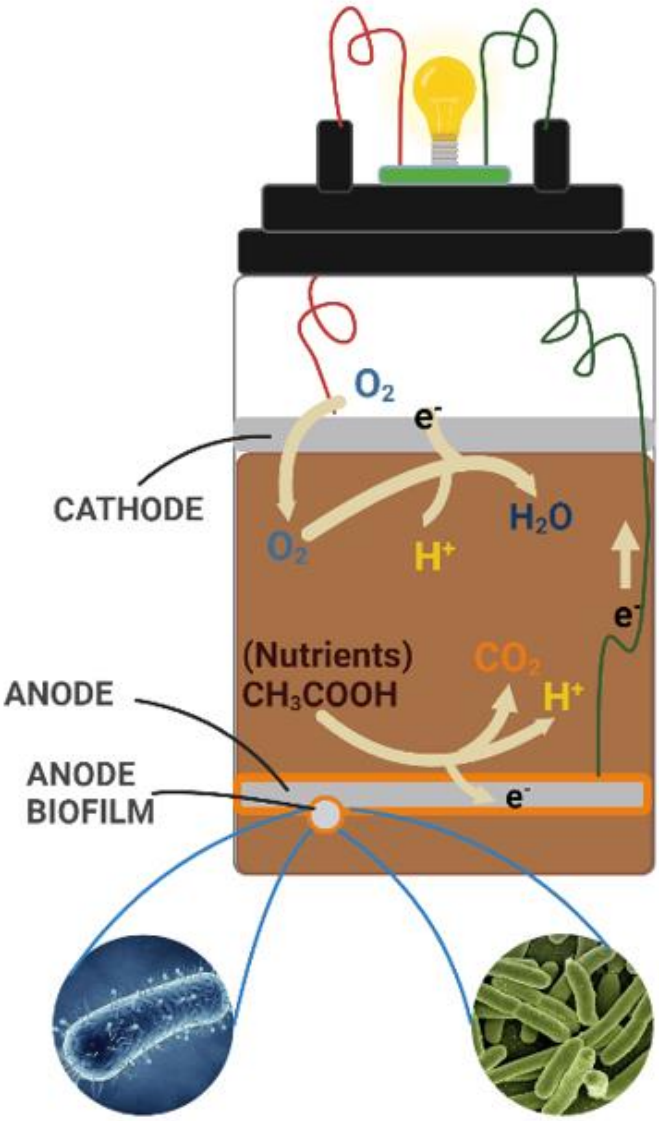

Figure 2.1. A diagram showing major oxidation and reduction reactions in a typical soil MFC. Reproduced from https://en.wikipedia.org/wiki/File:SoilMFC.png. Created using Biorender.com

2.4. Configuration of MFCs

Microbial fuel cells can broadly be divided into single-chambered and double-chambered MFCs. The difference between the two is the presence of a separate cathode chamber in the

double-chambered MFC and a requirement of a proton exchange membrane (PEM); whereas single-chambered MFC has cathode and anode compartments in the same system without physical separation and can function with or without PEM (Tamboli & Eswari, 2019).

2.4.1. Double-chambered MFC

Double-chambered MFC is the most common and the earliest attempt of creating a microbial fuel cell (Tamboli & Eswari, 2019). This conventional design consists of one anode chamber and one cathode chamber connected by a bridge and separated by a proton or cation exchange membrane. The purpose of PEM is to allow selective movement of protons generated from the organic substrate oxidation at the anode to the cathode chamber and to prevent diffusion of oxygen into the anode chamber. Liu and Logan (2004) demonstrated that when PEM was removed, oxygen transfer to the anode chamber increased substantially. In a typical double-chambered MFC, microorganisms grow on the anode or anolyte and transfer the electrons to the anode. Protons are transferred to the cathode chamber through the membrane. It is important to maintain anaerobic conditions in the anode chamber and oxygen-reducing conditions in the cathode chambers for the MFCs to operate. The first double chamber MFC was constructed by Potter (1911), which consisted of a glass jar and a porous cylinder inside. As a nutrient medium and electrolyte, glucose was added in both chambers and one platinum electrode in each. The modeling of MFCs was made by Zhang and Halme (1995). They had dual chambers; anode made of stainless-steel net packed with small graphite particles of 1.5 mm diameter. A cation exchange membrane was used to separate the anodic and cathodic chambers, and phosphate buffer was used as the catholyte. The mediator used was 2-hydroxy-l, 4- naphthoquinone. Nowadays, cathodes used for MFCs are carbon electrodes either coated with catalyst placed in water or a ferricyanide solution (Prakash, 2016).

2.4.2. Single-chambered MFC

Single-chambered MFC contains only one chamber with both anode and cathode, which may or may not be separated by a proton exchange membrane (PEM). There is no requirement for separate electrolytes for both chambers. This type is low-cost and easy to construct. Ghangrekar and Shinde (2007) reported that if the distance between the anode and cathode was less, it reduced internal ohmic resistance and thus increased the power density. However, in the membrane-less configuration, there are more chances of microbial contamination and crossover of oxygen from cathode to anode without PEM (Kim et al., 2008).

2.4.3. Stacked MFC

The theoretical maximum voltage an MFC can achieve is 1.2 V since the redox potential of reduced biomolecules (e.g., glucose in anodic reaction) is -0.4 V and the redox potential of oxygen vs standard hydrogen electrode (SHE) is 0.8 V (cathodic reaction) (Cooke et al., 2010; Tamboli & Eswari, 2019; Logan et al., 2006). Stacking is recommended to increase the power output. Stacking involves connecting MFCs in either series or parallel circuits. Connecting several fuel cells in series adds the voltage, while common current flows through them. Whereas, in parallel, the voltage averages and currents are added. Aelterman et al. (2006) connected six individual MFC units using cathode and anode of graphite granules and produced a maximum hourly average of 258 W m^{-3}.

2.4.4. Other configurations

The structural designs of MFCs can vary to a great extent depending on the purpose and in order to enhance power outputs. One example is the up-flow reactor which has received considerable attention. The up-flow microbial fuel cell, with sucrose as the substrate, was constructed by He et al. (2005) to generate electricity while treating wastewater, which recorded

a maximum power density of 170 W m^{-2}. Min and Logan (2004) designed a Flat Plate MFC (FPMFC) using a combined electrode/proton exchange membrane system operated in a continuous flow mode. The PEM (Nafion 117) was placed on the top of the anode and hot-pressed to the cathode like a sandwich between two polycarbonate plates and formed a PEM/electrode assembly (Min & Logan, 2004). Apart from this, miniature MFCs are becoming popular in both fundamental and applied studies because of their intrinsic advantages (Qian & Morse, 2011). Miniature MFCs can be developed from milliliter to microliter scale, and they are easy to fabricate and flexible in design (Qian & Morse, 2011). A miniature MFC (chamber volume of 1.2 mL) was developed by Ringeisen et al. (2006) using a biofilm developed from a pure culture of *Shewanella oneidensis*, featuring high surface area to volume (SAV) ratio chambers for enhanced proton diffusion, which produced a power density of 500 W m^{-3}.

2.5. Methods of electron transfer

Exoelectrogenic bacteria have the ability to transport electrons via two mechanisms, direct and indirect electron transfer. For direct electron transfer to happen, physical contact between the bacterial cell membrane and the electrode is necessary, for example, through nanowires and/or redox-active proteins (Slate et al., 2019). For indirect electron transfer, electron shuttling molecules, such as phenazine and flavins are involved (Lovely, 2012).

2.5.1. Direct transfer via conductive pili

A significant discovery in electromicrobiology was observed by Kim et al. (2002). They showed that electron transfer does not always require mediator compound molecules (Kim et al., 2002). The cell surface of specific isolated bacterial species, such as *Geobacter spp.* and *Shewanella spp*. have micrometer long proteinaceous filaments extending from their outer surface into the extracellular matrix that was also thought to be involved in the extracellular

transport of electrons (Slate et al., 2019). These appendages are referred to as microbial nanowires due to their long filament-like appearance and conductive properties revealed in a study by Reguera et al. (2005). In a study on microbial fuel cells performed by Nevin et al. (2009), it was found that deletion of PilA gene in *Geobacter sulfurreducens* reduced the microbial nanowire formation and considerably reduced the current density. Nanowires can be flagella or pili, both having very distinctive properties. Therefore, they are termed as "micro-nanowires" and "macro-nanowires" to describe pili and flagella, respectively, by Slate et al. (2019). Pili and flagella are homopolymers consisting of a single subunit whereas nanowires are a composite of different cytochromes, periplasmic, and membrane proteins (Sure et al., 2016). An MFC was operated for 5 months which led to the isolation of the KN400 strain of bacteria *Geobacter sulfurreducens*, producing an 8-fold increase in power density. This strain produced thinner biofilms with fewer outer surface cytochromes but more abundant electrically conductive nanowires giving insight into the complex mechanisms of electrode-microbe interactions (Yi et al., 2009).

2.5.2. Redox active proteins

Another electron transfer mechanism is through the redox-active proteins present on the outer cell membrane that facilitates short-range electron transfer (Yue et al., 2006). Kim et al. (2002) observed that electrochemical activity in *S. putrefacians* is due to direct electron transfer via electron carriers located on the cell surface, possibly the outer membrane cytochromes. Genome-scale gene expression studies have also shown that bacterial cells in direct contact with anode interact via c-type cytochromes on the outer cell surfaces (Kim et al., 2005; Holmes et al., 2006, 2008), while cells farthest from the electrode utilize long-range electron transfer via conductive nanowire network. (Franks & Nevin, 2010). The c-type cytochromes are multi-heme

proteins with a redox potential range of nearly 1 Volt and are stable against chemical modifications (as cited in Aiyer, 2020). These molecules are known for their primary function in mitochondria as these play a significant role in ATP synthesis (Ow et al., 2008). The MFCs which do not need any mediator to transfer electrons are classified as mediator-less MFCs (Parkash, 2016).

2.5.3. Presence of mediators

Some bacterial species cannot transfer electrons to the electrode due to the non-conductive nature of the cell surface structures. So, in that case, electrochemical mediators help in electron transfer from inside the bacterial cell to the anode (Prakash, 2016; Rabaey, 2005) and are often referred to as electron shuttles (Slate et al., 2019). In many studies, redox mediators such as neutral red, thionine methyl viologen, potassium ferric cyanide, and anthraquinone-2,6-disulfonate (AQDS) have been used. The mediators are required in high concentrations, and many of these are toxic chemicals; therefore, it becomes impractical to utilize these on a large scale to generate electricity (Liu et al., 2004). Some electroactive species can produce their own extracellular mediators, such as pyocyanin by *P. aeruginosa* and quinone by *S. oneidensis* (Tamboli & Eswari, 2019), that can eliminate the need for adding mediators (Liu et al., 2004). Some secondary metabolites produced by bacteria, such as flavins, have also acted as an electron shuttle when *S. oneidensis* biofilms were analyzed (Marsili et al., 2008). Mediators enter the cell in their oxidized form and get reduced by reducing agents inside the cell. The reduced mediators are permeable and capable of diffusing out of the cells to attach to the electrode surface, where these get electrochemically oxidized by transfer electrons. These oxidized mediators can start the cycle again. (Parkash, 2016).

2.6. Materials needed in MFC

2.6.1. Anode

At the anode, electroactive biofilm is created and bioelectrochemical reactions occur(Barbato & Gronwald, 2018). The anode should be placed in an anaerobic environment so that the necessary reaction can happen; for example, if it is in aerobic condition, oxidation of organic substrates (cellular respiration) will give carbon dioxide and water, whereas, in an anaerobic environment, oxidation of substrate will instead give carbon dioxide, protons, and electrons. The anode should have good conductivity, chemical stability, mechanical strength, and high surface area, and should be cost-effective. Apart from these, the anode in an MFC should also have i) high surface roughness; ii) biocompatibility; and iii) surface chemistry that promotes bacterial attachment and easy electron transfer (Cornejo et al., 2015). A variety of metals, including stainless steel, titanium, gold, copper, nickel, and carbon materials, such as carbon cloth, carbon paper, carbon graphite brush, carbon felt, biomass-derived porous carbon, have been used for the anode (Dong et al., 2012; Zhang et al., 2013; Logan et al., 2007). Among these materials, the most popular are the carbonaceous anodes, as these are cost-effective and promising for the large-scale applications of MFCs (Barbato & Gronwald, 2018).

2.6.2. Cathode

The cathodic compartment usually contains the cathode material, a catalyst to increase the consumption rate of electrons, and an electron acceptor (Zhang et al., 2012). The electrode materials mentioned above used for anodes can also be used as cathodes. Ferricyanide is used as a common electron acceptor due to its decent enactment in MFCs, and in its presence, a catalyst is not required at the cathode (Kumar et al., 2017). The advantage of using ferricyanide is that it generates low overpotential when using a plain carbon cathode, which results in cathode working

potential being close to the open circuit potential (Logan et al., 2006). Oxygen is one of the best alternatives for the electron acceptor due to its high redox potential, abundance, low price (free), sustainability, and because oxygen generates no harmful waste products (Logan et al., 2006, Bond & Lovely, 2003). However, oxygen reduction reactions on plain carbon are very slow and result in large overpotential; therefore, a catalyst is required. To increase the rate of the reaction (oxygen reduction), Pt catalysts are used (Kumar et al., 2017, Logan et al., 2006).

2.6.3. Proton exchange membrane

An ion exchange membrane is generally employed in an MFC between the anode and the cathode chamber e.g., a proton exchange membrane (PEM), which allows the exchange of only protons or specific cations from the anode to the cathode compartment. Exceptions to the MFC designs not using a PEM are the naturally separated systems, such as sediment MFCs (Reimers et al., 2001), and soil MFCs in which soil acts as the nutrient-rich anodic media, the inoculum, and the proton exchange membrane (Lovley & Nevin, 2008). The frequently used PEM is Nafion, while Ultrex CMI-7000 is also suitable for MFC applications, and it is more economical than Nafion (Kumar et al., 2017). However, PEM such as Nafion is reported to be permeable to oxygen, resulting in some oxygen diffusion from the cathode chamber to the anode chamber, which can cause loss of electron donor due to aerobic respiration by bacteria, lowering overall coulombic efficiency (Min & Logan, 2004). In addition, PEM can also be leaky to an anolyte, or in some cases to catholyte, which can considerably decrease MFC's performance (Kumar et al., 2017).

2.7. Microorganisms in MFCs

Microorganisms have the most crucial role to play in MFCs. They metabolize the organic substrates and transfer the electrons generated extracellularly to the electrode surface. Because

microbes in an MFC may fulfill all the energy and carbon requirements from the oxidation of the complex organic matter for their cellular growth and can conserve energy in this sense, MFC technology is thus referred to as self-sustaining (Lovley, 2006; Franks & Nevin, 2010). As long as the conditions remain favorable for current production, such as a regular supply of nutrients for the anodic microorganisms, an MFC has the potential to produce electricity indefinitely (Franks & Nevin, 2010). Microorganisms are found abundantly in marine sediment, soil, wastewater, freshwater sediment, and activated sludge (Zhang et al., 2006). One of the most popular and extensively studied microorganisms in the MFCs capable of producing high current densities is *Geobacter sulfurreducens* of any known pure culture and referred to as the "gold standard" (Slate et al., 2019).

Geobacter sulfurreducens is a gram-negative, rod-shaped, delta-proteobacterium and is a non-fermentative, obligate anaerobe with a flagella and type IV pili production. It belongs to the group of dissimilatory metal-reducing microorganisms, which reduces metal oxides under anaerobic conditions in soils and sediments and produces biologically useful energy in ATP (Du et al., 2007). The electrons are then transferred to the final electron acceptor, such as ferric oxide, primarily by direct contact of metal-reducing microorganisms to the mineral oxides (Lovley et al., 2004). In contrast, *Shewanella* and *Geothrix* species follow a different mechanism to transfer electrons. These species produce chelators that solubilize Fe (III) compounds and release electron shuttling compounds that facilitate the transfer of electrons from the cell surface to the surface of Fe (III) oxides which are not in direct contact with the cells (Lovley et al., 2004). The anodic reaction occurring in mediator-less MFCs involving metal-reducing bacteria belonging mainly to families of *Shewanella*, *Geobacter*, and *Rhodoferax* is similar to the process mentioned above because, in MFCs, the anodes act as the final electron acceptor just like the solid mineral

oxides (Du et al., 2007). Metal-reducing bacteria transfer the electrons to the anode through electrochemically active redox proteins located on the outer cell surface, allowing these to generate power in the absence of exogenous mediators. In MFCs using *Rhodoferax ferrireducens*, the observed power output was 33 mW m^{-2} with coulombic efficiency of 83% (oxidizing glucose). *Geobacter sulfurreducens* generated 14.7 mW m^{-2} with a coulombic efficiency of 96.8% (oxidizing acetate), and *Geothrix fermentans* had a coulombic efficiency of 94% oxidizing acetate (Oh & Logan, 2006; Franks & Nevin, 2010). Pure cultures capable of generating electricity belong to classes Firmicutes, Acidobacteria, four of the five classes of Proteobacteria as well as the yeast strains *Saccharomyces cerevisiae* and *Hansenula anomala* (as summarized by Franks & Nevin, 2010). Most of the mediator-less MFCs are operated with dissimilatory metal reducing microorganisms. An exception to this case was reported with *Clostridium butyricum*, which is a gram-positive fermentative bacterium (Park et al., 2001).

Mixed culture microbes are introduced in the anode chamber when marine sediments or anerobic sludge is inoculated in the MFCs (Du et al., 2007). Rabaey and Verstraaete (2005) reported mixed cultures had higher performances than isolated pure cultures with the advantage of much wider substrate utilization. This means that MFCs have much wider substrate specificity when having mixed cultures than pure cultures.

Ieropoulos et al. (2005) showed a relationship between power output and sulfur compounds (S-replete and S-deplete effects on power output) in an MFC containing anaerobic sludge. Their system was a mix of sulfate/sulfide mediator system and an anodophilic system due to naturally occurring levels of S-containing materials in sludge. They concluded that up to 70-80% of the power output was contributed by the sulfate/sulfide mediated system and only 20% by anodophilic microbial activity.

2.8. Applications of MFCs

2.8.1. Electricity generation

For many years, it has been known that electricity generated by microorganisms can be harvested in microbial fuel cells (Allen & Benetto, 1993; Du et al., 2007). The substrates that can be completely oxidized into electrons are particularly important to achieving high coulombic efficiency and high power output in MFCs (Kumar et al., 2017). In underdeveloped regions of the world, MFCs can serve as distributed power systems locally (Du et al., 2007). Microbial fuel cells are especially suitable for powering small devices for telemetry or sensing that have small power requirements such as transmitting signals like temperature to receivers in remote areas (Ieropoulos et al., 2005b).

2.8.2. Wastewater treatment

Generating power from wastewater while oxidizing organic or inorganic compounds is one of the most active areas of research in MFCs (Franks & Nevin, 2010). The MFCs can treat different kinds of wastewaters, such as industrial, urban, or domestic (Rhoads et al., 2005; Oh et al., 2010; Zhou et al., 2013). The first demonstration using domestic wastewater as a substrate in MFCs was reported by Liu et al. in 2004. They used single-chamber MFCs with a single air cathode and reported to remove 80% of the chemical oxygen demand (COD) of wastewater. The MFC studies usually involving wastewater treatment are coupled with power generation, although the coulombic efficiencies achieved from these systems are not that significant, varying from 3-12% (Liu et al., 2004). Wastewater components act as a substrate for microbial metabolism to produce protons and electrons and are a good inoculum source (Kumar et al., 2017). The fact that MFC can degrade a variety of environmental pollutants itself may be more valuable than the generation of electricity in specific settings, especially when MFC can be used

to clean up contaminants in the environment in situ (Franks & Nevin, 2010). MFCs may not be able to treat highly toxic wastewaters completely. However, these are capable enough to reduce the COD of wastewaters that can meet the discharge regulations before being released into the environment, and they have proven to remove up to 98% COD from the wastewater (Kumar et al., 2017; Oh et al., 2010).

2.8.3. Biosensors

Kim et al. (2003) first reported the use of MFC as biochemical oxygen demand (BOD) biosensor. They found a proportional correlation between the coulombic yield and the strength of the wastewater (Kim et al., 2003). The sensor was kept operational for five years without extra maintenance, far longer than any other type of BOD biosensor reported in the literature (Kim et al., 2003). The ability of MFC to act as a biosensor stems from the linear relationship between the coulombic yield of MFC and wastewater strength (Kumar et al., 2017). The MFC-based biosensor is expected to be one of the most promising applications of MFCs. It has been studied to test various parameters, including biochemical oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen (DO), toxicants, and microbial activity (Cui et al., 2019). There are several advantages of MFC type BOD sensors over other BOD sensors, such as excellent operational stability and good reproducibility and accuracy, and microorganism variety (Du et al., 2007; Parkash, 2016). In an MFC-based biosensor, the anodic biofilm plays the role of the bioreceptor, while the anode is the transducer (Cui et al., 2019). The biofilm responds to the disturbance that affects the electron transfer rate transduced to a measurable signal (Cui et al., 2019; Chauler and Lorenzo, 2015).

2.9. Challenges and future perspectives

The MFC technology has still not touched a commercial base despite intensive research in the last decades. There is still a lot to do to launch MFC in real-world applications. The major challenge in the large-scale application of MFCs is their low power output. As reported by Pietrelli et al. (2016), the power output of MFCs is too low to supply to low-power electronic devices, which require more than 3V continuously for normal operations. They observed a drastic power reduction after 2 days of operation. They named the period as "relaxation time" for microorganisms as they could not work in continuous mode for more than two days. It was also observed power obtained was not steady. Kim et. al (2007) reported that MFCs using similar microbial consortia and substrates had differences in the power density. Differences in MFC configurations, including cathode/anode/PEM materials, design and solution concentration, and chemistry, have impeded the progression of MFC technology because there is significantly less direct comparison available (Slate et al., 2019; Yang et al., 2017).

To enhance power density output, anodophilic microorganisms that can improve the electron transport rate to the anode are the need of the hour (Angenent et al., 2004). Lovley claimed that MFC's current could increase by ten thousand times if *Geobacter* transferred the electrons to the electrode at the same rate as its natural electron acceptor- ferric iron (Holzman et al., 2005). Biofouling is another performance-limiting factor in MFCs. Biofouling is the production of non-conductive debris, such as dead microbial cells on the electrode surface, which isolate the electrochemically active microbial biofilm from the electrode surface and leads to a potential reduction in the available surface area, thereby reducing current generation (Slate et al., 2019). There is a need to develop higher catalytic material with higher performance that limits biofouling, corrosion, and other degrading mechanisms of electrodes (Huang et al., 2011).

The low catalytic rate of the microbes also limits the scale-up of MFCs. Even if microbes work at their fastest growth rates, they are slow transformers (Du et al., 2007). Cross-over of organic compounds or electron acceptors from anode to cathode and vice versa has shown to be disrupting biofilm formation leading to biofilm inactivation, which can lead to forming mixed potentials, ultimately decreasing MFC performance (Slate et al., 2019). Kim et al. (2016) used dual anode in membrane-less MFC to provide a larger reaction surface to prevent the organic compounds crossover, which consequently enhanced the MFCs' performance. The literature also shows that the internal resistance also hinders the performance of MFCs from proton mass transfer and slow oxygen reduction kinetics at the cathode (Kim et al., 2007). Therefore, reducing the depth of the proton transfer is recommended to improve proton mass transfer from the anode to the cathode, which might be possible by using hollow fiber-type reactors or using a proton-specific membrane (Kim et al., 2007). While significant efforts by researchers are being made to improve the performance of MFCs by developing novel alternate designs, electrode materials, catalysts, and microorganisms, MFCs have a long way to go before they are commercialized (Do et al., 2018). Their energy output is still too low to be able to make an "energy-neutral operation" possible at the practical scale and, high capital and operational costs make it even more difficult (Do et al., 2018).

2.10. Potential and research gaps

Over the last decades, continuous efforts have improved MFC technology, focusing on advantages like environmental friendliness and energy production from wastewater and organic compounds. However, it is still considered a laboratory science rather than a useful technology because of the numerous limitations stated above. MFCs at the laboratory scale have been able to reach volumetric power densities as high as 2.15 kW m^{-3} and various studies show COD removal

efficiencies of over 90% (Waller & Trabold, 2013). Despite achieving high COD removal efficiency and power densities, significant research is required to make this technology a viable alternative for energy and other applications. Most MFC studies were laboratory-scale running on batch mode, which is impractical for many applications with steady flow rates (Waller & Trabold, 2013). Also, laboratory studies have used expensive materials that are not feasible for real-world settings. Additionally, most studies have focused on using pure bacteria cultures for energy generation, which does not represent a real-world scenario. Most researchers have limited themselves to synthetic wastewater, which is helpful for material selection and output but does not provide enough information on actual performance (Waller & Trabold, 2013). There are issues of scaling-up and choice of low-cost materials to enhance performance and other technical barriers that need to be solved at a small scale before expanding to industrial operation. More studies are needed to apply molecular biology or genetic engineering techniques to obtain higher specificity of microorganisms towards monitoring nutrients in biosensing techniques.

2.11. Review of soil-based MFCs

Soil-based MFCs were first explored in 2006, and it was shown that electricity could be generated from the microbes and the nutrients present in soil alone (Schamphelaire et al., 2008; Niessen et al., 2006). For MFCs utilizing natural media such as soil, sediments, or effluents, inoculation of specific electrogenic bacteria is not needed because these media already contain abundant electrogenic species. Because nutrients are constantly supplied/replenished in this media through plant and animal matter decay, soil-based MFCs have a theoretically indefinite lifespan. Additionally, the aerobic (oxygen consuming) microorganisms present in the soil act as an oxygen filter that is comparatively cheaper than the costly PEM materials used in laboratory MFC systems and cause the redox potential of the soil to decrease with increasing depth reaching

about -0.2V vs SHE (Cooke et al., 2010). However, microbial fuel cells studies based in soil are scarce, likely due to the complexity of the system. Here, I tried to compile some microbial fuel cell studies based on soil and how these can relate to the overall objectives of our studies.

2.11.1. Soil-based microbial fuel cells potential as a biosensor

Several studies in the past two decades have found significant relationships between electrical signals and microbial community, along with some soil properties. In a study by Cristiani et al. (2008), cathodic polarization detected as electrical signals was correlated with microbial growth and biofilm development on stainless steel electrodes when the electrochemical probes were directly inserted into the soil. They also highlighted the importance of soil humidity in the development of a stable biofilm, as well as it being the most critical and limiting factor for biofilm growth above ambient temperature and nutrient content. Wang et al. (2015) constructed MFC with five different paddy soils having distinct chemical properties. They found that paddy soil having higher DOC and ammonium concentration in porewater showed higher MFC performance. They also found that high-performance MFC was dominated mainly by Deltaproteobacteria. In contrast, low performing MFCs (with low DOC and ammonium concentrations) were dominated by Betaproteobacteria hence implying that soil properties have a significant effect on bacterial composition and thus MFC performance.

Another MFC-based sensor was recently proposed for in-field monitoring for early detection of eutrophication by Lorenzo et al. (2020). They developed a ceramic soil microbial fuel cell as a self-powered sensor for algal growth detection by monitoring the level of dissolved oxygen in water. This sensor was found to be correlated to algal concentration and dissolved oxygen levels (Lorenzo et al., 2020). Magotra et al. (2020) demonstrated electricity production

using waste urea as a fuel source for compost soil microbial fuel cells, which also would be a step towards curbing eutrophication.

A mathematical model has been proposed by Casula et al. (2020) to assess the effects of soil properties and system design on the performance of soil microbial fuel cells. Another study showed that when different concentrations of heavy metals were added to soil, the start-up time and coulomb quantity generated by soil exoelectrogenic bacteria were significantly correlated with soil dehydrogenase activity, a sensitive indicator of soil microbial activity (Deng et al., 2015; Jiang et al., 2015). Jiang et al. (2018) also found correlations between start-up time and soil dehydrogenase activity and soil organic matter content. Earlier, electrical signals and soil microbial activity was found to be affected by soil organic matter content and the microbial community in the soil (Dunaj et al., 2012). It was also found that current was correlated with temperature, which is also a vital factor influencing soil microbial activity (Deng et al., 2014; Gong et al., 2021).

All these studies lead to the fact that soil microbial community and nutrient levels affect microbial fuel cell performance. This is a new and wide area for research. Successful modeling of relationships between nutrient dynamics, microbial activity, and electrical signals can provide an MFC -based biosensor that can provide information on the availability of nutrients in real-time which is the bigger goal of our research. Our goal is to develop a soil-based MFC biosensor that tracks down changes in nutrient availability via changes in electrical signals.

2.12. References

Aelterman, P., Rabaey, K., Pham, T. H., Boon, N., Verstraete, W. (2006). Continuous electricity generation at high voltages and currents using stacked microbial fuel cells. *Environmental Science & Technology*, *40*, 3388-3394.

Aiyer, K. S. (2020). How does electron transfer occur in microbial fuel cells? *World Journal of Microbiology and Biotechnology*, *36*(2), 1-9. https://doi.org/10.1007/s11274-020-2801-z

Allen, R. M., & Benneto, H. P. (1993). Microbial Fuel Cells: Electricity Production from Carbohydrates. *Applied Biochemistry and Biotechnology*, *39*, 27-40.

Angenent, L. T., Karim, K., Al-Dahhan, M. H., Wrenn, B. A., & Domíguez-Espinosa, R. (2004). Production of bioenergy and biochemicals from industrial and agricultural wastewater. *TRENDS in Biotechnology*, *22*(9), 477-485.

Barbato, R., & Gronwald, F. (2018). A Study of Soil Based Microbial Fuel Cells. *International Journal of Scientific Research and Engineering Development*, *1*, 60-157.

Bennetto, H.P. (1990) Electricity generation by microorganisms. *Biotechnology Education*, *1(4)*,163-168.

Biffinger, J. C., Pietron, J., Ray, R., Little, B., & Ringeisen, B. R. (2007). A biofilm enhanced miniature microbial fuel cell using Shewanella oneidensis DSP10 and oxygen reduction cathodes. *Biosensors and Bioelectronics*, *22*(8), 1672-1679. https://doi.org/10.1016/j.bios.2006.07.027

Bond, D.R., & Lovley, D.R. (2003). Electricity production by *Geobacter sulfurreducens* attached to electrodes. *Applied and environmental microbiology*, *69(3)*, 1548-1555. https://doi.org/10.1128/AEM.69.3.1548-1555.2003

Casula, E., Kim, B., Chesson, H., Di Lorenzo, M., & Mascia, M. (2021). Modelling the influence of soil properties on performance and bioremediation ability of a pile of soil microbial fuel cells. *Electrochimica Acta*, *368*, 137568. https://doi.org/10.1016/j.electacta.2020.137568

Chouler, J., & Di Lorenzo, M. (2015). Water quality monitoring in developing countries; can microbial fuel cells be the answer? *Biosensors*, *5*(3), 450-470. http://dx.doi.org/10.3390/bios5030450

Cooke, K. G., Gay, M. O., Radachowsky, S. E., Guzman, J. J., & Chiu, M. A. (2010, May). BackyardNet: distributed sensor network powered by terrestrial microbial fuel cell technology. In *Unattended Ground, Sea, and Air Sensor Technologies and Applications XII* (Vol. 7693, p. 76931A). International Society for Optics and Photonics. https://doi.org/10.1117/12.853930

Cristiani, P., Franzetti, A., & Bestetti, G. (2008). Monitoring of electro active biofilm in soil. *Electrochimica Acta*, *54*, 41–46.

Cui, Y., Lai, B., & Tang, X. (2019). Microbial fuel cell-based biosensors. *Biosensors*, *9*(3), 92. https://doi.org/10.3390/bios9030092

Deng, H., Jiang, Y. B., Zhou, Y. W., Shen, K., & Zhong, W. H. (2015). Using electrical signals of microbial fuel cells to detect copper stress on soil microorganisms. *European Journal of Soil Science*, *66*(2), 369-377.

Do, M. H., Ngo, H. H., Guo, W. S., Liu, Y., Chang, S. W., Nguyen, D. D., ... & Ni, B. J. (2018). Challenges in the application of microbial fuel cells to wastewater treatment and energy production: a mini review. *Science of the Total Environment*, *639*, 910-920.

Du, Z., Li, H., & Gu, T. (2007). A state of the art review on microbial fuel cells: A promising technology for wastewater treatment and bioenergy. *Biotechnology Advances, 25,* 464–482.

Dunaj, S. J., Vallino, J. J., Hines, M. E., Gay, M., Kobyljanec, C., & Rooney-Varga, J. N. (2012). Relationships between soil organic matter, nutrients, bacterial community structure, and the performance of microbial fuel cells. *Environmental Science & Technology*, *46*(3), 1914-1922.

Franks, A. E., & Nevin, K. P. (2010). Microbial fuel cells, a current review. *Energies, 3*(5), 899-919.

Ghangrekar, M.M., & Shinde, V.B. (2007). Performance of membrane-less microbial fuel cell treating wastewater and effect of electrode distance and area on electricity production. *Bioresource Technology*, *98,* 2879–2885.

Gorby, Y.A., & Beveridge, T.J. (2005). Composition, reactivity, and regulation of extracellular metal-reducing structures (nanowires) produced by dissimilatory metal reducing bacteria. Presented at DOE/NABIR meeting, Warrenton, VA.

Habermann, W. (1991). Biological Fuel Cells with Sulphide Storage Capacity. *Applied microbiology and biotechnology,* 35.1, 128–133.

He Z, Minteer SD, Angenent LT. Electricity generation from artificial wastewater using an upflow microbial fuel cell. Environ Sci Technol. 2005 Jul 15;39(14):5262-7. doi: 10.1021/es0502876. PMID: 16082955.

Holmes, D. E., Chaudhuri, S. K., Nevin, K. P., Mehta, T., Methé, B. A., Liu, A., Ward, J.E.; Woodard, T.L.; Webster, J.& Lovley, D. R. (2006). Microarray and genetic analysis of

electron transfer to electrodes in Geobacter sulfurreducens. *Environmental Microbiology*, *8*(10), 1805-1815.

Holmes, D. E., Mester, T., O'Neil, R. A., Perpetua, L. A., Larrahondo, M. J., Glaven, R., Sharma, M.L.; Ward, J.E.; Nevin, K.P., & Lovley, D. R. (2008). Genes for two multicopper proteins required for Fe (III) oxide reduction in Geobacter sulfurreducens have different expression patterns both in the subsurface and on energy-harvesting electrodes. *Microbiology*, *154*(5), 1422-1435.

Holzman, D.C. (2005). Microbe power. *Environmental Health Perspectives*, *113*, A754-A757

Huan, D. E. N. G., Yi-Cheng, W. U., Zhang, F., HUANG, Z. C., Zheng, C. H. E. N., Hui-Juan, X. U., & Feng, Z. H. A. O. (2014). Factors affecting the performance of single-chamber soil microbial fuel cells for power generation. *Pedosphere*, *24*(3), 330-338.

Huang, L., Regan, J.M., & Quan, X. (2011). Electron transfer mechanisms, new applications, and performance of biocathode microbial fuel cells. *Bioresource Technology*, *102*, 316-323.

Huang, H., Cheng, C., Yang J., Li, C., Sun, Y., & Cen, K. (2018). Effect of nitrate on electricity generation in single-chamber air cathode microbial fuel cells. *Chemical Engineering Journal*, *337*, 661-670. https://doi.org/10.1016/j.cej.2017.12.150

Ieropoulos, I., Greenman, J., Melhuish, C., & Hart, J. (2005). Energy accumulation and improved performance in microbial fuel cells. *Journal of power sources*, *145*(2), 253-256.

Ieropoulos, I., Melhuish, C., Greenman, J., & Horsfield, I. (2005). EcoBot-II: An artificial agent with a natural metabolism. *International Journal of Advanced Robotic Systems*, *2*(4), 31.

Jiang, Y. B., Deng, H., Sun, D. M., & Zhong, W. H. (2015). Electrical signals generated by soil microorganisms in microbial fuel cells respond linearly to soil Cd2+ pollution. *Geoderma*, *255*, 35-41.

Jiang, Y., Huan, D. E. N. G., Hongyi, Q. I. N., Cheng, H. A. N., & Zhong, W. (2018). Indication of soil microbial activity by electrical signals of microbial fuel cells with re-vegetated red soils. *Pedosphere*, *28*(2), 269-276. https://doi.org/10.1016/S1002-0160(18)60009-8

Kim, B. H., Park, D. H., Shin, P. K., Chang, I. S., & Kim, H. J. (1999). *U.S. Patent No. 5,976,719*. Washington, DC: U.S. Patent and Trademark Office.

Kim, B. C., Leang, C., Ding, Y. H. R., Glaven, R. H., Coppi, M. V., & Lovley, D. R. (2005). OmcF, a putative c-type monoheme outer membrane cytochrome required for the expression of other outer membrane cytochromes in Geobacter sulfurreducens. *Journal of Bacteriology*, *187*(13), 4505-4513.

Kim, B. H., Chang, I. S., Cheol Gil, G., Park, H. S., & Kim, H. J. (2003). Novel BOD (biological oxygen demand) sensor using mediator-less microbial fuel cell. *Biotechnology letters*, *25*(7), 541-545.

Kim, H. J., Park, H. S., Hyun, M. S., Chang, I. S., Kim, M., & Kim, B. H. (2002). A mediator-less microbial fuel cell using a metal reducing bacterium, Shewanella putrefaciens. *Enzyme and Microbial technology*, *30*(2), 145-152.

Kim, I. S., Chae, K. J., Choi, M. J., & Verstraete, W. (2008). Microbial fuel cells: recent advances, bacterial communities and application beyond electricity generation. *Environmental Engineering Research*, *13*(2), 51-65.

Kim, J., Kim, B., An, J., Lee, Y.S., & Chang, I.S. (2016). Development of anode zone using dual anode system to reduce organic matter crossover in membrane less microbial fuel cells. *Bioresource Technology*, *213,* 140–145.

Kim, B. H., Chang, I. S., & Gadd, G. M. (2007). Challenges in microbial fuel cell development and operation. *Applied microbiology and biotechnology*, *76*(3), 485-494. https://doi.org/10.1007/s00253-007-1027-4

Kim, H.J., Hyun, M.S., Chang, I.S., & Kim, B.H. (1999). A microbial fuel cell type lactate biosensor using a metal-reducing bacterium, *Shewanella putrefaciens*. *Journal of Microbiology and Biotechnology*, *9 (3),* 365-367.

Kumar, R., Singh, L., & Zularisam, A. W. (2017). Microbial fuel cells: types and applications. In *Waste Biomass Management–A Holistic Approach* (pp. 367-384). Springer, Cham.

Logan, B.E., Hamelers, B., Rozendal, R., Schröder, U., Keller, J., Freguia, S., Aelterman, P., Verstraete, W., Rabaey, K. (2006). Microbial fuel cells: methodology and technology. *Environmental Science & Technology*. *40(17)*, 5181-92. https://doi/10.1021/es0605016

Lovley, D. R. (2012). Electromicrobiology. *Annual review of microbiology*, *66*, 391-409.

Lovley, D.R. (2006). Bug juice: harvesting electricity with microorganisms. *Nature Reviews Microbiology*, *4*, 497-508. https://doi.org/10.1038/nrmicro1442

Lovley, D. R., Holmes, D. E., & Nevin, K. P. (2004). Dissimilatory Fe (iii) and Mn (iv) reduction. *Advances in microbial physiology*, *49*(2), 219-286.

Lovley, D. R., & Nevin, K. P. (2008, May). Electricity production with electricigens. In *Bioenergy* (pp. 293-306). Washington, DC, USA: ASM Press.

Magotra, V. K., Kumar, S., Kang, T. W., Inamdar, A. I., Aqueel, A. T., Im, H., ... & Jeon, H. C. (2020). Compost soil microbial fuel cell to generate power using urea as fuel. *Scientific reports*, *10*(1), 1-9. https://doi.org/10.1038/s41598-020-61038-7

Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., & Bond, D. R. (2008). Shewanella secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences*, *105*(10), 3968-3973.

Min, B., & Logan, B. E. (2004). Continuous electricity generation from domestic wastewater and organic substrates in a flat plate microbial fuel cell. *Environmental science & technology*, *38*(21), 5809-5814.

Nevin, K. P., Kim, B. C., Glaven, R. H., Johnson, J. P., Woodard, T. L., Methé, B. A., ... & Lovley, D. R. (2009). Anode biofilm transcriptomics reveals outer surface components essential for high density current production in Geobacter sulfurreducens fuel cells. *PloS one*, *4*(5), e5628.

Niessen, J., Harnisch, F., Rosenbaum, M., Schröder, U., & Scholz, F. (2006). Heat treated soil as convenient and versatile source of bacterial communities for microbial electricity generation. *Electrochemistry Communications*, *8*(5), 869-873.

Oh, S. T., Kim, J. R., Premier, G. C., Lee, T. H., Kim, C., & Sloan, W. T. (2010). Sustainable wastewater treatment: how might microbial fuel cells contribute. *Biotechnology advances*, *28*(6), 871-881. http://dx.doi.org/10.1016/j.biotechadv.2010.07.008

Oh, S. E., & Logan, B. E. (2006). Proton exchange membrane and electrode surface areas as factors that affect power generation in microbial fuel cells. *Applied microbiology and biotechnology*, *70*(2), 162-169.

Oh, S., & Logan, B. E. (2004). Electricity generation using an air cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. *Environmental Science & Technology*, *38*, 4040-4046. https://doi.org/10.1021/es0499344

Olias, L.G., Otero, A.R., Cameron, P.J., & Lorenzo, M.D. (2020). Ceramic Soil Microbial Fuel Cells Sensors for Early Detection of Eutrophication. *Proceedings*, *60*, 64. https://doi.org/10.3390/IECB2020-07039

Ow, Y. L. P., Green, D. R., Hao, Z., & Mak, T. W. (2008). Cytochrome c: functions beyond respiration. *Nature reviews Molecular cell biology*, *9*(7), 532-542.

Pant, D., Van Bogaert, G., Diels, L., & Vanbroekhoven, K. (2010). A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. *Bioresource technology*, *101(6)*, 1533-1543. https://doi.org/10.1016/j.biortech.2009.10.017

Park, H., Kim, B., Kim, H., Kim, G., Kim, M., Chang, I., Park, Y. & Chang, H. (2001) A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to Clostridium butyricum isolated from a microbial fuel cell. *Anaerobe*, *7*, 297-306. http://doi.org/10.1006/anae.2001.0399

Parkash, A. (2016). Microbial fuel cells: a source of bioenergy. *Journal of Microbial & Biochemical Technology*, *8*(3), 247-255. https://doi.org/10.4172/1948-5948.1000293

Pietrelli, A., Ferrara, V., Khaled, F., Allard, B., Buret, F., & Costantini, F. (2016). Electrical characterization of MFC for low power applications. In *2016 IEEE 16th International Conference on Environment and Electrical Engineering (EEEIC)* (pp. 1-5). IEEE. http://doi.org/10.1109/eeeic.2016.7555624

Potter, M. C. (1911). Electrical effects accompanying the decomposition of organic compounds. *Proceedings of the royal society of London. Series b, containing papers of a biological character*, *84*(571), 260-276.

Qian, F., & Morse, D. E. (2011). Miniaturizing microbial fuel cells. *Trends in biotechnology*, *29*(2), 62-69. https://doi.org/10.1016/j.tibtech.2010.10.003

Reguera, G., McCarthy, K. D., Mehta, T., Nicoll, J. S., Tuominen, M. T., & Lovley, D. R. (2005). Extracellular electron transfer via microbial nanowires. *Nature*, *435*(7045), 1098-1101.

Reimers, C. E., Tender, L. M., Fertig, S., & Wang, W. (2001). Harvesting energy from the marine sediment− water interface. *Environmental science & technology*, *35*(1), 192-195.

Rhoads, A., Beyenal, H., & Lewandowski, Z. (2005). Microbial fuel cell using anaerobic respiration as an anodic reaction and biomineralized manganese as a cathodic reactant. *Environmental science & technology*, *39*(12), 4666-4671. http://doi.org/10.1021/es048386r

Schamphelaire, L. D., Bossche, L. V. D., Dang, H. S., Höfte, M., Boon, N., Rabaey, K., & Verstraete, W. (2008). Microbial fuel cells generating electricity from rhizodeposits of rice plants. *Environmental Science & Technology*, *42*(8), 3053-3058.

Sengodon, P., Steen, E., & Hays, D. B. (2012). Future Fuel Technologies, National Petroleum Council (NPC) Study.

Sure, S., Ackland, M. L., Torriero, A. A., Adholeya, A., & Kochar, M. (2016). Microbial nanowires: an electrifying tale. *Microbiology*, *162*(12), 2017-2028. https://doi.org/10.1099/mic.0.000382

Tamboli, E., & Eswari, J. S. (2019). Microbial fuel cell configurations: An overview. *Microbial Electrochemical Technology*, 407-435. https://doi.org/10.1016/B978-0-444-64052-9.00016-9

Waller, M.G., & Trabold, T.A. (2013). Review of Microbial Fuel cells for wastewater treatment: Large-Scale Applications, future Needs and current Research gaps. In *International Conference on Fuel Cell Science, Engineering and Technology* (Vol. 55522, p. V001T01A011). American Society of Mechanical Engineers. https://doi.org/10.1115/FuelCell2013-18185

Wang, N., Chen, Z., Li, H.B., Su, J.Q., Zhao, F., & Zhu, Y.G. (2015). Bacterial community composition at anodes of microbial fuel cells for paddy soils: the effects of soil properties. *Journal of Soils and Sediments*, *15*(4), 926-936. https://doi.org/10.1007/s11368-014-1056-4

Yang, W., Kim, K. Y., Saikaly, P. E., & Logan, B. E. (2017). The impact of new cathode materials relative to baseline performance of microbial fuel cells all with the same architecture and solution chemistry. *Energy & Environmental Science*, *10*(5), 1025-1033.

Yi, H., Nevin, K. P., Kim, B. C., Franks, A. E., Klimes, A., Tender, L. M., & Lovley, D. R. (2009). Selection of a variant of Geobacter sulfurreducens with enhanced capacity for current production in microbial fuel cells. *Biosensors and Bioelectronics*, *24*(12), 3498-3503.

Yue, H., Khoshtariya, D., Waldeck, D. H., Grochol, J., Hildebrandt, P., & Murgida, D. H. (2006). On the electron transfer mechanism between cytochrome c and metal electrodes. Evidence for dynamic control at short distances. *The Journal of Physical Chemistry B*, *110*(40), 19906-19913.

Zhang, X.-C., & Halme, A. (1995). Modelling of a microbial fuel cell process. Biotechnology Letters, 17(8), 809–814. https://doi.org/10.1007/bf00129009

Zhao, F., Harnisch, F., Schröder, U., Scholz, F., Bogdanoff, P., & Herrmann, I. (2006). Challenges and constraints of using oxygen cathodes in microbial fuel cells. *Environmental Science & Technology, 40 (17),* 5193-5199. https://doi.org/10.1021/es060332p

Zhou, M., Wang, H., Hassett, D. J., & Gu, T. (2013). Recent advances in microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) for wastewater treatment, bioenergy and bioproducts. *Journal of Chemical Technology & Biotechnology*, *88*(4), 508-518. http://dx.doi.org/10.1002/jctb.4004

Chapter 3 - Effect of changing nutrient levels on the performance of soil-based microbial fuel cells

Abstract

Microbial fuel cells (MFCs) are known to generate electricity from microbial oxidation of an organic substrate. The energy generation potential has been further explored for power generation, wastewater treatment, remediation of toxic compounds in soil and water, and biological oxygen demand biosensor. However, there is a lack of studies focusing on the biosensing potential of MFCs to available nutrients in the soil. Theoretically, the more nutrients present in the soil, the greater the microbial activity and decomposition, leading to more production of electrical signals, which can then be related to the nutrients present in the soil. Can a soil-based microbial fuel cell detect changes in nutrient availability and reflect this in voltage? To explore this idea, we conducted three studies. In these studies, we hypothesized that a change in the nutrient level, such as nitrogen, would produce a proportional microbial response, and hence a different electrical signal. The objective of the first study was to develop relationships between nitrogen availability, microbial activity, and MFC performance. The second study focused on organic carbon levels (25, 50, 100 mg C kg^{-1} soil) to see a change in the electrical signal. The focus of the third study was to estimate the differences in voltage produced in a plant microbial fuel cell with varying levels of nitrogen (20, 40, 80 mg N kg^{-1} soil). Soil-based MFCs were set up using natural, partially sterilized (a treatment in study 1), and sterilized (a treatment in study 2) soil at field capacity with nitrogen fertilizer (studies 1 and 3), and organic carbon treatments (study 2). The voltage generated was measured by a data logger and recorded every 15 minutes. Soil solution was analyzed for NO_3^-, NO_2^- and NH_4^+, dissolved organic carbon, pH, and electrical conductivity. Soil gas samples (CO_2) were collected periodically as a proxy for soil

microbial activity and soil organic carbon mineralization. In the first study, we found that MFC performance was better in control soil than in high nitrogen treatment (80N). This could be because of two reasons: a) negative effect of nitrogen addition on organic matter decomposition, or b) high levels of nitrate could undergo various reduction reactions diverting electrons from passing to the anode. In the second study, the voltage was not significantly different between organic C levels, likely due to non-significant changes in dissolved organic carbon levels. However, the voltage of sterilized soil was significantly low, suggesting that the voltages produced in the other treatments resulted from various biotic processes. In the third study, the voltage was significantly different initially and at the end. The 80N showed higher performance than 20N initially, whereas towards the end, the control treatment showed higher voltage compared to other nitrogen added treatments, which could be attributed to the effects of N addition on organic matter decomposition and the possibility of nitrate undergoing reduction, diverting electrons from the anode, and consequently decreasing the voltage. Overall, differences in voltage signal were observed, but depended on the soil nutrient composition, and complex soil processes.

3.1. Introduction

Soil-based MFC is a bioelectric device that can generate electricity from the oxidation of organic compounds by exoelectrogenic microorganisms present in the soil. A typical soil-based MFC consists of an anode and a cathode separated by soil which acts as a proton exchange membrane. At the anode, under anaerobic conditions, an organic substrate is oxidized by microorganisms that generate electrons, and protons, and produces carbon dioxide. The exoelectrogenic microorganisms can transfer these electrons outside their cells to the anode, from where these travel through an external circuit to the cathode. The cathode is placed in an

aerated environment where oxygen is the terminal electron acceptor. Electrons thereby combine with protons and oxygen to form water. Similarly, in a plant microbial fuel cell (PMFC) plant roots provide organic fuel by secreting rhizodeposits at the anode to the electrochemically active bacteria to generate electricity (Powell et al., 2014; Kabutey et al., 2019). Rhizodeposits include exudates like organic acids, sugars, etc., secretions (polymeric carbohydrates and enzymes), and lysates (Schamphelaire et al., 2008). This power generation from soil and plant MFCs can have many potential applications. One potential use of MFC is soil remediation from toxic compounds such as copper, chromium, and pollutants like petrol and phenol (Zhang et al., 2020; Guan-Xi Li, 2020; Huang et al., 2011; Wang et al., 2012). Electrochemically active microbes consume organic substrate and transfer electrons to the anode. The electrons flow through an external circuit to the cathode, where these get accepted by Cr(VI) which is reduced to Cr(III) (Wang et al., 2012; Huang et al., 2011). Soil-based MFCs have also been known to degrade organic contaminants when these become a fuel source. In an air-cathode MFC, the pollutant is the electron donor and is degraded by microorganisms. The electrons released get transferred to the anode and then ultimately to the cathode where oxygen acts as the terminal electron acceptor (Xiaojing et al., 2017). Apart from organic contaminants, some reduced inorganics such as Fe^{2+}, S^{2-}, NH_4^+, etc. can also be used as electron donors (Xiaojing et al., 2017). Zhang et al. (2020) found that the electricity generation in the MFC was linearly correlated ($R^2 = 0.93$) with the removal efficiency of heavy metals from the soil. Second, electrical properties of the MFCs such as start-up time, peak voltage, coulombic efficiency can be used to monitor pollutant toxicity and soil microbial activity (Deng et al., 2015; Jiang et al., 2015).

Among these applications, MFC-based biosensors are expected to be one of the most promising applications of MFCs. Kim et al. first reported using MFC as BOD biosensor and

found a proportional correlation between the coulombic yield and the strength of wastewater (Kim et al., 2003). Another MFC-based biosensor recently proposed is for the early detection of eutrophication for in-field monitoring. The ceramic soil microbial fuel cell sensor was found to be correlated to algal concentration and dissolved oxygen levels in water (Lorenzo et al., 2020). Over the years, MFCs have been studied to test various parameters, including biochemical oxygen demand (BOD), chemical oxygen demand (COD, dissolved oxygen (DO), toxicants, and microbial activity (Cui et al., 2019). However, the majority of these studies have been conducted using wastewater, and there is a dearth of studies focusing on the direct biosensing capability of MFCs for available nutrients in the soil.

Dunaj et al. (2012) and Jiang et al. (2018) have found relationships between microbial activity and composition and MFC performance in soil-based microbial fuel cells. Electrical signals and soil microbial activity were affected by soil organic matter content and the microbial community in the soil (Dunaj et al., 2012). A study by Deng et al. (2015) showed that when different concentrations of heavy metals were added to soil, the start-up time and coulomb quantity generated by electrogenic bacteria was significantly correlated with soil dehydrogenase activity which is a sensitive indicator of soil microbial activity. Jiang et al. (2018) also found correlations between start-up time and soil dehydrogenase activity and soil organic matter content. While it is clear that the kind and level of nutrients in soil affect microbial composition and activity and thereby MFC performance, hypothetically, the more nutrients microbes consume, the more it would translate into electrical signals. So, the question is, can we track the nutrient availability in the soil from the electrical signals generated, or is it far-fetched?

Nitrogen is an essential nutrient required by plants, animals, and microbes. Nitrogen fertilizers have enabled modern agriculture to produce sufficient food for the growing population

worldwide. Adding nitrogen to croplands is essential to sustain soil fertility and crop production. However, nitrogen is subject to numerous losses because of its nature to convert to inorganic forms quickly. First, nitrate can be lost through runoff and leaching, causing environmental problems such as eutrophication of freshwater bodies and estuaries, resulting in massive algal blooms and coastal dead zones. This can degrade the water quality severely and cause serious health problems in aquatic life, animals, and humans. Second, gaseous emissions from croplands include nitrous oxide, a potent greenhouse gas with a global warming potential 298 times higher than carbon dioxide. Therefore, there is a need to monitor nutrients like nitrogen on-site for improved efficiency and reduce losses. The other nutrient that is crucial for soil health and crop yield is soil organic carbon. Soil organic carbon improves water retention capacity, aeration, and enhance microbial growth. An increase in soil organic carbon results in a more stable carbon cycle and enhances overall agricultural productivity. Previous studies have shown good correlations between electrical properties and soil organic matter content. Therefore, nitrogen and organic carbon are our nutrients of choice.

To understand the biosensing ability of MFCs, we conducted soil-based microbial fuel cell studies and a plant-based microbial fuel cell study to develop an MFC-based biosensor that monitors changes in nutrient availability via changes in electrical signals. The objective of our first study was to develop relationships between nitrogen availability, microbial activity, and MFC performance. Our second study focused on different organic carbon level treatments to see a change in the electrical signal. Finally, the focus of our third study was to measure differences in voltage produced in a plant microbial fuel cell with varying nitrogen levels. We hypothesized that a change in the level of nutrients such as nitrogen or organic carbon would produce a different microbial response, generating a different electrical signal.

3.2. Materials and Methods

3.2.1. Soil collection and initial analysis

Topsoil was collected at 0-10 cm depth from an area near Westar Energy's Jeffrey Energy Center (JEC) located in Pottawatomie County, St. Marys, KS (39°17'10" N 96°07'01" W). The soil was classified as Clime (Fine, mixed, active, mesic Udorthentic Haplustolls)-Sogn (Loamy, mixed, superactive, mesic Lithic Haplustolls) complex silty clay. Upon bringing to the laboratory, the soil was air-dried, homogenized, and sieved through a 2-mm sieve. Initial analysis for various soil properties was performed. The characterization of soil is given in Table 3.1. Particle size (texture) was estimated by a modification of the Bouyoucos Hydrometer Method (Bouyoucos, 1962). Soil organic carbon and total nitrogen were determined by the LECO TruSpec CN Carbon/Nitrogen combustion analyzer (LECO Corporation, St. Joseph, MI, 2005) using the dry combustion method, which reports total levels (inorganic and organic) of C and N on a weight percent basis. Cation exchange capacity (CEC) was determined by the ammonium ion replacement method (Chapman, 1965) at the Soil Testing Lab, Kansas State University, Manhattan, KS. Soil pH was determined in a 1:5 soil:water slurry on a pH meter (Orion Star A111, Thermo Scientific, Waltham, MA). Maximum water holding capacity (MWHC) under the free gravity of soil was estimated (Jenkinson and Powlson, 1976). Soil inorganic nitrogen was extracted with 2M KCl (Mulvaney, 1996). The analysis of nitrate, nitrite, and ammonium was done using Lachat Quikchem 8500 Flow Injection Analyzer (Hach Company, Loveland, CO) at the Soil and Environmental Chemistry Laboratory, Kansas State University.

Table 3.1. Physicochemical properties of the soil material used in the experiment

Property	Values
Textural class	Silty clay
Sand, silt, and clay, %	4, 49, 47
pH (1:5 soil:water)	6.5 ± 0.1
Organic matter, g kg^{-1}	0.474
Total N, g kg^{-1}	0.027
C:N ratio	10:1
Cation exchange capacity, $cmol_c\ kg^{-1}$	19.5
Maximum water holding capacity, %	87

3.2.2. Study 1: Soil MFC with different levels of nitrogen

This study constituted of soil-based MFCs with varying levels of added inorganic nitrogen.

3.2.2.1. Experiment setup and MFC construction

Side-arm conical flasks (1000 mL) were used to construct MFCs. Each flask was gently packed with 600 g soil at 1.3 g cm^{-3} bulk density. The sidearm was used as a pathway for wires sealed with rubber septa (having holes for passing wires) and sealant. The electrodes were made of carbon felt material (C100 AvCarb® Soft Carbon Felt, Fuel Cell Earth, Woburn, MA) with anode and cathode of size 10 cm*2 cm. The anode was placed at the height of 2 cm from the bottom of the flask in the soil, and the cathode was placed at the top of the soil. The treatments (mentioned below) were added at the beginning of the experiment through water, and the moisture was maintained at 40% MWHC. One of the treatments required partial sterilization of microorganisms which was achieved by chloroform fumigation as the method described by Jenkinson and Powlson (1976) and incubated for 24 h before using in MFC. The MFCs were set

up in a growth chamber, maintaining 24°C temperature, 50% relative humidity, and 12 hours of light during the experiment. The top of the flasks was covered by parafilm to minimize moisture loss through evaporation, and the sides of the flasks with aluminum foil to avoid exposure to sunlight to prevent algal growth. The experiment used different levels of nitrogen (added as KNO_3) as per the following treatments in duplicates. Potassium concentrations were balanced by adding KCl for all treatments.

1. Control - **Control**
2. Sterile - **Sterile**
3. 20 mg N kg^{-1} soil (0.857 mol KNO_3)- **20N**
4. 40 mg N kg^{-1} soil (1.714 mol KNO_3)- **40N**
5. 80 mg N kg^{-1} soil (3.428 mol KNO_3)- **80N**

3.2.2.2. CO_2 sampling

Gas samples for CO_2 measurements from each MFC were taken 3 times during the incubation (7, 12, and 20 days). The overall experimental design structure for CO_2 was repeated measures in time. For drawing gas, the top of the flasks was fixed with a rubber stopper during measurement days with rubber septa inserted. Gas sampling was carried out at 0, 15, and 30 minutes. Before drawing gas, mixing of gas inside the flask was done using a syringe. Gas samples (10 ml) were drawn to 10 ml volume 20 mm headspace vials with grey butyl rubber septa. After each sampling, the MFCs were aerated to maintain aerobic conditions for the air cathode throughout the incubation. The gas analysis was done on a Shimadzu GC 8-A (Shimadzu Scientific Instruments Inc., Columbia, MD) with a thermal conductivity detector using 0.5 mL sample from each gas sample collected. The slope of the regression (linear regression) line was determined to get the rate of CO_2-C efflux from soils (μg CO_2-C g^{-1} min^{-1}).

3.2.2.3 Soil solution sampling

Soil solution samples were collected periodically from each MFC using rhizon samplers (Rhizon SMS soil moisture sampler, 5 cm porous tube, Rhizosphere Research Products, Wageningen, Netherlands) on days 5, 10, 17, 24, 32. The solution samples were preserved with a drop of concentrated H_2SO_4 (except for pH and electrical conductivity measurements) at 4°C until analysis. The samples were analyzed separately for each of the following: (1) NO_3^-, NO_2^-, and NH_4^+ on Lachat Quikchem 8500 Flow Injection Analyzer (Hach Company, Loveland, CO), (3) pH (Orion Star A111, Thermo Scientific, Waltham, MA), and (4) Electrical conductivity (EC; SevenEasy- Mettler Toledo, Columbus, OH)

3.2.3. Study 2: Soil MFC with different levels of organic carbon

In this study, soil-based MFCs were constructed with varying levels of organic carbon added through citric acid.

3.2.3.1. Experiment setup and MFC construction

The experiment setup was similar to study 1 except that one of the treatments was sterilized by adding mercuric chloride (550 mg $HgCl_2$ kg^{-1} dry soil) to the soil in ziplock bags and incubated for 48 h before using in MFC (Wolf and Skipper, 1994). This experiment used different levels of citric acid ($C_6H_8O_7$; F.W. 192.1 g mol^{-1}) as per the following treatments in triplicates:

1. Control – **Control**
2. Sterile- **Sterile**
3. 25 mg C kg^{-1} soil (0.0400 g citric acid)- **25C**
4. 50 mg C kg^{-1} soil (0.0800 g citric acid)- **50C**
5. 100 mg C kg^{-1} soil (0.1600 g citric acid)- **100C**

3.2.3.2. CO_2 sampling

Carbon dioxide gas samples from each MFC were taken 7 times during the incubation (7, 14, 21, 28, 35, 42, 49 days). At each gas sampling date, samples were taken at 0, 15, 30, 45, and 60 minutes.

3.2.3.3. Soil solution sampling

Soil solution samples were collected as described in study 1 on days 5, 10, 15, 20, 25, 30, 35, 40, and 45. The samples were analyzed separately for each of the following: (1) Dissolved organic carbon on TOC-L analyzer, (Shimadzu, Tokyo, Japan), (2) NO_3^-, NO_2^-, and NH_4^+ (total dissolved inorganic nitrogen), (3) pH, and (4) electrical conductivity as in study 1.

3.2.4. Study 3: Plant MFC with different levels of nitrogen

In this study, soil-based microbial fuel cells with plants (wheat) were constructed with varying levels of nitrogen added as KNO_3

3.2.4.1. Experiment setup and MFC construction

The experiment setup was similar to study 1 except that there were plants in the soil MFCs. Three weeks homogenous (similar height and color) wheat seedlings were selected and placed in the soil while packing. The top of the flasks was covered by parafilm initially but as the plants grew it was removed. Four different N levels were used in triplicates. Potassium concentrations were balanced by adding KCl to all treatments.

1. Control- **Control**
2. 20 mg N kg^{-1} soil (0.857 mol KNO_3)- **20N**
3. 40 mg N kg^{-1} soil (1.714 mol KNO_3)- **40N**
4. 80 mg N kg^{-1} soil (3.428 mol KNO_3)- **80N**

3.2.4.2. Soil solution sampling

Soil solution samples were collected from each MFC using rhizon soil moisture samplers on days 6, 12, and 18. After day 18, due to extensive root growth, it became difficult to extract pore water. So, at the end of the experiment (day 48), destructive sampling was done to extract water from MFCs by centrifugation after bringing them to MWHC. The samples were analyzed for parameters like described in study 2.

3.2.5. Voltage measurements

The voltage (single-ended voltage measurement) for all three studies was recorded at 15-minute intervals throughout the length of the experiment using a datalogger (CR1000), and the data was extracted using PC400 software. The voltage values were averaged for 8-hour intervals for convenience and easy visualization. The overall experimental design structure for voltage acquisition was repeated measures in time.

3.2.6. Statistical analysis

Statistical analysis was performed on SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, 2021) as a complete randomized design. Slopes were determined for CO_2 analysis using PROC REG. A PROC MIXED model was applied for repeated measures in time for voltage and CO_2 measurements, and multiple mean comparisons were determined using Tukey's HSD test at $\alpha=0.05$ as the level of significance unless otherwise stated. A PROC GLM model was used for total dissolved inorganic nitrogen and dissolved organic carbon measurements, and LSD was used to find least square mean differences at $\alpha=0.05$.

3.3. Results and Discussion

3.3.1. Study 1: Soil MFC with nitrogen addition

3.3.1.1. MFC performance (Voltage measurements)

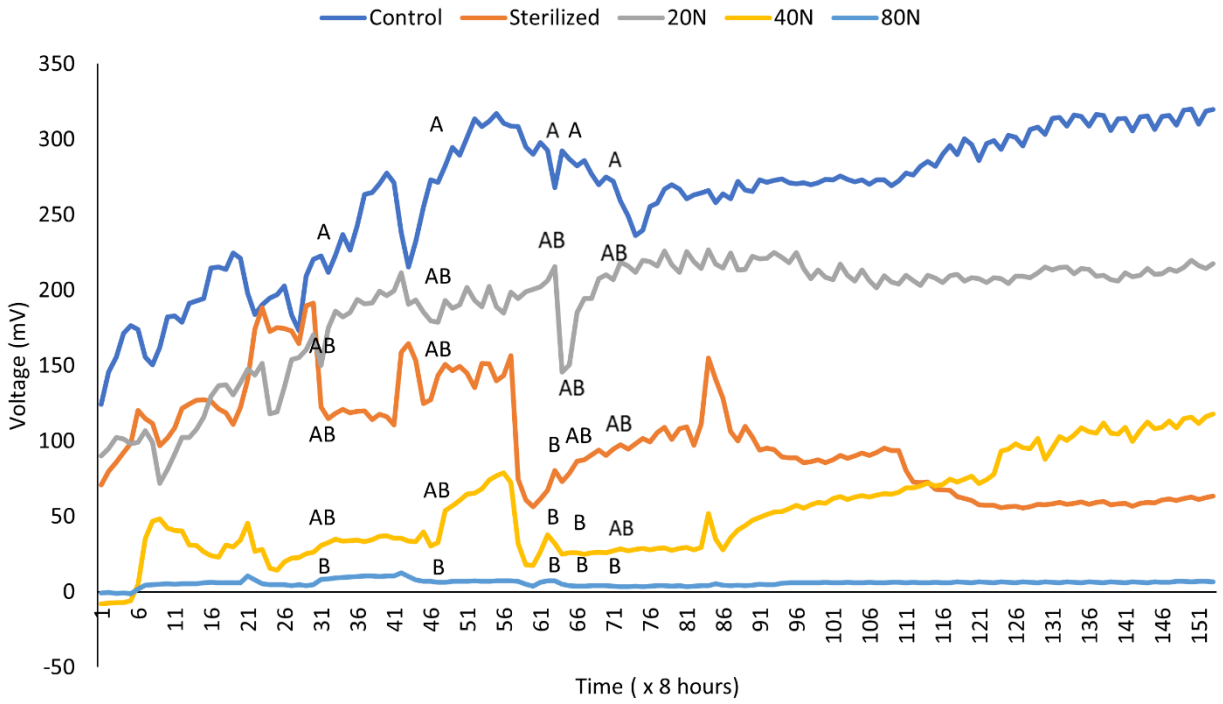

Figure 3.1. Voltage of the treatments averaged over 8-hour period for a total of 50 days (n=2) analyzed using PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N. Different letters show significant differences in means at $p<0.05$ according to Tukey-Kramer pairwise method.

The voltages of most treatments increased over time, except for the partially sterilized treatment where voltage decreased, and the voltage of treatment 80N was constant (Fig. 3.1). The voltage of treatment 80N remained close to 0 mV while the control treatment had a peak voltage of 300 mV around day 20. The differences were significant ($p<0.05$) between control and 80N treatment from day 10 to day 24.

The low voltage of 80N and 40N treatment as compared to control might be due to the accumulation of nitrate early in the system, primarily because of the nitrate addition as a treatment and mineralization of organic matter. The nitrate accumulation in the soil solution was evident from the total dissolved inorganic nitrogen. Nitrate can act as an electron acceptor in low

oxygen environments. It can be reduced to ammonium, which diverts the electron from going to the anode and utilizes it for its own reduction. Nitrate can also undergo other processes like denitrification, which uses up electrons, causing a decrease in electron availability for voltage production.

The high voltage obtained in the control treatment may be due to nitrogen mineralization rates in the control treatment (Fig. 3.3) and a spike in microbial respiration on day 12 (Fig. 3.2). This can also be a possible reason for the voltage being different in the control treatment compared to 80N. Previous studies on the effect of N addition on the mineralization of organic matter in grasslands and forest soil revealed that N addition could decrease microbial decomposition and respiration, either due to decreased oxidative enzyme activity (Fog, 1988; Craine et al., 2007 Waldrop et al., 2004) or increased microbial carbon use efficiency (Manzoni et al., 2012; Schimel and Weintraub, 2003). However, another study by Riggs and Hobbie (2016) revealed that decreased decomposition and SOM respiration were instead a result of a negative effect of N addition on microbial biomass in grasslands soil. In the partially sterilized treatment, voltage decreased over time, but it still showed a considerable amount of voltage in contrast to a study by Deng et al. (2015). Deng et al. (2015) observed a different voltage signal for chloroform-fumigated soil (below 1 mV) while all the other two-chamber soil microbial fuel cells treated with different levels of copper in the study showed much higher voltages (150-350 mV peak voltages). The sterilized treatment also did not show any electrochemical activity. They concluded that because sterilized treatment had no electrochemical activity, the electrical signals were a result of microbial processes instead of chemical reduction. This implies that our study may have had some contribution from chemical reduction as well in the production of voltage. However, it should be noted that because of the soil type and the fumigation method in general,

it was hard to achieve complete sterilization. Kale and Raghu (1982) found that chloroform failed to completely sterilize the soil, which could be due to incomplete permeation of the chemical due to soil structure. They also found that generally, soils with high organic matter content (clay loam and loam soil) were fairly resistant to all sterilization techniques except steam sterilization (Kale & Raghu, 1982). On the other hand, a study by Wolf et al. (1989) revealed that chloroform fumigation on soil significantly reduced fungal numbers but did not affect the bacterial population. Several other studies reported that chloroform fumigation reduced the microbial population but was not effective in eliminating all microorganisms (Jenkinson & Powlson, 1976; Shields et al., 1974; Kassim et al., 1981). However, a study also showed that soil fumigated with chloroform mineralized organic carbon more and evolved greater carbon dioxide initially as compared to non-fumigated grassland soil and over time reached the level of non-fumigated soil (Kemmitt et al., 2008). They introduced a Regulatory Gate hypothesis in which they theorized that the mineralization of organic matter would be the same irrespective of the microbial population size, diversity, and activity as the humified organic matter in soil is equally available to the small recolonizing colonies in chloroform fumigated soil and the larger population in non-fumigated soil (Kemmitt et al., 2008). All the studies above support the positive voltage observed in the sterilized treatment in more than one way.

3.3.1.2. Soil respiration (proxy for microbial activity)

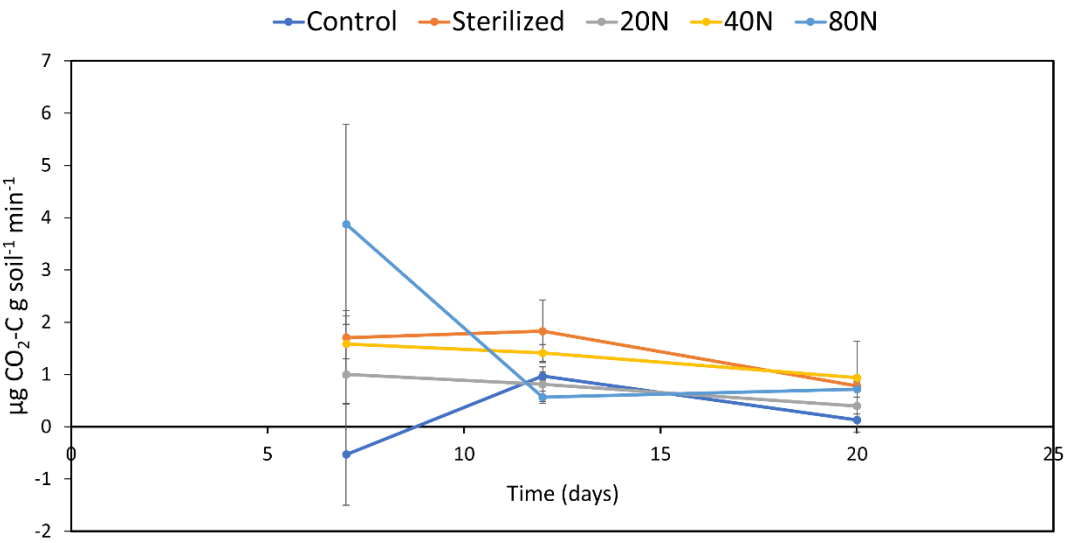

Figure 3.2. Mean rate of carbon dioxide evolution (CO2-C) on days 7, 12, and 20 (n=2). Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N. Error bars represent standard error of two replicates. Not significant at p<0.1 according to Tukey's pairwise comparison.

Carbon dioxide evolution in different treatments showed that initially at day 7, although not significantly different at alpha= 0.05 and 0.1, the 80N treatment had higher production of carbon dioxide as compared to the control, which signifies greater microbial activity present in 80N MFC. Treatment 40N, in general, had more evolved carbon dioxide than 20N treatment. The control treatment increased carbon dioxide production with time, indicating that microbial activity and mineralization increased over time. Carbon dioxide evolved from the sterilized treatment was higher than other treatments except for 80N on day 7 and 40N on day 20, and higher than all the treatments on day 12. From the trend, it seems that the production of carbon dioxide from microorganisms decreased over time implying that microbial activity decreased over time, with the decrease being the highest in 80N and the lowest in control. Previous studies support the decrease in respiration from high nitrogen added treatment (80N). Decreases in

organic matter mineralization in nitrogen added treatments could be a result of an inhibitory effect of N addition on oxidative enzyme activity (Fog, 1988; Craine et al., 2007 Waldrop et al., 2004) or increased microbial carbon use efficiency (Manzoni et al., 2012; Schimel & Weintraub, 2003) or reduction in microbial biomass (Riggs & Hobbie, 2016). The decrease in CO_2 evolved was not as much observed in other nitrogen added treatments 20N and 40N. The lowest decrease in the control treatment meant sustained microbial activity, although there are fewer sampling points to indicate that. Higher CO_2-C evolved from the sterilized treatment is also consistent with previous studies. Kemitt et al., (2008) observed a flush of CO_2 evolved after 24 h chloroform fumigation due to the mineralization of the fumigant-killed biomass which was also observed by Jenkinson and Powlson (1976) that was higher than non-fumigated grassland soil. Thereafter, the fumigated soil showed similar levels of CO_2-C evolved as the non-fumigated soils for over 62 days. In our case, we could not capture the flush of CO_2, as the first sampling point was on day 7, but the CO_2 evolved, in general, was similar to other non-fumigated soils with no significant differences throughout. Overall, the treatments decreased CO_2 production, most likely because of algal growth in the systems after day 15, which could consume CO_2 for photosynthesis.

3.3.1.3. Total dissolved inorganic nitrogen

The total dissolved inorganic nitrogen (sum of NO_3^-, NO_2^- and NH_4^+) in soil solution for different treatments on specified days are shown in figure 3.3. The sterilized treatment showed significantly lower levels of total dissolved inorganic nitrogen throughout the sampling days than the control.

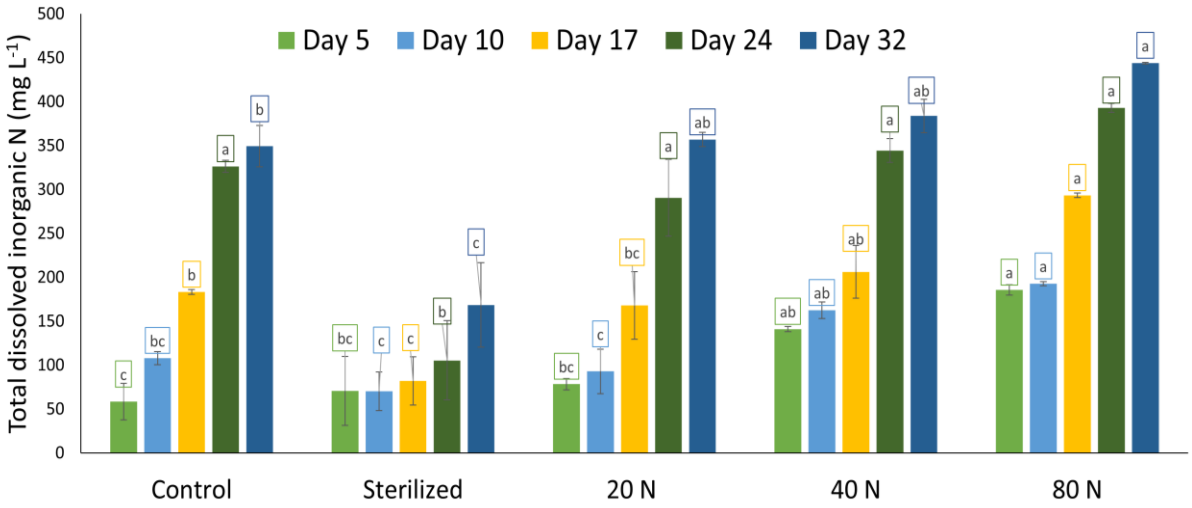

Figure 3.3. Mean of total dissolved inorganic nitrogen (sum of NO_3^-, NO_2^- and NH_4^+) analyzed from solution samples collected on days 5, 10, 17, 24, 32 (n=2) using PROC GLM. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N. Different letters show significant differences in means between treatments at α=0.05 according to LSD. Error bars represent the standard error of two replicates.

Control treatment also had significantly lower accumulated levels of dissolved nitrogen compared to the 80N treatment (except on day 24). The total dissolved inorganic nitrogen in 20N treatment was not different from the control treatment at any sampling point. Similarly, 40N had an amount of total dissolved inorganic nitrogen between 20N and 80N treatment as expected. The higher level of total dissolved inorganic nitrogen in the 80N treatment can be attributed to the addition of nitrogen in the form of nitrate (initially as a treatment) and the mineralization of the native organic nitrogen. The control treatment had a low net amount of inorganic N compared to the 80N treatment because microorganisms were utilizing nitrogen to reduce organic carbon giving higher voltage (Fig. 3.1.) with simultaneous mineralization. The sterilized treatment initially had a slightly higher amount of inorganic N (day 5) compared to the control because of the mineralization of the dead organisms. After day 5, the amount of inorganic N leveled out as observed in a previous study (Brookes et al., 1985). In all the treatments including

control, the trend shows that the levels of total dissolved inorganic nitrogen increased due to continuous mineralization of organic matter present in the soil attributed to the low C:N ratio of the soil.

3.3.1.4. Correlations between voltage and total dissolved inorganic nitrogen

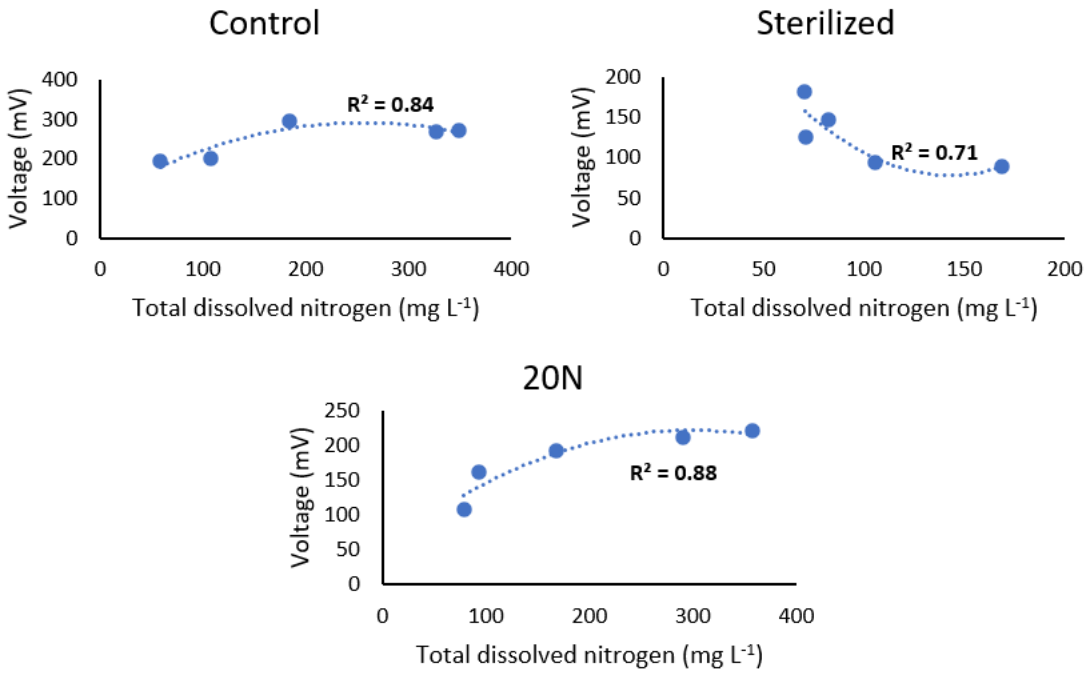

Figure 3.4. Correlations between total dissolved inorganic nitrogen and voltage on corresponding days 5, 10, 17, 24, 32. Control= no added N; 20N= 20 mg kg^{-1} N. Correlations significant at p<0.1 using two-tail T-test (n=5).

Correlation plots were obtained by plotting voltage and total dissolved inorganic nitrogen values at the same point of time over different days (e.g., voltage and total dissolved inorganic nitrogen on days 5, 10, 17, 24, and 32). The R^2 value was determined by fitting the appropriate regression model (linear or quadratic) with total dissolved inorganic nitrogen as the independent variable (x) and voltage as the dependent variable (y). T-statistic and p-value were calculated using R^2 value and degrees of freedom using a two-tail T-test. All correlations were significant at p-value <0.1. A quadratic model was chosen as it best fit the data and also we likely expected

the voltage to decrease when the total dissolved inorganic nitrogen reached a certain threshold. As the nitrate starts accumulating in the system, it can undergo reduction reactions that divert the electrons from going to the anode, which would decrease voltage. A significant amount of denitrification can also occur in the regions with less oxygen like around the anode, which could also decrease nitrogen. The dissolved nitrogen levels in the treatments (Fig. 3.4) show strong correlations with the voltage. The control and 20N treatments showed a strong positive correlation (R-square= 0.84 and 0.88, respectively). This indicates that the soil provided enough organic substrate for the MFC system, so as the nitrogen levels increased in the soil solution, the rate of mineralization and organic carbon oxidation increased, which contributed to an increase in voltage.

Sterilized treatment showed an opposite trend. The voltage decreased as total dissolved inorganic nitrogen levels increased because there were not enough microorganisms to break down organic substrate and use nitrate in the system.

3.3.2. Study 2: Soil MFC with organic carbon addition

3.3.2.1. MFC performance (Voltage)

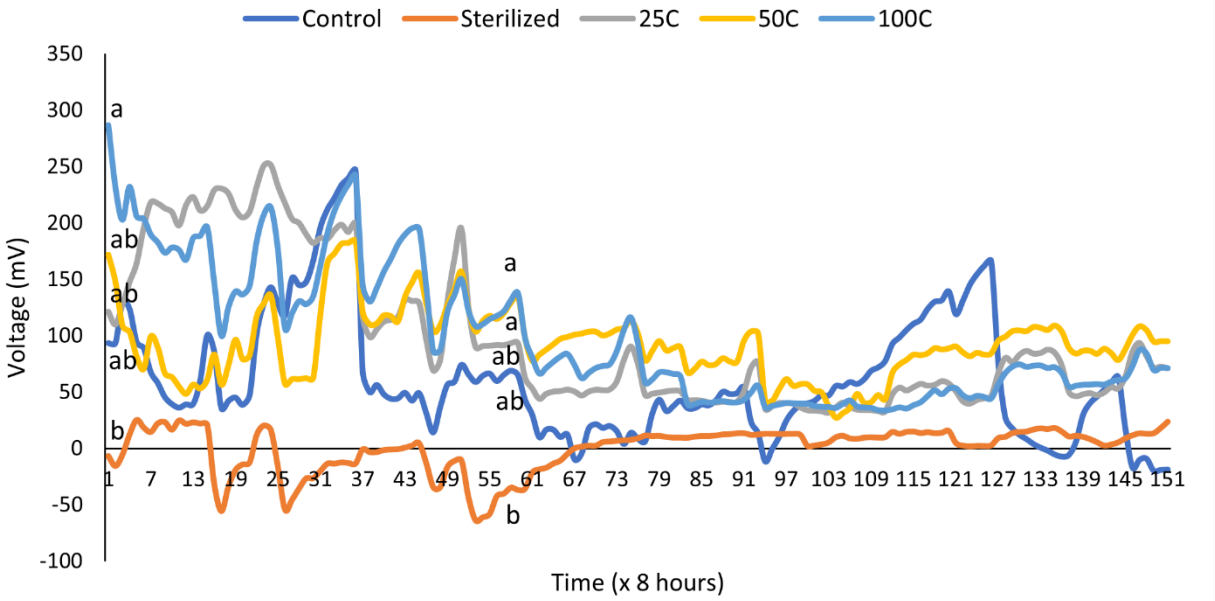

Figure 3.5. Voltage of the treatments averaged over 8-hour period for a total of 50 days (n=3) analyzed using PROC MIXED. Control= no added C; 25C= 25 mg kg^{-1} C; 50C= 50 mg kg^{-1}C; 100C= 100 mg kg^{-1} C. Different letters show significant differences in means at p<0.1 according to Tukey pairwise method.

The voltage for different treatments over time is shown in figure 3.5. The sterilized treatment showed significantly lower voltage than 100C initially (day 1) and in the later period (day 18). Also, the sterilized treatment had a different voltage pattern than the rest of the treatments (sudden dips and increases). Overall, in this study, the treatment voltages were very close to each other, and it was hard to differentiate between them. With time, the voltages of all treatments decreased, while the voltage of sterilized treatment remained consistently low. The sudden dips and increases pattern observed in sterilized treatment is different from others, revealing that other treatments were being affected by microbial processes. The consistently low voltage of sterilized treatment was also observed in a study by Deng et al. (2015). They used a

different method (chloroform fumigation) for sterilization and observed a voltage signal continuously below 1 mV while all the other two-chamber soil microbial fuel cells treated with different levels of copper in the study showed much higher voltages (150-350 mV peak voltages). The sterilized treatment also did not show any electrochemical activity in that study. They concluded that because sterilized treatment had no electrochemical activity, the electrical signals resulted from microbial processes instead of chemical reduction, which could be similar to our study. The similar voltages found in all the treatments could be because of the similar dissolved organic carbon concentrations over time. If more soluble carbon was available to microbes, it would stimulate more heterotrophic microbial activity and thus N mineralization as observed by Montaño et al. (2007). Several studies have shown a positive linear relationship between soil organic carbon content and soil respiration or variables describing microbial activity (Michel & Matzner 1999; Zhao et al. 2008; Peterson et al. 2013). Based on this, it can be inferred that similar levels of DOC could generate a similar level of microbial activity; therefore, differences in voltage were not observed. The decrease of the voltage of all the treatments with time indicated two pools of organic matter. One that was readily available (through the OC addition in the treatment and organic matter present in the soil)- in an easily degradable pool that got used up early in the system. The second one is the more resistant - slowly degrading pool that remained after the available carbon sources were used (Guo et al., 2014; Reichstein et al., 2005; Marzi et al., 2020). Marzi et al. (2020) explained that the period of slow decomposition continues until a soil equilibrium is achieved. In our study, mineralization and decomposition rates for all treatments were clear in the microbial respiration results (Fig. 3.7) and agree with the previous studies.

3.3.2.2. Dissolved organic carbon

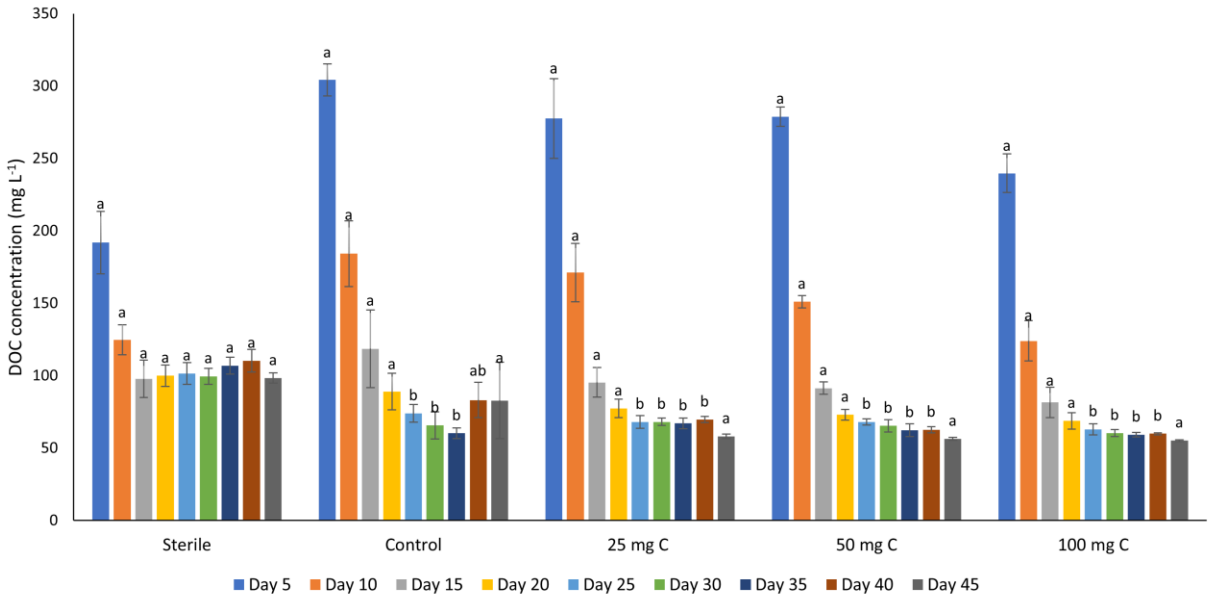

Figure 3.6. Dissolved organic carbon analyzed from solution samples collected on days 5, 10, 15, 20, 25, 30, 35, 40, and 45 (n=3) using PROC MIXED. Control= no added C; 25C= 25 mg kg^{-1} C; 50C= 50 mg kg^{-1} C; 100C= 100 mg kg^{-1} C. Different letters show significant differences in means at $p<0.05$ according to the Tukey-Kramer pairwise method. Error bars represent the standard error of three replicates.

The control treatment had no significant differences compared to the organic carbon added treatments indicating that the organic matter present in soil contributed significantly to dissolved organic carbon (Fig. 3.6). Therefore, the organic carbon additions could not impact DOC levels. This reveals that organic substrate was available in sufficient amounts to contribute to voltage initially (up to day 16, Fig. 3.5.). As the levels of DOC decreased and stabilized, voltages decreased. It appears that DOC became a limiting factor for producing the voltage in the later period for all treatments (i.e., after 16 days). This is consistent with the previous findings, which showed an initial fast DOC depleting period due to the presence of an easily degradable OM pool and stabilization soon after the available C sources are gone, and only resistant pool of OM remains (Guo et al., 2014; Reichstein et al., 2005; Marzi et al., 2020). The sterile treatment,

on the other hand, showed significantly higher dissolved organic carbon levels than other treatments, indicating that sterilization was effective and there were likely fewer microorganisms to consume the organic carbon. Mercuric chloride is a suitable inhibitor for preventing microbial activity resulting in minimal changes in chemical and physical properties (Trevors, 1996). Overall, the levels of dissolved organic carbon decreased over time due to consumption by microorganisms contributing to voltage.

3.3.2.3. Soil respiration

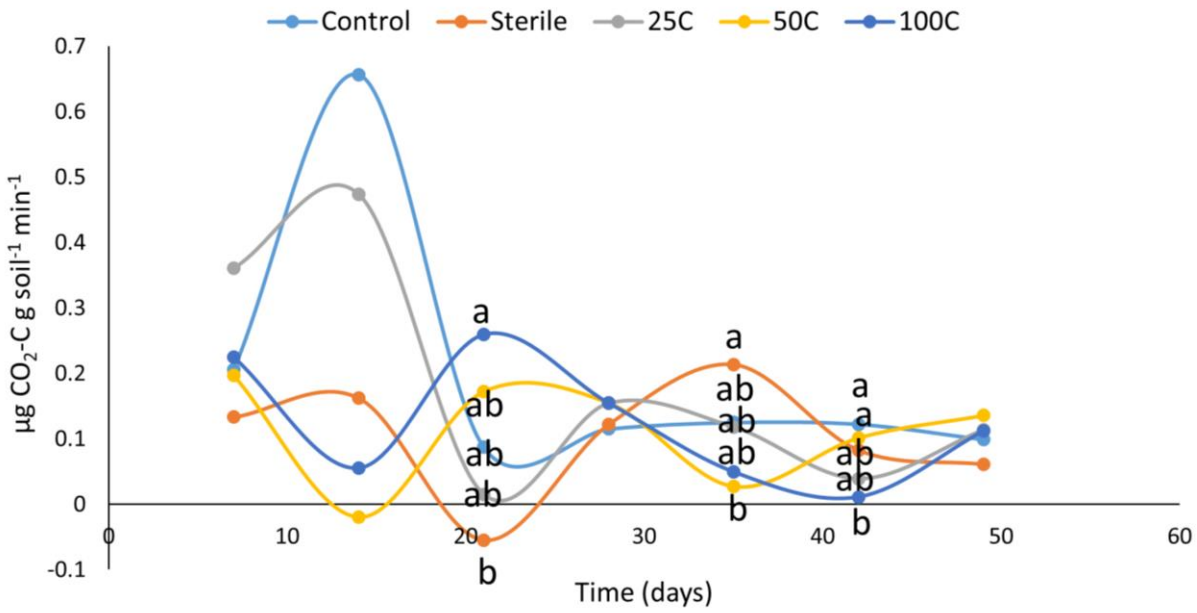

Figure 3.7. Mean rate of carbon dioxide evolution (CO_2-C) collected on day 7, 14, 21, 28, 35, 42, 49 using PROC REG. Control= no added C; 25C= 25 mg kg^{-1} C; 50C= 50 mg kg^{-1} C; 100C= 100 mg kg^{-1} C. Significant at p<0.1 according to Tukey's pairwise comparison.

The CO_2 production for different treatments over the study period is given in figure 3.7. The significant differences in CO_2 production between 100C treatment and sterile treatment were found on day 21 when 100C treatment was higher than sterile treatment. Other significant differences were observed on day 35, where sterile treatment showed higher CO_2 production than 50C, and on day 42, CO_2 production for 50C and control was higher than 100C. On day 35,

sterilized treatment may have shown higher respiration because of the sudden surge of microbial activity after a period of inactivity. Overall, this CO_2 curve agrees with the kinetics of carbon and nitrogen mineralization and ammonification as presented by Marzi et al. (2020) which showed a fluctuating trend because of the mineralization and immobilization at different times. In our study, the CO_2 curve shows a fluctuating trend depending on the stage of decomposition at which the gas sample was taken.

3.3.2.5. Total dissolved inorganic nitrogen

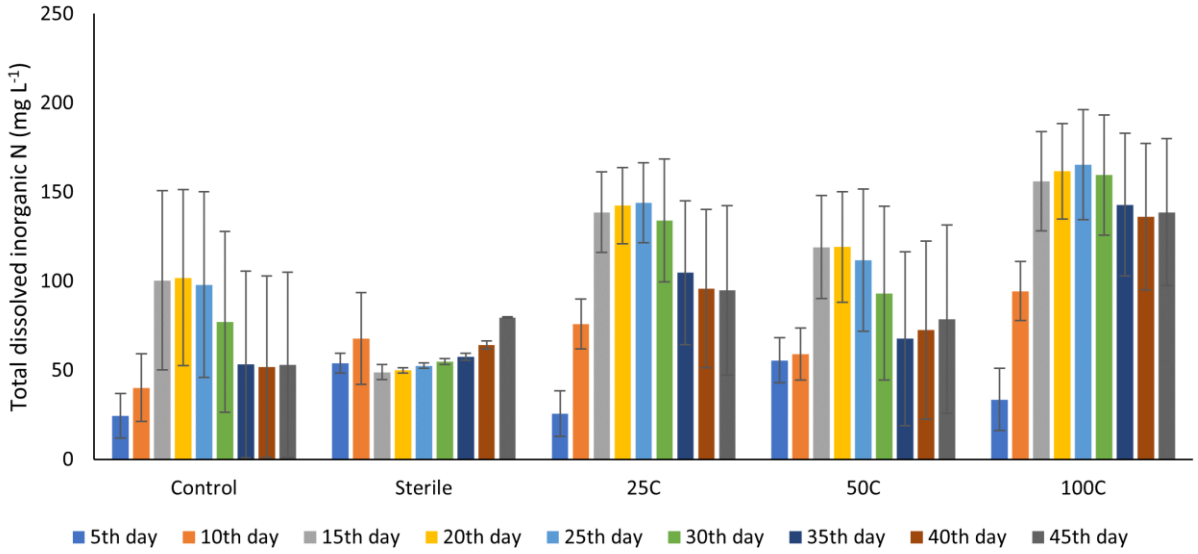

Figure 3.8. Total dissolved inorganic nitrogen (sum of NO_3^-, NO_2^- and NH_4^+) analyzed from solution samples day 5, 10, 15, 20, 25, 30, 35, 40, 45 (n=3) using PROC MIXED. Control= no added C; 25C= 25 mg kg^{-1} C; 50C= 50 mg kg^{-1} C; 100C= 100 mg kg^{-1} C. Not significant at $p<0.1$ according to Tukey-Kramer pairwise method. Error bars represent standard error of three replicates.

The sterilized treatment had a lower N mineralization rate than other treatments indicating that sterilization was effective at reducing microbial activity resulting in low voltage (Fig. 3.8). High variability resulted in no statistical differences in TDN levels between treatments. However, as expected, with more DOC in soil solution as in 100C treatment, the organic matter in the soil mineralized more; therefore, 100C treatment showed a relatively higher

level of dissolved nitrogen. Again, since dissolved nitrogen levels were not statistically different, it indicates that organic carbon oxidation and mineralization of organic matter were happening in all treatments, contributing to increasing voltage signals in the beginning. With time, as the rate of mineralization of organic nitrogen decreased, as apparent in the figure, the voltage of the treatments dropped as well (Fig. 3.5).

3.3.3. Study 3: Plant MFCs

3.3.3.1. MFC performance

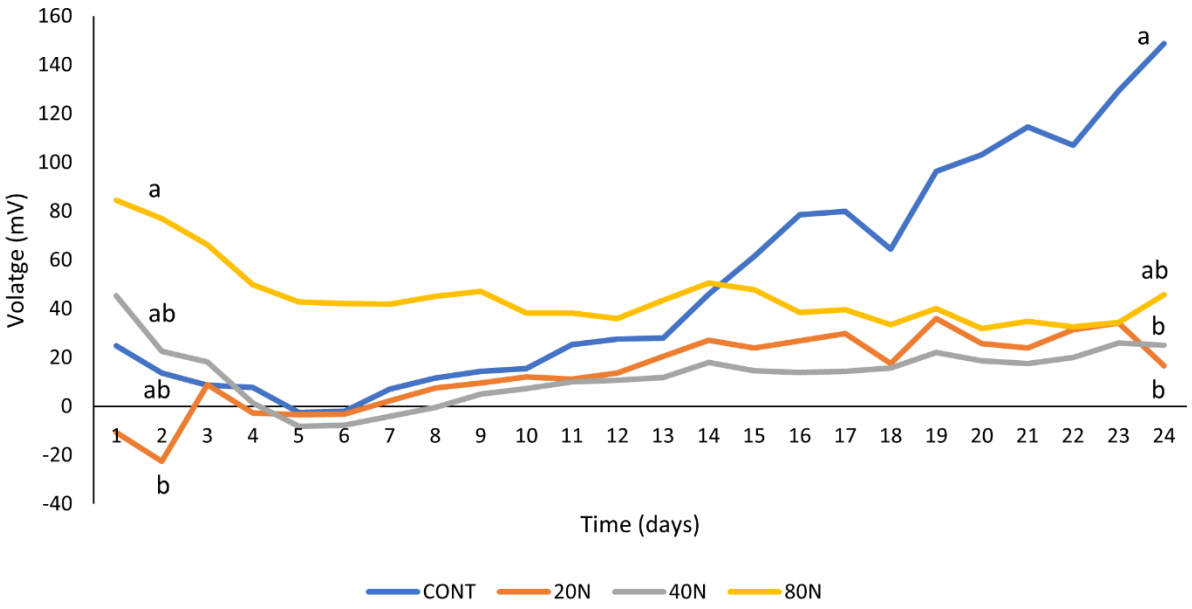

Figure 3.9. Voltage of the treatments averaged over 24-hour period for a total of 24 days (n=3) analyzed using PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N. Different letters show significant differences in means at p<0.1 according to Tukey-Kramer pairwise method.

The voltage produced for different treatments over 24 days is shown in figure 3.9. On day 2, 80N showed significantly higher voltage than 20N treatment, probably because of mineralization of organic matter by microorganisms as it had sufficient nitrogen in soil solution

due to nitrate addition (Fig. 3.10). As the wheat plants in the microbial fuel cells continued to utilize nitrogen for their growth, the voltage gradually decreased for 80N, while in other treatments voltage gradually increased over time. The increase in voltage production over time in all treatments (except in 80N, which showed a slight decrease) was most likely due to the surge in organic matter availability at the anode because of plant root biomass and its exudates which enhanced microbial oxidation (Schamphelaire et al., 2010; Cabezas et al., 2015; Kabutey et al., 2019). Based on the overall trend, there were no significant differences in the voltage production throughout the experimental period between the treatments because of the added biomass, which increased organic matter content and enhanced cellulase activity contributing to increased electricity (Song et al., 2014). However, towards the end, on day 24, the control treatment showed significantly higher voltage than the 20N and 40N treatments, indicating that organic carbon oxidation was enhanced with time due to the growth of the plants. In contrast, in nitrate-added treatments, nitrogen was used for other reduction processes that decreased the voltage (summarized in Fig. 4.10). The accumulation of nitrate in soil solution was evident from the total dissolved inorganic nitrogen results (Fig. 3.10). Nitrate can act as an electron acceptor in low oxygen environments. It can reduce to ammonium which diverts the electron from going to the anode and utilize them for its own reduction. Nitrate can also undergo other processes like denitrification, which uses up electrons, causing a decrease in electron availability for voltage production (Giles et al., 2012). Further, studies on the effect of N addition on mineralization of organic matter in grasslands soil like ours and forest soil have found that N addition could decrease microbial decomposition and respiration either due to decreased oxidative enzyme activity (Fog, 1988; Craine et al., 2007; Waldrop et al., 2004) or increased microbial carbon use

efficiency (Manzoni et al., 2012; Schimel & Weintraub, 2003). The negative effect of nitrogen addition on soil carbon mineralization was observed in our first study as well.

3.3.3.2. Total dissolved inorganic nitrogen

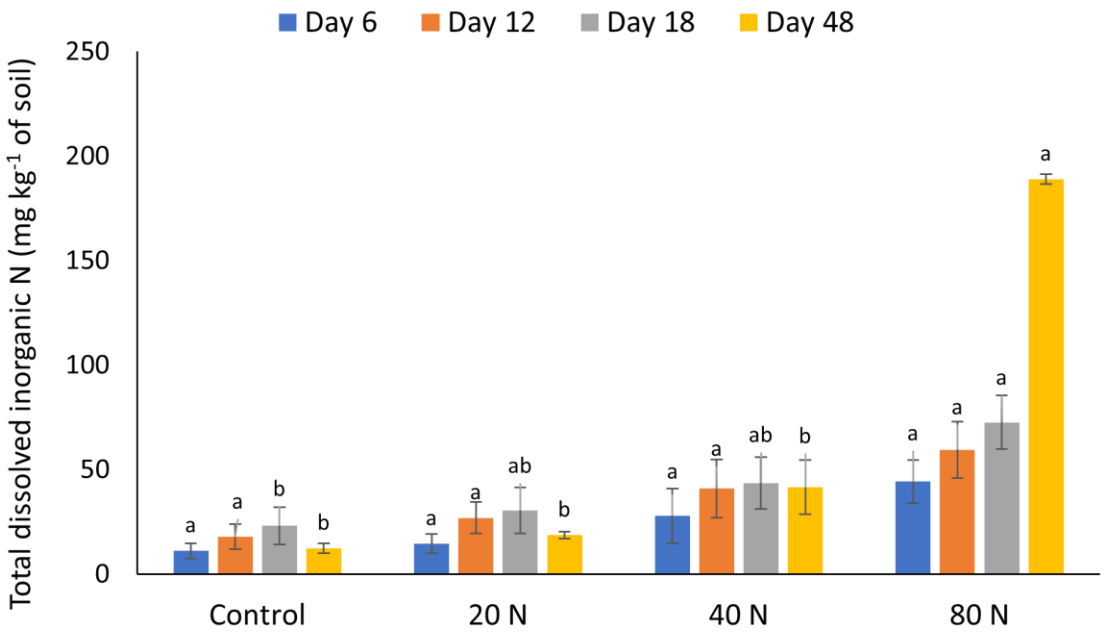

Figure 3.10. Total dissolved inorganic nitrogen (sum of NO_3^-, NO_2^- and NH_4^+) from solution samples collected on days 6, 12, 18, and 48 (n=3) using PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N. Different letters show significant differences in means between treatments at α=0.05 according to Tukey-Kramer pairwise comparison method. Error bars represent the standard error of three replicates.

The total dissolved inorganic nitrogen in soil solution for different treatments is shown in figure 3.10. For the first 12 days, the total dissolved inorganic nitrogen (TDN) level was not significantly different for all treatments. The levels of TDN were significantly different on day 18, with the control having lower TDN than the 80N treatment. Eventually, the TDN in destructive samples at day 48 was significantly higher in the 80N treatment than in the other treatments. The similar dissolved nitrogen levels in the soil for the first 12 days for all treatments

indicated that plants in the system had a significant effect on labile carbon availability to microbes, which mineralized it and released nitrogen continuously in the system. However, nitrogen could not get accumulated in the system as it was taken up by plants continuously. The nitrogen added treatments like 20N and 40N did not show a different effect on nitrogen mineralization than control as these added levels might not have greatly impacted the C:N ratio of the soil. On the other hand, 80N was able to mineralize organic matter present in the soil to a greater extent, accumulating significantly higher TDN levels than the rest of the treatments on day 48 showing that mineralization of organic matter was always more than immobilization. In a real-world situation, the excess nitrate would leach out from the system, but that could not happen in our system.

3.3.3.3. Dissolved organic carbon

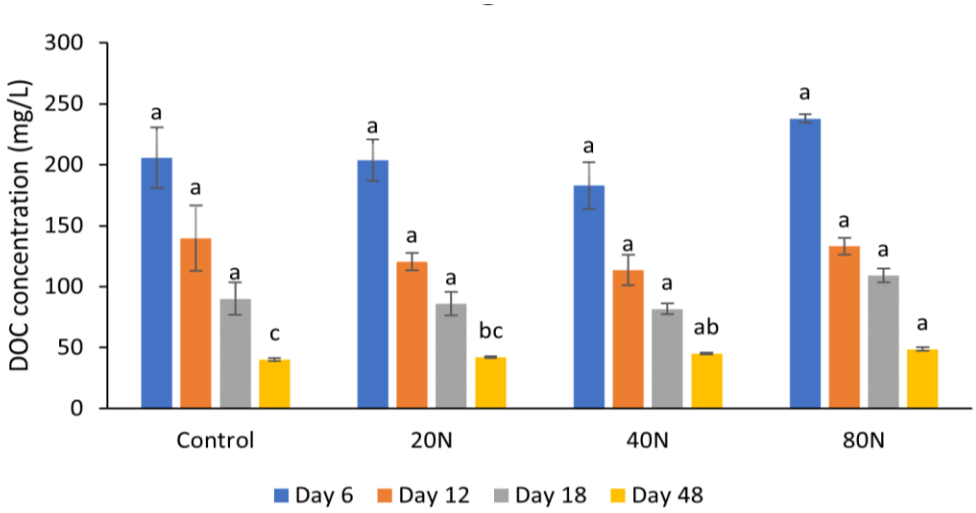

Figure 3.11. Dissolved organic carbon analyzed from solution samples collected on days 6, 12, 18, and 48 (n=3) using PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N. Different letters show significant differences in means between treatments at α=0.05 according to Tukey-Kramer pairwise comparison. Error bars represent the standard error of three replicates.

Dissolved organic carbon decreased more in control treatment on day 48 than in 40N and 80N treatments, which indicates that DOC was consumed more to increase the voltage in the control treatment. Similar was the case with 20N treatment to some extent. At other sampling points, no significant difference was found between the treatments indicating that mineralization was occurring at similar rates for all despite the difference in nitrogen levels in the treatments. Therefore, it was hard to distinguish between voltages for different treatments. The control treatment had a significantly high amount of soluble salts in the solution on day 6 compared to the 80N treatment as evident in table S3.6. (Supplementary info), providing that the plant in the control treatment contributed significantly in providing fuel (organic exudates) for microbes as well as solubilizing the soil organic matter.

3.4. Summary

In studies 1 and 3, we found significant differences in voltage between nitrogen added versus control soils. These differences were not significant with organic carbon additions as treatments (study 2) which could be because of the low C:N ratio of the soil and its high organic matter content. The voltage results were supported by the total dissolved inorganic nitrogen content and dissolved organic carbon content, indicating that monitoring these nutrients over time can provide useful information and a better understanding of the reactions occurring in soil. However, soil respiration does not generally indicate soil electrogenic activity, though it is important to know and relate with the dissolved organic carbon content in the soil. These studies have highlighted the potential soil-based microbial fuel cells hold as a biosensor. Further improvements in the setup and use of simpler soil where treatment addition effects can be seen visibly are the next steps and are discussed in the next chapter. Moreover, knowledge of the

bacterial community around the soil is also crucial in determining the effects of microorganisms on various reactions in the soil.

3.5. References

Brookes, P. C., Kragt, J. F., Powlson, D. S., & Jenkinson, D. S. (1985). Chloroform fumigation and the release of soil nitrogen: the effects of fumigation time and temperature. *Soil Biology and Biochemistry*, *17*(6), 831-835. https://doi.org/10.1016/0038-0717(85)90143-9

Cabezas, A., Pommerenke, B., Boon, N., & Friedrich, M. W. (2015). Geobacter, A naeromyxobacter and A naerolineae populations are enriched on anodes of root exudate-driven microbial fuel cells in rice field soil. *Environmental microbiology reports*, *7*(3), 489-497.

De Schamphelaire, L., Cabezas, A., Marzorati, M., Friedrich, M. W., Boon, N., & Verstraete, W. (2010). Microbial community analysis of anodes from sediment microbial fuel cells powered by rhizodeposits of living rice plants. *Applied and environmental microbiology*, *76*(6), 2002-2008.

Deng H., Jiang Y. B., Zhou Y. W., Shen K., & Zhong W. H. (2015). Using electrical signals of microbial fuel cells to detect copper stress on soil microorganisms. European Journal of Soil Science, *66*, 369–377. http://doi.org/10.1111/ejss.12215

Dunaj, S. J., Vallino, J. J., Hines, M. E., Gay, M., Kobyljanec, C., & Rooney-Varga, J. N. (2012). Relationships between soil organic matter, nutrients, bacterial community structure, and the performance of microbial fuel cells. *Environmental Science & Technology*, *46*(3), 1914-1922. http://doi.org/10.1021/es2032532

Guo, J., Yang, Y., Chen, G., Xie, J., & Yang, Z. (2014). Carbon mineralization of Chinese fir (Cunninghamia lanceolata) soils under different temperature and humidity

conditions. *Acta Ecologica Sinica*, *34*(1), 66-71. https://doi.org/10.1016/j.chnaes.2013.11.008

Huang, D. Y., Zhou, S. G., Chen, Q., Zhao, B., Yuan, Y., & Zhuang, L. (2011). Enhanced anaerobic degradation of organic pollutants in a soil microbial fuel cell. *Chemical engineering journal*, *172*(2-3), 647-653. http://doi.org/10.1016/j.cej.2011.06.024

Jenkinson, D.S., & Powlson, D.S. (1976). Effects of biocidal treatments on metabolism in soil-I. Fumigation with chloroform. *Soil Biology & Biochemistry*, *8*, 167–177. https://doi.org/10.2134/jeq1989.00472425001800010007x

Jenkinson, D.S., & Powlson, D.S. (1976). The effects of biocidal treatments on metabolism in soil: V. A. method for measuring soil biomass. *Soil Biology and Biochemistry*, *8*, 209-213.

Jiang, Y. B., Deng, H., Sun, D. M., & Zhong, W. H. (2015). Electrical signals generated by soil microorganisms in microbial fuel cells respond linearly to soil Cd2+ pollution. *Geoderma 255*, 35–41. http://doi.org/10.1016/j.geoderma.2015.04.022

Jiang, Y., Huan, D. E. N. G., Hongyi, Q. I. N., Cheng, H. A. N., & Zhong, W. (2018). Indication of soil microbial activity by electrical signals of microbial fuel cells with re-vegetated red soils. *Pedosphere*, *28*(2), 269-276. https://doi.org/10.1016/S1002-0160(18)60009-8

Kabutey, F. T., Zhao, Q., Wei, L., Ding, J., Antwi, P., Quashie, F. K., & Wang, W. (2019). An overview of plant microbial fuel cells (PMFCs): Configurations and applications. *Renewable and Sustainable Energy Reviews*, *110*, 402-414. https://doi.org/10.1016/j.rser.2019.05.016.

Kale, S. P., & Rachu, K. (1982). Efficacy of different soil sterilization methods. *Chemosphere*, *11*(12), 1243-1247. https://doi.org/10.1016/0045-6535(82)90038-8

Kemmitt, S. J., Lanyon, C. V., Waite, I. S., Wen, Q., Addiscott, T. M., Bird, N. R., ... & Brookes, P. C. (2008). Mineralization of native soil organic matter is not regulated by the size, activity or composition of the soil microbial biomass—a new perspective. *Soil biology and Biochemistry*, *40*(1), 61-73. https://doi.org/10.1016/j.soilbio.2007.06.021

Marzi, M., Shahbazi, K., Kharazi, N., & Rezaei, M. (2020). The influence of organic amendment source on carbon and nitrogen mineralization in different soils. *Journal of Soil Science and Plant Nutrition*, *20*(1), 177-191. https://doi.org/10.1007/s42729-019-00116-w

Michel, K., & Matzner, E. (1999). Release of dissolved organic carbon and nitrogen from forest floors in relation to solid phase properties, respiration and N-mineralization. *Journal of Plant Nutrition and Soil Science*, *162*(6), 645-652.

Montaño, N.M., García-Oliva, F. & Jaramillo, V.J. (2007). Dissolved organic carbon affects soil microbial activity and nitrogen dynamics in a Mexican tropical deciduous forest. *Plant Soil*, *295*, 265–277. https://doi.org/10.1007/s11104-007-9281-x

Peterson, M. E., Curtin, D., Thomas, S., Clough, T. J., & Meenken, E. D. (2013). Denitrification in vadose zone material amended with dissolved organic matter from topsoil and subsoil. *Soil Biology and Biochemistry*, *61*, 96-104.

Reichstein, M., Subke, J. A., Angeli, A. C., & Tenhunen, J. D. (2005). Does the temperature sensitivity of decomposition of soil organic matter depend upon water content, soil horizon, or incubation time?. *Global Change Biology*, *11*(10), 1754-1767.

Riggs, C. E., & Hobbie, S. E. (2016). Mechanisms driving the soil organic matter decomposition response to nitrogen enrichment in grassland soils. *Soil Biology and Biochemistry, 99*, 54-65. https://doi.org/10.1016/j.soilbio.2016.04.023.

Song, T. S., Wang, D. B., Han, S., Wu, X. Y., & Zhou, C. C. (2014). Influence of biomass addition on electricity harvesting from solid phase microbial fuel cells. *International journal of hydrogen energy*, *39*(2), 1056-1062. https://doi.org/10.1016/j.ijhydene.2013.10.125

Trevors, J. T. (1996). Sterilization and inhibition of microbial activity in soil. *Journal of Microbiological Methods*, *26*(1-2), 53-59. https://doi.org/10.1016/0167-7012(96)00843-3

Wang, X., Cai, Z., Zhou, Q., Zhang, Z., & Chen, C. (2012). Bioelectrochemical stimulation of petroleum hydrocarbon degradation in saline soil using U-tube microbial fuel cells. *Biotechnology and Bioengineering*, *109*(2), 426-433. http://doi.org/10.1002/bit.23351

Wolf, D.C., Dao, T.H., Scott, H.D. & Lavy, T.L. (1989). Influence of Sterilization Methods on Selected Soil Microbiological, Physical, and Chemical Properties. *Journal of Environmental Quality*, *18*, 39-44.

Wolf, D.C., & Skipper, H.D., (1994). Soil sterilization. In: Weaver, R.W., et al. (Eds.), *Methods of Soil Analysis Part 2: Microbiological and Biochemical Properties* (pp. 41–51). Soil Science Society of America, Madison.

Zhang, J., Cao, X., Wang, H., Long, X., & Li, X. (2020). Simultaneous enhancement of heavy metal removal and electricity generation in soil microbial fuel cell. *Ecotoxicology and Environmental Safety*, *192*, 110314. http://doi.org/10.1016/j.ecoenv.2020.110314

Zhao, M., Zhou, J., & Kalbitz, K. (2008). Carbon mineralization and properties of water-extractable organic carbon in soils of the south Loess Plateau in China. *European journal of soil biology*, *44*(2), 158-165.

3.6. Supplementary information

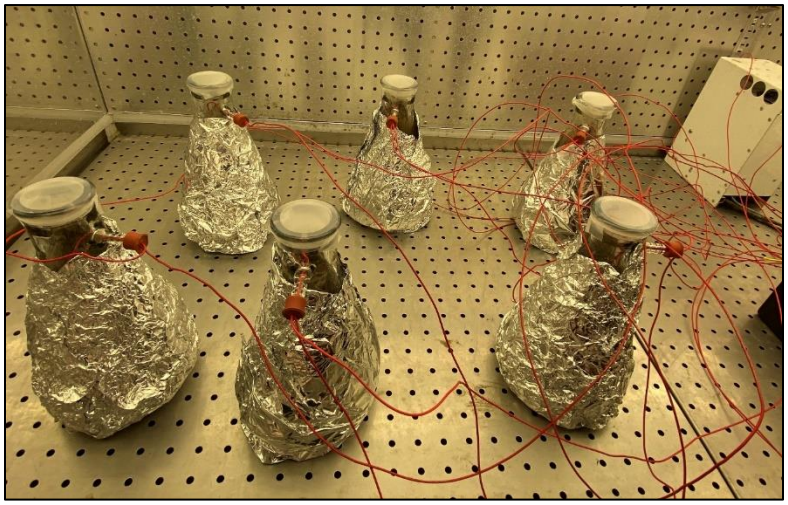

Figure S3. 1. MFC set up using 1000-mL flasks

3.6.1. pH and electrical conductivity (study 1)

Table S3. 1. Average pH of treatments analyzed from solution samples collected on day 5, 10,17, 24, and 32. Different letters show significant differences in means between treatments at α=0.05 using Tukey method of pairwise comparison and PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N

pH	day 5	day 10	day 17	day 24	day 32
Control	6.93	7.06	6.47	6.04	5.80 b
Sterile	7.14	7.05	6.69	6.59	6.32 a
20N	7.30	7.07	6.69	6.53	6.07 ab
40N	7.56	6.78	6.63	6.37	6.06 ab
80N	7.35	6.93	6.75	6.33	5.83 b

All the treatments showed a general decrease in soil pH over time, with control and 80N treatment having significantly low pH while sterile having the highest pH on day 32. Before that, none of the treatments' pH was significantly different from each other. The possible reason for observing low pH is the nitrification process that converts NH_4^+ to a more available form NO_3^- which releases two H^+. As sterile treatment had a small amount of mineralization happening compared to the control and 80N treatment, its pH was relatively higher than the other treatments.

Table S3. 2. Average EC (electrical conductivity) of treatments analyzed from solution samples collected on day 5, 10,17, 24, and 32 (n=2). Different letters show significant differences in means between treatments at α=0.05 using Tukey method of pairwise comparison and PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N

EC (mS/cm)	day 5	day 10	day 17	day 24	day 32
Control	2.89	3.51	3.95	4.81 a	4.85 a
Sterile	3.45	3.22	3.33	3.42 b	3.68 b
20N	2.91	2.99	3.42	4.18 ab	4.51 ab
40N	2.99	3.33	3.50	4.25 ab	4.48 ab
80N	2.78	2.93	3.21	3.72 b	4.40 ab

Electrical conductivity of all treatments, in general, increased over time, except the sterilized treatment as expected. From the trend, the control treatment had high electrical conductivity than the nitrogen added treatments. Electrical conductivity is the indicator/measure of dissolved ions (including available nutrients) in the soil, and ability of soil water to conduct electricity. It increased over time due to mineralization of organic matter, which released more nutrients/salts in the soil solution.

3.6.2. pH and electrical conductivity (Study 2)

Table S3. 3. Average pH of treatments analyzed from solution samples collected on days 5, 10, 15, 20, 25, 30, and 35 (n=3). Different letters show significant differences in means between treatments at α=0.05 using Tukey method of pairwise comparison and PROC MIXED. Control= no added C; 25C= 25 mg kg^{-1} C; 50C= 50 mg kg^{-1}C; 100C= 100 mg kg^{-1} C.

pH	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35
Control	6.74	6.86	7.10	6.84	6.94	6.96	7.22 a
Sterile	6.61	6.61	6.75	6.64	6.63	6.84	6.81 ab
25C	6.74	7.05	6.66	6.68	6.75	6.83	6.87 ab
50C	6.75	7.18	6.72	6.79	6.73	6.88	6.86 ab
100C	6.67	6.61	6.39	6.25	6.29	6.55	6.31 b

In general, soil pH of control MFCs stayed constant throughout the study period, except for Day 15 and Day 35, where the control treatment had a pH of 7.1 and 100C had a pH of 6.39 on day 10. On day 35, this pH comparison was significant, with control having pH of 7.22 and 100C having pH of 6.31.

Table S3. 4. Average EC of treatments analyzed from solution samples collected on days 5, 10, 15, 20, 25, 30, and 35 (n=3). Differences in means between treatments not significant at α=0.1 according to Tukey method of pairwise comparison and PROC MIXED. Control= no added C; 25C= 25 mg kg^{-1} C; 50C= 50 mg kg^{-1}C; 100C= 100 mg kg^{-1} C.

EC µS/cm	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35
Control	2092	1736	1809	1806	1695	1497	1319
Sterile	2883	1875	2075	2094	2088	2002	1771
25C	1747	1812	2080	2088	2059	1964	1680
50C	2084	2001	2021	1984	1944	1687	1466
100C	1855	2197	2160	2144	2107	2021	1770

3.6.3. pH and electrical conductivity (study 3)

Table S3. 5. Average pH of treatments analyzed from solution samples collected on days 6, 12, 18, 48 (n=3). Differences in means between treatments not significant at α=0.1 according to Tukey method of pairwise comparison and PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N

pH	Day 6	Day 12	Day 18	Day 48
Control	6.79	6.40	5.94	7.23
20N	7.21	6.67	6.2	7.31
40N	6.88	6.22	6.14	7.39
80N	7.08	6.54	6.29	7.43

Soil pH was high in the last sampling point because of destructive sampling in which all soil samples were brought to maximum water holding capacity.

Table S3. 6. Average EC of treatments analyzed from solution samples collected on days 6, 12, 18, 48 (n=3). Different letters signify differences in means between treatments significant at α=0.1 according to Tukey method of pairwise comparison and PROC MIXED. Control= no added N; 20N= 20 mg kg^{-1} N; 40N= 40 mg kg^{-1} N; 80N= 80 mg kg^{-1} N

EC mS/cm	Day 6	Day 12	Day 18	Day 48
Control	2.70 a	2.71	2.97	0.98 b
20N	2.47 ab	2.53	2.78	0.85 b
40N	2.42 ab	2.48	2.56	1.09 b
80N	2.03 b	2.28	2.58	1.84 a

Chapter 4 - Biofilm and nitrogen dynamics affect soil-based microbial fuel cell performance

Abstract

Microbial fuel cells are known to generate electricity with the help of exoelectrogenic microorganisms that consume nutrients and release electrons outside their cells. The most common and extensively studied exoelectrogenic bacteria is *Geobacter sulfurreducens,* which belongs to the group of dissimilatory metal-reducing microorganisms. With such bacteria effectively coated on anodes, we hoped to increase the sensitivity of our soil-based microbial fuel cells with different levels of nutrients to the voltage response and gain insight into complex microbial processes. To achieve this, we conducted two studies with a much simpler soil than before and an improved design for our soil-based microbial fuel cells. These studies had various nitrogen treatments with the *Geobacter* inoculum on the anode (fourth study), and pre-developed *Geobacter* anodic coating to provide a stable biofilm (fifth study). In addition to our previous hypotheses (Chapter 3), we hypothesized that treatments with *Geobacter* biofilm on anode would enhance electrogenic microbial activity and its ability to capture changes in nitrogen dynamics. Our objectives were i) to understand the effects of pre-developed anodic biofilm on MFC performance and detection of nitrogen availability, and ii) to develop relationships between nitrogen availability, microbial activity, and MFC performance in the presence of biofilm. The voltage generated (differential) was measured by a data logger and recorded every minute. Soil solution was analyzed to estimate NO_3^-, NO_2^- and NH_4^+, dissolved organic carbon, pH, and electrical conductivity. Soil gas samples (CO_2) were collected periodically as a proxy for soil microbial activity. At the end of the experiments, destructive soil sampling was done around the anode, and samples were immediately stored under -20°C until phospholipid fatty acid analysis

and subsamples at -80°C for further analysis. In the fourth study, we found no significant differences among the voltages of different treatments which was likely because of limited DOC levels in the soil and a similar rate of CO_2 release. In the fifth study, we found that nitrate added soil MFC (40N) produced significantly higher voltage than the control soil likely because of enhanced C mineralization with the N addition and stimulation of microbial community. The anodic biofilm coated treatments however had similar voltages owing to the various redox reactions simultaneously happening in the soil. The analysis of the nitrogen dynamics in the soil solution indicated that biofilm containing treatments "fixed" nitrogen in the system increasing the total dissolved inorganic nitrogen levels, but its effect on voltage is not known. Overall, these studies helped to give insight into the various complex processes happening in a soil-MFC and some findings and improvements to consider for future studies.

4.1. Introduction

Soil-based microbial fuel cells are known to generate electricity with the help of exoelectrogenic microorganisms that consume organic substrate and release electrons outside their cells. This extracellular respiration is commonly found in metal-reducing organisms such as *Geobacter spp*. and *Shewanella spp*. (Sure et al., 2016). These exoelectrogens can transfer electrons to the anode by electron mediators (Sund et al., 2007; Turick et al., 2003; Chui et al., 2003), nanowires (Gorby, 2005), or by direct membrane-associated transfer (Bond and Lovely, 2003). One of the most popular and extensively studied microorganisms in the MFCs capable of producing high current densities of any known pure culture is *Geobacter sulfurreducens* and considered as the "gold standard" (Slate et al., 2019). *Geobacter sulfurreducens* is a gram-negative, rod-shaped, delta-proteobacterium and is a non-fermentative, obligate anaerobe with flagella and type IV pili production. It belongs to the group of dissimilatory metal-reducing

microorganisms, which reduces metal oxides under anaerobic conditions in soils and sediments and produces biologically useful energy in ATP (Du et al., 2007).

An important factor in the efficient transfer of electrons in an MFC is the bacterial attachment and formation of a biofilm on an anode surface (Franks et al., 2010). A biofilm is an association of microorganisms made up of extracellular polymeric substances (EPS), such as proteins (<1-2%), polysaccharides (1-2%), DNA (<1%), RNA (<1%), and water (up to 97%) which is a major part of the biofilm and is responsible for the flow of nutrients inside the matrix (Jamal et al., 2015). *Geobacter sulfurreducens* is known to produce a biofilm greater than 50 µm in thickness in pure culture (Reguera et al., 2006; Franks et al., 2010). In MFCs using *Geobacter sulfurreducens*, the observed power output was 14.7 mW m^{-2} with coulombic efficiency of 96.8%, with acetate as the fuel (Oh & Logan, 2006; Frank & Nevin, 2010). However, it has also been reported that mixed cultures of microorganisms in the form of marine sediments or anaerobic sludge, when introduced into an MFC system, show higher performance than pure cultures. Rabaey and Verstraaete (2005) summarized and reported that mixed cultures showed higher performances than single bacterial species with the advantage of much broader substrate utilization. Ishii et al. (2008) also reported greater per biomass power density in the case of mixed consortium compared to the pure culture of *Geobacter sulfurreducens* in a single-chamber air cathode microbial fuel cell. However, there have been studies where a dual chamber MFC was used with ferric cyanide in the cathode chamber, and anodic biofilm of *G. sulfurreducens* generated more power than an enriched consortium of mixed species (Nevin et al., 2008). Since enrichment of biofilm is essential for the electron transfer processes to occur efficiently and *Geobacter spp.* is well known for its electrogenic activity in microbial fuel cells, we conducted studies containing *Geobacter* inoculum and pre-developed *Geobacter* coating on

anode along with the different levels of nitrogen treatments as being done previously in chapter 3. The motive is to increase the sensing ability of the system to the voltage produced and also, gain better insight into various processes in the soil as influenced by microbes as a further step in learning and developing a biosensor. The fourth and fifth studies were conducted with a much simpler soil than before and a different setup with *Geobacter* inoculum on the anode (fourth study) with various nitrogen treatments. The fifth study exclusively used pre-developed Geobacter anodic coating to provide a stable biofilm with different nitrogen treatments. In addition to our previous hypotheses (Chapter 3), we hypothesized that treatments with pre-developed biofilm on anode using *Geobacter* inoculum would enhance electrogenic microbial activity and its ability to capture changes in nitrogen dynamics. Our objectives were i) to understand the effects of pre-developed anodic biofilm on MFC performance and detection of nitrogen availability, and ii) to develop relationships between nitrogen availability, microbial activity, and MFC performance in the presence of biofilm.

4.2. Materials and Methods

4.2.1. Study 4: Soil MFC with enriched biofilm and different levels of nitrogen

4.2.1.1 Soil collection and initial analysis

The soil was collected to a depth of 0-20 cm from Ashland Bottoms Site, Manhattan, KS (39º 15’30” N, 96º 60’ 36” W). The soil at this site was classified as a rarely flooded Belvue silt loam. Upon bringing to the laboratory, the soil was air-dried, homogenized, and sieved through a 2 mm sieve. Initial analysis for various soil properties was performed as described in the previous chapter. The characterization of soil used for the study is given in table 4.1.

4.2.1.2. Biofilm development

Geobacter enriched biofilm (concentrated, 1.5 mL) was developed in an H-type microbial electrolysis reactor at the Civil Engineering Department, Kansas State University. The reactors were kept in a temperature-controlled environment maintained at 30°C while the anode was mixed at a rate of 100 rpm (Heronemus, 2021). Phosphate buffer media was prepared (Parameswaran et al., 2009; 2011). Acetate was then added as an electron donor to grow and condition the biofilm (Heronemus, 2021). The biofilm solution collected from this reactor was placed in a sealed falcon tube and brought to the Soil and Environmental Chemistry laboratory. The enriched inoculum was diluted 12 times with Milli-Q water to get enough volume to coat the anodes. The carbon felt anodes were dipped in the biofilm solution for 2 h in an oxygen-free environment ensured with a glove box chamber. The electrodes in contact with the biofilm were kept in sodium acetate solution in a closed circuit for 48 h to enrich the anode-respiring bacteria on the anode as a process of acclimatization before using these in soil. The electrodes were then deployed in the MFC.

Table 4.1. Physicochemical properties of the soil material used in the experiment

Property	Values
Textural class	Sandy loam
Sand, silt, and clay, %	76, 16, 8
pH (1:5 soil:water)	6.5
Total Organic Carbon, g kg^{-1}	0.044
Total N, g kg^{-1}	0.007
Maximum water holding capacity, %	40.2
NO_3^--N, NO_2^--N, NH_4^+-N, mg kg^{-1} soil	< d.l., 0.266, 3.10
Electrical conductivity, µS cm^{-1}	20.2

4.2.1.3. MFC construction

Specialized PVC columns (height= 26 cm, diameter= 7.7 cm) were constructed for this study (Supplementary information 4.6., Fig. S4.3.). The columns had a hole on the top right for wires to pass through, which was later covered with septa to make it airtight for gas sampling. Additionally, the columns had a water inlet in the bottom left to follow the bottom-up approach for watering the soil in the column with minimal disturbance to the packing. Each column was packed with 700 g soil at 1.2 g cm^{-3} bulk density, the soil was pre-moist with 13.5% of MWHC 2 days before packing in columns. The electrodes were cut out of carbon felt material (anode and cathode of size 7.5 cm* 2.66 cm). Before packing the soil, the inside bottom of the column was covered with glass fiber circles and nylon cloth to facilitate water movement through the soil and create a barrier between the soil above and the water inlet below. The anode was placed at the height of 2 cm from the soil, and the cathode was placed at 18 cm height. The top of the columns was covered by parafilm throughout the experiment to minimize moisture loss through evaporation. The MFCs were set up in a growth chamber under the same conditions as described in the chapter previously. Following setup, to compensate for the lost moisture through sampling or evaporation, nitrogen-bubbled Milli-Q water was added to the MFCs. The moisture content of 60% MWHC was maintained throughout the experiment.

The experiment had the following treatments in triplicates:

1. Control (no added nitrate)- **C**
2. Control (no added nitrate + **b**iofilm on anode)- **CB**
3. 20 mg N kg^{-1} soil (1 mole KNO_3)- **20N**

4. 20 mg N kg^{-1} soil (1 mole KNO_3 + biofilm on anode)- **20B**
5. 40 mg N kg^{-1} soil (2 moles KNO_3)- **40N**
6. 40 mg N kg^{-1} soil (2 moles KNO_3 + biofilm on anode)- **40B**

4.2.1.4. CO_2 sampling

Carbon dioxide gas samples from each MFC were taken periodically until the experiment was terminated. The overall experimental design structure for CO_2 was repeated measures in time. The repeated gas measurements were taken 8 times during the incubation (2, 7, 14, 21, 28, 35, 42, and 49 days). The MFC columns made of PVC were tightly sealed, with rubber septa on the side with a hole to allow wires to pass through. This was sealed with sealant. The top of the column was covered by a lid with a fixed rubber septum to draw samples from on the measurement days (Supplementary information 4.6., Fig. S4.3). After achieving complete sealing, gas sampling was carried out at 0, 15, 30, 45, and 60 minutes. The procedure of sampling and analyzing gas hereon is the same as described previously.

4.2.1.5. Soil solution sampling

Soil solution samples were collected periodically and closely coincided with the time gas samples were drawn on days 2, 7, 14, 21, 28, 35, 42, 49, and 54. The samples were analyzed separately for each of the following: (1) NO_3^-, NO_2^-, and NH_4^+ on Lachat Quikchem 8500 Flow Injection Analyzer (Hach Company, Loveland, CO), (3) pH (Orion Star A111, Thermo Scientific, Waltham, MA), (4) Electrical conductivity (EC; SevenEasy- Mettler Toledo, Columbus, OH), and 5) Dissolved organic carbon on TOC-L analyzer, (Shimadzu, Tokyo, Japan)

4.2.2. Study 5: Soil MFC with pre-developed anodic biofilm and different levels of nitrogen

4.2.2.1. Anodic coating

Geobacter biofilm was developed directly on carbon felt electrodes (C100 AvCarb® Soft Carbon Felt; Fuel Cell Earth, Woburn, MA) in a microbial electrolysis cell (MEC) reactor for 4 weeks in the Civil Engineering Dept., Kansas State University. As only 4 electrodes (anodes) could be enriched with biofilm due to the lack of the MEC reactors, the experiment had replicates that started at staggered times. Due to time variability and the variable nature of bacterial growth, biofilm coverage on the electrodes was not always uniform. We considered two biofilm levels based on biofilm coverage and named the treatments as "low biofilm" for electrodes showing 15-50% coverage and "high biofilm" for electrodes showing 85-100% coverage (See supplementary info 4.6, figure S4.2.). The biofilm-coated electrodes were brought to the lab in a sealed bottle containing phosphate buffer solution (supplementary info 4.6., figure S4.1.). Anoxic conditions were ensured using a glove bag filled with nitrogen, for handling and deployment of anodes in the MFC column. Later, the soils were packed in the columns as described in the next section.

4.2.2.2. MFC construction

The same soil and setup as in study 4 were used. The anode was placed at a height of 12.5 cm from the bottom. The cathode was placed at the top of the soil at 18 cm height, the distance between the electrodes being 5.5 cm. Also, to ensure MFCs operate continuously, a dissolved organic carbon substrate (5.4 mg citric acid) was added every 10 days. The moisture was maintained throughout the length of the experiment according to 62% of MWHC. Following setup, to compensate for the lost moisture through sampling or evaporation Milli-Q water was

added to the MFCs free of oxygen by bubbling N_2 for 20 seconds to eliminate chances of dissolved oxygen diffusion into the anodic environment.

The experiment had the following treatments in triplicates:

1. Control (no added nitrate)- **C**
2. Control (no added nitrate + **l**ow **b**iofilm on anode)- **CLB**
3. Control (no added nitrate + **h**igh **b**iofilm on anode)- **CHB**
4. 40 mg N kg^{-1} soil (2 moles KNO_3)- **40N**
5. 40 mg N kg^{-1} soil (2 moles KNO_3 + low biofilm on anode)- **40LB**
6. 40 mg N kg^{-1} soil (2 moles KNO_3 + high biofilm on anode)- **40HB**

4.2.2.3. Sample collection for phospholipid fatty acid analysis

After the experiment was terminated, the soil around the anode was extracted from all MFC systems. Soil at 0-1 cm above and below the anode was collected, composited, and homogenized as well as soil at 1-2 cm above and below the anode was also collected separately. A sub-sample from 0-1 cm around the anode soil of each MFC was immediately stored in an ultra-low temperature freezer at -80°C. The rest of the samples were stored at -20°C, which were later freeze-dried for 48 h, ground, and used for phospholipid fatty acid (PLFA) analysis.
The PLFA was analyzed using a modification of the White and Ringelberg (1998) and White and Rice (2009). The total lipids were determined by adding 5 mL of chloroform, 10 mL of methanol, and 4 mL of phosphate buffer (pH=7.4) on 5 g freeze-dried soil. Chloroform and water were added 3 hours after the extraction to separate the mixture into polar and nonpolar fractions, while total lipids remained in the nonpolar phase. Using silicic acid chromatography columns (Disposable BAKERBOND® SPE Columns, J.T. Baker®), phospholipids were separated from

neutral lipids and glycolipids and removed with methanol. The fatty acids were then cleaved from the glycerol backbone using KOH saponification (0.2 M KOH in methanol), and the harvested fatty acids were methylated to form fatty acid methyl esters (FAME) (Allison and Miller, 2005). The resulting FAMEs obtained from PLFA were analyzed using a Thermo Scientific Trace GC-ISQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a DB5-MS column (30 m x 250 μm inner diameter x 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA) using helium as the carrier gas. An internal standard of methyl nonadecanoate (C19:0) was used to calibrate the GC-MS and added to each sample as quality control. Standard nomenclature was used to explain the PLFAs. The total PLFA represents microbial biomass. The FAME peaks were identified by comparing with a total of 32 biomarkers, out of which 26 biomarkers were from a bacterial acid methyl esters mix (BAME; Pennsylvania, USA) and six additional FAMEs were custom made for 10Me16:0 (Cayman Chemical 24823; Cayman Chemical Company; Ann Arbor, MS), 10Me18:0 (Larodan 21-1810; Larodan AB; Solna, Sweden),), C16:1:11 (Matreya custom synthesis; Matreya LLC, Pleasant Gap, PA), a17:0 (Matreya 1614; Matreya LLC, Pleasant Gap, PA, C20:0 (MilliporeSigma 10941; MilliporeSigma; Burlington, St. Louis, MO), and C18:1:11:cis (MilliporeSigma 17264; MilliporeSigma; Burlington, St. Louis, MO). Peak concentration was estimated using the internal standard methyl nonadecanoate (19:0 FAME) (MilliporeSigma N5377; MilliporeSigma; Burlington, St. Louis, MO). Microbial groups were determined based on the features of biomarkers: (1) gram-positive (+) bacteria were determined using the following biomarkers: i15:0, a15:0, i16:0, i17:0, and a17:0; (2) gram-negative (-) bacteria were determined using biomarkers: 19:0:delta9,10; C18:1:11:cis; 17:0:delta9,10; C10:0:2-OH, C12:0:2-OH, C12:0:3-OH, C14:0:2- OH, C14:0:3-OH, C16:1:0:cis, C16:0:2-OH; (3) actinomycetes using: 10Me16:0

and 10Me18:0; (4) fungi using: C18:2:9,12; and (5) arbuscular mycorrhizal fungi (AMF) using C16:1:11 (White and Rice, 2009). Phospholipid fatty acid abundance was reported as nmol per gram of dry soil. Total microbial biomass was determined by summing all PLFA biomarkers and the common biomarkers in microbes with FAMEs for C11:0; C12:0; C13:0; C14:0; C15:0; C16:0; C17:0; C18:0; and C20:0.

4.2.2.4. CO_2 sampling

The repeated gas measurements were taken 15 times during the incubation (2, 8, 14, 19, 24, 32, 37, 45, 50, 55, 63, 70, 75, 80, 85 days) and analyzed as described in study 4.

4.2.2.5. Soil solution sampling

Soil solution samples were collected on days 2, 8, 14, 19, 24, 32, 37, 45, 50, 55, 63, 70, 75, 80, and 85. The samples were analyzed separately for each of the following: (1) NO_3^-, NO_2^-, and NH_4^+ on Lachat Quikchem 8500 Flow Injection Analyzer (Hach Company, Loveland, CO), (3) pH (Orion Star A111, Thermo Scientific, Waltham, MA), (4) Electrical conductivity (EC; SevenEasy- Mettler Toledo, Columbus, OH), and (5) Dissolved organic carbon on TOC-L analyzer, (Shimadzu, Tokyo, Japan)

4.2.3. Voltage measurements

The voltage (differential voltage measurement) was recorded at a 1-minute interval throughout the length of the experiment using data loggers (CR 1000, and CR23X; Campbell Scientific, Logan, UT), and the data was extracted using PC400 software. The voltage values were averaged over 8-hour intervals for convenience and easy visualization. The overall experimental design structure for voltage acquisition was repeated measures in time.

4.2.4. Statistical analysis

Statistical analysis was performed using SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, 2021) as a randomized complete block design using replicates as blocks. Slopes were determined for CO_2 analysis using PROC REG. A PROC MIXED model was applied for repeated measures in time for voltage and CO_2 measurements, and multiple mean comparisons were determined using Tukey's HSD test at α=0.05 as the level of significance. A PROC GLM model was used for total dissolved inorganic nitrogen measurements, and LSD was used to find least square mean differences at α=0.05.

4.3. Results and discussions

4.3.1. Study 4:

4.3.1.1 MFC performance (Voltage)

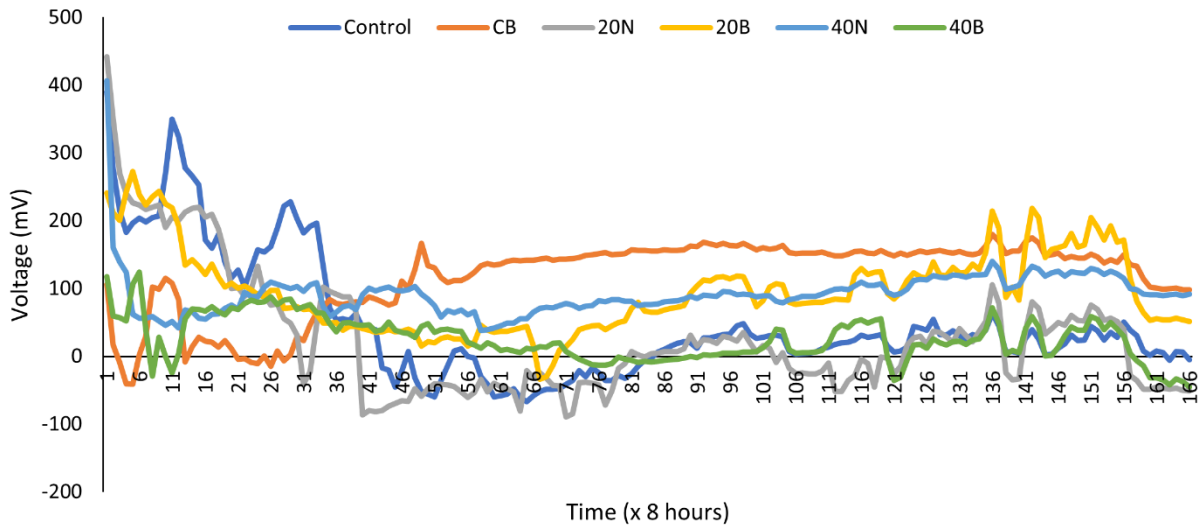

Figure 4.1. Voltage of the treatments averaged over 8-hour period for a total of 55 days (n=3) analyzed using PROC MIXED. Control= no added N; CB= control with biofilm; 20N= 20 mg kg^{-1} N; 20B= 20 mg kg^{-1} N with biofilm; 40N= 40 mg kg^{-1} N; 40B= 40 mg kg^{-1} N with biofilm. Treatments were not significant at p<0.1 according to Tukey-Kramer pairwise method.

The MFC performance over 55 days is shown in figure 4.1. Overall, the treatments were not significantly different from each other in voltage. The treatments, control, 20N, and 20B showed higher positive voltages initially (for up to 5 days), after which their voltages decreased, except for 20B, which increased in voltage after about 23 days, while treatment control with biofilm (CB) showed low voltage at the beginning (up to day 9) after which its voltage increased. The treatment 40B had a stable voltage slightly higher than zero, producing about 25-30 mV, whereas treatment 40N had a voltage consistently around 90 mV. The reason that a significant difference in the voltages of different treatments was not seen could be due to the dissolved organic carbon concentrations not being significantly different between treatments (except at day 54, Fig. 4.4). As there was no external supply of organic carbon, the concentration of soil organic matter was similar in all the treatments. However, the microbial community in the treatments could vary according to nutrient availability. Many studies have shown a positive linear relationship between soil organic carbon content and soil respiration or variables describing microbial activity (Michel & Matzner 1999; Zhao et al. 2008; Peterson et al. 2013). Based on this, it can be inferred that similar levels of DOC could generate a similar level of microbial activity, i.e., a similar rate of mineralization; therefore, differences in voltage were not observed. The treatment CB increased in voltage over time, which could be because of the addition of biofilm to the anode, which increased microbial activity, and organic substrate oxidation was more efficient, contributing to voltage production. The same happened with the 20B treatment, but at a much later stage (day 23) when the organic carbon oxidation started to contribute to voltage positively. In contrast, treatment 40B behaved differently. It constantly showed a low voltage value and no visible increase in voltage over time. This gives the insight that other reactions besides organic carbon oxidation were happening in the system decreasing voltage.

With enough nitrate present in the system, microbes, especially *Geobacter* could use nitrate as an electron acceptor instead of the anode, which caused a reduction in voltage. Kashima and Regan (2020) first described this competitive reaction occurring at the anode as the facultative nitrate reduction by *Geobacter metallireducens*. Apart from this, other electron-consuming reactions in anaerobic environments are denitrification, which also uses nitrate (Giles et al., 2012). Although not significantly different, 40B treatment had a concentration of total dissolved inorganic nitrogen less than the 40N treatment, which indicates that nitrate was being consumed (Fig. 4.3). In contrast, not having a *Geobacter* biofilm on 40N treatment proved beneficial for oxidation of organic carbon and producing a constant voltage of about 90 mV. Previous studies have shown positive effects of N addition on organic carbon mineralization, especially in agricultural soils like paddy (Zheng et al. 2007; Li et al. 2014; Zhou et al. 2014) and sandy soils (Azari et al., 2016, by stimulation of microbial communities). Several other studies have shown that fertilization in general increases the C and N mineralization by regulating the microbial biomass and diversity (Wu et al., 2020; Mandal et al., 2007; Kamaa et al., 2011). However, the same was not valid for the treatment 20N, which observed a low voltage throughout. It can be inferred that the nitrogen addition of 20 mg N kg^{-1} soil did not have a significant impact on the mineralization of organic carbon, which could be because of less microbial biomass and activity which could not contribute to the voltage much. Similar could be the case with control which had very low total dissolved inorganic nitrogen in the system (Fig. 4.3) to bring about a considerable change in the microbial community; therefore, the voltage decreased. In contrast, treatment 20B showed higher voltage because of stimulation of microbial activity from biofilm addition. Again, the voltages of the treatments were not significantly different because the similar organic carbon levels in the systems, as well as biofilm addition, did not bring a measurable difference as well.

All these prompted us to develop the next step of coating the biofilm on anodes properly to see an effect covered in the next study.

4.3.1.2. Microbial respiration

The microbial respiration for different treatments over time is shown in figure 4.2. On day 2, 20N and 40N showed higher production of carbon dioxide followed by control with biofilm, 20B, control, and 40B showing the least amount of carbon dioxide production. Between days 7 and 28, carbon dioxide release in 40B treatment increased greatly, followed by a decrease. Other treatments varied in their nature of carbon dioxide production but eventually, all decreased. Overall, the treatments did not vary in their microbial respiration and activity significantly (except on day 49 when carbon dioxide production in 20N was higher than control treatment), which could be another reason why a voltage difference was not observed.

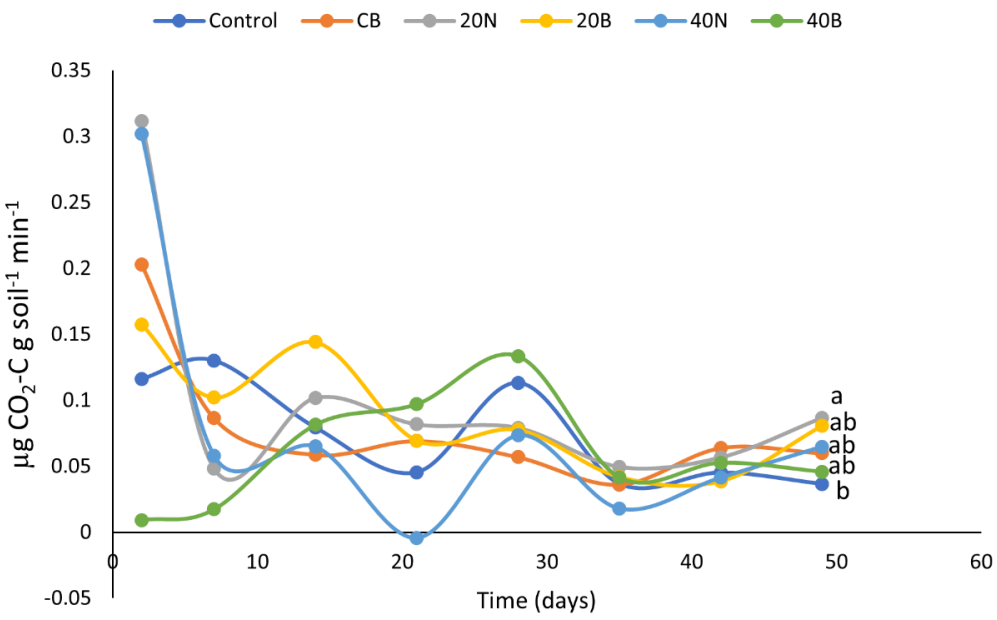

Figure 4.2. Mean rate of carbon dioxide evolution (CO_2-C) collected on days 2, 7, 14, 21, 28, 35, 42, and 49 (n=3). Control= no added N; CB= control with biofilm; 20N= 20 mg kg^{-1} N; 20B= 20 mg kg^{-1} N with biofilm; 40N= 40 mg kg^{-1} N; 40B= 40 mg kg^{-1} N with biofilm. Different letters show significant differences in means at $p<0.1$ according to Tukey's pairwise comparison.

Although the microbial activity in 40B increased over time, it did not reflect in the voltage, meaning that mineralization of organic C was happening, but electrons were not contributing to generating voltage. Other competitive reactions, such as facultative nitrate reduction and denitrification, were going on, which consumed the electrons produced from organic carbon oxidation (Kashima & Regan, 2020; Giles et al., 2012). Eventually, the microbial activity decreased over time for all treatments and showed similar levels, except that 20N showed a significantly higher respiration rate than the control. The very high respiration rate on day 2 for all treatments except 40B, and eventual decrease, in general, indicates the presence of two different pools of organic matter, one that was degraded early in the incubation and a resistant one that remained after all the available carbon sources were used (Reichstein et al., 2005). This decrease in respiration rate has been observed in other studies (Marzi et al., 2020; Guo et al., 2014), which suggests that microbially available carbon was low compared to the rates of respiration (Guo et al., 2014). Marzi et al. (2020) explained that the period of slow decomposition continues until a soil equilibrium is achieved.

4.3.1.3. Total dissolved inorganic nitrogen

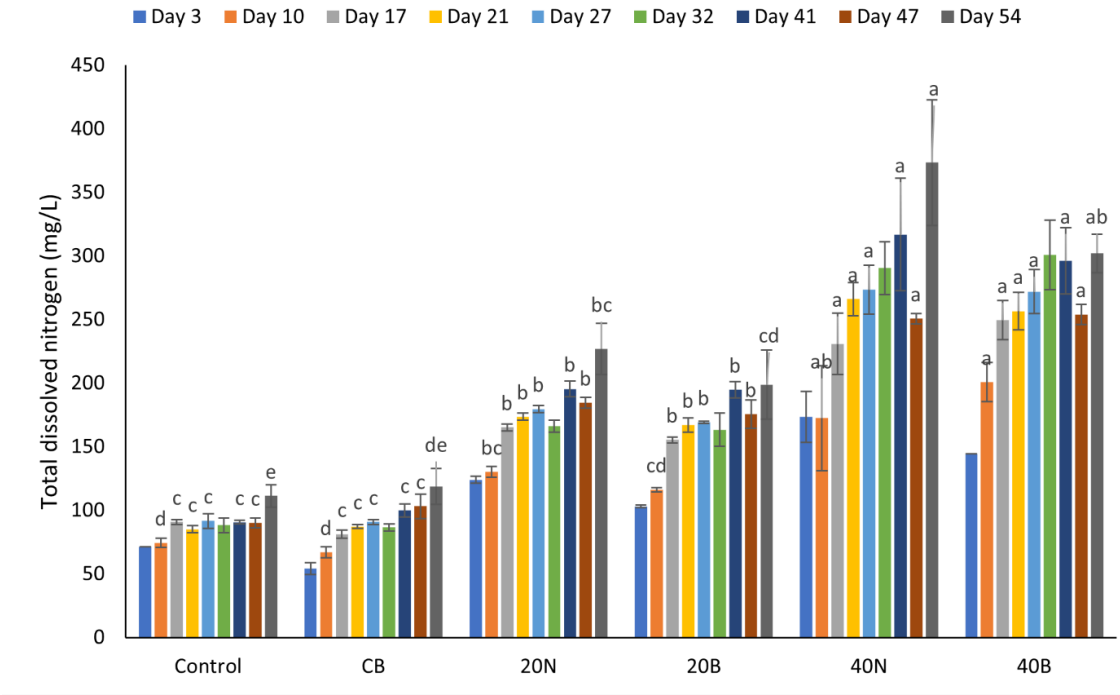

Figure 4.3. Mean of total dissolved inorganic nitrogen (sum of NO_3^-, NO_2^- and NH_4^+) analyzed from solution samples collected on days 3, 10, 17, 21, 27, 32, 41, 47, and 54 (n=3) using PROC MIXED. Control= no added N; CB= control with biofilm; 20N= 20 mg kg^{-1} N; 20B= 20 mg kg^{-1} N with biofilm; 40N= 40 mg kg^{-1} N; 40B= 40 mg kg^{-1} N with biofilm. Different letters show significant differences in means between treatments at α=0.05 according to the Tukey-Kramer pairwise method. Error bars represent the standard error of three replicates.

The total dissolved inorganic nitrogen in soil solution for different treatments generally showed that biofilm treatments had less total dissolved inorganic nitrogen in their soil solution than their respective non-biofilm counterparts (Fig. 4.3). Control and CB treatment showed lower levels of total dissolved inorganic nitrogen than other treatments. This was expected because no nitrogen was added in these systems, and the soil had less native N. Similarly, 20N and 20B treatments also showed significantly lower TDN levels than 40N and 40B treatments. The biofilm treatments with respect to the non-biofilm counterparts showing less dissolved nitrogen (not significant) could be attributed to *Geobacter*'s ability to reduce nitrate to

ammonium at the anode (Kashima & Regan 2020). Overall, the biofilm effect on the dissolved nitrogen levels was not markedly apparent. It could also be because biofilm survived in the soil for a very short period of time (3-4 days), or because of low/insufficient coverage on the electrodes.

4.3.1.4. Dissolved organic carbon

No significant differences were found between treatments except on day 54, where the control treatment had significantly lower dissolved organic carbon in solution than other treatments. In contrast, the 40N treatment had the highest amount of dissolved organic carbon. It is interesting to note that in the control treatment, the concentration of dissolved organic carbon generally decreased, whereas, in all other treatments, there was an increase in the organic carbon at some point in time, although not significantly different. The fact that dissolved organic carbon increased in these treatments despite considerable microbial activity (Fig. 4.2) and N addition in some treatments (20N, 20B, 40N, 40B), and that microbial composition and activity are altered in respective treatments is of particular interest (Shi et al., 2019). It was suggested that experimental N addition could alter the solute chemistry, such as the physical and chemical adsorption capacity of mineral soil by increasing ionic strength, soil nitrogen availability, and decreasing soil pH (Hagedorn et al., 2012).

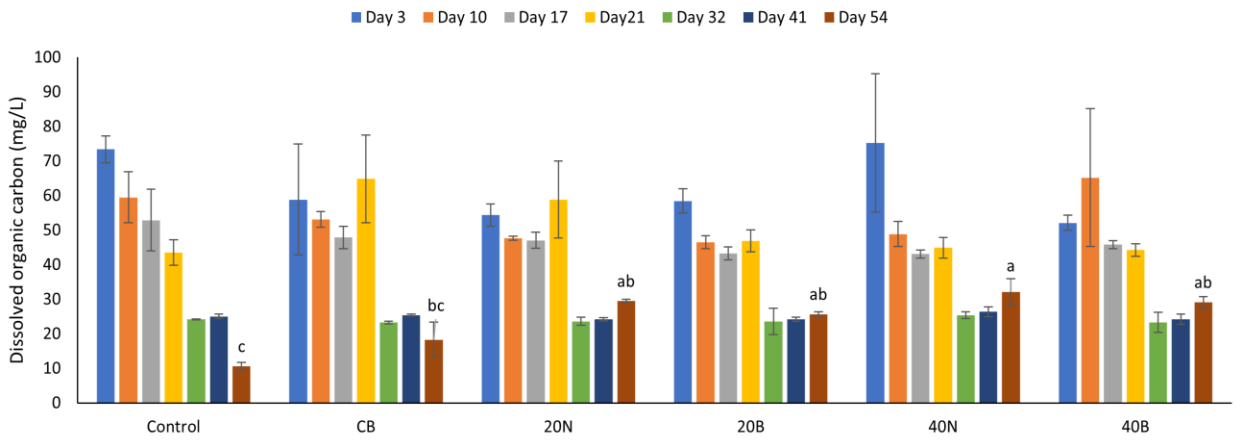

Figure 4.4. Dissolved organic carbon analyzed from solution samples collected on days 3, 10, 17, 21, 27, 32, 41, 47, and 54 (n=3) using PROC MIXED. Control= no added N; CB= control with biofilm; 20N= 20 mg kg^{-1} N; 20B= 20 mg kg^{-1} N with biofilm; 40N= 40 mg kg^{-1} N; 40B= 40 mg kg^{-1} N with biofilm. Different letters show significant differences in means at $p<0.05$ according to the Tukey-Kramer pairwise method. Error bars represent the standard error of three replicates.

A study has reported that sodium nitrate addition to the soil consistently increased DOC while ammonium salts addition decreased DOC (Evans et al., 2008; Shi et al., 2019). The study by Shi et al. (2019) also found that the three levels of nitrate additions (10, 20, and 40 kg of N $ha^{-1}yr^{-1}$) slightly increased DOC content compared to the control soil. However, the differences were not statistically significant.

4.3.2. Study 5:

4.3.2.1. MFC performance (voltage)

The voltage results for different treatments are given in figure 4.5. The voltage of 40N treatment showed significantly higher voltage than control treatment throughout the experiment, except when it was also higher than 40LB on day 1 and 40LB, 40HB, and CLB treatments around day 7 or 180th hour. The voltage of 40N remained relatively stable at an average of 300 mV. In comparison, the voltage of the control treatment was averaged around -350 mV in the beginning and later increased to -250 mV throughout the experiment. The voltage of CLB started

at high values but dramatically dropped at around the 80th hour and remained stable afterward. The voltages of all other treatments, CHB, 40LB, and 40HB, stayed in the middle.

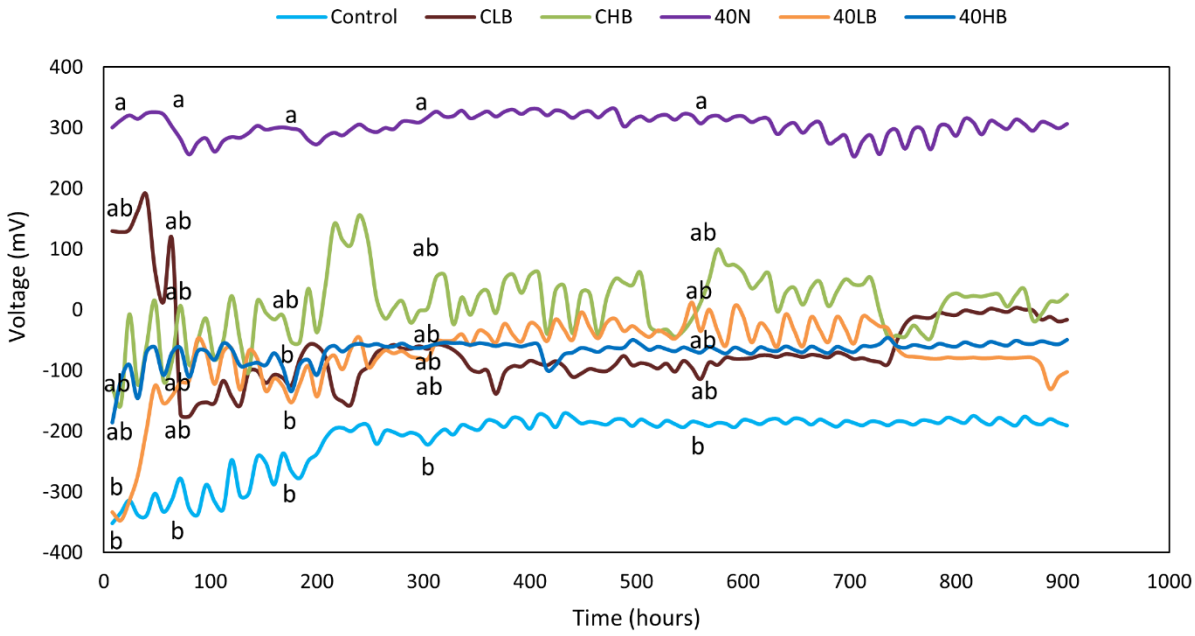

Figure 4.5. Voltage of the treatments averaged over 8-hour period for a total of 50 days (n=3) analyzed using PROC MIXED. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm. Different letters show significant differences in means at p<0.05 according to Tukey pairwise method.

The reason that higher voltage was observed in the 40N treatment could be attributed to enhanced C mineralization due to nitrogen addition in the soil. This enhanced C mineralization with nitrate addition agrees with our previous study (study 4) as well as with an incubation study conducted by Cao et al. (2021) where nitrogen addition in the form of NH_4NO_3 accelerated C mineralization in paddy soils while it had a negative impact on forest soil compared to the control soils. Several other studies on paddy soils also have shown positive effects of N addition on organic carbon mineralization (Zheng et al. 2007; Li et al. 2014; Zhou et al., 2014), while a study by Azari et al. (2016) showed stimulation of microbial communities with the addition of

nitrogen to sandy soils. Some studies have shown that fertilization in general increases the C and N mineralization by regulating the microbial biomass and diversity (Wu et al., 2020; Mandal et al., 2007; Kamaa et al., 2011). The treatments with biofilm on the anode, namely CLB, CHB, 40LB, 40HB, had voltages more than the control treatment because of the well-established *Geobacter* biofilm and other exoelectrogenic soil microorganisms were able to oxidize organic carbon by utilizing nitrogen available in soil solution and generating electrons contributing to voltage. These treatments showed less voltage than the 40N treatment which could be because *Geobacter* was also utilizing nitrate as an electron acceptor, reducing nitrate to ammonium (facultative nitrate reduction) thereby, using up the electrons generated from the organic carbon oxidation (Kashima & Regan, 2020) resulting in a decrease in voltage. Interestingly, the analysis of soil solution for total dissolved inorganic nitrogen in these treatments (Fig. 4.7) indicated that nitrogen was being "fixed" in all biofilm treatments, which was evident especially in CLB and CHB treatment. This nitrogen in soil solution could undergo various reactions like nitrate reduction and denitrification that could consume electrons generated from the organic carbon oxidation process and cause a reduction in voltage (Giles et al., 2012). The control treatment, however, had the lowest voltage produced, significantly lower than the 40N treatment, which could be because of use up of soil organic carbon very early in the system (up to day 8), which is supported by the lowest microbial activity observed in fig (at least up to day 19) and apparently no mineralization of soil N as seen in figure 4.7.

4.3.2.2. Microbial respiration

The microbial respiration over different days is shown in figure 4.6. It was observed that initially, on day 2, high biofilm treatments CHB and 40HB had more production of carbon dioxide followed by low biofilm treatments CLB and 40LB followed by control and 40N

treatment. This pattern changed over time, varying at each sampling point. A significant difference in carbon dioxide production was found on day 14, where CO_2 evolved more in the 40LB and 40HB treatment than in the control treatment. This shows that microorganisms in biofilm treatments were actively mineralizing organic carbon, although the time and extent varied for each. The control treatment had lower production of carbon dioxide initially up to day 14 and increased slightly later, showing that mineralization of organic carbon increased at a later stage. This time period also overlapped in the voltage results indicating that oxidation of organic carbon started contributing to voltage (slight increase in voltage) in the control treatment after the 14th day. However, it still remained the lowest in voltage compared to other treatments. Carbon dioxide evolved in 40N treatment was low in the beginning (day 2), which increased afterward, varied over time, and was higher at day 45 than other treatments, although not significantly different.

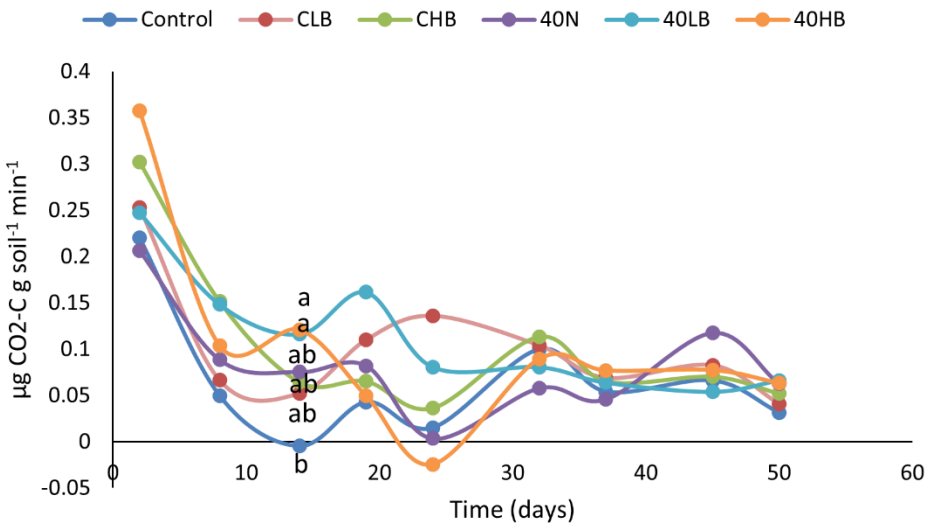

Figure 4.6. Mean rate of carbon dioxide evolution (CO_2-C) on days 2, 8, 14, 19, 24, 32, 37, 45, and 50 using PROC REG. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm. Different letters show significant differences at $p<0.05$ according to Tukey's pairwise comparison.

Even though 40N treatment had less carbon dioxide production overall than other treatments, the voltage of 40N showed that the mineralization of organic carbon contributed to voltage generation more in 40N treatment than in any other treatment. The carbon dioxide results thus indicate that although it depicts important information about microbial activity and mineralization of organic matter, it is not representative of electrogenic activity in the soil.

4.3.2.3. Total dissolved inorganic nitrogen

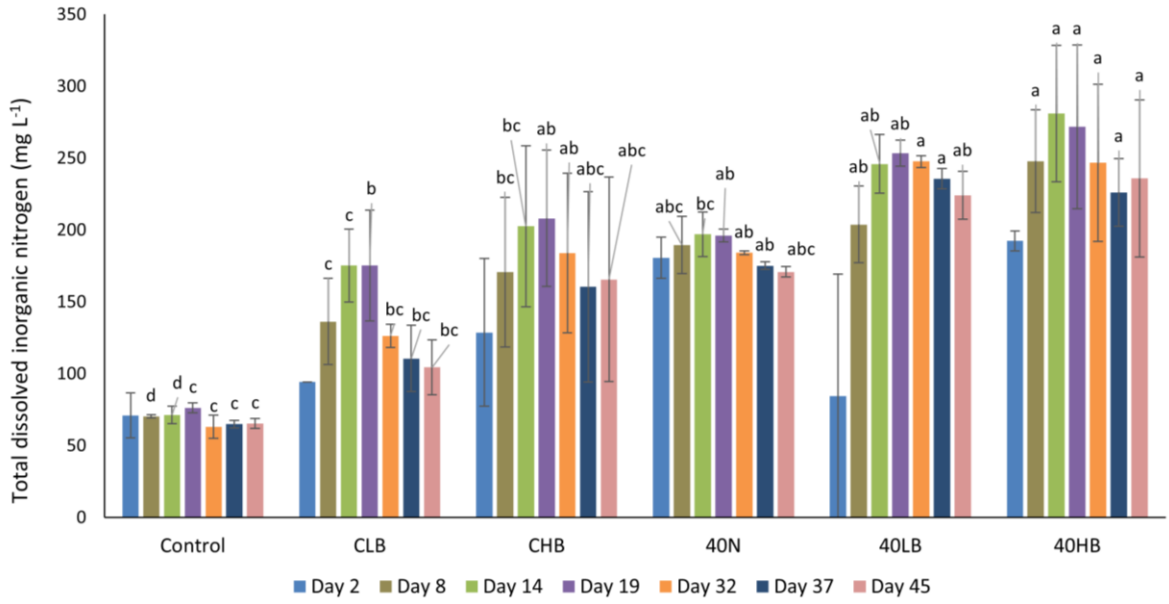

Figure 4.7. Total dissolved inorganic nitrogen (sum of NO_3^-, NO_2^- and NH_4^+) analyzed from solution samples on days 2, 8, 14, 19, 32, 37, and 45 (n=3) using PROC MIXED. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm. Different letters show significant differences at $p<0.05$ according to Tukey's pairwise comparison. Error bars represent the standard error of three replicates.

The soil solution analyzed for total dissolved inorganic nitrogen reveals that the control treatment had significantly lower levels of dissolved nitrogen compared to other treatments stagnating at around 75 mg L^{-1} (Fig. 4.7). In contrast, CLB and CHB interestingly, showed increasing total dissolved inorganic nitrogen levels indicating that nitrogen was being fixed in

these biofilm treatments compared to the control treatment. Similarly, 40LB and 40HB also showed significantly higher levels of total dissolved inorganic nitrogen compared to other treatments because of nitrate addition and nitrogen fixation, whereas 40N had apparently lower levels than 40LB and 40HB treatments as nitrogen fixation was not possible, but the dissolved nitrogen levels were higher than control because of nitrate addition. The results showed that the *Geobacter* biofilm was helping to fix nitrogen from the atmosphere in biofilm treatments. Several studies have highlighted the N_2-fixing ability of *Geobacter sulfurreducens* in a medium devoid of fixed N (Ueki & Lovley, 2010; Coppi et al., 2001, Jing et al., 2021). The study by Coppi et al. (2001) found that *Geobacter sulfurreducens* fixed nitrogen in a manner similar to other nitrogen-fixing microorganisms. Jing et al. (2021) found that nitrogen fixation by *G. sulfurreducens* did not impede coulombic efficiency, and that anode respiration was able to provide sufficient energy for nitrogen fixation while, in turn, nitrogen fixation enhanced anode respiration of the cell by increasing acetate catabolism and reducing acetate anabolism. Overall, total dissolved inorganic nitrogen analysis gave us insight into complex reactions occurring in soil because of a well-established biofilm.

4.3.2.4. Dissolved organic carbon

The dissolved organic carbon in soil solution for different treatments is shown in figure 4.8. The dissolved organic carbon was generally more in the biofilm treatments, significantly greater in CHB on day 24 than in control, 40N, and CLB. On day 50, it was significantly greater in 40HB and CHB treatments than in control. The point to note here is that at all points, the control treatment and 40N treatment were not significantly different from each other in their dissolved organic carbon levels; still, they differed in the voltage production. This shows that the

control treatment showed a lower voltage than the 40N treatment because nitrogen acted as a limiting factor in the mineralization of organic matter.

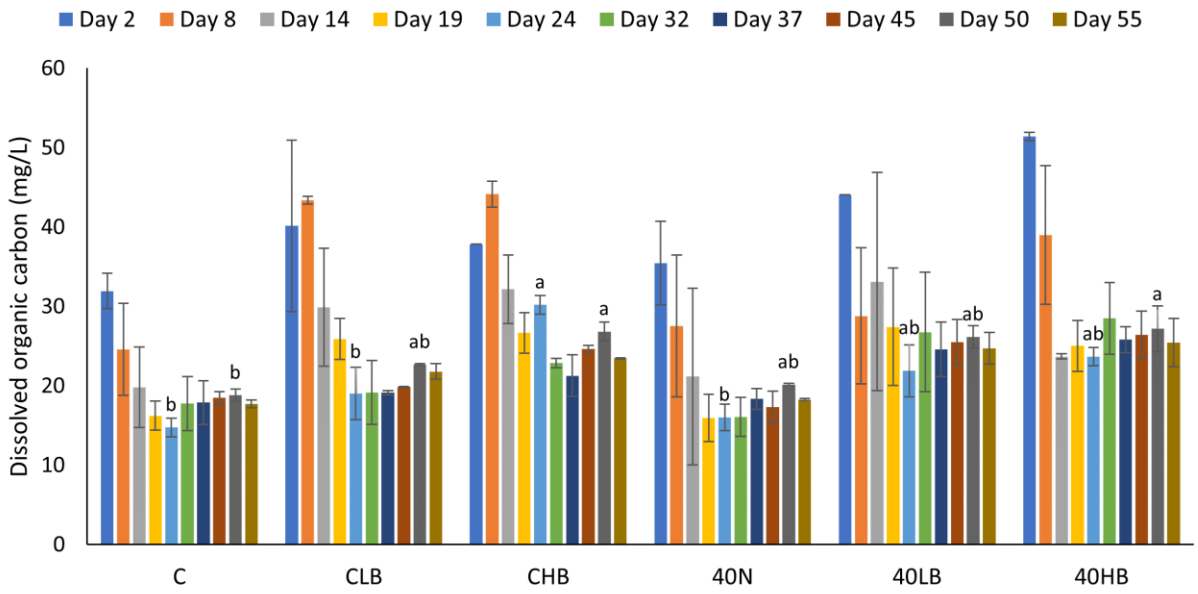

Figure 4.8. Dissolved organic carbon analyzed from solution samples collected on days 2, 8, 14, 19, 32, 37, and 45 (n=3) using PROC MIXED. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm. Different letters show significant differences in means between treatments at α=0.1 according to Tukey-Kramer pairwise comparison. Error bars represent the standard error of three replicates.

Studies have shown that both nitrogen and carbon are limiting factors of soil microbial activity (Azari et al., 2016; Schimel & Weintraub, 2003). The treatments with biofilm managed to increase the DOC levels by mineralization of organic matter. In this study, too, dissolved organic carbon increased in treatments despite considerable microbial activity (Fig. 4.6) and N addition in some treatments (40N, 40LB, & 40HB). It was suggested that experimental N addition could alter the physical and chemical adsorption capacity of mineral-form of soil organic matter by increasing ionic strength, soil N availability, and decreasing soil pH (Hagedorn et al., 2012; Shi et al., 2019), which could increase labile organic carbon content.

4.3.2.5. Correlations between total dissolved inorganic nitrogen and voltage

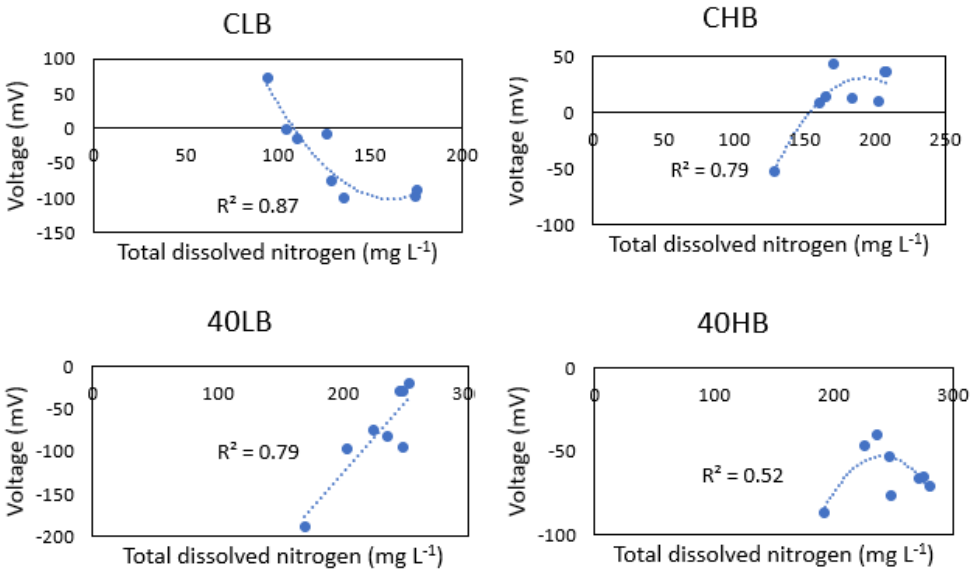

Figure 4.9. Correlations between total dissolved inorganic nitrogen and voltage on corresponding days 2, 8, 14, 19, 24, 32, 37, and 45. CLB= control with low biofilm; CHB= control with high biofilm; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm. Correlations significant at $p<0.05$ using the two-tail T-test (n=8).

Correlation plots were obtained by plotting voltage and total dissolved inorganic nitrogen values at the same time points over different days (e.g., voltage and total dissolved inorganic nitrogen on days 2, 8, 14, 19, 24, 32, 37, and 45; Fig. 4.9). The R^2 value was determined by fitting appropriate regression models (linear or quadratic) with total dissolved inorganic nitrogen as the independent variable (x) and voltage as the dependent variable (y). T-statistic and p-value were calculated using R^2 value and degrees of freedom using two-tail T-test. All correlations were significant at p-value <0.05. All biofilm-treated soils showed significant correlations between total dissolved N and voltage. It gave an idea about the dominant reaction occurring in each system. For example, in CLB, nitrate reduction and denitrification > organic carbon oxidation, and in CHB and 40LB organic carbon oxidation > nitrate reduction and denitrification.

4.3.2.5. Phospholipid fatty acid analysis

Table 4.2. Average phospholipid fatty acid representing microbial biomass analyzed from soil samples around anode at the end of the experiment using lmer function in R. Differences in means between treatments significant at α=0.05 according to Tukey method of pairwise comparison. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm.

	Tot. biomass	Gram+	Gram-	Actinomycetes	AMF	Fungi
	nmol PLFA g^{-1} soil					
C	4.52±1.92b	1.40±0.61b	0.49±0.24a	0.10±0.03a	0.04±0.02b	0.19±0.1a
CLB	5.60±2.18b	1.86±0.83ab	0.64±0.26a	0.11±0.05a	0.05±0.02ab	0.19±0.04a
CHB	9.51±1.40a	2.52±1.17a	2.77±1.41a	0.15±0.07a	0.06±0.03a	0.27±0.12a
40N	7.14±3.42ab	2.43±1.28a	0.83±0.43a	0.14±0.07a	0.06±0.03a	0.25±0.10a
40LB	6.09±2.64ab	1.64±0.67ab	0.57±0.20a	0.11±0.05a	0.05±0.02ab	0.20±0.06a
40HB	7.32±4.04ab	1.84±0.90ab	0.64±0.26a	0.12±0.04a	0.05±0.03ab	0.21±0.09a

Phospholipid fatty acid analysis was done on the soil samples taken around the anode after the termination of the experiment. Total microbial biomass (nmol g^{-1} soil) was estimated with this method along with various microbial groups (gram-positive bacteria, gram-negative bacteria, fungi, actinomycetes, and AMF; Table 4.3). Significant differences were found at $p<0.05$ between the treatments for total biomass, gram + bacteria, and AMF. Total microbial biomass was significantly higher in CHB treatment compared to control and CLB treatment (Fig. 4.10). Gram-positive bacteria (Fig. 4.11) and AMF were found to be significantly higher in CHB and 40N treatment compared to the control. However, no significant difference was observed in the gram-negative bacterial biomass between the treatments. It is possible that the *Geobacter*

biofilm got worn out towards the end of the experiment (bacterial decay) and the biomass during the start of the experiment was not the same as we obtained at the termination of the experiment. The interesting thing, however, is the accumulation of gram-positive bacteria around the anode which indicates that in our systems, gram-positive bacteria also had a role to play in the voltage production, especially in 40N and CHB treatments. Out of the microbial groups we tested, gram-positive bacteria contributed more than 50% in the group total and 25-35% in the total microbial biomass. Various studies based on soil microbial fuel cells have found that Firmicutes and Clostridia are the dominant phyla in the soil that positively relate to the voltage and electricity production (Jiang et al., 2016, 2018; Jimenez et al., 2020). The majority of the isolates of these phyla are gram-positive bacteria (Wells & Wilkins, 1996; Vesth et al., 2013). It is possible that the high voltage production obtained in 40N and CHB treatments could be because of the high biomass of gram-positive bacteria in our soil microbial fuel cell systems. Moreover, *Clostridium spp*. is also one of the first known free-living nitrogen-fixing bacteria (discovered by Winogradsky) which could also explain the "nitrogen-fixation" observed in our study. But further analysis of these species is required at the gene level to confirm their presence in our systems.

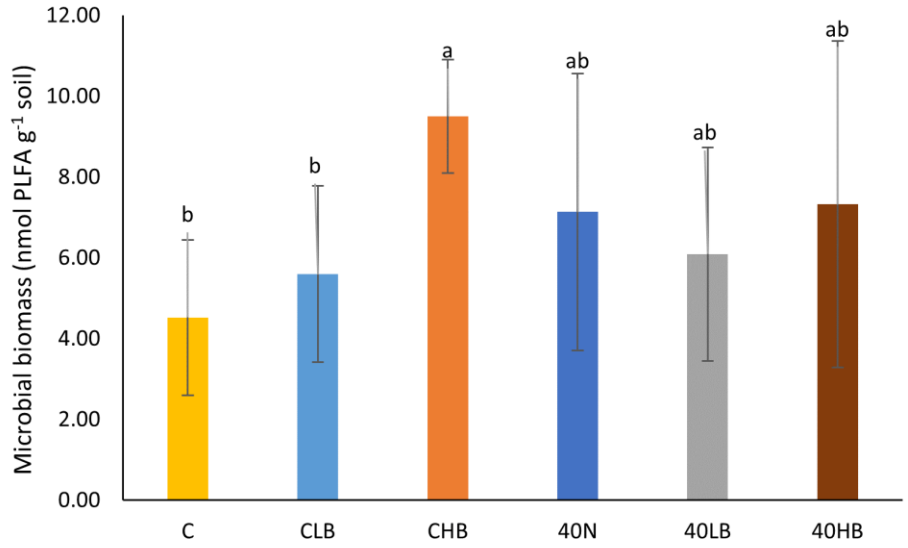

Figure 4.10. Total microbial biomass analyzed from soil samples around anode at the end of the experiment using lmer function in R. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm. Different letters show significant differences at p<0.05 according to Tukey's pairwise comparison. Error bars represent the standard error of the replicates.

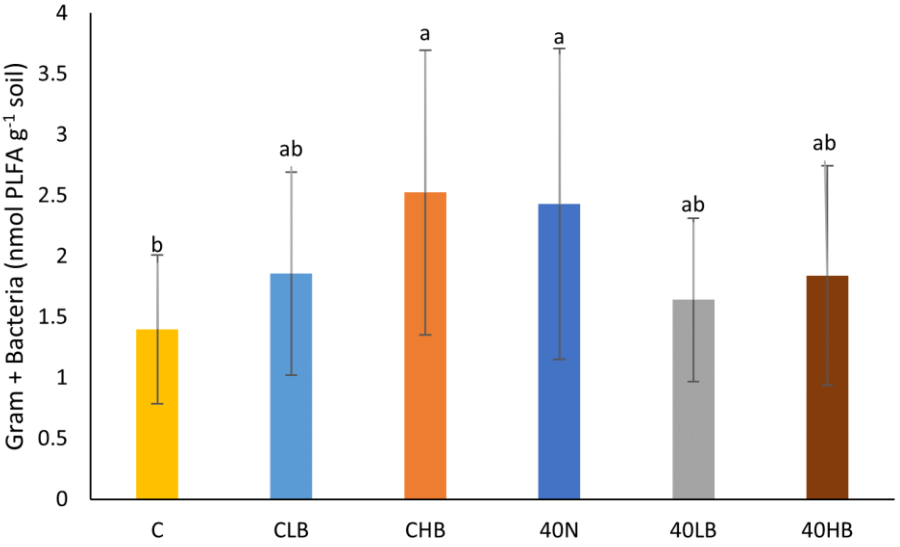

Figure 4.11**. Gram (+) bacteria analyzed from soil samples around the anode at the end of the experiment using lmer function in R. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm. Different letters show significant differences at p<0.05 according to Tukey's pairwise comparison. Error bars represent the standard error of the replicates.**

4.4. Summary

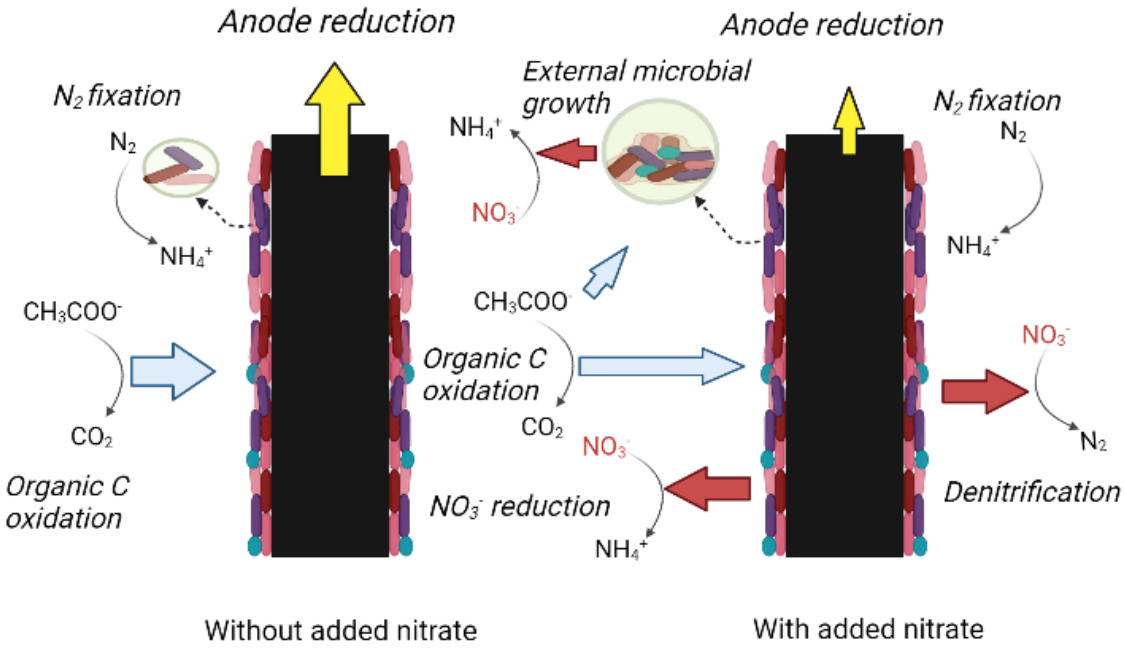

Figure 4.12. Diagram showing possible reactions occurring in an MFC with or without added nitrate leading to decrease/increase in voltage (Adapted and modified after Kashima and Regan, 2015; created using Biorender.com)

In these studies, complex reactions occurring in the soil were more pronounced. The differences in the voltage production were seen between control and 40N treatment in study 5, whereas in study 4, the differences were not significant. However, establishing a *Geobacter* biofilm on the anode helped to capture various nitrogen transformations better, providing insight into complex soil processes which are summarized in figure 4.12. The reaction that increases the voltage is the organic matter oxidation. With enough nitrate present in the solution, it can act as an electron acceptor in the presence of *Geobacter* and can reduce it to ammonium, leading to a decrease in voltage. So, the electrons instead of going to the anode, get used up in this process. Another reaction that can cause a reduction in voltage is denitrification. We conducted some preliminary sampling for N_2O and found that denitrification was happening in our systems (Table 4.2).

Table 4.3. Average N_2O emissions based on two-spots testing

Treatments	C	CHB	40LB	40HB
N_2O (ppm)	0.46	0.85	0.60	0.65

Apart from that, we also found nitrogen fixation occurring in the system, but its effect on voltage production is not known. The phospholipid fatty acid analysis confirmed high microbial biomass in 40N and CHB treatments, and the dominance of gram-positive bacteria around the anode. However, further analysis at the gene level is required to clearly state if these had any role in soil-based MFC's electrogenic activity or not. As there was an indication of wearing out of *Geobacter* biofilm over time, for future studies, a polymer coating is required to sustain its activity for a longer time and to protect from exogenous soil microorganisms.

4.5. References

Allison, V. J., & Miller, R. M. (2005). Soil grinding increases the relative abundance of eukaryotic phospholipid fatty acids. *Soil Science Society of America Journal*, *69*(2), 423-426.

Azari Moghaddam, H., & Abu Bakar, N. K. (2016). Carbon dynamic in sandy soil artificially contaminated with petroleum in the presence of various forms of inorganic nitrogen for bioremediation. *Environmental Earth Sciences*, *75*(3), 1-13. https://doi.org/10.1007/s12665-015-4927-0

Bond, D. R., & Lovley, D. R. (2003). Electricity production by Geobacter sulfurreducens attached to electrodes. Applied and environmental microbiology, 69(3), 1548-1555. https://doi.org/10.1128/AEM.69.3.1548-1555.2003

Bouyoucos, G. J. (1962). Hydrometer method improved for making particle size analyses of soils 1. *Agronomy journal*, *54*(5), 464-465.

Cao, R., Chen, L., Hou, X., Lü, X., & Li, H. (2021). Nitrogen addition reduced carbon mineralization of aggregates in forest soils but enhanced in paddy soils in South China. *Ecological Processes*, *10*(1), 1-11. https://doi.org/10.1186/s13717-021-00319-z

Chapman, H.D. (1965). Cation Exchange Capacity. In: Black, C.A., Ed., *Methods of Soil Analysis* (891-901). American Society of Agronomy, Madison

Choi, Y. J., Kim, N. J., Kim, S. H., & Jung, S. H. (2003). Dynamic behaviors of redox mediators within the hydrophobic layers as an important factor for effective microbial fuel cell operation. *Bulletin of the Korean Chemical Society*, *24*(4), 437-440.

Evans, C. D., Goodale, C. L., Caporn, S. J., Dise, N. B., Emmett, B. A., Fernandez, I. J., ... & Sheppard, L. J. (2008). Does elevated nitrogen deposition or ecosystem recovery from

acidification drive increased dissolved organic carbon loss from upland soil? A review of evidence from field nitrogen addition experiments. *Biogeochemistry*, *91*(1), 13-35. https://doi.org/10.1007/s10533-008-9256-x

Franks, A. E., Malvankar, N., & Nevin, K. P. (2010). Bacterial biofilms: the powerhouse of a microbial fuel cell. *Biofuels, 1(4)*, 589–604. https://doi.org/10.4155/bfs.10.25

Franks, A. E., & Nevin, K. P. (2010). Microbial fuel cells, a current review. *Energies*, *3*(5), 899-919. https://doi.org/10.3390/en3050899

Giles, M. E., Morley, N. J., Baggs, E. M., & Daniell, T. J. (2012). Soil nitrate reducing processes–drivers, mechanisms for spatial variation, and significance for nitrous oxide production. *Frontiers in microbiology*, *3*, 407. https://doi.org/10.3389/fmicb.2012.00407

Gorby, Y.A., & Beveridge, T.J. (2005). Composition, reactivity, and regulation of extracellular metal-reducing structures (nanowires) produced by dissimilatory metal reducing bacteria. Presented at DOE/NABIR meeting, Warrenton, VA.

Hagedorn, F., Kammer, A., Schmidt, M. W., & Goodale, C. L. (2012). Nitrogen addition alters mineralization dynamics of 13 C-depleted leaf and twig litter and reduces leaching of older DOC from mineral soil. *Global Change Biology*, *18*(4), 1412-1427.

Heronemus, E. (2021). Phosphorus recovery from anaerobic membrane bioreactor (AnMBR) permeate by chemical coagulation and bioelectrochemical processes. Master's thesis, Kansas State University, Man7hattan, KS.

Ishii, S. I., Watanabe, K., Yabuki, S., Logan, B. E., & Sekiguchi, Y. (2008). Comparison of electrode reduction activities of Geobacter sulfurreducens and an enriched consortium in an air-cathode microbial fuel cell. *Applied and environmental microbiology*, *74*(23), 7348-7355.

Jenkinson, D. S., & Powlson, D. S. (1976). The effects of biocidal treatments on metabolism in soil-V: a method for measuring soil biomass. *Soil biology and Biochemistry*, *8*(3), 209-213.

Jiang, Y., Huan, D. E. N. G., Hongyi, Q. I. N., Cheng, H. A. N., & Zhong, W. (2018). Indication of soil microbial activity by electrical signals of microbial fuel cells with re-vegetated red soils. *Pedosphere*, *28*(2), 269-276. https://doi.org/10.1016/S1002-0160(18)60009-8

Jiang, Y.B., Zhong, W.H., Han, C., &Deng H. (2016). Characterization of Electricity Generated by Soil in Microbial Fuel Cells and the Isolation of Soil Source Exoelectrogenic Bacteria. *Frontiers in Microbiology*, *7*, 1776. https://doi.org/10.3389/fmicb.2016.01776

Jimenez, L., Kulko, M., Kim, R., Jashari, T., & Choe, T. (2020). 16S rRNA analysis of electrogenic bacterial communities in microbial fuel cells developed from temperate soils. *BIOS*, *91(1)*, 9-20. https://doi.org/10.1893/BIOS-D-18-00006

Jing, X., Liu, X., Zhang, Z., Wang, X., Rensing, C., & Zhou, S. (2022). Anode respiration-dependent biological nitrogen fixation by Geobacter sulfurreducens. *Water research*, *208*, 117860. https://doi.org/10.1016/j.watres.2021.117860

Kamaa, M., Mburu, H., Blanchart, E., Chibole, L., Chotte, J. L., Kibunja, C., & Lesueur, D. (2011). Effects of organic and inorganic fertilization on soil bacterial and fungal microbial diversity in the Kabete long-term trial, Kenya. *Biology and Fertility of Soils*, *47*(3), 315-321.

LECO Corp. (2005). Carbon and Nitrogen in Soil and Sediment. Organic Application Note: TruSpec CN (Form No. 203-821-275). St. Joseph.

Li, Y.K., Chen, M.P., Mei, X.R., Xia, X., Guo, W.Z., Li, H.R., & Hao, W.P. (2014). Effects of soil moisture and nitrogen addition on organic carbon mineralization in a high-yield cropland soil of the North China plain. *Acta Ecologica Sinica*, *34*, 4037–4046.

Mandal, A., Patra, A. K., Singh, D., Swarup, A., & Masto, R. E. (2007). Effect of long-term application of manure and fertilizer on biological and biochemical activities in soil during crop development stages. *Bioresource technology*, *98*(18), 3585-3592.

Michel, K., & Matzner, E. (1999). Release of dissolved organic carbon and nitrogen from forest floors in relation to solid phase properties, respiration and N-mineralization. *Journal of Plant Nutrition and Soil Science*, *162*(6), 645-652.

Muhsin, J., Ufaq, T., Tahir, H., & Saadia, A. (2015). Bacterial biofilm: its composition, formation and role in human infections. *J Microbiol Biotechnol*, *4*, 1-14.

Mulvaney, R.L. (1996). Nitrogen—Inorganic Forms. In Sparks, A. Page, P. Helmke, R. Loeppert, P.N. Soltanpour, M.A. Tabatabai, C.T. Johnston, & M.E. Sumner (eds D.), *Methods of soil analysis: Part 3 Chemical methods*, *5*, 1123-1184. https://doi.org/10.2136/sssabookser5.3.c38

Nevin, K. P., Richter, H., Covalla, S. F., Johnson, J. P., Woodard, T. L., Orloff, A. L., ... & Lovley, D. R. (2008). Power output and columbic efficiencies from biofilms of Geobacter sulfurreducens comparable to mixed community microbial fuel cells. *Environmental microbiology*, *10*(10), 2505-2514.

Oh, S. E., & Logan, B. E. (2006). Proton exchange membrane and electrode surface areas as factors that affect power generation in microbial fuel cells. *Applied microbiology and biotechnology*, *70*(2), 162-169.

Parameswaran, P., Torres, C. I., Lee, H.-S., Krajmalnik-Brown, R., & Rittmann, B. E. (2009). Syntrophic interactions among anode respiring bacteria (ARB) and Non-ARB in a biofilm anode: Electron balances. *Biotechnology and Bioengineering*, *103(3)*, 513–523. https://doi.org/10.1002/bit.22267

Parameswaran, P., Torres, C. I., Lee, H.-S., Rittmann, B. E., & Krajmalnik-Brown, R. (2011). Hydrogen consumption in microbial electrolysis systems (MXCs): The role of homoacetogenic bacteria. *Bioresource Technology*, *102(1)*, 263–271. https://doi.org/10.1016/j.biortech.2010.03.133

Peterson, M. E., Curtin, D., Thomas, S., Clough, T. J., & Meenken, E. D. (2013). Denitrification in vadose zone material amended with dissolved organic matter from topsoil and subsoil. *Soil Biology and Biochemistry*, *61*, 96-104.

Rabaey, K., & Verstraete, W. (2005). Microbial fuel cells: novel biotechnology for energy generation. *TRENDS in Biotechnology*, *23*(6), 291-298.

Reichstein, M., Subke, J. A., Angeli, A. C., & Tenhunen, J. D. (2005). Does the temperature sensitivity of decomposition of soil organic matter depend upon water content, soil horizon, or incubation time? *Global Change Biology*, *11*(10), 1754-1767.

Reguera, G., Nevin, K. P., Nicoll, J. S., Covalla, S. F., Woodard, T. L., & Lovley, D. R. (2006). Biofilm and nanowire production leads to increased current in Geobacter sulfurreducens fuel cells. *Applied and environmental microbiology*, *72*(11), 7345-7348.

Schimel, J. P., & Weintraub, M. N. (2003). The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. *Soil Biology and Biochemistry*, *35*(4), 549-563

Shi, L., Dech, J. P., Yao, H., Zhao, P., Shu, Y., & Zhou, M. (2019). The effects of nitrogen addition on dissolved carbon in boreal forest soils of northeastern China. *Scientific Reports*, *9*(1), 1-9. https://doi.org/10.1038/s41598-019-44796-x

Slate, A. J., Whitehead, K. A., Brownson, D. A., & Banks, C. E. (2019). Microbial fuel cells: An overview of current technology. *Renewable and sustainable energy reviews*, *101*, 60-81. https://doi.org/10.1016/j.rser.2018.09.044

Sund, C. J., McMasters, S., Crittenden, S. R., Harrell, L. E., & Sumner, J. J. (2007). Effect of electron mediators on current generation and fermentation in a microbial fuel cell. *Applied Microbiology and Biotechnology*, *76*(3), 561-568. https://doi.org/10.1007/s00253-007-1038-1

Sure, S., Ackland, M. L., Torriero, A. A., Adholeya, A., & Kochar, M. (2016). Microbial nanowires: an electrifying tale. *Microbiology*, *162*(12), 2017-2028. https://doi.org/10.1099/mic.0.000382

Turick, C. E., Caccavo Jr, F., & Tisa, L. S. (2003). Electron transfer from Shewanella algae BrY to hydrous ferric oxide is mediated by cell-associated melanin. *FEMS microbiology letters*, *220*(1), 99-104.

Ueki, T., & Lovley, D. R. (2010). Novel regulatory cascades controlling expression of nitrogen-fixation genes in Geobacter sulfurreducens. *Nucleic acids research*, *38*(21), 7485-7499. https://doi.org/10.1093/nar/gkq652

Vesth, T., Ozen, A., Andersen, S. C., Kaas, R. S., Lukjancenko, O., Bohlin, J., Nookaew, I., Wassenaar, T. M., & Ussery, D. W. (2013). Veillonella, Firmicutes: Microbes disguised as Gram negatives. Standards in genomic sciences, 9(2), 431–448. https://doi.org/10.4056/sigs.2981345

Wateson, M.E. and Brown, J.R. (1998). In Brown J.R (ed.) *Recommended Chemical Soil Test Procedures for the North Central Region* (pp. 13-14). University of Missouri Agricultural Experiment Station, Columbia, MO.

Wells, C. L., & Wilkins, T. D. (1996). Clostridia: Sporeforming Anaerobic Bacilli. In S. Baron (Ed.), *Medical Microbiology*. (4th ed.). University of Texas Medical Branch at Galveston. https://www.ncbi.nlm.nih.gov/books/NBK8219/

White, D. C., & Ringelberg, D. B. (1998). Signature lipid biomarker analysis. *Techniques in Microbial Ecology*, *255*, 255-272.

White, P. M., & Rice, C. W. (2009). Tillage effects on microbial and carbon dynamics during plant residue decomposition. *Soil Science Society of America Journal*, *73(1)*, 138-145. https://doi.org/10.2136/sssaj2007.0384

Wu, H., Cai, A., Xing, T., Huai, S., Zhu, P., Xu, M., & Lu, C. (2021). Fertilization enhances mineralization of soil carbon and nitrogen pools by regulating the bacterial community and biomass. *Journal of Soils and Sediments*, *21*(4), 1633-1643. https://doi.org/10.1007/s11368-020-02865-z

Zhao, M., Zhou, J., & Kalbitz, K. (2008). Carbon mineralization and properties of water-extractable organic carbon in soils of the south Loess Plateau in China. *European journal of soil biology*, *44*(2), 158-165.

Zheng, J., Zhang, X., Li, L., Zhang, P., & Pan, G. (2007). Effect of long-term fertilization on C mineralization and production of CH4 and CO2 under anaerobic incubation from bulk samples and particle size fractions of a typical paddy soil. *Agriculture, ecosystems & environment*, *120*(2-4), 129-138. https://doi.org/10.1016/j.agee.2006.07.008

Zhou, L., Zhou, X., Zhang, B., Lu, M., Luo, Y., Liu, L., & Li, B. (2014). Different responses of soil respiration and its components to nitrogen addition among biomes: a meta-analysis. *Global Change Biology*, *20(7)*, 2332–2343. https://doi.org/10.1111/gcb.12490

4.6. Supplementary information

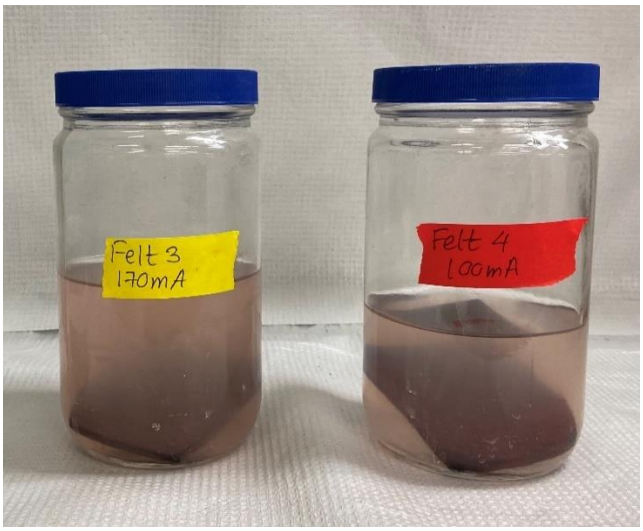

Figure S4.1. Carbon felt anodes brought to the lab in a jar containing phosphate buffer solution

Figure S4.2. Photo of second replicate of *Geobacter* coated electrodes, coverage on first (left) photo was considered 85%-high biofilm treatment, while on second (right) photo was considered 45%-low biofilm treatment by visual analysis

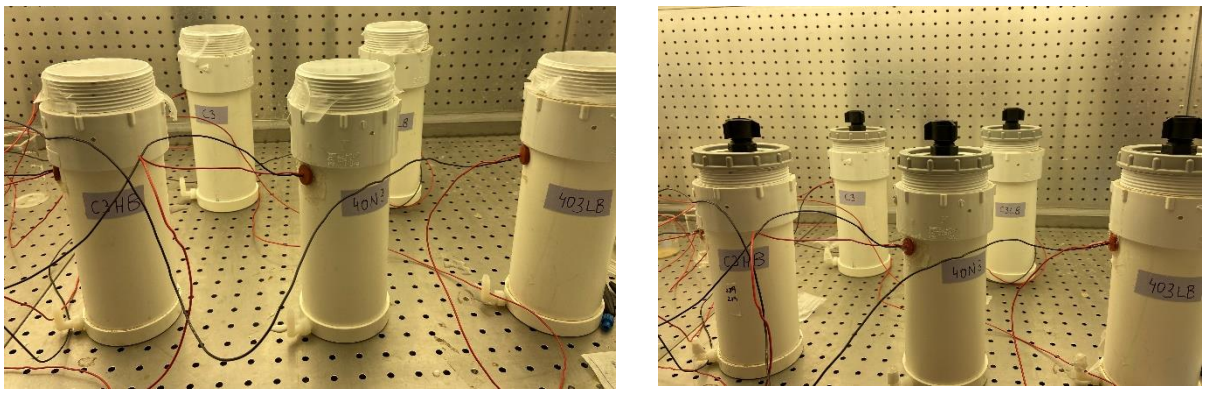

Figure S4.3. PVC column design: Right figure shows sealing achieved by using lids (having septa on top) to cover/seal the top properly for gas sampling, photo taken in growth chambers

Table S4.1. Average pH of treatments analyzed from solution samples collected on days 2, 8, 14, 19, 24, 32, 37, 45, 50 (n=3) of study 5. Differences in means between treatments not significant at α=0.1 according to Tukey method of pairwise comparison. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm.

Days	2	8	14	19	24	32	37	45	50
Control	6.13	6.07	6.09	5.60	5.92	5.66	5.79	6.02	6.07
CLB	5.85	5.68	5.65	5.50	5.87	5.67	5.90	5.94	5.99
CHB	5.86	5.64	5.54	5.92	5.61	5.49	5.62	5.63	5.59
40N	6.26	6.50	5.95	5.94	5.86	5.61	5.71	5.98	5.98
40LB	6.10	6.01	5.72	5.72	5.78	5.71	5.71	5.84	5.93
40HB	6.40	5.59	5.69	5.90	6.16	5.42	5.74	6.10	5.97

Table S4.2. Average electrical conductivity (µS cm^{-1}) of treatments analyzed from solution samples collected on days 2, 8, 14, 19, 24, 32, 37, 45, 50 (n=3) of study 5. Differences in means between treatments not significant at α=0.1 according to Tukey method of pairwise comparison. Control= no added N; CLB= control with low biofilm; CHB= control with high biofilm; 40N= 40 mg kg^{-1} N; 40LB=40 mg kg^{-1} N with low biofilm; 40HB=40 mg kg^{-1} N with high biofilm.

Days	2	8	14	19	24	32	37	45	50
Control	1536	1706	1711	1779	1727	1780	1659	1699	1583
CLB	1812	2078	2520	2429	1951	1963	2007	1595	1697
CHB	na	2096	na	2300	2740	2522	2210	2396	2318
40N	1494	1589	1642	1641	1578	1562	1491	1495	1460
40LB	na	1598	2110	2135	2080	2135	2115	2665	1953
40HB	1657	2127	2475	2298	2303	2167	1937	2055	1971

Chapter 5 - Summary and recommendations

Summary

Electrogenic activity in soil-based microbial fuel cells can depend on the soil type, nutrient status of the soil, microbial community, and composition, and environmental conditions. In this thesis, a series of experiments on soil-based microbial fuel cells were conducted in order to study a voltage difference in response to changing nutrient levels, i.e., organic carbon or nitrate, and the addition of biofilm to the anode using two different soils. Soil 1 (used for studies 1, 2, & 3) was classified as Clime (Fine, mixed, active, mesic Udorthentic Haplustolls)-Sogn (Loamy, mixed, superactive, mesic Lithic Haplustolls) complex silty clay with 4.74% organic matter and 0.27% total N. Soil 2 (used for studies 4 & 5) was a sandy loam soil classified as a rarely flooded Belvue silt loam with 0.44% organic matter, 0.07% total N and low dissolved N. In the first and third studies, we found significant differences in voltage where the control treatment had a significantly higher voltage than the nitrogen added treatment. However, in the second study, which focused on addition of different organic carbon levels, no significant differences in voltage were found most likely because of the similar level of organic carbon found in soil solution throughout the experiment despite the treatments. Studies 4 and 5 used much simpler soil (soil 2) and an improved design of soil MFC (specialized PVC columns, bottom-up approach for watering the soil, uniform soil packing, etc.). These studies had various nitrogen treatments with the *Geobacter* inoculum on the anode (study 4), and pre-developed *Geobacter* anodic coating to provide a stable biofilm (study 5). In the fourth study, no significant differences among the voltages of different treatments were found which could be because of the similar DOC concentrations in the soil solution throughout, a similar rate of CO_2 release and insufficient coverage of inoculum on anode. The fifth study however, found significant

differences in voltage as well as the microbial community between control and 40N treatment, whereas voltages of biofilm treatments tended to stay in the middle of these two. The phospholipid fatty acid analysis for study 5 indicated the dominance of gram-positive bacteria around the anode (significantly higher in CHB and 40N compared to the control), some species of which are also known to generate electricity. However, further analysis of microbial structure at the gene level is required to understand the role of the specific microbial community in producing a voltage response. These studies have highlighted the potential soil-based microbial fuel cells hold, apart from electricity generation, as a biosensor. Since research on soil-based microbial fuel cells is very limited, our studies had its own challenges which we tried to overcome over the course of our experiments, like maintaining uniform moisture content, gas measurements, significant design improvements, biofilm attachment to the anode, uniform packing of the soil, and voltage measurements. There is a need for the related scientific community to come to a consensus about the microbial fuel cell design and voltage/electrical measurements so that the application of MFC technology to disciplines like soil science become easy.

Future research

The major challenge that remains in our research is to deconvolute the multiple and conflicting signals arising from the soil. To achieve a better separation of the signal, future research should be done by choosing an electrogenic organism that responds to a specific nutrient that can provide a reliable, reproducible, and consistent voltage signal under various conditions. Moreover, protecting this microorganism (increasing its longevity as a biofilm on the anode) with a polymer-based anode coating should also be a step into consideration. This polymer coating will help inhibit fouling from exogenous soil microorganisms and control

against abrupt changes in pH, thereby improving the longevity and function of electroactive biofilms on anode. Another possibility could be to build a polymer around the anode specific to the nutrient which allows only the nutrient in question to pass through. This will ensure specificity of the anode signal which will be reliable and consistent. Whether or not these techniques will help separate out signals between a single nutrient at multiple levels (for example, 20 mg N kg^{-1}, 40 mg N kg^{-1} and 80 mg N kg^{-1}) is still a question to be explored and should be researched further.

As indicated before, microbial community structure analysis is indispensable to this research to know what kind of microbes are involved in the soil apart from the microbes we inoculate or coat on the anode. This can determine on a deeper level (using functional gene analysis, rRNA gene sequencing, metagenome sequencing, etc.) how microorganisms interact with each other and utilize nutrients leading to power generation, which in turn would determine the health and nutrient status of the soil, which could be another step towards making of this incredible technology. Future studies could also utilize isotopic tracing techniques to track carbon dioxide production directly from electrogenic microorganisms in the soil to further enhance understanding of organic carbon oxidation and its response to the electrical signal.