



Article

16S rRNA Analysis of Electrogenic Bacterial Communities from Soil Microbial Fuel Cells

Ana Rumora, Liliana Hopkins, Kayla Yim, Melissa F. Baykus, Luisa Martinez and Luis Jimenez *

Biology and Horticulture Department, Bergen Community College, 400 Paramus Road, Paramus, NJ 07652, USA; arumora@me.bergen.edu (A.R.); llhopkins145705@me.bergen.edu (L.H.); kyim142129@me.bergen.edu (K.Y.); mbaykus@me.bergen.edu (M.F.B.); lmartinez144129@me.bergen.edu (L.M.)

* Correspondence: ljimenez@bergen.edu

Abstract: Electrogenic bacteria present in bioelectrical devices such as soil microbial fuel cells (SMFCs) are powered by the oxidation of organic and inorganic compounds due to microbial activity. Fourteen soils randomly selected from Bergen Community College or areas nearby, located in the state of New Jersey, USA, were used to screen for the presence of electrogenic bacteria. SMFCs were incubated at 35–37 °C. Of the 14 samples, 11 generated electricity and enriched electrogenic bacteria. The average optimal electricity production by the top 3 SMFCs was 152 microwatts. The highest electrical production was produced by SMFC-B1C and SMFC-B1B, with 162 and 152 microwatts, respectively. Microbial DNA was extracted from the biofilm grown on the anodes, followed by PCR analysis of the 16S rRNA V3–V4 region. Next-generation sequencing was performed to determine the structure and diversity of the electrogenic microbial community. The top 3 MFCs with the highest electricity production showed a bacterial community predominantly composed of bacteria belonging to the Bacillota and Pseudomonadota phyla with a significant presence of Euryarcheota members of methanogenic archaea. SMFC-B1C showed a more diverse electrogenic community, followed by SMFC-B1B and SMFC-B1. When analyzing the top 10 bacteria in the SMFCs, 67 percent belonged to the class Clostridia, indicating that anaerobic conditions were required to enrich electrogenic bacterial numbers and optimize electrical production. The ongoing optimization of SMFCs will provide better production of electricity and continuous enhancement of microbial activity to sustain longer operational times and higher levels of electrogenesis. The characterization of electrogenic microbial communities will provide valuable information to understand the contribution of different populations to the production of electricity in bioelectrical devices.

Keywords: soil microbial fuel cells; 16S rRNA analysis; electrogenic bacteria; Bacillota; Pseudomonadota; Euryarcheota; bioelectrical devices



Citation: Rumora, A.; Hopkins, L.; Yim, K.; Baykus, M.F.; Martinez, L.; Jimenez, L. 16S rRNA Analysis of Electrogenic Bacterial Communities from Soil Microbial Fuel Cells. *Appl. Microbiol.* **2024**, *4*, 918–933. <https://doi.org/10.3390/applmicrobiol4020062>

Academic Editor: Francisco Jesús Fernández Morales

Received: 20 May 2024

Revised: 1 June 2024

Accepted: 3 June 2024

Published: 5 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

There is no other environment on earth as complex as soil [1,2]. Microorganisms are crucial for soil fertility and plant productivity by the recycling of carbon, nitrogen, and phosphorus [3,4]. For instance, there are some biochemical reactions that are only performed by microorganisms such as nitrogen fixation and cellulose biodegradation. The decomposition of organic compounds in soils is based upon the oxidation of different substances such as carbohydrates, fats, and proteins supporting microbial metabolism to sustain growth and survival in a very dynamic and competitive matrix [5,6]. The microbial decomposition of organic matter under aerobic conditions results in the complete mineralization of organic materials to carbon dioxide and water. However, when anaerobic conditions are present, microbial activity is driven by processes such as anaerobic respiration, fermentation, and methanogenesis. Based upon 16S rRNA analyses of soils around the world, bacterial communities in soils are mostly composed of the phyla Acidobacteriota, Actinomycetota, Bacteroidota, and Pseudomonadota. Other phyla such as Bacillota, Chloroflexota, Planctomycetota, and Verrucomicrobiota occurred in lower numbers [5,7]. A large percentage

of soil bacteria did not match with any known bacterial phylum, nor they have been isolated and biochemically characterized in the laboratory [5–7]. Previous studies by our laboratory reported that bacteria belonging to the phyla Acidobacteriota, Actinomycetota, Chloroflexota, Planctomycetota, and Pseudomonadota were the dominant types in soils at Bergen Community College (BCC) [8]. The phyla Actinomycetota or Pseudomonadota were always the top two types in soils from different locations. The contributions of these bacterial phyla to the cycling of carbon and nitrogen in soils are well documented [6–8].

Energy generation around the world mostly relies on non-renewable sources such as fossil fuels [9]. Unfortunately, the use of fossil fuels has led to serious environmental contamination problems and global warming [10]. Because of the continuous growth of human populations and the quick industrialization of underdeveloped countries, the demand for fossil fuels has increased. This leads to more energy consumption and the fast depletion of non-renewable energy sources such as coal, gas, and oil. Evidently, there is a need to develop renewable energy sources to reduce the dependence on non-renewable sources and decrease environmental pollution and global warming. Technologies such as wind, hydro, and solar power provide clean and sustainable sources of energy [10]. One of the technologies that is currently being developed to provide sustainable and renewable energy via the microbial oxidation of organic compounds is microbial fuel cells (MFCs) [11].

In MFCs, the transfer of electrons to the anode electrodes is carried out by membrane cytochromes, pili, nanowires, and protein complexes [11]. Furthermore, some microorganisms carry out the transfer indirectly using environmental or self-produced electron mediators [12]. Once the electrons are transferred to the anode, they flow to the cathode, producing an electrical current. In the cathode, electron acceptors react with both electrons and protons, producing reduced chemical compounds such as water. There are several publications demonstrating electrical productions by soil microbial fuel cells (SMFCs) using different formats [13–16]. Aerobic and anaerobic soil microbial fuel cells (SMFCs) have been constructed with single and multiple chambers, optimizing electricity generation and microbial activity. Double-chamber cells consist of two chambers with the anode and cathode separated and joined by a cation-exchange membrane.

Single-chamber cells have the electrodes at opposite ends, with the anode buried in the soil and cathode on top. Different types of graphite felt electrodes have been tested as the anode in an air-cathode and membraneless SMFC [17]. The composition of the cathode has been also shown to be critical for optimizing electrical production [18]. For instance, graphite felt electrodes provided optimal electrogenesis with higher voltage and catalytic activity. One of the problems during the SMFC operation is the eventual depletion of the organic carbon present in the soil with the gradual reduction in and termination of electrogenesis. However, hybrid SMFCs with plants and soils have been shown to sustain long-term activity by continuously supplying photosynthetic products to the microbial community [19]. The depletion of organic matter in the SMFC can also be avoided by the addition of external carbon sources such as compost, cellulose, or glucose to replenish the materials lost during microbial oxidation [20,21].

Soil chemistry is one of the major factors affecting electrogenesis in SMFCs [14,22,23]. Certain types of organic compounds such as polyphenols may limit the production of electrical power by bacterial communities. SMFCs developed from forest and agricultural soils were shown to sustain an electrogenic bacterial community where agricultural SMFCs generated 17 times more electricity and 10 times higher respiration rates than forest SMFCs [14]. High-clay-content SMFCs demonstrated optimal electrogenesis and longer operational times by providing a less porous and permeable matrix, optimizing anaerobic conditions in the anode [22]. Furthermore, faster start-up times and electrogenic activity were reported with high organic matter. Start-up operational times are defined as the enrichment process when bacteria adapt to anaerobic conditions and develop an optimal biomass on the anode surface, resulting in electrical output through the oxidation of natural substances.

The ability to generate electricity from the oxidation of organic compounds in soils is widespread through different bacterial phyla. Bacteria belonging to the phyla Pseu-

domonadota, Bacillota, Actinomycetota, and Bacteroidota were shown to be electrogenic in MFCs [11]. As of now, *Geobacter sulfurreducens* has been shown to be the most electrogenic microorganisms [11,24]. Pure and mixed cultures of *G. sulfurreducens* have been used to generate electricity in SMFCs. *G. sulfurreducens* has been shown to reduce Fe^{+3} using a variety of organic compounds as electron donors. Bacteria from the phylum Bacillota such as *Bacillus* and *Clostridium* have been shown to be important members of the electrogenic bacterial community in SMFCs [8,11,14].

Previous studies in our laboratory demonstrated a sustainable electrical production lasting a maximum of 23 days with a power output of 73 microwatts [8]. The maximum power output by SMFCs was reported to be 80 microwatts, but it lasted only 12 days. 16S rRNA analysis showed that the most abundant bacteria in the anodes were members of Pseudomonadota, Bacillota, Actinomycetota, Chloroflexota, and Planctomycetota. SMFCs lacking large numbers of bacteria belonging to Bacillota did not generate electricity. However, only six soil samples from different locations were used to build the SMFCs. Additional soil locations were later analyzed, and a higher electrical output was reported to be 143 microwatts after 15 days at 37 °C [25]. The predominant bacteria in the electrogenic community were shown to belong to the phylum Bacillota of the class Clostridia. The major objectives of this study were to continue sampling additional soil locations around BCC to determine if they have the potential to generate higher electrical output and compare and analyze the electrogenic bacterial community using 16S rRNA analysis at different taxonomical levels.

2. Materials and Methods

2.1. Soil Sampling

Fourteen surface soils were collected from different locations at BCC and two from Saddle River County Park located in the city of Paramus, NJ, USA. Samples were aseptically taken, as previously described [8]. Each soil type was immediately used to make mud suspensions, as described below.

2.2. Microbial Fuel Cell Assembly

Mud suspensions in deionized water were constructed using soils. The mud suspensions were placed into the MudWatt cells [8]. SMFC-B1C and SMFC-B1D were built from the same soil location, but dry leaves were mixed with mud to make SMFC-B1C. Mud suspensions from the same soil location were split to build SMFC-B1A and SMC-B1B. SMFC-B1A was built by mixing the content of a blood agar plate with mud. A different soil sample was also used to develop SMFC-2A and SMFC-2B.

The electrodes were constructed from a circular carbon cloth. The cylindrical MFC was made of a plastic material with a plastic lid. For each SMFC, about 1 cm of mud was placed at the bottom of the plastic container before installing the anode; additional soil was added on top of the anode until the SMFC was 90% full. The cathode was placed on top of the mud. The hacker board was placed on the indentation of the lid (Figure 1). The board has a microchip that will take the power generated by the MFC and convert the voltage to 2.4 V in short bursts, which will power the light-emitting diode (LED). The anode and cathode were connected to the hacker board, and the lid was attached to seal the container. Finally, the LED and capacitor were connected to the hacker board, and the SMFCs were incubated at 35–37 °C.



Figure 1. Soil microbial fuel cells.

2.3. Electricity and Electrogenic Bacteria Measurements

The electrical power output and number of electrogenic bacteria were measured using an application (App) downloaded into an iPhone 14 as previously described [8]. The App was developed by Keego Technologies (<http://www.mudwatt.com>, accessed on 10 November 2023) and was freely available from the Apple App Store.

2.4. DNA Extraction and PCR Analysis of Bacterial 16S rRNA Genes in SMFC Samples

Approximately 250 milligrams of the biofilm grown on the anode from SMFC-B1, SMFC-B1B, and SMFC-B1C was aseptically added separately to BashingBead™ Lysis Buffer (Zymo Research, Irvine, CA, USA). Samples were mixed for 5 min at maximum speed. After mixing, centrifugation was performed at $10,000 \times g$ for 3 min. DNA was extracted as described by Rumora et al. [25].

After the extraction was completed, DNA concentration was determined by using the Qubit® dsDNA HS assay, as previously described by Jimenez et al. [26]. PCR amplification of extracted DNA was performed using primers 341f (CCTACGGGNGGCWGCAG) and 785r (GACTACHVGGGTATCTAATCC), which amplified the V3–V4 fragment of the 16S rRNA gene with a size of approximately 465 base pairs (bps). Reaction conditions were as follows: 95 °C for 5 min, followed by 25 cycles consisting of denaturation at 95 °C for 40 s, annealing at 55 °C for 2 min, and extension at 72 °C for 1 min. After the 25 cycles were completed, a final extension step at 72 °C for 7 min was added to the reaction [27]. Ready-To-Go (RTG) PCR beads (GE Healthcare, Buckinghamshire, UK) were used for each PCR reaction volume, as previously described [26]. Reaction mixtures were added to the T100™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) or Mastercycler thermal cycler (Eppendorf Scientific, Westbury, NY, USA). After PCR amplification, amplicon detection was analyzed through gel electrophoresis using the FlashGel system (Lonza Inc., Rockland, ME, USA) [26]. A FlashGel DNA Marker (Lonza Inc., Rockland, ME, USA) with fragment sizes ranging from 100 bp to 4 kilo bases (kbs) was used to determine the presence of the correct DNA fragments.

2.5. DNA Sequencing Analysis of 16S rRNA Genes in SMFC Samples

To determine the electrogenic bacterial community present in the anodes of the SMFCs, the V3–V4 region (465 bp) of the 16S rRNA gene was amplified using PCR, as previously described [25]. Next-generation sequencing was performed using an Illumina MiSeq protocol (Illumina, San Diego, CA, USA). Bioinformatic analysis was performed as described by Jimenez et al. [8].

2.6. Analysis of Cellulase Genes in SMFC Samples

DNA was extracted from the anode biofilm grown in SMFCs with the highest electrical output and electrogenic bacteria, as described above. The presence of cellulase genes belonging to glycoside hydrolase (GH) family 48 was determined using a PCR reaction, as previously described [28]. After the PCR reaction was completed, amplicon detection was analyzed via gel electrophoresis using the FlashGel system (Lonza Inc., Rockland, ME, USA) [26]. A FlashGel DNA Marker (Lonza Inc., Rockland, ME, USA) with fragment sizes ranging from 100 bps to 4 kilo bases (kbs) was used to determine the presence of the correct DNA fragments. The quantification of the PCR fragments was performed using the Qubit® dsDNA HS assay, as previously described by Jimenez et al. [26].

3. Results

3.1. Electricity Generation and Electrogenic Bacteria by SMFCs

Eleven out of fourteen SMFCs generated some electricity and enriched electrogenic bacteria (Tables 1 and 2). The average highest electrical generation between all fourteen SMFC was 63 microwatts. Start-up days for SMFCs ranged from 1 to 11 days. Only SMFC1 SMFC-AT, and SMFC-T1 did not show any electrical production or any electrogenic bacteria. The fastest generation of electricity was obtained after 1 day by SMFC-CT, SMFC-B1, and SMFC-T4. Of all three, SMFC-CT showed the highest output with 15 microwatts of electricity with 3.19×10^8 electrogenic bacteria. SMFC-B1 showed electrical output after 1 day. However, it was half the value detected by SMFC-CT. The highest electricity produced at the start-up day was produced by SMFC-B1D with 45 microwatts and 9.49×10^8 electrogenic bacteria.

Table 1. Electrical output by SMFCs.

Name	S	Microwatts	H	Microwatts
SMFC1	0	0	0	0
SMFC2	5	6	7	20
SMFC3	3	13	12	80
SMFC-B1	1	7	14	143
SMFC-B2A	11	2	26	50
SMFC-B2B	11	24	21	31
SMFC-B1A	8	1	16	24
SMFC-B1B	9	3	16	152
SMFC-CT	1	15	1	15
SMFC-AT	0	0	0	0
SMFC-B1C	6	7	14	161
SMFC-B1D	6	45	8	88
SMFC-T1	0	0	0	0
SMFC-T4	1	2	7	111

S = electricity start day; H = electricity highest day.

The time for maximum electrical output by SMFCs ranged from 1 to 21 days. The highest electrical output was detected by SMFC-B1C with 161 microwatts after 14 days. The number of electrogenic bacteria for SMFC-B1C was 3.37×10^9 . The second highest electrical output was shown by SMFC-B1B, with 152 microwatts and 3.17×10^9 electrogenic bacteria after 16 days.

SMFC-B1C showed the longest sustainable production of electricity with values over 100 microwatts (Figure 2). After 6 days, electrical output increased dramatically until reaching a maximum of 161 at 14 days, leveling off for one more day and decreasing down to 135 microwatts after 21 days. At 22 days, there was a slight increase to 140 microwatts, followed by a steady decrease to 115 microwatts after 29 days. It was the only SMFC with

a double-digit electrical output after 20 days. The sample was stopped after 29 days to determine the bacterial community.

Table 2. Number of electrogenic bacteria in SMFCs.

Name	EBS	EBH
SMFC1	0	0
SMFC2	1.37×10^8	4.33×10^8
SMFC3	2.71×10^8	1.67×10^9
SMFC-B1	1.51×10^8	2.99×10^9
SMFC-B2A	5.43×10^7	1.06×10^9
SMFC-B2B	5.08×10^8	6.53×10^8
SMFC-B1A	3.70×10^7	5.15×10^8
SMFC-B1B	8.14×10^7	3.17×10^9
SMFC-CT	3.19×10^8	3.19×10^8
SMFC-AT	0	0
SMFC-B1C	1.55×10^8	3.37×10^9
SMFC-B1D	9.49×10^8	1.84×10^9
SMFC-T1	0	0
SMFC-T4	4.71×10^7	2.31×10^9

EBS = electrogenic bacteria start day; EBH = electrogenic bacteria highest day.

SMFC-B1D built with soil from the same location but without leaves did not produce double-digit values, reaching an optimal electrical output of 88 microwatts after 8 days. After 8 days, electrical output by SMFC-B1D decreased to 20 microwatts (15 days), followed by a sudden spike of 52 microwatts at 16 days. No electrical output was detected after 21 days.

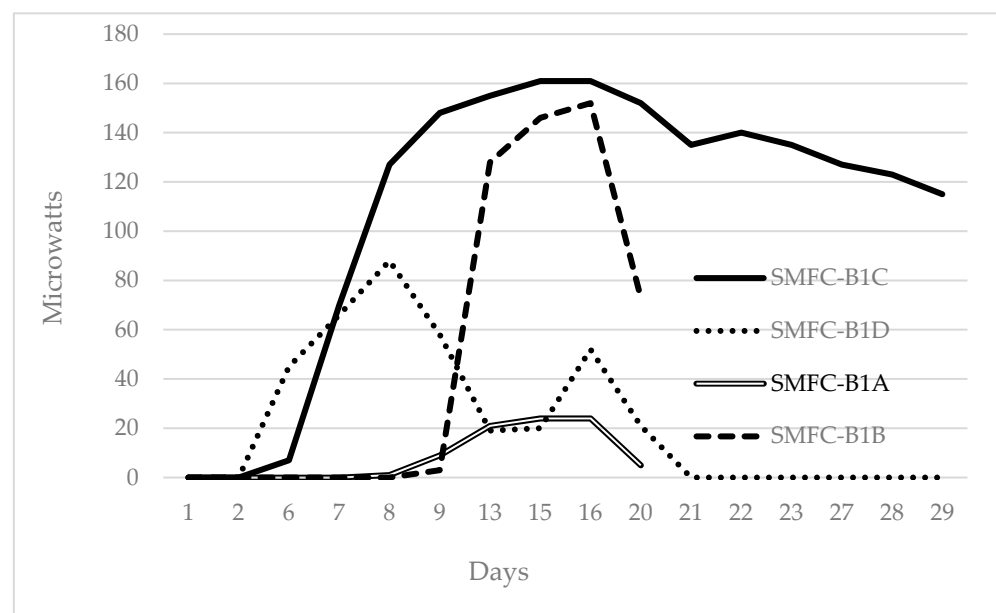


Figure 2. Electricity (microwatts) generation over days by SMFCs.

SMFC-B1A built from the same soil as SMFC-B1B but amended with blood agar never generated as much electricity. It reached a maximum of 24 microwatts after 16 days, going down to 5 microwatts after 20 days. However, SMFC-B1B reached an optimal electrical output of 152 microwatts after 16 days. The first significant increase was detected after

13 days with 128 microwatts. To determine the bacterial community in SMFC-B1B, the sample was stopped after 20 days when electricity was measured to be 73 microwatts.

Different results were detected with SMFC-2A and SMFC-2B, which were built from soils from the same location but with a different setup. With blood agar added to SMFC-2A, the electrical output reached a high of 50 microwatts compared to 31 microwatts generated by SMFC-2B (Table 1).

3.2. Diversity, Bacteria and Archaea Phyla in SMFCs with High Electrical Output

Alpha diversity is a measurement of the microbial diversity of each SMFC. Figure 3 shows the number of observed species in the samples. Observed species as operational taxonomic units (OTUs) were detected based upon minimum 97% identity criteria. Greater bacterial diversity was found in SMFC-B1C (746), followed by SMFC-B1B (637), and B1B (232).

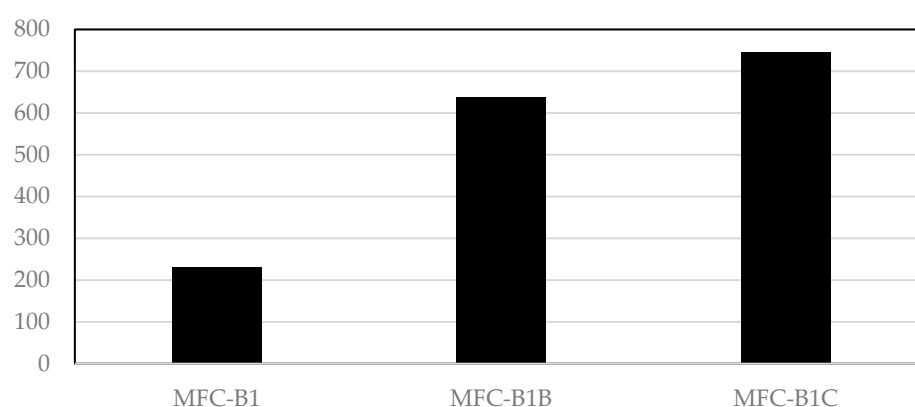


Figure 3. Alpha diversity of SMFCs. (number of species per sample).

The analysis of 16S rRNA genes from the anode of SMFC-B1 showed 13 bacteria phyla (Figure 4). Bacteria belonging to the phylum Bacillota accounted for 63.5% of the anode bacterial community, followed by unclassified bacteria (13.8%), Pseudomonadota (9.4%), Actinomycetota (2.9%), Acidobacteriota (1%), Chloroflexota (1%), Plantomycetota (1.6%), and Mycoplasmatota (0.7%). All other phyla showed values of less than 0.5%. Two archaea phyla were detected. These were Euryarcheota (4.1%) and Crenarcheota (0.5%).

The electrogenic community of SMFC-B1B showed 12 bacteria phyla (Figure 4). Bacteria belonging to the phylum Bacillota accounted for 79.6% of the anode bacterial community, followed by Pseudomonadota (6.7%), Mycoplasmatota (2.7%), Plantomycetota (1.5%), unclassified bacteria (1.1%), Actinomycetota (0.7%), Bacteroidota (0.6%), Acidobacteriota (0.5%), Chloroflexota (0.5%), Verrucomicrobiota (0.5%), and the rest with less than 0.4%. Two archaea phyla were detected. These were Euryarcheota (4%) and Crenarcheota (0.9%).

The electrogenic community of SMFC-B1C showed 17 bacteria phyla (Figure 4). Bacteria belonging to the phylum Bacillota accounted for 55.5% of the anode bacterial community, followed by Pseudomonadota (19.1%), Actinomycetota (2.2%), Bacteroidota (5.0%), Acidobacteriota (3.2%), Chloroflexota (2.4%), Planctomycetota (1.6%), Verrucomicrobiota (1.4%), and unclassified bacteria (1.2%), with all other phyla being below 0.7%. Three archaea phyla were detected. The Euryarcheota accounted for 4.7%, followed by Chrenarcheota (0.7%) and Thaumarcheota (0.1%).

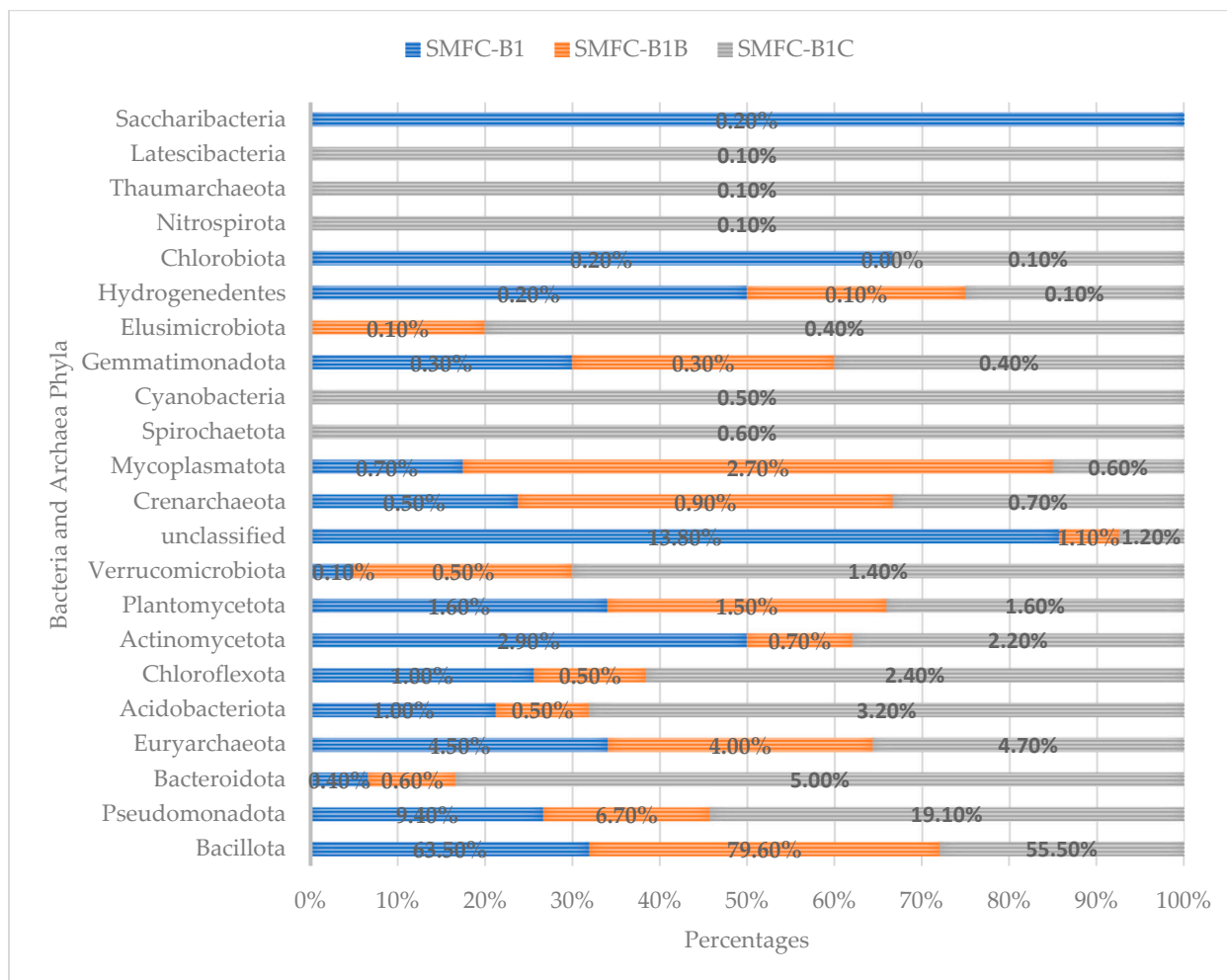


Figure 4. Relative abundances of bacteria and archaea phyla in SMFCs.

3.3. Distribution of Bacteria and Archaea in SMFC with High Electrical Output

When lower taxonomical levels were analyzed from the anode in SMFC-B1, 8 of the 10 most abundant bacteria belonged to the Bacillota phylum (Table 3). Only one other phylum, e.g., Pseudomonadota, was represented in the top ten bacteria. This sequence was identified as part of the genus *Magnetospirillum* with 1.7% of the sequences. The most abundant bacteria in SMFC-B1 were unidentified, e.g., 13.8%, since it did not match any bacteria at any taxonomical level. Three bacteria were identified as members of the family Ruminococcaceae. Only two Bacillota bacteria were identified at the genus level with *Caldicoprobacter* and *Mobilitalea*. Two bacteria were classified as members of the Bacillota without any possible match at a lower taxonomic level.

All top 10 sequences in SMFC-B1B were identified as members of the phylum Bacillota, with 6 out of the 10 belonging to the order Clostridiales (Table 4). Four of the six were members of the family Ruminococcaceae, and one was identified as Heliobacteriaceae. Only one sequence was identified at the genus level as *Gracilibacter*. There were four sequences that belonged to the Bacillota but did not match any taxonomical level below phylum. The most abundant bacteria in SMFC-B1 were a sequence related to the class Clostridia, order Clostridiales, family Ruminococcaceae, e.g., 6.7%.

Table 3. Dominant 16S rRNA sequences in the SMFC-B1 anode.

Phylum, Class	Order, Family	Genus	Percent
Unclassified	NM *	NM *	13.8
Bacillota, Clostridia	Clostridiales, Heliobacteriaceae	NM *	8.4
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	3.1
Bacillota, Clostridia	Clostridiales, Caldicoprobacteraceae	<i>Caldicoprobacter</i>	3.0
Bacillota,	NM *	NM *	2.6
Bacillota, Clostridia	Clostridiales, Lachnospiraceae	<i>Mobilitalea</i>	2.2
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	2.0
Pseudomonadota, Alphaproteobacteria	Rhodospirillales, Rhodospirillaceae	<i>Magnetospirillum</i>	1.7
Bacillota	NM *	NM *	1.7
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	1.6

* No match.

Table 4. Dominant 16S rRNA sequences in the SMFC-B1B anode.

Phylum, Class	Order, Family	Genus	Percent
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	6.7
Bacillota, Clostridia	Clostridiales, Gracilibacteraceae	<i>Gracilibacter</i>	6.3
Bacillota, Clostridia	Clostridiales, Heliobacteriaceae	NM *	5.0
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	3.8
Bacillota, NM *	NM *	NM *	2.5
Bacillota, NM *	NM *	NM *	2.2
Bacillota, NM *	NM *	NM *	2.1
Bacillota, NM *	NM *	NM *	2.1
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	2.0
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	2.0

* No match.

In SMFC-B1C, 8 of the top 10 bacteria belonged to the phylum Bacillota. The other two were members of the Pseudomonadota (Table 5). The number one sequence was identified to be a member the Pseudomonadota genus *Azospira*. Another Pseudomonadota was identified at the genus level as *Bdellovibrio*. The Bacillota order Clostridiales accounted for seven of the bacteria, with two identified as part of the genera *Mobilitalea* and *Gracilibacter*. Two bacteria were identified as members of the family Ruminococcaceae. One sequence was related to the class Clostridia, order Clostridiales but did not match any known bacteria at lower taxonomical levels. One other sequence was identified as belonging to the class Clostridia but did not match any known taxonomic level below.

Table 5. Dominant 16S rRNA sequences in the SMFC-B1C anode.

Phylum, Class	Order, Family	Genus	Percent
Pseudomonadota, Alphaproteobacteria	Rhodocyclales, Rhodocyclaceae	<i>Azospira</i>	6.8
Bacillota, Clostridia	Clostridiales, Lachnospiraceae	<i>Mobilitalea</i>	6.7
Bacillota, Clostridia	Clostridiales, Gracilibacteraceae	<i>Gracilibacter</i>	3.5
Bacillota, Clostridia	Clostridiales, NM *	NM *	3.5
Pseudomonadota, Deltaproteobacteria	Bdellovibrionales, Bdellovibrionaceae	<i>Bdellovibrio</i>	3.2
Bacillota, Clostridia	Clostridiales, Christensenellaceae	NM *	2.6
Bacillota, Clostridia	NM *	NM *	2.3
Bacillota, Clostridia	Clostridiales, NM *	NM *	2.3
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	2.0
Bacillota, Clostridia	Clostridiales, Ruminococcaceae	NM *	1.9

* No match.

Archaea were the third most abundant species in SMFC-B1B (4.0%) and SMFC-B1C (4.7%) and fourth in SMFC-B1 (4.5%) (Figure 4). Higher archaea diversity was detected in SMFC-B1C (Figure 5). Most archaea were members of the phylum euryarchaeota (80%). The genera represented were *Methanobacterium*, *Methanocella*, *Methanosarcina*, *Methanomassiliicoccus*, family Methanocellaceae, and order Thermoplasmatales (Figure 5). Four sequences were related to the phylum Chrenarcheota, but no matches were found with any known archaea at lower taxonomical levels. Higher frequencies of archaea were found in SMFC-B1C than in the other two cells. A sequence related to the family Methanocellaceae (1.6%) was the most abundant in SMFC-B1C but was also found in the other two cells. Different species of the genera *Methanosarcina* were widely distributed in all three cells. *Methanobacterium* was also found in all three cells. One of the Crenarcheota sequences, e.g., 1381, was detected in all SMFCs.

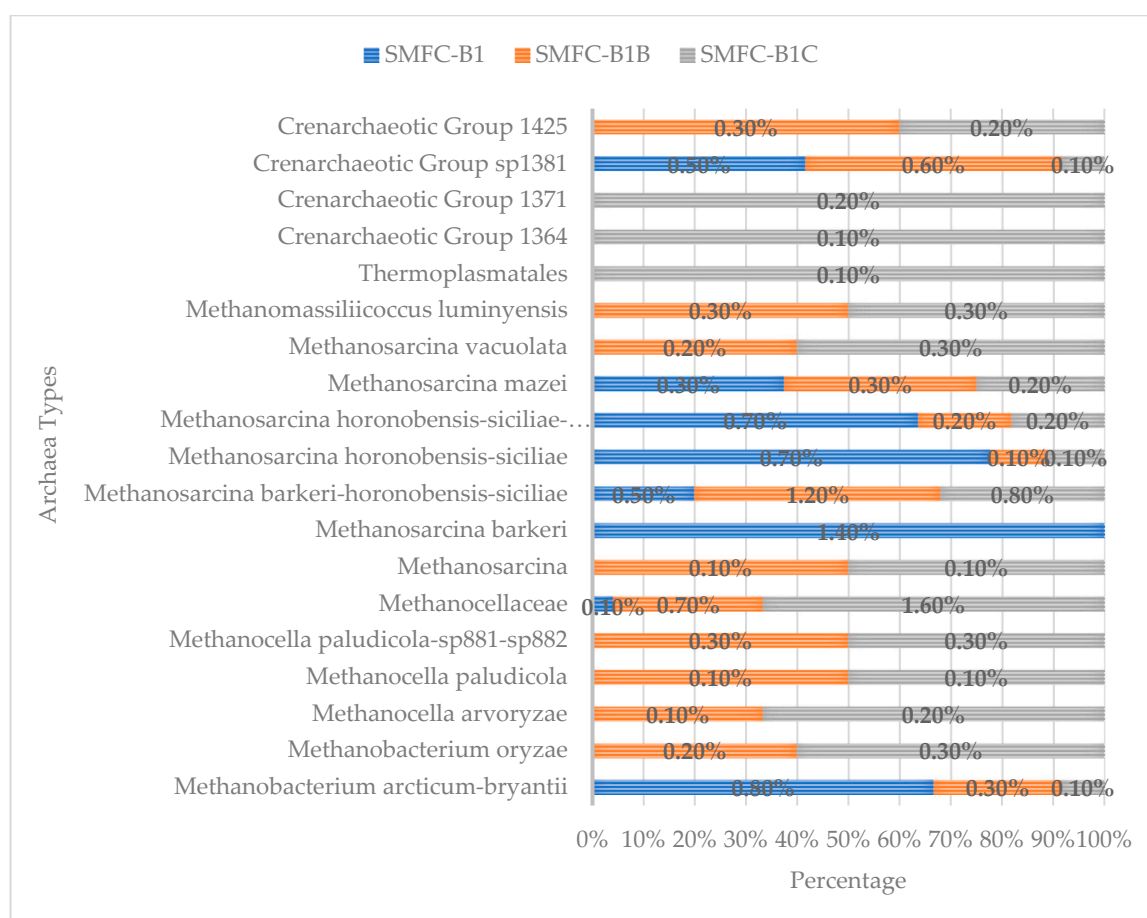


Figure 5. Relative abundance of archaea in SMFCs.

3.4. PCR Detection and Quantification of Cellulase Genes in SMFCs

The presence of cellulase genes from family GH48 was analyzed in all three SMFCs with the highest electrical production and electrogenic bacteria. Figure 6 shows the gel electrophoresis results for SMFC-B1, SMFC-B1B, and SMFC-B1C. The presence of a 430 bp fragment indicated a positive result. The intensity of the PCR band was higher in SMFC-B1C, which was the sample with leaves. The quantitation of the DNA fragment demonstrated that the concentration in SMFC-B1C was more than twice the amount detected in SMFCs with no cellulose (Table 6).

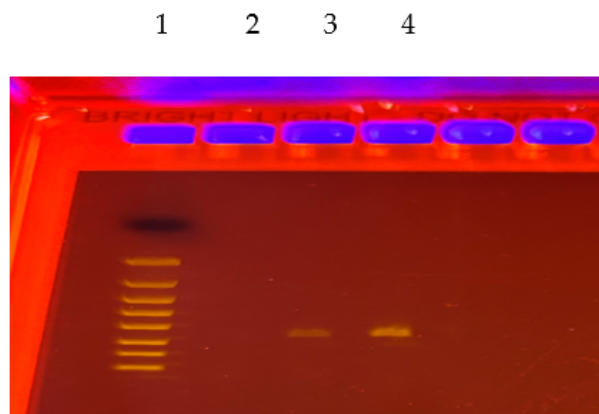


Figure 6. Gel electrophoresis analysis of GH48 genes in SMFCs. Lane 1: molecular-weight markers. Lane 2: SFMC-B1. Lane 3: SMFC-B1B. Lane 4: SMFC-B1C.

Table 6. The quantitation of GH48 genes in SMFCs.

Sample	DNA Concentration Micrograms/mL
SMFC-B1	1.59
SMFC-B1B	1.95
SMFC-B1C	4.38

4. Discussion

Fourteen SMFCs were constructed using soils from different locations at BCC. The oxidation of organic compounds in the SMFC's provided the electrons needed to generate an electrical power to sustain electrical production by most of the cells. However, some SMFCs did not generate any electrical power, and neither did we detect the presence of electrogenic bacteria. Previous studies by our laboratory demonstrated that the presence of a significant number of bacteria belonging to the phylum Bacillota was needed to generate significant electrical output due to the fact that the anaerobic conditions in the SMFC-enriched electrogenic bacteria did not use oxygen as the last electron acceptor for the degradation of organic matter [8,25]. Most soil locations tested were able to support the development of a viable and sustainable electrogenic bacterial community. In those samples, the oxidation of organic substances provided electrons to the anode to produce electricity, while protons migrate to the cathode through the soil. For each electron produced as an electrical current, a proton is also produced. Compared to other studies, two new samples, SMFC-B1B and SMFC-B1C, produced higher electrical power and electrogenic bacterial numbers. They produced 6% and 11% more electricity than SMFC-B1, which was previously reported to be the SMFC with the highest electrical output [25]. Of the three SMFCs, only SMFC-B1C exhibited a sustainable electrical production with double-digit values after 21 days of operational time. This is the first time that we recorded a SMFC with such a high and sustainable electrical production. Electrical production relied on the oxidation of native organic substances in the SMFCs. The addition of leaves (cellulose) to the mud to develop SMFC-B1C might have provided additional carbon sources to sustain a longer operational time and higher electrical output compared to any other SMFC. Furthermore, cellulase genes such as GH48 were found to be significantly increased by qualitative and quantitative analyses such as PCR amplification and the quantitation of the amplified fragment. GH48 genes were widely distributed in soils at BCC, with bacteria from the Pseudomonadota and Actinomycetota comprising most of the community [28]. Cellulases of the GH48 family are one of the most important GHs responsible for the biodegradation of crystalline cellulose to glucose. Most cellulose in the environment is found in plant material biomass based upon crystalline cellulose bound to hemicellulose and lignin. The presence of GH48 cellulases is essential to breakdown cellulose to glucose. The biodegradation of cellulose will provide additional organic compounds such as cellobiose and glucose for the electrogenic bacteria

to enhance electron transfer to the anode. A different carbon source, e.g., blood agar, added to the mud to construct SMFC-B1A and SMFC-2A did not produce a significant increase in sustainable electrical production.

Initial electrogenic activity in the SMFCs might have relied on the oxidation of the native organic substances in soil. But, those substances were significantly depleted, and it was not until other members of the bacterial community were capable of producing metabolic intermediates such as organic acids that electrogenic bacteria had additional organic substances to serve as electron generators to the anode. Soil organic carbon, mineralization rates, and bacterial community structure have been demonstrated to impact the performance of SMFCs [11,14,23]. Because of the closed system used in this study, the depletion of natural substrates led to the eventual reduction in electricity production and electrogenic bacterial numbers. This depletion was overcome in SMFC-B1C through the addition of cellulose, providing additional carbon sources for bacterial oxidation, resulting in higher electrical production and longer operational times. Acetate is a major intermediate to cellulose biodegradation and can be used for electrogenesis [21]. Future studies in our laboratory will develop new SMFCs with different concentrations of cellulose to optimize electrogenesis. We will also clone the GH48 genes to characterize the bacterial community responsible for cellulose biodegradation in the SMFCs.

Another strategy to overcome the depletion of carbon sources is the development of self-contained hybrid plant–soil MFCs (PSMFCs). These PSMFCs were shown to provide continuous addition of carbon substances via photosynthesis to compensate for the loss of organic matter and subsequent decay of electrical production [19]. Other studies demonstrated that electrode spacing and the addition of external organic carbon can also optimize electrical output and electrogenic bacterial numbers [22,29,30]. Substrate addition into the SMFC to compensate for the loss of organic material was observed to be better applied when electricity generation was decreasing instead of having a continuous system [22,29]. The quality of the available organic matter in soil affected the performance of SMFCs constructed from agricultural and forest soils [14]. SMFCs from agricultural soils showed 17 times more electricity than forest soils with respiration rates 10 times higher. Higher concentration of water-soluble polyphenols in forest soils compared to agricultural soils may have reduced the availability of organic matter to optimize microbial activity. Furthermore, in another study, soils with high clay contents and organic matter concentrations supported faster and higher electrogenic activity [22]. High clay content provided a stronger barrier to prevent the diffusion of oxygen into the anode, facilitating the development of anaerobic conditions. Soil characteristics related to electrical production by electrogenic bacteria will be further investigated by analyzing the chemical and physical compositions of the soils used to generate SMFC-B1B and SMFC-B1C.

To understand the compositions of the electrogenic bacteria communities enriched in SMFC-B1, SMFC-B1B, and SMFC-B1C, 16S rRNA analysis was performed on extracted microbial DNA from all anodes. Bacterial communities in soils at BCC were shown to be predominantly composed of the phyla Actinomycetota, Pseudomonadota, Chloroflexota, Acidobacteriota, and Planctomycetota [8]. These soils never showed a significant number of bacteria belonging to the phylum Bacillota. The highest percentage ever reported in soils at BCC was 1.23% [8]. We used single-chamber cells with the anode buried in the SMFC and the cathode on top. Samples were taken from the biofilm on the anode. The bacterial diversity in SMFC-B1C was the highest, followed by SMFC-B1B and SMFC-B1. We detected 20 bacteria and archaea phyla with the phylum Bacillota representing more than half of the bacteria in all cells. The only other bacterial phylum with double-digit numbers was the Pseudomonadota in SMFC-B1C. Bacteria belonging to the phylum Bacillota were the predominant classes, orders, families, and genera in SMFC-B1, SMFC-B1B, and SMFC-B1C. We found similar results with other SMFCs previously reported, where the increase in Bacillota bacteria compared to soils was required to generate electricity [8,25]. The 16S rRNA sequences with the highest frequencies in SMFC-B1 were unclassified bacteria, demonstrating the inability of current databases to identify some environmental

communities [31,32]. The predominant bacteria in SFMC-B1B were found to be members of the class Clostridia family Ruminococcaceae. Unfortunately, no matches were found at lower taxonomical levels. In SMFC-B1C, the most abundant bacteria were found to be members of the Pseudomonadota genus *Azospira*. The genus *Azospira* showed significant frequencies in the anodes of a wastewater MFC [33]. They can denitrify under anaerobic conditions with very high electrogenic activity. An uncultured *Azospira* sp. clone accounted for 65% of the community in MFCs with anodes built with granular graphite [34]. Different anode materials such as granulated activated carbon and carbon felt cube led to a bacterial community that consisted mostly of the genus *Geobacter*. The chemical composition of the electrodes influenced the formation of biofilms, affecting the adhesion and growth of electrogenic bacteria.

Overall, members of Bacillota, class Clostridia, family Ruminococcaceae were widely distributed in the three SFMCs. Bacteria from the class Clostridia were previously shown to be important contributors in SMFCs during electricity generation, either by directly generating electrons transferred to the anode via the oxidation of organic substances or by producing organic acids that were subsequently oxidized by other electrogenic bacteria within the anerobic environment of the SMFCs [8,14,15,21,24,25,30,35]. Furthermore, the isolation of electrogenic bacteria from SMFCs built with soils from seven sites in China demonstrated that 11 of the 15 bacteria were phylogenetically related to the genus *Clostridium* [35].

In addition to bacteria from the phylum Bacillota, two other Pseudomonadota genera were found to be very important members of the electrogenic bacterial community in SMFC-B1 and SMFC-B1C. They were *Magnetospirillum* sp. and *Bdellovibrio*. *Magnetospirillum* sp. was previously reported to be an important member of the electrogenic bacterial community in SMFC-B1 [25]. *Magnetospirillum* sp. can ingest iron and proteins inside the cells, interacting with it to produce magnetite that is located inside membranous structures called magnetosomes. Most electrogenic bacteria such as *Clostridium* sp. and *Magnetospirillum* sp. showed the ability to reduce Fe(III). However, some can also reduce nitrate. *Magnetospirillum* sp. was also shown to be predominantly present in the anodic biofilms developed by an anaerobic sludge-MFC [36]. Furthermore, when present along with *Clostridium* sp. these bacteria optimized electricity generation. SMFCs developed from Chinese soils showed a predominantly electrogenic bacterial community composed of bacteria belonging to the class Clostridia that were capable of reducing Fe(III) [35]. They reported that the family Clostridiaceae were the predominant electrogenic bacteria in soils. SMFCs constructed with German soils also showed an increase in the abundance of bacteria belonging to the Bacillota phylum when electricity production was the highest [30]. In that study, they found that the electrode materials were the most important factor for sustainable electrical generation. Modified stainless steel produced optimal electrical generation compared to carbon felt. They concluded that microbial diversity and soil chemistry were not as important during the optimization of electrical production.

The other bacterial genus belonging to the phylum Pseudomonadota found to be a predominant member of the electrogenic bacterial community in the anode of SMFC-B1C was *Bdellovibrio*. This is the first report of these bacteria to be members of electrogenic bacterial communities in SMFCs generating significant electrical outputs. *Bdellovibrio* species are predatory bacteria that are widely distributed in soil and aquatic systems that prey upon Gram-negative bacteria [37]. However, Gram-positive bacteria are not affected by them. The high frequency of these bacteria in some environments was positively correlated with higher microbial alpha-diversity in activate sludge. They might have been actively preyed on highly abundant bacterial species opening up specific niches for rare taxa in SMFC-B1C [38]. The lysis of Gram-negative bacteria by *Bdellovibrio* might have also increased the carbon and nitrogen concentration in the cell. Opposite results were reported with the protozoan grazing of bacteria in MFCs built from marine sediments where electricity generation and community diversity were reduced [39]. Protozoa were consuming *Geobacter sulfurreducens*, reducing the electrical output by up to 91%. However,

protozoa are not as selective as *Bdellovibrio* and prey on both Gram-negative and Gram-positive bacteria. Therefore, *Bdellovibrio* is a more selective predator of specific types of bacteria such as Gram-negative bacteria, allowing Gram-positive bacteria such as the Bacillota phylum to occupy different niches in the SMFCs.

Different taxonomical levels of archaea were detected as part of the predominant electrogenic community in all SMFCs. Most of them were methanogenic bacteria. Methanogenesis is a form of anaerobic respiration that produces methane as the final product. In hydrogenotrophic methanogenesis, hydrogen is used for the reduction of carbon dioxide to produce methane. However, in acetoclastic methanogenesis, hydrogen is used to reduce acetate and produce methane. Most of the archaea detected in the SMFCs are acetoclastic methanogens such as *Methanosarcina* and *Methanobacterium*, with only the genus *Methanocella* capable of hydrogenotrophic methanogenesis. Because of the anaerobic conditions detected in SMFCs, organic substances were decomposed by either hydrolysis, acidification, fermentation, or methanogenesis. Methanogenesis was previously shown to compete for electrons against electrogenesis in MFCs [21,40–42]. A high frequency of *Methanobacterium* spp. was frequently detected in SMFCs fed with cellulose [21]. However, a higher concentration of archaea in SMFCs may not be beneficial to electrogenesis. Furthermore, the final product of the anaerobic decomposition of organic substrates in soils is methane, but partial decomposition during fermentation or methane oxidation by methanotrophic bacteria produced compounds such as formate and acetate that are used by either methanogens to produce methane or by electrogenic bacteria from the class Clostridia, which will oxidize these compounds and transfer the electrons to the anode [21,41,43]. Furthermore, reverse methanogenesis was previously demonstrated with different genera of archaea such as *Methanobacterium*, *Methanosarcina*, and *Methanospirillum* [43,44]. This resulted in the production of acetate, which can be used by bacteria of the class Clostridia to oxidize these compounds for electrogenesis. Anaerobic methanotrophic archaea were cultivated in bioelectrical systems with methane-dependent currents related to the enrichment of *Methanoperedens* on the anode [45]. Extracellular electron transfer (EET) to an electrode was demonstrated through the use of multiheme c-type cytochromes. Our results showed that *Methanoperedens* was capable of the electrogenic anaerobic oxidation of methane with or without the collaboration of other electrogenic bacteria such as *Geobacter* [45].

5. Conclusions

Additional soils from different locations at BCC enriched electrogenic bacteria and archaea communities in SMFC-B1B and SMFC-B1C, which led to higher electricity generation and longer operational times compared to previous reports by our laboratory. Bacterial and archaea communities were predominantly composed of the class Clostridia and different genera of methanogenic archaea such as *Methanobacterium*, *Methanosarcina*, and *Methanocella*. However, bacteria from the phylum Pseudomonadota were needed to optimize the performances of the cells in two of the three cells. The addition of cellulose prolonged the operational times and enhanced the electrical output to levels never previously reported. Future studies will sample additional soil locations at BCC to ascertain their potential to develop SMFCs with higher electrogenic potentials and longer operational times.

Author Contributions: L.J. conceptualized, directed, and supervised the research. L.J. wrote the original draft. L.J., A.R., L.H. and K.Y. designed the experiments. L.J., A.R., L.H., K.Y., M.F.B. and L.M. performed experiments and analyzed results. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Falkowski, P.G.; Fenchel, T.; Delong, E.F. The microbial engines that drive Earth's biogeochemical cycles. *Science* **2008**, *320*, 1034–1038. [[CrossRef](#)] [[PubMed](#)]
2. Fierer, N.; Jackson, R.B. The diversity and biogeography of soil bacteria communities. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 626–631. [[CrossRef](#)] [[PubMed](#)]
3. Ray, A.E.; Zhang, E.; Terauds, A.; Ji, M.; Kong, W.; Ferrari, B.C. Soil microbiomes with the genetic capacity for atmospheric chemosynthesis are widespread across the poles and are associated with moisture, carbon, and nitrogen limitation. *Front. Microbiol.* **2020**, *11*, 1936. [[CrossRef](#)] [[PubMed](#)]
4. Bickel, S.; Or, D. Soil bacterial diversity mediated by microscale aqueous-phase processes across biomes. *Nat. Commun.* **2020**, *11*, 116. [[CrossRef](#)] [[PubMed](#)]
5. Delgado-Baquerizo, M.; Oliverio, A.M.; Brewer, T.E.; Benavent-Gonzalez, A.; Eldridge, D.J.; Bardgett, R.D.; Maestre, F.T.; Singh, B.K.; Fierer, N. A global atlas of the dominant bacteria found in soil. *Science* **2018**, *359*, 320–325. [[CrossRef](#)] [[PubMed](#)]
6. Zhou, Z.; Wang, C.; Luo, Y. Meta-analysis of the impacts of global change factors on soil microbial diversity and functionality. *Nat. Commun.* **2020**, *11*, 3072. [[CrossRef](#)] [[PubMed](#)]
7. Llado, S.; Lopez-Mondejar, R.; Baldrian, P. Forest soil bacteria: Diversity, involvement in ecosystem processes, and response to global change. *Microbiol. Mol. Biol. Rev.* **2017**, *81*, e00063-16. [[CrossRef](#)] [[PubMed](#)]
8. Jimenez, L.; Kulko, M.; Kim, R.; Jashari, T.; Choe, T. 16S rRNA analysis of electrogenic bacterial communities in microbial fuel cells developed from temperate soils. *BIOS* **2020**, *91*, 9–20. [[CrossRef](#)]
9. Ruijven, B.J.; De Cian, E.; Wing, I.S. Amplification of future energy demand growth due to climate change. *Nat. Commun.* **2019**, *10*, 2762. [[CrossRef](#)]
10. Al-Ghussain, L. Global warming: Review on driving forces and mitigation. *Environ. Prog. Sustain. Energy* **2019**, *38*, 13–21.
11. Wang, J.; Ren, K.; Zhu, Y.; Huang, J.; Liu, S. A review of recent advances in microbial fuel cells: Preparation, operation, and application. *BioTech* **2022**, *11*, 44. [[CrossRef](#)]
12. Rabaey, K.; Boon, N.; Siciliano, S.D.; Verhaege, M.; Verstraete, W. Biofuel cells select for microbial consortia that self-mediate electron transfer. *Appl. Environ. Microbiol.* **2004**, *70*, 5373–5382. [[CrossRef](#)] [[PubMed](#)]
13. Bond, D.R.; Holmes, D.E.; Tender, L.M.; Lovley, D.R. Electrode-reducing microorganisms that harvest energy from marine sediments. *Science* **2002**, *295*, 483–485. [[CrossRef](#)]
14. Dunaj, S.J.; Vallino, J.J.; Hines, M.E.; Gay, M.; Kobyljanec, C.; Rooney-Varga, J.N. Relationships between soil organic matter, nutrients, bacterial community structure, and the performance of microbial fuel cells. *Environ. Sci. Technol.* **2012**, *46*, 1914–1922. [[CrossRef](#)] [[PubMed](#)]
15. Wang, N.; Chen, Z.; Li, H.B.; Su, J.Q.; Zhao, F.; Zhu, Y.G. Bacterial community composition at anodes of microbial fuel cells for paddy soils: The effects of soil properties. *J. Soils Sediments* **2015**, *15*, 926–936. [[CrossRef](#)]
16. Zhao, J.; Li, X.; Ren, Y.; Wang, X.; Jian, C. Electricity generation from Taihu Lake cyanobacteria by sediment microbial fuel cells. *J. Chem. Technol. Biotechnol.* **2012**, *87*, 1567–1573. [[CrossRef](#)]
17. Kaur Dhillon, S.; Dziegielowski, J.; Patit Paban, P.; Di Lorenzo, M. Functionalized graphite felt anodes for enhanced power generation in membrane-less soil microbial fuel cells. *R. Soc. Chem. Sustain.* **2023**, *1*, 310–325. [[CrossRef](#)]
18. Nandy, A.; Farkas, D.; Pepio-Tarrega, B.; Martinez-Crespiera, S.; Borrás, E.; Avignone-Rossa, C.; Di Lorenzo, C. Influence of carbon-based cathodes on biofilm composition and electrochemical performance in soil microbial fuel cells. *Environ. Sci. Ecotechnol.* **2023**, *16*, 100276. [[CrossRef](#)] [[PubMed](#)]
19. Uria-Molto, N.; Costa, R.D.; Nunziata, C.; Santiago, S.; Guirado, G.; Munoz-Berbel, X.; Kowalski, L. Self-contained and integral microbial fuel cells as portable and sustainable energy sources for low-power field devices. *Electr. J. Biotechnol.* **2022**, *57*, 44–51. [[CrossRef](#)]
20. Greenman, J.; Thorn, R.; Willey, N.; Ieropoulos, I. Energy harvesting from plants using hybrid microbial fuel cells; potential applications and future exploitation. *Front. Bioeng. Biotechnol.* **2024**, *12*, 1276176. [[CrossRef](#)]
21. Ishii, S.; Shimoyama, T.; Hotta, Y.; Watanabe, K. Characterization of a filamentous biofilm community established in a cellulose-fed microbial cell. *BMC Microbiol.* **2008**, *8*, 6. [[CrossRef](#)] [[PubMed](#)]
22. Dziegielowski, J.; Mascia, M.; Metcalfe, B.; Di Lorenzo, M. Voltage evolution and electrochemical behavior of soil microbial fuel cells operated in different quality soils. *Sustain. Energy Technol. Assess.* **2023**, *56*, 103071.
23. Hodgson, D.M.; Smith, A.; Dahale, S.; Stratford, J.P.; Li, J.V.; Gruning, A.; Bushell, M.E.; Marchesi, J.R.; Avignone-Rossa, C. Segregation of the anodic microbial communities in a microbial fuel cell cascade. *Frontiers Microbiol.* **2016**, *7*, 699. [[CrossRef](#)] [[PubMed](#)]
24. Cao, Y.; Mu, H.; Liu, W.; Zhang, R.; Guo, J.; Xian, M.; Liu, H. Electrigens in the anode of microbial fuel cells: Pure cultures versus mixed communities. *Microb. Cell Factories* **2019**, *18*, 39. [[CrossRef](#)]
25. Rumora, A.; Hopkins, L.; Yim, K.; Baykus, M.F.; Martinez, L.; Jimenez, L. Detection and characterization of electrogenic bacteria from soils. *BioTech* **2023**, *12*, 65. [[CrossRef](#)] [[PubMed](#)]

26. Jimenez, L.; Jashari, T.; Vasquez, J.; Zapata, S.; Bochis, J.; Kulko, M.; Ellman, V.; Gardner, M.; Choe, T. Real-Time PCR detection of *Burkholderia cepacia* in pharmaceutical products contaminated with low levels of bacterial contamination. *PDA J. Pharm. Sci. Technol.* **2018**, *72*, 73–80. [[CrossRef](#)] [[PubMed](#)]
27. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glockner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2012**, *41*, e1. [[CrossRef](#)]
28. Jimenez, L.; Vazquez, S.; Turku, A.; Pincus, L. PCR detection, cloning, and genetic identification of microbial cellulases in soils. *BIOS* **2022**, *93*, 31–48. [[CrossRef](#)]
29. Simeon, M.I.; Freitag, R. Influence of electrode spacing and fed-batch operation on the maximum performance trend of a soil microbial fuel cell. *Int. J. Hydrogen Energy* **2022**, *47*, 12304–12316. [[CrossRef](#)]
30. Simeon, M.I.; Weig, A.; Freitag, R. Optimization of soil microbial fuel cell for sustainable bio-electricity production: Combined effects of electrode material, electrode spacing, and substrate feeding frequency on power generation and microbial community diversity. *Biotechnol. Biofuels Bioprod.* **2022**, *15*, 124. [[CrossRef](#)]
31. Wang, Y.; Gallagher, L.A.; Andrade, P.A.; Liu, A.; Humphreys, I.R.; Tukarslan, S.; Cutler, K.J.; Arrieta-Ortiz, M.L.; Li, Y.; Radey, M.C.; et al. Genetic manipulation of *Patescibacteria* provides mechanistic insights into microbial dark matter and the epibiotic lifestyle. *Cell* **2023**, *186*, 1–15. [[CrossRef](#)] [[PubMed](#)]
32. Francioli, D.; Lentendu, G.; Lewin, S.; Kolb, S. DNA metabarcoding for the characterization of terrestrial microbiota-pitfalls and solutions. *Microorganisms* **2021**, *9*, 361. [[CrossRef](#)] [[PubMed](#)]
33. Yang, Z.; Li, H.; Li, N.; Fahad Sardar, M.; Song, T.; Zhu, H.; Xing, X.; Zhu, C. Dynamics of a bacterial community in the anode and cathode of microbial fuel cells under sulfadiazine pressure. *Int. J. Environ. Res. Public Health* **2022**, *19*, 6253. [[CrossRef](#)] [[PubMed](#)]
34. Sun, Y.; Wei, J.; Liang, P.; Huang, X. Electricity generation and microbial community changes in microbial fuel cells packed with different anodic materials. *Bioresour. Technol.* **2011**, *102*, 10886–10891. [[CrossRef](#)] [[PubMed](#)]
35. Jiang, Y.B.; Zhong, W.H.; Han, C.; Deng, H. Characterization of electricity generated by soil in microbial fuel cells and the isolation of soil source exoelectrogenic bacteria. *Front. Microbiol.* **2016**, *7*, 1776. [[CrossRef](#)] [[PubMed](#)]
36. Xiao Min, L.; Cheng, K.Y.; Selvam, A.; Wong, J.W.C. Bioelectricity production from acidic food waste leachate using microbial fuel cells: Effect of inocula. *Process Biochem.* **2013**, *48*, 283–288.
37. Feng, S.; Tan, C.H.; Constancias, F.; Kohli, G.S.; Cohen, Y.; Rice, S.A. Predation by *Bdellovibrio bacteriovorus* significantly reduced viability and alters the microbial community composition of activated sludge flocs and granules. *FEMS Microbiol. Ecol.* **2017**, *93*, 1093. [[CrossRef](#)] [[PubMed](#)]
38. Johnke, J.; Fraune, S.; Bosch, T.C.G.; Hentschel, U.; Schulenburg, H. *Bdellovibrio* and other like organisms are predictors of microbiome diversity in distinct host groups. *Microb. Ecol.* **2020**, *79*, 252–257. [[CrossRef](#)] [[PubMed](#)]
39. Holmes, D.E.; Nevin, K.P.; Snoeyenbos-West, O.L.; Woodward, T.L.; Strickland, J.N.; Lovely, D.R. Protozoan grazing reduced the current output of microbial cells. *Bioresour. Technol.* **2015**, *193*, 8–14. [[CrossRef](#)]
40. Yamasaki, R.; Maeda, T.; Wood, T.K. Electron carriers increase electricity production in methane microbial fuel cells that reverse methanogenesis. *Biotechnol. Biofuels* **2018**, *11*, 211. [[CrossRef](#)]
41. MacAnulty, M.J.; Poosarla, V.G.; Kim, K.Y.; Jasso-Chavez, R.; Logan, B.E.; Wood, T.K. Electricity from methane by reversing methanogenesis. *Nat. Commun.* **2017**, *8*, 15419. [[CrossRef](#)] [[PubMed](#)]
42. Barbato, R.A.; Foley, K.L.; Toro-Zapata, J.A.; Jines, R.M.; Reynolds, C.M. The power of soil microbes: Sustained power production in terrestrial microbial fuel cells under various temperature regimes. *Appl. Soil Ecol.* **2017**, *109*, 14–22. [[CrossRef](#)]
43. Guerrero-Cruz, S.; Vaksmaa, A.; Horn, M.A.; Niemann, H.; Pijuan, M.; Ho, A. Methanotrophs: Discoveries, environmental relevance, and a perspective on current and future applications. *Front. Microbiol.* **2021**, *12*, 678057. [[CrossRef](#)] [[PubMed](#)]
44. Gao, Y.; Lee, J.; Neufeld, J.D.; Park, J.; Rittmann, B.E.; Lee, H.S. Anaerobic oxidation of methane coupled with extracellular electron transfer to electrodes. *Sci. Rep.* **2017**, *7*, 5099. [[CrossRef](#)]
45. Ouboter, H.T.; Mesman, R.; Sleutels, T.; Postma, J.; Wissink, M.; Jetten, M.S.M.; Ter Heijne, A.; Berber, R.; Welte, C.U. Mechanisms of extracellular electron transfer in anaerobic methanotrophic archaea. *Nat. Commun.* **2024**, *15*, 1477. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.