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Comparative Genomic and Molecular Characteristics of Bacteria in Frostburg, Maryland Soil

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Abstract: The microorganisms within the soil hold an essential role in the global cycling of elements and nutrient content available to support ecosystems. The biological fertility of soil is a highly complex and dynamic component of soil productivity and is the least well-understood component of soil functions. The main objective of this research was to identify bacterial communities in Frostburg soil and conduct further studies to understand their benefits for the ecosystems they live in. Twenty soil samples were collected from mature forests, grass lawns, forest swamps, meadows, and shrub swamps. The soil samples were homogenized, and two replicates were transported to the microbiology laboratory at Frostburg State University, Maryland for identification. The element composition of soil samples was detected by using the XRF and nitrate levels were measured with a nitrate ion selective electrode. DNA extraction from bacteria was performed using earth microbiome 16S Illumina sequencing protocol. The purity of the DNA was measured using nanodrop and gel electrophoresis. The average percentage of Fe in all the samples is over 57%, and Cr, K, S, and Ca are the other elements most abundant in the soil samples. Whereas nitrate levels in mature forest, grass lawn, forest swamp, meadow, and shrub swamp were 87, 121, 48, 127, and 88ppm, respectively. Nanodrop reading of A260/A280 were in the range of 1.85-1.87, and gel electrophoresis results had only one band per sample around 350bp. Bacteria were identified using the NCBI-BLAST tool and Metagenomics. The alpha and beta diversities were conducted using Qiime 2 with $p<0.05$. According to the BLAST analysis, many more uncultured bacteria were detected in the soil samples collected from the forest and grass lawn than in wetlands. The most common bacterial genera found in all samples were *Shingomonas*, *Acidobacteria*, *Chloroflexi*, and *Bradyrhizobium*, which are benefited in many ways including bioremediation, biodegradation, and nitrogen fixation. The Shannon-Wiener Index curve plot indicated sufficient sequencing depth to characterize microbial diversity. The comparison of genomics and molecule characteristics of bacteria in Frostburg, Maryland soil provided baseline data for further studies in relation to understanding the benefits of microbial growth, including the growth of plants.

Keywords: Soil Bacteria, 16S Gene, Illumina Gene Sequencing

1. Introduction

Microbial communities are essential for many important soil properties, including water quality, nutrient availability, nitrogen fixation, decomposition, and oxygen-carbon dioxide balance. Soil microorganisms are directly involved in nutrient cycles, such as carbon, nitrogen, and phosphorus cycles [1].

Soil organisms have been studied for many years, but the diversity remains unclear due to their abundance [2]. Soil bacteria interact with plant roots and mycorrhizal fungi as commensals and respond to the effects of global change, such as climate warming and nitrogen fixation [1].

Microorganisms in the soil work as an index of soil

fertility for plants [3]. Especially they manipulate the hormonal signals of plants to increase the growth, outcompeting pathogens, and increase the bioavailability of soil nutrients [4-6]. They convert organic molecules to bioavailable, preferred nutrient forms for plants, such as nitrate, phosphate, and ammonium [6]. Biodiversity can be disturbed by most human activities, such as agriculture, urbanization, and the emission of pollutants. Physical disturbances of soil including tillage distract soil microbial balance by decreasing carbon levels through the oxidation of carbon into carbon dioxide and release to the atmosphere [7]. Promoting microorganisms in the soil helps increase active carbon, which will be benefited by other organisms, including plants [8]. More studies are needed to understand soil microbial diversity and its ecological contributions because it was shown that the diversity of soil microbial communities is higher than previously estimated based on identification tests such as genetic analysis [9].

Laboratory media can be effectively used to identify bacteria isolated from clinical samples with staining, cultural, and biochemical techniques. Yet, due to the magnitude of microbial diversity, molecular methods have broadly been used in identifying culturable and non-culturable microorganisms in soil [10]. Many of the soil bacteria have not been cultivated or have no similar genes for the preexisting gene databases, and most soil microbial taxa and metabolic capabilities remain unknown [2]. Bacteria rDNA consists of 23S, 16S, and 5S regions, where 16S has been used primarily on gene analysis [11].

Identifying dominant microbial communities in various ecosystems in the Frostburg area helps with understanding its terrestrial microbial diversity and conducting further studies to understand the benefits of the microorganisms to the ecosystems they live in.

2. Materials and Method

2.1. Sampling Technique

All the sampling sites were at least 60 ft away from the roads, in order to collect fair representation of soil samples. Plant residues were removed from the surface at each sample site, and evenly spaced soil samples were collected using the grid sampling technique [12]. A soil core sampler probe (Forestry suppliers, Jackson, MS) was inserted vertically and collected soil samples within 10 cm depth, The samples were transferred into labeled sample bags with the name of the sampling site and the date.

2.2. Sample Collection

Twenty homogenized soil samples from each site were collected from the mature forest, grass lawn, forest swamp, and shrub swamp located in Frostburg, Maryland and transported on ice to the microbiology laboratory at Frostburg State University, Maryland to extract DNA from bacteria. The transported soil samples were stored at 4°C until the DNA was extracted.

2.3. Soil Composition Test

The X-ray fluorescence spectrometer analyzer (Niton-XRF, Thermo Scientific, Southgate, MI) was used to measure the soil composition of samples. The sample cup was filled with soil and covered with the Myler sheet before running the program with the XRF.

2.4. Nitrate Test

The nitrate ion selective electrode method was used [13] to measure the nitrate in the soil samples. The soil samples were dried with forced air flow overnight and grinned in a motor and pestle to homogenize sample. For the test, 20g of soil was funneled into an Erlenmeyer flask, and 50 ml extracting solution (20ml Ionic Strength (2M ammonium sulfate) and 10ml preservative solution (1M boric acid) were added. The flask shook for 15 minutes at 200 oscillations per minute. Next, the sample was vacuum filtered, and added 2 mL of ISA which was added to 100 mL of distilled water. The calibration curve was made after performing the slope test with the assembled nitrate electrodes and measured nitrate levels of the samples.

2.5. DNA Extraction and Purification

The DNA extraction and purification were conducted according to the earth microbiome project protocols [14]. The DNA extraction was performed using the DNeasy PowerSoil Pro Kit (Qiagen, Valencia, CA). A 250g homogenized soil sample and 800µl of CD 1 solution were added to the PowerBead Pro tube and vortexed for 10 min. Then, centrifuge the PowerBead Pro tube at 14,000 x g for 1min at room temperature. Then, 600µl of the supernatant of the mixture was transferred to a Microcentrifuge tube and added 200µl of the CD2. Next, vortexed and centrifuged the tube with the same centrifuging specification. The other reagents, CD3-CD6, were also added according to the manufacturer's protocol, and DNA was extracted from the soil.

2.6. DNA Amplification

Polymerase Chain reaction (PCR, MJ Research, St Bruno, Canada) was performed using nucleotide oligos with the 806R reverse primer and 515S barcode primer for bacteria.

2.7. The Reaction Mixture for the PCR

The reaction mixture was prepared according to the earth microbiome protocol with PCR master mix, forward primer, reverse primer, template DNA, and PCR grade water to get a total of 25µl of the mixture.

2.8. PCR Reaction Cycles

Denaturation, annealing, and extension were performed at 94, 50, and 72°C for 45, 60, and 90 seconds and amplification underwent for 35 cycles. The amplicons were stored at 4°C until cleaning up the amplicons with the PCR clean-up kit (Qiagen, Valencia, CA).

2.9. The DNA Concentration and Purity

The amplicon concentration and purity were measured using the nanodrop spectrophotometer (Thermo scientific, MA) and the gel electrophoresis.

2.10. DNA Quality Checks with the Nanodrop

When using the nanodrop spectrometer, the sample was subjected to ultraviolet light for 5 seconds and got the readings. The dsDNA (factor 50) assay type from the home screen was selected and established a blank using 2µl of distilled water. A 2 µl of amplified and cleaned DNA was added onto the pedestal of the Nanodrop and the DNA concentration in ng/µl and A260/A280 ratio was recorded. For best sequencing results, at least 500ng and 1.8-2.0 of the A260/280 were required.

2.11. DNA Quality Checks with DNA Fingerprinting

Gel electrophoresis was performed with 1% agarose under a voltage of 100V for 40min to confirm the fragment length of the V4 region of the bacterial 16S. The expected length of the fragment of bacteria was around 300-350bp.

2.12. Next-Generation Sequencing

After the quality control checks, all the amplicons from the soil samples were shipped overnight to Azenta Technologies for DNA sequencing with the Illumina MiSeq sequencing platform [9].

2.13. Data Analysis

The data analysis was performed using Qiime 2 [15] and the NICB-BLAST [16] database. Significance for the analysis was defined as $p<0.05$.

3. Results

The soil samples were collected from mature forest, grass lawn, forest swamp, meadow, and shrub swamp located in Frostburg, Maryland. The soil element composition and the nitrate test gave more insight into the environment where the bacteria DNA was extracted.

Two replicates of the homogenized soil collected from each site were used for DNA extraction. The quality of the extracted DNA was checked using the nanodrop spectrometer and gel electrophoresis. Metagenomics and the NCBI-BLAST tool were used to analyze DNA after DNA next generation sequencing.

To measure soil composition, a sampling cup was filled with homogenized soil and measured the element percentage using the XRF. Ferrous (Fe) was the highest percentage of the element in all the sites (Table 1) with 58.42, 67.44, 42.56, 66.19, and 54.1 in mature forest, grass lawn, forest swamp, meadow, shrub swamp, respectively. The Chromium (Cr) was the second highest element of all the sites except the grass lawn. The Chromium percentages were 9.45, 6.66, 13.43, 7.95, and 11.32; Potassium percentages were 8.78, 7.52, 11.44, 4.46, and 9.51; Sulfur (S) percentages were 3.95, 3.93, 9.8, 4.1, and 6.4. and Calcium (Cd) percentages were 3.44, 2.23, 4.81, 2.96, and 4.3 in mature forest, grass lawn, forest swamp, meadow, and shrub swamp, respectively (Table 1).

Table 1. *Element composition of soil samples.*

Element	% in MF	%in GL	% in FS	% in M	% SS
Fe	58.42±1	67.44±1	42.56±1.	66.19±1	54.1±1.5
Cr	9.45±0.3	6.66±0.3	13.43±0.5	7.95±0.4	11.32±0.5
K	8.78±1.4	7.52±1.2	11.44±2.1	4.46±1.5	9.51±2.1
S	3.95±2.1	3.93±1.8	9.8±3.4	4.1±2.4	6.44±3.2
Ca	3.44±0.6	2.23±0.4	4.81±0.9	2.96±0.6	4.3±0.8
Cu	3.15±0.3	2.29±0.2	4.55±0.4	2.37±0.2	3.15±0.3
W	2.66±0.4	1.53±0.3	3.3±0.6	1.74±0.4	2.33±0.5
Zn	2.28±0.2	1.85±0.1	3.65±0.3	2.58±1.2	2.77±0.2
Zr	2.26±0.1	2.28±0.1	2.76±0.1	2.29±0.1	1.36±0.1
Co	2.16±0.5	1.7±0.4	<LOD	2.04±0.4	1.09±0.5
V	1.36±0.2	0.98±0.2	1.78±0.3	1.16±0.2	1.41±0.3

*F-Forest; GL-Grass lawn; FS-Forest swamp; M-Meadow; SS-Shrub swamp; LOD-Limit of detection.

Table 2. *Nitrate levels of the soil samples.*

Sample locations	Nitrate levels (ppm)
Mature forest	87
Grass lawn	121
Forest swamp	48
Meadow	127
Shrub swamp	88

A forced air was used to dry soil samples before the nitrate test, and homogenized samples were used for the test to avoid inaccurate readings with the moisture and large soil aggregations. The nitrate levels were 87, 121, 48, 127, and 88 ppm in samples collected from mature forest, grass lawn, forest swamp, meadow, and shrub swamp, respectively (Table 2).

The Nanodrop and the gel electrophoresis readings (Table 3) were collected after the extracted DNA was amplified by using the PCR and purified with the PCR cleaning kit. Furthermore, quality control checks of the DNA were conducted using the nanodrop and gel electrophoresis by adapting 16S earth microbiome protocols for high throughput next generation gene sequencing of bacteria DNA extracted.

All the nanodrop readings for the soil samples were within the required range of A260/A280 ratio of 1.8 to 2, and the results were 1.85, 1.86, 1.87, 1.85, and 1.86 for the DNA

extracted from the in mature forest, grass lawn, forest swamp, meadow, shrub swamp; respectively, as shown in Table 3.

Table 3. *Nanodrop Readings of the soil samples.*

Sample locations	A260/A280
Mature forest	1.85
Grass lawn	1.86
Forest swamp	1.87
Meadow	1.85
Shrub swamp	1.86

For the gel electrophoresis, cleaned DNA from bacteria was loaded on agarose gel, and the electrophoresis was conducted for 30 minutes with the DNA markers. DNA bands of bacteria approximately 350 bp, which met the requirement of the base pairs for sequencing 300-350 bp and the bands were significantly thick for the high concentration of DNA.

The DNA samples were sequenced using the Illumina MiSeq chemistry following the manufacturer's protocols at the Azenta laboratory in Burlington, MA. The alpha and beta diversities were conducted using Qiime 2 [15] with $p<0.05$ to better visualize microbiome data. Shannon-Wiener (Figure 2) and observed feature rarefaction curves (Figure 3) were calculated based on the selection of amplicon sequence variants using DADA2.

The Alpha and beta diversity analysis were rarefied to 2500 sequences. Alpha diversity refers to the diversity within an area. The Shannon-Wiener Index curve (Figure 2) reaches a plateau at approximately 200 sequences indicating sufficient sequencing depth to characterize microbial diversity. Beta diversity is an analysis of the microbial community structure to compare the relativeness of species collected from the sites. A principal coordinate analysis (Figure 4) is performed to find the beta diversity and visualize microbial communities' relationship between areas.

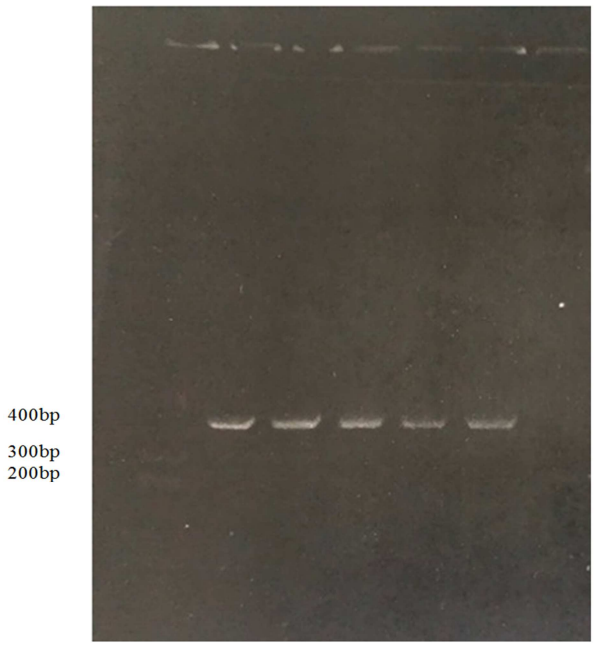

Figure 1. *Gel electrophoresis image. From left to right: DNA marker, Bacterial DNA extracted from the Mature forest, Grass lawn, Forest swamp, Meadow, Shrub swamp.*

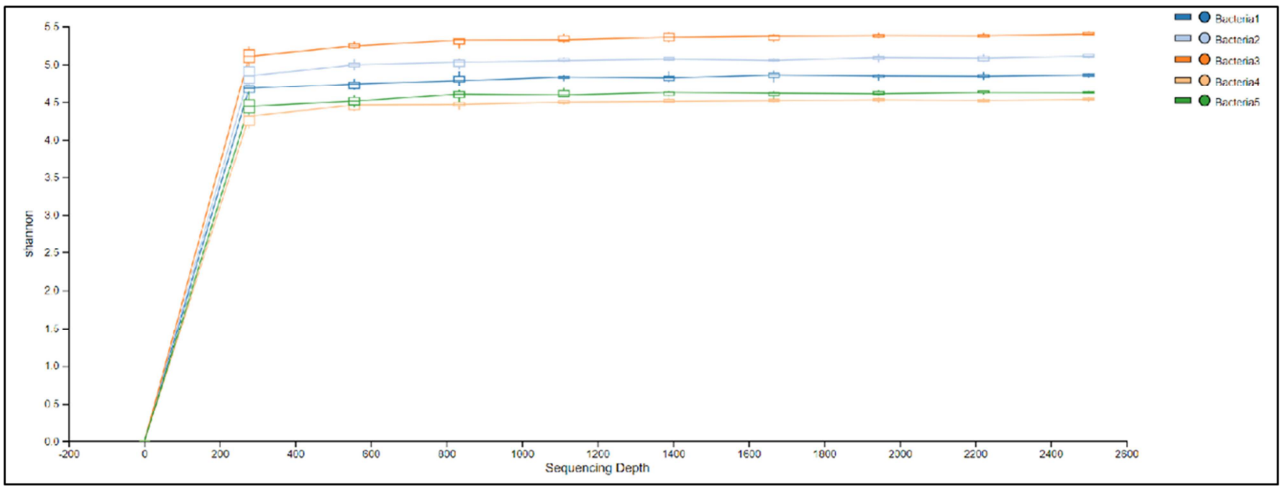

Figure 2. *Shannon-Wiener curve. 1. Forest swamp, 2. Grass lawn, 3. Mature Forest, 4. Meadow, 5. Shrub swamp.*

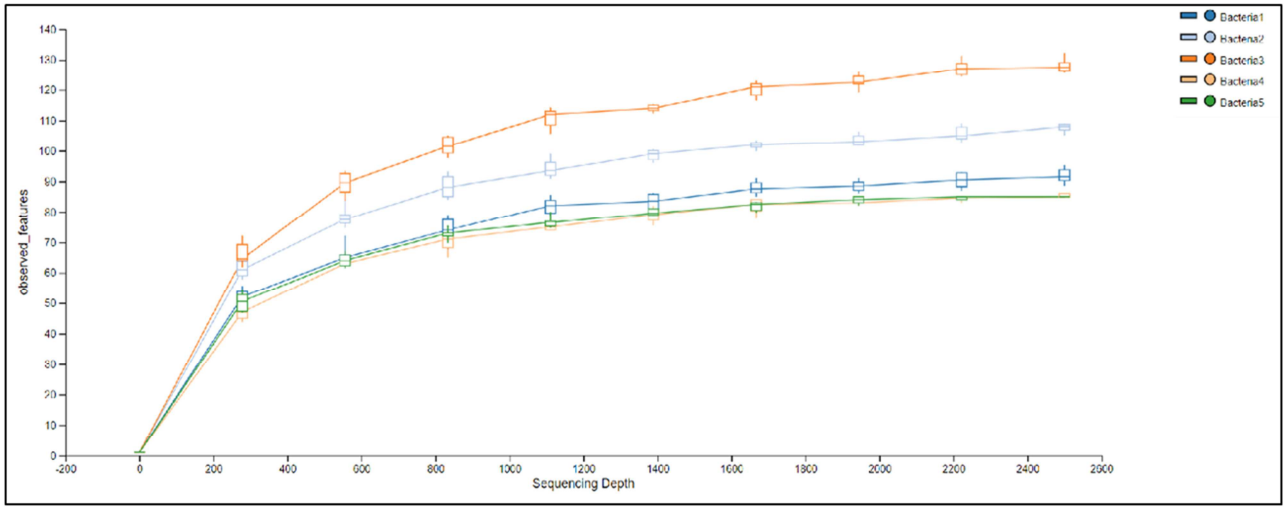

Figure 3. *Observed feature rarefaction curve. 1. Forest swamp, 2. Grass lawn, 3. Mature Forest, 4. Shrub swamp, 5. Meadow.*

Beta diversity was represented by the principal coordinate analysis plot (Figure 4). The results showed the microorganisms in the meadow and shrub swamp were distance from the remaining samples**,** mature forest, grass lawn and forest swamp.

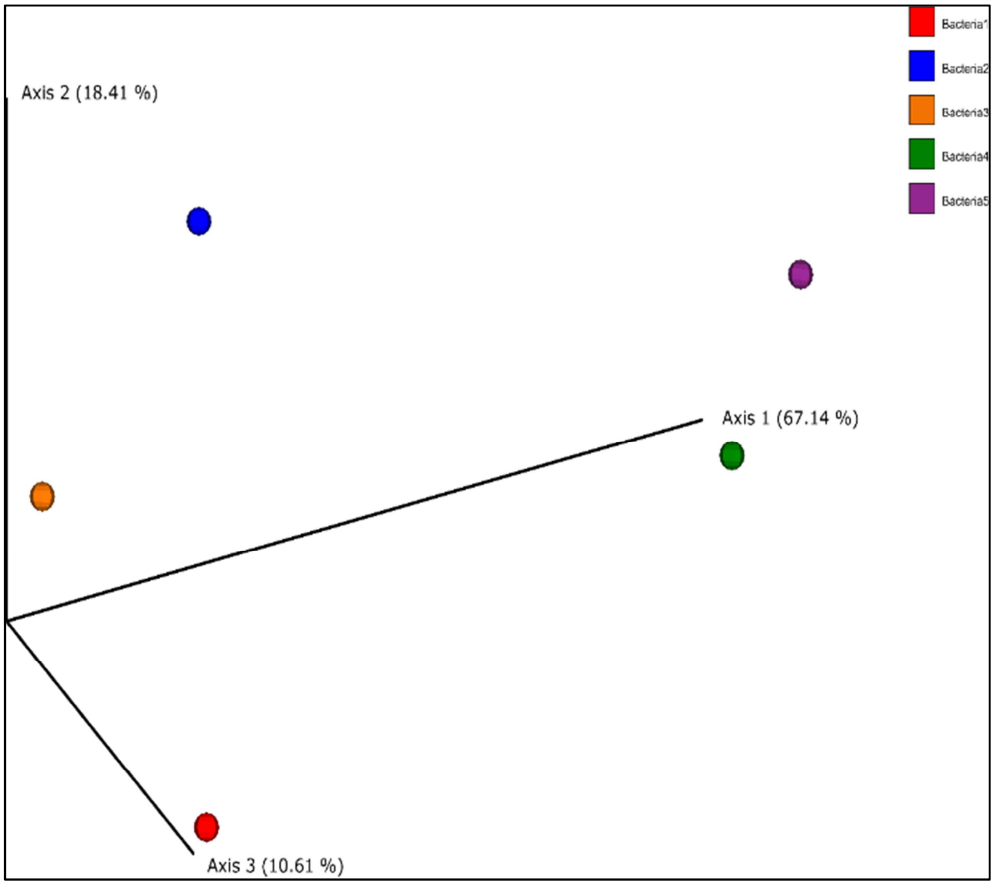

Figure 4. *Principal coordinate plot of weighted UniFrac data.*

According to the BLAST analysis, more uncultured bacteria were in the soil samples collected from the forest and grass lawn than in wetlands. The most common bacterial genera found in all samples were *Shingomonas*, *Acidobacteria*, *Chlorofexi*, *Bradyrhizobium*, and *Candidaturs*. A comparison of genomics and molecule characterization of bacteria in Western Maryland soil provided baseline data for further studies to understand the benefits of microbial growth.

4. Discussion

The soil samples were collected after removing humus from mature forest, grass lawn, forest swamp, meadow, and shrub swamp at 10 cm depth. The grid sampling method was

used to collect twenty representative soil samples from each site and homogenized the samples to get representative samples.

Soil composition results obtained from XRF showed that the area has the highest percentage of Fe. The Fe in coal mine lands catalyzes pyrolysis, which makes gases such as NH_3 and HCN. It was found that Fe in coal mines can inhibit the formation of NH_3 and HCN [17, 18]. The average percentage of Fe in the samples is over 57%, and Cr, K, S, and Ca were the other elements significantly present in soil samples.

The dried and sieved soil samples were used for the nitrate test to get homogenized samples and avoid obtaining inaccurate reading from largely aggregated soil. Then, the soil samples were exposed to forced air at ambient temperature overnight because the soil nitrate level can fluctuate in warm and moist conditions, especially after samples were removed from the cooler. In addition, excess moisture in soils collected from the swamps might lack oxygen and decrease soil nitrate through denitrification reactions [19].

DNA isolation, PCR, nanodrop and get electrophoresis were conducted before sending samples for sequencing. The nanodrop readings of A260/A280 ratio was within the required range of 1.8-2, and the DNA bands on the gel electrophoresis were around 350bp for bacteria.

This study obtained estimates of bacteria diversity in soil samples using the Qiime 2 and the NCBI-BLAST analysis. The alpha and beta diversities were conducted using Qiime 2 with $p<0.05$. The Shannon-Wiener Index curve reaches a plateau at approximately 200 sequences indicating sufficient sequencing depth to microbial diversity. According to the BLAST analysis, there were more uncultured bacteria in the soil samples collected from the forest and grass lawn than in wetlands. The most common bacterial genera found in all samples were *Shingomonas*, *Acidobacteria*, *Chlorofexi*, *Bradyrhizobium*, and *Candidaturs*, which are benefited to ecosystems in many ways including bioremediation, biodegradation, and nitrogen fixation.

5. Conclusion

Two replicates of homogenized soil samples collected with a soil core sampler tube at a depth of 10cm from the mature forest, grass lawn, forest swamp, and shrub swamp located in Frostburg, Maryland were used for soil testing.

The soil composition and nitrate concentration were tested at Frostburg State University, Maryland. The average percentage of Fe in the samples is over 57%, and Cr, K, S, and Ca were the other elements significantly present in soil samples. High Fe percentage is tied to the coal mine lands located in Frostburg, Maryland. The electrode readings for the nitrate levels of soils collected from mature forest, grass lawn, forest swamp, meadow, and shrub swamp were 87, 121, 48, 127, and 88ppm, respectively. The quality checks of DNA for next generation sequencing were performed using Nanodrop and gel electrophoresis. Nanodrop readings of A260/A280 ranged from 1.85 to 1.87, and gel electrophoresis results had only one band per sample, around 350bp. Bacteria were identified using the NCBI-BLAST tool and Metagenomics. The alpha and beta diversities were conducted using Qiime 2 with $p<0.05$. The Shannon-Wiener Index curve plot indicated sufficient sequencing depth to characterize microbial diversity. According to the BLAST analysis, there were many more uncultured bacteria in the soil samples collected from the forest and grass lawn than in wetlands. The most common bacterial genera found in all samples were *Shingomonas*, *Acidobacteria*, *Chlorofexi*, *Bradyrhizobium*, and *Candidaturs*, *which* are benefited in bioremediation, biodegradation, and nitrogen fixation. Comparison of genomics and molecule characterization of bacteria in Frostburg, Maryland soil provided baseline data for further studies in relation to the growth of plants, including crops.

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