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Weak correlations between soil properties and bacterial diversity at reforested sites in North America

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SUMMARY Soil microbial populations are interconnected in complex ways with soil properties and ecological processes. Ecological disturbances, such as timber harvesting, alter the equilibrium between these environmental factors. Therefore, it is crucial to understand how the relationships between soil properties and microbial diversity change in response to these disturbances. Using bioinformatics analyses, the relationships between bacterial alpha diversity and total carbon, total nitrogen, pH, and moisture content were explored. Based on previous literature, we hypothesized that correlations would exist between bacterial alpha diversity and total carbon, total nitrogen, and pH, but not with moisture content. Surprisingly, we observed no correlations between Shannon's Diversity index and total carbon, total nitrogen, and pH while a weak correlation was found for moisture content. In addition, we discovered that separating the data based on organic matter removal treatment revealed new correlation patterns at the most intense level of organic matter removal for total carbon and total nitrogen. However, we observed that the different intensities of organic matter removal did not alter the overall bacterial community structure and composition. Therefore, this study provides a valuable insight into the potential effects of different organic matter removal intensities on the relationship between soil properties and bacterial alpha diversity.

INTRODUCTION

Soil sustains a vast number of microorganisms that drive organic matter decomposition and biogeochemical cycling that promotes ecosystem productivity and stability (1). Nutrient breakdown and cycling processes are facilitated by microbial populations with highly diverse functional potentials. Processes including organic matter decomposition that are performed by functionally redundant microbial groups are less susceptible to ecological disturbances that alter bacterial diversity, such as timber harvesting (1). In contrast, processes that are conducted by smaller groups of microorganisms are more vulnerable to these disturbances (1). These processes include carbon mineralization and nitrification (2). An understanding of how changes in soil microbial diversity influence ecosystem stability and productivity is particularly important with regards to land management and climate change (1). The long-term effects of organic matter removal are not well-understood, but the removal of organic matter from poorly managed forest sites contributes to changes in nutrient availability, moisture, carbon, nitrogen content, and pH and may significantly alter soil microbial diversity (3). Thus, understanding the relationships between soil properties and bacterial diversity may provide an appreciation for the ecological impacts of timber harvesting and reforestation.

The data that was analyzed for this project was collected over a period of 7 years, as part of the Long-Term Soil Productivity Study (4). The soil samples were obtained from reforested sites following timber harvesting in North America in distinct ecozones. Each site was subject to varying intensities of organic matter (OM) removal treatments.

Soil properties, such as total carbon, total nitrogen, pH, and moisture content, have profound effects on microbial communities. Therefore, we were interested in exploring these specific properties further. Previous literature shows that organic carbon availability in soil impacts bacterial community structure and functional diversity (5, 6). The results indicate that additions of organic carbon are associated with a significant increase in the number of OTUs of soil bacterial communities (5, 6). In contrast, a soil ecosystem with a decreased amount of organic carbon results in a bacterial community profile exhibiting reduced functional diversity (7).

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Previous studies demonstrate that when high concentrations of nitrogen are added to the soil, the microbial alpha diversity decreases as the competitiveness of certain nitrogen cycling species increases (8–10). The abundances of nitrogen cycling species within the genera *Nitrobacter, Nitrospira*, and *Nitrosospira* increase when the total nitrogen in soil increases (8, 9). Lastly, soil amendments with nitrogen can increase the ammonium concentration, which consequently decreases bacterial OTU richness as nitrophilous populations expand (10).

Several studies have reiterated the relationship between soil pH and microbial diversity. In studies that used various sequencing techniques to analyze soil samples from North and South America, soil pH is observed to be correlated with microbial diversity and predict community composition (11, 12). At near-neutral pH, the diversity was found to be the greatest. However, as pH diverges from neutrality, diversity decreases. In a soil plot where a pH gradient is established and other environmental variables are constant, an increase in soil pH leads to an increase in bacterial OTUs and abundance (13).

Previous studies illustrate that soil moisture content does not influence the bacterial alpha diversity. Research that examined how various soil parameters affect microbial communities found no significant correlation between moisture content and alpha diversity (12, 14). A study showed that soil moisture content is not a predictor of bacterial community structure and does not significantly correlate with the alpha diversity of the most abundant bacterial phyla (12).

Based on these findings, we formulated our hypothesis in which 1) increases in the total carbon in the soil will correlate with an increase in the bacterial alpha diversity; 2) increases in the total nitrogen in the soil will correlate with a decrease in the bacterial alpha diversity; 3) increases in soil pH will correlate with bacterial alpha diversity (within the pH range of the samples in the metadata); and 4) there is no correlation between soil moisture and bacterial alpha diversity. This study presents a surprising finding in which Shannon's Diversity index weakly correlates with moisture content but does not correlate with total carbon, total nitrogen, and pH when we analyzed based on OM removal intensities. Additionally, we discovered novel correlation patterns for total carbon and total nitrogen at a specific OM removal intensity. We also found that bacterial community composition does not vary significantly with OM removal levels therefore, we deduced that selection of certain classes of bacteria are not driving the observed correlations.

METHODS AND MATERIALS

Soil data collection. Soil data was collected by Wilhelm et al. as part of the Long-Term Soil Productivity (LTSP) study (4). From 2008 to 2014, data was collected from soil samples obtained from both the organic and mineral layers which have a soil depth of 10 and 30 cm, respectively. Soil samples were obtained from 18 reforested sites following varying intensities of timber harvesting treatments (REF, OM1, OM2, and OM3) in 6 distinct North American ecozones: IDF_{BC} (interior Douglas-fir), SBS_{BC} (sub-boreal spruce), PP_{CA} (ponderosa pine), BS_{ON} (black spruce), JP_{ON} (jack pine) and LP_{TX} (loblolly pine). REF refers to an unharvested site, OM1 refers to the debranching of trees, OM2 refers to the removal of both trunks and branches, and OM3 refers to the removal of trunks, branches and top-soil. Triplicate samples from single plots were collected from all ecozones except for BS_{ON} and JP_{ON}. At these two ecozones, replicate samples were taken from three plots. At the time of data collection, the reforested sites were between 11 and 17 years old. The samples were subject to Illumina high throughput sequencing and the dataset consisted of 724 16s rRNA bacterial and 658 ITS2 fungal amplicon libraries, 133 shotgun metagenomic libraries, stable isotope probing amplicon libraries, and soil property metadata.

Bioinformatic analysis. Here we provide a summary of the computational methods. Detailed descriptions of the commands used in QIIME 2™ and R are provided as supplementary text files.

We used the QIIME 2™ bioinformatics software, which is a pipeline used for microbiome analysis from raw sequencing data to conduct our analysis as outlined in QIIME 2™ moving pictures tutorial (https://docs.qiime2.org/2020.11/tutorials/moving-pictures/). The collected data was imported to QIIME 2™ for diversity analysis. Using the QIIME 2™ DADA2

algorithm, the reads containing sequencing errors were detected and corrected. The samples containing no data for total carbon, total nitrogen, pH, and moisture content were filtered out. The filtered data was used to generate a feature table. A phylogenetic diversity analysis tree was generated to assess the alpha diversity of the bacterial samples using Shannon's Diversity index. An alpha rarefaction plot was created to verify sampling depth and ensure that the pool of amplicon reads represented all of the potential observed features in the samples. We subjected taxonomic output files to ASV-based filtering, which removed Archaea, chloroplasts, mitochondria, and ultra-rare ASVs from the data, allowing for the analysis of only bacterial ASVs. Then, we exported our data to R (version 4.0.3) and performed statistical analysis using the following packages: ggplot2, vegan, tidyverse, and phyloseq. Correlation plots between each of our soil properties and Shannon's diversity index, log2 fold change plots, relative abundance plots, and Weighted UniFrac Principal Component Analysis (PCoA) were generated using R. The *P*-values, *R*-values, and adjusted R^2-values of the correlation plots were determined in R.

RESULTS

No correlation observed between four soil properties and Shannon's Diversity index. In order to visualize the relationship between the four soil properties and Shannon's Diversity index, we performed a correlational analysis in R. For this correlational analysis, as well as subsequent analyses, we considered Shannon's Diversity index which is a quantitative measure of community richness that takes bacterial abundance into account. In order to be consistent with previous literature and to verify their findings, we chose to examine this diversity metric. The entire metadata pertaining to total carbon, total nitrogen, pH, and moisture content was used to generate the correlation plots. The data showed no correlation between Shannon's Diversity index and total carbon, total nitrogen, pH, and moisture content as demonstrated by the low R^2-value (Fig. 1A-D). Upon initial analysis, there appeared to be no correlations between the soil properties and Shannon's Diversity index, which urged us to further investigate the metadata.

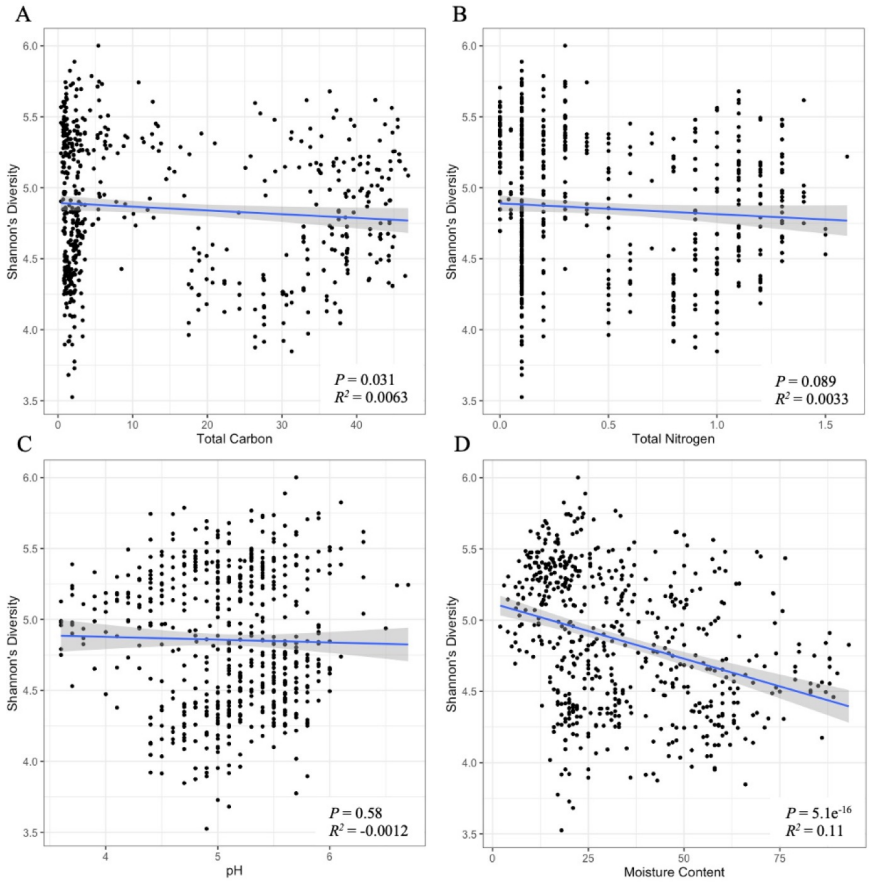

FIG. 1 No correlation between Shannon's Diversity index and total carbon, total nitrogen, pH, and moisture content. Correlation plot for Shannon's Diversity index and total carbon (A), total nitrogen (B), pH (C), moisture content (D).

Total carbon is weakly correlated with Shannon's Diversity index at the OM3 treatment level. Following the initial analysis, we wanted to control for the possible effects of OM removal on our chosen soil properties, which may subsequently affect the bacterial diversity. To investigate this, we generated correlation plots separating the data based on OM treatment levels. The reference (REF) site served as a control and there was no correlation observed (Fig. 2A). At the OM1 and OM2 treatment levels, we did not observe a correlation between total carbon and Shannon's Diversity index (Fig. 2B, 2C). In contrast, there was a weak, positive correlation between diversity and total carbon (Fig. 2D). By separating the metadata based on OM removal, we identified a new correlation between total carbon and Shannon's Diversity index at the OM3 treatment level.

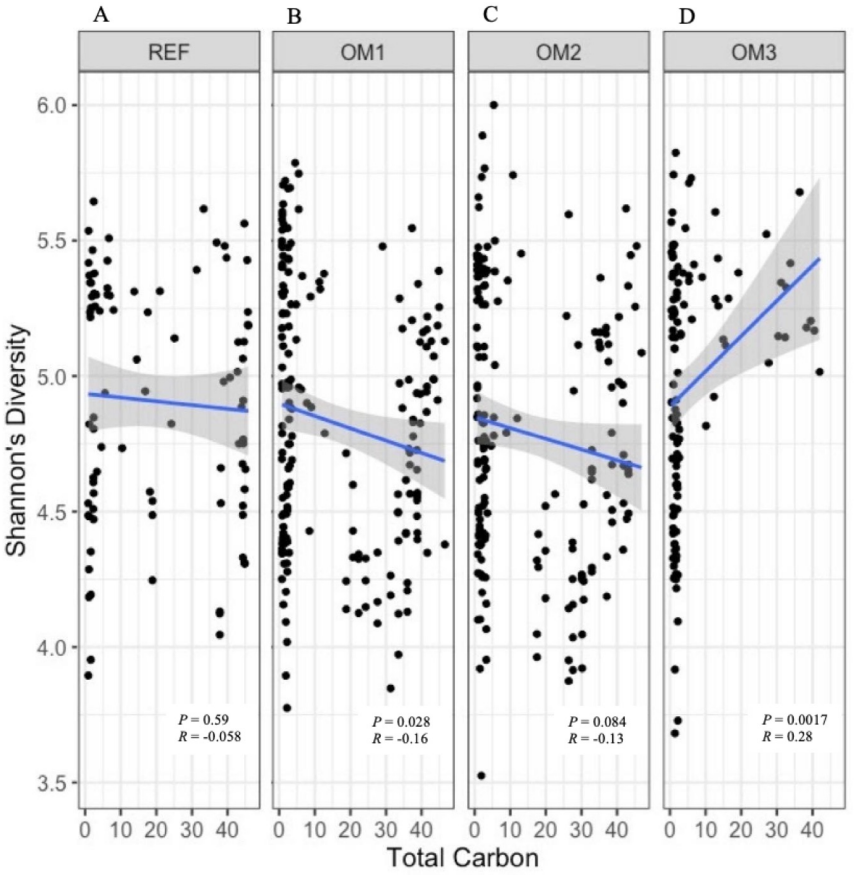

FIG. 2 Weak correlation between Shannon's Diversity index and total carbon at OM3 treatment level. Correlation plot for Shannon's Diversity index and total carbon at the REF site (A), OM1 (B), OM2 (C), OM3 (D) treatment levels.

Total nitrogen is weakly correlated with Shannon's Diversity index at the OM3 treatment level. In a similar approach, we wanted to see if a new correlation could be found between total nitrogen and diversity by separating based on OM removal. There were no correlations observed at the reference (REF) site, OM1, and OM2 treatment levels (Fig. 3A-C). As previously found in our analysis of total carbon, we discovered a novel, weak positive correlation between diversity and total nitrogen (Fig. 3D). This approach led to the discovery of different correlations between total carbon and total nitrogen nutrients with Shannon's Diversity index at the OM3 treatment level.

pH is not correlated with Shannon's Diversity index. Following the analysis of nutrient soil properties, we decided to look at a chemical property, pH, and see if we could unearth a novel relationship between this property and Shannon's Diversity index. Consistent with our previous analyses, we produced correlation plots for each OM condition. Once again, no correlation was observed at the REF plot (Fig. 4A). In addition to this, correlations were not seen at any of the OM treatment levels (Fig. 4B-D). Overall, we did not find any correlation between soil pH and Shannon's Diversity index in any of our analyses.

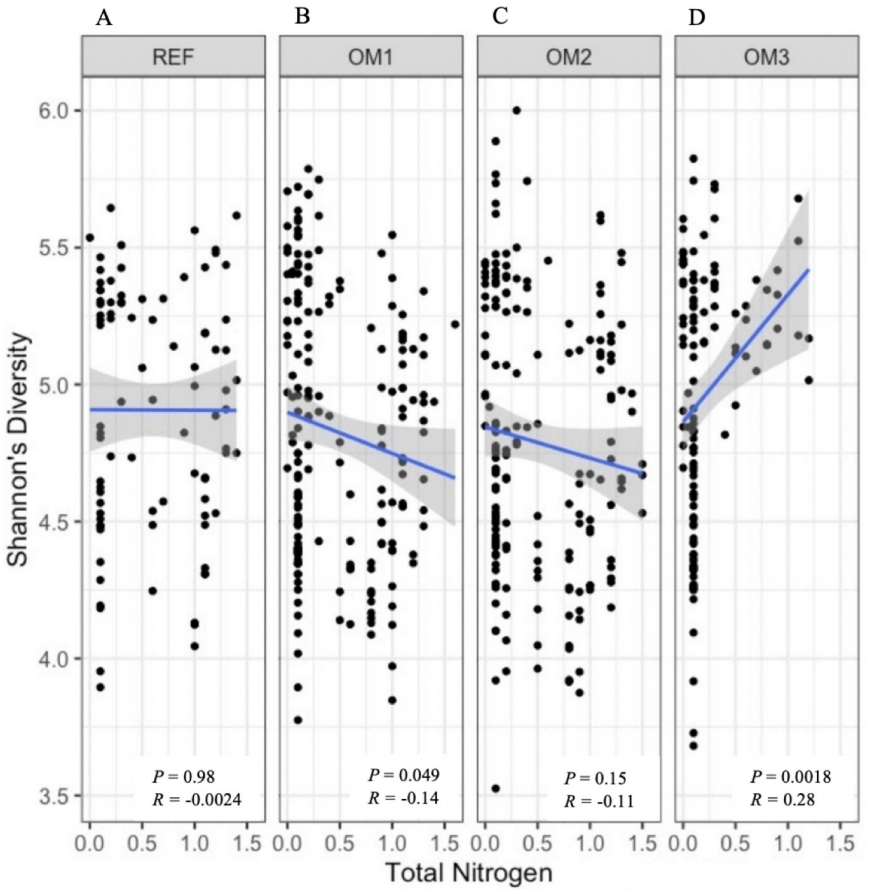

FIG. 3 Weak correlation between Shannon's Diversity index and total nitrogen at OM3 treatment level. Correlation plot for Shannon's Diversity index and total nitrogen at the REF treatment (A), OM1 (B), OM2 (C), OM3 (D) treatment levels

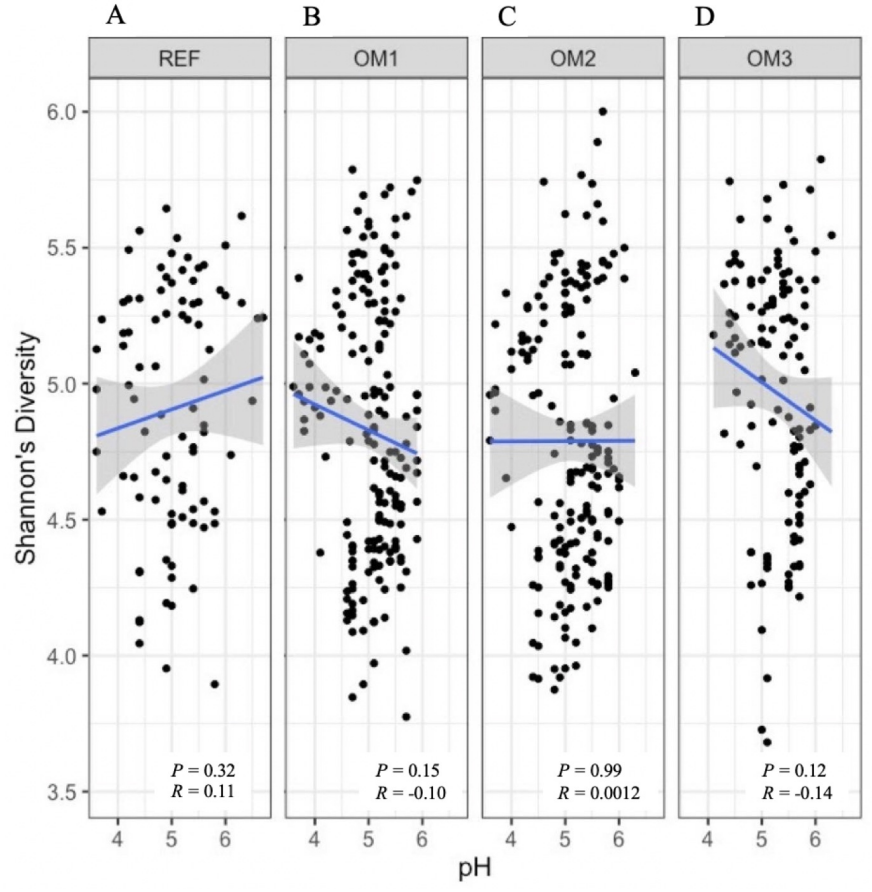

FIG. 4 No correlation between Shannon's Diversity index and pH at all OM treatment levels. Correlation plot for Shannon's Diversity index and pH at the REF (A), OM1 (B), OM2 (C), OM3 (D) treatment level.

Moisture content is weakly correlated with Shannon's Diversity index. To further investigate the relationship of non-nutrient abiotic factors with Shannon's Diversity index, we focused on moisture content. As formerly described, we constructed a correlation plot for each OM treatment level. Unlike the other soil properties, we observed a weakly negative correlation in the REF plot for moisture content (Fig. 5A). For OM1 and OM2 treatment levels, we observed very similar, weakly negative correlations (Fig. 5B, C). This correlation was not evident at the OM3 treatment level (Fig. 5D). In contrast to earlier findings with nutrient properties, we observed a correlation at all OM treatment levels except for OM3.

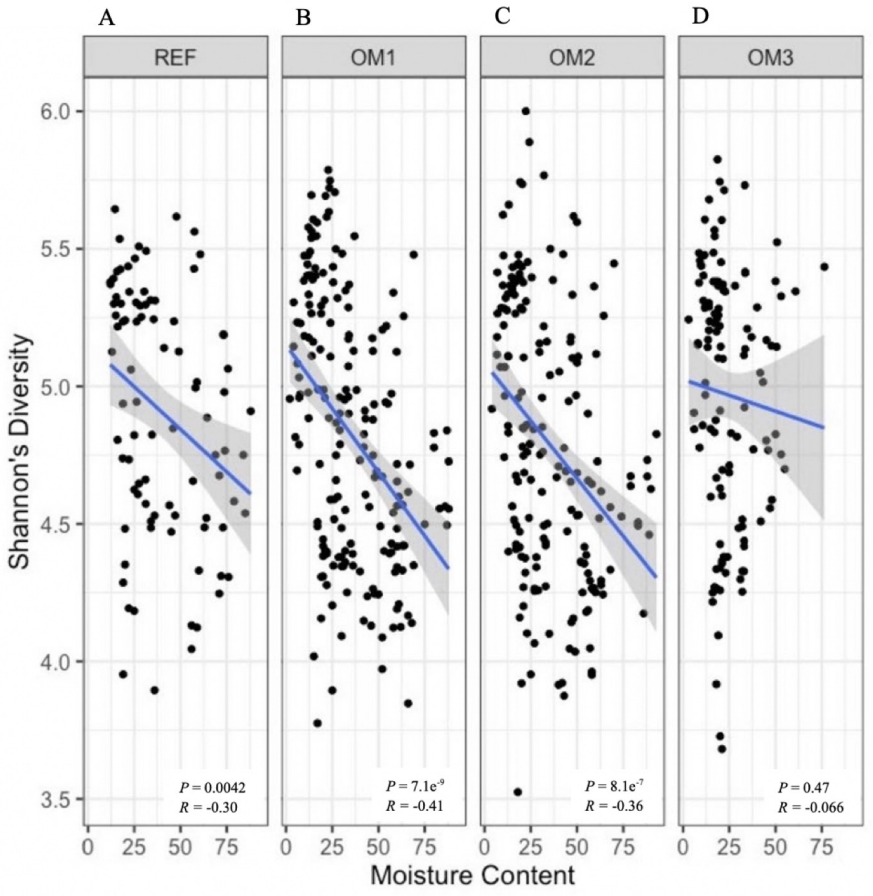

FIG. 5 Weak correlation between Shannon's Diversity index and moisture content at REF, OM1 and OM2. Correlation plot for Shannon's Diversity index and moisture content at the REF (A), OM1 (B), OM2 (C), OM3 (D) treatment level.

Relative abundance of Actinobacteria does not significantly differ across OM treatment levels. Based on the correlation plots generated for total carbon (Fig. 2), total nitrogen (Fig. 3), and moisture content (Fig. 5), the trends observed at the OM3 treatment levels for each of these three soil properties diverged from what was observed at the OM1 and OM2 treatment levels. This encouraged us to investigate if OM treatment selected for a certain class of bacteria. More specifically, we wanted to determine if one class of bacteria dominated the community at the OM3 treatment level, thereby shifting the correlation pattern. We chose to explore this using a relative abundance analysis which considers the evenness of species distribution within a community. Upon analyzing the log2 fold change of different classes of bacteria across OM treatment levels, we identified that Actinobacteria undergo the most prominent fold change (data not shown; see Fig. S1 in the supplemental material). However, the relative abundance plot shows that the mean relative abundance of Actinobacteria does not vary across OM treatment levels suggesting that the aforementioned selection does not occur (Fig. 6).

Bacterial community composition does not change following varying levels of organic matter removal. Although we found that a particular class does not dominate at the various OM treatments, we wanted to determine if OM treatment has an effect on the overall bacterial community composition. To investigate this, we generated a PCoA plot to compare the beta

diversity between sites of varying OM treatment. We were interested in the differences based on bacterial relatedness and abundance, which led us to use a Weighted UniFrac in our analysis. The results showed that the bacterial community composition is not significantly different at different OM treatment levels, and this is clearly illustrated through the common clustering of data points within the red, orange, green, and blue ellipses (Fig. 7).

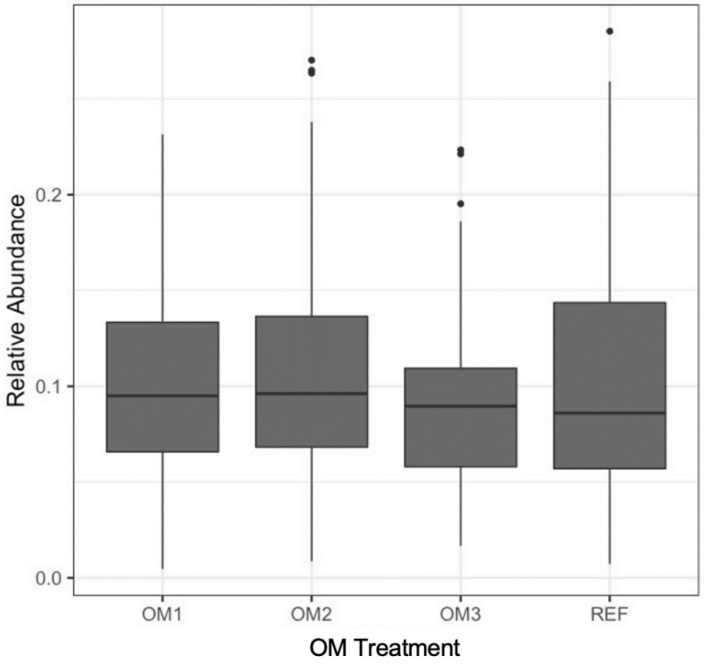

FIG. 6 Relative abundance of Actinobacteria does not significantly differ across OM treatment levels. A box and whisker plot of relative abundance of Actinobacteria based on OM treatment.

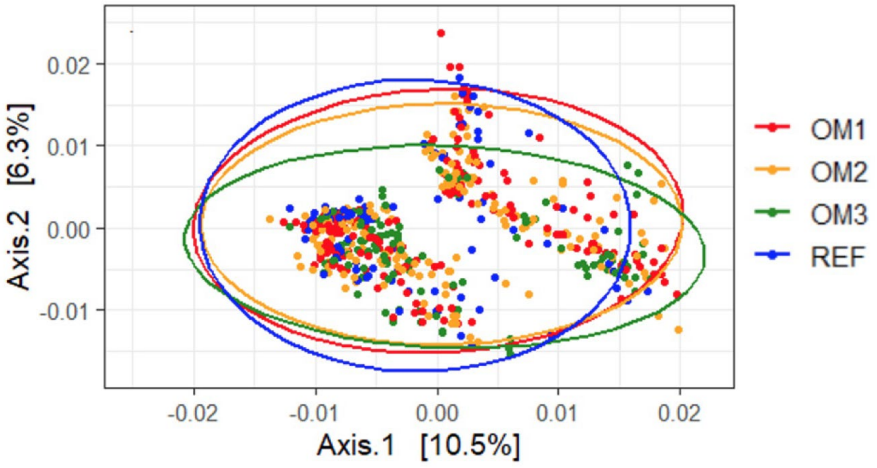

FIG. 7 Bacterial community composition does not change following varying levels of organic matter removal. A Weighted UniFrac Principal Component Analysis (PCoA) was generated based on OM treatment.

DISCUSSION

The main finding of this study was that total carbon, total nitrogen, pH, and moisture content are not the sole contributing factors to changes in bacterial alpha diversity when forest soils are subjected to organic matter removal. We determined this through the lack of strong correlations between bacterial alpha diversity and each of our four chosen soil properties, and this was surprising in comparison to the established relationships from previous research. With the exception of moisture content, extensive work has been conducted to understand the relationship between bacterial alpha diversity and total carbon, total nitrogen, and soil pH. A general consensus among the scientific community has been established wherein changes in the total carbon, total nitrogen, and soil pH are associated with distinct changes in bacterial alpha diversity. Previous studies on organic carbon availability in soil indicate a positive correlation between total carbon and bacterial alpha diversity (5, 7). Additionally, several studies illustrate that increases in the total soil nitrogen can decrease microbial alpha diversity

by enhancing the competitiveness of certain species involved in nitrogen cycling (8-10). It was stated that at near-neutral pH, the diversity is the greatest and it decreases as pH moved out of this range (12). The relationship between soil moisture and alpha diversity has not been extensively studied previously as the main topic of research. However, the few studies that examined how soil moisture affects alpha diversity found no relation (12, 14).

In disagreement with our hypothesis, we consistently observed no clear correlational trends between bacterial alpha diversity and total carbon, total nitrogen, pH, and moisture content even when samples were separated by OM treatment (Fig. 1-5). This suggests that OM treatment intensity does not influence bacterial alpha diversity with respect to each of the four soil properties. This surprising finding forced us to reconsider the robustness of our experimental design. When we compared our study to previous studies that investigated the relationships between bacterial alpha diversity and various soil properties, we noticed key differences in the sample collection methods (5-14). While previous studies have established clear relationships between bacterial alpha diversity and total carbon, total nitrogen, and pH, these studies were also designed such that sample collection sites were not subject to an intense ecological disturbance such as organic matter removal or soil compaction (5-13). This ultimately controlled for the effect of other environmental factors due to ecological disturbances on bacterial alpha diversity. In addition to this, other studies may have presented different findings due to differences in soil sample collection depths and geographical sites (5-14). In contrast, our study employed a full-factorial design in order to illustrate the combined effects of organic matter removal and soil compaction on the long-term soil productivity (15). These significant differences in experimental design may account for the differences in the trends observed.

When we separated samples based on OM treatment level, we observed divergent trends at OM3 for total carbon, total nitrogen, and moisture content (Fig. 2, 3, 5). This drove us to investigate relative abundances and beta diversity based on OM treatment. We expected the differential abundances and beta diversity to differ at the OM3 treatment level in comparison to OM1 and OM2 treatments. When we subsequently analyzed the differential abundance of Actinobacteria (Fig. 6) which was the bacterial class that exhibited the greatest log2 fold change based on OM treatment (data not shown; see Fig. S1 in the supplemental material), the mean relative abundances among OM treatments were not significantly different. Surprisingly, this indicates that OM treatment has no significant associations with changes in the relative abundances of Actinobacteria. This further suggests that the relative abundance of other classes which show less fold change do not vary significantly across OM treatment levels. When we further investigated the beta diversity of bacterial communities at the various OM treatment levels through a PCoA (Fig. 7), we did not observe any significant differences in community composition. This indicates that organic matter removal does not influence beta diversity. However, because we observed low percent variation on each axis of the PCoA, this suggests that the PCoA is unable to resolve differences in beta diversity based on OM treatment level (Fig.7). This consequently weakens the reliability of our results and we suspected that the low percent variation was due to the fact that our data was not filtered by ecozone. In contrast, studies that used the same dataset but separated the data based on ecozone found that OM treatment levels do not greatly alter the bacterial community composition in soil, which suggests an inherent resilience within the bacterial community in response to ecological disturbances (16, 17). Instead, it was found that the ecozone and soil layer contribute the most to the observed differences in beta diversity between bacterial communities, where the ecozone and soil layer accounted for 64% and 18% of the variation, respectively (16). Although we were able to deduce that the prevalence of certain bacteria and community composition were not responsible for the diverging trends at the OM3 treatment level, we could not determine what was driving this novel phenomenon due to time constraints.

Further support of the significance of soil layer on microbial alpha diversity comes from a study which demonstrates that soil bacterial and fungal communities are distinct at each soil layer (18). Each soil layer is observed to have a specific microbial profile as illustrated by the presence of unique OTUs, which indicates that microbial community changes with soil layer (18). In addition, variability in ecozone-specific factors such as soil characteristics, climate, and tree species are found to be the predominant factors contributing to differences in soil

microbial communities (17). Because we did not consider the impacts of soil layer and ecozone when filtering our metadata, these factors may have consequently confounded our data.

Lastly, the correlation patterns between alpha diversity and total carbon and total nitrogen are similar across the OM treatment levels. We suspect that this is because nitrogen and carbon are both crucial nutrients involved in bacterial processes. However, because the metadata was collected for only these two nutrients, we were unable to definitively determine if these similarities are nutrient-specific.

Limitations A limitation of our study was that we only filtered our data to remove samples without soil property metadata. As a result, we observed very low percent variation on both axes of the PCoA (Fig. 7). Following literature research, we found that ecozone and soil depth are implicated in the alpha and beta diversity of the soil microbiome. Therefore, filtering based on ecozone or soil depth may improve the ability to resolve the differences in diversity based on OM removal treatment, increase the percent variation, and observe clearer trends.
A limitation associated with our metadata was that data was only collected for two nutrients, total carbon and total nitrogen. Due to this limitation, we were unable to determine if the similarity in correlation patterns will be observed across other soil nutrients or if an undiscovered variable is responsible for this.

Conclusions In conclusion, we did not observe clear correlational trends between bacterial alpha diversity and our four soil properties. More specifically, we observed a weak correlation in total carbon and total nitrogen at the OM3 treatment level but we were unable to determine a robust model. Additionally, we did not observe any significant correlations between bacterial alpha diversity and pH at all OM treatment levels. As for moisture content, we observed weak correlations at all OM treatment levels except for OM3. We deduced that the diverging patterns at the OM3 treatment level were not due to changes in relative abundance or bacterial community composition.

Future Directions Future work should consider analysis using beta and alpha diversity through different metrics. Specifically, alpha diversity analysis with Faith's Phylogenetic Diversity could provide further insight into the importance of phylogenetic distances between species. Also, the dataset can be filtered for different soil layers or ecozones as these may be confounding variables that affect bacterial diversity. These directions could help provide a clearer relationship between bacterial diversity and soil properties.

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CONTRIBUTIONS

All authors contributed equally to this project and manuscript.

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