The Effects of Long Term Andisol Fertilization on the Diversity of Prokaryotic Genera

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Abstract

It is widely known that the long term use of fertilizers has an effect on both crop yield rates and the structure of the soil microbiome. Their specific effects on Andisols, however, is unknown. Thus, using metagenomic samples originating from long term experimental Andisol plots a diversity analysis was performed to determine the chronic effects of fertilizers. It was discovered that while the alpha diversities of the samples varied by little, the beta diversities revealed significant ($P \leq 0.05$) differences in their metagenomic structures. The presence of underlying variables beyond fertilizer usage was also identified. Future work is needed to concretely determine the long-term effects of fertilizer as well as identifying the underlying latent variables in the metagenomic profiles.

Introduction

Andisols are a type of soil originating from volcanic ejecta or – in rare cases – due to the weathering of primary aluminosilicates [1]. Unsurprisingly, Andisols are primarily located in areas which host active or recently extinct volcanoes, with a distribution pattern that encompasses the Pacific Ring of Fire, the western coast of Italy, and Iceland amongst others [1]. They are also capable of maintaining a high carrying capacity [2] and are considered as being a highly fertile soil [1]. Despite containing a high yield rate, Andisols have a low nitrogen and phosphorus mineralization rate and are sensitive to agricultural tilling [3]. Improper agricultural practices can result in the significant loss of fertility, increased deterioration, infection of rooting systems, and the frequent application of fertilizer to maintain yield rates [4].

While research has been typically performed on the effects of fertilizer on yield rates [3, 5, 6, 7, 8], there is growing interest in the effect of the soil microbiome on plant yield [9]. As current fertilizers are obtained from finite resources and have a negative effect on the environment [10], it has been hypothesized that manipulating the microbiome could lessen our dependence on these fertilizers and with little loss on plant yield [11]. Research has already demonstrated that the effects of the soil microbiome are reproducible [12] and thus can be

leveraged to increase plant yields. While there are studies concerning the effects of long-term fertilization on the soil microbiome [13, 14] none are specific to its effects on the Andisol microbiome.

Thus, this exploratory study aims to determine the effects of long term fertilization on the diversity and composition of the microbiome present in Andisol plots. This will be achieved by studying the microbiome of soils sampled from the Nagano Prefecture Vegetable and Ornamental Crops Experiment Station in Japan [15]. These plots were continuously fertilized using the same soil management practices as when they were first established in 1938 [16]. This will allow the study to determine the long-term effects of organic (compost) and inorganic (Nitrogen, Phosphorus, and Potassium) fertilizers along with their concentrations [15].

Material & Methods

Preparing the Amplicon Sequences

The amplicon sequences used in [15] were obtained from the Sequence Read Archive under the accession number *DRA007565* as paired-end FASTQ files. There were 12 different fertilizing regimes with three samples each, giving a total of 36 different sequencing files. The three samples for each treatment, however, originated from the same plots as replication was not established at the experimental station [16].

The sequences were then analyzed using *FastQC* (`https://www.bioinformatics.babraham.ac.uk/projects/fastqc/`) to determine their overall quality and the amount of trimming required. Trimming was then performed with *Trimmomatic* [17] using the default settings. The adapter sequences used during trimming is located at `http://bioinformatics.cvr.ac.uk/blog/illumina-adapter-and-primer-sequences/`. The successfully trimmed and paired reads were then merged using *SeqPrep* `https://github.com/jstjohn/SeqPrep`. String deduplication was then performed using *prinseq* [18]. Specifically, sequences that were either exact duplicates or were reverse complements were filtered from the analysis.

Metagenomic Processing

Despite identifying the sequences as 16S rRNA amplicons, KrakenUniq [19] was used to assign taxonomic information to each read. This is largely due to two considerations. The first is that while OTU clustering is fairly common method for identifying taxa, it has been shown that Kraken performed significantly better [20]. While it has been shown that there is loss of sensitivity and specificity at the deepest taxonomic levels (species, subspecies, etc.) [21], this study only endeavours to identify reads to the genus level. This is due to the increased false positive rate at the deepest taxonomic levels [22]. The second reason why OTU clustering was not used in this study was to determine the robustness of the metagenomic profiles identified by *Mise et al.*. Their study determined metage-

nomic profiles using amplicon sequence variants determine by DADA2 [23] and annotations provided by the RDP Classifier [24] trained with Greengenes 13_8 [25] which was clustered with a 97% identity threshold [15]. Thus, by using a different approach to identify the genera present in the samples, the robustness of the metagenomic profiles identified by *Mise et al.* is tested.

Once the reads were identified, abundance filtering was performed to account for any false positives in the data. Specifically, despite using 16S rRNA data, KrakenUniq [19] still managed to identify several reads as originating from Fungi. While Fungi are definitely present in the soil [26, 27] and have a significant role in nutrient-use efficiency [28], 16S rRNA cannot be used to accurately identify their presence [29]. Thus, a minimum abundance of 1% was required to consider a genera as a true positive [30]. This filtering step removed $\approx 52\%$ of reads identified at the genus level. The vast majority ($\approx 96\%$) of genera identified by KrakenUniq [19] were also removed using the abundance filter.

Statistical Analysis

The resulting read counts were then transformed to account for potential heteroscedasticity in the variances and to ensure that the samples could be accurately compared. Two normalization methods were used separately on the data. In the first method, read counts were converted into proportional abundances. This allows the study to accurately determine whether there are any differences between the microbiome profiles identified by *Mise et al.* and those determined in this study. Using proportional abundances, however, removes any information about the total size "size" of the microbiome as the read counts are abstracted. To account for this loss of information the samples were also normalized using the inverse hyperbolic sine function ($arcsinh$). This has been shown to variance stabilize read counts accurately [31] as metagenomic data typically follows a gamma-Poison mixture model [32].

Upon transforming the read counts, the metagenomic profiles were pooled and analyzed at the phylum and genus taxonomic levels using *phyloseq* [33]. The phylum level was analyzed to ensure that the metagenomic structures were comparable to those discovered in the original announcement [15]. Results similar to those found in [15] would indicate that the results from this study are robust and broadly applicable to the dataset as whole. Heat maps of the genera abundances were also analyzed to qualitatively determine any differences in the bacteria which form the structure of each sample. This would give a coarse understanding of how the different fertilizer regimes effect the microbiome. This was done using the $arcsinh$ transformed results.

A Principle Coordinate Analysis (PCoA) was also performed on the metagenomic profiles to determine the relationships between treatments. Samples were labelled based on their treatment (ie. the combination of fertilizers used) as well as the individual fertilizers used (ie. was Nitrogen added). This was done to better visualize the differences caused by the individual fertilizers and determine whether the individual fertilizers had any affect. The Bray-Curtis dissimilarity statistic was used to create the PCoAs as well as to perform the network anal-

ysis below. It was chosen as it demonstrated a) a large difference between the proportional and *arcsinh* transformations, and b) the explained variances were approximately average when compared to the other 39 distances.

Networks were also created to identify the relationships between the different samples. Samples were with an edge if they had a Bray-Curtis distance ≤ 0.25. This maximum distance was chosen as it was the highest value that contained a meaningful (ie. not all interconnected) network. This network was used to determine whether communities created by the individual fertilizer components – Nitrogen, Phosphorus, Potassium, and Compost – were significant. This was determined through a permutation test consisting of $10,000$ replicates using communities sizes identical to those found in the data. The values used for the permutation tests were the modularities of randomly created clusters [34]. The permutation test was modified to ensure that the minimum p-value was ≥ 0. While oftentimes small, it has been shown that the p-value can be understated without this correction [35]. Furthermore, a walktrap algorithm as implemented in the R package *igraph* [36] was used to blindly create communities. The optimal community algorithm was not used due to recent changes making it impossible to run (see `https://github.com/igraph/rigraph/issues/273`) in recent versions of R.

The alpha diversities of the samples were also calculated. This was done to determine whether the fertilizer regimes affected the overall composition of each sample. Specifically, the Shannon index [37] was used to determine the overall diversity while the Simpson index [38] calculated the concentration of the samples. The samples were then compared using the Tukey method [39] to determine if the treatments were significantly different. Despite the warnings raised by *phyloseq* [33], the abundance filtered data was used due to the concerns outlined above. The results from the untrimmed data can be found in the supplemental figures (see Figure SF.1).

Results & Discussion

Taxonomic Abundances

Prior to any analysis of the results, a comparison between the phyla identified in the original announcement [15] and the phyla detected using KrakenUniq [19]. Overall, all but one of the major phyla (Thaumarchaeota) identified by *Mise et al.* were also detected in this study as shown in Table 1. In contrast, KrakenUniq [19] detected four additional phyla. These phyla are not considered as unique to KrakenUniq, however, as the profiles released by *Mise et al.* grouped phyla with smaller abundances into a single category (*Others*) with no indication of their identity. Furthermore, the proportional abundances of the shared phyla are similar to each other, despite abundances not adding to 100% in this study (see Figure 1). This is due to the presence of three candidate genera which are not allocated a phylum as they have not been poublished under the criteria stipulated in the *International Code of Nomenclature of Bacteria* [40].

At the genus level, the metagenomic profiles become much more apparent. Using the *asinh* transformed values, Figure 2 indicates the overall trends in each microbiome. Each pooled sample includes a core community consisting of *Bacillus, Chondromyces, Gemmatimonas*, and *Sphingomonas* amongst other genera. It is the presence or absence of other genera such as *Thermobacillus* and *Hyphomicrobium* that defines the pooled samples. In particular the samples Compost PK, Compost, and Low NPK are remarkably similar to each other. The same can be said for NP, NPK, and PK. While these two groups could potentially indicate the presence of clustering within the results, they also indicate that the presence and absence of few genera are required to differentiate the samples. This is especially true for Compost NP and Compost NK which only differ by three genera (*Caldilinea, Chloroflexus,* and *Dehalogenimonas*).

Nitrogen and Compost Drive Metagenomic Profiles

Upon identifying the possibility of clusters in the data, a PCoA was created to further inspect this assumption. Using the genera as our variables, a total of 65.9% of the variance was explained by the first two axes. Furthermore, the overall shape of the resulting PCoA (see Figure 3) is a horseshoe which is indicative of niche differentiation and species turnover through one or more environmental gradients [41]. Patterns similar to those found in Figure 2 could also be identified in the PCoA giving further credence to this assumption. The samples consisting of Compost PK, Compost, and Low NPK are located in the same general area along with outliers from Compost NK and Compost NP. Compost NP and Compost NK are also occupying the same area along with High Compost NPK. A few notable outliers were also identified; the sampling group Very High NPK was separated from the rest of the samples. The same visual analysis was performed using the networks formed by the PCoA. As expected, similar clustering profiles are indicated (see Figure 4). The relationship between NP and NPK is further highlighted by the distinct lack of outgoing edges. Furthermore, evidence that Very High NPK possesses a unique profile is also recorded due to the single node connecting it to the rest of the network. The outlier samples from Compost NK and Compost NP, however, are completely separated from the rest of the network.

Since the results could be due to environmental factors, the PCoA and network results were then labelled based on the presence and absence of a particular fertilizer. Using these labels it becomes evident that clusters present in the data are most likely defined by the absence of a fertilizer rather than their presence (Figures 5 and 6). In an attempt to determine which communities were significantly distinct, the modularities and their respective p-values were calculated. As seen in Table 2, the individual fertilizing methods contribute differently to the overall metagenomic profiles. In particular, it is indicated that fertilizing with Compost and Nitrogen has a significant ($P < 0.05$) effect whereas the presence of Phosphorus and Potassium matters little. This is further seen in the modularities of the resulting clusters as Phosphorus and Potassium have values ≈ 0. While it is expected that the presence of Nitrogen could have a significant

effect due to its low mineralization rate in Andisol, the same was thought to be true for Phosphorus [3]. This deviation could be due to Phosphorus inhibiting the growth of the microbiome [42]. These effects, however, are only detected when Phosphorus was added individually [42]. The clustering results for Nitrogen and Compost can be seen in Figure 7 and Figure 8 whereas Phosphorus and Potassium are in the Supplemental Figures (see Figures SF.2 and SF.3).

Due to the highly significant clusterings, a walktrap algorithm was employed to blindly determine communities present in the samples. The resulting community structure, as seen in Figure 9, is much more varied and pronounced than those resulting from a single fertilizer. Furthermore, when compared to the communities created by the individual fertilizers, the walktrap algorithm resulted in a higher modularity score (see Table 2). While this may be due to the binary state of the fertilizers, these results are also potentially indicative of an underlying environmental variable. For example, these latent variables could be responsible for the creation of the NP–NPK complex and the separation of the Very High NPK sample, which are their own communities with this unbiased approach. Furthermore, the communities created by this algorithm hint at potential interactions between Compost and Nitrogen due to the presence of a Compost + Nitrogen cluster and a Compost without Nitrogen cluster. Potential issues arise with the walktrap algorithm, however, as three communities consisting of one to two samples were identified.

Little Difference Detected in Alpha Diversities

After investigating the PCoA and its resulting networks, alpha diversities of the pooled treatments were analyzed with the goal of potentially identifying it as one of the latent variables in the data. As seen in Figure 10, however, this was not case. While both the Shannon [37] and Simpson [38] indices strengthened the argument that Very High NPK is significantly different from the rest of our samples, the overall results demonstrate little in the way of significant clusters. If the hypothesis that alpha diversity is one of the latent variables were true, one would expect that NP and NPK would be significantly different from every treatment except for one another. What occurs instead is that while NP and NPK are within the same Tukey group they are accompanied by PK, Low NPK, and High Compost NPK, resulting in the rejection of the hypothesis.

Beyond determining whether alpha diversity is a latent environmental variable, it is evident that the overall diversity changes very little between the treatments. The Shannon index [37], a measurement of the overall diversity within a treatment does not contain any stratification nor clustering of the samples. Furthermore, the absence of a particular fertilizer or compost does not impart a significant effect on the overall diversity as seen in Figure 10. The same is true for the Simpson index [38], which contained identical treatment groups using Tukey's.

Conclusion

Andisols are a common soil type known to be capable of maintaining a high carrying capacity [2] and is extremely fertile [1]. This is despite Andisols having a low Nitrogen and Phosphate mineralization rate and being physically fragile [3]. Even with this knowledge very little research has gone into determining Andisol microbiome, much less the long-term effects of fertilizer on Andisol. With the understanding that previous studies had shown significant differences in community composition before and after long-term fertilization [13, 14], this study endeavoured to determine their effects on Andisol. This was done using recently published microbiome data originating from the Nagano Prefecture Vegetable and Ornamental Crops Experiment Station in Japan [15].

Very little change was noted in terms of the alpha diversities for each fertilizer regime. As shown by Figure 10 as well as Figure SF.1, there were no clear strata nor clusters of the treatments using Tukey's Method [39]. The only statistically meaningful results arising from this is that treatment group Very High NPK is a clear outlier. It is likely, therefore, that the application of organic or inorganic fertilizers has little effect on the overall diversity of the microbiome. This conclusion, however, should be tempered by the fact that technical replicates were used in this study, along with the lack of negative controls.

In light of this, a beta diversity analysis was performed to determine whether the presence or absence of a fertilizer was significant. Using a combination of PCoA and network analyses, it was observed that the presence of Nitrogen or Compost imparted a significant ($P \leq 0.05$) difference in the microbiome structure. Surprisingly, however, was that the addition of Phosphorus did not have a significant impact on the microbiome structure despite it having a poor mineralization rate in Andisols [3]. While it has been been previously shown that Phosphorus can inhibit the growth of the microbiome [42], further study is needed to determine whether it was the root cause of the lack of significance.

More work is required before determining the overall effects of long-term fertilization in Andisols. While the analyses performed in this study may indicate that Compost and Nitrogen have the largest effects in differentiating microbiome profiles, other latent variables are likely to be present. This is shown by the horseshoe effect in Figures 3 and 4 indicating a potential environmental gradient which was not reported [41]. The possibility that the alpha diversity may be one of these variables was rejected by the lack of significance and clear strata. Further exploration using probabilistic latent variable methods [43] may identify the number and significance these hidden characteristics. Additionally, evidence of possible interactions between fertilizers is hinted at by the communities formed with the walktrap algorithm in Figure 9.

Furthermore, the experimental design used in this study is potentially hampering the analysis. While the treatments are blocked such that the effect of each fertilizer can be measured through its absence, explicit negative controls are lacking. The use of negative controls will allow researchers to compare natural soil to those which were fertilized. This would reveal the overall long-term

effects of fertilizer on the diversity and composition of the soil microbiome, irregardless of the specific fertilizer used. The use of an experimental design such as that found in the study conducted by *Ai et al.* will allow for a greater confidence in these results.

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Figures

Table 1: Phyla detected either through ASV analysis as done in *Mise et al. 2019* or detected in this study using KrakenUniq [19]. Checkmarks indicate that the phylum was detected.

Phylum	**Mise et al. 2019**	**This Study**
Acidobacteria	✓	✓
Actinobacteria	✓	✓
Bacteroidetes	✓	✓
Chloroflexi	✓	✓
Firmicutes	✓	✓
Gemmatimonadetes	✓	✓
Kiritimatiellaeota		✓
Nitrospirae		✓
Planctomycetes	✓	✓
Proteobacteria	✓	✓
Spirochaetes		✓
Thaumarchaeota		✓
Verrucomicrobia	✓	

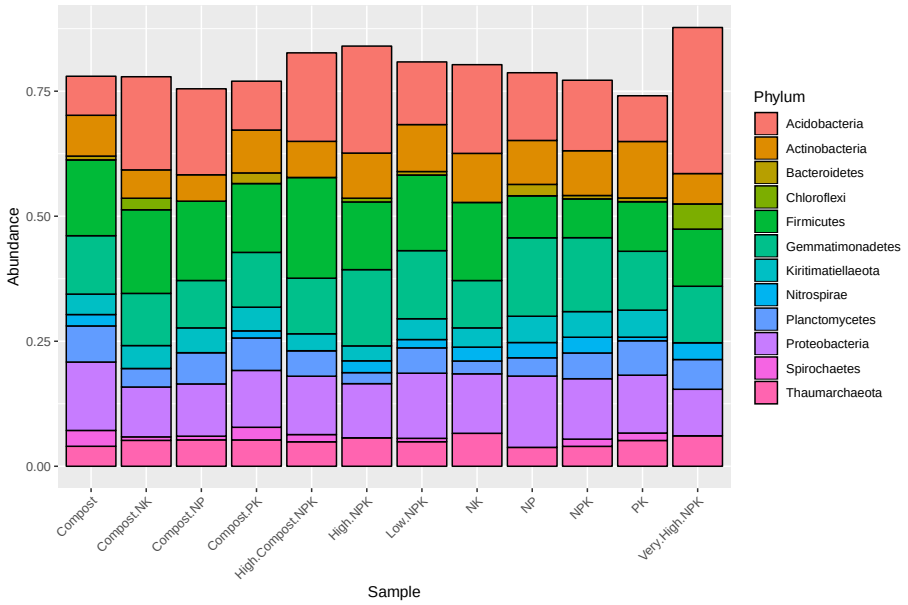

Figure 1: Proportional abundances of the pooled fertilizer treatments at the Phyla level. Abundances below 100% indicate the presence of genera which are not assigned a phylum.

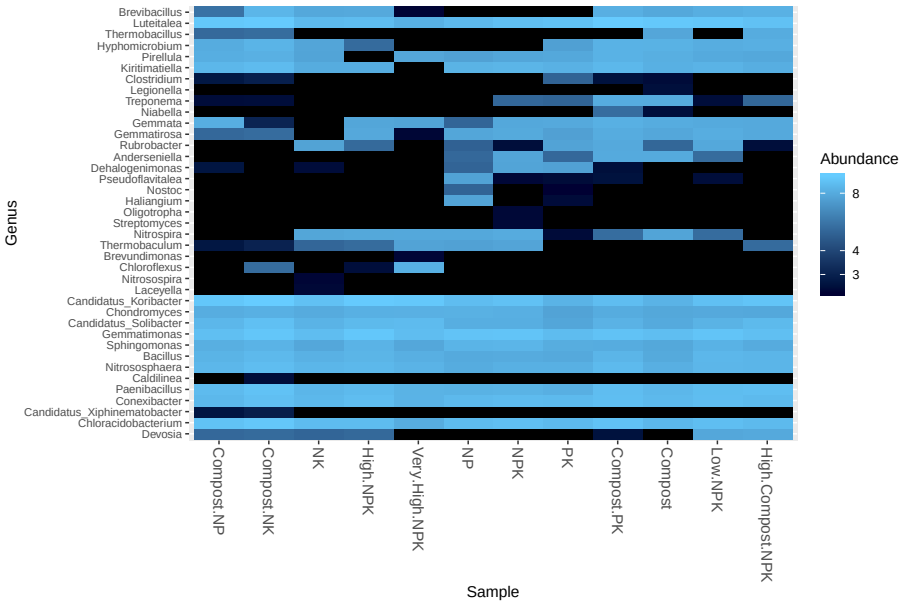

Figure 2: Heatmap of the pooled *arcsinh* transformed genera abundances. The genera and samples were ordered using the PCoA algorithm.

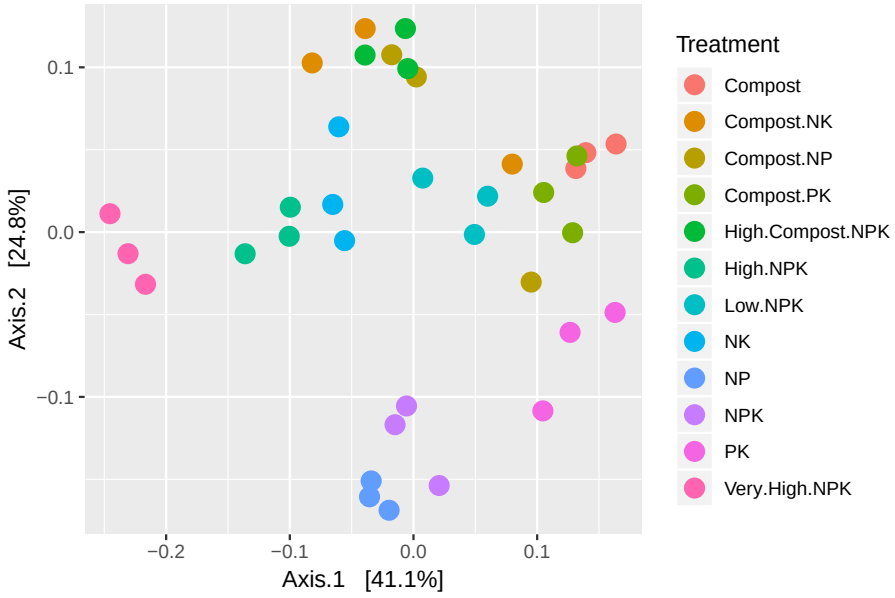

Figure 3: A PCoA of the samples using the overall treatment as the label.

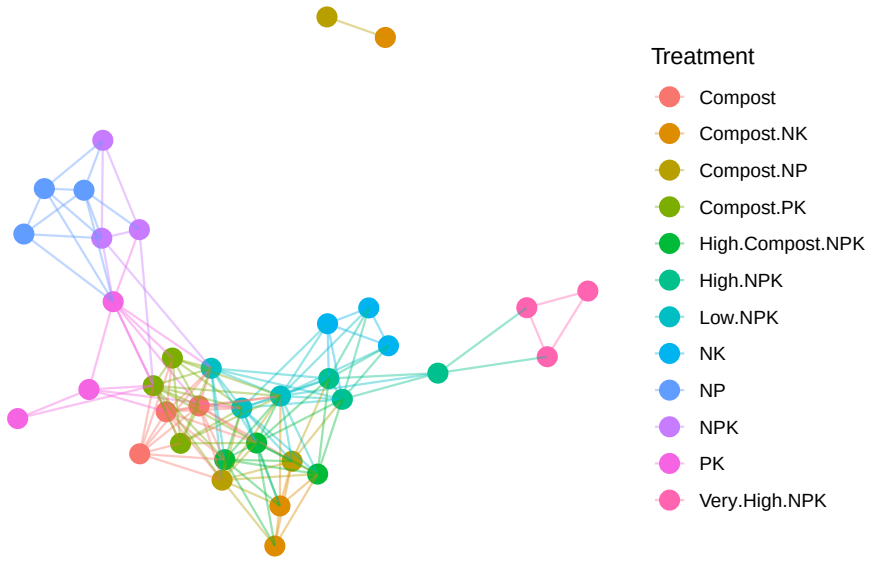

Figure 4: Network of microbiome samples. Edges indicate that the connecting samples have a Bray-Curtis dissimilarity ≤ 0.25.

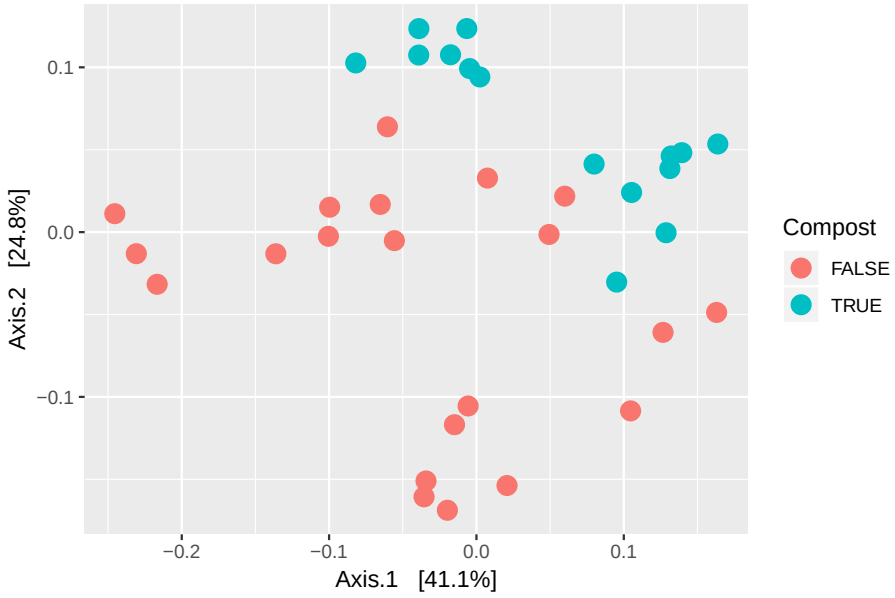

Figure 5: A PCoA of the samples using the presence of compost as the label.

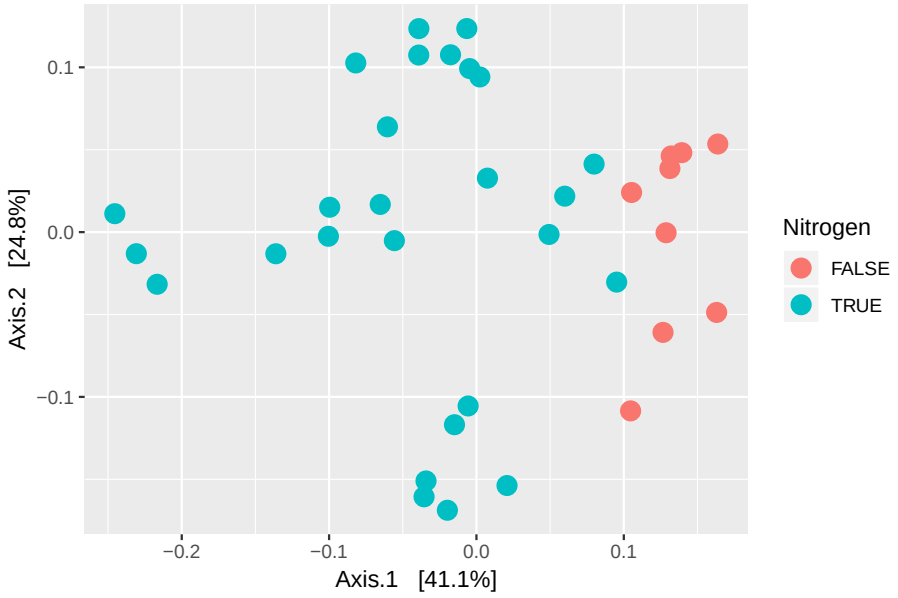

Figure 6: A PCoA of the samples using the presence of Nitrogen as the label.

Table 2: The calculated modularities using the presence of the individual fertilizers (Nitrogen, Phosphorus, Potassium, and Compost) as the communities. Modularities ≥ 0 indicate communities with more edges remaining within their clusters than expected. In contrast, modularities ≤ 0 indicate communities with more edges leaving their clusters than expected. P-values are calculated through a permutation test which calculates modularities using communities of identical sizes.

Community	**Modularity**	**P-Value**
Compost	0.196	9.99×10^{-5}
Nitrogen	0.159	9.99×10^{-5}
Phosphorus	-0.002	0.265
Potassium	0.023	0.061
Walktrap	0.304	—

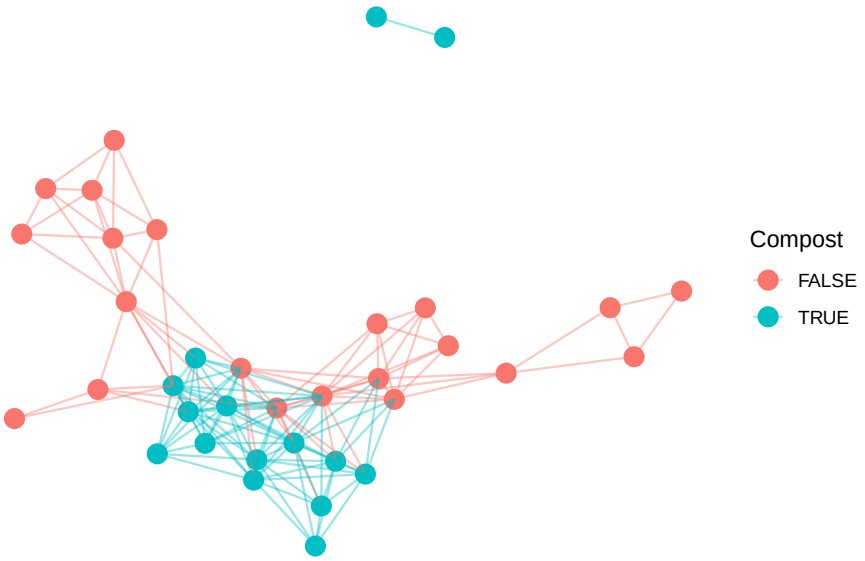

Figure 7: Network of microbiome samples using the presence of compost as the label. Edges indicate that the connecting samples have a Bray-Curtis dissimilarity ≤ 0.25.

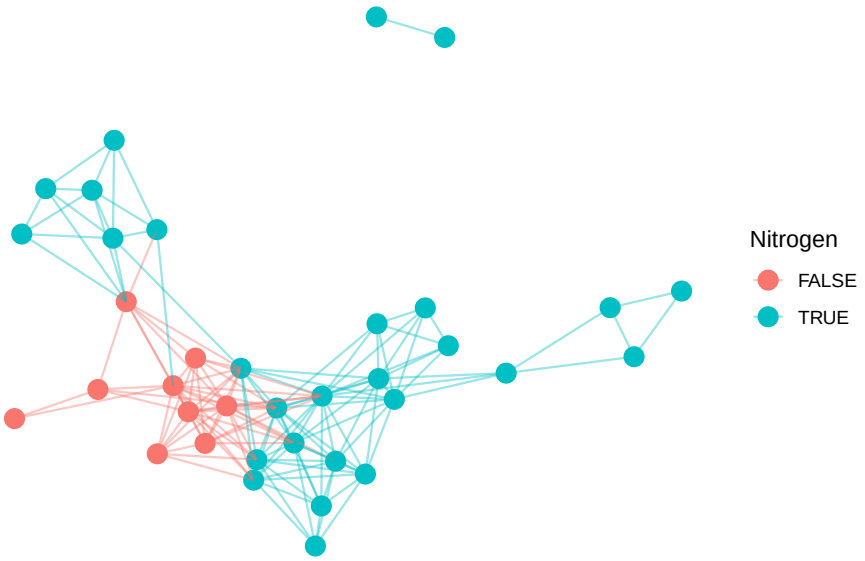

Figure 8: Network of microbiome samples using the presence of Nitrogen fertilizer as the label. Edges indicate that the connecting samples have a Bray-Curtis dissimilarity ≤ 0.25.

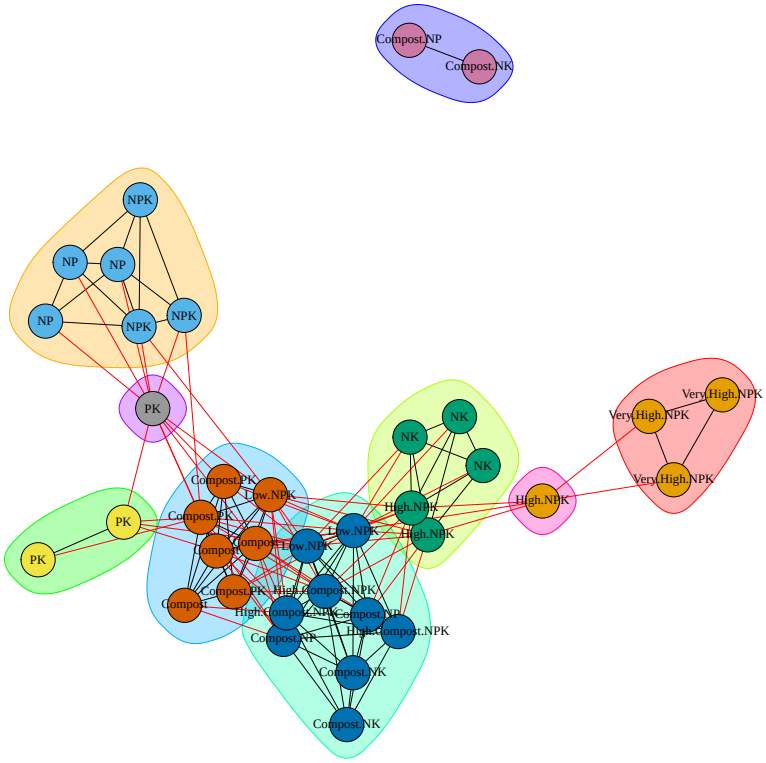

Figure 9: Network of microbiome samples clustered using a walktrap algorithm. Edges indicate that the connecting samples have a Bray-Curtis dissimilarity ≤ 0.25. Red edges indicate connections spanning two communities whereas black edges indicate connections within a community.

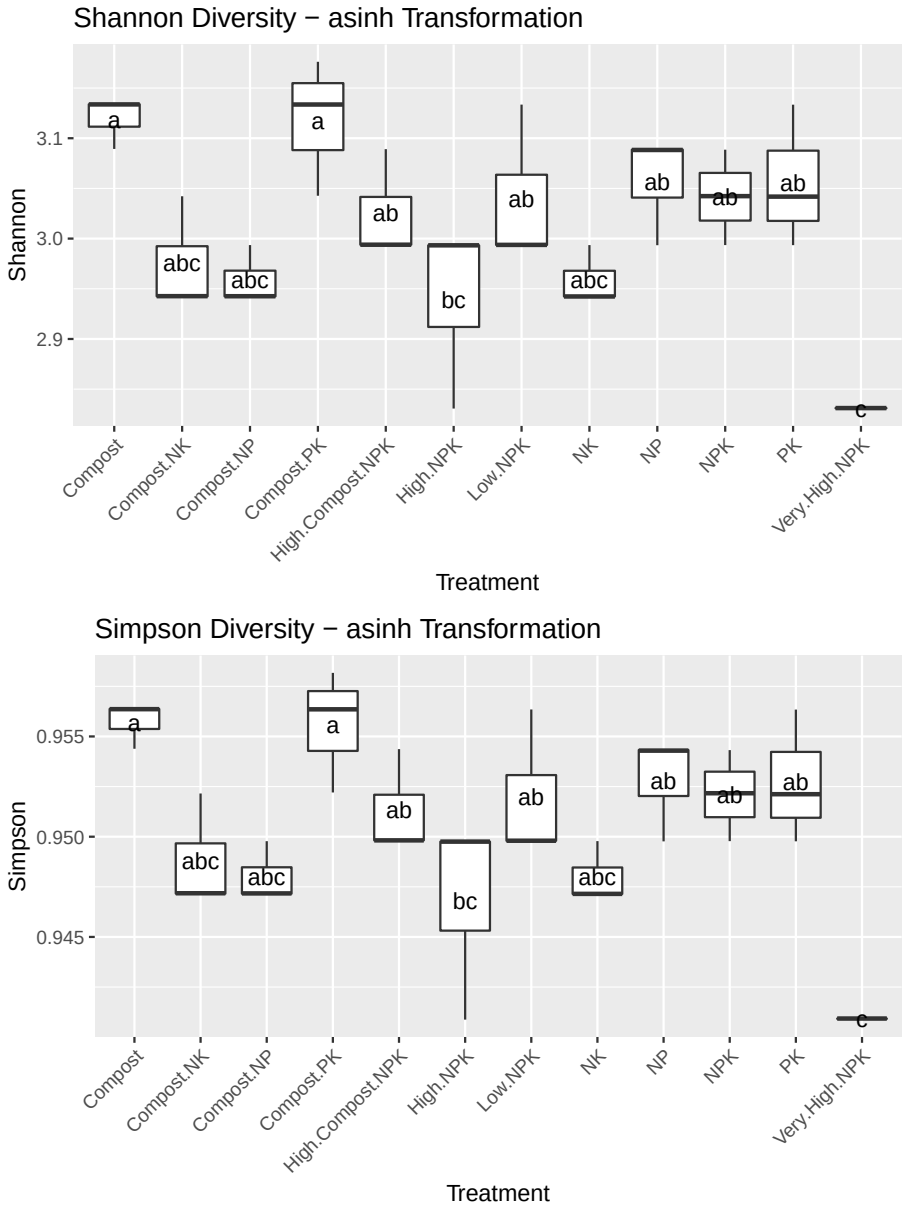

Figure 10: Alpha diversity results using trimmed, *arcsinh* transformed data.

Supplementary Figures

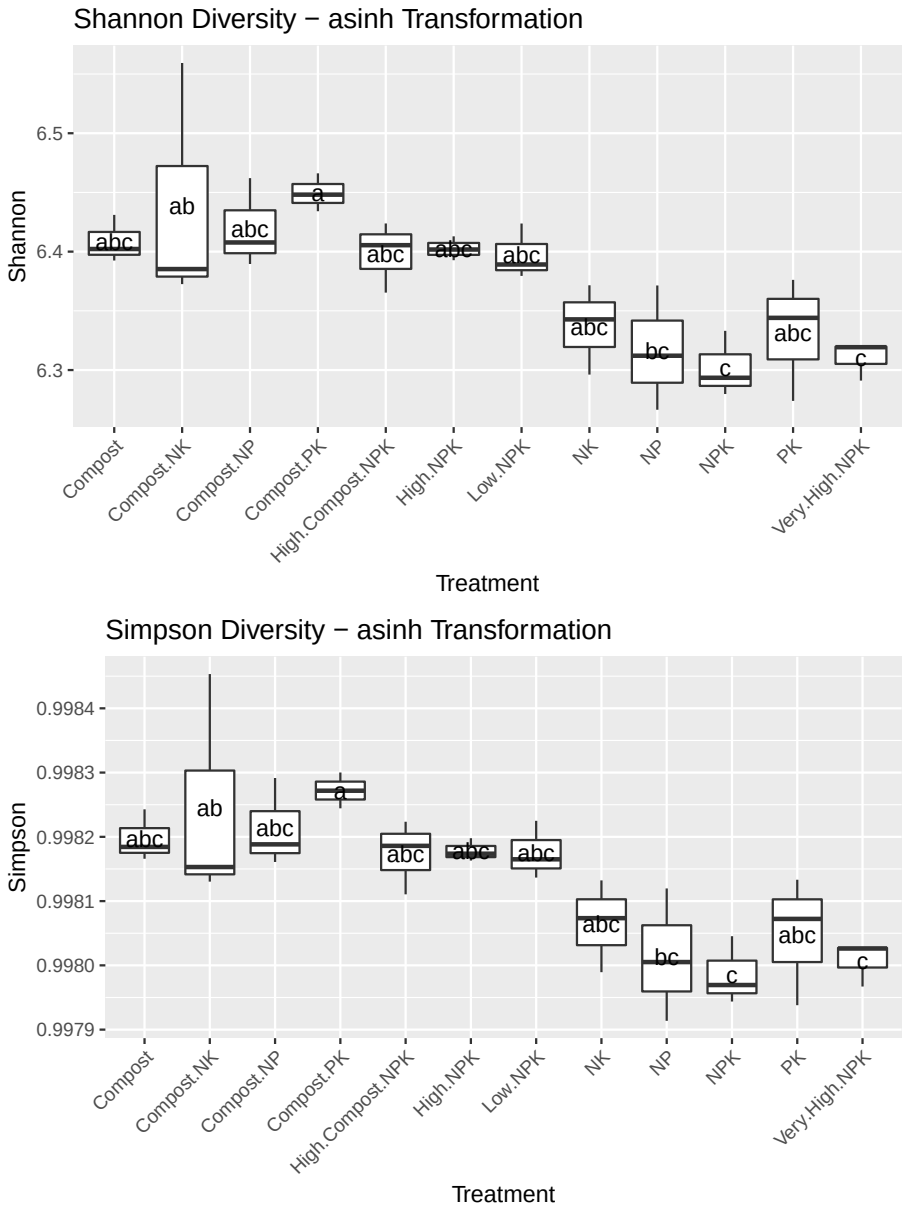

Figure SF.1: Alpha diversity results using untrimmed, *arcsinh* transformed data.

Figure SF.2: Network of microbiome samples using the presence of Phosphorus fertilizer as the label. Edges indicate that the connecting samples have a Bray-Curtis dissimilarity ≤ 0.25.

Figure SF.3: Network of microbiome samples using the presence of Potassium fertilizer as the label. Edges indicate that the connecting samples have a Bray-Curtis dissimilarity ≤ 0.25.

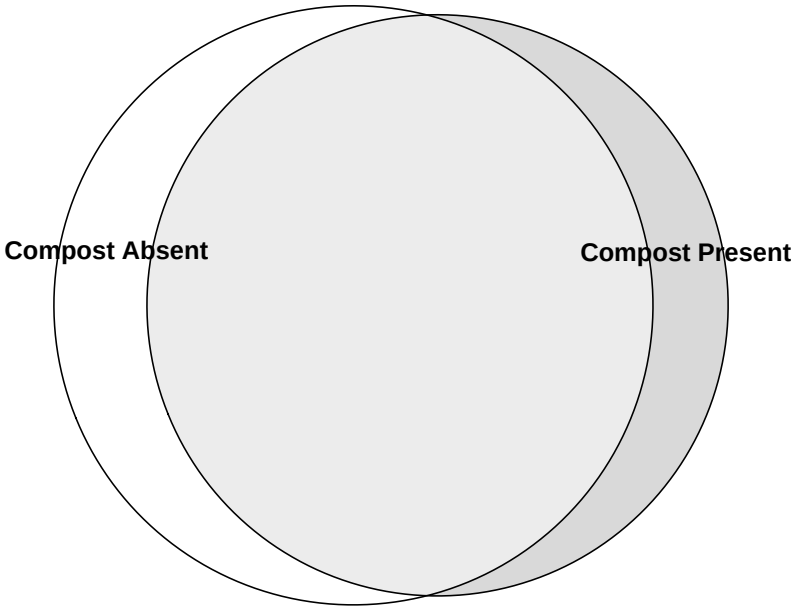

Figure SF.4: Eulerr diagram indicating the proportion of shared genera between samples fertilized with or without compost.

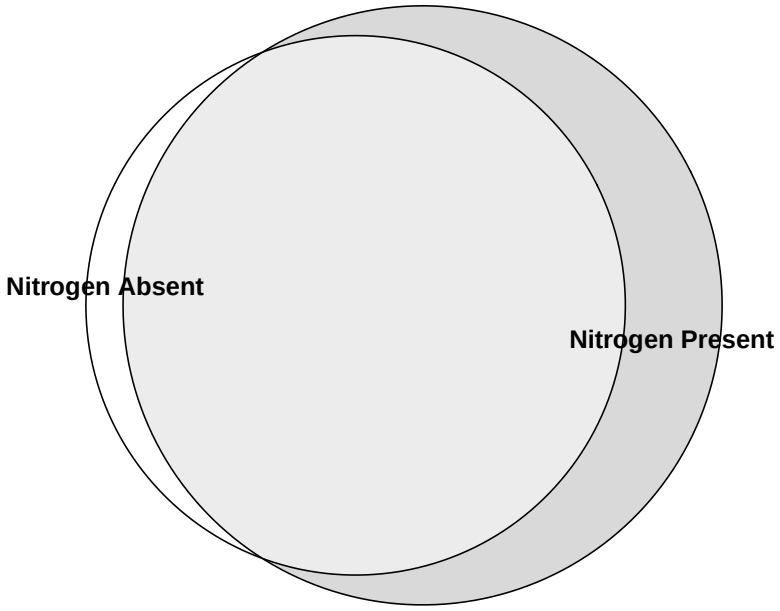

Figure SF.5: Eulerr diagram indicating the proportion of shared genera between samples fertilized with or without Nitrogen.