**1. Introduction**

Practicing agriculture in a way that benefits the environment while supporting the human population requires efficient use of both biological and synthetic nitrogen to prevent environmental degradation from over-use and losses (Tilman et al. 2002)⁠. Legumes offer a sustainable source of fertility to growers, these crops are able to fix abundant atmospheric N through symbiotic relationships with soil bacteria. Legume-rhizobia symbioses are responsible for the fixation of 33-46 Tg of fixed N annually from agricultural systems (Herridge, Peoples, and Boddey 2008)⁠. When legumes such as alfalfa or clover are grown in the year or two prior to maize, the biomass generated releases nitrogen when incorporated and decomposed in the soil. Efficient use of this source of nitrogen is possible when soil microbes successfully decompose and mineralize the organic nitrogen from biomass to meet crop fertility needs. Complex interactions between soil biota and biomass challenge the predictability of mineralization in these systems, limiting efficiency and therefore use of legumes as biological nitrogen sources. To facilitate the use of legumes as sustainable nitrogen sources, research into the bacterial species stimulated in soils during decomposition is needed. Identification of unique bacterial groups responding to amendments will inform our ability to predict and potentially manipulate these communities for improved efficiency and increased use of biological nitrogen sources.

Mineralization and depolymerization of organic nitrogen from legume biomass is a largely biologically mediated process controlling the release of nitrogen in soils. N-bearing polymers must be depolymerized into smaller and lighter forms that can be assimilated by microbes before mineralization of nitrogen can occur. Depolymerization is a function of enzyme production by a range microbial species, the response of which is influenced by the microbial composition and nutrient availability of the soil and amendment. Augmentation and stimulation of species, particularly those involved in decomposition and nutrient cycling, are potential approaches for improving nutrient use efficiency. There is a knowledge gap associated with identification of bacterial response to amendments in alfalfa soils, specifically the response unique to amendment. Identification of bacteria responding during decomposition of amendments and mixtures of amendments will inform our understanding of biologically mediated nutrient cycling. Particularly beneficial is the characterization of the bacterial community at multiple time points during a decomposition event, capturing the temporal dynamics. Additionally, efforts to describe bacteria responding during decomposition will facilitate the generation of target species for bio augmentation and stimulation.

Bacterial response to amendment is shaped by the interaction between soil-enriched and amendment-enriched bacteria and the quality and nutrient availability of the amendment. Despite knowledge of different amendments influencing community structure, there is a knowledge gap regarding the different responses stimulated by amendments in the same soils. Additionally, the temporal dynamics of the bacterial community response during decomposition are not well known. It is likely that a range of species will respond to amendment and these will change over time as compounds are decomposed and nutrients are cycled, shifting the levels of available nutrients and potentially altering the bacterial communities. Further, given the promise of bio-engineering environmental microbiomes through stimulation and augmentation, more knowledge is needed regarding the colonization of soil communities by species originating from amendment. Accurate characterization of responding bacterial communities over time will advance our understanding of how taxonomic groups shift with decomposition, informing bio-engineering approaches.

In this study, we aimed to understand specifically the soil microbial response to organic amendments. Improving our understanding of the biological players involved in nutrient release could help improve agricultural production while still maximizing environmental benefits from complex amendments. We hypothesize that distinct microbial communities respond to varying organic amendments. In this study, we study the impacts of amendments of both alfalfa and compost, provided at 3 equal rates of total nitrogen, to a soil but with differing C: N ratios ….let’s work on this paragraph together!.... immobilization (compost) or mineralization (alfalfa). We characterize both the chemical and microbial response to these amendments and hypothesize that specific microbial communities will respond to initial nitrogen and carbon availability and that this membership will be specific to amendment. We expect that these distinct microbial responders will dominate soil microbial communities in response to organic amendments and will vary in abundance through time. Our objective was to characterize these responding microbial communities for various organic amendments and to identify potential microbial membership within organic amendments that may be involved in plant nutrient availability through amendment decomposition.

**2. Methods**

Three organic nitrogen amendments plus control were chosen based on current use in agriculture and predicted effects on soil nitrogen cycling: (1) an amendment of alfalfa residue with low C: N ratio, simulating plow down of alfalfa hay as a nitrogen source before maize production; (2) stable composted horse manure with a high C: N ratio used as an amendment replicating the use of composted manure on many organic farms; (3) an amendment consisting of a mixture of alfalfa residue and compost was constructed to represent a neutral C: N ratio; and (4) a control treatment receiving no amendment included in the study as a reference.

Alfalfa hay samples were collected following hay harvest and processed by passing fresh hay through a grinder and then through a 2mm mesh screened cyclone mill. Dry alfalfa was then stored in an air-tight vessel prior to use as amendment. Compost samples were collected on September 23rd 2015 from a large windrow of composted horse manure and saw dust bedding, the mixture was composted by the ISU Compost Facility, located at 52274 260th St. Ames, Iowa. Compost was dried, processed, and stored in the same manner as the alfalfa amendment. Soil originated from the USDA-ARS Organic Water Quality (OWQ) research site, situated near Boone, Iowa; on the ISU Agronomy Research Farm. Surface soil (0-15 cm) was taken from alfalfa plots that were in a four-year corn- soybean-oat/alfalfa/alfalfa rotation under organic management. The site was located on the Clarion-Nicollet-Webster soil association with fine-loamy texture soils. A total of 25 kg of soil was taken and processed through a 2 mm sieve, allowed to air-dry and stored in air-tight vessels before use.

Microcosms were constructed by mixing soil and each amendment treatment. Each microcosm consisted of 50g of air dried 2mm soil plus amendment, applied at a rate of 134.55 kg/ha of total nitrogen. The amended and control soil samples were incubated for 97 days under aerobic conditions at 300C in 3.79 l glass jars. During the incubation, samples were aerated every 24 hours by removing the lid and kept moist via addition of deionized water to the bottom of the 1-gallon jar. Soil samples were wetted to 60% water-filled pore capacity and placed into the incubator for a pre-incubation at 300 C before analysis of the first samples on day seven. Samples were subsequently analyzed on day 14, 21, 35, 45, and 97 yielding 336 incubated samples for the four treatments with n = 12 for replicates. Samples representative of Day 0 conditions were constructed by extracting DNA from un- wetted soils mixed in the same ratios as incubated samples.

The bacterial community was characterized by phylotyping using DNA sequencing. Microcosm soils were homogenized during destructive sampling and a sub sample of soil was frozen immediately using dry ice, for preservation until extraction could be performed. DNA extraction was performed using the HTP 96 well power soil kit from Qiagen using 0.25 g of soil. Following extraction, 16S rRNA genes were sequenced on an Illumina MiSeq using 16S V4 primers at Argonne National Lab in Lemont, Illinois. Mothur version 1.41.0 pipeline was used for sequence processing of the 151 bp paired end reads and operational taxonomic units (OTUs) were defined based on 97% genomic similarity. Taxonomic assignment of OTUs was completed by alignment to the most similar representative gene in the Silva 16S ribosomal database version 123.

Microbial biomass was quantified using moist microcosm soil subsampled during destruction of microcosms on sampling days. Microbial biomass carbon was calculated and measured using standard soil fumigation-extraction methods modified for a 20 g sample. Briefly, two sub-samples of 20 g of moist soil from each microcosm were weighed into 50 ml beakers and 125 ml bottles. The 125 ml bottles with 20 g of soil were extracted with 0.5 M K2SO4 and carbon was quantified. This soil fraction represents the non-fumigated portion of the microbial biomass. The 50 ml beakers with 20 g of soil were placed into a fumigation chamber and fumigated with chloroform overnight and extracted after 24 hours with 0.5 M K2SO4. This soil fraction represents the fumigated portion of microbial biomass carbon. Dissolved organic carbon in the filtrate was determined using flow injection technology using a Torch TOC Combustion analyzer (Teledyne Tekmar, Mason, Ohio) and carbon associated with microbial biomass was calculated using the correction factor (k=0.33).

Inorganic nitrogen was determined by extraction with 2.0 M potassium chloride from homogenized moist microcosm soil. Concentrations of NO3 and NH4 were quantified in the filtrate using Lachat Instruments flow injection analyzer (Lachat Instruments, Milwaukee, WI).

Total carbon and nitrogen in microcosm soils was determined using dry combustion analysis of 2 g of air-dry, soil ground with mortar and pestle. Dry homogenized soil was combusted using Thermo Scientific FLASH Elemental Analyzer (Thermo Fisher Scientific, Waltham, MA). The pH of soils was measured potentiometric ally in a 2:1 soil-to-water slurry using a dual electrode pH meter. Soil water content was determined gravimetrically with overnight drying at 105°C.

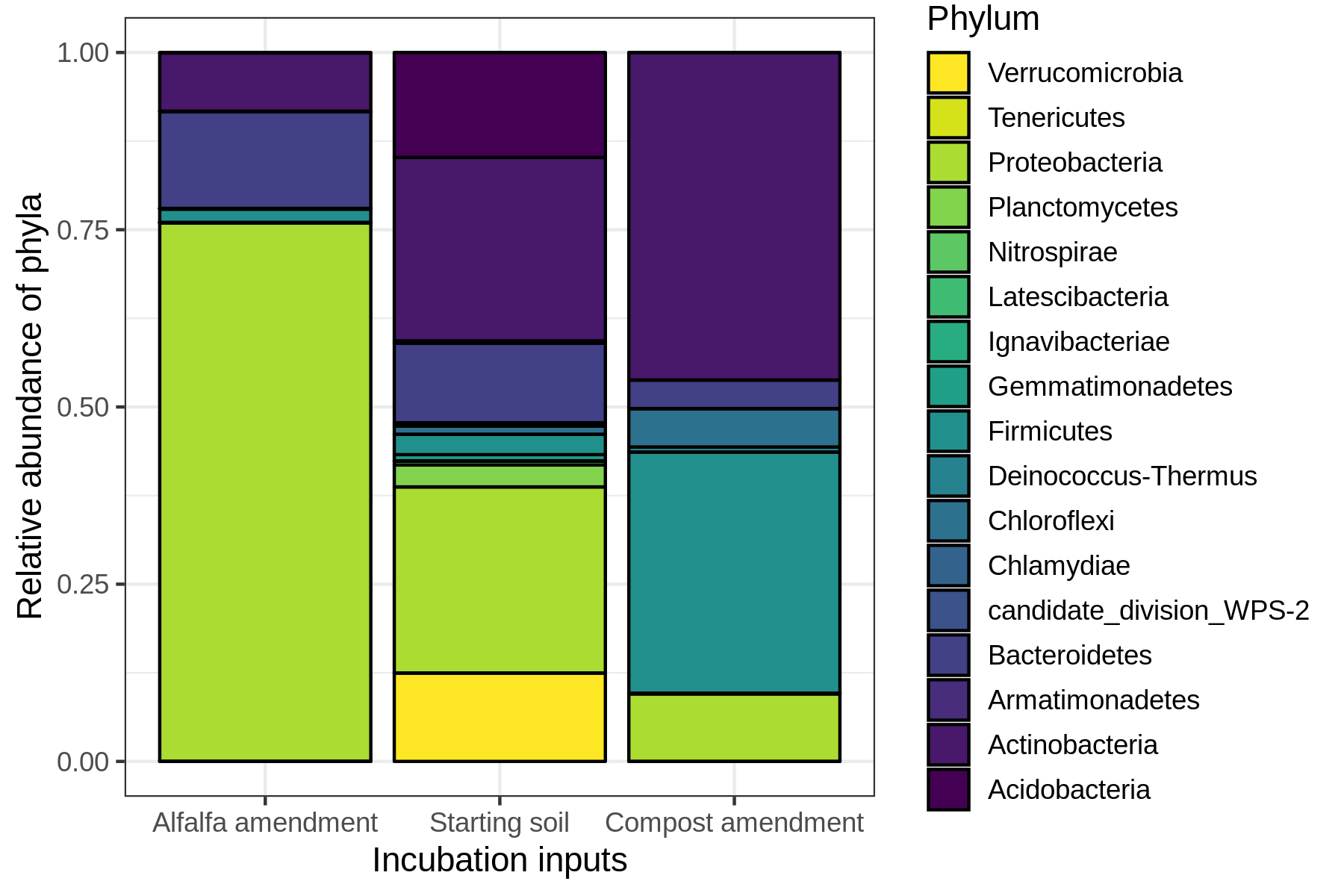
Dissimilarities in the composition of sample bacterial communities were visualized by NMDS ordination of the Bray-Curtis distances between samples. Samples included both the microcosm inputs (i.e., the organic amendments) and the incubated microcosms (i.e., amended soils). NMDS ordinations were performed using the metaMDS() function from the Vegan package in R and were visualized with the plot\_ordination() function from the Phyloseq package. To test if the composition of OTUs was different in samples, we used the adonis() function from the vegan package in R. Adonis() uses a non-parametric multivariate analysis of variance method to test the null hypothesis that there are no differences in microbial communities.

To characterize the impact that environmental variables had on the dissimilarities of communities in each treatment we performed CAP (Canonical Analysis of Principal coordinates) analysis of the environmental variables of inorganic nitrogen, microbial biomass and C: N ratio in conjunction with the Bray-Curtis distance matrix. CAP seeks to display ordinations along with explanatory variables to reduce dimensionality and can reveal patterns in multivariate data with reference to a priori hypotheses.

All environmental variables were fit to a linear mixed effects model using the function lme() from the nlme package in R. Treatment and day were set as fixed effects in the linear model and means were estimated using the emmeans() function from the emmeans package in R. Comparisons were performed between each treatment by each day (i.e., treatment x day). ANOVA was performed on the results from the linear model. A post-hoc test for the ANOVA analysis was performed Tukeys HSD and are reported in a table found in the supplementary data.

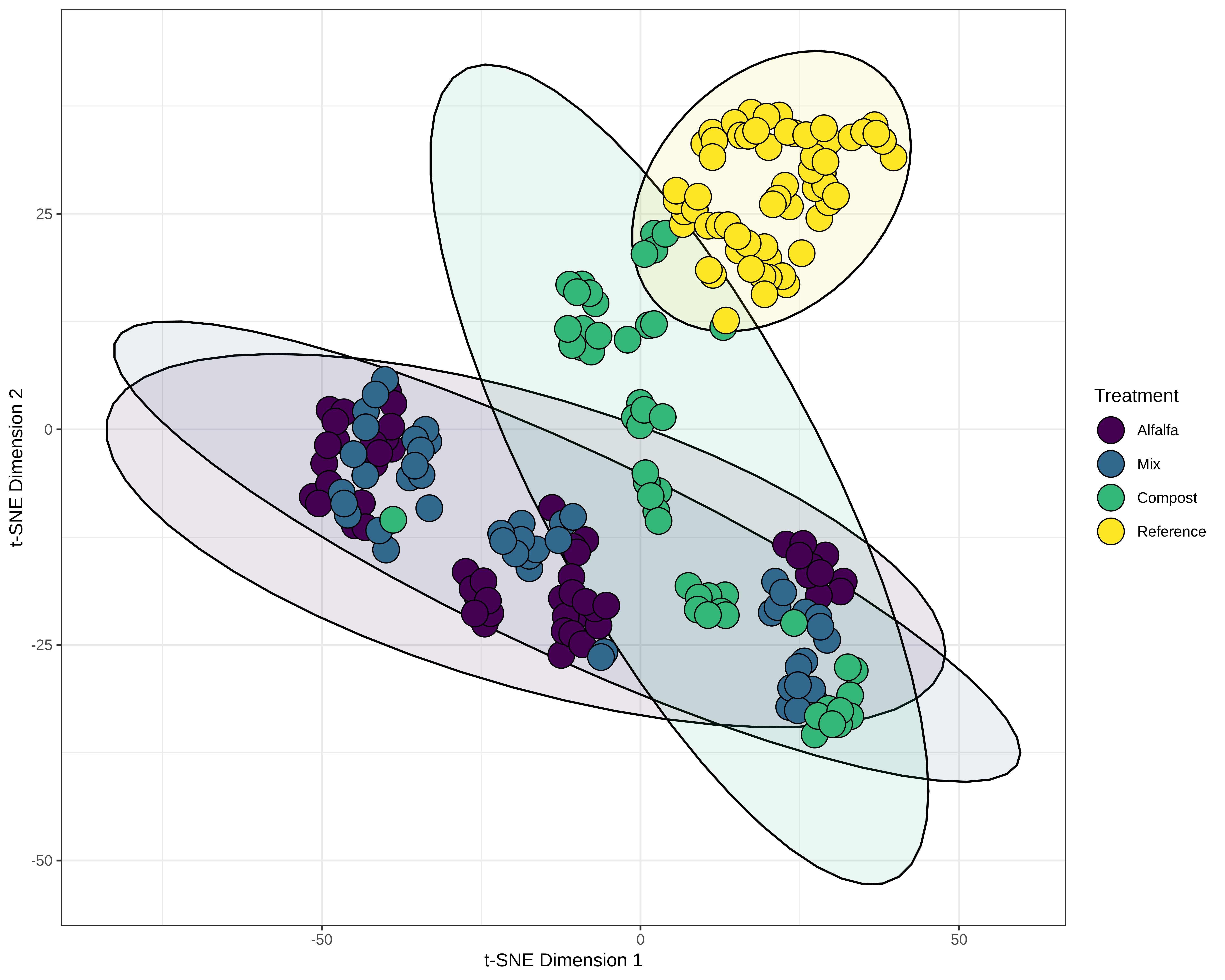
**3. Results**

The nutrients available for each amendment in this study were characterized by the amount of inorganic N, total C, and total N. The alfalfa amendment was highest in inorganic nitrogen concentration at 25.53 ppm and lowest in carbon to nitrogen ratio with 20.14 parts carbon to one-part nitrogen. The compost amendment was lowest in inorganic nitrogen at 13.91 ppm and had the highest C: N ratio at 28.97. It has been shown that a C: N ratio below 25:1 will result in net mineralization while greater than 25:1 will result in net immobilization, suggesting that the alfalfa amendment is expected to result in nitrogen mineralization, and a net increase in inorganic nitrogen compared to reference soils, while the compost amendment is expected to result in nitrogen immobilization and a net decrease in inorganic nitrogen compared to reference soils. Corresponding to differences in C and N content, we observed differences in the soil microbiomes, as defined by 16S rRNA phylogeny, associated with each amendment. Comparing the microbiomes of each amendment, we observed significantly different communities for alfalfa and compost amendments [p-value=0.001, adonis()]. The distribution of phyla from OTUs with abundance greater than 2% in the alfalfa amendment is dominated by unclassified bacteria (sharing no homology to any known phyla) and Proteobacteria, while the compost amendment is dominated by unclassified bacteria, Actinobacteria, and Firmicutes. These amendments are also distinct from the incubation soil used in this study, which is primarily dominated by Acidobacteria, Actinobacteria, and Proteobacteria [[Figure 2](#Image2|graphic)].



Overall, the level of inorganic N in the starting soils were similar and increased over time, with Alfalfa soils had significantly higher inorganic N concentrations on days 14 to 97 than other treatments, and by day 97 had 2-fold greater inorganic N compared to reference soils. Mixed soils initially were observed with lower concentrations of inorganic N but increased to levels comparable to reference soils by day 97. Compost amendments resulted in a significantly lower concentration on all days of the incubation compared to the reference soil and had inorganic N concentrations less than 1 ppm on days 21, 35, and 45.

Microbial biomass provides an indication of the size of the active pool of soil organic matter and increases in microbial biomass indicate microbial utilization of nutrients for metabolic growth and is correlated with nutrient availability. All amended microcosms yielded significantly higher MBC levels than reference control soils. Alfalfa had the most significant positive difference, with a two-three fold higher MBC than reference and achieving the most MBC within the experiment, 780 mg MBC C/kg dry wt. soil on day 14. Reference soils nearly doubled in microbial biomass on day 21 before declining to baseline concentrations on day 97. Mix amended soils had consistently decreasing MBC levels throughout the experiment.

 Differences were observed in the bacterial community composition of incubated microcosms over time and by treatment. For each incubated microcosm, we compared the Bray-Curtis dissimilarity indices between all bacterial communities associated with each sample ([Figure 3](#Image3|graphic)), showing that there are distinct clusters of samples by community similarity for each amendment. These results reveal that the microbial community in reference soils are dissimilar from amended soils, with compost-amended soils being the most similar in composition to reference soils. The alfalfa and mixed amendments had community compositions that were the most comparable throughout the incubation. ~~The vertical spread~~

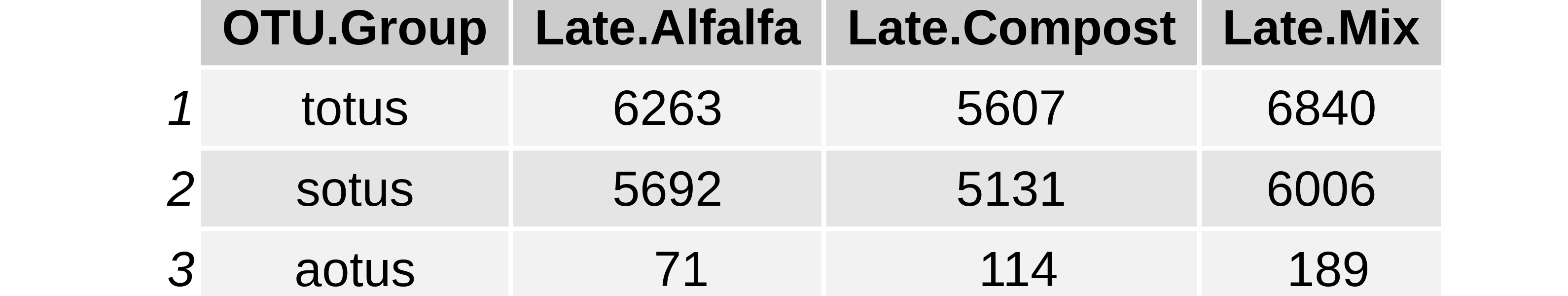
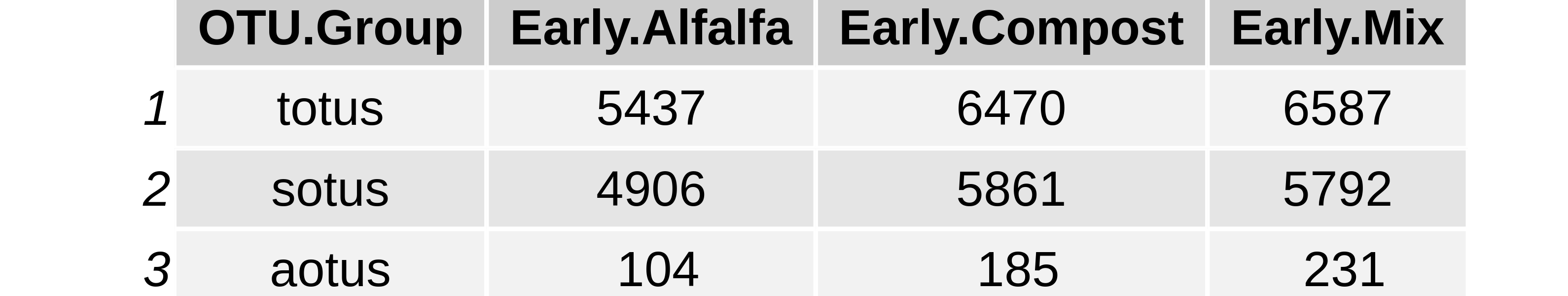
~~of samples along axis 1 suggests that amendment type explains the dissimilarity between treatments, while the clustering of samples by day on the horizontal axis shows that time in incubation also explains variation.~~ Talk about adonis results here, how much dissimilarity was due to treatment vs. day?

To better understand the influence of the various environmental variables that were measured on the microbial community, canonical analysis of principle coordinates was performed on the Bray-Curtis distance matrix, and environmental variable scores were fitted to this ordination. We observed that measurements of C: N ratios and inorganic N concentrations were correlated to community variation along an axis, CAP1 (up to 20% variance explained in mixed alfalfa microcosms). In addition, alfalfa-amended soils also had MBC correlated with this axis. The second CAP axis (CAP2) had weak associations with pH and MBC in all treatments except alfalfa. However, CAP2 was much lower in

percentage explained, between 3.1% to 5.1% for all amendments.

Generally, each amendment is associated with similar microbial communities representing early and late time points within the study. For all incubated microcosms day 7 communities were observed to be the most dissimilar to day 97 communities and are most similar to day 14 and day 21 communities. As the incubation progressed, the dissimilarity between sampling times became more pronounced regardless of treatment. Further, the first three sampling points in time showed the most similar communities, with increasingly different communities after day 21. As a consequence of these observations, we have combined samples for “early” and “late” microbiomes for each amendment, where sample days 7, 14, 21 were defined as early responders and 35, 49, 97 are defined as the late responders (Supplementary data fig 1-4). [need to add some stats to back this up]

We next characterized the microbial composition of early and late responders for each amendment. Additionally, we aimed to differentiate the source of the bacteria, from either the soils or the amendment. After associating samples with either a early or late response group with hierarchical clustering, we next classified OTUs as soil-enriched or amendment-enriched. **Soil enriched is defined as OTUs observed in the reference microcosm communities and not observed in any of the amendment communities. Amendment enriched is defined as OTUs observed in the amendment and not observed in the reference communities**. We used soil or amendment enriched to classify OTUs from the early and late responding communities in the treated and incubated microcosms. Taxa observed less than five times are not included in this characterization and samples were not rarefied to an even depth. The number of total OTUs (totus) observed in each amendments response group in addition to the number of OTUs originating from soil (sotus) or amendment (aotus) are displayed in tables 2 and 3 below.



### Start focus on responding OTUs (lfc > 2 compared to reference) and incorporating some sort of tree

~~A total of 25 and 21 OTUs specific to alfalfa and 43 and 71 specific to compost in the early and late response groups, respectively (Figure 4, tree, barchart?). The presence of these amendment-specific early responding OTUs was next compared across all treatments. We identified OTUs whose membership were significantly more abundant in early and late treated microcosms from each treatment compared to paired reference control soil samples, with at least a log 2-fold relative abundance increase.~~

~~We observed the phylogenetic distribution of OTU to be different across response~~

~~group and treatment for both alfalfa and compost amended microcosms. The 25 OTUs~~

~~unique to alfalfa in the early response group were dominated by OTUs from the~~

~~Proteobacteria and Firmicutes. The late response group in alfalfa was dominated by OTUs~~

~~from Proteobacteria and unclassified bacteria, Firmicutes were not observed in the late~~

~~response group. The compost response groups were also dominated by Proteobacteria in~~

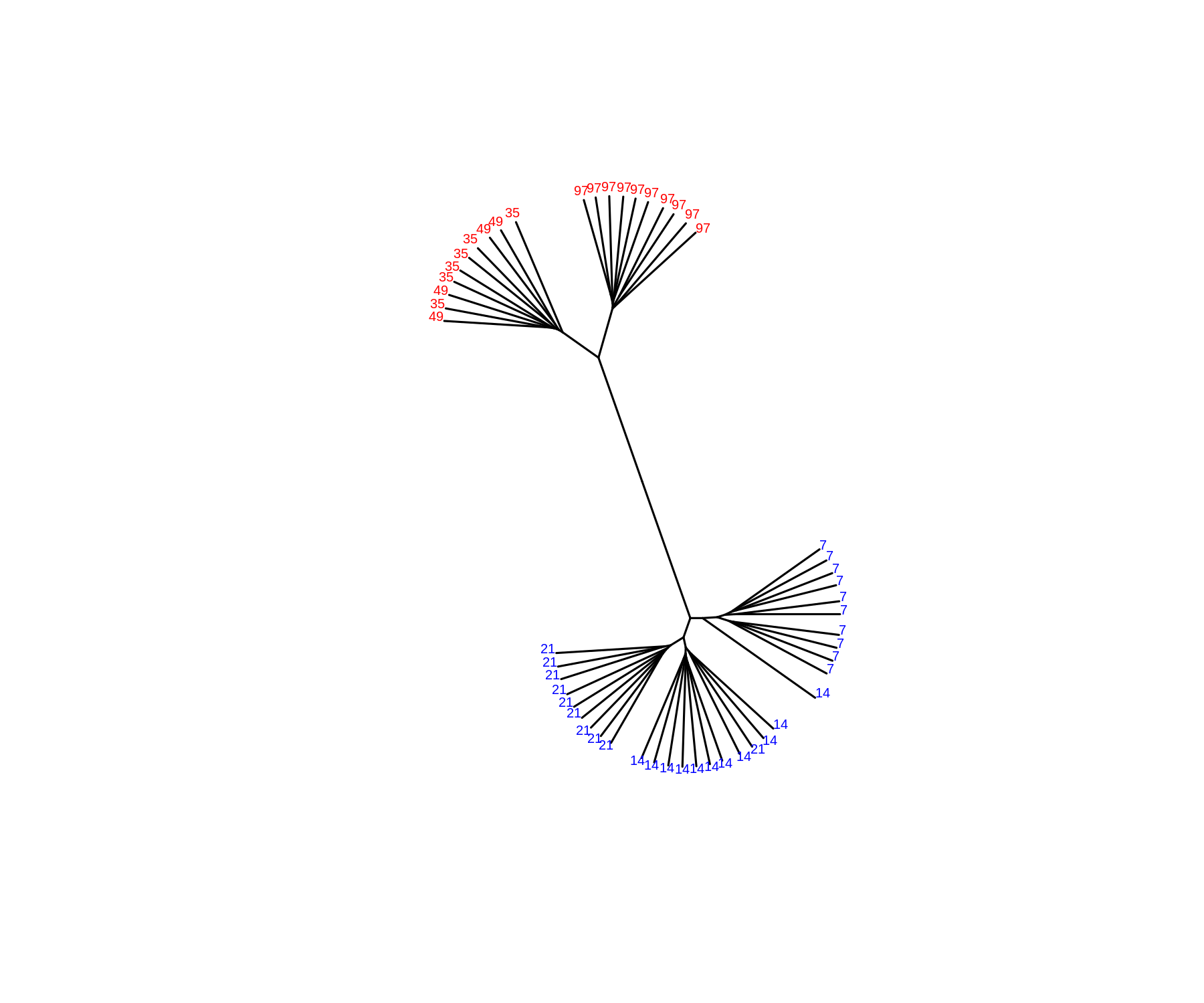
~~both early and late, while Bacteroidetes made up a greater portion of the early responders~~

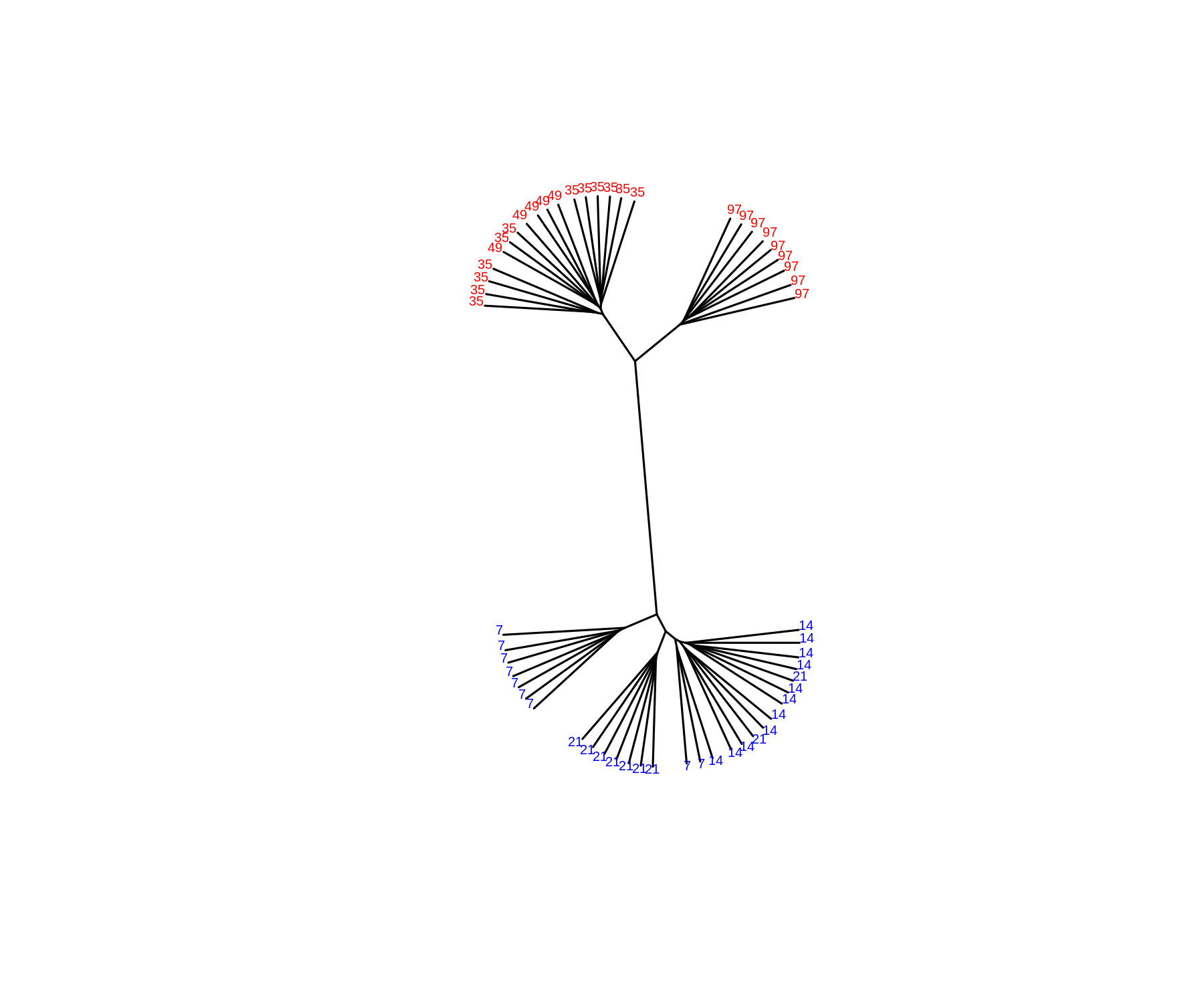
~~than late in the compost. Unclassified bacteria were a large percentage of both response~~

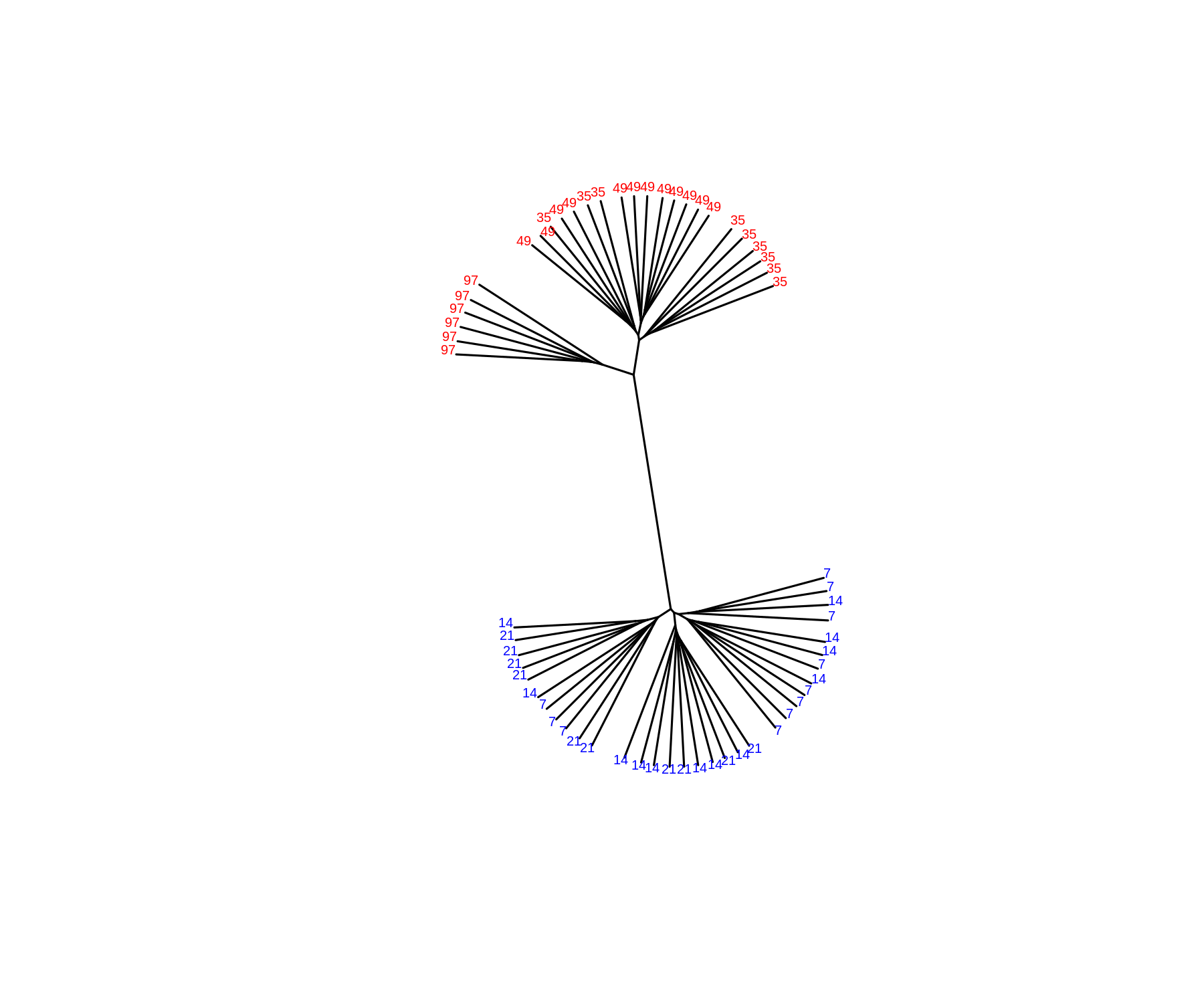
~~groups in the compost treated soil.~~

**4. Discussion**

**5. Supplementary Data**

  
Illustration 2: Compost clustering by day into early and late groups

  
Illustration 3: Alfalfa clustering by day into early and late groups

  
Illustration 4: Reference clustering