

Deciphering Soil Microbial Dynamics in Pennsylvania Grasslands with Goldenrods (*Solidago* sp)

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Abstract

Grasslands are important centers of biodiversity, however these ecosystems have been in decline. Although many methods for grassland restoration have been developed, the abundant microbial communities in these regions are understudied and could be used to assist in these efforts. In this study, we aimed to understand how microbial communities varied by soil type, grassland site, and environmental conditions. Samples were taken from rhizosphere soil, proximal soil (close to the plant roots) and from bulk cores at Ricketts Glen State Park and Nescopeck State Park in Northeastern Pennsylvania during June and August of 2021 and 2022. Rhizosphere soil samples were taken from the common grassland plant, *Solidago rugosa*. 16S rRNA gene sequencing revealed that pH as well as soil type (bulk, proximal, or rhizosphere) significantly influenced the microbial community composition and taxonomic composition of each soil. Each soil type had its own distinct microbial communities, and proximal soil was identified as a transition zone between rhizosphere and bulk microbial communities. We also observed that the rhizosphere communities are dependent upon geography, as these communities were significantly different between grasslands even though the plant species remained the same. Our results highlight the complex nature of soil microbial communities and how many factors, including pH, soil type, and geography, are able to layer together and impact microbial community composition. Results showcase potential avenues for conservation and rehabilitation of diminished grassland regions through modification and regulation of specific soil microbial communities.

Introduction

An important topic in conservation science and ecology is the global decline of grassland ecosystems. Recently these biomes have become one of the most endangered ecosystems in North America [1]. These regions provide essential ecosystem services, such as carbon sequestration, biofuel generation, and water storage and filtration [2]. Grasslands also contain cultural value, provide areas for recreational usage, and are centers of biodiversity, specialized to house various species of birds, small mammals, reptiles, and insects [2]. However, grasslands have begun to decline as a result of agriculture, cattle grazing, urbanization, and global climate change. In the northeastern United States, grasslands have become particularly rare from the result of a lack of disturbance (e.g., fire and grazing), which results in the conversion of grasslands into forests [3]. Due to this decline only about 20% of North American grasslands are still intact [4]. Pennsylvania grasslands are currently a high priority region for restoration and much effort to restore and maintain them is underway [5].

Restoration of grasslands can be aided by understanding the relationship between soil microbial communities and grassland plants [6, 7]. Studies have shown that grassland environments have a high abundance of soil microbes [8, 9]. A study conducted by Szoboszlay et al. (2017) indicated that grassland soils have significantly more bacterial numbers than croplands and in a similar study conducted by Kaiser et al. (2016) it was shown that bacterial communities were significantly more diverse in grasslands than in forests [8, 9]. Within grassland soils, microbes are able to regulate soil organic matter content through decomposition and nutrient cycling, they can impact soil stability, plant

productivity, and overall biodiversity [9–14]. Soil microbes are essential for soil health, and in turn plant health, as microbes partake in up to 90% of soil ecosystem functions [15]. Previous studies have found that increases in microbial biomass and activity in grassland soils cause an increase in the organic matter and biodegradability within the soil [16]. However, soil microbial communities can be sensitive to changes in the environment and their composition and diversity are highly variable based on a variety of environmental factors such as temperature, soil depth, soil nutrient concentrations, and pH [17–21]. Previous studies have shown that out of all soil factors, pH contributes the most to variation in bacterial richness and diversity (45.9%) and there is a significant negative correlation between soil pH and bacterial community diversity in low depth soils (0-20cm) [22].

Due to the dynamic role that soil microbial communities play in soil environments they are able to form a complex relationship with grassland plants where they are able to influence plant diversity and productivity [23]. Microbes are able to affect nutrient acquisition in soil environments which allows them to regulate primary nutrients available to various species of plants [24]. Soil microbes supply up to 20% of nitrogen and 90% of phosphorus that the plant will need [24]. The microbes not only enhance plant productivity in grasslands by two-fold but also help protect the plant against heavy metal toxicity and drought [25]. In addition to soil microbial communities influencing plant diversity, studies have shown that aboveground plant communities strongly impact the belowground microbial communities, which varies based on vegetation type, plant diversity, and productivity [26, 27].

Studies on soil microbial communities and plants show the diverse interactions that occur between them are concentrated in the rhizosphere region. The rhizosphere is the area of soil that is directly influenced by the root exudates of plants and is commonly associated with soil microbes [23, 28]. The rhizosphere microbial community composition is determined by bulk soil, soil collected with a soil corer that is not associated with any one plant [29, 30]. The root exudates will recruit certain microbes from the surrounding bulk soil in higher concentration in the rhizosphere region, where many will form a mutualistic relationship. The rhizosphere region has a different physical, chemical, and biological composition than the surrounding bulk soil, which contributes to the different species of microbes found in this region [23]. The roots release substances such as sugars, amino acids, glycosides, organic acids, vitamins and enzymes [31, 32] which are utilized by the associated microbes, stimulating growth and production [23, 31]. However, root exudates vary by species, stage of growth, and environmental conditions, impacting the rhizosphere composition [31, 32]. Consequently, changes in plant community structure can affect the size, composition, and functionality of the rhizosphere associated microbial communities [27].

Much remains unknown about the complex relationship between soil microbial communities and plants in grassland ecosystems. Our study examines the composition and abundance of soil microbes in northeastern Pennsylvania grasslands in relation to the grassland plant, *Solidago rugosa*, commonly known as wrinkleleaf or rough-stemmed goldenrod, under varying environmental conditions. *S. rugosa* was selected because it is a common North American grassland plant that was present at all sampling locations. This study investigates how microbial community composition and diversity changed spatially

within a grassland from rhizosphere, to proximal (close to plant roots), to bulk soil. We also wanted to determine if the rhizosphere microbial community associated with *Solidago rugosa* was consistent across grasslands and how environmental factors, such as soil pH and organic matter content, influenced community composition in this region.

Methods

Site Descriptions

Soil samples were collected from two grasslands in northeastern Pennsylvania, Ricketts Glen State Park located at 41.3354° N, 76.3014° W and Nescopeck State Park located at 41.0911° N, 75.8798° W. Samples from Ricketts Glen State Park were collected in June and August of 2021 and 2022. Samples from Nescopeck State Park were collected in June and August of 2022. Both sites were located on lands that were previously pastures. However, Ricketts Glen State Park is ~ 700 m above sea level and Nescopeck State Park is at ~ 300 m above sea level. Woody vegetation is not actively controlled at Ricketts, though fires occasionally burn the vegetation at the site (*personal communication, park employees*). At Nescopeck, encroaching woody vegetation is primarily composed of two invasive species, Russian olive (*Elaeagnus angustifolia*) and Tatarian honeysuckle (*Lonicera tatarica*), which are controlled by targeted herbicides. The primary cover at Ricketts Glen State Park is a mixture of graminoids and herbaceous plants, whereas Nescopeck State Park is primarily covered in warm season grasses [33].

Field collection of plants and soil microbes

Within each grassland there were two sampling sites each 10 m by 10 m, for a total of four sites. At each site, three distinct soil types were collected: bulk, proximal, and rhizosphere. Bulk soil was defined as soil with no particular association with one plant. Within each site three bulk soil samples were collected using a sterilized soil corer up to 10 cm deep. Next, three *Solidago rugosa* plants were randomly selected from within the 10 m by 10 m plots, extracted using a sterile shovel, and placed inside a sterile WhirlPak bag. *Solidago rugosa* plants were identified using a standard field guide, Newcomb's Wildflower Guide [34]. To collect the proximal soil, each extracted plant was vigorously shaken to remove excess soil from the roots. This soil was also placed into a WhirlPak bag. To collect the rhizosphere soil, sterile scissors were used to cut the roots of each plant, which were placed inside a sterile 50 mL conical tube filled with a prepared epiphyte buffer solution of 6.75 g of KH_2PO_4 , 8.75 g of K_2HPO_4 , and 1 mL of Triton X-100 [35]. Roots, proximal soil, and soil cores were placed on ice in the field and then transferred to the - 80°C freezer for storage.

For the bulk soil samples, soil pH and percent organic matter content were measured. To measure pH, 15 g of soil and 15 mL of DI water were placed in a sterile 50 mL conical tube and allowed to acclimate for 15 minutes. A standardized pH probe was then used to measure pH. To measure the percent organic

matter in each sample, 1g of soil was placed in a weighted crucible and dried for 24 hours at 105°C. Samples were then reweighted and placed in a muffle furnace for 16 hours at 450°C. Percent organic matter content was then calculated based on the mass lost after drying. To determine plant species abundance at each sampling site, eight 1 m x 1 m quadrats were placed inside each sampling site. Each plant species present within the quadrats was identified using a standard field guide and average percent abundance of each species, including *S. rugosa*, was documented.

DNA Extraction and Amplification

A sterilized 2 mm sieve was used to sieve proximal soil prior to DNA extraction. Bulk soil cores were separated into two depths: 0–5 cm and 5–10 cm and a 4 mm sieve was used to sieve samples prior to DNA extraction. To extract the rhizosphere soil, the 50 mL conical tubes containing the plant roots and buffer were shaken for 45 minutes at 150 RPM at 20°C. After shaking, flame sterilized tweezers were used to remove the roots from the buffer solution and the tube was resealed. The tubes were then centrifuged for 10 min at 3000 rpm to pellet the soil. After soil was pelleted, the excess liquid was removed and DNA was extracted. DNA was extracted from bulk, proximal, and rhizosphere samples using the Qiagen Powersoil Pro kit (Qiagen, Germantown, Maryland) following manufacturer instructions.

PCR was performed in triplicate to prepare samples for 16S rRNA gene sequencing. The primers used for amplification focused on the variable V4 region of the 16S rRNA gene (515F and 806R) [36]. Samples were run using the following thermocycling program: initial denature at 94°C for 3 minutes, then 35 cycles of 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 1.5 minutes and the final extension at 72°C for 10 minutes. The concentration of PCR reactions were determined for each sample using a Qubit fluorometer and samples were then combined into a single tube in equimolar concentrations and sent for sequencing at University of Connecticut Center for Genome Innovation. A total of 122 samples were sent for sequencing, including 82 samples from Ricketts Glen State park and 40 samples from Nescopeck State park. This included 50 bulk soil samples, 36 proximal soil samples, and 36 rhizosphere soil samples. Two samples were removed after sequencing due to low quality sequences. These were both from Nescopeck state park: SS428 from the proximal soil and SS363 from the rhizosphere soil.

Amplicon Sequence Analysis

The 16S rRNA gene sequence data was analyzed using the R environment (v4.3.3) [37]. The DADA2 package in R was used to quality check sequences, remove chimeras, and determine ASVs (Amplicon Sequence Variants) [38]. ASVs were classified using the SILVA rRNA database (v. 132) [39]. After taxonomic classification, sequences identified as chloroplast and mitochondria were removed. An ASV table was constructed to determine the abundance of each ASV across samples. Samples with a low number of sequences were removed from analyses. Alpha and beta diversity (Bray-curtis) metrics were run using the Vegan package in R [40]. All samples were rarified to the sample with the lowest number of sequences (n = 4100). Permutational Multivariate Analysis of Variance (PERMANOVA) tests were run

with the pairwise function to determine significance between groups using the Vegan package in R [40]. Similarity Percentage (SIMPER) analysis was used to determine which ASVs were driving any significant differences that were observed using the Indicspecies package in R with minsta = 0.6 [41]. Complete workflow for data analysis in R is available at: <https://github.com/liliez13/Soil-Sample-R-Script.git>. Raw sequence data are available through the NCBI SRA database under study accession number PRJNA1146467.

Results

Overall, 5,870,875 sequences were obtained from 120 samples after removal of samples with a low number of sequences. This resulted in 35,526 ASVs. Our results showed that each soil type, sampling site, and soil pH range had their own distinct microbial communities. The rhizosphere communities of *Solidago rugosa* were not the same across grassland sampling sites, as differences in geographic location and soil conditions shaped these microbial communities.

Fifty bulk soil samples were averaged across all months and years to get the basic soil conditions at each site. These basic soil conditions were variable across the four sampling sites (Ricketts Site 1 & 2 and Nescopeck Site 1 & 2). The pH of Ricketts Site 2 (pH:4.3 ± 0.2) was the most acidic compared to the other three (pH: 5.5 ± 0.3). Percent organic matter content was variable across the sampling locations and throughout the sampling season (June vs. August).

Table 1
Average grassland soil conditions of pH and percent organic matter content of each sampling site taken from bulk soil only.

Sampling Site (# of Samples)	pH	Organic Matter Content June (%)	Organic Matter Content August (%)
Ricketts Site 1 (23)	5.8 ± 0.6	14.8 ± 4.6	15.7 ± 7.9
Ricketts Site 2 (11)	4.3 ± 0.2	5.4 ± 3.8	11.1 ± 10.1
Nescopeck Site 1 (8)	5.3 ± 0.4	20.7 ± 2.3	34.5 ± 4.7
Nescopeck Site 2 (8)	5.5 ± 0.3	18.31 ± 3.8	27.8 ± 9.4

Plant species abundance data also showed variation across grassland sampling sites. Ricketts Site 2 was the only site with lowbush blueberry (*Vaccinium angustifolium*) and Nescopeck Site 1 and 2 were the only sites with Big Bluestem (*Andropogon gerardii*), a warm season grass. Analysis of the plant species at each sampling location showed that Goldenrod (*Solidago sp.*) were present at all sampling locations (Figure S1). Goldenrod species accounts for 6.5% of the community at Ricketts Site 1, 16.2% of the community at Ricketts Site 2, 60.5% of the community at Nescopeck Site 1 and 40.3% of the community at Nescopeck Site 2.

Taxonomic composition of the microbial communities was determined for each soil type and sampling site (Fig. 1). Figure 1a represents the bacterial phyla identified. In general, there was a higher relative abundance of Acidobacteriota in the bulk soil (22.8%) compared to the proximal soil (16.2%) and rhizosphere soil (14.5%), and a higher relative abundance of Proteobacteria in the rhizosphere soil (34.0%) than the proximal soil (27.5%) and bulk soil (26.7%). Since there was similarity at the phylum level, we also examined the order level to get a finer scale analysis (Fig. 1b). In general, we saw a higher relative abundance of *Rhizobiales* in the bulk soil (15.6%) than the proximal soil (11.7%) and rhizosphere soil (12.6%). Within the sampling sites we saw that there was a higher relative abundance of *Chthoniobacterales* in Nescopeck Site 1 and 2 than Ricketts Site 1 and 2. It was also determined that Ricketts Site 2 had a higher relative abundance of *Isosphaerales* and Acidobacteria Subgroup 2 than Ricketts Site 1 and Nescopeck Site 1 and 2. Subgroup 2 Acidobacteria and *Isosphaerales* prefer more acidic environments making Ricketts Site 2 optimal for these bacteria [44]. The bulk soil at Ricketts Site 2 had the highest abundance of *Esterales* compared to all other soil types and sampling sites. Unfortunately, little is documented on *Esterales* and its soil environment preferences. Ricketts Site 1 had a higher relative abundance of *Burkholderia* than Ricketts Site 2 and Nescopeck Site 1 and 2. *Burkholderia* is a common soil bacterium that prefers soils with a higher pH [45].

Shannon diversity and species richness were calculated for each sample. Results showed no significant difference across soil types (Shannon Diversity: $p = 0.423$, $df = 2$, Species Richness: $p = 0.521$, $df = 2$), pH ranges (Shannon Diversity: $p = 0.234$, $df = 1$, Species Richness: $p = 0.342$, $df = 1$), or sampling site locations (Shannon Diversity: $p = 0.128$, $df = 3$, Species Richness: $p = 0.266$, $df = 3$) (Table S2). Interestingly, even though Shannon diversity was not significantly different across soil types, a lower diversity can be observed from bulk to proximal to rhizosphere soil, as evidenced in Fig. 2. The same trend is seen for species richness (data not shown). These results are consistent with the knowledge that the plant is recruiting certain bacteria from the bulk soil for the rhizosphere soil (Table S2).

Beta diversity analysis of all 120 soil samples collected showed that microbial community composition was significantly different across the four sampling sites (Figure S2, PERMANOVA: $p = 0.01$, $df = 3$). After running a pairwise PERMANOVA it was revealed that the driving factor behind this difference was Ricketts Site 2, which had a significantly different microbial community from all other sites. This difference in community composition between Ricketts Site 2 and the other sites can be attributed to pH, as this site has a distinctly lower pH (4.3 ± 0.2) than Ricketts Site 1 (5.8 ± 0.6), Nescopeck Site 1 (5.3 ± 0.4) and Nescopeck Site 2 (5.5 ± 0.3). To test this, samples were separated based on bulk soil pH. The pH range of proximal and rhizosphere soil was assumed based on the bulk soil from that site, as previous studies have revealed only slight differences between rhizosphere and bulk soil pH from the same sampling location [42, 43]. There was a significant difference in the microbial community composition based on pH (Figure S2, PERMANOVA: $p = 0.03$, $df = 1$). A spearman rank correlation also showed that bulk soil pH strongly influenced microbial community composition ($r_s=0.619$).

Analysis of all 120 samples also showed a significant difference in microbial community composition based on soil type (PERMANOVA: $p = 0.002$, $df = 2$). Specifically, between bulk and proximal soil

(PERMANOVA: $p = 0.008$) and bulk and rhizosphere soil (PERMANOVA: $p = 0.003$). However there was not a significant difference in composition between proximal and rhizosphere soil (Fig. 3a). To account for the influence of soil pH, an additional analysis was run separating the samples into the pH categories, $pH \geq 5$ and $pH < 5$. Similar to the analysis of all samples, analysis of the 88 samples with $pH \geq 5$ showed that there was a significant difference between soil types (PERMANOVA: $p = 0.001$, $df = 2$). Pairwise analysis revealed that there was a significant difference between bulk and proximal soil ($p = 0.003$) and bulk and rhizosphere soil ($p = 0.0003$), however there was not a significant difference between proximal and rhizosphere soil (Fig. 3b). Analysis of the 32 samples with $pH < 5$ showed that there is a significant difference between soil types (PERMANOVA: $p = 0.042$, $df = 2$). The nMDS showed this separation, however pairwise PERMANOVAs were not significant (Fig. 3c; Table S1).

Overall microbial community composition was significantly different between the four sampling sites (PERMANOVA, $p = 0.02$, $df = 3$), but pairwise comparisons did not show significance (Supplementary Table 1). However, when samples were first separated by soil type (bulk, proximal, rhizosphere), the geographic differences in community composition were more clear. For example, within bulk soil communities, microbial community composition was significantly different across the four sampling sites (PERMANOVA, $p = 0.004$, $df = 3$). The same was seen across the rhizosphere (PERMANOVA, $p = 0.001$, $df = 3$) and proximal (PERMANOVA, $p = 0.001$, $df = 3$) groups (Table S1).

To better visualize the microbial community composition, ASVs were classified by soil type and sampling location based on maximum average relative abundance in pooled sequences (Figure S3). For example, if an ASV's average relative abundance was highest in bulk soil it was classified as a bulk ASV. These results revealed that each soil type had their own distinct ASVs, however each class of ASV was occurring in all soil types. Notably there was a decrease in bulk soil ASVs as soil changed from the bulk soil (79.99%) into proximal soil (14.72%) and finally into rhizosphere soil (6.35%). Additionally, there was a decrease in rhizosphere ASVs as the soil changed from rhizosphere soil (90.10%) to proximal soil (34.58%) and then to bulk soil (16.23%) (Figure S3a). The results also revealed that each grassland had its own distinct ASVs. This was most evident in Ricketts Site 2, the site with the lowest pH. At Ricketts Site 2, 89.6% of the microbial community was comprised of Ricketts Site 2 ASVs. At Ricketts Site 1, 86.1% of the microbial community was comprised of Ricketts Site 1 ASVs. Interestingly, Nescopeck Site 1 and 2 shared more ASVs than the Ricketts Sites, potentially due to more similar environmental conditions between sites (Table 1). Nescopeck Site 1 was composed of 66.0% Nescopeck Site 1 ASVs and 17.4% Nescopeck Site 2 ASVs. Nescopeck Site 2 was composed of 84.5% Nescopeck Site 2 ASVs and 4.5% Nescopeck Site 1 ASVs (Figure S3b).

Analysis of the abundance of specific ASVs across samples using an indicator analysis (SIMPER) allowed for a more in-depth analysis of the different microbes identified across soil types and sampling sites. Specifically looking at soil type, ASV303 and ASV110 were found to have a higher relative abundance in the rhizosphere soil compared to the proximal or bulk soil (Fig. 4). ASV303 was identified as a microbe from the phylum Latescibacterota and ASV110 was identified as *Starkeya sp.* from the phylum Proteobacteria based on BLAST searches of the representative sequences for each. Even though

we discovered that pH was contributing to differences in microbial community composition, these two ASVs were ubiquitous and transcend soil pH (ASV303 average relative abundance: pH $\geq$ 5 1.026%, pH < 5: 0.754%; ASV110 average relative abundance: pH $\geq$ 5: 0.369%, pH < 5: 1.024%), showing these microbes were able to survive varying environmental conditions and thus are important for the rhizosphere of *Solidago rugosa* plants.

Since only two indicator ASVs were identified in the rhizosphere soil across all samples, we separated samples by pH (pH $\geq$ 5 or pH < 5) to determine soil type indicator ASVs. This revealed distinct indicators for the rhizosphere, proximal, and bulk soil in each of the pH categories. In soils with a pH $\geq$ 5 there were 17 indicators for rhizosphere soil, 7 indicators for proximal soil, and 1 indicator for bulk soil. In soils with a pH < 5 there were 7 indicators for rhizosphere soil, 57 indicators for proximal soil, and 59 indicators for bulk soil. Some rhizosphere indicators for soils with a pH $\geq$ 5 were ASV406, ASV236, and ASV116 (Fig. 5a). After BLAST analysis of each representative sequence, ASV406 was identified as a member of the family *Chitinophagaceae*, which belongs to the phylum Bacteroidetes, ASV236 was identified as *Candidatus Udaeobacter* from the phylum Verrucomicrobia and ASV116 was identified as *Pseudarthrobacter* sp. from the phylum Actinobacteria. Some rhizosphere indicators for soils with a pH < 5 were ASV463, ASV904 and ASV818 (Fig. 5b). After BLAST search of each representative sequence, ASV463 was identified as *Pseudomonas* sp. from the phylum Proteobacteria, ASV904 was identified as *Succinivibrio* sp. from the phylum Proteobacteria and ASV818 was identified as a member of the family *Rhodospirillaceae* from the phylum Proteobacteria.

Analysis was also performed on the entire dataset to determine if there were any ASV indicators for each grassland. There were 33 ASV indicators within both Nescopeck sampling sites and 3 ASV indicators within both of the Ricketts sampling sites (Fig. 6). Some of the indicators for the Nescopeck sites were identified as ASV24, ASV30 and ASV31. After a BLAST search of the representative sequences, ASV24 was identified as *Bacillus* sp., ASV30 was identified as *Stenotrophomonas*, and ASV31 was identified as *Acinetobacter*. The indicators at the Ricketts sites were identified as ASV16, ASV10 and ASV82. After a BLAST search of the representative sequences, ASV16 was identified as *Candidatus Xiphinematobacter*, ASV10 was identified as *Xanthobacteraceae* and ASV82 was identified as *Elsterales*. These results indicate that each grassland has its own distinct microbial community composition.

Discussion

This study revealed many of the interactions between grassland plants and their soil microbial communities. The focus of this study was on the microbial community composition and diversity associated with *Solidago rugosa* across different grasslands, soil conditions (pH and organic matter content) and soil types (bulk, proximal, and rhizosphere). Our results highlight the complex nature of soil microbial communities and how many factors layer together to influence microbial community composition within soil environments. The key findings of our study showed that soil pH is one of the most important environmental factors influencing microbial community composition. However, when samples were separated by pH, there was a significant difference in microbial communities based on

soil type, with proximal soil communities being the transition zone between bulk and rhizosphere soil environments. Additionally, when samples were then separated by soil type, geography (grassland site) was shown to impact microbial community composition and abundance allowing us to highlight that *Solidago rugosa* recruits microbes from the surrounding bulk soil into the rhizosphere. Together, these results show that although the plant species remained the same, the rhizosphere microbial communities significantly changed and these variations were based on the composition of the bulk soil from each grassland site.

Our results indicate that soil pH is one of the most influential factors behind microbial community composition and abundance ($r_s=0.619$). Even across the different soil types and geography, pH has a dominating influence on soil microbial community composition and abundance [46–49]. Our results were congruent with previous studies that have shown that pH has a strong influence on microbial communities [48–52]. Within the sampling sites with a pH below 5 there was a higher abundance of Isosphaerales, Acidobacteriales, and Acidobacteria Subgroup 2 (Fig. 1b). This was consistent with our indicator analysis that revealed ASV387 (Isosphaerales), ASV102 (Acidobacteriales) and ASV281 (Acidobacteria Subgroup 2) to be more abundant in soils with a pH below 5. Isosphaerales is an order of Planctomycetes that is commonly found in low pH soils [53] and Acidobacteriales and Acidobacteria Subgroup 2 are both orders of Acidobacteria and are commonly associated with lower pH environments [54–58]. Acidobacteria Subgroup 2 was more specifically found in higher abundances in the bulk soil for samples with a pH below 5. Previous studies have shown that Acidobacteria subgroups tend to be more abundant in bulk soil environments compared to the rhizosphere soil, however we lack a better understanding of the functions of a majority of Acidobacteria Subgroups [57–59]. Our results also revealed that the pH at Ricketts Site 2 ($\text{pH}: 4.3 \pm 0.2$) was the most acidic compared to the other three sites ($\text{pH}: 5.5 \pm 0.3$) which influenced the difference we observed in microbial community composition and plant species abundance at Ricketts Site 2. Once the samples were separated by pH, only then did soil type (bulk, proximal, and rhizosphere) appear to have had an impact upon microbial community composition and abundance.

We observed that Shannon diversity and species richness of the microbial communities did change across soil type. Shannon diversity and richness was highest in the bulk soil and decreased in the rhizosphere soil. Although these changes were not found to be significant, they still indicate that the rhizosphere community is selected from surrounding bulk soil populations, with the proximal soil community acting as a transition between the two. Additionally, there was a significant difference between the microbial community composition of bulk and rhizosphere soils and also bulk and proximal soils within all soil samples. This is congruent with previous studies that found significant differences in the composition of microbial communities of bulk and rhizosphere soils across multiple environments, such as grasslands and forests [50, 51, 62–65]. Vieira et al. (2019) found that the rhizosphere bacterial communities within temperate grassland environments were significantly different from the bacterial communities in the bulk soil and Xu et al. (2023) found that the microbial community composition, abundance and diversity were significantly different among the bulk and rhizosphere soils within temperate forests [62, 64].

When comparing the taxonomic differences across soil types, we observed a higher relative abundance of bacteria of the phylum Acidobacteria in the bulk soil compared to rhizosphere and proximal soils (Fig. 1a). This is supported by our indicator analysis that found ASV147 (Acidobacteria Subgroup 2) to be in higher abundance in bulk soil. Previous studies have also identified certain Acidobacteria subgroups to be more common in the bulk soil than the rhizosphere soil [57–59]. Additionally, we identified a higher abundance of Rhizobiales in the bulk soil which was congruent with our findings from an indicator analysis that found ASV138 (*Rhizobiaceae*) and ASV371 (*Hyphomicrobium*) to be in bulk soils (Fig. 1b). *Rhizobiaceae*, a member of the Rhizobiales order, has been documented in higher abundance in bulk soil compared to rhizosphere soil and root surfaces [66, 67]. Yousef et al. (2024) and Macey et al. (2020) identified *Hyphomicrobium* (Order: Rhizobiales) to be in higher abundance in bulk soil than rhizosphere soils [68, 69]. Yousef et al. (2024) identified *Hyphomicrobium* as using ammonium as a nitrogen source and being a methanol-using bacteria in these bulk soil environments and with the presence of this bacterium in bulk soil potentially linked to fertilizers [68].

Our results also identified proximal soil as an area of transition between the rhizosphere and bulk soil microbial communities, where a microbial community significantly different from bulk soil thrives. This was evident in Figure S3, where although bulk and rhizosphere assigned ASVs were present, the majority of the microbial community was proximal associated populations. ASV330 was also identified as a proximal soil indicator no matter the soil pH. ASV330 was identified as *Nocardioides* from the phylum Actinobacteria. Some species of *Nocardioides* have been found in rhizosphere soil environments and are able to thrive in nutrient poor environments [70, 71]. The identification of ASV330 as a proximal ASV could be due to the fact that it may have been in the process of being actively recruited to the rhizosphere region at the time of sampling.

For rhizosphere soil, we identified specific ASVs within each pH range. For samples with a pH > 5 some indicators were ASV406 (*Galbitalea*), ASV236 (*Gemmataceae*) and ASV116 (*Variovorax*) and for samples with a pH below 5 some indicators were ASV462 (Sphingobacteriales), ASV904 (*Granulicella*) and ASV818 (*Haliangium*). Since not all rhizosphere indicators were consistent across geographic location and pH, these results show that *Solidago rugosa*, like other plants, selects its microbial communities based on the available microbes in the bulk soil. This selection is also evident by the decrease in Shannon diversity and species richness in the rhizosphere soil compared to the bulk soil (Fig. 2). Additionally, we observed a significant difference in rhizosphere community composition across grassland sites (Table S1). These results indicate that not all *Solidago rugosa* plants have the same rhizosphere community composition and in fact rhizosphere communities of *Solidago rugosa* are distinct across grassland sites (Fig. 6; Table S1). However, these communities are likely functionally similar as functional redundancy is common across a variety of taxonomic groups [72–76]. For example, Chen et al. (2022) showed that bacterial species within the phyla: Firmicutes, Bacteroidetes, Plantomycetes, and Actinobacteria have similar functions [75]. Additionally, Yu et al. (2020) showed that different species of Deltaproteobacteria have similar functions and Meyer et al. (2004) showed that different species of *Pseudomonas* (Phylum: Pseudomonadota) have metabolic versatility [73, 76]. So

although the rhizosphere community composition was significantly different across grasslands, the microbial populations are likely providing the same necessary services to the plant.

Although we observed distinct *Solidago rugosa* rhizosphere communities across grassland sites, we did also observe two rhizosphere indicators that were common across all rhizosphere samples, ASV110 and ASV303 (Fig. 4). ASV303 is in the phylum Latescibacterota and is a common soil bacterium that has a preference for higher pH environments, but can thrive in varying ranges and studies have identified Latescibacterota to be associated with both bulk soils and rhizosphere soils [77–82]. ASV110 was identified as *Starkeya* sp. from the class Alphaproteobacteria. This bacteria has been identified in the rhizosphere in previous studies and is a bacteria that can withstand varying environmental conditions, such as arid, semi-arid, and sahara conditions and nitrogen rich environments [83–85]. The presence of these two ASVs indicates that they survive across different environmental conditions and geographic locations. Moreover, these results show that although the rhizosphere communities differed based on pH and grassland site, there are still cosmopolitan populations that transcend these environmental and geographic differences and may play an important role in all rhizosphere communities, regardless of plant species.

Grassland conservation and restoration are important, and many efforts are currently underway to restore these environments [86–89]. This study reveals important information about how the microbial community composition varies from bulk to rhizosphere soil. We also documented proximal soil (which is not typically measured) as a transition zone from bulk to rhizosphere, however it still contains its own distinct ASVs. We also showed the rhizosphere microbial community of *Solidago rugosa* differed depending on pH and grassland, as the composition of these communities were influenced and selected from the surrounding bulk soil. Although the rhizosphere communities were taxonomically different, they are likely functionally similar, but further research needs to be done on the metabolic potential of these communities. Although the rhizosphere communities of *Solidago rugosa* were distinct, there were also specific populations that were present in all rhizosphere samples, regardless of location or environmental conditions. These cosmopolitan microbial populations may play an important role in plant-microbe interactions of the rhizosphere. Together, both the specific and cosmopolitan microbial populations identified in this study could potentially be used in grassland restoration efforts to promote plant diversity and growth, leading to conservation of these declining ecosystems.

Declarations

We declare that we did not utilize generative artificial intelligence (AI) tools in the composition of our manuscript, nor, did any part of this work involve the use of such tools. We declare that we have no conflict of interest.

Author Contribution

L.K. and C.F. wrote the main manuscript text. L.K. ran statistical analysis and prepared all figures. Z.A. assisted with felid collection and PCR prep. J.S. offered support and assistance with statistics. All authors reviewed the manuscript.

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Data Availability

Raw sequence data are available through the NCBI SRA database under study accession number PRJNA1146467. Complete workflow for data analysis in R is available at: <https://github.com/liliez13/Soil-Sample-R-Script.git>.

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Figures

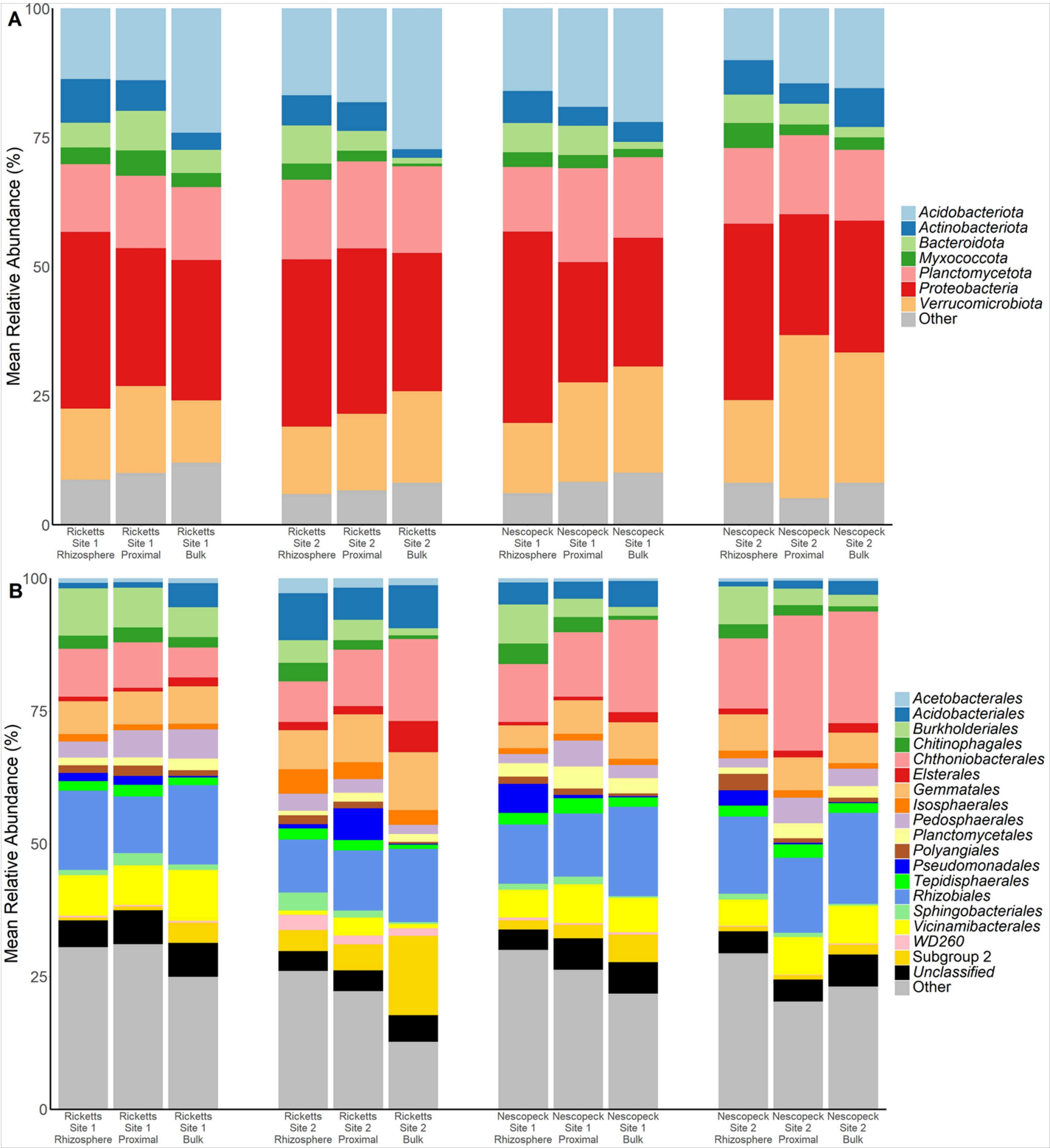


Figure 1

Microbial community taxonomy. a) Microbial community taxonomy on the phylum level within soil types, “other” is bacteria that had a mean relative abundance under 4%. **b)** Microbial community taxonomy on the order level within soil types, “other” is bacteria that has a mean relative abundance under 1.5%.

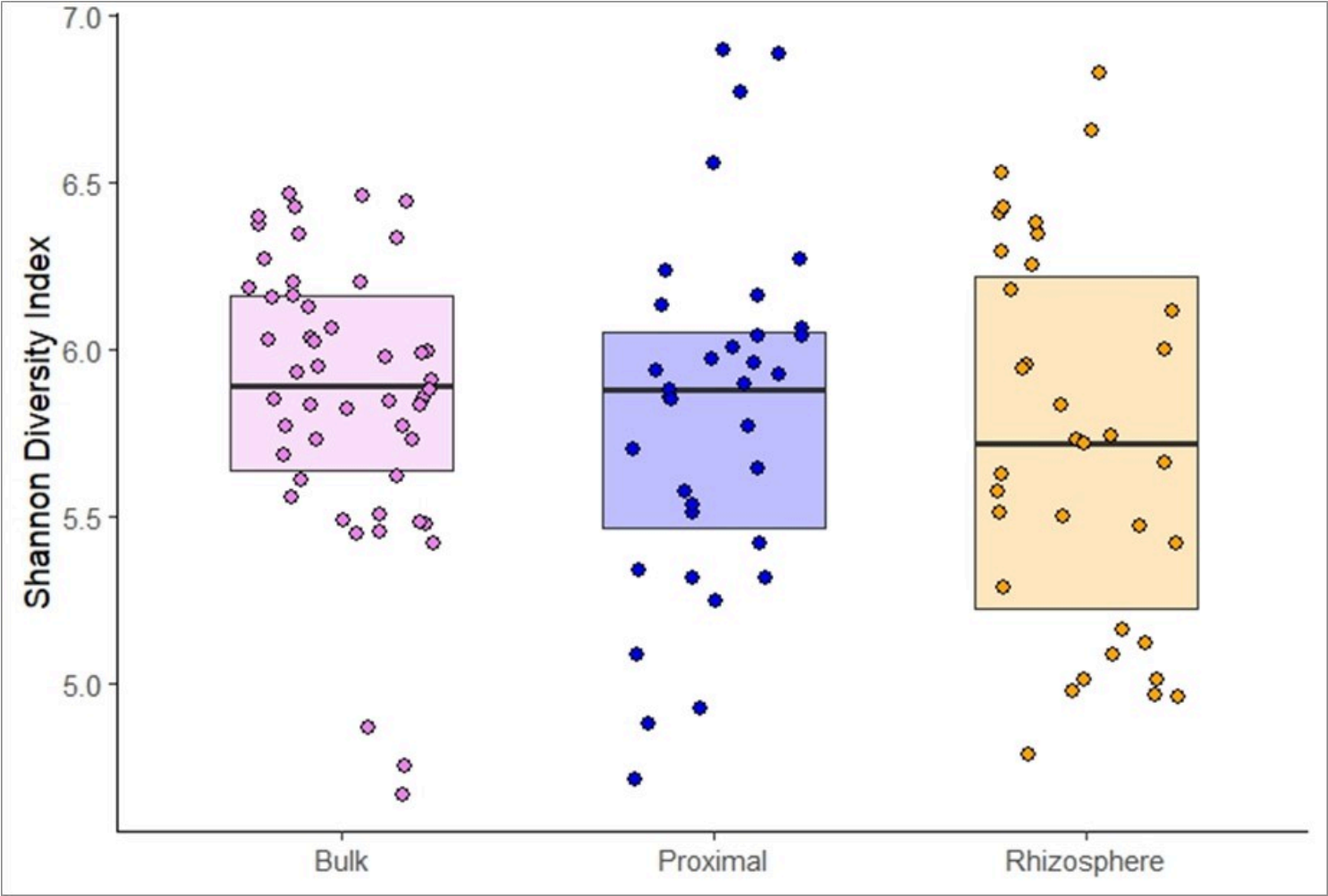


Figure 2

Box Jitter Plots of Shannon Diversity. Box and jitter plot separated by soil type. The black line represents the mean Shannon diversity for each soil type.

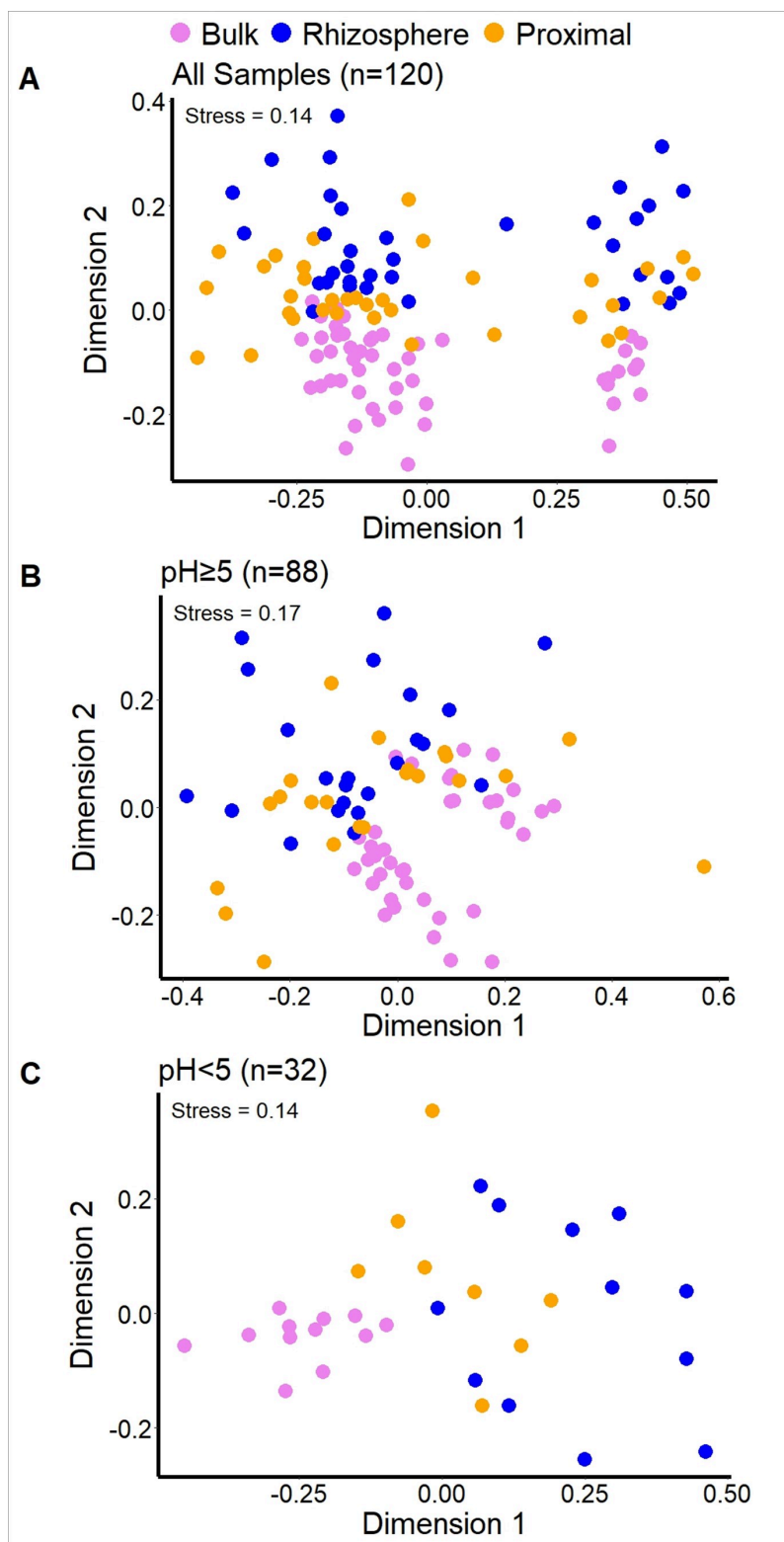


Figure 3

Microbial community composition across soil types. Each color represents a different soil type. **a)** All 120 soil samples. **b)** Samples with a pH of 5 or higher. **c)** Samples with a pH below 5.

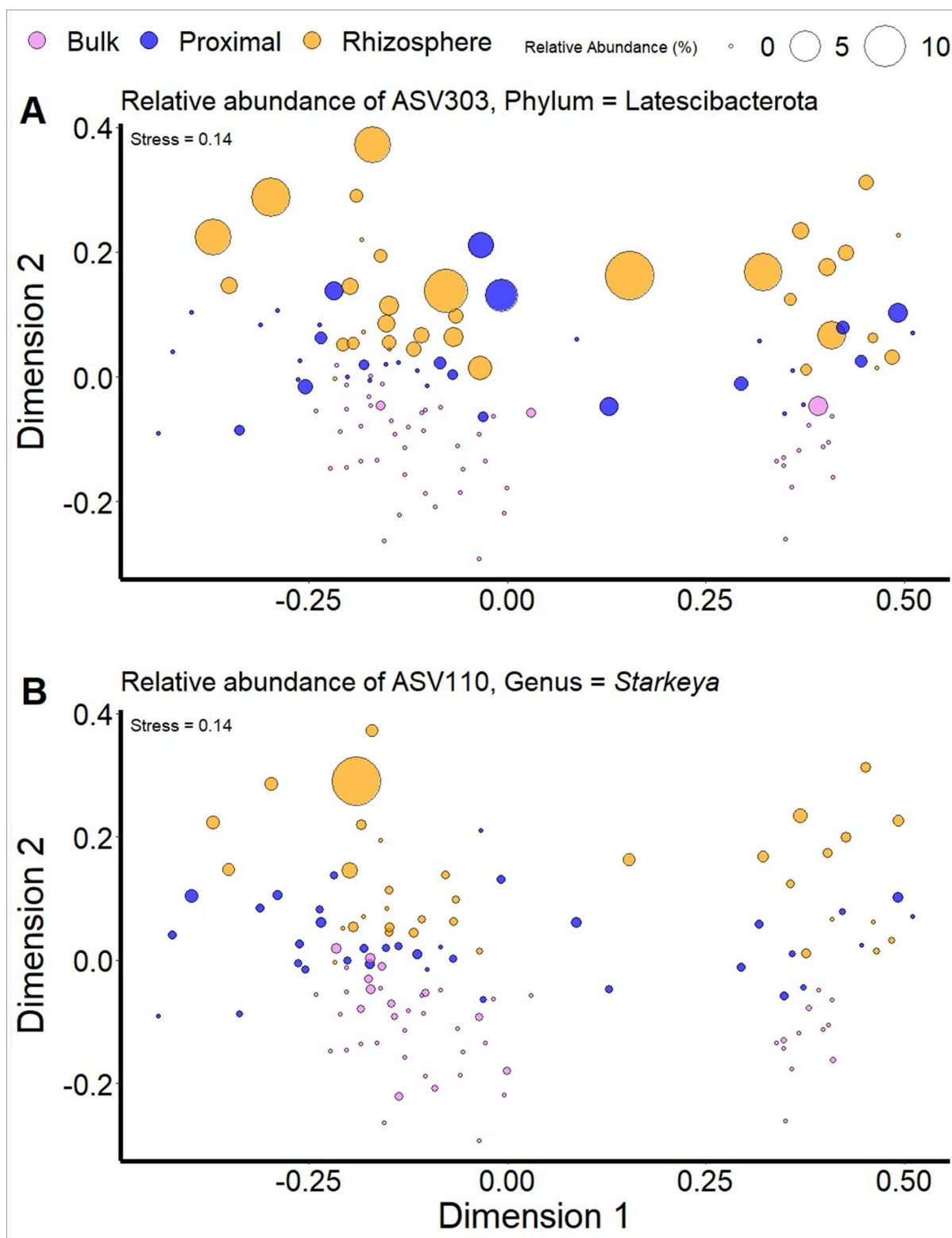


Figure 4

Relative abundance of ASV303 and ASV110. **a)** Bubble plot nMDS diagram of all 120 samples representing the abundance of ASV303. The larger dots indicate a higher abundance, and the smaller dots indicate a lower abundance. There is a higher abundance of ASV303 in the rhizosphere than in the bulk and proximal soil. **b)** Bubble plot nMDS diagram of all 120 samples representing the abundance of

ASV110. The larger dots indicate a higher abundance, and the smaller dots indicate a lower abundance. There is a higher abundance of ASV110 in the rhizosphere than in the bulk and proximal soil.

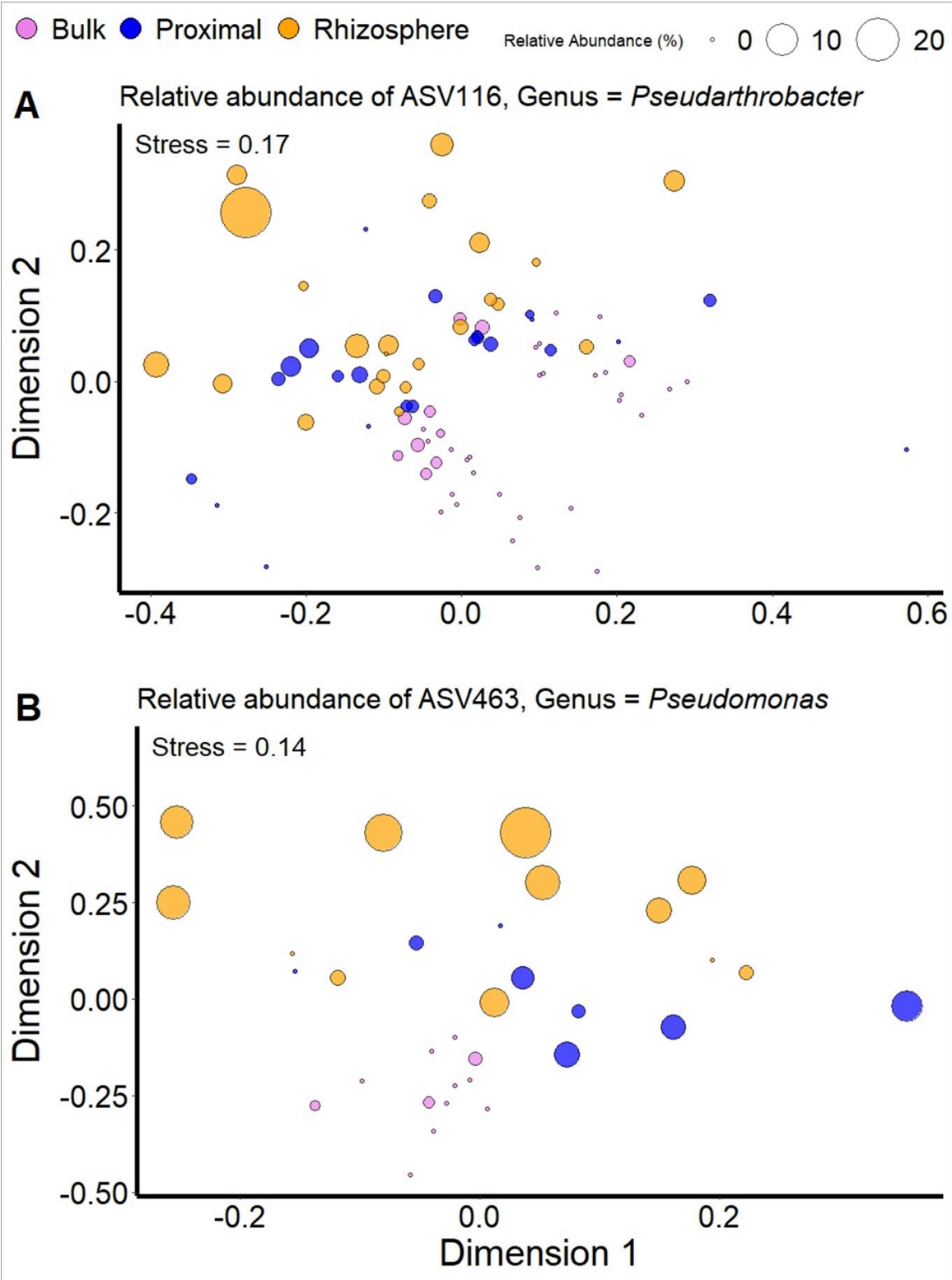


Figure 5

Relative abundance of ASV116 and ASV463. Bubble plot nMDS diagram representing the abundance of ASV116 and ASV463. The larger dots indicate a higher abundance, and the smaller dots indicate a lower

abundance. **a)**Indicator ASV116 in all 88 samples with a pH above 5. **b)** Indicator ASV463 in all 32 samples with a pH below 5.

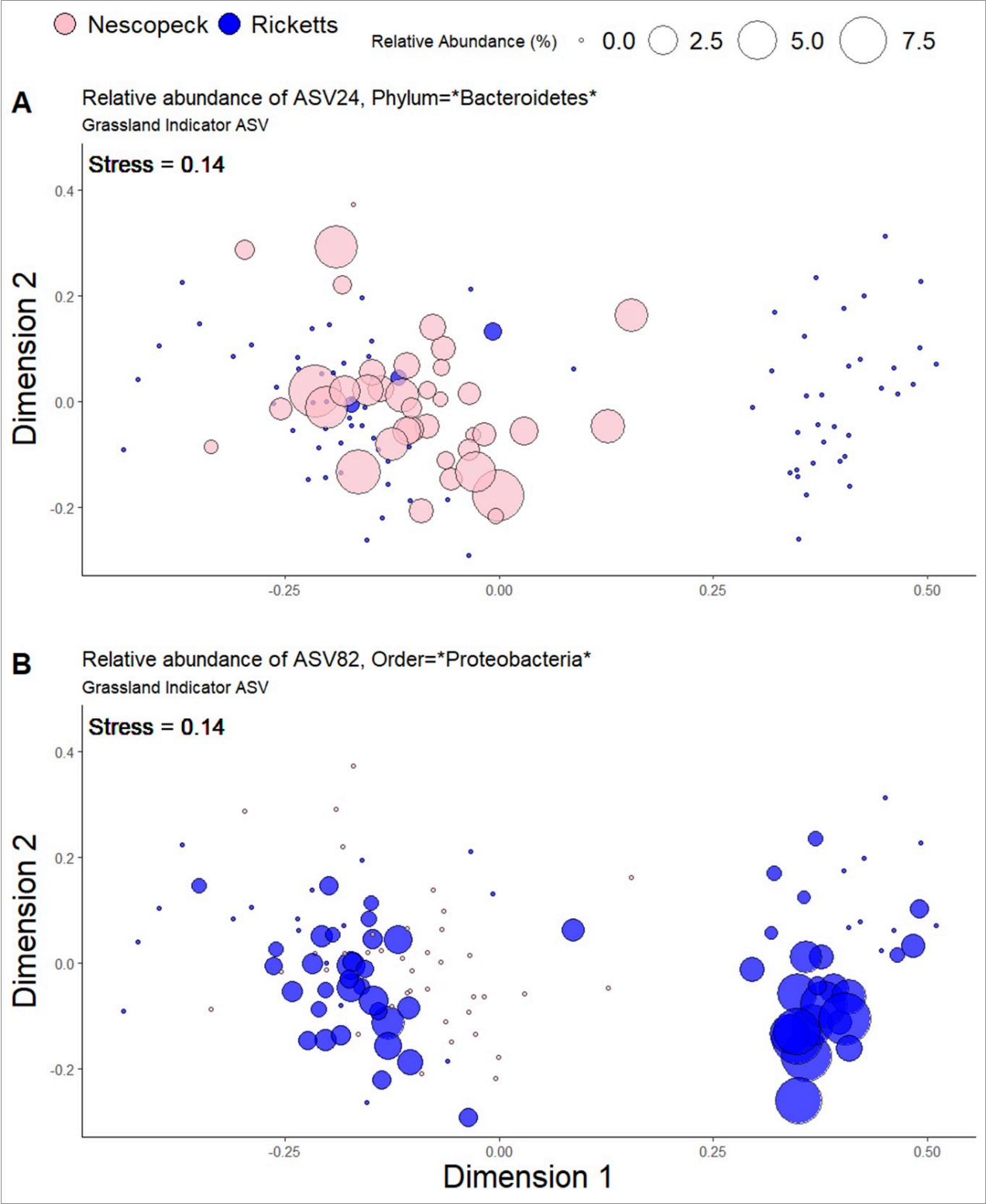


Figure 6

Relative abundance of ASV24 and ASV82. Bubble plot nMDS diagram representing the abundance of ASV24 and ASV82. The larger dots indicate a higher abundance, and the smaller dots indicate a lower

abundance. **a)**Indicator ASV24 in all 120 samples across both grasslands. **b)** Indicator ASV82 in all 120 samples across both grasslands.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [KelleherMetadata.csv](#)
- [Supplement.Fig.1.tiff](#)
- [Supplement.Fig.2.tiff](#)
- [Supplement.Fig.3.tiff](#)
- [Kelleheretal.Supplement.docx](#)
- [KelleherASVTable.csv](#)