



Changes in fungal and bacterial diversity over a peanut soil moisture gradient

Laura Rodríguez Rodríguez · Morgan Bragg ·
Kira L. Bowen · Zachary A. Noel

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Abstract

Background and aims The soil microbiome is vital to plant health. Drought can affect the abundance of soil microbial communities, plant productivity, and plant health. Peanut (*Arachis hypogaea*) is an important crop worldwide that is at risk of infection with *Aspergillus flavus* during heat and drought, which increases the risk of carcinogenic aflatoxin contamination. We hypothesized that varying soil moisture would shift microbial composition and reveal drought-adapted microbes possibly capable of competing with *A. flavus*.

Methods Two soils from fields with a history of peanut production were collected at Wiregrass Research and Extension Center, Headland, Alabama. The soils were placed in polyvinylchloride tubes inside a growth chamber at 29 °C. Five water regimes in a gradient from dry to wet were applied to the soils for nine weeks. They were sampled every other week, and the ITS1 for fungi and 16S rRNA gene for

bacterial amplicons were sequenced to determine the effect of the moisture treatments on the microbial communities.

Results Time and moisture treatments impacted both bacterial and fungal community structure and composition. Bacterial and fungal richness and evenness decreased and never fully recovered over nine weeks. Additionally, bacterial and fungal communities were also influenced by time and moisture treatments. Specifically, the bacterial phylum Actinobacteria thrived in drier treatments, while Proteobacteria were more abundant in moist treatments.

Conclusion We concluded that dry moisture treatments do influence bacterial and fungal communities in peanut soils, with Actinobacteria as potential microbes of interest to support peanut growth, increase drought tolerance, and disease resilience.

Keywords Peanut · Soil · Drought · Bacteria · Fungi

Abbreviations

BSA	Bovine serum albumin
ACC	1-Aminocyclopropane-1-carboxylic acid
ITS	Internal transcribed spacer region
EPS	Exopolysaccharides
WGREC	Wiregrass Research and Extension Center
PVC	Polyvinylchloride
OTUs	Operational taxonomic units

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L. Rodríguez Rodríguez · M. Bragg · K. L. Bowen ·
Z. A. Noel (✉)
Entomology and Plant Pathology, Auburn University,
Auburn, AL, USA
e-mail: zan0002@auburn.edu

USDA	United States Department of Agriculture
PCR	Polymerase chain reaction
fOTUs	Fungal operational taxonomic units
bOTUs	Bacterial operational taxonomic units
PERMANOVA	Permutational multivariate analysis of variance
PERMDISP	Permutational multivariate analysis of dispersion
ANCOMBC	Analysis of compositions of microbiomes with bias correction

Introduction

Drought is a severe environmental stress affecting plant productivity and health (Seleiman et al. 2021). Drought can be defined as long-term exposure to water deficit. Over time, drought directly interferes with the normal function of the plant (Shao et al. 2008). Plants under water stress will have a decline in photosynthesis, morphological changes, and a decrease in physiological functions (Sun et al. 2020). Peanuts (*Arachis hypogaea*) are an economically important legume crop that can be detrimentally affected by drought particularly because 80% of the peanuts produced in the United States are grown in the southeast, i.e., states including Georgia, Alabama, Florida, North Carolina, and Texas (Zhang et al. 2022) and, peanut production in these states tends to occur in sandy or sandy loam soil under rainfed conditions (Zhang et al. 2022). Periodic drought can affect peanut size, quality, and yield (Rosas-Anderson et al. 2014; Zhang et al. 2022), and annual losses from drought can reach up to \$50 million (Dai et al. 2019; Zhang et al. 2022). Moreover, peanuts can become more susceptible to colonization by fungi like *Aspergillus flavus*, with subsequent contamination with aflatoxins, especially when stressed with drought combined with increased temperatures (Uppala et al. 2013).

Aspergillus flavus is an opportunistic fungal soil saprotroph known to be associated with several economically important crops such as maize, cotton, rice, wheat, sorghum, and peanuts (Amaike and Keller 2011; Kebede et al. 2012). This fungus is ubiquitous, being found in agricultural soils, storage areas, and processing facilities (Tola and Kebede 2016). Some

A. flavus strains and other species in sections *Flavi* and *Ochraceorosei* can produce aflatoxins, which are highly carcinogenic and mutagenic (Amaike and Keller 2011). These fungal species produce toxins B1, B2, G1, and G2, with the most potent carcinogen being aflatoxin B1 (Bhatnagar-Mathur et al. 2015). Infection of peanuts with this fungus does not necessarily reduce yield, but its production of aflatoxin contaminates the grain, nuts, and seeds, preventing sales and causing significant economic losses (Uppala et al. 2013). The southeast United States peanut industry can lose over \$25 million annually due to aflatoxin contamination (Amaike and Keller 2011; Robens and Cardwell 2003). Consequently, the U.S. Department of Agriculture (USDA) Federal Grain Inspection Service has highly regulated guidelines for total aflatoxins in food or feed, with a max of 20 parts per billion (ppb) in food or feed for human or livestock consumption and 0.5 ppb in milk (Alam et al. 2020). Aflatoxin contamination from *A. flavus* is a major problem exacerbated by drought.

As plants direct their energy to osmotic adjustments during water deficit, the roots expand and penetrate the soil, looking for water and nutrients. Root microbial communities have been shown to interact with stressed plants and aid plants by improving nutrient circulation and water acquisition, producing compounds such as secondary metabolites and hormones that can protect the plant from biotic and abiotic stresses (Chen et al. 2022; Oppenheimer-Shaanan et al. 2022). However, the microbial community structure and abundance can be affected by drought. Some bacteria are known to aid plants under drought stress by the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, formation of exopolysaccharides (EPS), osmotic adjustment by the production of hormones, and antioxidant defenses, among others (Saberri Riseh et al. 2021). In the case of fungi, evidence suggests they can aid the plant through the regulation of hormones, solubilization and mineralization of nutrients, evasion of pathogenic infections by producing volatile organic compounds and antimicrobial compounds, regulation of plant defense responses, and aiding in abiotic stresses tolerance like drought (Hossain and Sultana 2020). Plant roots with active microorganisms promoting plant nutrient uptake may change that microbial community. Several studies propose that plants grown

in arid regions and drought-affected environments are more selective in their microbial counterparts (Naylor and Coleman-Derr 2018; Saberi Riseh et al. 2021).

Due to the threat that drought and heat pose to peanut production and the risks associated with aflatoxin contamination, in this study, we focused on detecting changes in bacterial and fungal communities of two peanut soils under five different water regimes over nine weeks. We hypothesize that applying different water regimes to peanut soils will alter the microbial composition over time and reveal target microbes that thrive under dry conditions. Identification of potential target microbes provides future avenues of research that could help plants tolerate drought conditions or compete with undesirable microbes such as *Aspergillus flavus*.

Materials and methods

Study site and sample collection

Two soils (designated soil A and soil B) with peanuts were collected from two fields approximately 2.19 km apart at the Wiregrass Research and Extension Center (WGREC) in Headland, Alabama, USA on 29 June 2021. The soils were collected in a "w" transect subsampled at regular intervals, 12.7 cm deep for each location, placed in a bucket, mixed, and then sieved through a 1 mm screen for uniform particle size. The depth of 12.7 cm was used because it is a major root zone and to capture the soil zone with the highest amount of microbial activity during early stages of plant growth (Meisner and Karnok 1992; Zhao et al. 2021). Soil was sieved through the 1 mm screen to remove coarse material and sizeable organic matter, which may impact the efficacy of the moisture gradient, to ensure homogeneous soils across the experiment. A subset of each soil was sent to the Auburn University Soil Testing Laboratory for percent sand, percent silt, percent clay, soil organic matter, nutrient levels (phosphorus, potassium, magnesium, and calcium), and pH, textural class, and water availability. Soil classification for soil A was Lucy Loamy Sand (thermic Arenic Kandiudults) and soil B was Orangeburg Sandy Loam (thermic Typic Kandiudults).

Growth chamber soil moisture regimes

Approximately 574 cm³ of sieved soil was added to a polyvinylchloride (PVC) cylinder 7.5 cm diameter and 15.24 cm length to simulate a soil core. A mesh screen with 1 mm openings was affixed to the bottom to prevent soil disturbance while allowing drainage. The PVC tubes were arranged in a completely randomized design with six replicates and five soil moisture treatments. Five water regimes were established to represent a gradient of dry to wet conditions and were as follows: 1) never watered, 2) 20 ml of water once per week, 3) 20 ml of water twice a week, 4) 50 ml of water once per week, and 5) 50 ml of water twice per week. The wettest moisture treatment (50 ml twice per week) does reflect average weekly rainfall in Alabama during peanut growth (NOAA NCEI 2025). The experiment ran for nine weeks at a temperature of 29 °C inside a growth chamber with no light. While this does not replicate field conditions, we did not use light to mimic the rhizosphere conditions as best as possible because no plants were used in this experiment. Sterile water for the treatments was added using a micropipette Repeater M4 (Eppendorf, USA). Each week before watering, 2 to 3 g of soil were collected in a sterile coin envelope, and 250 mg of soil was added to 2 ml disruptor tubes from OMEGA E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). To verify the water regime was altering soil moisture as expected for each treatment, one gram of soil was weighed before and after drying at 105 °C for 24–48 h (Robertson and VanderWulp 2019) (Supplementary Fig. 1). With five treatments, six replicates, and two soils sampled over five time-points, the total number of samples collected was equal to 300.

Genomic DNA extraction and library preparation

DNA was extracted from soils at weeks 0, 2, 5, 7, and 9 using OMEGA E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) and quantified using a Nanodrop (Thermo Scientific, USA) or a Qubit 3.0 fluorometer (Thermo Fisher, USA). Amplicon libraries were then constructed targeting the internal transcribed spacer (ITS) region using primers ITS 1F (5'-CTTGGT CATTAGAGGAAGTAA-3') and ITS 4 (5'-AGC CTCCGCTTATTGATATGCTTAART-3') of the fungal rRNA gene. The V4 region of the 16S rRNA gene

using primers 515F (5'-GTGCCAGCMGCOGCG GTAA-3') and 806R (5'-GGACTACHVGGGTWT CTAAT-3') following. The fungal and bacterial amplicon libraries were prepared according to modified protocols from Lundberg et al. (2013), as explained in Noel et al. (2022), Olofintila et al. (2021), and Longley et al. (2020). Briefly, library preparation composed of three rounds of polymerase chain reaction (PCR), followed by normalization with the Invitrogen SequalPrep kit (Thermo Fisher, USA) kit, concentrated using the Amicon® Ultra 0.5 mL filters (EMDmillipore, Germany), and clean-up using AMPure beads (Beckman Coulter, USA) at 0.7X before sequencing. The PCR steps, reagents, primers, and cycling conditions are disclosed in Supplemental Tables 2, 3, and 4, which were adapted from Noel et al. (2022). At least one negative extraction control, a PCR negative control, and bacterial and fungal mock communities as positive controls were included in each 96-well plate and sequenced. The pooled amplicon libraries were sent to SeqCenter (Pittsburgh, PA) for sequencing using Illumina MiSeq 2×300 bp V3 chemistry.

Bioinformatics and statistical analysis

Bacterial raw sequences were merged using VSEARCH 2.14.1 (Rognes et al. 2016). Fungal and bacterial primers were removed using cutadapt 4.0 (Martin 2011). Sequences were filtered using an expected error threshold of 1.0 for fungi and 0.5 for bacteria and trimming to 250 bp length. For fungi, the conserved 18S region was trimmed from the 5' region of the reads, leaving just ITS1. Qualified reads were then dereplicated using VSEARCH 2.14.1, and then chimeras were removed and clustered into operational taxonomic units (OTUs) at traditional 97% identity using USEARCH v11.0.667 (Rognes et al. 2016). OTU sequences were then assigned taxonomy with the SINTAX algorithm for bacteria and the NCB algorithm implemented through DADA2 1.26.0 for fungi (Chen et al. 2021). Fungal ITS1 sequences were compared against the UNITE v 9 Eukaryote database and bacteria with SILVA 138.1 database. We are aware of recent nomenclature changes for the bacterial phyla (i.e., Actinobacteria to Actinomycetota) (Oren 2024); however, we will refer to the phylum as Actinobacteria to stay consistent with the database used to generate taxonomy for the current study.

Data analysis

The R packages phyloseq v134.0 (McMurdie and Holmes 2013) and vegan 2.5–7 (Oksanen et al. 2020) were primarily used for the analysis. Unclassified taxonomy at the kingdom level and OTUs matching positive control mock sequences were filtered out. Additionally, OTUs found in the negative controls above a threshold of 0.1 were removed using the R package decontam 1.10.0 (Davis et al. 2018). Four fungal OTUs (fOTUs) and 25 bacterial OTUs (bOTUs) were identified as contaminants and were filtered out. Samples with lower than 5,000 reads per sample were discarded due to low sequence coverage. Two samples were removed due to low fungal sequencing coverage and seven samples were removed due to low bacterial sequencing coverage. Microbiome composition differed between soil A and B (Supplementary Fig. 2); therefore, we separated these data by soil type, and statistical analyses were conducted separately on each data set. Differences in alpha diversity were examined by measuring species richness and evenness for fungi and bacteria, plus Faith's phylogenetic diversity for bacteria only. Levene's test (function *leveneTest*, package 'car') was used to determine homogeneity of variance across groups, and variance inflation factor (function *vif*, package 'car') was used to verify that week and treatment were not confounded variables. There were equal variances in fungal and bacteria species richness ($p < 0.05$), evenness ($p < 0.05$), and bacteria Faith's phylogenetic diversity ($p < 0.05$) among weeks and treatments in both soil types. Further, week and treatment were not confounding variables across both soil types. We utilized linear mixed models (function *lmer*, package "lmerTest") with species richness, evenness (fungi and bacteria) or Faith's phylogenetic diversity (bacteria only) as the response variable and week, moisture treatment, and their interaction as the explanatory variables with replicate as a random effect to account for repeated sampling of the same pot. Pairwise comparisons were conducted if appropriate (function *emmeans* and *pairs*, package "emmeans"). To assess differences in fungal and bacteria community composition between moisture treatments and over time, counts were normalized based on cumulative sum scaling and permutational multivariate analysis of variance (PERMANOVA; function *adonis2*, package "vegan") were run with Bray–Curtis, Jaccard (fungi

and bacteria) and Unweighted UniFrac (bacteria only) distance matrices and replicate as a random effect to account for repeated sampling of the same pot. If necessary, pairwise comparisons (function *pairwise.adonis2*, package “pairwiseAdonis”, Martinez Arbizu 2020) were applied. Dispersion of the fungal and bacteria communities between moisture treatments and over time was tested with PERMDISP (function *betadisper*, package “vegan”). We rooted the bacterial phylogenetic tree using the longest branch as the outgroup before running a PERMANOVA and PERMDISP using Unweighted UniFrac distance matrix. Differential abundance of fungi and prokaryotes between moisture treatment 1 (never watered) and treatment 5 (50 ml twice a week) was conducted using analysis of compositions of microbiomes with bias correction (ANCOM-BC2; function *ancombc2*, package “ANCOMBC”). Before running ANCOM-BC2, we filtered fOTUs and bOTUs only to include taxa that were present in 15 or more samples (25% of total samples) to reduce the false discovery rate and remove sparsely abundant OTUs (Mandal et al. 2015). P values were adjusted using the Holm-Bonferroni method, and OTUs were considered significant if the *p*-value was below 0.05.

Results

Soil analysis

Soil analysis showed that the two soils had similar sand content, pH, water availability, and organic matter but differed in silt, calcium, phosphorous, potassium, and magnesium (Table 1). Soil A had higher silt content, phosphorus, and potassium, while soil B had higher calcium and magnesium than soil A.

Overall sequencing output

To understand the microbial shifts from the peanut soils under different water treatments, fungi and bacteria were sequenced for both soils (A and B). We retained 23,084,763 fungal reads post filtering across 298 samples and 9,073,713 bacterial reads post filtering across 291 samples. A total of 3,441 fOTUs and 12,154 bOTUs were obtained. Rarefaction analysis indicated that the median read depth per sample captured most OTUs in each soil (Supplementary Fig. 3).

Table 1 Field collection and soil properties description for soil A and soil B

Soils	Year	Coordinates	Sampled	Soil Classification	Taxonomic Classification	% Sand	% Silt	% Clay	pH	% Organic matter	Calcium ^a (ppm)	Phosphorus (ppm)	Potassium (ppm)	Magnesium (ppm)
WGREC (A)	2021	31.373, -85.317	29-Jun	Lucy Sandy Loam	thermic Arenic Kandiuults	79.38	1.88	18.75	6	1.4	69.5	20.5	118	9
WGREC (B)	2021	31.354, -85.323	29-Jun	Orangeburg Sandy Loam	thermic Typic Kandiuults	79.38	0.63	20	6	1.9	287	13.5	56	31.5

^aThe addition of calcium to the soil is in the form of gypsum

Alpha-diversity analysis

Soil A: Species richness and evenness were calculated for fungi and bacteria, and Faith's phylogenetic diversity was calculated for bacteria only. In general across all treatments, fungal and bacteria richness was highest at the start of the experiment then declined from weeks 2 through 5 followed by a slight increase during week 7 and week 9 (Fig. 1). However, diversity metrics present at week 9 never fully recovered to levels recorded at week 0. Specifically, fungal species richness differed over time (Fig. 1a); there was a decrease in fungal species richness in weeks 2, 5, 7 and 9 compared to week 0 ($p < 0.001$), week 5 ($p = 0.03$) compared to week 2 but an increase in week 9 compared to week 5 ($p = 0.009$). Additionally, fungal species richness significantly interacted between week 2 and treatment 4 ($p = 0.03$). Lastly,

there was a decrease in fungal evenness in week 5 compared to week 0 ($p = 0.003$). Bacterial species richness also differed over time (Fig. 1b). Specifically, there was a decrease in bacterial species richness in weeks 2, 5, 7, and 9 compared to week 0 ($p < 0.006$) and weeks 5 ($p = 0.002$) and 9 compared to week 2 ($p = 0.02$). Similarly, Faith's phylogenetic diversity differed over time (Fig. 1b). A decrease in phylogenetic diversity was observed in weeks 2, 5, 7, and 9 compared to week 0 ($p < 0.006$) and weeks 5 and 9 compared to week 2 ($p < 0.009$). Bacterial species evenness significantly interacted between week 5 and treatment 5 ($p = 0.04$).

Soil B: In soil B, similar patterns were observed; in general, fungal richness decreased after week 0 and began to gradually increase after week 5 without fully recovering to richness seen at week 0. For bacterial richness and bacterial phylogenetic diversity, a

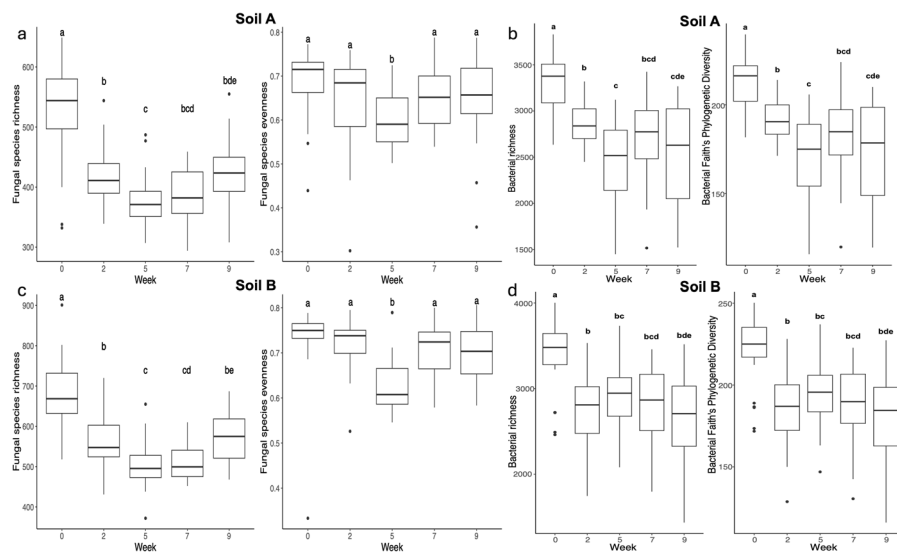


Fig. 1 Fungal and bacterial diversity was affected by time in different soils. Week of sample is on the x-axis and either species richness, evenness, or Faith's phylogenetic diversity is on the y-axis. Letters on the plot visually represent significant differences between the weeks; different letters indicate a significant difference. **(a)** Soil A fungal species richness over time. Higher fungal species richness was documented in week 0 compared to weeks 2, 5, 7, and 9; week 2 compared to weeks 5 and 7; and week 9 compared to weeks 5 and 7. Higher fungal species evenness was documented in week 0 compared to week 5. **(b)** Soil A bacterial species richness and Faith's phylogenetic diversity over time. Higher bacterial species richness was documented in week 0 compared to weeks 2, 5, 7, and 9; and week 2 compared to weeks 5 and 9; and week 7 compared to weeks 5 and 9; and week 9 compared to week 5. Faith's phy-

logenetic diversity was higher in week 0 compared to weeks 2, 5, and 7; and week 2 compared to weeks 5, 7, and 9; and week 3 compared to week 7. **(c)** Soil B fungal species richness and evenness over time. Higher fungal species richness was documented in week 0 compared to weeks 2, 5, 7, and 9; and week 2 compared to weeks 5, 7, and 9; and week 9 compared to weeks 5 and 7. An increase in fungal species evenness was documented in week 0 compared to weeks 5 and 7; and week 3 compared to week 5. **(d)** Soil B bacterial species richness and Faith's phylogenetic diversity over time. Higher bacterial species richness was documented in week 0 compared to weeks 2, 5, 7, and 9; and week 3 compared to week 5. Faith's phylogenetic diversity was increased in week 0 compared to weeks 2, 5, 7, and 9.

decrease was noted at week 2 with a slight increase in week 5 that plateaued through week 7 and 9. Fungal species richness and evenness differed over time (Fig. 1c), with a decrease in species richness reported in weeks 2, 5, 7, and 9 compared to week 0 ($p < 0.001$), weeks 5 ($p = 0.01$) and 7 ($p = 0.03$) compared to week 2, while an increase in richness was documented in weeks 5 and week 7 compared to week 9 ($p < 0.001$). A decrease in fungal species evenness was observed in week 5 ($p = 0.0001$) compared to week 0, week 5 compared to week 2 ($p > 0.0001$), but an increase in week 7 and 9 compared to week 5 ($p < 0.0001$). Additionally, fungal species richness significantly interacted between week 7 and treatment 4 ($p = 0.04$). Bacterial species richness differed over time (Fig. 1d). Specifically, there was a decrease in bacterial species richness in weeks 2, 5, 7 and 9 compared to week 0 ($p < 0.0001$) and week 9 compared to week 5 ($p = 0.04$). Moreover, bacterial species richness significantly interacted between week 2 and treatment 3 ($p = 0.02$) and week 9 and moisture treatments 3 ($p < 0.0001$), 4 ($p = 0.005$) and 5 ($p = 0.02$). Faith's phylogenetic diversity differed over time (Fig. 1d), with a decrease in phylogenetic diversity observed in weeks 2, 5, 7 and 9 compared to week 0 ($p < 0.001$) and week 9 compared to week 5 ($p = 0.02$). Faith's phylogenetic diversity also had significant interactions between week 2 and treatment 3 ($p = 0.02$) and week 9 and moisture treatments 3 ($p < 0.0001$), 4 ($p = 0.005$) and 5 ($p = 0.02$). Lastly, for bacterial species evenness, there was a significant interaction between moisture treatment 5 and weeks 5 ($p = 0.008$) and 7 ($p = 0.04$) but no relationship with week or treatment.

Beta-diversity analysis

Soil A: Principal coordinate ordinations for soil A fungi and bacteria are shown in Fig. 2. Changes in fungal composition and structure were seen over time and among treatments (Fig. 2a and b; Bray–Curtis PERMANOVA: Week $p = 0.001$, $R^2 = 11.8\%$, $F_{4,143} = 4.66$, Treatment $p = 0.001$, $R^2 = 3\%$, $F_{4,143} = 1.18$; Jaccard PERMANOVA: Week $p = 0.001$, $R^2 = 9.1\%$, $F_{4,143} = 3.53$, Treatment $p = 0.001$, $R^2 = 2.9\%$, $F_{4,143} = 1.12$). Differences in fungal composition and structure were seen among week 0 and weeks 5, 7 and 9 ($p = 0.001$), week 2 and weeks 5, 7 ($p = 0.001$) and 9 ($p < 0.02$), and week 5 and weeks 7 ($p < 0.02$) and 9 ($p < 0.002$). Differences

in fungal composition were seen among treatment 1 and treatment 4 ($p < 0.04$) and 5 ($p < 0.03$). There was similar dispersion in the fungal community across treatments but dissimilar dispersion over time (PERMDISP: Bray–Curtis $p = 0.009$; Jaccard's $p = 0.007$).

Bacterial community composition and structure changed over time and across treatments (Fig. 2c and d; Bray–Curtis PERMANOVA: Week: $p = 0.001$, $R^2 = 12.8\%$, $F_{4,139} = 5.08$, Treatment: $p = 0.001$, $R^2 = 4.7\%$, $F_{4,139} = 1.86$; Jaccard PERMANOVA: Week: $p = 0.001$, $R^2 = 10.8\%$, $F_{4,139} = 4.16$, Treatment: $p = 0.001$, $R^2 = 4.3\%$, $F_{4,139} = 1.65$; Unweighted UniFrac PERMANOVA: Week $p = 0.001$, $R^2 = 7.4\%$, $F_{4,139} = 2.77$; Treatment: $p = 0.001$, $R^2 = 3.7\%$, $F_{4,139} = 1.41$). Differences in bacterial community composition and structure were reported between week 0 and weeks 2, 5, 7 and 9 ($p < 0.006$), week 2 and week 5, 7, 9 ($p < 0.01$) and week 5 and week 7 and 9 ($p < 0.05$). Bacterial community composition and structure was different between treatments 1 and treatments 2 ($p < 0.05$ Bray–Curtis and Jaccard only), 3, 4 and 5 ($p < 0.02$) and treatments 2 and treatment 5 ($p < 0.05$). There was similar dispersion in the bacterial community composition among treatments but dissimilar dispersion over time (PERMDISP: Bray–Curtis, Jaccard and Unweighted UniFrac $p = 0.001$).

Soil B: Principal coordinate ordinations for soil B fungi and bacteria are shown in Fig. 3. Similar to soil A, changes in fungal composition and structure were seen over time and between treatments (Fig. 3a; Bray–Curtis PERMANOVA: Week $p = 0.001$, $R^2 = 15\%$, $F_{4,145} = 6.52$, Treatment $p = 0.001$, $R^2 = 4.2\%$, $F_{4,145} = 1.85$; Jaccard PERMANOVA: Week $p = 0.001$, $R^2 = 11.2\%$, $F_{4,145} = 4.63$, Treatment $p = 0.001$, $R^2 = 3.7\%$, $F_{4,145} = 1.55$). Differences in fungal composition and structure were seen among week 0 and weeks 2, 5, 7 and 9 ($p = 0.001$), week 2 and weeks 5, 7 and 9 ($p < 0.02$) and week 5 and weeks 7 and 9 ($p = 0.001$). Fungal community composition and structure was different between treatment 5 and treatments 1 ($p < 0.007$), 2 ($p = 0.002$), and 3 ($p = 0.05$). There was similar dispersion in the fungal community composition over time and among treatments.

Bacterial community composition and structure changed over time and across treatments (Fig. 3b; Bray–Curtis PERMANOVA: Week $p = 0.0001$, $R^2 = 13\%$, $F_{4,142} = 5.68$, Treatment $p = 0.001$,

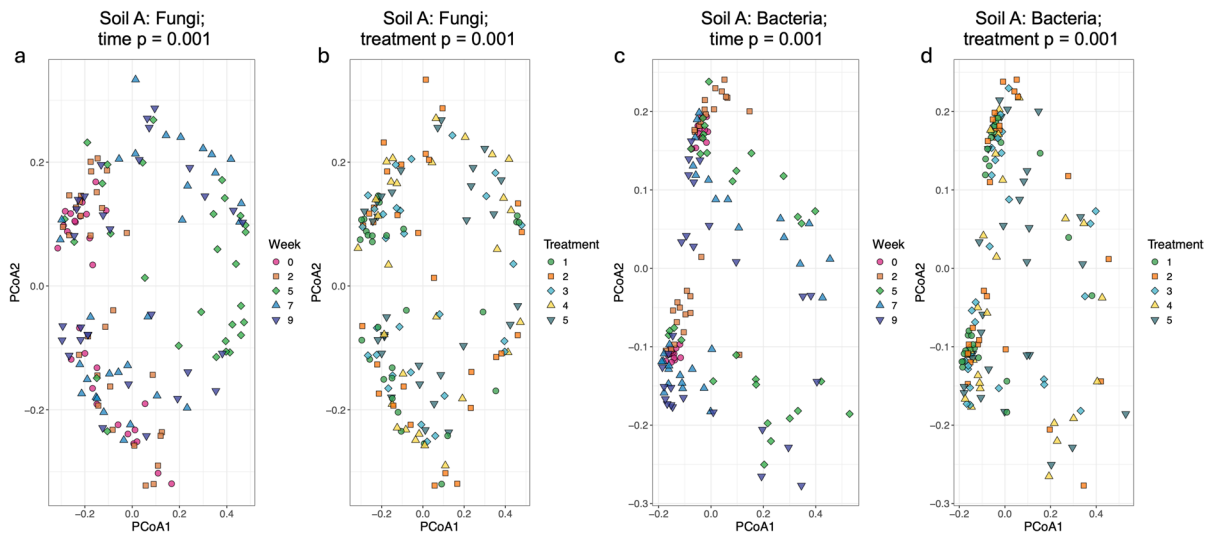


Fig. 2 Principal coordinate analysis (PCoA) of soil A fungal and bacterial community compositions over time and moisture treatments. Each point represents the community composition of one sample. In panel (a), Soil A fungal Bray-Curtis distance matrix points are colored by week, while in panel (b), they are colored by moisture treatments. Differences in fungal community composition were observed between week 0 and weeks 5, 7, and 9; week 2 and weeks 5, 7, and 9; week 3 and weeks 7 and 9; and between week 7 and week 9. Additionally, differences were documented between treatment 1 and treat-

ments 2 and 4. Panel (c) shows Soil A bacterial Bray-Curtis distance matrix points colored by week, and panel (d) shows them colored by treatments. Differences in bacterial community composition were observed between week 0 and weeks 2, 5, 7, and 9; week 2 and weeks 5, 7, and 9; week 3 and weeks 5, 7, and 9; and between week 5 and week 9. Furthermore, differences were documented between treatment 1 and treatments 2, 3, and 5. Reported significance values are shown above each plot ($\alpha = 0.05$)

$R^2 = 7.4\%$, $F_{4,142} = 3.25$; PERMANOVA Jaccard: Week $p = 0.001$, $R^2 = 11.1\%$, $F_{4,142} = 4.69$, Treatment $p = 0.001$, $R^2 = 5.9\%$, $F_{4,142} = 2.49$; PERMANOVA Unweighted UniFrac: Week $p = 0.001$, $R^2 = 7.2\%$, $F_{4,142} = 2.82$; Treatment Unweighted UniFrac: $p = 0.001$, $R^2 = 3.9\%$, $F_{4,142} = 1.53$). Additionally, bacterial community composition had a significant interaction between week and treatment (PERMANOVA: Jaccard $p = 0.03$, $R^2 = 10\%$, $F_{16,130} = 1.11$, Unweighted UniFrac $p = 0.001$, $R^2 = 11\%$, $F_{16,130} = 1.07$). Differences in bacterial community composition and structure were reported between week 0 and 2, 5, 7 and 9 ($p < 0.002$), week 2 and week 5, 7, 9 ($p < 0.005$), week 5 and weeks 7 ($p = 0.04$ Jaccard only) and 9 ($p < 0.05$) and week 7 and week 9 ($p < 0.02$ Jaccard and Unweighted UniFrac only). Bacterial community composition and structure was different between treatment 5 and treatments 1, 2, 3 and 4 ($p < 0.05$), treatment 4 and treatments 3 (Bray-Curtis only), 2, and 1 ($p < 0.05$ Bray-Curtis and Jaccard only) and treatment 3 and treatment 1 ($p = 0.02$). There was dissimilar dispersion in the bacterial community

composition among treatments (PERMDISP: Bray-Curtis $p = 0.01$, Jaccard $p = 0.03$, Unweighted UniFrac $p = 0.04$) and over time (PERMDISP: Bray-Curtis $p = 0.005$, Jaccard $p = 0.001$, Unweighted UniFrac $p = 0.005$).

Fungi associated with specific moisture treatments

Since we set out to understand mainly the effects of moisture treatments on the soil microbiome, we conducted differential abundance analysis for each soil between treatment 1, completely dry, and treatment 5, watered twice per week with 50 ml. Volcano plots depicting the differently abundant taxa are shown in Figs. 4 and 5.

Soil A: Differential abundance analysis highlighted two fOTUs with significant increases in relative abundance associated with treatment 5, simulating well-watered conditions, and no fOTUs associated with treatment 1, simulating drought conditions (Fig. 4a). The fungal order *Rhizophlyctidales*

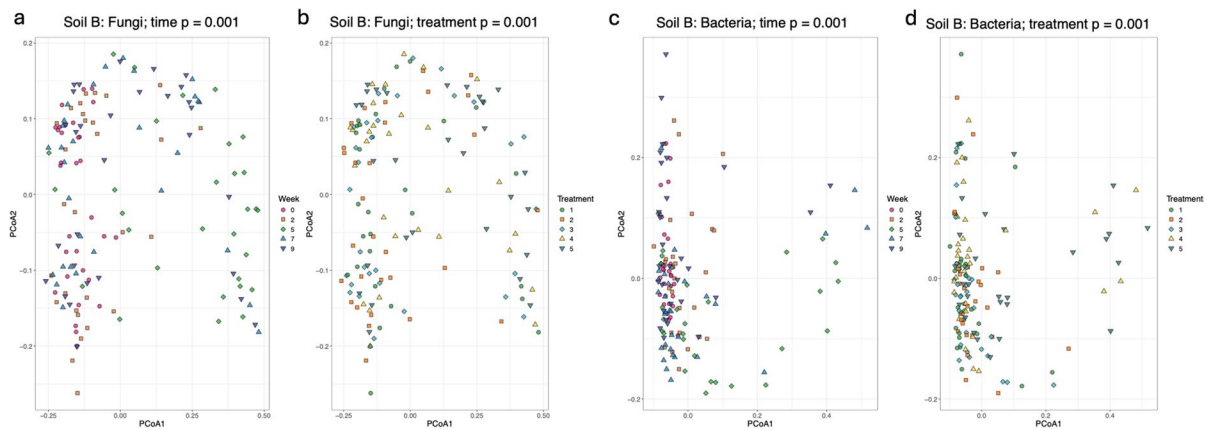


Fig. 3 Principal coordinate analysis (PCoA) of soil B fungal and bacterial community compositions over time and moisture treatments. Each point represents the community composition of one sample. In panel (a), Soil B fungal Bray-Curtis distance matrix points are colored by week, and in panel (b), by moisture treatments. Differences in fungal community composition were observed between week 0 and weeks 2, 5, 7, and 9; week 2 and weeks 5, 7, and 9; and between week 5 and weeks 7 and 9. Additionally, differences were documented between treatment 1 and treatments 1 through 4. Panel (c) shows Soil B bac-

terial Bray-Curtis distance matrix points colored by week, and panel (d) by treatments. Differences in bacterial community composition were observed between week 0 and weeks 2, 5, 7, and 9; week 2 and weeks 5, 7, and 9; and between week 5 and weeks 7 and 9. Furthermore, differences were documented between treatment 1 and treatments 1, 2, 3, and 4; between treatment 3 and treatment 1; and between treatment 4 and treatments 1, 2, and 3. Reported significance values are shown above each plot ($\alpha = 0.05$)

(fOTU 1343) was more abundant in treatment 5 than in treatment 1, indicating an association with wetter soils (ANCOMBC: p adj=0.001). An additional fOTU (fOTU 4029) representing the kingdom Fungi had increased abundance in treatment 5 compared to 1; however, there was no taxonomic resolution past the kingdom level (ANCOMBC: p adj<0.0005). No fOTUs were more abundant in treatment 1 compared to treatment 5.

Soil B: Differential abundance analysis identified three fOTUs that were more abundant in treatment 5 compared to treatment 1 (Fig. 4b). Similar to soil A, there was higher relative abundance of two fOTUs representing the order Rhizophlyctidiales (ANCOMBC: fOTU 38 p adj=0.0001, fOTU 1343 p adj=0.0002) and one fOTU representing the kingdom Fungi with no further taxonomic resolution (fOTU 170; ANCOMBC p adj=0.0001) in treatment 5. In contrast, two species *Alternaria tenuissima* (fOTU 25; ANCOMBC p adj=0.00004) and *Alternaria alternata* (fOTU 789; ANCOMBC p adj=0.00009) were more abundant in treatment 1 versus treatment 5 indicating an association with dryer soils.

Bacteria associated with specific water treatments

Soil A: We identified 22 bOTUs that were more abundant in the well-watered treatment (treatment 5) compared to the dry treatment (treatment 1; Fig. 5a). The enriched taxa belong to the phyla Proteobacteria (27%, $n=6$), Planctomycetota (18%, $n=4$), Myxococcota (14%, $n=3$), Bacteroidota (14%, $n=3$), Firmicutes (9%, $n=2$), Acidobacteriota (5%, $n=1$), Actinobacteria (5%, $n=1$), Cyanobacteria (5%, $n=1$) and Elusimicrobiota (5%, $n=1$). Regarding the Proteobacteria, there were three bOTUs unresolved at the family level including an uncultured Methylophilaceae (bOTU 212; ANCOMBC p adj<0.0001), an uncultured Oxalobacteraceae (bOTU 118; ANCOMBC p adj<0.0001) and an unidentified bacterium from Rhodocyclaceae (bOTU 1592; ANCOMBC p adj<0.0001). The three-remaining bOTUs from Proteobacteria were unresolved at the genus level, including, *Rudaea* (bOTU 986; ANCOMBC p adj<0.0001), *Cupriavidus* (bOTU 46; ANCOMBC p adj=0.02), and an uncultured *Duganella* (bOTU 8697; ANCOMBC p adj=0.04). Within phylum Planctomycetota, one

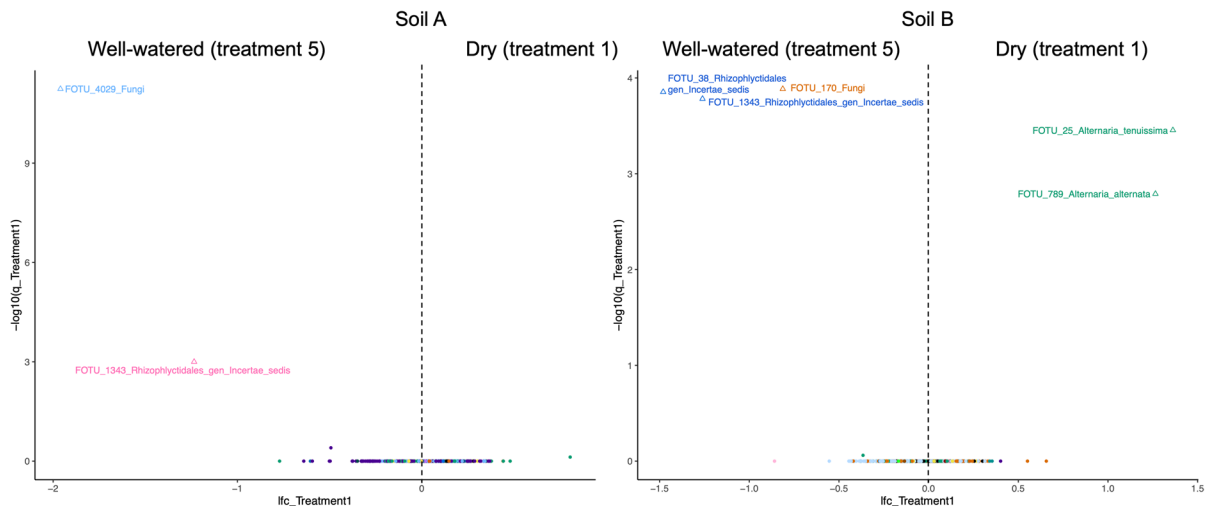


Fig. 4 Differential abundance of fungal OTUs in response to moisture. For both panels (a and b), positive values represent an enrichment in relative abundance in the dry treatment (treatment 1), while negative values indicate enrichment under well-watered conditions (treatment 3). Each point represents an OTU, with labeled points identifying significantly abundant taxa ($p < 0.05$). The color of each point indicates the Phylum

to which the OTU belongs. ANCOM-BC2 was used to calculate p-values, which were adjusted using the Holm-Bonferroni method. The x-axis shows log fold change (Lfc) as the effect size, and the y-axis shows log10-transformed adjusted p-values in relation to treatment. Panel (a) displays differential abundance of fungal OTUs in soil A, and panel (b) shows differential abundance in soil B

bOTU represented an uncultured bacterium from the order Planctomycetales (bOTU 2453; ANCOMBC $p \text{ adj} < 0.0001$) and three bOTUs belonging to the family Gemmataceae consisting of an uncultured bacterium from the genus *Gemmata* (bOTU 977; ANCOMBC $p \text{ adj} = 0.005$), an unidentified (bOTU 12128; ANCOMBC $p \text{ adj} = 0.03$) and uncultured bacterium (bOTU 2093; ANCOMBC $p \text{ adj} = 0.04$). The phylum Myxococcota was comprised of three bOTUs unresolved at the genus level, *P3OB-42* (bOTU 1014; ANCOMBC $p \text{ adj} < 0.0001$), *Phaselicystis* metagenome (bOTU 885; ANCOMBC $p \text{ adj} = 0.0003$) and an unidentified *Phaselicystis* (bOTU 1239; ANCOMBC $p \text{ adj} = 0.002$). The phylum Bacteroidota was represented by three bOTUs, an uncultured bacterium from the family Chitinophagaceae (bOTU 907; ANCOMBC $p \text{ adj} = 0.004$) and an uncultured bacterium from the genus *Kapabacteriales* (bOTU 1727; ANCOMBC $p \text{ adj} = 0.002$) and env.OPS_17 (bOTU 724; ANCOMBC $p \text{ adj} = 0.03$). Within Firmicutes, two bOTU representing unidentified bacterium from the genus *Paenibacillus* (bOTU 2326 ANCOMBC $p \text{ adj} = 0.0002$; bOTU 5393 ANCOMBC $p \text{ adj} = 0.0005$). For the remaining phyla, Acidobacteriota was represented by an uncultured bacterium

from the order Gaiellales (bOTU 10815; ANCOMBC $p \text{ adj} < 0.0001$), and an uncultured bacterium from the order Acidobacteriales (bOTU 1283; ANCOMBC $p \text{ adj} < 0.0001$), Cyanobacteria was represented by an uncultured bacterium from the genus *Sericytochromatia* (bOTU 3316; ANCOMBC $p \text{ adj} < 0.0001$) and *Elusimicrobiota* was represented by an uncultured bacterium from the genus *Lineage IV* (bOTU 1923; ANCOMBC $p \text{ adj} < 0.0001$).

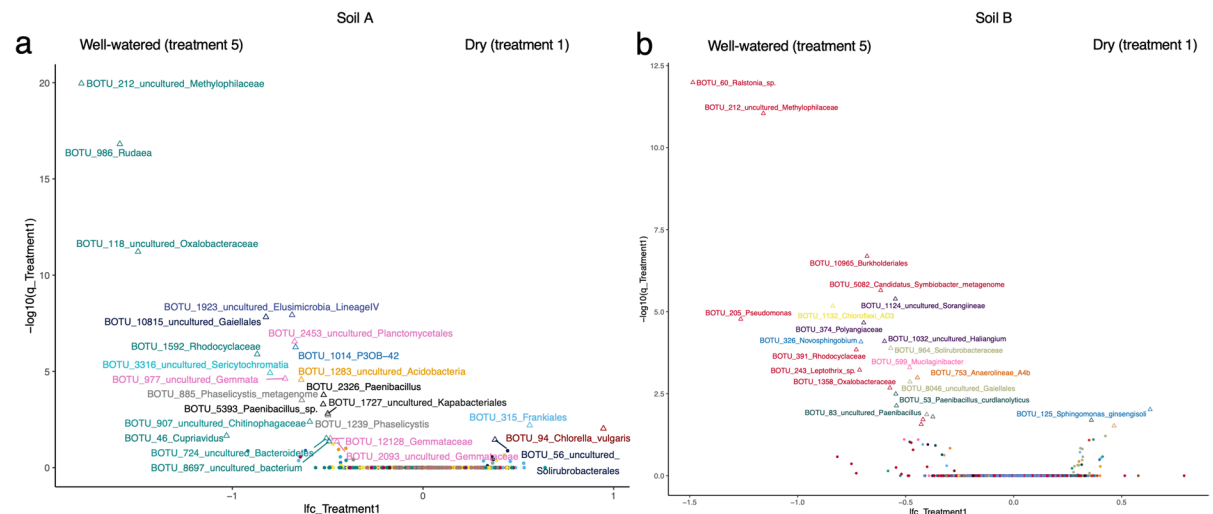
There were three bOTUs enriched in treatment 1 compared to treatment 5 (Fig. 5a). The more abundant taxa were members of the phyla Actinobacteria (67%, $n = 2$) and Cyanobacteria (33%, $n = 1$). Within Actinobacteria, two bOTUs were unidentified at the order level including Frankiales (bOTU 315; ANCOMBC $p \text{ adj} = 0.006$) and uncultured Solirubrobacterales (bOTU 56; ANCOMBC $p \text{ adj} = 0.04$). Lastly, Cyanobacteria included *Chlorella vulgaris* (bOTU 94; ANCOMBC $p \text{ adj} = 0.009$).

Soil B: There were 19 bOTUs with higher relative abundance in treatment 5 compared to treatment 1 (Fig. 5b). The enriched bOTUs belonged to the phyla Proteobacteria (47%, $n = 8$), Myxococcota (16%, $n = 3$), Firmicutes (11%, $n = 2$), Chloroflexota (11%, $n = 2$) and Actinobacteria (11%, $n = 2$). Within

the phylum Proteobacteria, Burkholderiales (bOTU 10965; ANCOMBC p adj < 0.0001) was unidentified at the order level, three bOTUs were unidentified at the family level which included Methylophilaceae (bOTU 212; ANCOMBC p adj < 0.0001), Rhodocyclaceae (bOTU 391; ANCOMBC p adj = 0.0001), Oxalobacteraceae (bOTU 1358; ANCOMBC p adj = 0.002) and five bOTUs were unidentified at the genus level including *Ralstonia* (bOTU 60; ANCOMBC p adj < 0.0001), *Candidatus Symbiobacter* (bOTU 5082; ANCOMBC p adj < 0.0001), *Pseudomonas* (bOTU 205; ANCOMBC p adj < 0.0001), *Novosphingobium* (bOTU 326; ANCOMBC p adj < 0.0001) and *Leptothrix* (bOTU 243; ANCOMBC p adj = 0.0006). The phylum Myxococcota was represented by one bOTU that was unclassified at the suborder level (*Sorangiiineae* bOTU 1124; ANCOMBC p adj < 0.0001), the family level (Polyangiaceae bOTU 374; ANCOMBC p adj < 0.0001) and at the genus level (*Haliangium* bOTU 1032; ANCOMBC p adj = 0.0008). Within Firmicutes there were two bOTU highlighted, *Paenibacillus curdlanolyticus* (bOTU 53; ANCOMBC p adj = 0.004) and an uncultured *Paenibacillus* (bOTU 83; ANCOMBC p adj = 0.008).

The phylum Chloroflexota was comprised of two bOTUs, with one unidentified AD3 cluster (bOTU 1132; ANCOMBC p adj < 0.0001) and an uncultured bacterium from the family A4b (bOTU 753; ANCOMBC p adj = 0.001). Actinobacteria was represented by an uncultured bacterium from the order Giaeallales (bOTU 8046; ANCOMBC p adj = 0.001) and an unidentified bacterium from the Solirubrobacteraceae family (bOTU 964; ANCOMBC p adj = 0.0001). Lastly, there was one bOTU enriched in treatment 1 compared to treatment 5, *Sphingomonas ginsengisoli* (Phylum Proteobacteria; bOTU 125; ANCOMBC p adj = 0.03).

Following this trend of Actinobacteria OTUs more abundant in the dry treatment and Proteobacteria more abundant in the wet treatments, we saw this following the moisture gradient with treatment 1 (dry) having the highest relative abundance in soil A with increasing divergence from the well-watered treatment over time (Fig. 6). Proteobacteria was the opposite trend, having the least relative abundance in the dry treatment and most in the well-watered treatment. Overall, this analysis expands upon the trends seen with the differential abundance analysis and gives us more confidence that our



moisture regimes affected the relative abundances of microbes at the phylum level.

Discussion

In this study, we aimed to understand the changes in peanut soil microbial communities varying across a moisture gradient since peanuts under drought conditions are more prone to colonization with mycotoxin-producing *A. flavus*. Despite the similar geographical location of the peanut soils, just more than 2 km apart, both fungal and bacterial communities had different community compositions in the two soils, demonstrating that fields can have different microbial compositions and thus may not be impacted by environmental conditions like moisture in a similar manner. The moisture treatment implemented throughout the nine-week study impacted the microbial composition, especially the bacteria. Some taxa groups were strongly associated with moisture changes. For example, we observed that Actinobacteria and OTUs in the fungal genus *Alternaria* increased their relative abundance in the drier treatment and thus provide future research paths to investigate if these taxa may be able to compete with detrimental organisms that also thrive under similar conditions, like *Aspergillus flavus*.

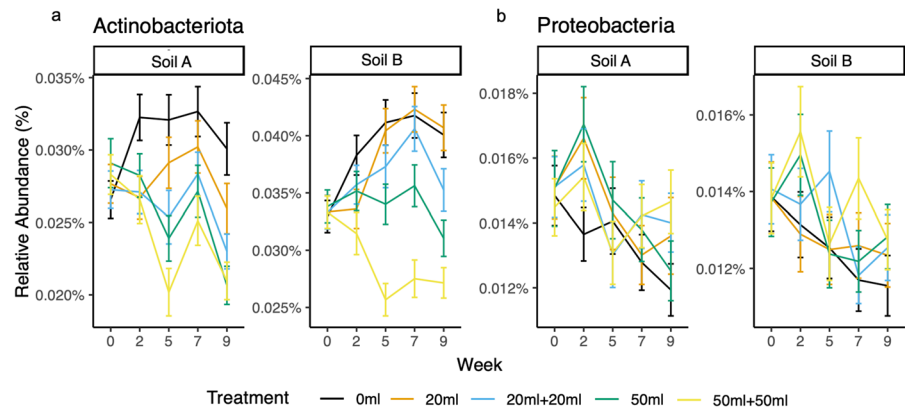
Changes in soil moisture can impact the microbial composition of soils (Xie et al. 2021). Bacterial taxa were more associated with moist treatments than drier ones. Proteobacteria were found at greater relative abundance in treatments containing 50 ml of water twice weekly (treatment 5) compared to the non-watered treatment. Proteobacteria have previously been associated with irrigated and moist soils and respond to rewetting events (Hartmann et al. 2017; Meisner et al. 2018; Naylor and Coleman-Derr 2018). Proteobacteria play a prominent role in carbon, nitrogen, and sulfur cycling in soils (Abdul Rahman et al. 2021; Hartmann et al. 2017). For example, the genus *Pseudomonas* (phylum Proteobacteria) has been identified as a plant growth-promoting rhizobacteria that can help the plant withstand abiotic or biotic stressors and increase nutrient availability in the soil (Wang et al. 2025). Similarly, *Paenibacillus* (phylum Proteobacteria) is a broad bacterial genus that can withstand extreme conditions, is a known plant growth-promoting rhizobacteria that can encourage

root growth and plant productivity, and several species within this genus have nitrogen fixing capabilities (Wu et al. 2024; Liu et al. 2019). Interestingly, the genus *Ralstonia* (phylum Proteobacteria) was associated with the highest moisture treatment. This genus contains the pathogen *Ralstonia solanacearum*, the causative agent of bacterial wilt in peanuts and several other plants. Jiang et al. (2021) found that soil moisture, specifically an increase, was the most important factor for predicting bacterial wilt disease outcomes in tomato (*Solanum lycopersicum*) plants. It is possible that a similar link exists between *Ralstonia solanacearum*, increased soil moisture, and peanuts but additional studies are required to elucidate this relationship.

In contrast to our results, Proteobacteria have also been found as core members of root microbiomes under drought and the most abundant phylum in soil, bringing into question the selection of these microbes by the plant in drought conditions (Xie et al. 2021). Expansion of our current study to sample roots under similar situations would be needed to test this hypothesis in peanuts. For example, the gram-negative nitrogen-fixing bacteria *Rhizobium* infects legumes through root hairs and forms nodules (Gage 2004). This source of nitrogen aids in plant fitness and stress tolerance (Abdul Rahman et al. 2021).

The Actinobacteria results show an increase in relative abundance in the drier treatments (dry and moderate). Actinobacteria are Gram-positive bacteria positively associated with drought environments due to its fast stress response. The adapted tolerance to moisture-limited soils from Actinobacteria might come as life strategies from certain bacterial groups, drought response mechanisms due to host specificity, or fast responses to stressors (Evans et al. 2020; Frene et al. 2022; Hartmann et al. 2017; Naylor and Coleman-Derr 2018; Xie et al. 2021), leading to adaptation to dry environments and conferring tolerance to other hosts such as plants. This phylum is known to have a dense cell wall and tends to have an oligotrophic lifestyle (Frene et al. 2022; Naylor and Coleman-Derr 2018). They are a widespread phylum known for producing stress proteins and osmoprotectants conferring stress resistance for themselves and for other hosts, such as plants (Xie et al. 2021). Some groups have filamentous growth (Actinobacteria), biofilm formation (EPS), production of stress-resistant spore-forming ability (exospores and

Fig. 6 Changes in Actinobacteria and Proteobacteria relative abundance follow a moisture gradient. **(a)** Relative abundance of all Actinobacteria OTUs in different moisture treatments in soil A and soil B. **(b)** Relative abundance of all Proteobacteria OTUs in different moisture treatments in soil A and soil B



endospores), and aid in aggregate formation in soils (Abdul Rahman et al. 2021; Frene et al. 2022; Hartmann et al. 2017; Xie et al. 2021). Hartmann et al. (2017) and Naylor and Coleman-Derr (2018) mention Actinobacteria as having genes for complex carbon degradation, hence facilitating degradation and utilizing recalcitrant carbon (important for the soil carbon cycle) in dry environments. In drought environments, plant selection has also been observed in many rhizospheric microbial studies (Xie et al. 2021). Actinobacteria are one of the most abundant and enriched phyla in the rhizosphere, and studies have detected plant growth promotion and alleviation of different stresses such as desiccation, higher temperatures, and salinity (Abdul Rahman et al. 2021; Finkel et al. 2020; Hartmann et al. 2017). For example, Actinobacteria have been associated with impeding the dissemination and development of plant pathogens such as *Agrobacterium tumefaciens* (crown gall disease) (Abdul Rahman et al. 2021), providing ammonium fixation (Abdul Rahman et al. 2021; Naylor and Coleman-Derr 2018), phosphorus mobilization (Naylor and Coleman-Derr 2018; Vargas Hoyos et al. 2021), degradation of cellulose and lignin, production and regulation of phytohormones (leading to changes in cell division, elongation, and differentiation), antibiotics, vitamins, and amino acids (Abdul Rahman et al. 2021). *Streptomyces*, a genus under the phylum Actinobacteria, have been shown to have plant growth-promoting properties and provide drought tolerance (Nazari et al. 2023). Hu et al. (2020) inoculated the rhizosphere of tomato plants with *Streptomyces* sp. TOR3209 and reported a shift in the prokaryotic community and increased abundance of beneficial prokaryotic taxa, which could lead to plant

growth promotion, inhibition of phytopathogens and increased phosphorus solubilization, phytohormone production and nitrogen fixation. Further, this genus has been documented to produce antimicrobial properties against *Phytophthora palmivora* (Nonthakaew et al. 2022), increase corn (*Zea mays*) biomass under normal and saline conditions (Nozari et al. 2021), and confer drought stress tolerance in *Sorghum* plants (Abdul Rahman et al. 2021). Utilizing species within this phylum to antagonize *Aspergillus flavus* in peanut settings could be a direction in future studies.

Unlike the bacterial communities, it was harder to parse strong trends in fungal community change due to the moisture treatments. Evans et al. (2020) reported that bacterial communities responded more to drought treatments, while fungi responded less. Generally, Ascomycota is known to dominate over Basidiomycota in drought conditions (Hartmann et al. 2017). Here, we found that two OTUs in the genus *Alternaria* (phylum Ascomycota) were more associated with the dry conditions but only in soil B, demonstrating the soil-specific responses to environmental conditions. *Alternaria* is often considered a leaf-spotting plant pathogen under warm, humid climates, but varied lifestyles such as nonpathogenic endophytes also exist (DeMers 2022). They also produce exorbitant conidia under warm, dry conditions, possibly reflecting that our data detected spores rather than growing mycelium (Grewling et al. 2022). In other studies, the Eurotiomycetes, a class in the phylum Ascomycota, had a higher abundance under drought (Frene et al. 2022; Meisner et al. 2018). In our study, Ascomycota were found in both soils and in moist, dry, and moderately dry treatments. In contrast, the wet treatment selected fungi with biological

predispositions to wet conditions. Rhizophlyctidales, an order in the phylum Chytridiomycota, need moisture to prompt zoosporulation (Hanrahan-Tan et al. 2023). Though we were not able to identify the specific species of these OTUs in this study, a species in that order, *Rhizophlyctis rosea*, is a known soil-inhabiting cellulose-degrading fungus, perhaps demonstrating its role as a decomposer of organic matter in soils under wet conditions.

In conclusion, drought poses a significant threat to agriculture and particularly to peanut production. Overall, we identified changes in the microbial community associated with changes in peanut soil moisture, which may help pinpoint potential antagonists against problematic fungi like *Aspergillus flavus*, and better understand how microbiomes change under stressful conditions. For example, we identified Actinobacteria as a bacterial phylum significantly associated with drier soil conditions. Future directions include repeating studies under field conditions with rain-restricting shelters and sequencing different peanut habitats, like roots and pods, which will determine if these trends stand under realistic field conditions.

Author contributions *Laura Rodríguez Rodríguez*: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing. *Morgan Bragg*: Conceptualization, Methodology, Validation, Formal Analysis, Visualization, Data Curation, Writing – original draft, Writing – review & editing. *Kira L. Bowen*: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision. *Zachary A. Noel*: Conceptualization, Methodology, Validation, Resources, Data Curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Data availability Raw sequencing data generated are available on the National Center for Biotechnology Information (NCBI) Sequence Read Archive at the bioproject ID PRJNA1150584. Code used to perform analysis and generate figures is located here: <https://github.com/Noel-Lab-Auburn/PeanutSoilDroughtMicrobiome> and on Zenodo with the following doi (<https://doi.org/10.5281/zenodo.13388411>).

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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