Nutrient effects on soil fungal communities in a coastal plain wetland ecosystem

By

Scott W. Siebor

December 2024

Director of Thesis: Ariane L. Peralta PhD

Major Department: Department of Biology

ABSTRACT

Anthropogenic influences such as urbanization and intensive agriculture practices modify nutrient and water cycles in significant ways. These activities can cause disruptions to microbial mutualisms, especially in low-nutrient wetland ecosystems. Due to ongoing alterations to nutrient and moisture variations, the composition of fungal microbe communities may be shifting in unexpected ways. In this study, I investigated how long-term fertilization, and hydrologic alterations affect soil fungal microbial communities in a historically low-nutrient coastal plain wetland. I hypothesized that long-term fertilization and varying water conditions through ditching influenced patterns in fungal communities to different degrees. I tested this hypothesis at a long-term nutrient addition (N-P-K fertilizers) and disturbance (mowing) experiment (established in 2003) located at East Carolina University's West Research Campus in Greenville, North Carolina. I specifically examined fungal microbe communities (based on amplicon sequencing of the internal transcribed spacer (ITS) region between the small and large subunits of the ribosomal RNA gene) in mowed plots undergoing and not undergoing nutrient enrichment. Results revealed that nutrient enrichment and ditch effects influenced fungal community composition, with community evenness being higher in wetter, unfertilized soils than drier fertilized soils over time. Additionally, soil treatments were more strongly associated with specific subsets of less abundant fungal families.

Nutrient effects on soil fungal communities in a coastal plain wetland ecosystem

A Thesis

Presented to the Faculty of the Department of Biology

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Biology

By

Scott W. Siebor

December 2024

Director of Thesis: Ariane L. Peralta, PhD

Thesis Committee Members

Michael Brewer, PhD

Carol Goodwillie, PhD

Table of Contents

LIST OF TABLES

LIST OF FIGURES

Introduction

Microbial mutualisms are critical for the maintenance of ecosystem functions, specifically, nutrient and carbon cycling. Microbial cross-kingdom species interactions (e.g., bacteria, archaea, fungi) and associations with plant partners are especially important. Mutualisms are established between microbes and plants through beneficial interactions, such as nutrient and resource exchange or antibiosis (Estrela et al. 2012; Isah 2019). An example of these mutualisms between plants, bacteria and fungi have existed since plant life first established terrestrial roots in the Devonian period of the Paleozoic era (Selosse &Tacon 1998; Pirozynski and Malloch 1975). For example, complex fungal structures associated with plant roots, known as the mycorrhiza, facilitate nutrient and water acquisition beyond the plant root boundary in exchange for fixed carbon provided by the plant. This mutualism helped drive the proliferation and diversification of terrestrial plant life with nutrients that were otherwise unavailable, along with altering the patterns of natural selection on plants by increasing tolerances to environmental stressors (Hawkins et al. 2023; Lau & Lennon 2011).

Bacterial, fungal and plant mutualisms facilitate nutrient exchange of available carbon- (C), nitrogen- (N) and phosphorus- (P) based metabolites. The consequent resource partitioning can be direct, such as bacteria fixing nitrogen for legume partners or fungal hyphae accessing nutrients beyond the plant's roots, or indirect, such as microbial antibiosis leading to protection of plant hosts from pathogens. An example of this can be seen in the roots of legumes, where bacterial genera such as *Rhizobium*, *Bradyrhizobium*, or *Sinorhizobium* associate with mycorrhizal families Bankeraceae, Hygrophoraceae and saprotrophic fungal families, such as Trimorphomycetaceae, establishing plant root and substrate connectivity as well as offering protection from bacterial pathogens (Kumari et al. 2021; Liu et al. 200; Sharma et al. 2020). However, environmental changes can influence these relationships in unexpected ways.

Enrichment of historically low nutrient ecosystems is becoming increasingly common, leading to direct and indirect effects on plant, bacterial, and fungal community composition. (Bledsoe et al. 2020; Goodwillie et al. 2020; Guignard et al. 2017). Most notably, human influence, such as industrialization, has altered the flow of nutrients such as N and P. These can be seen in land use modifications such as deforestation and industrial agricultural practices, which have led to increases in atmospheric emissions and eventual deposition of C, N and P to ecosystems that are adjacent to or far away from the land use change (Wang et al. 2015). Ongoing nutrient enrichment alterations in community structures of plants and microbes within soils is known to directly change the storage potential (Kearns et al. 2018). In coastal plain wetland plant communities, we know that nutrient enrichment has detrimental effects on biodiversity, steadily decreasing it over time (Goodwillie et al. 2020). Whereas the inverse can be seen in coastal plain wetland bacterial communities, with nutrient enrichments increasing overall biodiversity (Bledsoe et al. 2020).

However, despite extensive research on the impacts of nutrient enrichment on plants and bacterial communities of coastal plain wetlands, the effects on fungal community composition remain largely underexplored. This gap in understanding is particularly important due to the crucial roles that fungi play in soil nutrient cycling, decomposition, and plant interactions. There exists evidence of biodiversity decreases in soil fungal communities when exposed to hydrological stress in grasslands, as well as shifts in structure, relative to plant response to excess N and P nutrient addition (deVries et al 2018; Leff et al. 2015). In coastal plain wetlands, such as salt marshes, fungal community composition has been altered via increasing prevalence of fungal denitrifiers when exposed to prolonged nutrient enrichment (Kearns et al. 2018). A deeper investigation into how nutrient enrichment influences fungal diversity and function is essential for developing a more comprehensive understanding of the soil microbiome, especially in sensitive ecosystems like coastal plain wetlands.

Exploring this relationship can offer new insights into ecosystem health, resilience, and nutrient dynamics in coastal wetlands. As such, this study examined the long-term effects of fertilization on community composition patterns of soil fungal communities. I characterized the extent that long-term fertilization of low nutrient wetlands influence (1) patterns in soil fungal community composition, and (2) relative abundance of a subset of commonly observed fungal taxa. I hypothesize that if fungal communities are sensitive to nutrient enrichment compared to ambient conditions, then fungal diversity will decrease, and composition will shift in fertilized/mowed compared to unfertilized/mowed conditions. I also hypothesize that if overall fungal community structure is dependent upon a water saturated environment, then fungal diversity will be lower in dry conditions, and fungal composition will differ in dry compared to wet conditions.

Methods

Research Site:

This study was conducted on the long-term fertilization and disturbance by mowing experiment (established in 2003) at East Carolina University's West Research Campus (WRC) (Figure 1). Two treatments, fertilization and simulated fire disturbance via mowing, were replicated on eight 20 x 30m plots, following a 2-by-2 factorial design, which included two levels of fertilization (fertilized and unfertilized) and two levels of mowing (mowed or unmowed). Because of the presence of a ditch adjacent to half of the replicate blocks, a hydrologic gradient was observed, with drier soil conditions found in blocks adjacent to the ditch compared to those blocks away from the ditch. At the fertilized plots, an N-P-K (10-10-10) granular fertilizer was applied three times annually (yielding an annual application rate of 45.4 kg ha^{-1}). For the disturbance by mowing, aboveground vegetation was mowed and raked annually to simulate a previous fire disturbance in the historically wet-pine savannah ecosystem (Goodwillie et al., 2020). For this study, I focused on data collected during the 2019 through 2023 field seasons in the mowed plots only.

Fungal Sequencing Workflow:

For fungal sampling, composite soil cores were collected annually at the WRC. At each treatment plot, two soil samples (3.1 cm diameter, 12 cm depth) were collected near to each of the three permanent 1-m^2 quadrats established for plant community sampling (Bledsoe et al., 2020). The six soil cores per plot were combined, passed through a 4-mm sieve, homogenized, subsampled into sterile whirlpack bags, and stored at -20 °C for DNA extraction. Genomic DNA (gDNA) was extracted using the Qiagen PowerLyzer PowerSoil kit according to the manufacturer's protocol. Initial DNA concentrations were measured using a nanodrop

spectrophotometer, and samples were diluted using molecular grade PCR water to a uniform concentration of 10 ng/uL in preparation for amplification via Polymerase Chain Reaction (PCR)

Fungal Amplicon Sequencing:

Fungal community amplicon sequencing was completed in triplicate using primers specific to the internal transcribed spacer (ITS) region between the small and large subunits of the ribosomal RNA gene (White et al. 1990). Each DNA sample was amplified by PCR in triplicate (50 µL PCR libraries) for more concentrated reads using the Eppendorf Flexlid Nexus Gradient model MasterCycler. The PCR reactions were conducted using the following settings: 5 min of initialization at 95 ℃, 30 sec of denaturation at 95 ℃, 30 sec of annealing at 52 ℃, 60 sec of extension at 72 ℃, 10 min of final elongation at 72 ℃, and lastly a final hold at 4 ℃. PCR libraries were prepared by combining 34.75µL molecular grade water, 5µL Amplitaq gold 360 10x buffer, 5 µL MgCl2 (25 mmol/L), 1 µL dNTP (10 mM), 1 µL ITS-1F forward barcode primer (10 µmol/L), 1µL ITS-2R reverse barcode primer (10 µmol/L), 2µL DNA template (10 ng/µL), 0.25 µL Amplitaq gold taq-polymerase.

The ITS amplicon length was approximately 250-500bp. After confirming the quality of three replicates per gDNA sample, replicate PCR reactions were combined and concentrated using AxyGen magnetic beads, with a final elution of 20 µL. Each sample was quantified using the Qubit dsDNA BR assay to determine the DNA concentration. All samples were pooled in equimolar concentrations and submitted to the Duke Center for Genomic and Computational Biology Sequencing Core. All samples were pooled in equimolar concentrations of 5 ng/µL and sequenced using the Illumina MiSeq platform, chosen primarily due to accessibility but also its fast run time. Other studies in microbial community analysis use this platform for its short read length; therefore, the machine's high throughput/run (>25 million reads or 15Gb per lane) reduces costs significantly and provides greater coverage, at the cost of short-read length,

overcome by pair-end reads (Hert et al., 2008). This means that the target DNA can be sequenced in a forward and reverse direction, thus improving the ability to identify the position in the read and structural errors such as deletions, insertions, and repetitive regions.

I formatted sequences prior to downstream processing. First, primers were removed from each sequence using GitBash terminal commands. Additional commands were then applied to remove any 'N' calls in the sequences, replacing the standard DADA2 cutadapt steps with this custom processing. Then, I initiated the DADA2 workflow in the R statistical environment (Callahan et al. 2016). I confirmed primer removal using the primer() function and proceeded with sequence identification. Forward and reverse sequences were identified, and error rates were calculated using the learnErrors() function. The sequences were then merged into single contiguous sequences via the mergers() function. Sequence tables were generated using the makeSequenceTable() function, and chimeras were removed using the function removeBimeraDenovo(). Finally, taxonomy was assigned using the UNITE database (Kõljalg et al 2020), and CSV files were exported for downstream analysis of the non-chimeric sequence. Prior to downstream diversity calculations and statistics, I evaluated the total number of sequences per sample, and I omitted five samples with total sequences of less than ten. In addition, Amplicon Sequence Variant (ASV) counts that totaled less than 10 across all samples were omitted from the data set.

Statistical Analyses

All statistical analyses, diversity calculations, and graphing were conducted in the R environment (RStudio 2024.04.2+764 "Chocolate Cosmos"). I used various packages such as phyloseq (McMurdie and Holmes, 2013) for data import and initial community analysis, such as construction of sequence tables, ggplot2 (Wickham, 2016) for graphing species diversity and composition, tidyverse (Wickham et al., 2019) for data importing, data wrangling and

manipulating, and data visualization, and finally, vegan (Oksanen et al., 2012) for computing diversity metrics and conducting multivariate analyses.

Prior to calculating diversity metrics, I rarefied the data to 29,090, and I calculated the following soil fungal diversity metrics: Shannon diversity H', using the diversity() function and Pielou's Evenness Index J' using the shannon/log() function, both from the vegan package and ASV richness, where I summed ASV counts based on the presence/absence matrix. Plotting of the diversity metrics were performed using the ggplot package. Species diversity examines the composition of a community and considers every unique organism in the area (species richness), as well as how each species relates to the others in terms of relative abundance (species evenness). Shannon diversity considers both the number of distinct species (i.e., species richness) and their distribution within the community, with more sensitivity towards rare species (Shannon & Weaver 1963). Pielou's evenness index measures how evenly sequences are distributed among the different species (or taxa) within a community (Pielou 1969). It was here that two additional samples were removed for low sequence counts that were skewing the boxplot interquartile ranges. I used analysis of variance (ANOVA) to measure the extent that fertilization, proximity to ditch, and year influences variation in fungal diversity, evenness, and ASV richness. I also summed the relative abundance according to fertilization treatment and hydrology to examine patterns of dominant fungal taxa and compared relative abundances using an ANOVA.

To characterize patterns of the total soil fungal community, I used permutational analysis of variance (PERMANOVA) using the adonis() function in the vegan package to measure the variation in soil fungal community explained by fertilization, year, ditch effect, and their interaction. Community composition was visualized using the ordination technique Principal Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity of the relative abundance of the fungal communities using the vegan and ggplot packages. I used ordination plots to

evaluate patterns in fungal community composition according to fertilization, year, and ditch effect. Then, I subset the data to focus on the most abundant family-level taxa, which were Bankeraceae, Hygrophoraceae and Trymorphomycetaceae. For each of the three families, I ran a series of PERMANOVAs and PCoA ordination plots. The PERMANOVA also focused on explaining variation due to fertilization, year, and the interaction. The proximity to ditch was excluded from the final model for the family-level analyses because it was not significant in the full model. Finally, I examined an additional subset of the data to focus on the top three indicator families Herpotrichiellaceae, Tympanidaceae, and Hyaloscyohaceae. The indicspecies package was used to calculate fidelity (i.e., the degree to which the species is associated with the treatment group) and constancy (i.e., the consistency to which the species is identified across samples within the treatment group) for the indicator values (De Caceres & Legendre 2009). For each of these three families, I also ran a series of PERMANOVAs and PCoA ordination plots. The PERMANOVA also focused on explaining variation due to fertilization, hydrology, year, and the interactions.

Results

Fertilization, hydrology, and year influenced fungal species diversity to different degrees

The average Shannon diversity was higher in unfertilized compared to fertilized plots over time, except for 2023 Shannon diversity, which was higher in fertilized compared to unfertilized for wet plots only (ANOVA, $F_{1,54}$ = 15.1428, P= 0.0003; Figure 2, Table 1). The treatment differences between fertilized and unfertilized plots were larger in wet plots compared to the dry plots (ANOVA, Treatment: $F_{1,54}$ = 2.0566, P= 0.1573; Figure 2, Table 1). The main effect of year and the interaction of treatment, hydrology, and year also revealed a significant effect on diversity (ANOVA Year: $F_{4,54}$ = 3.8932, P= 0.0075; Treatment x Hydrology x Year: $F_{4,54}$ = 3.0966, P= 0.0229; Figure 2, Table 1). The pattern in Pielou's evenness was similar to the Shannon diversity trends, with communities in the unfertilized plots representing higher evenness than fertilized plots in all years (ANOVA, Treatment: $F_{1,54}$ = 28.3076, P= $2.04e^{-6}$; Figure 3, Table 2). Further, differences were more pronounced in the wetter (i.e., away from the ditch) plots compared to dry plots across all years (ANOVA, Treatment x Hydrology: $F_{1,54}$ = 12.4950, P = 0.0008; Figure 3, Table 2). In addition, the main effect of hydrology and the interaction between treatment and year were also significantly affecting evenness (ANOVA, Hydrology, $F_{1,54}$ = 10.3028, P = 0.0022; Treatment x Year, $F_{4,54}$ = 3.7804, P = 0.0088 Figure 3, Table 2) Finally, only year influenced ASV richness, where a decrease in richness was observed for 2023 ($F_{4,54}$ = 7.8215, P = 4.77e-5; Figure 4, Table 3).

Fertilization and year influenced fungal community composition

Fertilization, hydrology, and year significantly influenced variation in fungal community composition (PERMANOVA, Treatment: R^2 = 0.1131, F = 10.5109, P = 0.001; Hydrology: R^2 = 0.0460, F = 4.2693, P = 0.001; Year: R^2 = 0.0971, F = 2.2566, P = 0.001; Figure 5, Table 4). To a

lesser degree, the interaction of hydrology and treatment explained variation in fungal community composition (PERMANOVA, Hydrology x Treatment: $R^2 = 0.0322$, $F = 2.9923$, $P = 0.002$; Figure 5, Table 4). For the most abundant fungal family (based on relative abundance), Bankeraceae, year and fertilization treatment explained variation in community composition (PERMANOVA, Treatment: $R^2 = 0.0337$, $F = 2.5894$, $P = 0.0563$; Year: $R^2 = 0.0931$, $F_{4,64} = 1.7909$, $p = 0.0580$; Figure 6, Table 5). For the second most abundant fungal family Hygrophoraceae, year and to a lesser extent fertilization treatment significantly explained variation in community composition (PERMANOVA, Year: $R^2 = 0.1191$, $F_{4,60} = 2.2444$, $p < 0.0001$; Treatment: $R^2 = 0.0300$, $F_{1,60} = 2.2624$, $p = 0.0054$; Figure 7, Table 6). Finally, for the third most abundant fungal family Trimorphomycetaceae, year (but not fertilization treatment) explain variation in community composition (PERMANOVA, Year: $R^2 = 0.2416$, $F_{4,63} = 6.1075$, $p = 9.99e\text{-}5$; Figure 8). To a lesser degree, the interaction of treatment and year influenced Trimorphomycetaceae community composition (PERMANOVA, Treatment x Year: $R^2 = 0.1095$, $F_{4,63} = 2.7643$, $p = 0.0170$; Figure 8, Table 7). Further, for the most abundant fungal family, the sum of the relative abundances of Bankeraceae ASVs were higher in fertilized compared to unfertilized plots (ANOVA, Treatment: $F_{1,794} = 3.729$, $p = 0.0538$; Figure 13, Table 12). For the second most abundant fungal family, Hygrophoraceae, the summed relative abundances of ASVs were higher in unfertilized compared to fertilized for the dry plots only and the opposite pattern for wet plots (ANOVA, Treatment x Hydrology: $F_{1,2792} = 7.980$, $p = 0.0048$; Figure 13, Table 13). The summed relative abundances for the Trimorphomycetaceae ASVs were similar across fertilization and hydrology (Figure 13, Table 14).

Species indicator analysis revealed three unique fungal families that are associated with fertilization. The ASV with the highest species indicator occurrence belonged to the Hyaloscyphaceae family and associated with unfertilized plots. When all ASVs in the Hyaloscyphaceae family were examined, fertilization treatment and to a lesser degree, the

interaction between treatment and hydrology significantly explained patterns in community composition (PERMANOVA, Treatment: $R^2 = 0.1121$, $F_{1,50} = 3.5898$, $p = 9.9e^{-5}$; Treatment x Hydrology: $R^2 = 0.0837$, $F_{1,50} = 1.7276$, $p = 0.0579$ Figure 11, Table 10). The ASV with the second highest fungal indicator value belonged to the family Tympanidaceae and also associated with unfertilized plots. When we subset all ASVs in the Tympanidaceae family, the main effects of fertilization treatment, year, hydrology and the interaction of treatment and year, as well as treatment and hydrology significantly explained variation in community composition (PERMANOVA, Treatment: $R^2 = 0.1286$, $F_{1,52} = 12.4111$, $p = 9.9e^{-5}$; Year: $R^2 = 0.0760$, $F_{4,52} = 1.8344$, $p = 0.0016$; Hydrology: $R^2 = 0.0514$, $F_{1,50} = 4.9554$, $p = 9.9e^{-5}$; Treatment x Year: $R^2 = 0.0582$, $F_{4,52} = 1.4044$, $p = 0.0365$; Treatment x Hydrology: $R^2 = 0.0600$, $F_{1,52} = 5.7891$, $p = 9.9e$-5; Figure 10, Table 9). Lastly, we subset ASVs from Herpotrichiellaceae family, which was the only indicator family associated with fertilized plots only and identified that fertilization treatment, year, hydrology and the interaction of fertilization treatment and year, treatment and hydrology, as well as to a lesser degree, hydrology and year explained variation in community composition (PERMANOVA, Treatment: $R^2 = 0.1561$, $F_{1,52} = 20.8558$, $p = 9.9e^{-5}$; Year: $R^2 = 0.1390$, $F_{4,52} = 4.6415$, $p = 9.9e^{-5}$; Hydrology: $R^2 = 0.1218$, $F_{1,52} = 16.2765$, $p = 9.9e^{-5}$; Treatment x Year: $R^2 = 0.0243$, $F_{4,52} = 3.1806$, $p = 9.9e^{-5}$; Treatment x Hydrology: $R^2 = 0.0243$, $F_{1,50} = 3.2433$, $p = 0.0074$; hydrology & year: $R^2 = 0.0463$, $F_{4,50} = 1.5458$, $p = 0.0578$ Figure 9, Table 8). It is important to note that these three indicator taxa represented families that were not the top three ASVs identified in the indicator species analysis table, but rather the first three ASVs that were able to be classified to the family level based on UNITE database.

Discussion

Changes to abiotic conditions such as nutrient enrichment and hydrology modify nutrient and water cycles, which results in changes to plant communities (Goodwillie et al. 2020) and soil microbial communities (Bledsoe et al. 2020, Koceja et al. 2021). In this study, results revealed that long-term fertilization and changes to hydrology modified fungal species diversity and community composition in unexpected ways. Specifically, I examined the extent that fertilization and hydrologic conditions affected (1) patterns in soil fungal species diversity and community composition, (2) relative abundance of a subset of dominant fungal taxa, and (3) fungal species diversity and community composition of a subset of fungal families over time (2019 to 2023).

Nutrient Enrichment Modifies Fungal Species Diversity and Community Composition to Different Degrees

In our study, we observed that fertilization decreased fungal diversity and evenness (Figure 3, Table 2). Regarding other ecosystems, the observed results from this study were similar to findings in a long-term phosphorus fertilization study, where there was a negative enrichment effect on fungal diversity in a prairie ecosystem (Beauregard et al. 2009). Additionally, nutrient enrichment was observed to decrease fungal diversity in forest and grassland environments (Anthony et al 2021; Leff et al. 2015). Conversely, nutrient enriched soils of tropical forests and marshlands resulted in higher Shannon diversity of fungal communities compared to control soils (Kerekes et al. 2013). Fertilization also positively influenced fungal species diversity in wetland marsh soils (Kearns et al. 2018). Our findings partially support our hypothesis that nutrient enrichment negatively affected fungal diversity and further highlight the context-specific nature of these shifts, particularly in coastal

plain wetlands, where increases in fertilization via atmospheric deposition due to anthropogenic influence interacts to reshape soil microbial community patterns. This suggests that land-use practices, even far from the initial source, could have nuanced and regionally specific impacts on fungal diversity, with implications for ecosystem services such as nutrient cycling and carbon storage.

Hydrology Modifies Fungal Species Diversity and Community Composition

Fungal Shannon diversity was similar between wet and dry plots. However, hydrology did significantly decrease Pielou's evenness, especially in unfertilized plots (Figure 3, Table 2). This aligns with previous research on wetland ecosystems, which emphasizes water availability as a critical factor for microbial life and supports our hypothesis that water limitation has negative implications for microbial diversity (Bogati & Walczak 2022). In addition, research in other wetlands has demonstrated that hydrology, particularly gradients of low versus high water saturation, significantly influences microbial populations involved in processes like anaerobic decomposition, further highlighting the importance of water availability in maintaining microbial diversity and specifically fungal diversity in wetlands (Ma et al. 2018; Maietta et al. 2020)

The moisture levels in the unfertilized, wet plots likely support a more diverse composition of fungal species, whereas the drier conditions may stress microbial communities, leading to decreased fungal species evenness. Our findings also contrast what has been seen in other ecosystems, such as grasslands, where post-drought conditions resulted in increased fungal richness and evenness (de Vries et al., 2018). This is also similar to other studies, such as in agricultural wheat fields, which showed little to no diversity effects for fungal communities under restricted water availability (Kundel et al., 2020). These results suggest that the availability of water in wetlands, specifically, could be buffering changes to fungal community shifts due to fertilization.

We also observed that hydrology influenced fungal evenness but not richness or diversity. In wet plots, where water availability is relatively higher than plots adjacent to a drainage ditch, fungal species are less drought-stressed, allowing more species to maintain similar population levels (Chen et al. 2023). This moisture status supports evenness by mitigating stressors that could otherwise favor only the most drought-tolerant species. In dry conditions, the community may shift to favor species adapted to moisture stress, resulting in fewer abundant species that dominate the landscape, thus reducing evenness without altering overall richness (Kaiserman et al. 2015). Alternatively, studies, such as those in wetland and riparian zones, indicate that fungi adapt to moisture gradients by adjusting community interactions rather than changing species richness. For example, moisture can enable a variety of niche adaptations and metabolic strategies, allowing many species to coexist (Ma et al. 2018). However, in dry conditions, only a few highly competitive or stress-tolerant fungi might dominate, resulting in an uneven structure while leaving the richness unchanged (Kundel et al., 2020).

Fungal Community Composition Effects are Independent of Dominant Taxa

When focused on the community composition of the dominant taxa (i.e., highest relative abundance fungal families), Bankeraceae, Hygrophoraceae, and Trimorphomycetaceae consistently remained the top three families across all five years. Notably, Bankeraceae maintained higher relative abundances in fertilized compared to unfertilized plots under both wet and dry conditions, while Hygrophoraceae exhibited higher abundance specifically in unfertilized dry plots and fertilized wet plots (Figure 13, Table 12, Table 13). To further evaluate taxa-level associations with the fertilization effect, we used indicator ‘species’ analysis (based on ASVs at the family level) to explore fertilization effects on specific ASVs. It was observed that these indicator families represented lower relative abundance ASVs (Figure 9, Table 8; Figure 10,

Table 9; Figure 11, Table 10). While there was no initial prediction of this outcome, it does provide insight to the role that less dominant fungal taxa have in dictating community patterns.

A consistent set of dominant families was found in all five years of samples, despite treatment differences resuling in distinct environmental conditions. This consistent pattern suggests that these families possess phylogenetic and taxonomic diversity that enable the dominance of these taxa despite the environmental variability from the experimental treatments. Notably, a substantial proportion of the fungal ASVs remain unidentified each year, highlighting the limitations of current taxonomic databases and sequencing techniques and suggests that our understanding of the community composition may be incomplete, possibly obscuring less dominant but ecologically relevant families.

Although the dominant families were relatively abundant across years, the indicator species analysis highlights a different set of families consistently associated with changing environmental conditions. This suggests that a different subset of taxa is responsive to environmental changes introduced by the treatments. While less dominant taxa may not be as frequent or abundant due to unideal conditions or more specialized roles, their presence within the fungal community still contributes valuable ecosystem functions by interacting synergistically with dominant families, facilitating nutrient cycling, enhancing moisture and nutrient exchange, especially as environmental conditions fluctuate (Morón-Ríos 2017).

Putative Roles of the Dominant Fungal Taxa

I performed an analysis of ASVs representing the most abundant fungal families; ASVs from the families Bankeraceae, Hygrophoraceae, and Trimorphomycetaceae were dominant across all five years. In prior studies, these fungal families played essential roles in nutrient cycling and plant health. Bankeraceae, primarily comprising ectomycorrhizal fungi, form symbiotic relationships with tree roots, helping plants absorb critical nutrients like nitrogen and

phosphorus in exchange for carbon compounds from the host. This nutrient exchange is especially valuable in nutrient-poor soils, where Bankeraceae fungi help sustain forest health by driving nutrient cycling and supporting tree growth (Holec & Kučera 2018). This aligns with the broader body of research by emphasizing the role of ectomycorrhizal fungi like Bankeraceae in nutrient acquisition in nutrient-poor soils, further supporting their previously defined role in forest ecosystems (Holec & Kučera 2018).

The fungal families Hygrophoraceae and Trimorphomycetaceae both contribute significantly to nutrient cycling and organic matter decomposition within soil ecosystems. Hygrophoraceae, largely represented by waxcap fungi, thrive in grasslands and woodland soils, where they act as saprotrophs, breaking down organic matter and enriching soil fertility. These fungi are indicators of stable, low-disturbance habitats, reflecting conditions that promote balanced ecosystem processes and fungal diversity (Lodge et al. 2014). Trimorphomycetaceae similarly aids in nutrient turnover, particularly in nutrient-rich or disturbed soils where rapid microbial activity is supported. Members of this family decompose organic material, playing an active role in carbon cycling and contributing to soil health, especially in areas with high nutrient inputs (Liu et al. 2015). The roles of Hygrophoraceae and Trimorphomycetaceae in soil ecosystems underscore the importance of saprotrophic fungi in nutrient turnover and stability, consistent with findings on fungal contributions to low-disturbance habitats (Lodge et al. 2014).

Putative Roles of Indicator Fungal Taxa

We identified ASVs from the families Hyaloscyphaceae, Herpotrichiellaceae, and Tympanidaceae that were associated with specific treatment effects over all five years. Hyaloscyphaceae consists primarily of saprotrophic fungi that thrive on decomposing plant material, particularly on dead wood, leaf litter, and other organic matter. These fungi produce a range of enzymes that allow them to break down complex compounds like cellulose and lignin in

plant debris. By converting organic matter into simpler nutrients, Hyaloscyphaceae fungi help maintain soil fertility and support other soil organisms, contributing to balanced nutrient cycles in their ecosystems (Vohnik et al. 2022). The Herpotrichiellaceae family includes a variety of fungi, some of which are saprotrophic, while others are pathogenic or form endophytic relationships within plant tissues. Many species in this family contribute to organic matter decomposition, particularly leaf litter and decaying wood, aiding in nutrient turnover and soil health while also having medical significance due to their potential pathogenicity in humans and animals (Crous et al. 2007). The Tympanidaceae family includes primarily saprotrophic fungi, which play a crucial role in nutrient cycling by decomposing dead wood, leaf litter, and other plant debris, and contain some strains of plant pathogens. These fungi help break down lignin and cellulose, which are complex compounds in plant materials, thereby releasing essential nutrients back into the soil. This decomposition process is particularly important for ecosystems, where it contributes to soil fertility and promotes healthy nutrient turnover by recycling organic matter into forms accessible to other soil organisms (Quijada et al 2020).

All three indicator fungal families appear to be primarily saprotrophic, favoring wood and other forms of plant matter as their substrate. This aligns with observations from the annual WRC plant surveys, which have documented an increase in the relative abundance of woody plant species, such as blackberry and maple and sweetgum saplings, as indicated by higher stem counts and increased percent coverage in sampling quadrats. While a direct causal relationship has yet to be established, the presence of these saprotrophic families may be influenced by the growing abundance of woody plants at the WRC, suggesting a potential connection worth exploring further. Dead root material, resulting from mowing treatments, is a key factor driving fungal community composition. The high turnover rate of plant matter in these plots creates conditions favorable to saprotrophic fungi, as confirmed by the indicator species analysis. While other fungal families also contributed to the observed community structures, the

top three families remained functionally consistent across all years, dominated by taxa associated with decomposition. This consistency underscores the significant role of dead plant material in shaping fungal communities, as well as the functional importance of saprotrophs in these ecosystems.

Results from this study align with the broader literature, which highlights the roles of saprotrophic and mutualistic fungi in supporting nutrient turnover and nutrient acquisition for plants. The consistent association of Hyaloscyphaceae, Herpotrichiellaceae, and Tympanidaceae with specific treatments suggests that these families are responsive to environmental changes, playing pivotal roles in nutrient and carbon cycling under varying conditions. This supports previous research on the ecological importance of both saprotrophic and arbuscular mycorrhizal fungi, underscoring their contributions to soil fertility and nutrient cycling across diverse ecosystems (Quijada et al 2020; Vohnik et al. 2022; Stürmer 2012)

Time and Plant Material as a Driving Factor of the Experiment

I anticipated a time effect on fungal community composition, as time contributes to the assembly of biological communities. This expectation is further supported by data from the EPA CASTNET system and the National Atmospheric Deposition Program, which indicates consistent nitrogen deposition contributing to ambient nutrient enrichment throughout the study period of 2019 through 2023 (National Atmospheric Deposition Program 2024; US Environmental Protection Agency 2024). Such enrichment creates a dynamic baseline against which other treatment effects can be evaluated, making time an essential factor to include in the analysis. The decision to examine five years of data was deliberate and grounded in multiple objectives. First, the study aimed to capture not only the effects of nutrient enrichment and hydrological conditions on fungal communities but also how these effects manifest and change over time. Lastly, observing temporal shifts in fungal communities alongside complementary

plant and bacterial data offers insights into how the ecosystem's functionality may be changing and provides a basis for predicting future shifts. Given the increasing ambient nutrient deposition, understanding these temporal dynamics is critical to contextualizing the results within broader ecological trends.

Limitations and Future Directions

One limitation of this study is the reliance on current sequencing technologies and available taxonomic databases, which left a considerable proportion of fungal ASVs unidentified. This taxonomic uncertainty limits our ability to capture the full diversity and potential functional roles of the fungal community, particularly among less dominant fungal ASVs. Despite these limitations, the analyses performed revealed fungal community shifts across fertilization treatment, hydrology and time. These findings underscore the variation in taxa-level responses to environmental differences, where the dominant taxa remain similar across treatment differences and a subset of taxa and relatively lower abundance were sensitive to the abiotic treatment differences.

Future studies would benefit from ongoing efforts to expand fungal taxonomic databases, which would help reduce the number of unidentified ASVs and enable a more comprehensive assessment of fungal diversity. Another limitation of this study was that it was entirely DNA based, and future studies could consider RNA-based sequencing of fungal ribosomal RNA (rRNA) regions, such as the small (SSU) and large (LSU) ribosomal subunits, to distinguish metabolically active members of the community instead of non-transcribable ITS regions (Xue et al 2019). These rRNA regions would allow researchers to assess which taxa are actively responding to environmental treatments, providing a more dynamic view of fungal community functionality (Raja et al. 2017; Xue et al. 2019).

Additionally, incorporating a broader range of experimental treatments could yield further insights into the adaptability and resilience of these communities. For instance, testing varied nutrient addition types or concentrations could help identify specific responses among fungal families to a gradient of nutrient levels. Quantifying hydrologic dynamics alongside community shifts would enable the study of nuanced responses to water availability. Additional factors such as pH adjustments, temperature shifts, or light exposure could also be examined to explore how fungal communities respond to a variety of environmental changes. Finally, additional longitudinal ecological studies would help elucidate longer-term responses and shifts within fungal communities, providing a more complete picture of community dynamics over time.

References

Anthony, M. A., Knorr, M., Moore, J. A., Simpson, M., & Frey, S. D. (2021). Fungal community and functional responses to soil warming are greater than for soil nitrogen enrichment. Elem Sci Anth, 9(1), 000059.

Beauregard, M. S., Hamel, C., & St-Arnaud, M. (2010). Long-term phosphorus fertilization impacts soil fungal and bacterial diversity but not AM fungal community in alfalfa. Microbial ecology, 59(2), 379-389.

Bledsoe, R. B., Goodwillie, C., & Peralta, A. L. (2020). Long-Term Nutrient Enrichment of an Oligotroph-Dominated Wetland Increases Bacterial Diversity in Bulk Soils and Plant Rhizospheres. mSphere, 5(3), e00035-20. https://doi.org/10.1128/mSphere.00035-20

Bogati, K., & Walczak, M. (2022). The impact of drought stress on soil microbial community, enzyme activities and plants. Agronomy, 12(1), 189.

Bradshaw, M. J., Braun, U., & Pfister, D. H. (2022). Phylogeny and taxonomy of the genera of Erysiphaceae, part 1: Golovinomyces. Mycologia, 114(6), 964–993. https://doi.org/10.1080/00275514.2022.2115419

Bridgham, S. D., Megonigal, J. P., Keller, J. K., Bliss, N. B., & Trettin, C. (2006). The carbon balance of North American wetlands. *Wetlands*, *26*(4), 889–916. https://doi.org/10.1672/0277-5212(2006)26[889:TCBONA]2.0.CO;2

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621–1624. https:// doi.org/10.1038/ismej.2012.8.

Chen, X., Wang, Y., Wang, Y., Zhang, Y., Shen, Y., He, X., & Xiao, C. (2023). A natural moisture gradient affects soil fungal communities on the South Shore of Hulun Lake, Inner Mongolia, China. Journal of Fungi, 9(5), 549.

Crous, P. W., Schubert, K., Braun, U., De Hoog, G. S., Hocking, A. D., Shin, H. D., & Groenewald, J. Z. (2007). Opportunistic, human-pathogenic species in the Herpotrichiellaceae are phenotypically similar to saprobic or phytopathogenic species in the Venturiaceae. Studies in Mycology, 58(1), 185-217.

Debeljak, P., & Baltar, F. (2023). Fungal diversity and community composition across ecosystems. Journal of Fungi, 9(5), 510.

De Cáceres M, Legendre P (2009). “Associations between species and groups of sites: indices and statistical inference.” _Ecology_, *90*, 3566-3574. doi:10.1890/08-1823.1 <https://doi.org/10.1890/08-1823.1>.

deVries, F. T., Griffiths, R. I., Bailey, M., Craig, H., Girlanda, M., Gweon, H. S., Hallin, S., Kaisermann, A., Keith, A. M., Kretzschmar, M., Lemanceau, P., Lumini, E., Mason, K. E., Oliver, A., Ostle, N., Prosser, J. I., Thion, C., Thomson, B., & Bardgett, R. D. (2018). Soil bacterial networks are less stable under drought than fungal networks. Nature Communications, 9(1), 3033. https://doi.org/10.1038/s41467-018-05516-7

Estrela, S., Trisos, C. H., & Brown, S. P. (2012). From Metabolism to Ecology: Cross-Feeding Interactions Shape the Balance between Polymicrobial Conflict and Mutualism. *The American Naturalist*, *180*(5), 566–576. https://doi.org/10.1086/667887

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes—Application to the identification of mycorrhizae and rusts. Molecular Ecology, 2(2), 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Goodwillie, C., McCoy, M. W., & Peralta, A. L. (2020). Long-term nutrient enrichment, mowing, and ditch drainage interact in the dynamics of a wetland plant community. Ecosphere, 11(10), e03252. https://doi.org/10.1002/ecs2.3252

Guignard, M. S., Leitch, A. R., Acquisti, C., Eizaguirre, C., Elser, J. J., Hessen, D. O., Jeyasingh, P. D., Neiman, M., Richardson, A. E., Soltis, P. S., Soltis, D. E., Stevens, C. J., Trimmer, M., Weider, L. J., Woodward, G., & Leitch, I. J. (2017). Impacts of Nitrogen and Phosphorus: From Genomes to Natural Ecosystems and Agriculture. Frontiers in Ecology and Evolution, 5. https://www.frontiersin.org/articles/10.3389/fevo.2017.00070

Hawkins, H.-J., Cargill, R. I. M., Van Nuland, M. E., Hagen, S. C., Field, K. J., Sheldrake, M., Soudzilovskaia, N. A., & Kiers, E. T. (2023). Mycorrhizal mycelium as a global carbon pool. *Current Biology*, *33*(11), R560–R573. https://doi.org/10.1016/j.cub.2023.02.027

He, C., Ruan, Y., & Jia, Z. (2024). Effects of Nitrogen Addition on Soil Microbial Biomass: A Meta-Analysis. Agriculture, 14(9), 1616.

Hert, D.G., Fredlake, C.P., Barron, A.E., 2008. Advantages and limitations of next-generation sequencing technologies: A comparison of electrophoresis and non-electrophoresis methods. electrophoresis 29, 4618–4626. doi:10.1002/elps.200800456

Holec, J., & Kučera, T. (2018). Hydnoid fungi of the family Bankeraceae–their assemblages and vegetation ecology in Central Europe, Czech Republic. Fungal ecology, 32, 40-48.

Huang, R., McGrath, S. P., Hirsch, P. R., Clark, I. M., Storkey, J., Wu, L., Zhou, J., & Liang, Y. (2019). Plant–microbe networks in soil are weakened by century-long use of inorganic fertilizers. *Microbial Biotechnology*, *12*(6), 1464–1475. https://doi.org/10.1111/1751-7915.13487

Isah, T. (2019). Stress and defense responses in plant secondary metabolites production. *Biological Research*, *52*(1), 39. https://doi.org/10.1186/s40659-019-0246-3

Kaisermann, A., Maron, P. A., Beaumelle, L., & Lata, J. C. (2015). Fungal communities are more sensitive indicators to non-extreme soil moisture variations than bacterial communities. Applied Soil Ecology, 86, 158-164.

Kearns, P. J., Bulseco-McKim, A. N., Hoyt, H., Angell, J. H., & Bowen, J. L. (2019). Nutrient Enrichment Alters Salt Marsh Fungal Communities and Promotes Putative Fungal Denitrifiers. *Microbial Ecology*, *77*(2), 358–369. https://doi.org/10.1007/s00248-018-1223-z

Kerekes, J., Kaspari, M., Stevenson, B., Nilsson, R. H., Hartmann, M., Amend, A., & Bruns, T. D. (2013). Nutrient enrichment increased species richness of leaf litter fungal assemblages in a tropical forest. Molecular Ecology, 22(10), 2827-2838.

Koceja, M. E., Bledsoe, R. B., Goodwillie, C., & Peralta, A. L. (2021). Distinct microbial communities alter litter decomposition rates in a fertilized coastal plain wetland. Ecosphere, 12(6), e03619. https://doi.org/10.1002/ecs2.3619.

Kõljalg U, Nilsson HR, Schigel D, Tedersoo L, Larsson K-H, May TW, Taylor AFS, Jeppesen TS, Frøslev TG, Lindahl BD, Põldmaa K, Saar I, Suija A, Savchenko A, Yatsiuk I, Adojaan K, Ivanov F, Piirmann T, Pöhönen R, Zirk A, Abarenkov K. The Taxon Hypothesis Paradigm—On the Unambiguous Detection and Communication of Taxa. Microorganisms. 2020; 8(12):1910. DOI: 10.3390/microorganisms8121910

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 79:5112–5120. https://doi.org/10.1128/ AEM.01043-13.

Kumari, P., Singh, A., & Kharwar, R. N. (2021). Phytostimulation and ISR responses of fungi. In *Fungi Bio-Prospects in Sustainable Agriculture, Environment and Nano-Technology* (pp. 459–473). Elsevier. https://doi.org/10.1016/B978-0-12-821394-0.00018-4

Kundel, D., Bodenhausen, N., Jørgensen, H. B., Truu, J., Birkhofer, K., Hedlund, K., Fliessbach, A. (2020). Effects of simulated drought on biological soil quality, microbial diversity and yields under long-term conventional and organic agriculture. FEMS microbiology ecology, 96(12), fiaa205.

Lamprecht, S. C., Crous, P. W., Groenewald, J. Z., Tewoldemedhin, Y. T., & Marasas, W. F. O. (2011). Diaporthaceae associated with root and crown rot of maize. IMA Fungus, 2(1), 13–24. https://doi.org/10.5598/imafungus.2011.02.01.03

Lau, J. A., & Lennon, J. T. (2011). Evolutionary ecology of plant–microbe interactions: Soil microbial structure alters selection on plant traits. *New Phytologist*, *192*(1), 215–224. https://doi.org/10.1111/j.1469-8137.2011.03790.x

Leake, J. R., Donnelly, D. P., & Boddy, L. (2002). Interactions between ecto-mycorrhizal and saprotrophic fungi. Mycorrhizal ecology, 345-372.

Leff, J. W., Jones, S. E., Prober, S. M., Barberán, A., Borer, E. T., Firn, J. L., ... & Fierer, N. (2015). Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. Proceedings of the National Academy of Sciences, 112(35), 10967-10972. https://doi.org/10.1073/pnas.1508382112

Lekberg, Y., Arnillas, C. A., Borer, E. T., Bullington, L. S., Fierer, N., Kennedy, P. G., Leff, J. W., Luis, A. D., Seabloom, E. W., & Henning, J. A. (2021). Nitrogen and phosphorus fertilization consistently favor pathogenic over mutualistic fungi in grassland soils. *Nature Communications*, *12*(1), 3484. https://doi.org/10.1038/s41467-021-23605-y

Liu, J., Maldonado-Mendoza, I., Lopez-Meyer, M., Cheung, F., Town, C. D., & Harrison, M. J. (2007). Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots: Local and systemic alterations in transcript profiles in AM symbiosis. *The Plant Journal*, *50*(3), 529–544. https://doi.org/10.1111/j.1365-313X.2007.03069.x

Liu, X. Z., Wang, Q. M., Göker, M., Groenewald, M., Kachalkin, A. V., Lumbsch, H. T., ... & Bai, F. Y. (2015). Towards an integrated phylogenetic classification of the Tremellomycetes. Studies in mycology, 81(1), 85-147.

Lodge, D. J., Padamsee, M., Matheny, P. B., Aime, M. C., Cantrell, S. A., Boertmann, D., ... & Hattori, T. (2014). Molecular phylogeny, morphology, pigment chemistry and ecology in Hygrophoraceae (Agaricales). Fungal Diversity, 64, 1-99.

Ma, Y., Li, J., Wu, J., Kong, Z., Feinstein, L. M., Ding, X., ... & Wu, L. (2018). Bacterial and fungal community composition and functional activity associated with lake wetland water level gradients. Scientific reports, 8(1), 760.

Maietta, C. E., Hondula, K. L., Jones, C. N., & Palmer, M. A. (2020). Hydrological conditions influence soil and methane-cycling microbial populations in seasonally saturated wetlands. Frontiers in Environmental Science, 8, 593942.

McMurdie, P.J., Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8, e61217. doi:10.1371/journal.pone.0061217.

Morón-Ríos, A., Gómez-Cornelio, S., Ortega-Morales, B. O., De la Rosa-García, S., Partida-Martínez, L. P., Quintana, P., ... & González-Gómez, S. (2017). Interactions between abundant fungal species influence the fungal community assemblage on limestone. PLoS One, 12(12), e0188443.

National Atmospheric Deposition Program. (n.d.). *National Atmospheric Deposition Program (NADP)*. Retrieved 2024, from https://nadp.slh.wisc.edu/

Parrent, J. L., Morris, W. F., & Vilgalys, R. (2006). Co2-Enrichment and Nutrient Availability Alter Ectomycorrhizal Fungal Communities. *Ecology*, *87*(9), 2278–2287. https://doi.org/10.1890/0012-9658(2006)87[2278:CANAAE]2.0.CO;2

Pielou, E. C. (1969). An introduction to mathematical ecology.

Pirozynski, K. A., & Malloch, D. W. (1975). The origin of land plants: A matter of mycotrophism. *Biosystems*, *6*(3), 153–164. https://doi.org/10.1016/0303-2647(75)90023-4

Quijada, L., Tanney, J. B., Popov, E., Johnston, P. R., & Pfister, D. H. (2020). Cones, needles and wood: Micraspis (Micraspidaceae, Micraspidales fam. et ord. nov.) speciation segregates by host plant tissues. Fungal Systematics and Evolution, 5(1), 99-112.

Roulet, N. T. (2000). Peatlands, carbon storage, greenhouse gases, and the Kyoto Protocol: Prospects and significance for Canada. *Wetlands*, *20*(4), 605–615. https://doi.org/10.1672/0277-5212(2000)020[0605:PCSGGA]2.0.CO;2

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: opensource, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. https://doi.org/10.1128/AEM.01541-09.

Selosse, M. A., & Tacon, F. L. (1998). The land flora: A phototroph-fungus partnership? *Trends in Ecology & Evolution*, *13*(1), 15–20. https://doi.org/10.1016/S0169-5347(97)01230-5

Shannon, C. E., Weaver, W. (1963). The mathematical theory of communication. University of Illinois Press.

Sharma, M. P., Grover, M., Chourasiya, D., Bharti, A., Agnihotri, R., Maheshwari, H. S., Pareek, A., Buyer, J. S., Sharma, S. K., Schütz, L., Mathimaran, N., Singla-Pareek, S. L., Grossman, J. M., & Bagyaraj, D. J. (2020). Deciphering the Role of Trehalose in Tripartite Symbiosis Among Rhizobia, Arbuscular Mycorrhizal Fungi, and Legumes for Enhancing Abiotic Stress Tolerance in Crop Plants. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.509919

Stürmer, S. L. (2012). A history of the taxonomy and systematics of arbuscular mycorrhizal fungi belonging to the phylum Glomeromycota. Mycorrhiza, 22(4), 247-258.

Treseder, K. K. (2008). Nitrogen additions and microbial biomass: A meta-analysis of ecosystem studies. Ecology letters, 11(10), 1111-1120.

U.S. Environmental Protection Agency. (Year). Total deposition maps. Retrieved from https://www.epa.gov/castnet/total-deposition-maps

Vohník, M., Figura, T., & Réblová, M. (2022). Hyaloscypha gabretae and Hyaloscypha gryndleri spp. nov.(Hyaloscyphaceae, Helotiales), two new mycobionts colonizing conifer, ericaceous and orchid roots. Mycorrhiza, 32(1), 105-122.

Wang, R., Balkanski, Y., Boucher, O., Ciais, P., Peñuelas, J., & Tao, S. (2015). Significant contribution of combustion-related emissions to the atmospheric phosphorus budget. Nature Geoscience, 8(1), 48–54. https://doi.org/10.1038/ngeo2324

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: a guide to methods and applications (pp. 315–322). New York: Academic Press.

Xue, C., Hao, Y., Pu, X., Ryan Penton, C., Wang, Q., Zhao, M., ... & Tiedje, J. M. (2019). Effect of LSU and ITS genetic markers and reference databases on analyses of fungal communities. Biology and Fertility of Soils, 55, 79-88.

List of Tables

Table 1: Summary of Shannon diversity ANOVA results comparing fungal diversity across fertilization, hydrology, year and their interactions.

Soil Factor	Df	SumSq	Mean Sq	F	Pr(>F)
Treatment	1	4.0233	4.0233	15.1428	0.0003
Hydrology	1	0.5464	0.5464	2.0566	0.1573
Year	4	4.1376	1.0344	3.8932	0.0075
Treatment: Hydrology	1	0.2573	0.2573	0.9684	0.3295
Treatment: Year	4	1.7224	0.4306	1.6207	0.1824
Hydrology: Year	4	1.3901	0.3475	1.3080	0.2787
Treatment: Hydrology: Year	4	3.9474	0.8227	3.0966	0.0229
Residual	54	14.3474	0.2657		

Table 2: Summary of Pielou's evenness ANOVA results comparing fungal diversity across fertilization, hydrology, year and their interactions.

Soil Factor	Df	SumSq	Mean Sq	F	Pr(>F)
Treatment	1	0.0161	0.1061	28.3076	2.04e-6
Hydrology	1	0.0386	0.0386	10.3028	0.0022
Year	4	0.0185	0.0046	1.2318	0.3083
Treatment: Hydrology	1	0.0468	0.0468	12.4950	0.0008
Treatment: Year	4	0.0567	0.0142	3.7804	0.0088
Hydrology: Year	4	0.0166	0.0042	1.1095	0.3616
Treatment: Hydrology: Year	4	0.0059	0.0015	0.3948	0.8115
Residual	54	0.2025	0.0037		

Table 3: Summary of ASV Richness ANOVA results comparing fungal across fertilization, hydrology, year and their interactions.

Soil Factor	Df	SumSq	Mean Sq	F	Pr(>F)
Treatment	1	8919	8919	0.0684	0.7947
Hydrology	1	60994	60994	0.4677	0.4970
Year	4	4080448	1020112	7.8215	4.77e-5
Treatment: Hydrology	1	56945	56945	0.4366	0.5116
Treatment: Year	4	114763	28691	0.2200	0.9262
Hydrology: Year	4	461099	115275	0.8838	0.4799
Treatment: Hydrology: Year	4	600538	150135	1.1511	0.3426
Residual	54	7042928	130425		

Table 4: Summary of multivariate analyses PERMANOVA comparing total ITS fungal microbial community composition across fertilization, hydrology, year and their interactions.

Soil Factor	Df	SumSq	R^2	F	Pr(>F)
Treatment	1	2.5193	0.1131	10.5109	0.001
Hydrology	1	1.0233	0.0460	4.2693	0.001
Year	4	2.1635	0.0971	2.2566	0.001
Treatment: Hydrology	1	0.7172	0.0322	2.9923	0.002
Treatment: Year	4	0.9785	0.0440	1.0206	0.387
Hydrology: Year	4	0.8234	0.0370	0.8589	0.893
Treatment: Hydrology: Year	4	0.8727	0.0392	0.9103	0.746
Residual	52	13.182	0.5917		

Table 5: Summary of multivariate analyses PERMANOVA comparing ITS fungal community composition across fertilization, hydrology, year and their interactions, specifically for the Bankeraceae family.

Soil Factor	**Df**	**SumSq**	**R^2**	**F**	**Pr(>F)**
Treatment	1	0.6596	0.0337	2.5894	0.0563
Year	4	1.8248	0.0931	1.7909	0.0580
Treatment: Year	4	0.8045	0.0411	0.7795	0.6474
Residual	64	19.5918	0.8321		

Table 6: Summary of multivariate analyses PERMANOVA comparing ITS fungal community composition across fertilization, hydrology, year and their interactions, specifically for the Hygrophoraceae family.

Soil Factor	**Df**	**SumSq**	**R^2**	**F**	**Pr(>F)**
Treatment	1	0.8598	0.0300	2.2624	0.0054
Year	4	3.4120	0.1191	2.2444	9.99e-5
Treatment: Year	4	1.5757	0.0550	1.0365	0.3914
Residual	60	22.8033	0.7959		

Table 7: Summary of multivariate analyses PERMANOVA comparing ITS fungal community composition patterns across fertilization, hydrology, year and their interactions, specifically for the Trimorphomycetaceae family.

Soil Factor	**Df**	**SumSq**	**R^2**	**F**	**Pr(>F)**
Treatment	1	0.1400	0.0250	2.5271	0.0926
Year	4	1.3536	0.2416	6.1075	9.99e-5
Treatment: Year	4	0.6126	0.1095	2.7643	0.0170
Residual	63	3.4907	0.6237		

Table 8: Summary of multivariate analyses PERMANOVA comparing ITS fungal community composition across fertilization, hydrology, year and their interactions, specifically for the Herpotrichiellaceae family.

Soil Factor	**Df**	**SumSq**	**R^2**	**F**	**Pr(>F)**
Treatment	1	2.4029	0.1561	20.8558	9.9e-5
Year	4	2.1390	0.1390	4.6415	9.9e-5
Hydrology	1	1.8753	0.1218	16.2765	9.9e-5
Treatment: Year	4	1.4658	0.0952	3.1806	9.9e-5
Treatment: Hydrology	1	0.3737	0.0243	3.2433	0.0074
Hydrology: Year	4	0.7124	0.0463	1.5458	0.0578
Treatment: Hydrology: Year	2	0.6612	0.0430	1.4348	0.0877
Residual	52	5.7606	0.3743		

Table 9: Summary of multivariate analyses PERMANOVA comparing ITS fungal community composition across fertilization, hydrology, year and their interactions, specifically for the Tympanidaceae family.

Soil Factor	**Df**	**SumSq**	**R^2**	**F**	**Pr(>F)**
Treatment	1	3.0165	0.1286	12.4111	9.9e-5
Year	4	1.7834	0.0760	1.8344	0.0016
Hydrology	1	1.2044	0.0514	4.9554	9.9e-5
Treatment: Year	4	1.3654	0.0582	1.4044	0.0365
Treatment: Hydrology	1	1.4070	0.0600	5.7891	9.9e-5
Hydrology: Year	4	1.0318	0.0440	1.0614	0.3350
Treatment: Hydrology: Year	4	1.0079	0.0430	1.0367	0.3897
Residual	52	12.6385	0.5388		

Table 10: Summary of multivariate analyses PERMANOVA comparing ITS fungal microbial community across fertilization, hydrology, year and their interactions, specifically for the Hyaloscyphaceae family.

Soil Factor	**Df**	**SumSq**	**R^2**	**F**	**Pr(>F)**
Treatment	1	1.4688	0.1121	3.5898	9.9e-5
Year	4	1.4663	0.1119	0.8959	0.6911
Hydrology	1	0.6849	0.0523	1.6739	0.0666
Treatment: Year	4	1.0964	0.0837	0.8932	0.6581
Treatment: Hydrology	1	0.7069	0.0540	1.7276	0.0579
Hydrology: Year	4	1.1157	0.0851	0.9098	0.6475
Treatment: Hydrology: Year	2	0.8352	0.0637	1.0206	0.4386
Residual	50	5.7284	0.4372		

Table 11: The first 50 fungal ASVs representing significant indicator species analysis according to fertilization treatment. Cluster 1 represents mowed/unfertilized plots (M) and cluster 2 represents mowed/fertilized plots (MF).

OTU	Cluster	IndVal	Prob	TRT	Domain	Phylum	Class	Order	Family	Genus	Species
ASV_166	1	0.926071486	0.005	M	Fungi	Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Glutinomyces	NA
ASV_580	1	0.914858661	0.005	M	Fungi	Ascomycota	NA		NA	NA	NA
ASV_44	2	0.911903281	0.005	MF	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_217	2	0.90895044	0.005	MF	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_158	1	0.902686999	0.005	M	Fungi	Ascomycota	Leotiomycetes	Leotiales	Tympanidaceae	Tympanidaceae_gen_Incertae	NA
ASV_222	1	0.901135793	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_118	1	0.896601289	0.005	M	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiales_fam_Incertae	Leohumicola	NA
ASV_463	1	0.889482586	0.005	M	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiales_fam_Incertae	Cistella	NA
ASV_359	2	0.888566945	0.005	MF	Fungi	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Cladophialophora	chaetospira
ASV_351	1	0.884333426	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_136	1	0.883408409	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_83	1	0.879923391	0.005	M	Fungi	Ascomycota	Archaeorhizomycetes	Archaeorhizomycetales	Archaeorhizomycetaceae	Archaeorhizomyces	NA
ASV_605	1	0.877430334	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_314	1	0.868687877	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_369	1	0.867538889	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_141	1	0.867103798	0.005	M	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiales_fam_Incertae	Leohumicola	atra
ASV_396	2	0.861457574	0.005	MF	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiales_fam_Incertae	Cistella	NA
ASV_399	1	0.859856617	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_71	1	0.857823412	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_129	1	0.849549778	0.005	M	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae	Arnium	NA
ASV_356	1	0.847370452	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_137	1	0.845242566	0.005	M	Fungi	Ascomycota	Sordariomycetes	NA	NA	NA	NA
ASV_194	1	0.844579099	0.01	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_98	1	0.841615494	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_188	1	0.841044029	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_466	1	0.837958445	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_750	2	0.837571643	0.005	MF	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Sarocladiaceae	Sarocladium	strictum
ASV_414	2	0.833394136	0.005	MF	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pyrenochaetopsidaceae	Pyrenochaetopsis	leptospora
ASV_215	1	0.832454376	0.005	M	Fungi	Ascomycota	Archaeorhizomycetes	Archaeorhizomycetales	Archaeorhizomycetaceae	Archaeorhizomyces	NA
ASV_421	1	0.830472333	0.005	M	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pyrenochaetopsidaceae	Pyrenochaetopsis	NA
ASV_76	2	0.829823524	0.005	MF	Fungi	Ascomycota	Archaeorhizomycetes	Archaeorhizomycetales	Archaeorhizomycetaceae	Archaeorhizomyces	NA
ASV_475	1	0.828662155	0.005	M	Fungi	Basidiomycota	Agaricomycetes	Hymenochaetales	Rickenellaceae	Peniophorella	NA
ASV_140	2	0.82492097	0.005	MF	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_281	1	0.82086608	0.005	M	Fungi	Ascomycota	Leotiomycetes	NA	NA	NA	NA
ASV_315	1	0.820222715	0.005	M	Fungi	Ascomycota	Leotiomycetes	Leotiales	NA	NA	NA

ASV_79	2	0.819308792	0.005	MF	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_530	1	0.818424594	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_296	1	0.817885629	0.005	M	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Agaricales_fam_Incertae	NA	NA
ASV_610	2	0.817225113	0.005	MF	Fungi	Rozellomycota	Rozellomycota_cls_Incertae	Rozellomycota_ord_Incertae	Rozellomycota_fam_Incertae	Rozellomycota_gen_Incertae	NA
ASV_148	1	0.816671946	0.005	M	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_170	2	0.816335209	0.005	MF	Fungi	NA	NA	NA	NA	NA	NA
ASV_198	1	0.815018671	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_420	2	0.809355704	0.005	MF	Fungi	Ascomycota	Leotiomycetes	Helotiales	Mollisiaceae	Phialocephala	NA
ASV_173	2	0.807609399	0.005	MF	Fungi	Ascomycota	NA	NA	NA	NA	NA
ASV_154	1	0.805832114	0.005	M	Fungi	Ascomycota	Leotiomycetes	Leotiomycetes_ord_Incertae	Leotiomycetes_fam_Incertae	Leotiomycetes_gen_Incertae	NA
ASV_138	1	0.805488043	0.005	M	Fungi	Ascomycota	Sordariomycetes	NA	NA	NA	NA
ASV_686	2	0.805116375	0.005	MF	Fungi	Ascomycota	Sordariomycetes	Chaetosphaeriales	Chaetosphaeriaceae	Dictyochaeta	lithocarpi
ASV_333	1	0.80418416	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_143	1	0.802885281	0.005	M	Fungi	NA	NA	NA	NA	NA	NA
ASV_683	1	0.802089906	0.005	M	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Stachybotryaceae	Alfaria	caricicola

Table 12: Summary of ANOVA results comparing relative abundance of the Bankeraceae family across fertilization, hydrology, year, and their interactions.

Soil Factor	**Df**	**SumSq**	**Mean Sq**	**F**	**Pr(>F)**
Treatment	1	0.0020	0.0020	3.7290	0.0538
Hydrology	1	0.0001	0.0001	0.2540	0.6145
Year	4	0.0017	0.0004	0.8050	0.5219
Treatment: Hydrology	1	0.0000	1.22e-5	0.0220	0.8808
Treatment: Year	4	0.0015	0.0004	0.7160	0.5811
Hydrology: Year	4	0.0006	0.0001	0.2770	0.8932
Treatment: Hydrology: Year	4	0.0009	0.0002	0.4320	0.7853
Residual	794	0.4291	0.0005		

Table 13: Summary of ANOVA results comparing relative abundance of the Hygrophoraceae family across fertilization, hydrology, year, and their interactions.

Soil Factor	Df	SumSq	Mean Sq	F	Pr(>F)
Treatment	1	0.0001	0.0001	1.0250	0.3114
Hydrology	1	0.0003	0.0003	3.0980	0.0785
Year	4	0.0004	0.0001	0.9920	0.4106
Treatment: Hydrology	1	0.0008	0.0008	7.9800	0.0048
Treatment: Year	4	0.0004	0.0001	1.0520	0.3787
Hydrology: Year	4	0.0001	1.95e-5	0.1920	0.9426
Treatment: Hydrology: Year	4	0.0001	1.91e-5	0.1850	0.9463
Residual	2792	0.2880	0.0001		

Table 14: Summary of ANOVA results comparing relative abundance of the Trimorphomycetaceae family across fertilization, hydrology, year, and their interactions.

Soil Factor	Df	SumSq	Mean Sq	F	Pr(>F)
Treatment	1	0.0001	9.612e-5	0.6730	0.4130
Hydrology	1	0.0002	1.627e-5	1.1390	0.2860
Year	4	0.0005	1.280e-4	0.8960	0.4660
Treatment: Hydrology	1	3.0e-5	3.148e-5	0.2200	0.6390
Treatment: Year	4	0.0003	8.276e-5	0.5790	0.6780
Hydrology: Year	4	0.0003	6.891e-5	0.4820	0.7490
Treatment: Hydrology: Year	4	0.0001	1.279e-5	0.0900	0.9860
Residual	572	0.0818	1.429e-4		

List of Figures

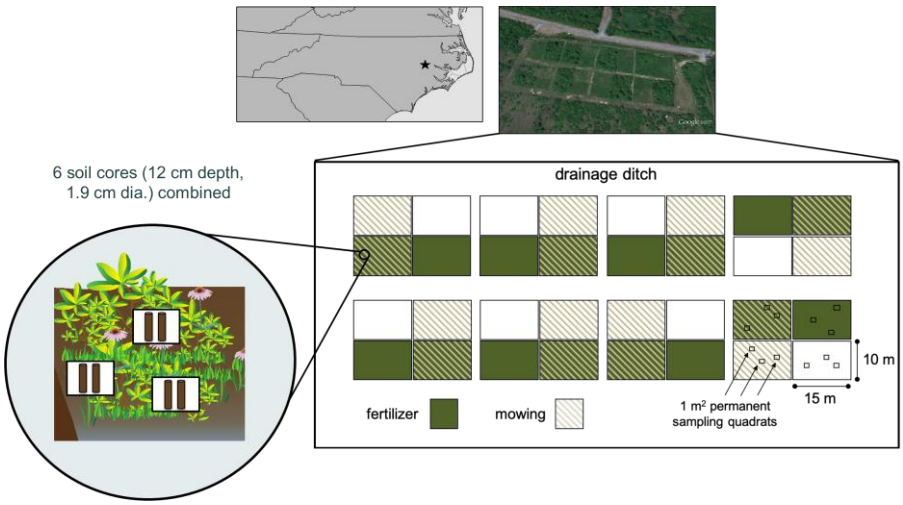

Figure 1: Experimental design of the long-term ecological experiment. Since 2003, fertilization and disturbance by mowing plots at East Carolina University's West Research Campus located in Greenville, North Carolina, USA.

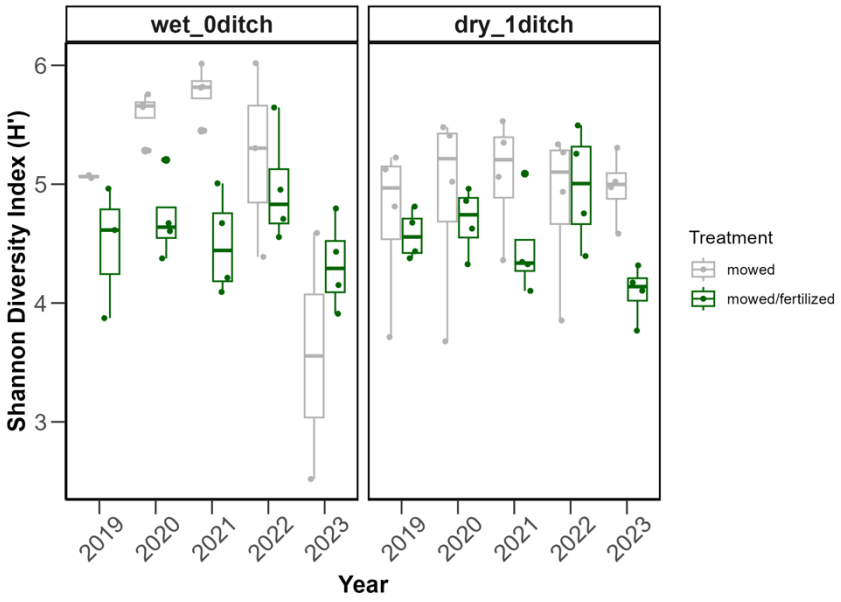

Figure 2: Summary of fungal community diversity. Boxplots representing fungal Shannon diversity in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch, left) and closer (dry_1ditch, right) to the drainage ditch over time. The boxplot is a visual representation of five key summary statistics: the median, the 25% and 75% percentiles, and the whiskers which represent the feasible range of the data as determined by 1.5 × the interquartile range. Symbols represent individual raw data points from four replicate samples.

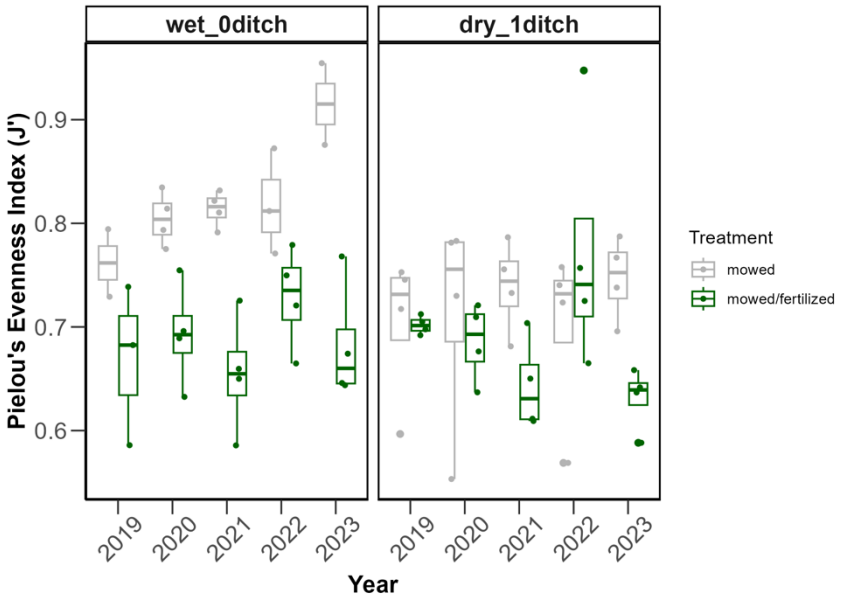

Figure 3: Summary of fungal community evenness. Boxplots representing fungal Pielou's Evenness Index measurements in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch, left) and closer (dry_1ditch, right) to the drainage ditch over time. The boxplot is a visual representation of five key summary statistics: the median, the 25% and 75% percentiles, and the whiskers which represent the feasible range of the data as determined by 1.5 × the interquartile range. Symbols represent individual raw data points from four replicate samples.

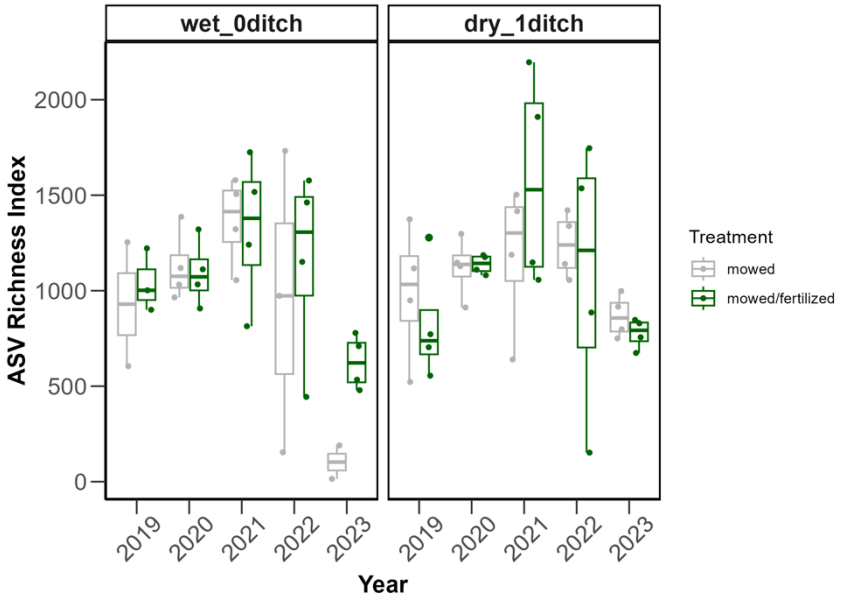

Figure 4: Summary of fungal community ASV richness. Boxplots representing fungal ASV richness measurements in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch, left) and closer (dry_1ditch, right) to the drainage ditch over time. The boxplot is a visual representation of five key summary statistics: the median, the 25% and 75% percentiles, and the whiskers which represent the feasible range of the data as determined by 1.5 × the interquartile range. Symbols represent individual raw data points from four replicate samples.

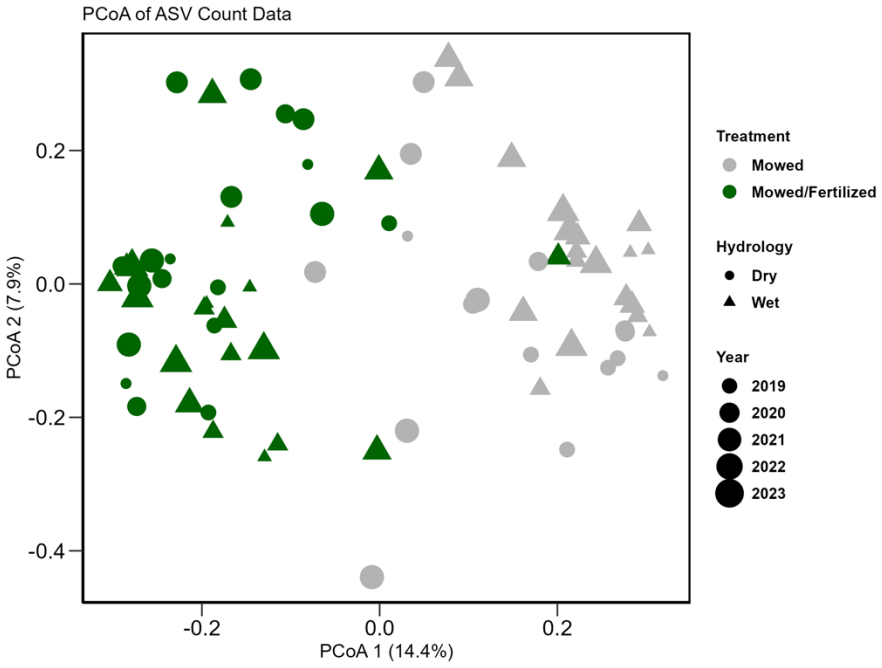

Figure 5: Ordination based on principal coordinates analysis (PCoA) depicting total fungal community composition based on ITS amplicon sequencing. Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized), shapes represent hydrology based on proximity to ditch (circle = dry plots near ditch, triangle = wet plots away from ditch), and size of symbol increases with year (from 2019 to 2023).

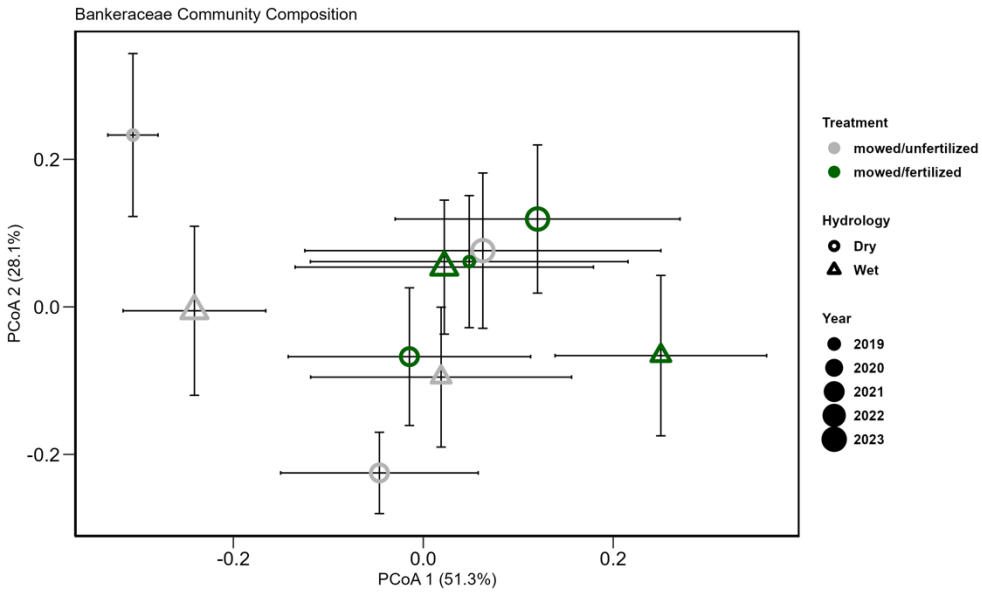

Figure 6: Ordination based on principal coordinates analysis (PCoA) depicting total fungal community composition based on ITS amplicon sequencing of ASVs belonging to the Bankeraceae family only. Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized), shapes represent hydrology based on proximity to ditch (circle = dry plots near ditch, triangle = wet plots away from ditch), and size of symbol increases with year (from 2019 to 2023).

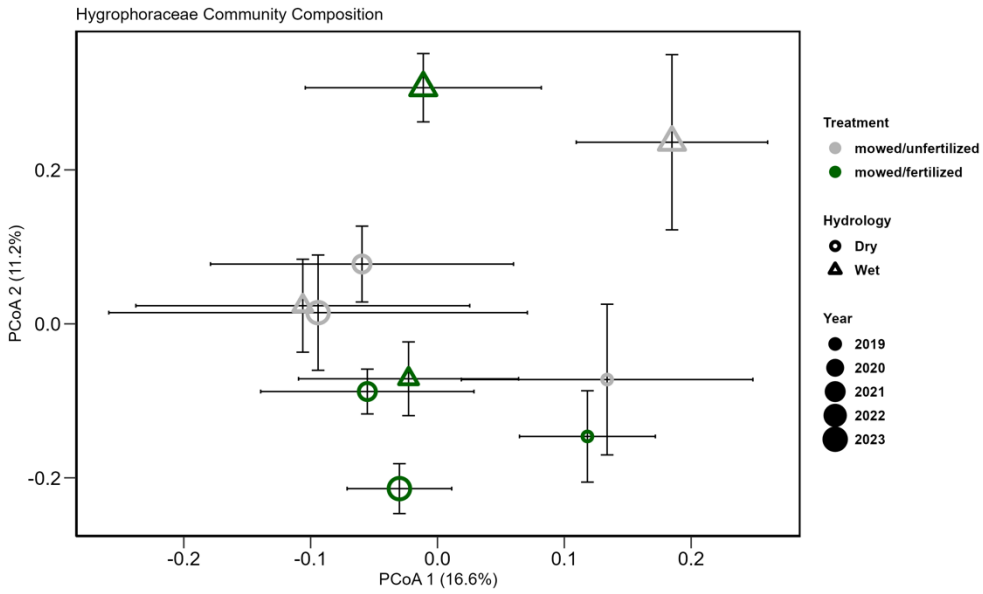

Figure 7: Ordination based on principal coordinates analysis (PCoA) depicting total fungal community composition based on ITS amplicon sequencing of ASVs belonging to the Hygrophoraceae family only. Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized), shapes represent hydrology based on proximity to ditch (circle = dry plots near ditch, triangle = wet plots away from ditch), and size of symbol increases with year (from 2019 to 2023).

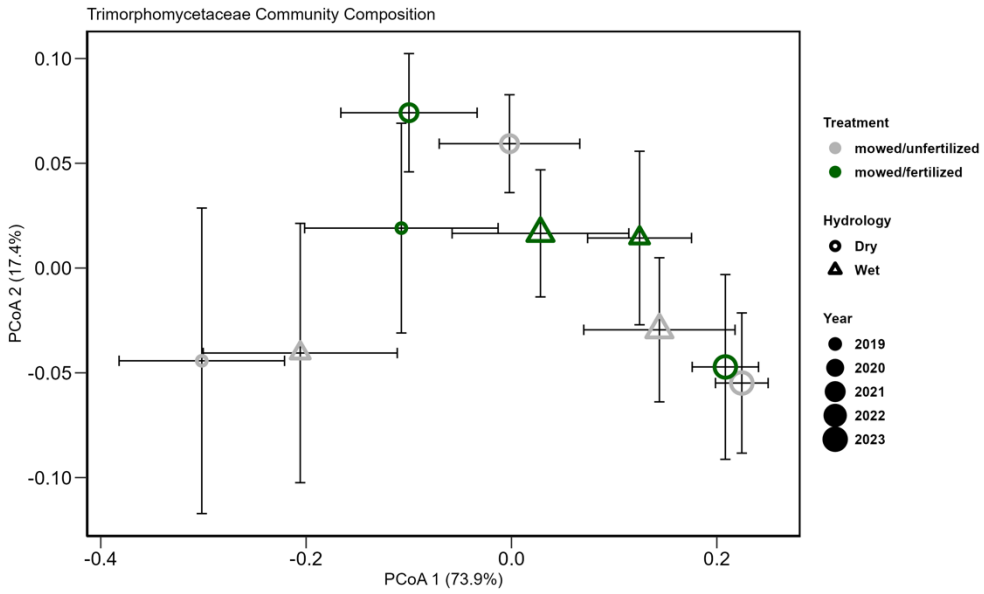

Figure 8: Ordination based on principal coordinates analysis (PCoA) depicting total fungal community composition based on ITS amplicon sequencing of ASVs belonging to the Trimorphomycetaceae family only. Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized), shapes represent hydrology based on proximity to ditch (circle = dry plots near ditch, triangle = wet plots away from ditch), and size of symbol increases with year (from 2019 to 2023).

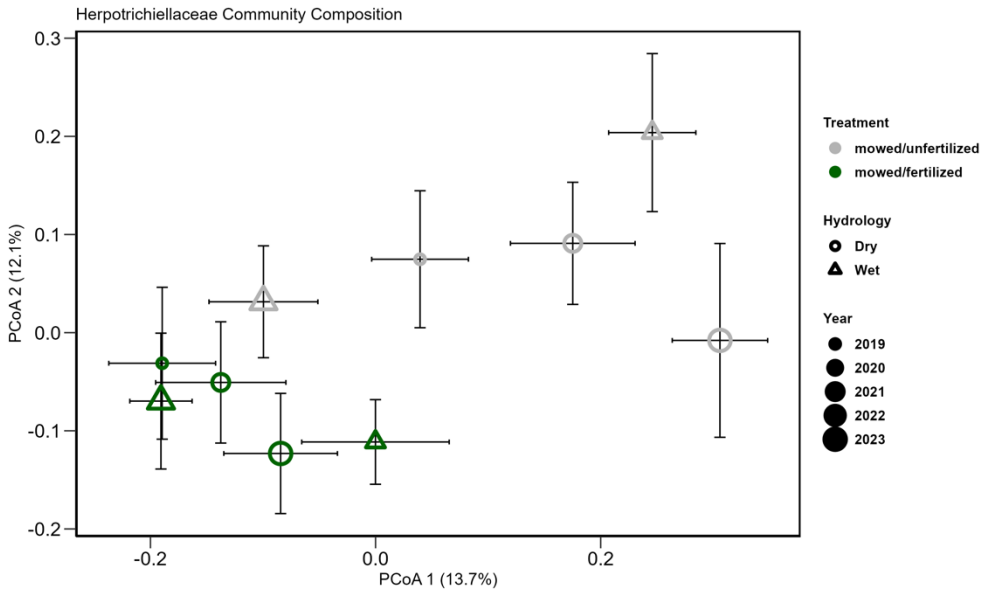

Figure 9: Ordination based on principal coordinates analysis (PCoA) depicting total fungal community composition based on ITS amplicon sequencing of ASVs belonging to the Herpotrichiellaceae family only. Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized), shapes represent hydrology based on proximity to ditch (circle = dry plots near ditch, triangle = wet plots away from ditch), and size of symbol increases with year (from 2019 to 2023).

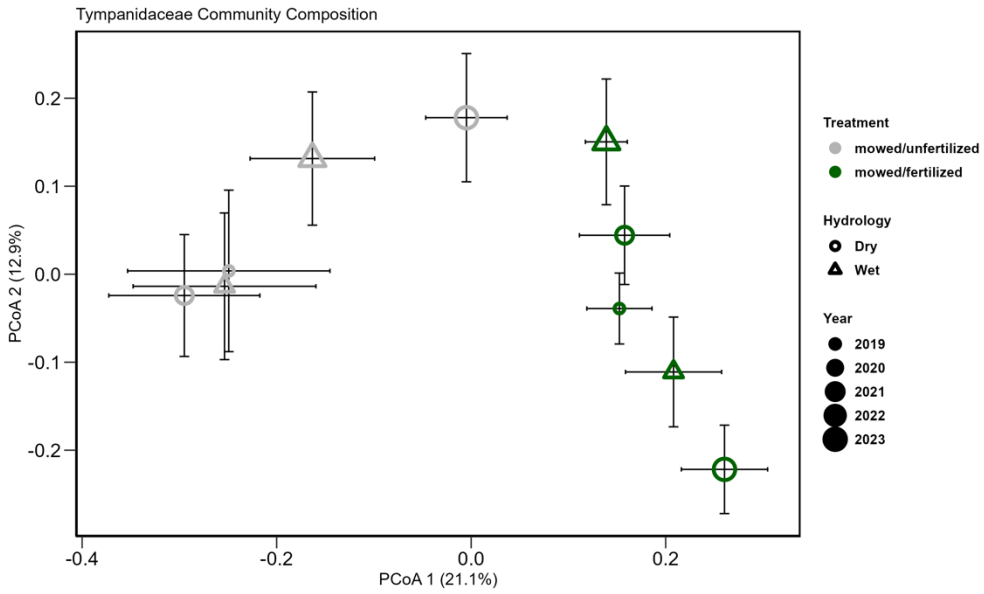

Figure 10: Ordination based on principal coordinates analysis (PCoA) depicting total fungal community composition based on ITS amplicon sequencing of ASVs belonging to the Tympanidaceae family only. Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized), shapes represent hydrology based on proximity to ditch (circle = dry plots near ditch, triangle = wet plots away from ditch), and size of symbol increases with year (from 2019 to 2023).

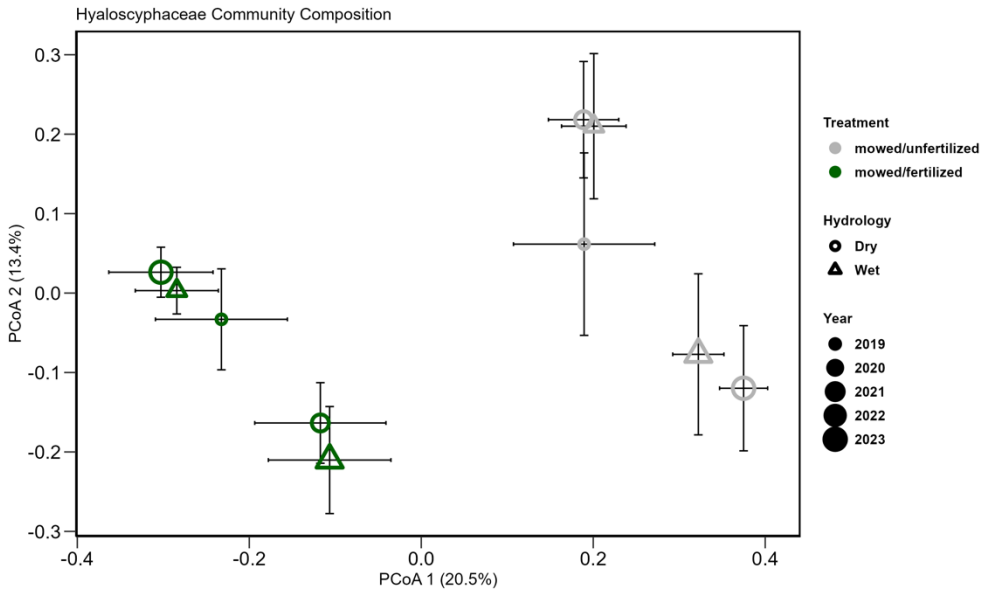

Figure 11: Ordination based on principal coordinates analysis (PCoA) depicting total fungal community composition based on ITS amplicon sequencing of ASVs belonging to the Hyaloscyphaceae family only. Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized), shapes represent hydrology based on proximity to ditch (circle = dry plots near ditch, triangle = wet plots away from ditch), and size of symbol increases with year (from 2019 to 2023).

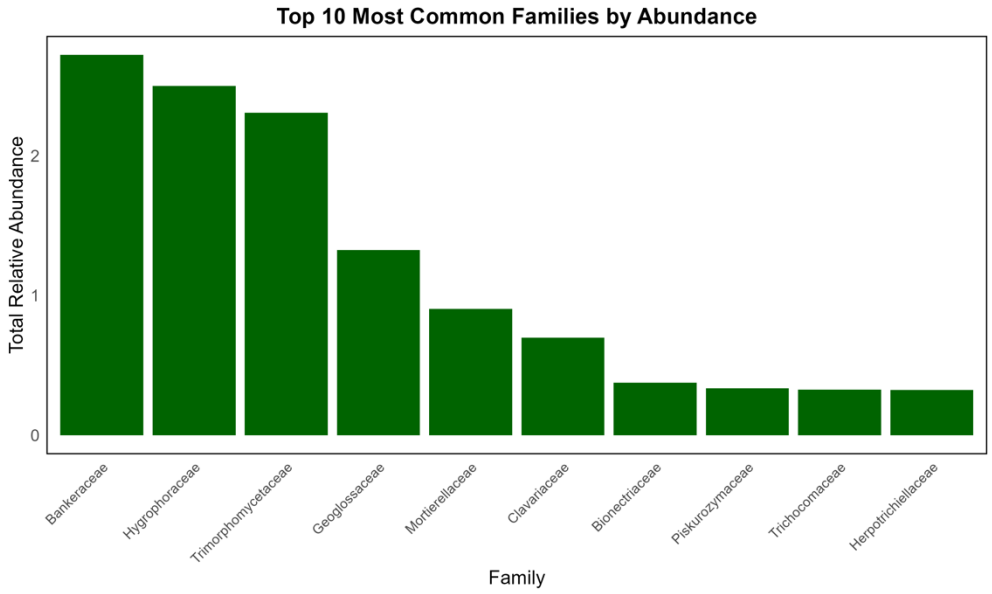

Figure 12: Bar graph depicting the fungal families among all five years along the x-axis and total relative abundances of the sum of all ASVs identified to the family-level. Families are sorted in descending order.

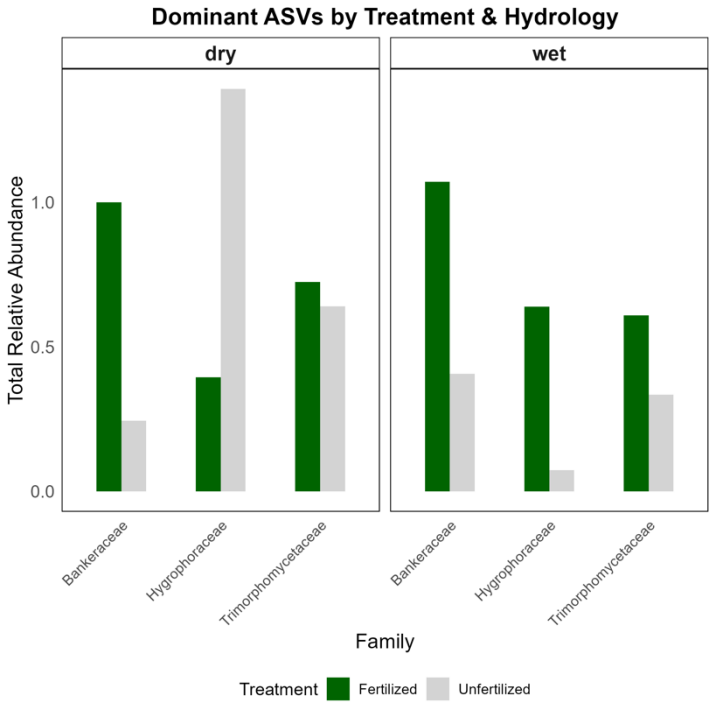

Figure 13: Bar graph depicting the dominant family of ASVs according to fertilization treatment and hydrology. The total relative abundance (sum of all ASVs representing each family) of the three most dominant fungal families according to fertilization treatment (fertilized/mowed, unfertilized mowed) and hydrology (dry, wet). Colors represent fertilization treatment (gray = mowed, green = mowed/fertilized).