LONG-TERM FERTILIZATION AND SOIL MOISTURE INTERACT TO INFLUENCE

PLANT AND BACTERIAL COMMUNITIES IN A LOW NUTRIENT WETLAND

By

Daniya S. Stephens

December, 2022

Director of Thesis: Ariane L. Peralta, PhD

Major Department: Department of Biology

ABSTRACT

Human activities such as urbanization and intensive agriculture modify nutrient and water cycles in significant ways. Changes to nutrient and water cycles can cause disruptions to plant-microbe mutualisms, especially in low-nutrient wetland ecosystems. Due to ongoing nutrient and moisture variations, the succession of plant and microbial communities may diverge in unexpected ways. In this study, I investigated how long-term fertilization and hydrologic alterations affect associations between wetland plants and soil microbial communities in a historically low-nutrient coastal plain wetland. I hypothesized that long-term fertilization and drier ditched conditions influenced patterns in plant and bacterial 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. Specifically, I examined the relationship between plant communities and bacterial communities (based on amplicon sequencing of the 16S rRNA gene) in mowed plots undergoing nutrient enrichment and varying soil moisture conditions from 2014 to 2020. Results revealed that nutrient enrichment and ditch effects influenced plant and bacterial community succession to different degrees. In addition, bacterial diversity was higher in wetter fertilized soils than drier

unfertilized soils over time. Plant communities were distinct due to hydrology, especially in unfertilized plots, while fertilization influenced bacterial communities more than hydrology. In addition, total soil carbon was correlated to bacterial community patterns. Because of nutrient enrichment and drying conditions, changes to wetland plants and soil bacterial community patterns could imply an increasingly competitive rather than cooperative relationship between plants and soil microbes.

LONG-TERM FERTILIZATION AND SOIL MOISTURE INTERACT TO INFLUENCE

PLANT AND BACTERIAL COMMUNITIES IN A LOW NUTRIENT WETLAND

A Thesis

Presented to the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Masters of Science in Biology

By

Daniya S. Stephens

December, 2022

Director of Thesis: Ariane L. Peralta, PhD

Thesis Committee Members:

Carol Goodwillie, PhD

Rachel Gittman, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Symbiotic species contribute to the functioning and stability of communities that are responsible for ecosystem functions (i.e., biogeochemical cycles, carbon sequestration, water purification) and the generation and maintenance of biodiversity. But novel and dynamic environmental conditions can disrupt the interdependence amongst interacting species (Wagg et al., 2014). As a result, community assembly patterns and associated ecosystem functions are altered. These modifications to biodiversity result in unpredictable changes to ecosystem functions. For example, plants and root-associated microbes have co-evolved under historically nutrient poor conditions. Plants convert atmospheric carbon into organic carbon required for heterotrophic microbes, and microbes transform bound mineral and organic nutrients in the soil for plants. While these plant-microbe interactions are happening, humans are also manipulating elemental cycling in ways that disrupt plant-microbe relationships (Guignard et al., 2017). Although the effects of biodiversity loss are most noticeable for species with known population declines, it is more difficult to track the loss of microbial biodiversity and the effects it has on microbially-controlled ecosystem functions. Therefore, a deeper understanding of how changes to microbial diversity and composition relate to known changes in plant communities will enable improved prediction of microbial responses to environmental changes (Nielsen et al., 2011; Wagg et al., 2014).

Fertilization of historically nutrient-poor ecosystems can interrupt the plant-microbe associations that enhance nutrient acquisition for microbes and plants. Nutrient enrichment from industrial and urban activities (i.e., point source), fossil fuel combustion, and agricultural production (i.e., non-point source) are common sources of nutrient pollution (Rabalais et al., 2009). For instance, the use of nitrogen and phosphorus-based fertilizers has increased significantly on a

global scale, and plant species may have differing rates of nutrient uptake, increasing the possibility of leaching and perhaps leading to eutrophication in aquatic ecosystems. The combination of modifying elemental cycles (via fertilization) and variation in organismal response to environmental stressors makes it challenging to predict environmental change effects across different ecosystems (Cavicchioli et al., 2019). While there are ongoing efforts to study ecosystem responses to environmental change, such as the long-term enrichment studies in terrestrial grassland ecosystems (Dickson and Gross, 2013; Huang et al., 2019), nutrient enrichment studies at the terrestrial-aquatic interface could have fundamentally different outcomes, especially with regards to the distinct carbon storage potential and climate change mitigation functions that these ecosystems provide.

An influx of nutrients can disrupt plant-microbe interactions, whereby fast growing (copiotrophic) species that flourish in nutrient-rich conditions outcompete and dominate over slower-growing (oligotrophic) species. This mechanism, known as competitive exclusion, has the potential to significantly decrease diversity in both above- and below-ground communities. (Stanton, 2003; Bauer et al., 2012). Positive interactions are therefore expected to occur when many symbionts are associated with a single host, are functionally distinct, and provide the host unique benefits. This expected increase in copiotroph to oligotroph ratios following nutrient enrichment could serve as a proxy for how soil nutrient supply influences community structure. Furthermore, soil physicochemical features such as soil acidity may change over time, allowing tolerant species to emerge in this new stress-induced habitat (Bobbink et al., 1998). As nutrient concentrations increase, so does microbial growth, biomass, and turnover rate of soil microbes (Prommer et al., 2020). In comparison to plants and their root-associated microbes in unfertilized

soil, previous studies found that beneficial plant-microbe interactions with increased nutrients became less mutualistic (Weese et al., 2015; Huang et al., 2019).

While most previous studies have focused on plant community succession, my work examines how the change in plant communities could directly or indirectly affect the structure and function of belowground microbial communities (Jacoby et al., 2017). The relationships between soil physicochemical properties and microbial communities influence the geographic distribution of microorganisms as well as their structure and function. Therefore, it is possible to forecast how future soil and environmental (or human-induced) change will affect how they respond to these pressures (Wang et al., 2013). Bacterial community changes were identified in two stages during a 160-year research of bacterial succession under grassland conditions: a rapid increase in bacteria abundance and community structure at first, followed by a long slow stable plateau that spanned the rest of the study (Liu et al., 2020; Zhang et al., 2021). Soil microbes can respond to selection pressure faster than plants because of their short generation cycles, resulting in ecological changes over time in terms of the community assemblages and functional connections with plants (for example, plant immunity or resource partitioning) (Jones et al., 2019; Dastogeer et al., 2020; Howard et al., 2020). When nutrient concentrations vary or other disturbances occur, the previous symbiotic plant-microbe association may shift into a more competitive/parasitic one when there are abundant resources accessible and/or nutrients are disrupted unevenly (Weese et al., 2015; Cavicchioli et al., 2019). The intensity and frequency of the input or disturbance determines the severity of these shifts, which has the potential to alter the bacterial community's functional (i.e. metabolic) performance and composition (Berga et al., 2012). For example, more disturbed soils provide less plant-derived resources that support diverse soil biological communities (Bardgett and Caruso, 2020). In the event that communities are disturbed, having a diverse community can

increase the likelihood that functionally redundant traits will be preserved, and ecosystem functions remain stable (Nielsen et al., 2011).

This study examined how ongoing environmental change influenced soil bacterial community patterns and plant-microbe relationships in a coastal plain wetland, where plots are chronically fertilized and mowed and raked annually to mimic previous fire-disturbance features of the wet pine savannah ecosystem. The plots are also hydrologically distinct, where plots adjacent to a drainage ditch are drier than plots away from the ditch. A previous study conducted at the same research site found that interacting environmental conditions (nutrient enrichment, mowing, and soil moisture) altered plant community composition over time (Goodwillie et al., 2020). Wetter, unfertilized plots had the slowest succession rates, while drier fertilized plots were associated with faster turnover of plant communities (Goodwillie et al., 2020). Fertilization and proximity to a drainage ditch (wet or dry plots) influenced the rate of plant succession in the absence of the mowing disturbance, and fertilized plant communities shifted more than unfertilized plant communities (Goodwillie et al., 2020). As such, the dynamic plant-derived compounds from plant community shifts are expected to indirectly influence the belowground bacterial community. While prior work provides some insight into plant responses to these long-term disturbances, it is unclear how these changes affect belowground bacterial communities.

This study addressed the question: to what extent does fertilization and soil moisture influence (1) soil environmental parameters, (2) succession of soil bacterial communities and (3) plant-bacterial associations in a low-nutrient wetland ecosystem over time? I hypothesized that long-term fertilization influenced rates of plant community succession leading to changes in soil carbon resources associated with shifts in soil bacterial communities, while direct effects of fertilization change the soil environment and affect bacterial communities to different degrees. I predicted that

(i) fertilization effects on soil properties associate with bacterial community patterns, (ii) fertilization treatment influences bacterial community composition more than the hydrologic effect over time , and (iii) fertilization weakens plant-bacterial community associations over time and the effect is more pronounced in the drier plots. I leveraged a long-term fertilization experiment (est. 2003) and examined how nutrient starved soil microbial communities in a coastal plain wetland respond to long-term fertilization and hydrology (Bledsoe et al., 2020) and how these patterns related to ongoing plant community changes. To test this hypothesis, I (i) measured the strength of the relationship between soil properties (pH, total carbon, total nitrogen, soil moisture) and bacterial community composition due to fertilization and hydrology, (ii) examined the extent that fertilization and hydrology influence soil bacterial community composition over time, and (iii) measured the correlation between plant and bacterial community dissimilarities (based on the Bray-Curtis dissimilarity matrix) according to fertilization and hydrology over time in mowed plots.

METHODS

Experimental design

I collected bulk soil samples from the wet pine savannah wetland long-term experimental site located at East Carolina's West Research Campus (Figure 1). Fertilization and disturbance (by mowing) treatments were replicated on eight 20 x 30 m plots following a factorial design, with two levels of fertilization (fertilized, unfertilized) crossed with two levels of disturbance by mowing (mowed, unmowed). Half of the plots are drier and adjacent to a drainage ditch; the other half of the plots are wetter and further from the ditch which creates a hydrological gradient. A nitrogen-phosphorus-potassium (N-P-K 10-10-10) granular fertilizer was applied three times annually (February, June, October) for a total of 45.4 kg ha^{-1} for each nutrient to the soil. Plots were also mowed and raked annually to simulate a previous fire disturbance and better depict the features of the aboveground plant community (Goodwillie et al., 2020). Since 2004, plant communities have been taxonomically classified and from 2014 bacterial communities have been examined. For this study, I focused on mowed plots, where plant community patterns continue to change over time.

Sample collection and storage

I used plant community data from ongoing annual plant surveys (Goodwillie et al., 2020), in which stem counts and percent cover of each plant species were assessed in three randomly placed 1-m^2 quadrats per plot. I concentrated on mowed plots sampled from 2014 to 2020 because they better represent the historical fire-maintained plant communities at the site in comparison to the woody tree dominated unmowed plots. For bacterial community sample process, the lab sampled two soil cores at each of the three permanent sampling plots and combined 6 soil cores (annually

in October to November). Soils were passed through a 4 mm sieve to remove large roots, homogenized, and subsampled for storage at –80 °C for molecular analyses, at 4 °C for culturing, and air dried for physical and chemical analyses.

Soil physical and chemical properties

Soil moisture for each subsample (previously passed through a 4 mm sieve) was measured by using a gravimetric method. The field-moist soil was weighed (20 g – 30 g) and then oven-dried at 105 °C overnight. Soil moisture was then calculated by dividing the weight of the water (field moist – oven dried) by the weight of oven-dried soil. Then, a subsample of air-dried soils was further ground to a fine powder for total soil organic carbon and nitrogen analyses using an elemental combustion analyzer at the Environmental and Agricultural Testing Service Laboratory at North Carolina Statement University. Soil pH was measured by mixing a 1:1 ratio of air-dried soil and deionized water (10g soil: 10mL deionized water), vortexing the mix to a slurry and taking the average of three readings using a pH meter (GeneMate).

Genomic DNA extraction

I used the Qiagen Powerlyzer Powersoil Kit and followed the manufacturer's protocol to extract the genomic DNA from 0.25 g of each soil sample. The target cells were mechanically lysed with power beads and spun down in the centrifuge to separate the target material from any unwanted debris and cell particles. Many buffers were used to remove inhibitors and to obtain high concentration and integrity of the samples. Genomic DNA was eluted using 50 µL of TE buffer. The DNA was quantified with the spectrophotometer (Nanodrop 2000 UV-vis spectrophotometer) and diluted (~10 ng/µL) so that each sample was at the same level of concentration. Each DNA

sample was amplified by PCR in triplicate (50 µL PCR libraries) for more concentrated reads using the Eppendorf Flexilid MasterCycler. The PCR reactions were conducted using the following settings: 3 min of initialization at 94 °C, 45 sec of denaturation at 94 °C, 30 sec of annealing at 50 °C, 90 sec of extension at 72 °C, 10 min of final elongation at 72 °C, and lastly a final hold at 4 °C. PCR libraries were prepared by combining 35.75µL molecular grade water, 5µL Amplitaq gold 360 10x buffer, 5 µL MgCl$_2$ (25 mmol/L), 1 µL dNTP (10 mM), 1 µL 515 forward barcode primer (10 µmol/L), 1µL 806 reverse barcode primer (10 µmol/L), 1µL DNA template (10 ng/µL), 0.25 µL Amplitaq gold polymerase. Each DNA sample had a reverse primer (bacterial 806R) in the mix but a unique, barcoded forward primer (bacterial 515F). The Earth Microbiome Project first created both primers to specifically target the V4 region of the bacterial 16S component of the rRNA gene (Caporaso et al., 2012).

The PCR products were checked for successful amplification of the V4 region of the 16S rRNA gene with agarose gel electrophoresis. The 16S rRNA gene is used because it is highly studied and ubiquitous in all microorganisms; the hypervariable regions are used to identify and classify individual isolates. Expected band sizes for 515F-806R is ~300-350 bp; a single bright band indicates a successful amplification. To purify the sample, I used the magnetic bead PCR clean up protocol (Axygen) and then Qubit dsDNA BR fluorometric assay to determine the concentration of DNA for each sample (Koceja et al., 2021).

Bacterial 16S rRNA sequencing

Each PCR library was combined in equimolar concentration of 5 ng/uL into one tube and sequenced using the Illumina MiSeq platform. This platform was chosen primarily due to accessibility but also its relatively fast run time. Many other studies that are focused on various

microbial communities use this platform because of their short read length; therefore, the machine's high throughput/run (>25 million reads or 15Gb per lane) reduces costs significantly and in turn provides greater coverage. Although one drawback to using this platform is the short-read length, this can be 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.

Mothur, an open-source software package for bioinformatics data processing was used to analyze the sequences obtained from Illumina Miseq along with determining the error rate of the sequences using a mock community. The software converted the paired end reads into one contig (sequences overlapping one another). Singletons, or operational taxonomic units (OTUs) represented by a single unique sequence across the whole dataset, were discarded. A few commands were used to trim, get rid of any ambiguous reads, and merge duplicate sequences into one unique sequence. The sequences were run through SILVA, a known reference database so that the sequences were binned into operational taxonomic units (OTUs) based on 97% similarity cutoff.

Statistical analyses

All statistical analyses, diversity calculations, and graphing were conducted, and graphs made in the R environment (R version 4.2.2, 2022; R Studio version 2022.07.1, 2022). I calculated the following soil bacterial diversity metrics: OTU richness, Shannon diversity H', and Inverse Simpson's Diversity Index. 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

9

the number of distinct species (i.e., species richness) and their distribution within the community, with more sensitivity towards rare species. Bacterial Shannon diversity values less than five were omitted for the calculations due to relatively low sequence reads. Bacterial species evenness is considered by the Inverse Simpson's diversity index, but with a stronger focus on dominance and common species. I focused on Shannon diversity and Inverse Simpson's diversity for the OTU diversity analysis. Even though we make an effort to take sample bias into account (i.e., using rarefaction curves), counting taxonomic units on its own does not reveal information about rare and dominant taxa (Willis, 2019).

I used analysis of variance (ANOVA) to measure the extent that fertilization, proximity to ditch, and year influences variation in soil physicochemical properties using the aov() function. I identified which soil variable(s) ($P < 0.05$) to incorporate into a distance-based dissimilarity matrix as vectors, using the envfit() function in the vegan package to measure the extent that the soil property explained soil bacterial community composition (Objective 1). I also used R packages to perform generalized linear mixed models (GLMMs) (DHARMa, glmmTMB (Hartig, 2017), aods3 (Lesnoff and Lancelot, 2022), ggplot2 (Wickham, 2016), and gridExtra (Auguie, 2017)). Other R packages I used to examine multivariate analyses of microbial communities were phyloseq (McMurdie and Holmes, 2013), dplyr (Wickham et al., 2022), tidyverse (Wickham and Girlich, 2022), vegan (Oksanen et al., 2022), reshape2 (Wickham, 2007), lme4 (Bates et al., 2015), car (Fox and Weisberg, 2019), ade4 (Thioulouse et al., 2018), agricolae (Mendiburu, 2021) and pls (Liland et al., 2022)

These tools allowed me to use model diagnostics (i.e., residuals, conditional modes, QQ plots), statistical significance analyses, and data visualization to examine how interactions between fertilizer, year, and soil moisture (proximity to ditch) influence soil bacterial species richness and

relative abundances. I also visualized community patterns using a principal coordinates analysis (PCoA ordination) (based on the Bray-Curtis dissimilarity matrix) to examine bacterial and plant communities over space and time. The centroid of four replicate samples from mowed plots is represented in the PCoA (Principal Coordinates Analysis) ordination figures. The closer the points are in space, the more similar the community composition is between samples, and the further away the points are in ordination space, the more dissimilar the communities. I conducted permutational multivariate analysis (PERMANOVA) using the adonis() function in the vegan package to measure the variation in soil bacterial community explained by fertilization, year, ditch effect, and their interactions (Objective 2). To measure plant-bacterial relationships, I measured dissimilarity matrix correlations within fertilization and hydrology treatments using a series of matrix correlations using Mantel tests (based on plant and bacteria Bray-Curtis dissimilarity matrices) (Objective 3).

RESULTS

Fertilization alters the soil environment

Fertilizer, proximity to ditch, and year influenced soil factors. Soil moisture was measured at a single time point across all plots. The interactions across year, fertilization, and ditch influenced soil moisture to different degrees. The 3-way interaction of fertilization*ditch*year ($F_{6,78} = 2.94$, $P = 0.012$) and the 2-way interactions of fertilization*ditch ($F_{1,78} = 4.00$, $P = 0.049$) and ditch*year ($F_{6,78} = 2.51$, $P = 0.028$) modified soil moisture (Fig. 2, Appendix S1: Table S1). The main effects of year ($F_{6,78} = 43.89$, $P < 0.001$) and to a lesser extent ditch ($F_{1,78} = 12.98$, $P < 0.001$) also influenced soil moisture over time in the mowed plots (Fig. 2, Appendix S1: Table S1). Regardless of the fertilization treatment in both wet and dry plots, the years 2016 and 2017 showed the highest percent moisture (~40% to 60%), while 2019 and 2020 measured the lowest (<20%).

In both the mowed/fertilized and mowed/unfertilized plots, the soil pH steadily increased from 3.0 to 4.0 ($F_{1,78} = 10.71$, $P = 0.002$). Proximity to ditch ($F_{1,78} = 19.13$, $P < 0.001$) and year ($F_{6,78} = 37.45$, $P < 0.001$) also influenced soil pH (Fig. 3, Appendix S1: Table S1). On average, mowed/fertilized plots were slightly less acidic in pH than mowed/unfertilized plots across both wet and dry plots (Fig. 3, Appendix S1: Table S1). In both wet and dry plots, the total soil carbon increased by nearly three times from 2014 and 2020 ($F_{6,78} = 39.81$, $P < 0.001$). Despite the proximity to the drainage ditch ($F_{1,78} = 6.04$, $P = 0.016$), or whether fertilizer was applied ($F_{1,78} = 17.21$, $P < 0.001$), total soil carbon increased from ~2% to ~6%. On average, total soil carbon concentrations in mowed/fertilized plots were slightly higher than mowed/unfertilized (Fig. 4A, Appendix S1: Table S1). Drier wetland conditions also minimized the fertilization effect seen in the wetter plots (Fig. 4A, Appendix S1: Table S1). Total soil nitrogen increased in both mowed/fertilized and mowed/unfertilized plots (ANOVA: year: $F_{6,78} = 18.16$, $P < 0.001$;

fertilization: $F_{1,78} = 13.54$, $P < 0.001$) (Fig. 4B, Appendix S1: Table S1). Mowed/fertilized plots on average had slightly higher soil nitrogen concentrations compared to mowed/unfertilized. The soil carbon to nitrogen (C:N) ratio increased in both mowed/fertilized and mowed/unfertilized plots over time (ANOVA: year: $F_{6,78} = 27.40$, $P < 0.001$; ditch: $F_{1,78} = 8.88$, $P = 0.004$) (Fig. 4C, Appendix S1: Table S1). In both wet and dry plots, soil C:N ratio was lowest, ~11-13 at the start of the monitoring (2014) and increased to ~18-20 towards the end (2020). In the wet plots, soil C:N ratio in both treatments reached its peak in 2017 (~20).

Fertilization and ditch effects modify soil bacterial diversity and composition

In contrast to the effects seen in plant diversity (Fig. S1, Appendix S1: Table S5), fertilization increased bacterial Shannon diversity ($F_{1,78} = 28.48$, $P < 0.001$) and decreased Inverse Simpson's diversity ($F_{1,78} = 12.36$, $P < 0.001$) (Fig. 5A, Appendix S1: Table S2) and was more pronounced in the wet (further from the ditch) compared to dry plots (closer to the ditch). In contrast to the wetter plots, the bacterial diversity in the drier plots showed significantly less variation between treatments. In both wet and dry plots, the bacterial communities from the mowed/fertilized plots increased over time, in contrast to those from the mowed/unfertilized plots which decreased over time (Fig. 5A, Appendix S1: Table S2).

In the mowed plots, fertilization decreased bacterial Inverse Simpson's diversity in wet and dry plots (Fig. 5B, Appendix S1: Table S2). In the dry plots, bacterial Inverse Simpson's diversity was similar across fertilized and unfertilized plots except 2015, 2018 and 2020, where Inverse Simpson's diversity in unfertilized plots was higher than fertilized plots (Fig. 5B, Appendix S1: Table S2). The magnitude of the increase in bacterial Inverse Simpson's diversity in the unfertilized plots was dampened in the drier compared to the wetter plots.

13

Year ($R^2 = 0.179$, $P < 0.001$), fertilization ($R^2 = 0.084$, $P < 0.001$), then ditch effect (hydrology) ($R^2 = 0.049$, $P < 0.001$) strongly influenced bacterial 16S rRNA community composition, where 20.2% of the bacterial community variation can be explained by PCoA axis 1 and 12.8% can be explained by PCoA axis 2 (Fig. 6C, Appendix S1: Table S3). The bacterial communities in the fertilized plots were distinct from the communities in the unfertilized plots and are further distinguished based on their proximity to the drainage ditch (Fig. 6C, Appendix S1: Table S3). The interaction of fertilization and year explained more variation ($R^2 = 0.069$, $P < 0.001$) in bacterial community composition compared to ditch effect alone (Fig. 6C, Appendix S1: Table S3). In addition, total soil carbon concentrations were related to mowed/fertilized plots than mowed/unfertilized ($R^2 = 0.06$, $P = 0.035$) (Fig. 6C, Appendix S1: Table S3).

Plant and bacterial community patterns varied across space and time. Plant communities were distinct due to hydrology ($F_{1,60} = 22.76$, $P = 0.001$), especially in unfertilized plots, while fertilization influenced bacterial communities ($F_{1,82} = 12.50$, $P < 0.001$) (Fig. 6) more than hydrology ($F_{1,82} = 7.29$, $P < 0.001$) (Fig. 7). When bacterial and plant community dissimilarities were weakly correlated (2015 fertilized/wet, 2016 unfertilized/dry), the variation in plant and bacterial community differed (Fig. S2). For example, the error bars representing variation along PCoA axes 1 and 2 were longer (i.e., more variation) for plant community composition compared to bacterial community composition in fertilized/wet plots in 2015 (Fig. 8A, 8B). Variation along PCoA axes 1 and 2 was higher for bacterial community composition compared to plant community composition in unfertilized/dry in 2016 (Fig. 8C, 8D). In contrast, for fertilized/wet plots, variation in plant and bacterial communities were observed to have similar variation in dissimilarity patterns and represented strong and positive mantel R correlations (2016, 2017, 2018, 2020) (Fig. 8, Fig. S2).

DISCUSSION

Climate change and human activities modify nutrient and water cycles, resulting in changes to plant-microbe relationships, species diversity, community composition, and ecosystem functions in unexpected ways. Although wetlands are known for their enormous capacity to store carbon, ongoing environmental changes such as atmospheric deposition of nutrients affect the composition of plant and bacterial communities and carbon storage potential. After 10 years of manipulation, we examined the extent that fertilization and hydrologic conditions affected (1) soil environmental parameters, (2) the succession of soil bacterial communities, and (3) plant-bacterial associations in a low-nutrient wetland ecosystem over time. Even though fertilization, year, and ditch effects influenced soil factors, the results showed that none of the total soil parameters examined—soil pH, total soil carbon, total soil nitrogen, and soil moisture—could meaningfully account for the variations in bacterial Shannon diversity. Further, fertilization treatment more than the hydrologic gradient influenced bacterial community patterns. Results also revealed that fertilization increased total soil carbon content and was more correlated with the total bacterial community composition from mowed/fertilized plots compared to mowed/unfertilized regardless of the ditch effect. The findings of this study demonstrated that, to varied degrees, the manipulation of nutrients interacted with an established hydrologic gradient to modify the wetland soil physicochemical properties, bacterial and plant communities, and bacterial-plant associations.

Nutrient enrichment modifies the wetland soil environment

As expected, the fertilization treatment significantly affected soil physicochemical properties over time in the low-nutrient wetland ecosystem. Long-term fertilization increased total soil carbon content over time regardless of hydrologic effects (Fig. 4A, Appendix S1: Table S1). Since the

main sources of carbon entering the soil are compounds from plant litter and root exudates, diverse bacteria can form close associations with plant partners owing to the diversity of possible sugar resources provided by plants (Millard and Singh, 2010). Such chemical substances are secreted by plants to preferentially choose and exclude bacteria from establishing root associations among the vast diversity of soil microorganisms. This may account for the substantial differences in bacterial communities between mowed/fertilized and mowed/unfertilized plots (Fig. 4A, Appendix S1: Table S1), as well as the increased association of fertilized plots with total soil carbon (Fig. 6C, Appendix S1: Table S3). Due to the absence of a geographical barrier between fertilized and unfertilized soils and a physical barrier from ongoing atmospheric deposits, it is also likely that fertilization effects in mowed/unfertilized plots are being observed (Fig. 4A, Appendix S1: Table S1) (Tian and Niu, 2015; Li et al., 2022; R.-T. Zhang et al., 2022). Over time, excess nutrients from fertilized plots may leach into the unfertilized plots (Schimel, 2018). Past studies showed that N-P-K fertilization corresponded with increases in total soil carbon accumulation compared to unfertilized soils (Cenini et al., 2015; Poeplau et al., 2018; Poeplau, 2021; L. Zhang et al., 2022; Watson et al., 2022). In addition, inorganic fertilizers applied in isolation (i.e., N, P, K) rather than together (i.e., NP, NK, PN, PK, NPK) resulted in more carbon accumulation (Fornara et al., 2013). Past studies have also evaluated the effects of chronic nitrogen inputs on soil carbon transport in forest ecosystems and showed that fertilization effects promote soil carbon gains (Saiya-Cork et al., 2002; Hobbie, 2008). Nitrogen fertilizer also slows lignin decomposition by suppressing lignin-degrading enzymes while boosting cellulose-degrading enzymes. This mechanism results in less organic matter being recycled and more recalcitrant carbon accumulating, according to short-term results that are consistent across ecosystems. However, less is known about the long-term effects of these microbial functional alterations (Cenini et al., 2015; Chen et al., 2018). If

microbial communities with essential enzymatic functions are disturbed, significant ecosystem functions, such as carbon sequestration and nutrient availability, could be disrupted (Wagg et al., 2014).

I anticipated that the fertilization treatment would decrease soil pH over time, but I observed that the soil pH of the mowed/fertilized and mowed/unfertilized plots increased (pH ~ 3.0 to 4.0) (Fig. 3, Appendix S1: Table S1). Since soil sampling began in 2014 after the experiment had been ongoing for ten years and since coastal plain wetland soils are typically acidic, soil pH changes during early years of the experiment were not measured. I observed that soil pH was not a strong predictor or related to changes in the bacterial communities from mowed plots (Fig. 3, Appendix S1: Table S3; Appendix S1: Table S7), in contrast to many previous long-term fertilization studies (Rousk et al., 2010; Dai et al., 2018). Previous nutrient enrichment studies have identified the response of soil bacteria to nitrogen input varies among ecosystems, although soil pH acidification is more consistent (Yuan et al., 2016; Xu et al., 2020). Soil pH is a very strong environmental filter and best predictor of bacterial structural and functional changes on both small and large spatial scales (Rousk et al., 2010). Additionally, edaphic characteristics, notably pH, may indirectly alter the quantity and quality of various plant-derived sugars in the root exudates (Lauber et al., 2009; Dickson and Gross, 2013; Hortal et al., 2017; Zhang et al., 2017). Given the observed fertilization effects on soil organic carbon but not soil pH (Fig. 6C, Appendix S1: Table S3), these results indicate that bacterial community responses to long-term nutrient inputs may be mediated more by changes in the composition of the plant community than by changes in the environmental conditions of the soil. Our findings imply that soil pH was not the dominant factor as anticipated, even though soil pH is known to be a strong environmental filter influencing the assembly of bacterial communities (Fierer et al., 2007).

Fertilization effects on bacterial diversity and community composition

Long-term fertilization of a coastal plain wetland increased bacterial diversity (Fig. 5A, Appendix S1: Table S2) and modified bacterial composition (Fig. 6C, Appendix S1: Table S3). I observed that the unfertilized and wet conditions (i.e., the historical coastal plain wetland status) exhibited increased bacterial Inverse Simpson's diversity and decreased bacterial Shannon diversity. At this wetland ecosystem, the combined drier conditions masked fertilization effects observed in wet conditions. Physical and chemical changes in the soil can reduce belowground biodiversity, which leads to more species displaying traits that are essential for stress tolerance instead of growth (Choi et al., 2017). It is possible that the stressor created by drier plots decreased nutrient access and inhibited bacterial growth (Schimel, 2018; Siebielec et al., 2020). Dry and fertilized conditions limit nutrient availability throughout the belowground soil ecosystem, whereas wetter conditions could replenish the soil and mobilize nutrients via diffusion and transport (Schimel, 2018). Such environmental stressors may interact to have a synergistic effect, affecting the bacterial community simultaneously, as opposed to more temporally separate perturbations that might cause a different response (Rudgers et al., 2020). Instead of growing and reproducing, more energy expenditure is put into surviving.

The relative abundance of species with stricter oxygen demands could also contribute to the variation observed in wet and dry plots (Fig. 5A, Fig. 5B, Appendix S1: Table S2). In contrast to wet environments, which would promote more facultative and anaerobic microbial processes, drier conditions would favor more facultative and obligate aerobic microbes (Bledsoe and Peralta, 2020; Koceja et al., 2021). Species that cannot tolerate or sustain unfavorable conditions (i.e., soil moisture, nutrient limited, soil acidity) will be outcompeted. However, those individuals that are

less sensitive to perturbations (increased resistance) or have a quicker recovery rate (increased resilience) would persist and become dominant in the community (Berga et al., 2012).

Results supported the hypothesis that, over time, fertilization more than hydrology influenced the bacterial community composition over time. Year, fertilization, and then ditch effect (hydrology) strongly influenced the patterns observed in the bacterial community (Fig. 6, Appendix S1: Table S3). Since total soil carbon was more correlated to total bacterial community composition in fertilized compared to unfertilized plots (Fig. 6C, Appendix S1: Table S3), it is possible that the soil bacterial community could take advantage of increases nutrients and carbon resources, especially under wet conditions.

By adding nutrients or carbon resources to soils, soil respiration will increase due to increased microbial activity as well as encourage the growth of copiotrophs (Fierer et al., 2007; Bledsoe et al., 2020; Koceja et al., 2021). Most of the bacteria in the nutrient-limited coastal plain wetland are slow-growing oligotrophs as defined by their taxonomy (Bledsoe et al., 2020). Long-term fertilization increased the relative abundance of oligotrophs and decreased copiotrophs, according to a more recent publication at the same experimental site, which contrasted with previous studies' findings (Bledsoe et al., 2020). The copiotroph to oligotroph ratio was not examined in this work, although the ramifications of this change at the community level may have a significant impact on future carbon storage capability, especially in light of increased microbial carbon usage rates measured in mowed/fertilized compared to mowed/unfertilized (Bledsoe et al., 2020). Additionally, over time, the fertilizer treatment compared to unfertilized, increased soil carbon concentrations, soil nitrogen concentrations, and soil C:N ratios (Fig.4B, Fig. 4C, Appendix S1: Table S1). Perhaps, the combined effects of fertilizer and soil moisture promoted plant productivity thus increasing organic matter inputs which contribute to the increase in total carbon percent (Fig.

4A, Appendix S1: Table S1) (Cenini et al., 2015; Poeplau et al., 2018; Poeplau, 2021; L. Zhang et al., 2022; Watson et al., 2022).

Fertilization effects on plant-bacteria associations

Human disturbances have resulted in major shifts to plant communities across terrestrial and aquatic ecosystems. These modifications are known to affect litter quality and quantity thus affecting soil nutrient availability and shaping bacterial community composition. Therefore, it was anticipated that the addition of nutrients would result in opposing plant and bacterial diversity patterns based on prior studies, including one from the same experimental site (Bledsoe et al., 2020), and results from this study confirm this ongoing trend. Plant diversity decreased (Fig. S1, Appendix S1: Table S5) while bacterial diversity increased (Fig. 5A, Appendix S1: Table S2). Competition for available nutrients and light are implicated for the decline in plant diversity in low-nutrient environments since mowing lessens the plants' ability to outcompete one another for sunlight (Goodwillie et al., 2020). In comparison to species that develop slower, shorter, and with smaller leaves, those that can grow taller and/or generate larger leaves could influence the belowground community more than slow growing plant species (Dickson and Gross, 2013; Huang et al., 2019; Goodwillie et al., 2020).

The interaction of changes to the soil environment and the plant community influence soil bacteria to varying degrees. In one long-term fertilization study, nitrogen's direct effects decreased soil nutrient limitations, but its indirect effects changed bacterial populations by affecting the pH of the soil and plant-derived substrates (Yuan et al., 2016). Previous work also showed that belowground bacterial community succession is driven more by indirect effects of plant community composition than by direct effects of climate-induced effects (Lange et al., 2014;

Classen et al., 2015; Leff et al., 2015). It is also recognized that indirect effects of climate-induced changes are more noticeable over longer time scales (i.e., decades to centuries), whereas direct effects of community changes are more noticeable over a shorter time scale (Wootton, 2002; Bardgett et al., 2008).

It is widely acknowledged that a healthy soil microbiome offers significant advantages to its plant partners. Beneficial microbial communities and functional traits that support plant development and defense mechanisms against stressors can be affected by any shifts in soil characteristics (pH, oxygen, nutrients, temperature), environmental conditions (drought, flood, fire disturbance), plant host genetics, and land use changes (Abdul Rahman et al., 2021). However, a shift in soil microbial patterns could have unintended consequences, where bacterial communities that rely on carbon from plants change in composition to bacterial communities that can more effectively use soil organic carbon (Ramirez et al., 2012; Ai et al., 2015). In this low-nutrient wetland, bacterial (Fig. 6, Appendix S1: Table S3) and plant (Fig. 7, Appendix S1: Table S6) communities responded differently to the hydrologic gradient and similarly to the fertilization treatment, indicating similar abilities to uptake nutrient inputs. Drying wetland conditions appeared to have a stronger impact on plants compared to bacteria, and fertilization strongly influences both bacterial and plant communities. A bacterial community shift to more antagonistic plant partnerships could also make plants more vulnerable to perturbations and less likely to recover quickly from stressors that would have been mediated by beneficial microorganisms (Huang et al., 2019). This work showed that interacting soil physicochemical characteristics (such as nutrient concentrations and moisture) restrict bacterial diversity and community patterns in distinct ways. Because of nutrient enrichment and drying conditions, changes to wetland plants

and soil bacterial community patterns could imply an increasingly competitive rather than cooperative relationship between plants and soil microbes.

Limitations of ecological experiments

While long-term ecology experiences are critical for understanding ecological responses to environmental changes, experimental manipulations are simplified. For example, the mowing and raking component represents the fire regimes that maintained wet pine savannah coastal plain wetlands. The mowing and raking does not capture the biochar addition, which would feed microbes and hasten the breakdown of plant litter and debris (Minamino et al., 2019; Guo et al., 2020). In addition, multiple stressor effects that mimic real-world disturbances are challenging to scale up, replicate, and maintain at the ecosystem-level but are recognized as important for examining complex nutrient feedbacks on carbon cycling. Ecosystem-scale studies that examine ecosystem responses to two levels of environmental drivers still offer important insight to understand mechanistic underpinnings of plant-bacterial associations on biogeochemical functions (Collins et al., 2022).

Implications of findings and future directions

This study examined how human disturbances such as nutrient enrichment and hydrologic fluctuations influenced soil bacterial and plant communities at the terrestrial-aquatic interface. Changes to mutualistic wetland plant and soil bacterial community patterns may indicate an increasingly competitive rather than cooperative connection between plants and soil microorganisms due to nutrient enrichment and drying conditions of the soil environment. The amount of nitrogen and phosphorus that is being deposited from the atmosphere is rising, but it is

less known how this nutrient enrichment may affect natural ecosystems that are poor in nutrients yet extremely productive carbon sinks. This work emphasizes how long-term enrichment in a wetland system, which has received less attention, is crucial for accurately predicting nutrient feedback effects on carbon cycling.

Additional research may be required to explain unexpected community patterns. The overall trajectory of soil carbon feedbacks from terrestrial ecosystems to the atmosphere and vice versa has been debated in a number studies on climate change and its effect on litter decomposition rates (Classen et al., 2015; Koceja et al., 2021). When estimating carbon sequestration dynamics as nutrients passively enter an ecosystem, a number of factors must be taken into account, including the substrate composition being degraded, the habitat being studied, the rate of fertilizer application, the amount of fertilizer added, and the interaction of abiotic and biotic factors (Hobbie, 2008; Koceja et al., 2021).

As our attention shifts to more inclusive methods and approaches for analyzing complex assemblages of interacting species, a better comprehension of these global environmental effects could improve future predictions of soil health and the preservation of plant-microbe ecological and evolutionary functions (Rudgers et al., 2020). The gaps in our understanding of the mechanisms by which climate change disturbs plant-microbe interactions to various degrees are addressed by the knowledge gained from this research. Significant ecological functions are driven and regulated by soil microorganisms. This foundational information is significant to the fields of restoration, agricultural, and restoration ecology as well as microbial, ecosystem, and community ecology.

References

Abdul Rahman, N.S.N., Abdul Hamid, N.W., Nadarajah, K., 2021. Effects of abiotic stress on soil microbiome. International Journal of Molecular Sciences 22, 9036. doi:10.3390/ijms22169036

Ai, C., Liang, G., Sun, J., Wang, X., He, P., Zhou, W., He, X., 2015. Reduced dependence of rhizosphere microbiome on plant-derived carbon in 32-year long-term inorganic and organic fertilized soils. Soil Biology and Biochemistry 80, 70–78. doi:10.1016/j.soilbio.2014.09.028

Auguie, B., 2017. gridExtra: Miscellaneous functions for "Grid" graphics_. R package.

Bardgett, R.D., Caruso, T., 2020. Soil microbial community responses to climate extremes: resistance, resilience and transitions to alternative states. Philosophical Transactions of the Royal Society B: Biological Sciences 375, 20190112. doi:10.1098/rstb.2019.0112

Bardgett, R.D., Freeman, C., Ostle, N.J., 2008. Microbial contributions to climate change through carbon cycle feedbacks. The ISME Journal 2, 805–814. doi:10.1038/ismej.2008.58

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67. doi:10.18637/jss.v067.i01

Bauer, J.T., Kleczewski, N.M., Bever, J.D., Clay, K., Reynolds, H.L., 2012. Nitrogen-fixing bacteria, arbuscular mycorrhizal fungi, and the productivity and structure of prairie grassland communities. Oecologia 170, 1089–1098. doi:10.1007/s00442-012-2363-3

Berga, M., Székely, A.J., Langenheder, S., 2012. Effects of disturbance intensity and frequency on bacterial community composition and function. PLoS ONE 7, e36959. doi:10.1371/journal.pone.0036959

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. doi:10.1128/mSphere.00035-20

Bledsoe, R.B., Peralta, A.L., 2020. Dynamic hydrology and plant-mediated effects reduce greenhouse gas emissions and alter wetland microbial communities (preprint). Ecology. doi:10.1101/2020.06.29.178533

Bobbink, R., Hornung, M., Roelofs, J.G.M., 1998. The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation. Journal of Ecology 86, 717–738. doi:10.1046/j.1365-2745.1998.8650717.x

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal 6, 1621–1624. doi:10.1038/ismej.2012.8

Cavicchioli, R., Ripple, W.J., Timmis, K.N., Azam, F., Bakken, L.R., Baylis, M., Behrenfeld, M.J., Boetius, A., Boyd, P.W., Classen, A.T., Crowther, T.W., Danovaro, R., Foreman, C.M., Huisman, J., Hutchins, D.A., Jansson, J.K., Karl, D.M., Koskella, B., Mark Welch, D.B., Martiny, J.B.H., Moran, M.A., Orphan, V.J., Reay, D.S., Remais, J.V., Rich, V.I., Singh, B.K., Stein, L.Y., Stewart, F.J., Sullivan, M.B., van Oppen, M.J.H., Weaver, S.C., Webb, E.A., Webster, N.S., 2019. Scientists' warning to humanity: microorganisms and climate change. Nature Reviews Microbiology 17, 569–586. doi:10.1038/s41579-019-0222-5

Cenini, V.L., Fornara, D.A., McMullan, G., Ternan, N., Lajtha, K., Crawley, M.J., 2015. Chronic nitrogen fertilization and carbon sequestration in grassland soils: evidence of a microbial enzyme link. Biogeochemistry 126, 301–313. doi:10.1007/s10533-015-0157-5

Chen, J., Luo, Y., van Groenigen, K.J., Hungate, B.A., Cao, J., Zhou, X., Wang, R., 2018. A keystone microbial enzyme for nitrogen control of soil carbon storage. Science Advances 4, eaaq1689. doi:10.1126/sciadv.aaq1689

Choi, S., Song, H., Tripathi, B.M., Kerfahi, D., Kim, H., Adams, J.M., 2017. Effect of experimental soil disturbance and recovery on structure and function of soil community: a metagenomic and metagenetic approach. Scientific Reports 7, 2260. doi:10.1038/s41598-017-02262-6

Classen, A.T., Sundqvist, M.K., Henning, J.A., Newman, G.S., Moore, J.A.M., Cregger, M.A., Moorhead, L.C., Patterson, C.M., 2015. Direct and indirect effects of climate change on soil microbial and soil microbial-plant interactions: What lies ahead? Ecosphere 6, art130. doi:10.1890/ES15-00217.1

Collins, S., Whittaker, H., Thomas, M.K., 2022. The need for unrealistic experiments in global change biology. Current Opinion in Microbiology 68, 102151. doi:10.1016/j.mib.2022.102151

Dai, Z., Su, W., Chen, H., Barberán, A., Zhao, H., Yu, M., Yu, L., Brookes, P.C., Schadt, C.W., Chang, S.X., Xu, J., 2018. Long-term nitrogen fertilization decreases bacterial diversity and favors the growth of *Actinobacteria* and *Proteobacteria* in agro-ecosystems across the globe. Global Change Biology 24, 3452–3461. doi:10.1111/gcb.14163

Dastogeer, K.M.G., Tumpa, F.H., Sultana, A., Akter, M.A., Chakraborty, A., 2020. Plant

>microbiome–an account of the factors that shape community composition and diversity.

>Current Plant Biology 23, 100161. doi:10.1016/j.cpb.2020.100161

Dickson, T.L., Gross, K.L., 2013. Plant community responses to long-term fertilization: changes

>in functional group abundance drive changes in species richness. Oecologia 173, 1513–

>1520. doi:10.1007/s00442-013-2722-8

Fierer, N., Bradford, M.A., Jackson, R.B., 2007. Toward an ecological classification of soil

>bacteria. Ecology 88, 1354–1364. doi:10.1890/05-1839

Fornara, D.A., Banin, L., Crawley, M.J., 2013. Multi-nutrient vs. nitrogen-only effects on carbon

>sequestration in grassland soils. Global Change Biology 19, 3848–3857.

>doi:10.1111/gcb.12323

Fox, J., Weisberg, S., 2019. An R companion to applied regression, Third edition. ed. SAGE,

>Los Angeles.

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.

>doi: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. doi:10.3389/fevo.2017.00070

Guo, X., Liu, H., Zhang, J., 2020. The role of biochar in organic waste composting and soil improvement: A review. Waste Management 102, 884–899. doi:10.1016/j.wasman.2019.12.003

Hartig, F., 2017. Hierarchical (multi-level / mixed) regression models. R package.

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

Hobbie, S.E., 2008. Nitrogen effects on decomposition: a five-year experiment in eight temperate sites. Ecology 89, 2633–2644. doi:10.1890/07-1119.1

Hortal, S., Lozano, Y.M., Bastida, F., Armas, C., Moreno, J.L., Garcia, C., Pugnaire, F.I., 2017. Plant-plant competition outcomes are modulated by plant effects on the soil bacterial community. Scientific Reports 7, 17756. doi:10.1038/s41598-017-18103-5

Howard, M.M., Kao-Kniffin, J., Kessler, A., 2020. Shifts in plant–microbe interactions over community succession and their effects on plant resistance to herbivores. New Phytologist 226, 1144–1157. doi:10.1111/nph.16430

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, 1464–1475. doi:10.1111/1751-7915.13487

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., Kopriva, S., 2017. The role of soil microorganisms in plant mineral nutrition-current knowledge and future directions. Frontiers in Plant Science 8, 1617. doi:10.3389/fpls.2017.01617

Jones, P., Garcia, B.J., Furches, A., Tuskan, G.A., Jacobson, D., 2019. Plant host-associated

    mechanisms for microbial selection. Frontiers in Plant Science 10, 862.

    doi:10.3389/fpls.2019.00862

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. doi:10.1002/ecs2.3619

Lange, M., Habekost, M., Eisenhauer, N., Roscher, C., Bessler, H., Engels, C., Oelmann, Y.,

    Scheu, S., Wilcke, W., Schulze, E.-D., Gleixner, G., 2014. Biotic and abiotic properties

    mediating plant diversity effects on soil microbial communities in an experimental

    grassland. PLoS ONE 9, e96182. doi:10.1371/journal.pone.0096182

Lauber, C.L., Hamady, M., Knight, R., Fierer, N., 2009. Pyrosequencing-based assessment of

    soil pH as a predictor of soil bacterial community structure at the continental scale.

    Applied and Environmental Microbiology 75, 5111–5120. doi:10.1128/AEM.00335-09

Leff, J.W., Jones, S.E., Prober, S.M., Barberán, A., Borer, E.T., Firn, J.L., Harpole, W.S.,

    Hobbie, S.E., Hofmockel, K.S., Knops, J.M.H., McCulley, R.L., La Pierre, K., Risch,

    A.C., Seabloom, E.W., Schütz, M., Steenbock, C., Stevens, C.J., 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,

    10967–10972. doi:10.1073/pnas.1508382112

Lesnoff, M., Lancelot, R., 2022. aods3: analysis of overdispersed data using S3 methods.

Li, T., Cui, L., Liu, L., Wang, H., Dong, J., Wang, F., Song, X., Che, R., Li, C., Tang, L., Xu, Z.,

    Wang, Y., Du, J., Hao, Y., Cui, X., 2022. Characteristics of nitrogen deposition research

within grassland ecosystems globally and its insight from grassland microbial community changes in China. Frontiers in Plant Science 13, 947279. doi:10.3389/fpls.2022.947279

Liland, K., Bjorn-Helge, M., Wehrens, R., 2022. pls: Partial least squares and principal component.

Liu, J., Jia, X., Yan, W., Zhong, Y., Shangguan, Z., 2020. Changes in soil microbial community structure during long-term secondary succession. Land Degradation & Development 31, 1151–1166. doi:10.1002/ldr.3505

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

Mendiburu, F. de, 2021. agricolae: statistical procedures for agricultural research.

Millard, P., Singh, B.K., 2010. Does grassland vegetation drive soil microbial diversity? Nutrient Cycling in Agroecosystems 88, 147–158. doi:10.1007/s10705-009-9314-3

Minamino, Y., Fujitake, N., Suzuki, T., Yoshitake, S., Koizumi, H., Tomotsune, M., 2019. Effect of biochar addition on leaf-litter decomposition at soil surface during three years in a warm-temperate secondary deciduous forest, Japan. Scientific Reports 9, 16961. doi:10.1038/s41598-019-53615-2

Nielsen, U.N., Ayres, E., Wall, D.H., Bardgett, R.D., 2011. Soil biodiversity and carbon cycling: a review and synthesis of studies examining diversity-function relationships. European Journal of Soil Science 62, 105–116. doi:10.1111/j.1365-2389.2010.01314.x

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R.B., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H.,

FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahiti, L., McGlinn, D., Ouellette, M., Riberio, C.E., Smith, T., Stier, A., Ter Braak, C., Weedon, J., 2022. vegan: community ecology package_. R package.

Poeplau, C., 2021. Grassland soil organic carbon stocks along management intensity and warming gradients. Grass and Forage Science 76, 186–195. doi:10.1111/gfs.12537

Poeplau, C., Zopf, D., Greiner, B., Geerts, R., Korvaar, H., Thumm, U., Don, A., Heidkamp, A., Flessa, H., 2018. Why does mineral fertilization increase soil carbon stocks in temperate grasslands? Agriculture, Ecosystems & Environment 265, 144–155. doi:10.1016/j.agee.2018.06.003

Prommer, J., Walker, T.W.N., Wanek, W., Braun, J., Zezula, D., Hu, Y., Hofhansl, F., Richter, A., 2020. Increased microbial growth, biomass, and turnover drive soil organic carbon accumulation at higher plant diversity. Global Change Biology 26, 669–681. doi:10.1111/gcb.14777

Rabalais, N.N., Turner, R.E., Díaz, R.J., Justić, D., 2009. Global change and eutrophication of coastal waters. ICES Journal of Marine Science 66, 1528–1537. doi:10.1093/icesjms/fsp047

Ramirez, K.S., Craine, J.M., Fierer, N., 2012. Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. Global Change Biology 18, 1918–1927. doi:10.1111/j.1365-2486.2012.02639.x

Rousk, J., Bååth, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. The ISME Journal 4, 1340–1351. doi:10.1038/ismej.2010.58

Rudgers, J.A., Afkhami, M.E., Bell-Dereske, L., Chung, Y.A., Crawford, K.M., Kivlin, S.N., Mann, M.A., Nuñez, M.A., 2020. Climate disruption of plant-microbe interactions. Annual Review of Ecology, Evolution, and Systematics 51, 561–586. doi:10.1146/annurev-ecolsys-011720-090819

Saiya-Cork, K.R., Sinsabaugh, R.L., Zak, D.R., 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biology and Biochemistry 34, 1309–1315. doi:10.1016/S0038-0717(02)00074-3

Schimel, J.P., 2018. Life in dry soils: effects of drought on soil microbial communities and processes. Annual Review of Ecology, Evolution, and Systematics 49, 409–432. doi:10.1146/annurev-ecolsys-110617-062614

Siebielec, S., Siebielec, G., Klimkowicz-Pawlas, A., Gałązka, A., Grządziel, J., Stuczyński, T., 2020. Impact of water stress on microbial community and activity in sandy and loamy soils. Agronomy 10, 1429. doi:10.3390/agronomy10091429

Stanton, M.L., 2003. Interacting guilds: moving beyond the pairwise perspective on mutualisms. The American Naturalist 162, S10–S23. doi:10.1086/378646

Thioulouse, J., Dray, S., Dufour, A.-B., Siberchicot, A., Jombart, T., Pavoine, S., 2018. Multivariate analysis of ecological data with ade4. Springer New York, New York, NY. doi:10.1007/978-1-4939-8850-1

Tian, D., Niu, S., 2015. A global analysis of soil acidification caused by nitrogen addition. Environmental Research Letters 10, 024019. doi:10.1088/1748-9326/10/2/024019

Wagg, C., Bender, S.F., Widmer, F., Heijden, M.G.A. van der, 2014. Soil biodiversity and soil community composition determine ecosystem multifunctionality. Proceedings of the National Academy of Sciences 111, 5266–5270. doi:10.1073/pnas.1320054111

Wang, G., Post, W.M., Mayes, M.A., 2013. Development of microbial-enzyme-mediated decomposition model parameters through steady-state and dynamic analyses. Ecological Applications 23, 255–272. doi:10.1890/12-0681.1

Watson, E.B., Rahman, F.I., Woolfolk, A., Meyer, R., Maher, N., Wigand, C., Gray, A.B., 2022. High nutrient loads amplify carbon cycling across California and New York coastal wetlands but with ambiguous effects on marsh integrity and sustainability. PLOS ONE 17, e0273260. doi:10.1371/journal.pone.0273260

Weese, D.J., Heath, K.D., Dentinger, B.T.M., Lau, J.A., 2015. Long-term nitrogen addition causes the evolution of less-cooperative mutualists: nitrogen addition destabilizes mutualism. Evolution 69, 631–642. doi:10.1111/evo.12594

Wickham, H., 2016. ggplot2: elegant graphics for data analysis, 2nd ed. 2016. ed, Use R! Springer International Publishing : Imprint: Springer, Cham. doi:10.1007/978-3-319-24277-4

Wickham, H., 2007. Reshaping data with the reshape package. Journal of Statistical Software 21. doi:10.18637/jss.v021.i12

Wickham, H., Francois, R., Henry, L., Muller, K., 2022. dplyr: A Grammar of Data Manipulation.

Wickham, H., Girlich, M., 2022. tidyr: tidy messy data. https://tidyr.tidyverse.org.

Willis, A.D., 2019. Rarefaction, alpha diversity, and statistics. Frontiers in Microbiology 10, 2407. doi:10.3389/fmicb.2019.02407

Wootton, J.T., 2002. Indirect effects in complex ecosystems: recent progress and future challenges. Journal of Sea Research 48, 157–172. doi:10.1016/S1385-1101(02)00149-1

Xu, Q., Ling, N., Chen, H., Duan, Y., Wang, S., Shen, Q., Vandenkoornhuyse, P., 2020. Long-term chemical-only fertilization induces a diversity decline and deep selection on the soil bacteria. MSystems 5, e00337-20. doi:10.1128/mSystems.00337-20

Yuan, X., Knelman, J.E., Gasarch, E., Wang, D., Nemergut, D.R., Seastedt, T.R., 2016. Plant community and soil chemistry responses to long-term nitrogen inputs drive changes in alpine bacterial communities. Ecology 97, 1543–1554. doi:10.1890/15-1160.1

Zhang, L., Zhao, L., Yi, H., Lan, S., Chen, L., Han, G., 2022. Nitrogen addition alters plant growth in China's Yellow River Delta coastal wetland through direct and indirect effects. Frontiers in Plant Science 13, 1016949. doi:10.3389/fpls.2022.1016949

Zhang, Q., Liu, K., Shao, X., Li, H., He, Y., Sirimuji, Wang, B., 2021. Microbes require a relatively long time to recover in natural succession restoration of degraded grassland ecosystems. Ecological Indicators 129, 107881. doi:10.1016/j.ecolind.2021.107881

Zhang, R.-T., Liu, Y.-N., Zhong, H.-X., Chen, X.-W., Sui, X., 2022. Effects of simulated nitrogen deposition on the soil microbial community diversity of a Deyeuxia angustifolia wetland in the Sanjiang Plain, Northeastern China. Annals of Microbiology 72, 11. doi:10.1186/s13213-022-01666-8

Zhang, Y., Shen, H., He, X., Thomas, B.W., Lupwayi, N.Z., Hao, X., Thomas, M.C., Shi, X., 2017. Fertilization shapes bacterial community structure by alteration of soil ph. Frontiers in Microbiology 8, 1325. doi:10.3389/fmicb.2017.01325

FIGURES

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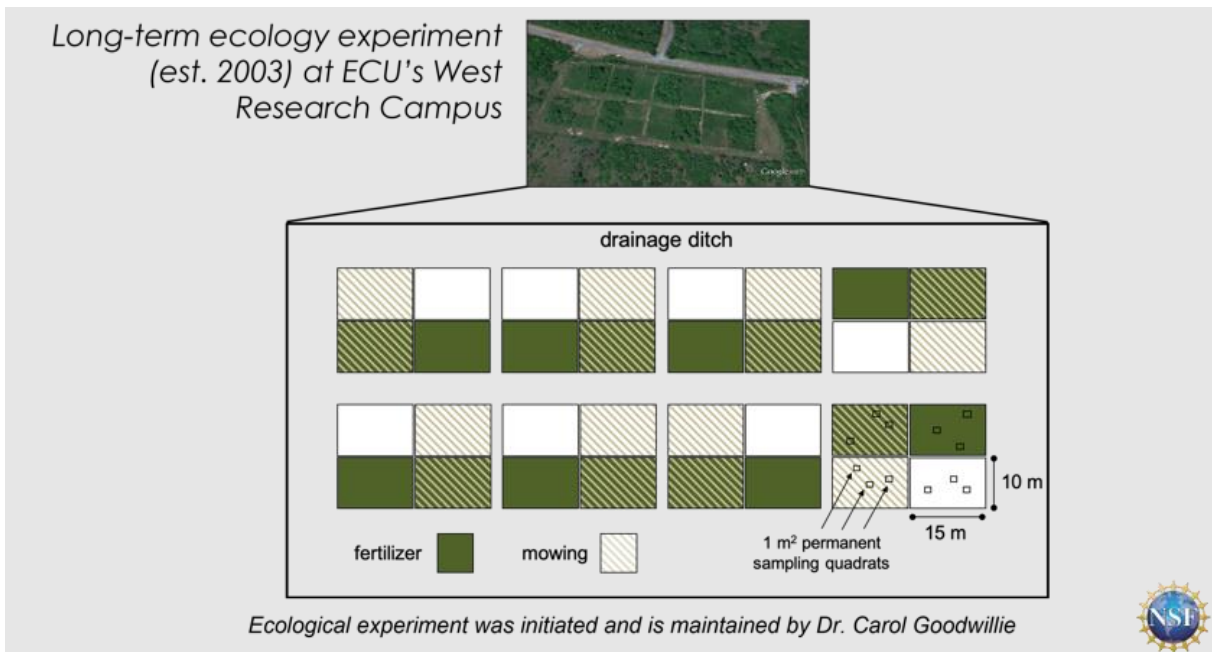
Figure 1. Experimental design of the long-term ecological experiment. Since 2003, fertilization and disturbance by mowing plots at East Carolina University's West Research Campus located in Greenville, North Carolina, USA.

Figure 2
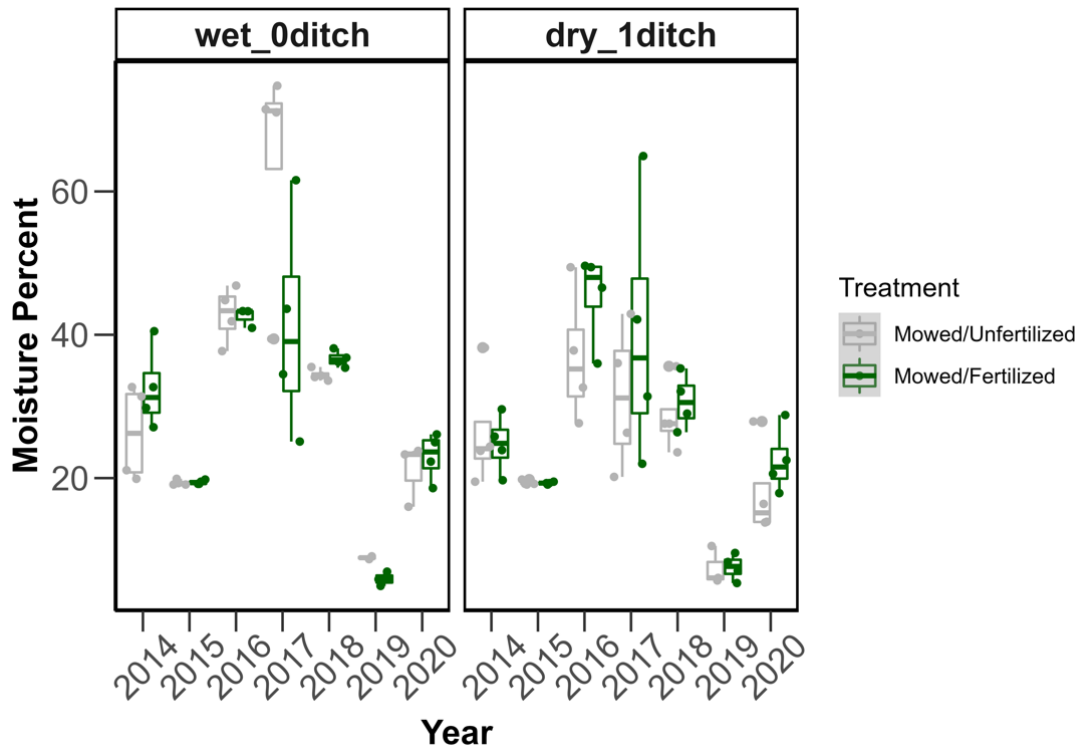
Figure 2. Summary of gravimetric soil moisture content measured annually. Boxplots representing total moisture percent in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch or left) and closer (dry_1ditch or right) to the drainage ditch. The boxplot is a visual representation 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. Summary of statistical output in Appendix S1: Table S1.

Figure 3

Figure 3. Summary of soil pH measured annually. Boxplots representing soil pH in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch or left) and closer (dry_1ditch or right) to the drainage ditch. 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 $1.5 \times$ the interquartile range. Symbols represent individual raw data points from four replicate samples. Summary of statistical output in Appendix S1: Table S1.

Figure 4

Total soil C, N, C:N

A

B

C

Figure 4. Summary of soil carbon and nitrogen measured annually. Boxplots representing total soil carbon (A), total nitrogen (B), and soil C:N ratio (C) in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch or left) and closer (dry_1ditch or right) to the drainage ditch. 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 $1.5 \times$ the interquartile range. Symbols represent individual raw data points from four replicate samples. Summary of statistical output in Appendix S1: Table S1.
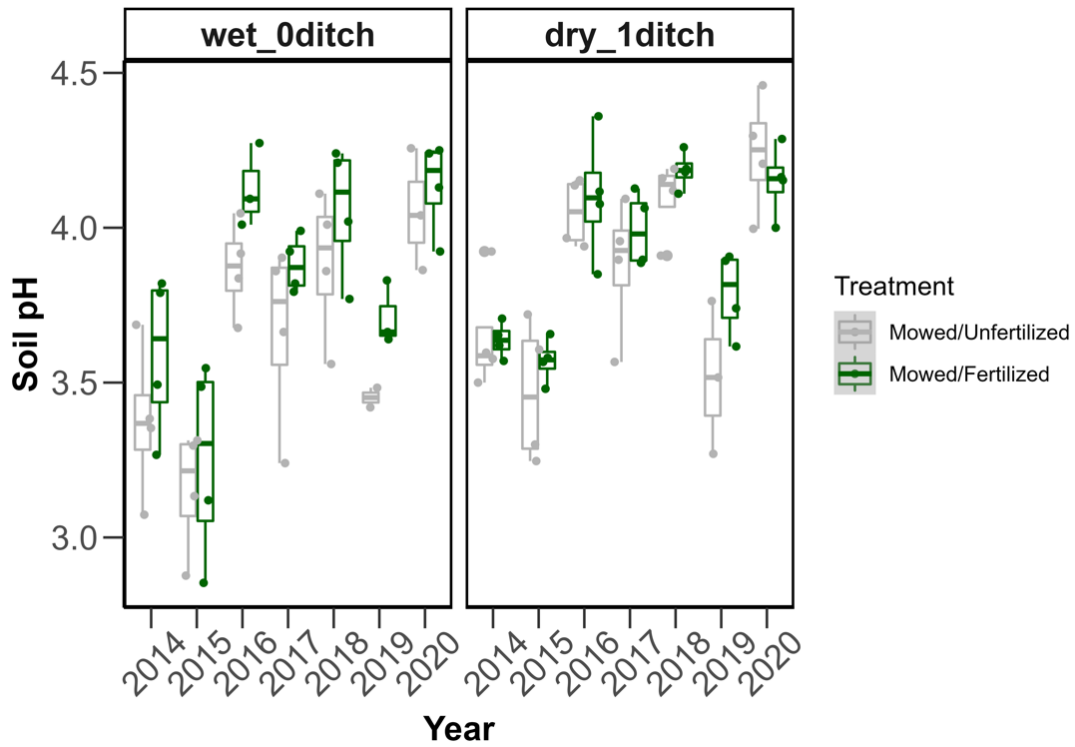
Figure 5

Bacterial Diversity

Figure 5. Summary of soil bacterial diversity metrics. Boxplots representing bacterial Shannon diversity (A) and Inverse Simpson's diversity (B) in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch or left) and closer (dry_1ditch or right) to the drainage ditch. 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 $1.5 \times$ the interquartile range. Symbols represent individual raw data points from four replicate samples. Summary of statistical output in Appendix S1: Table S2.
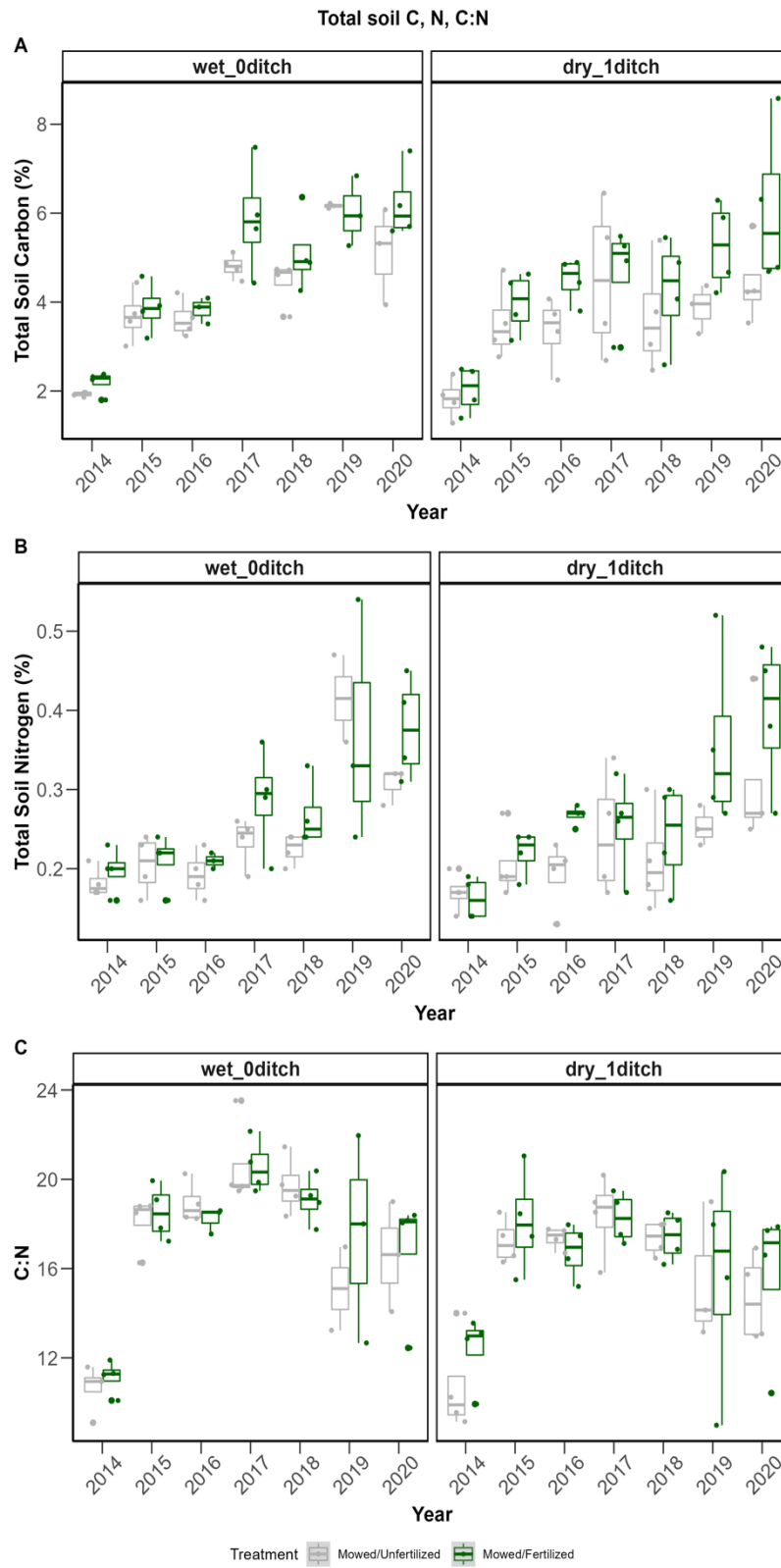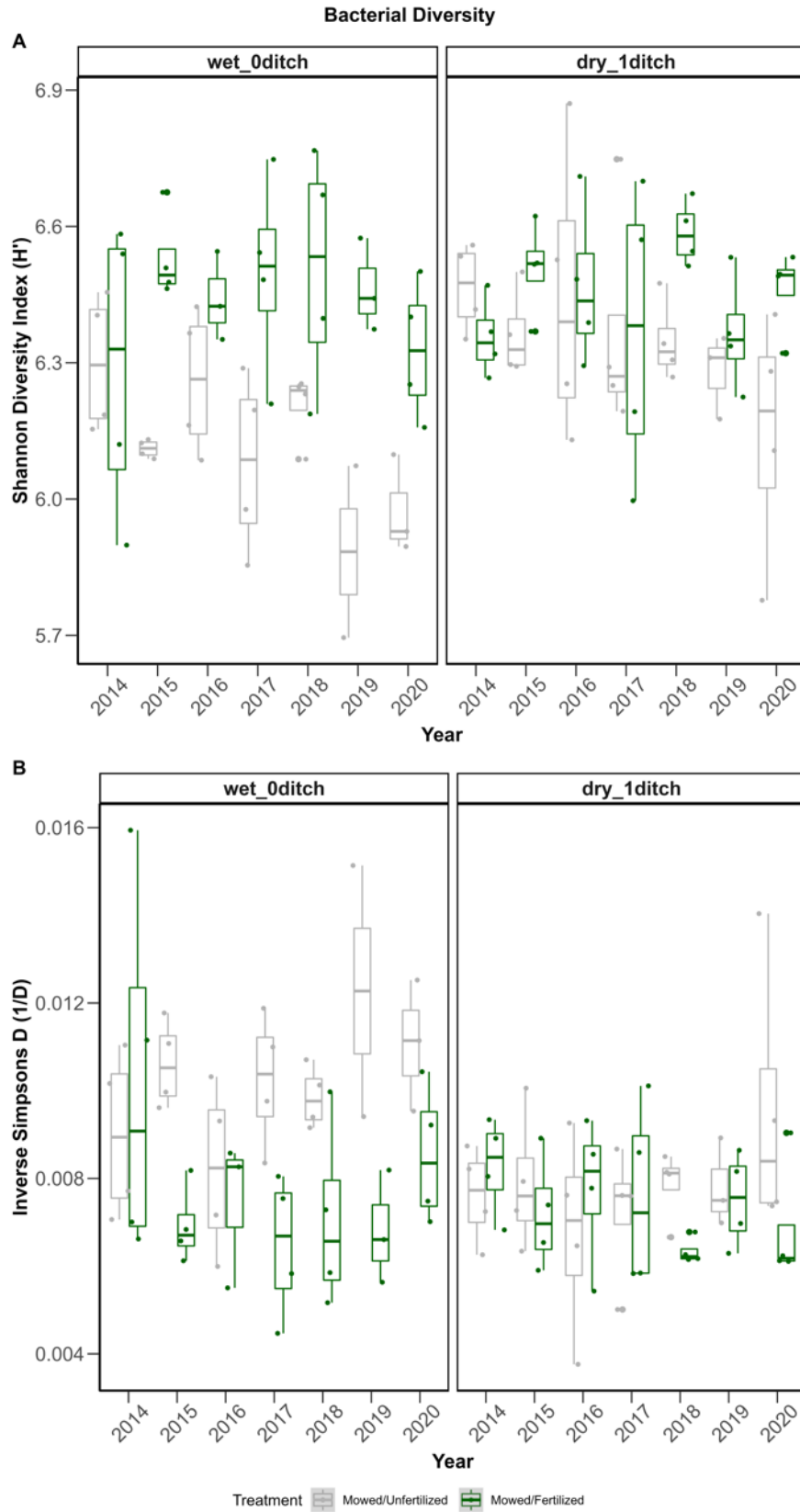
Figure 6

Bacterial 16S rRNA Community Composition

Figure 6. Summary of soil bacterial community composition. Ordination based on a Principal Coordinates Analysis depicting bacterial community composition according to fertilization and hydrology and displayed according to unfertilized plots (A), fertilized plots (B). The direction of the vector in (C) indicates the soil factor that is most correlated with patterns in bacterial community composition. Symbols are colored according to fertilization treatment (gray = mowed/unfertilized, green = mowed/fertilized) at drier mowed plots situated close to the drainage ditch (open triangles) compared to wetter mowed plots (filled circles) over time (symbols increase in size from 2014 to 2020). The centroid and standard error bars (along axes 1 and 2) were calculated for four replicate plots. Summary of statistical output in Appendix S1: Table S3.

Figure 7
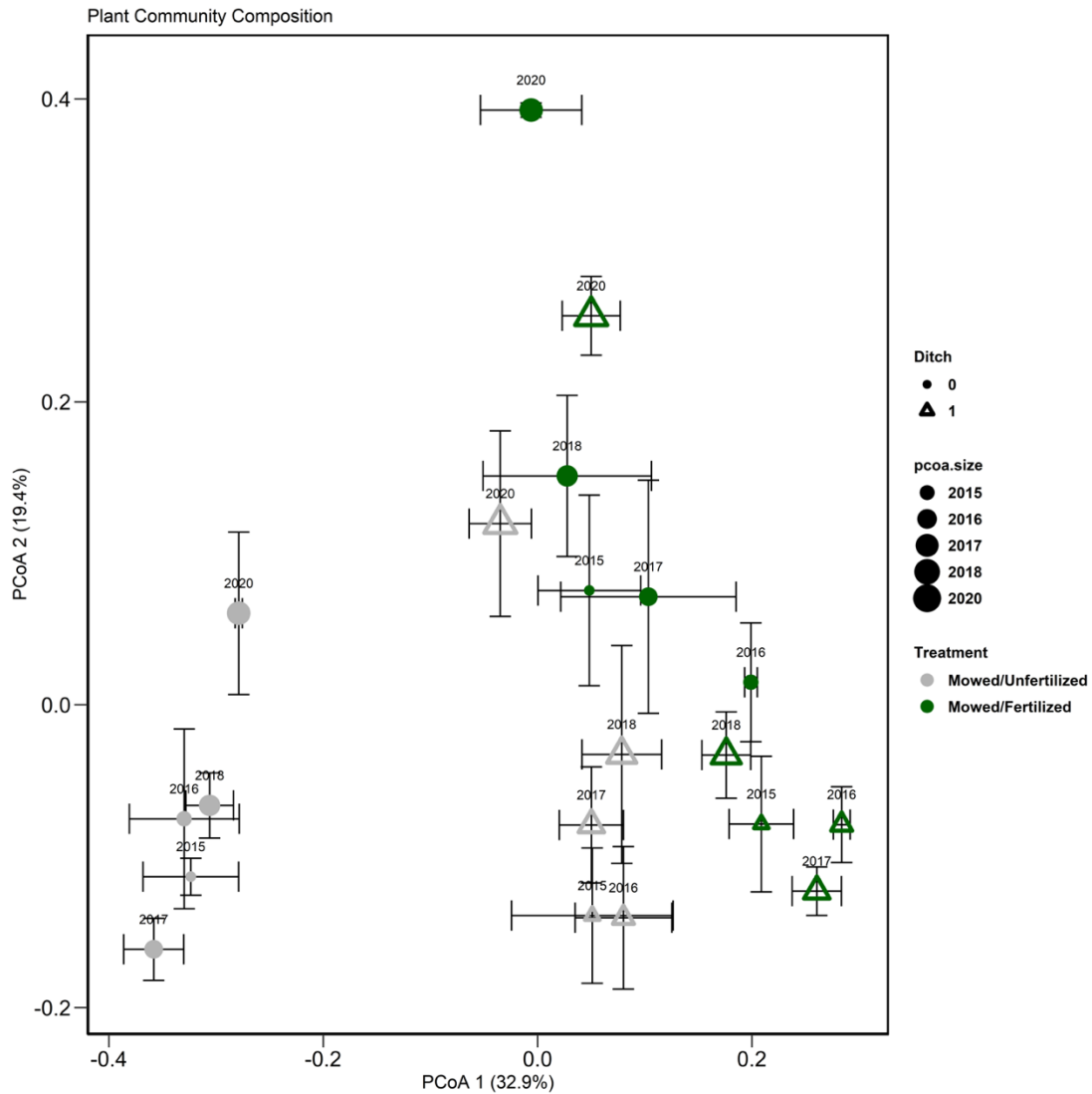
Figure 7. Summary of plant community composition. Ordination based on a Principal Coordinates Analysis depicting plant community composition according to fertilization and hydrology. Symbols are colored according to fertilization treatment (gray = mowed/unfertilized, green = mowed/fertilized) at drier mowed plots situated close to the drainage ditch (open triangles) compared to wetter mowed plots (filled circles) over time (symbols increase in size from 2015 to 2020). The centroid and standard error bars (along axes 1 and 2) were calculated for four replicate plots. Summary of statistical output in Appendix S1: Table S6.

Figure 8

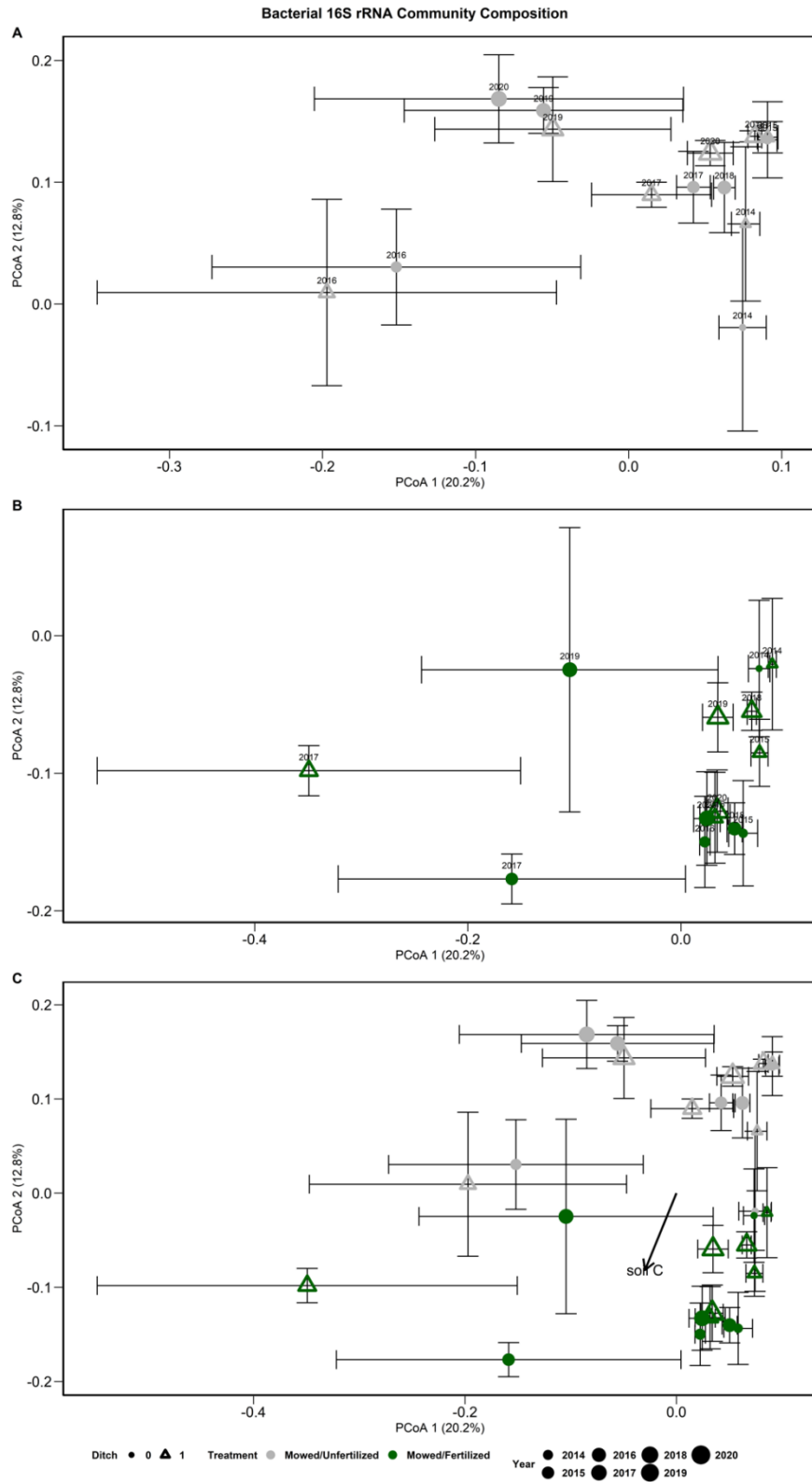Plant and Bacterial Community Composition

45
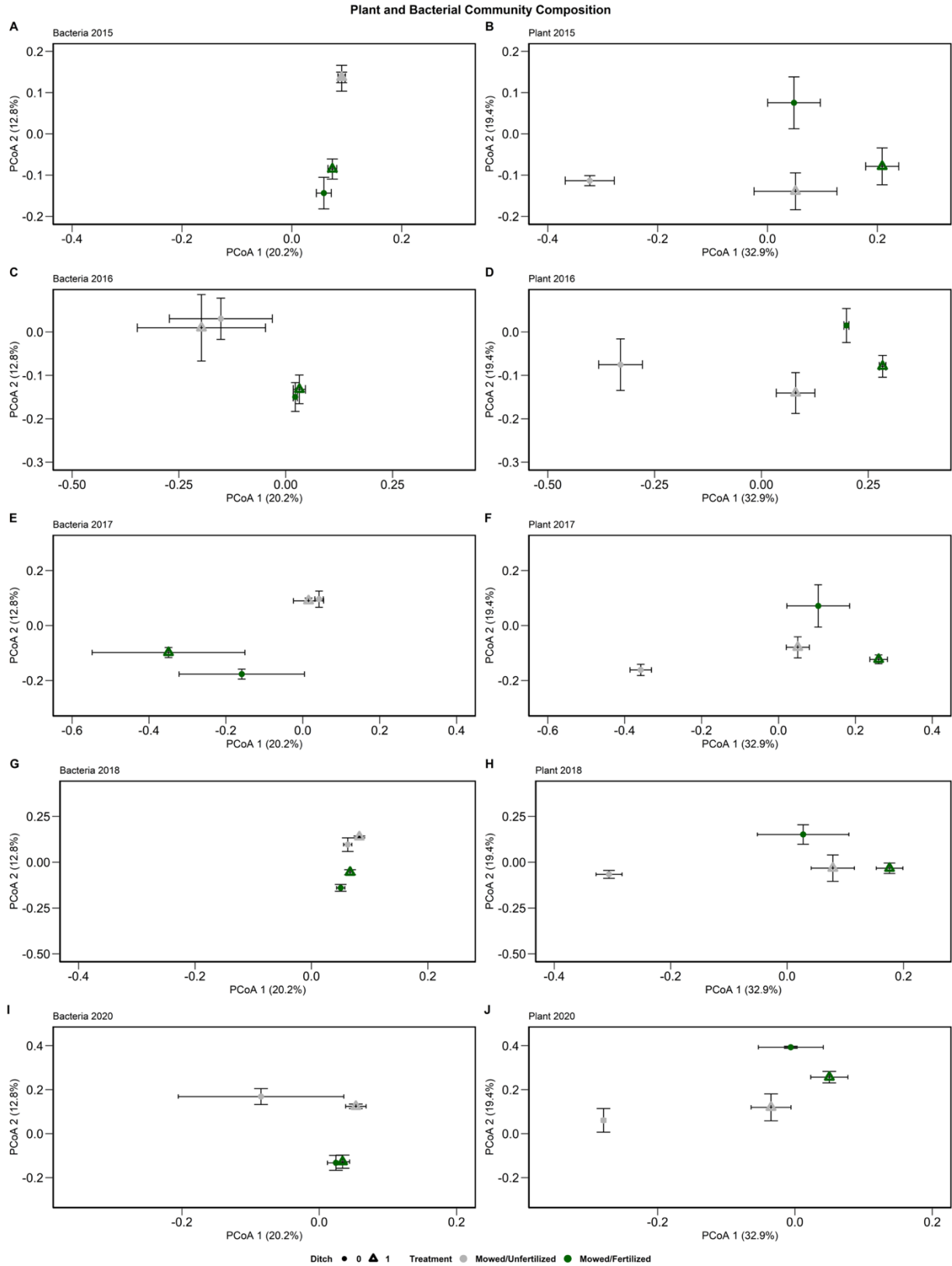
Figure 8. Summary of plant and bacterial community composition. Ordination based on a Principal Coordinates Analysis depicting plant and bacterial community composition according to fertilization and hydrology. Symbols are colored according to fertilization treatment (gray = mowed/unfertilized, green = mowed/fertilized) at drier mowed plots situated close to the drainage ditch (open triangles) compared to wetter mowed plots (filled circles) over time (A,B = 2015; C, D = 2016; E, F = 2017; G, H = 2018; I, J = 2020). The centroid and standard error bars (along axes 1 and 2) were calculated for four replicate plots. Summary of statistical output in Appendix S1: Table S3 and Table S6.

APPENDIX 1

Supplemental information

Figure S1

Plant and Bacterial Diversity
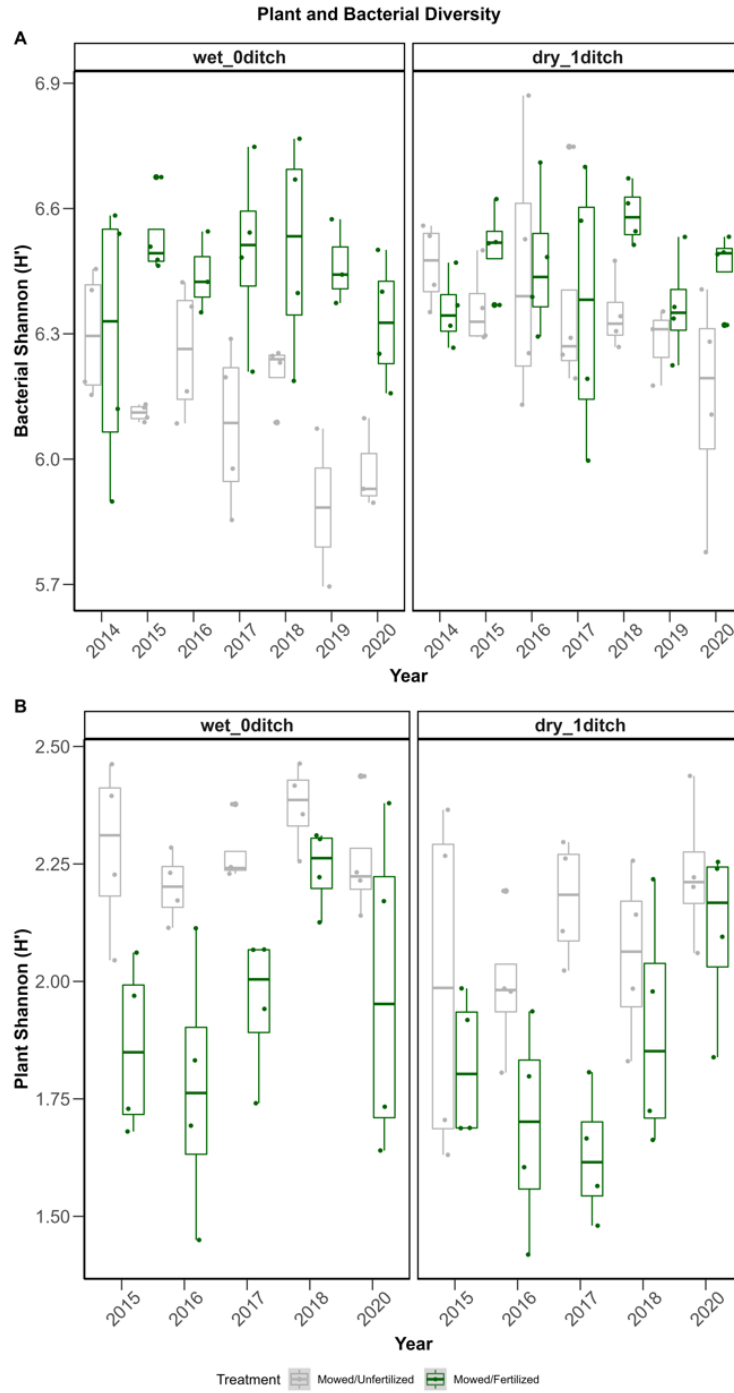
Figure S1. Summary of plant and bacterial diversity metrics. Boxplots representing bacterial Shannon diversity (A) and plant Shannon diversity (B) in mowed/fertilized (green) and mowed/unfertilized (grey) plots further (wet_0ditch or left) and closer (dry_1ditch or right) to the drainage ditch over time (plant data were not collected in 2014 and 2019). 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 $1.5 \times$ the interquartile range. Symbols represent individual raw data points from four replicate samples. Summary of statistical output in Appendix S1: Table S2 and Table S5.

Figure S2
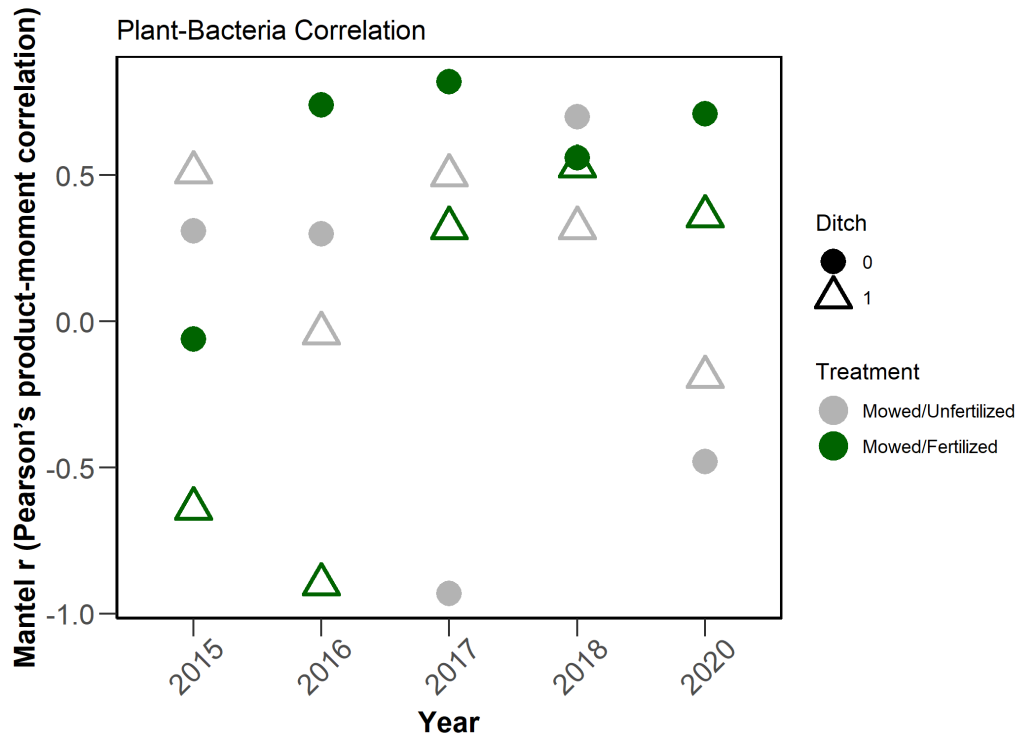
Figure S2. Plant and bacterial community associations. Mantel r test to measure plant and bacterial community associations across space and time. Correlations measured according to fertilization treatment in mowed/unfertilized (grey), mowed/fertilized (green) and according to proximity to ditch, where plots close to the ditch are relatively dry (open triangles) and plots away from the ditch are relatively wet (filled circles). Summary of statistical output in Appendix S1: Table S4.

Table S1. Summary of analysis of variance table comparing soil properties: (A) soil moisture (B) soil pH (C) total soil carbon (D) total soil nitrogen (E) soil C:N ratio between mowed/unfertilized and mowed/fertilized treatments.

(A) Soil Moisture

Fixed Effect	Df	SumSq	MeanSq	F-value	Pr(>F)
Fertilization	1	10.3	10.27	0.1896	0.664
Ditch	1	703.3	703.31	12.9858	**<0.001**
Year	6	14262.7	2377.12	43.8907	**<0.001**
Fertilization:Ditch	1	216.6	216.64	4.0000	**0.049**
Fertilization:Year	6	350.2	58.37	1.0778	0.383
Ditch:Year	6	817.4	136.23	2.5153	**0.028**
Fertilization:Ditch:Year	6	955.6	159.26	2.9406	**0.012**
Residuals	78	4334.5	54.16		

(B) Soil pH

Fixed Effect	Df	SumSq	MeanSq	F-value	Pr(>F)
Fertilization	1	0.3810	0.3810	10.7192	**0.002**
Ditch	1	0.6802	0.6802	19.1384	**<0.001**
Year	6	7.9883	1.3314	37.4595	**<0.001**
Fertilization:Ditch	1	0.0844	0.0845	2.3760	0.127
Fertilization:Year	6	0.1416	0.0236	0.6639	0.679
Ditch:Year	6	0.1521	0.0254	0.7131	0.640
Fertilization:Ditch:Year	6	0.0509	0.0085	0.2388	0.962
Residuals	78	2.7723	0.0355		

(C) Total Soil Carbon

Fixed Effect	Df	SumSq	MeanSq	F-value	Pr(>F)
Fertilization	1	0.7764	0.7764	17.2138	**<0.001**
Ditch	1	0.2728	0.2728	6.0483	**0.016**
Year	6	10.7739	1.7957	39.8105	**<0.001**
Fertilization:Ditch	1	0.0336	0.0336	0.7445	0.391
Fertilization:Year	6	0.0749	0.0125	0.2766	0.946
Ditch:Year	6	0.2575	0.0429	0.9514	0.464
Fertilization:Ditch:Year	6	0.1301	0.0217	0.4809	0.821
Residuals	78	3.5182	0.0451		

(D) Total Soil N

Fixed Effect	Df	SumSq	MeanSq	F-value	Pr(>F)
Fertilization	1	0.0460	0.0464	13.5480	**<0.001**
Ditch	1	0.0011	0.0011	0.3290	0.5680
Year	6	0.3731	0.0622	18.1666	**<0.001**
Fertilization:Ditch	1	0.0016	0.0016	0.4653	0.497
Fertilization:Year	6	0.0144	0.0024	0.7007	0.650
Ditch:Year	6	0.0210	0.0035	1.0213	0.418
Fertilization:Ditch:Year	6	0.0194	0.0032	0.9463	0.467
Residuals	78	0.2670	0.0034		

(E) Soil C:N ratio

Fixed Effect	Df	SumSq	MeanSq	F-value	Pr(>F)
Fertilization	1	1.98	1.981	0.4596	0.500
Ditch	1	38.29	38.291	8.8860	**0.004**
Year	6	708.66	118.109	27.4093	**<0.001**
Fertilization:Ditch	1	0.45	0.450	0.1045	0.747
Fertilization:Year	6	10.68	1.781	0.4132	0.868
Ditch:Year	6	23.01	3.835	0.8899	0.507
Fertilization:Ditch:Year	6	5.48	0.913	0.2118	0.972
Residuals	78	336.11	4.309		

Table S2. Summary of analysis of variance table comparing bacterial Shannon diversity (A) and Inverse Simpson's diversity (B) between mowed/unfertilized and mowed/fertilized treatments.

(A) Bacterial Shannon diversity

Fixed Effect	Df	SumSq	MeanSq	F-value	Pr(>F)
Fertilization	1	0.9537	0.9537	28.4812	**<0.001**
Ditch	1	0.2654	0.2654	7.9256	**0.006**
Year	6	0.4327	0.0721	2.1536	0.056
Fertilization:Ditch	1	0.3114	0.3114	9.2987	**0.003**
Fertilization:Year	6	0.4234	0.0706	2.1074	0.062
Ditch:Year	6	0.0172	0.0029	0.0855	0.998
Fertilization:Ditch:Year	6	0.1964	0.0327	0.9777	0.446
Residuals	78	2.6118	0.0335		

(B) Bacterial Inverse Simpson's diversity

Fixed Effect	Df	SumSq	MeanSq	F value	Pr(>F)
Fertilization	1	4.41E-05	4.41E-05	12.3680	**<0.001**
Ditch	1	3.58E-05	3.58E-05	10.0414	**0.002**
Year	6	2.56E-05	4.26E-06	1.1954	0.318
Fertilization:Ditch	1	2.62E-05	2.62E-05	7.3444	**0.008**
Fertilization:Year	6	4.39E-06	7.31E-06	2.0497	0.069
Ditch:Year	6	2.72E-05	4.53E-06	0.2051	0.974
Fertilization:Ditch:Year	6	2.78E-04	3.57E-06	1.2716	0.280
Residuals	78	2.78E-04	3.57E-06		

Table S3. Summary of multivariate analyses PERMANOVA comparing total 16S rRNA bacterial community composition patterns between mowed/unfertilized and mowed/fertilized treatments (A) and correlation of soil factors and bacterial community composition (B).

(A) Total 16S rRNA bacterial community composition patterns

Fixed Effect	Df	SumSq	R²	F-value	Pr(>F)
Fertilization	1	1.4103	0.0843	12.5080	**<0.001**
Ditch	1	0.8230	0.0492	7.2990	**<0.001**
Year	6	3.0023	0.1794	4.4380	**<0.001**
Fertilization:Ditch	1	0.1915	0.0115	1.6985	0.064
Fertilization:Year	6	1.1629	0.0695	1.7190	**<0.001**
Ditch:Year	6	0.4800	0.0287	0.7096	0.987
Fertilization:Ditch:Year	6	0.4167	0.0249	0.6160	1.000
Residuals	82	9.2455	0.5526		

(B) Correlation of soil factors and bacterial community composition

Vectors	Dim1	Dim2	R²	Pr(>F)
pH_avg	-0.7696	-0.6386	0.0365	0.149
moist_percent	-0.4301	-0.9028	0.0243	0.241
c_percent	-0.3422	-0.9396	0.0616	**0.035**
n_percent	-0.0046	-0.1000	0.0381	0.141

Table S4. Summary of plant-bacterial correlations. Plant and bacterial dissimilarity matrix comparison based on Mantel matrix correlations according to year, ditch, and fertilization treatment.

Plant and bacterial correlation patterns

Year	Ditch	Treatment	Mantel_r	Pr(>F)	Cumulative precip. (in.)
2015	0	M	0.31	0.203	46.69
2015	0	MF	-0.06	0.593	46.69
2015	1	M	0.51	**0.030**	46.69
2015	1	MF	-0.64	0.879	46.69
2016	0	M	0.30	0.253	59.92
2016	0	MF	0.74	0.300	59.92
2016	1	M	-0.04	0.482	59.92
2016	1	MF	-0.90	1	59.92
2017	0	M	-0.93	1	40.56
2017	0	MF	0.82	**0.048**	40.56
2017	1	M	0.50	0.312	40.56
2017	1	MF	0.32	**0.001**	40.56
2018	0	M	0.70	**0.001**	56.83
2018	0	MF	0.56	0.163	56.83
2018	1	M	0.32	0.291	56.83
2018	1	MF	0.53	0.255	56.83
2020	0	M	-0.48	0.780	49.11
2020	0	MF	0.71	0.087	49.11
2020	1	M	-0.19	0.658	49.11
2020	1	MF	0.36	0.221	49.11

Table S5. Summary of analysis of variance table comparing plant Shannon diversity between mowed/unfertilized and mowed/fertilized treatments.

Plant Shannon diversity

Fixed Effect	Df	SumSq	MeanSq	F-value	Pr(>F)
Fertilization	1	1.6514	1.6514	42.6124	**<0.001**
Ditch	1	0.5198	0.5198	13.4122	**<0.001**
Year	4	0.6507	0.1627	4.1978	**0.005**
Fertilization:Ditch	1	0.0156	0.0156	0.4031	0.528
Fertilization:Year	4	0.2181	0.0545	1.4072	0.243
Ditch:Year	4	0.3067	0.0767	1.9786	0.109
Fertilization:Ditch:Year	4	0.1374	0.0344	0.8864	0.478
Residuals	60	2.3253	0.0388		

Table S6. Summary of multivariate analyses for plant community composition. PERMANOVA comparing plant community composition patterns between mowed/unfertilized and mowed/fertilized treatments.

Plant community composition

Fixed Effect	Df	SumSq	R²	F value	Pr(>F)
Fertilization	1	2.0256	0.1822	26.8804	**0.001**
Ditch	1	1.7154	0.1543	22.7629	**0.001**
Year	4	1.3576	0.1221	4.5037	**0.001**
Fertilization:Ditch	1	0.6985	0.0628	9.2690	**0.001**
Fertilization:Year	4	0.3914	0.0352	1.2984	0.164
Ditch:Year	4	0.2156	0.0194	0.7152	0.837
Fertilization:Ditch:Year	4	0.1897	0.0171	0.6294	0.933
Residuals	60	4.5214	0.4068		

Table S7. Summary of linear regression estimating the extent that soil properties explain bacterial

Shannon diversity.

Bacterial Shannon diversity and soil properties

Soil Factor	Df	SumSq	MeanSq	F value	Pr(>F)
pH_avg	1	0.1395	0.1395	2.8107	0.097
moist_percent	1	0.0467	0.0467	0.9405	0.334
c_percent	1	0.0093	0.0093	0.1874	0.660
n_percent	1	0.0047	0.0047	0.0950	0.759
Residuals	101	5.0117	0.0496		