Title: Nutrient enrichment increases soil bacterial diversity and decomposition rates of different litter types in a coastal plain wetland

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ABSTRACT

Anthropogenic disturbances have led to increased deposition of nitrogen (N) and phosphorus (P) into soils. Nutrient enrichment of soils is known to increase plant biomass and also increase rates of microbial litter decomposition. Thus, examining plant-soil-microbial interactions and their influence on decomposition rates is essential for predicting microbial contributions to carbon (C) cycling as atmospheric deposition persists. This study explores how changes in organic C, nitrogen (N), and phosphorus (P) caused by long-term nutrient enrichment and known litter-type composition influence soil microbial community structure and function. It is hypothesized that long-term nutrient enrichment causes shifts in soil microbial community structure that lead to higher rates of litter decomposition. Further, plant litter with a lower C: N ratio (compared to high C:N ratio litter) is expected to decompose faster due to an available N source provided to nutrient-starved microbes. We leverage a long-term experimental fertilization and disturbance by mowing experiment to test how nutrient enrichment affects decomposition and soil bacterial community structure in a nutrient poor coastal plain wetland. In each of eight replicate mowed/fertilized and mowed/unfertilized plots, bags of two different litter types (high C:N ratio rooibos tea and low C:N ratio green tea) were buried for 111 days. We characterized litter-associated and bulk soil microbiomes using 16S rRNA bacterial sequencing and quantified litter mass losses. As predicted, the green tea litter decomposed faster than the rooibos tea litter. Results also revealed that distinct bacterial communities were involved in decomposing rooibos tea litter (higher C:N ratio) more quickly in fertilized compared to unfertilized especially under drier soil conditions, while decomposition rates of green tea litter (lower C:N ratio) were similar between fertilized and unfertilized plots. Bacterial community structure in part explained litter decomposition rates, and long-term fertilization and drier hydrologic status affected bacterial

diversity and increased decomposition rates. This study provides insight into long-term fertilization effects on soil bacterial diversity and composition, related rates of decomposition, and the increased potential for soil carbon loss as nutrient enrichment continues to affect historically low nutrient ecosystems.

1. Introduction

Humans are constantly transforming their environments as industrialization and urbanization continue. Some ways in which humans modify their landscapes are through fossil fuel burning, deforestation, and intense agricultural activity (Fowler et al., 2013; Vitousek et al., 2010). These anthropogenic disturbances have led to increased atmospheric deposition of nitrogen (N) and phosphorus (P) onto historically nutrient-limited ecosystems (Guignard et al., 2017). Since N and P are important limiting nutrients in many ecosystems, this increased nutrient deposition can cause a fertilization effect on plant-microbial interactions due to increased biomass and shifts in plant and microbial community structure (Cherif Mehdi and Loreau Michel, 2009; Harpole et al., 2016; Leff et al., 2015). Changes in community composition of plants and microbes directly influence ecosystem functions associated with carbon (C) cycling. This can alter the balance of C stored in soils and plant biomass and C released as carbon dioxide (CO_2) through respiration. Specifically, this fertilization effect can increase plant biomass C, which fuels heterotrophic microbial growth and leads to increased respiration of CO_2 (Hoosbeek et al., 2004). Thus, it is important to explore how changes in plant litter composition and plant biomass affect the functioning of microbes that are dependent on C resources and nutrients (such as N and P) accessed from their environment. If increased nutrient deposition ultimately leads to

more C loss through respiration than C fixed through primary production and C stored in soils, then climate change is exacerbated.

Human modifications to physical landscapes cause drastic alterations to elemental cycles. Elemental cycles can change as a consequence of variations in C to N to P (C:N:P) concentrations due to changes in community composition, physical movement of elements that subsidize nutrient-poor areas, changes in predation, and a multitude of other means (Leroux Shawn J. et al., 2012; Rappaport et al., 2018). While human alteration of the environment and its effect on C storage and release is relatively well-documented, the mechanisms governing the interactive effects of nutrient enrichment on plant-soil-microbial relationships are not fully understood. This makes it challenging to predict microbial responses to environmental change in ecosystem models. Specifically, we question how changes in internal C:N:P plant litter stoichiometry and external C:N:P additions affect soil microbiomes and carbon cycling. The rate of heterotrophic decomposition is limited by N and P availability (Su et al., 2015; Van Der Heijden et al., 2008); therefore, plant and soil C:N:P composition affects rates of biogeochemical processes such as decomposition. Changes in the nutrient stoichiometry of both plants and surrounding soils are expected to affect microbial community composition and function, leading to changes in elemental cycling.

One way in which plant composition is influenced by nutrient addition is through its biomass. Prior studies have shown that long-term nutrient addition enhances plant biomass and increases C inputs into soils (e.g., Harpole et al. 2016). These increased plant inputs also increase CO_2 outputs via microbial respiration (Hoosbeek et al., 2004; Lange et al., 2015). If microbes are not limited by N and P, the increase in plant derived organic C inputs are more quickly metabolized and soil CO_2 fluxes increase (Peralta and Wander, 2008). In addition to plant

biomass, plant biomass could also vary in C:N or C:P ratios, which could also cause changes in microbial nutrient use and respiration rates. Thus, it is important to understand how changes in available nutrients within plant litter alter microbial communities and CO_2 respiration rates (Hoosbeek et al., 2004; Lange et al., 2015).

This study examines how long-term nutrient enrichment and litter-type composition influence decomposition rates. We hypothesize that high quality litter with a low C:N ratio (compared to low quality, high C:N ratio litter) would lead to higher decomposition rates in both nutrient limited or enriched conditions. This is due to an available N source of the low C:N litter provided to nutrient-starved microbes. We further hypothesize that long-term nutrient additions will cause shifts in soil microbial community structure that lead to higher rates of litter decomposition. Before controlled experiments can be conducted on how the nutrient addition effects on the nutrient stoichiometry of plant communities influence microbial function, however, a small-scale model is necessary. A small-scale model will allow us to understand if indeed differences in single species plant litter stoichiometry affect microbial function. This study's small-scale model can later be expanded upon to measure how shifts in plant community composition cause ecosystem-scale changes in CO_2 fluxes from soils. In attempt to further understand the effects of microbial communities on C storage and release, we employed a universal protocol to measure decomposition rate in any type of environment. The Tea Bag Index (TBI) compares the decomposition rates of two different plant litters, green and rooibos Lipton™ tea bags (Keuskamp et al., 2013). Green tea has a measured mean C:N ratio of 12.229 while rooibos tea is measured to have a much higher mean C:N ratio of 42.870. Considering both the established method for measuring decomposition and the model plant litter with known C:N ratios, we used the TBI protocol to examine how long-term fertilization affects decomposition

rates in a nutrient-limited coastal plain wetland ecosystem. We also characterized the bacterial communities (using amplicon sequencing) associated with the litter decomposition of two differing litter types (green and rooibos teas) occurring in wetland soils exposed to fertilization or not. This study provides evidence that the direct effects of nutrient enrichment on soil microbial communities can interact with nutrient chemistry of the plant community, which could be fundamentally changing decomposition rates in these valuable carbon sinks.

2. Materials and methods

2.1. Experimental design of a long-term ecological experiment

Initiated in 2003, a long-term ecological experiment started at East Carolina University's West Research Campus (WRC) (35.6298N, -77.4836W) examines the effects of mowing and fertilization on coastal plain wetland plant and microbial communities. This study site is classified as a jurisdictional wetland, and the plant community has been described as a mosaic of wet pine flatwood habitat, pine savanna, and hardwood communities (Chester, 2004). The soils were characterized as fine, kaolinitic, thermic Typic Paleaquults (Coxville series), fine-loamy, siliceous, semiactive, thermic Aeric Paleaquults (Lynchburg series), and fine-loamy, siliceous, subactive, thermic Aquic Paleudults (Goldsboro series). These soils are moderately to poorly drained ultisols (USDA NRCS 2019). The annual mean temperature is 16.4 °C and annual precipitation is 126 cm (U.S. Climate Data 2019). Fertilization and mowing treatments are replicated on eight 20×30 m blocks in a full factorial design, and the N-P-K 10-10-10 pellet fertilizer is applied 3× per year (February, June, and October) for a total annual supplementation of 45.4 kg ha^{-1} for each nutrient. Plots are mowed by bush-hog annually to simulate fire

disturbance (Goodwillie et al., In Review). The plant community in plots that are mowed is dominated by perennial forbs in two major genera of the Asteraceae—*Eupatorium* and *Solidago*—and graminoid species. The relative abundance of major grass species differs between mowed and mowed/fertilized plots, with switchcane (*Arundinaria tecta*) most dominant in fertilized plots and broomsedge (*Andropogon virginicus*) found almost exclusively in unfertilized plots. Sedges (e.g., *Rhychospora* and *Carex* spp.) and species of *Juncus* are common in (wet) plots away from, but not in (dry) plots adjacent to the drainage ditch. This hydrologic gradient is caused by a roadside drainage ditch such that four blocks near the ditch are drier and four blocks away from the ditch are wetter (Goodwillie et al. In Review). This hydrologic filter drives variation in both plant and soil bacterial community composition. Within each plot, annual soil and plant sampling takes place at three randomly-placed quadrats (Fig. 1). This hydrologic gradient has not yet been characterized by modeling flow according to water levels over time. However, in 2018, volumetric soil moisture content (measured by capacitance), was >2 times wetter in blocks away from the ditch compared to blocks adjacent to the ditch. The ditch was not intentionally manipulated as part of our experimental design, but it contributes an important ecological variable to the study (Goodwillie et al. In Review). For this study, tea litter decomposition experiments were focused in the mowed plots only.

2.2. Soil sampling

We collected composite soil samples from each mowed plot, which represented two soil cores (12 cm depth, 3.1 cm diameter) adjacent to each of three permanently installed 1 m^2 quadrats where plant community data are annually collected. Each composite bulk soil sample was passed

through a 4 mm sieve and homogenized before further analysis. This bulk soil sampling occurred on 14 November 2019, about three months after the tea litter bags were collected from the field.

2.3. Soil physico-chemical analyses

We measured gravimetric soil moisture by weighing 20-30 g of field-moist soil, drying at 105 °C overnight, and then re-weighing. We calculated percent moisture as difference between field-moist and dried soils divided by the oven-dried soil weight. In addition, we measured pH of oven-dried soil by mixing a 1:1 soil:water solution and using a pH probe (Genemate-Bioexpress; Kaysville, Utah; USA). We measured total soil organic carbon and total nitrogen on finely ground dried soil using an elemental analyzer (2400 CHNS Analyzer; Perkin Elmer; Waltham, Massachusetts, USA) at the Environmental and Agricultural Testing Service laboratory (Department of Crop and Soil Sciences at NC State). Extractable inorganic N for each soil sample was measured on approximately 5 g of field moist soil. We added 45 ml of 2 M KCl to soil, shook the sample for about 1 hour, and gravity filtered and collected soil extracts. Ammonium (NH_4^+) and nitrate (NO_3^-) ions in soil extracts were colorimetrically measured using a SmartChem 200 auto analyzer (Unity Scientific Milford, Massachusetts, USA) at the East Carolina University Environmental Research Laboratory.

2.4. Field experimental methods

The field protocol for this experiment was adapted from the Tea Bag Index Protocol (Keuskamp et al., 2013) and applied within the eight-replicate mowed/fertilized and mowed/unfertilized plots of the WRC. The experimental setup involved three bags of Lipton™ green tea and three bags of Lipton™ rooibos tea per replicate plot (3 bags × 8 blocks × 2

treatment plots = 48 bags per each tea bag type). In each treatment plot, we prepared six holes about 20 cm apart and 8 cm deep using a hand trowel. We weighed green and rooibos tea bags and buried them in separate holes. Soil was lightly packed around the tea bags, while keeping the labels visible above the soil. Tea bags were recovered after 111 days (May 21–September 09, 2018) using hand trowels to loosen soil adjacent to tea bag locations. Two of three green tea bags at each replicate plot were measured for mass loss. The third green tea bag was placed into a sterile Whirl-pak bag and stored at -20 °C for tea-associated bacterial community analysis. The rooibos tea was processed following the same procedure. Upon returning to the lab, the tea bags for decomposition analysis were separated from adhered soil particles, placed into individual aluminum weighing dishes, and oven-dried at 70 °C for 48 hours. To quantify mass loss, the tea bags were reweighed following drying and compared to their initial weight prior to soil incubation.

2.5. Soil and tea-associated bacterial 16S rRNA sequencing

Following freezing, tea litter was removed from tea bags, and DNA was extracted from samples using the Qiagen DNeasy PowerSoil kit. We also extracted DNA from soils using the Qiagen DNeasy PowerSoil Kit. For each mowed or mowed/fertilized treatment, DNA was extracted from green tea (15 total), rooibos tea (14 total), and bulk soil samples (16 total). Three litter samples were not retrieved from the field. Following extraction, sample DNA was used as template in PCR reactions with a bacterial 515F/806RB barcoded primer set originally developed by the Earth Microbiome Project to target the V4-V5 region of the bacterial 16S subunit of the rRNA gene (Apprill A et al., 2015; Caporaso et al., 2012; Parada et al., 2016). Three 50 µL PCR libraries were combined for each sample and cleaned using the AMPure XP magnetic bead

protocol. Following cleaning, PCR product were quantified using Quant-iT dsDNA BR (broad-range) assay (Thermo Scientific, Waltham, MA, USA). Libraries were pooled in equimolar concentration of 5 ng μL^{-1}after being diluted to a concentration of 10 ng μL^{-1}. The Indiana University Center for Genomics and Bioinformatics sequenced the pooled libraries using the Illumina MiSeq platform using paired-end reads (Illumina Reagent Kit v2, 500 reaction kit).

Following sequencing, processing utilized a standard mothur pipeline (v1.40.1) (Kozich et al., 2013). Contigs were assembled from paired end reads and quality trimmed using a moving average quality score (minimum score of 35 bp). Sequences were aligned to the SILVA rRNA gene database (v.128) (Quast et al. 2013) and chimeric sequences were removed using the VSEARCH algorithm (Rognes et al., 2016). Formation of operational taxonomic units (OTUs) involved dividing sequences based on taxonomic class and then binning into OUTs with a 97% sequence similarity level. OTUs were classified using the SILVA database (Glöckner et al., 2017; Yilmaz et al., 2014).

2.6. Statistical analyses

All statistical calculations were completed in the R environment (R v3.6.0, R Core Development Team 2019). Decomposition was measured through mass loss of buried tea bags. Initial and final tea bag weights were used to determine percent of loss. We ran a linear mixed effects model with ‘source’, ‘treatment’, and ‘ditch proximity’ as fixed effects and ‘block’ as a random effect using the lmer() function in the lmerTest package (Kuznetsova et al. 2017). For the mass loss response variable, a linear mixed model was fit by REML and Type II ANOVA and the Kenward-Roger's degrees of freedom method using the lmerModLmerTest() function. Calculations of decomposition rate (k) and stabilization factor (S) for each tea bag were

accomplished according to formulas found within the TBI protocol. Decomposition rate, *k*, is a parameter representing short-term dynamics of new input decomposition, while stabilization factor, *S*, is a parameter characterizing long-term carbon storage. These values were used to draw comparisons between *k* and *S* values found within the WRC wetland ecosystem to those of global ecosystems submitted to the Tea Bag Index protocol (Keuskamp et al., 2013). Outcomes of these calculations were plotted on a graph, and locations of groupings were compared to those of the larger multi-ecosystem index (Keuskamp et al., 2013).

For the bulk soil factors measured and the computed alpha diversity (OTU richness, Shannon Diversity, Simpson's Evenness) metrics for soil and tea litter associated bacterial communities, we ran a series of linear mixed models. Since 'ditch' was did not significantly explain variation in soil factors and alpha diversity metrics, we ran a linear mixed effects model with 'source' and 'treatment' as fixed effects and 'block' as a random effect and fitted by the REML criterion using the lmer() function in the lmerTest package (Kuznetsova et al. 2017). Statistical inferences for fixed effects were calculated from type II analysis of variances tables and the Kenward-Roger's degrees of freedom method using the lmerModLmerTest() function.

To visualize the community responses to fertilization and litter type, we used principal coordinates analysis (PCoA) of bacterial community composition based on the Bray-Curtis dissimilarity. We used a permutational multivariate analysis of variance (PERMANOVA) to examine among-treatment differences in bacterial communities. We also include a comparison of the bulk soil and tea litter associated bacterial communities (PERMANOVA, PCoA ordination, Supplemental Table S1, Fig. S1), but focused on the specific tea litter associated microbiome patterns. To identify which bacterial species were most representative of each litter type, we ran an indicator species analysis. For the indicator species analysis, we only included bacterial taxa

with a relative abundance greater than 0.05 when summed across all plots. We performed PERMANOVA using the *adonis* in the vegan package (Oksanen et al., 2017) and the indval() function in the indicspecies package (Caceres and Jansen, 2016).

To examine structure-function relationships between the tea-associated bacterial community and the mass loss of the tea litter, we ran a linear regression to measure the relationship between mass loss as a function of alpha diversity (mass loss ~ OTU richness + Shannon Diversity + Simpson's Evenness). In addition, we conducted a distance-based partial least squares regression (dbplsr) measure how much variation in mass loss of litter (function) was explained by the tea-associated bacterial community composition (structure) (mass loss ~ bacterial community). We used the Bray-Curtis dissimilarity matrix and the generalized cross-validation estimate of the prediction error ('gcv' method). We performed a distance-based partial least squares regression analysis using the dbplsr() function in the dbstats and pls packages (Boj Del Val et al., 2007; Boj et al., 2017; Mevik et al., 2019).

3. Results

Long-term fertilization strongly influenced this nutrient-limited wetland soil environment. A subset of soil factors was distinct between fertilized and unfertilized soils in the mowed plots. Total and water extractable phosphorus concentrations were higher in fertilized compared to unfertilized soils (Table 1). In addition, total soil C and N, soil pH and moisture were higher in fertilized compared to unfertilized plots (Table 1). These differences in fertilized compared to unfertilized soils led to increased litter decomposition rates and shifts in bacterial community structure.

To examine how litter type and nutrient addition influenced decomposition rates, we measured mass loss of green and rooibos tea bags. Following an 111-day incubation, there was a significant litter type (source) × fertilization treatment interaction (Fig. 2, Table 2). The mass loss of green tea litter was ~24% higher than the overall mass loss of rooibos tea litter (Fig. 2, Table 2), averaged across fertilized and unfertilized soils. Both fertilization and proximity to a drainage also increased the rate of mass loss (Fig. 2, Table 2). Across all tea types and drainage proximities, fertilized soils showed an average mass loss of ~7.5% more than that of unfertilized soils. Tea bags in closer proximity to drainage had ~6% more mass loss averaged across all litter and treatment types.

We also compared the decomposition rates (*k*) and stabilization factors (*S*) from the long-term fertilization experiment to a broader context of ecosystems (Keuskamp et al., 2013). The most variable *S* and *k* values were measured in the wetter, fertilized plots (Fig. 3). The mowed/unfertilized compared to fertilized samples were generally higher along the stabilization factor (*S*) axis. Samples that were buried in the wetter, fertilized plots clustered along the higher end of the stabilization factor (*S*), while litter buried in the drier plots (fertilized and unfertilized) had lower estimated stabilization factor (*S*) (Fig. 3). Upon comparison with other ecosystems, a large number of mowed/fertilized treatment samples grouped near the grassland-ambient ecosystem in Iceland and the forest ecosystem in the Netherlands (Keuskamp et al., 2013). Mowed treatment samples, however, grouped near the peat-disturbed and peat-undisturbed ecosystems of Iceland and the peat ecosystem of the Netherlands (Keuskamp et al., 2013).

Fertilization effects on soils and decomposition rates were related to shifts in bulk soil and litter-associated bacterial communities. Fertilization effect, tea type, and proximity to ditch (i.e., wetter vs. drier plots) influenced bacterial diversity in various ways. Specifically, tea type

explained the most variation in bacterial community composition, while the interaction of tea type and proximity to ditch explained bacterial community variation to a lesser degree (Fig. 4, Table 3) (see circle vs triangle). The proximity to ditch (proxy for hydrology) also explained variation in bacterial community composition (Fig. 4, Table 3) (see opened vs filled). Within tea type, fertilization treatment explained variation in bacterial community composition (Fig. 4, Table 3) (see green vs gray). When the bulk soil and tea-associated bacterial communities were analyzed together, bulk soil or tea explained the most variation in bacterial community composition (Fig. S1, Table S1). In addition to these observed beta diversity patterns, alpha diversity was generally higher in fertilized compared to unfertilized plots, especially in the drier plots associated with the drainage ditch (Fig. 5, Table 4). Specifically, OTU richness values were highest in bulk soil compared to tea types (Fig. 5A, Table 4A). Shannon diversity values were highest in bulk soil compared to tea type in fertilized plots (Fig. 5A, Table 4A). Lastly, bacterial evenness was similar across fertilization treatment, source type, and proximity to ditch (Fig. 5C, Table 4C).

To further examine bacterial associations with litter decomposition, we used indicator species analysis to identify a subset of bacterial taxa that represented each of the bulk soil or tea associated microbiomes. The unclassified OTU within the class Spartobacteria and within the order Acidobacteria Gp1 represented mowed/unfertilized bulk soil bacterial community, while an unclassified OTU within the order Solirubrobacterales represented bulk soils in the mowed/fertilized, dry plots. In wetter plots, an unclassified OTU within the order Rhodospirillales represented mowed/unfertilized plots while unclassified taxa within the orders Acidobacteria Gp1, Gp2, and Gp6 signified mowed/fertilized bulk soils (Tables S2, S3). In addition, the unclassified OTU within the Acidobacteria Gp3 and *Dongia* spp. represented the

green tea-associated (low C:N litter) bacterial communities in the mowed, dry plots while *Conexibacter* spp. were represented in the mowed/fertilized, dry plots. In the wetter plots, *Phenylobacterium* spp. represented green tea-associated bacterial communities in mowed plots and *Legionella* spp. in mowed/fertilized plots. The OTUs *Acidisoma* spp. in mowed/unfertilized plots and *Dyella* spp. in mowed/fertilized characterized rooibos tea associated bacterial communities in the dry plots. Lastly, the OTU *Lacibacterium* spp. represented mowed plots, while *Dokdonella* spp. and an unclassified OTU within the genus Microbacteriaceae represented rooibos tea associated communities in the wet plots (Tables S2, S3).

When examined together, the link between bacterial community structure and decomposition function was relatively weak. No relationship between litter mass loss and alpha diversity was detected (R^2_{adj} = -0.038, p-value = 0.574). However, when the total bacterial community was considered, the tea-associated bacterial community accounted for ~64 % of the variation in litter mass loss (dbplsr, Component 1 R^2_{adj} = 63.56, Component 2 R^2_{adj} = 87.09, Component 3 R^2_{adj} = 93.96) (Table 5).

4. Discussion

Overall, distinct bacterial communities decomposed the low C:N ratio litter faster than the high C:N ratio litter. The results obtained from the litter mass lost following the field incubation indicate that distinct soil bacterial communities are capable of decomposing rooibos tea litter (higher C:N ratio) more quickly in fertilized compared to unfertilized plots due to nutrient enrichment from the fertilizer treatment. However, green tea litter (lower C:N ratio) decomposition rates were similar among fertilized and unfertilized plots. This suggests that green tea itself is providing a N source to microbes regardless of external soil nutrient conditions,

whereas rooibos tea has a much higher C:N ratio and is reliant on N solely from the fertilized soil in order to have increased rates of decomposition and litter mass lost. The interaction between source (tea type) and treatment influenced mass loss of litter provides evidence that both nutrient composition of plant litter and nutrient additions to soil play a role in rate of decomposition. The green (low C:N) tea litter and fertilized soil had the highest rate of decomposition, followed by green tea litter in unfertilized soil, rooibos (high C:N) tea litter in fertilized soil, and rooibos tea litter in unfertilized soil. This trend confirms that both soil and plant litter composition interact to play a significant role in decomposition rates.

The comparative results from the decomposition rates (*k*) and stabilization (S) factors of the WRC and the TBI indicates that the tea litter bags buried in the mowed/unfertilized treatment had *k* and S values similar to that of peatland ecosystems (Keuskamp et al., 2013). These results reflect the coastal plain wetland environment of the West Research Campus, offering additional evidence that wetland environments store a disproportionate amount of C compared to other ecosystems (Nahlik and Fennessy, 2016; Sutfin et al., 2016). Lower rates of decomposition occurred in unfertilized plots (the ambient state of this nutrient-poor habitat). When looking specifically at high C:N ratio litter, less C was being released. This provides evidence that C storage potential within these plots resembles that of peatlands (Hill et al., 2018). Wetland ecosystems that are disconnected and isolated from pulses of nutrients from natural processes or agricultural or urban runoff are more commonly nutrient limited by N and P (Vitousek et al., 2010). The plant species that are adapted to these low-nutrient ecosystems can maintain positive population growth and contribute to organic C additions to soils. Further, flooded environments, such as wetlands, are also known to support anaerobic microbial processes, which result in slower rates of decomposition (Collins et al., 2015). Taken together, low nutrient environments

can be sites of high plant biodiversity leading to organic C inputs and balanced with slower decomposition rates, which contribute to long-term C storage in soils (Hooper et al., 2005; Kleber et al., 2011).

The mowed/fertilized plots, however, had *k* and *S* values that grouped closer towards grassland and forest ecosystems, which have lower C storage potential because more available nutrients and oxic environments support aerobic respiration and higher decomposition rates. Thus, the mowed/fertilizer environment decomposition status is more similar to that of a grassland or forest when compared using the TBI (Riggs et al., 2015). Results from this study provide support that nutrient enrichment can have a lasting influence on plant-soil-microbe interactions that affect C storage potential of wetland ecosystems (Allison et al., 2014; Hartman et al., 2017; Lambers et al., 2009).

Patterns in bacterial community composition (i.e., beta diversity) but not the combined OTU richness, Shannon diversity, Simpson's evenness diversity metrics (i.e., alpha diversity), significantly explained decomposition rates. Further, resource quality followed by the fertilization and ditch effects determined bacterial community composition. A trend of higher decomposition rates was associated with high bacterial alpha diversity, especially in the fertilized compared to unfertilized plots. Higher diversity has been associated with higher decomposition rates across different ecosystems (Lange et al., 2015; Trivedi et al., 2016). Shifts in plant and microbial communities can fuel decomposition rates and ultimately carbon losses from historically low nutrient ecosystems that experience atmospheric nutrient deposition (Sardans and Peñuelas, 2012; Wieder et al., 2015).

When bulk and tea-associated microbiomes were considered together, the bulk soil bacterial communities were very distinct from the tea-associated bacterial communities. Within

the bulk soil, bacterial communities were distinct across mowed/fertilized and unfertilized dry plots, and indicator taxa were phylogenetically distinct at the phylum level (Proteobacteria vs. Acidobacteria). While results revealed similarity in bulk soil bacterial composition in the mowed/fertilized and mowed/unfertilized wet plots, the indicator taxa also represented distinct phyla (mowed/unfertilized: Verrucomicrobia + Acidobacteria, mowed/fertilized: Actinobacteria). In contrast, the indicator taxa for tea-associated bacterial communities that overlapped in composition were similar at the phylum level. Specifically, similar bacterial assemblages of rooibos tea-associated (high C:N litter) microbiomes in the mowed/fertilized and mowed/unfertilized, dry plots were associated with Proteobacteria (Table S2, unfertilized: Proteobacteria/Alphaproteobacteria/Rhodospirillales/Rhodospirillaceae/Lacibacterium, fertilized: Proteobacteria/Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae/Dokdonella). Indicator bacterial taxa representing rooibos tea-associated microbiomes buried in mowed/fertilized, dry plots were *Dokdonella spp.* (Proteobacteria phylum) and unclassified Microbacteriaceae (Actinobacteria phylum). The genus *Dokdonella* has been involved in heterotrophic denitrification (Figueroa-González et al., 2016; Palma et al., 2018), while members of the family Microbacteriaceae have been predominantly found in terrestrial and aquatic ecosystems with putative functions related to plant pathogenicity (Glöckner et al., 2000; Lory, 2014; Young, 2008). Indicator taxa within the order Alphaproteobacteria represented the similar bacterial communities of the rooibos tea-associated microbiomes in unfertilized (indicator OTU *Acidisoma spp.*), wet plots and green tea-associated microbiomes in unfertilized, dry plots (indicator OTU *Phenylobacterium spp.*). In addition, the community similarity between green tea-associated microbiomes in unfertilized, wet plots and rooibos tea-associated microbiomes in fertilized, wet plots were represented by taxa within different Proteobacterial classes.

Specifically, *Dongia spp.* (within Alphaproteobacteria class) and *Dyella spp.* (within Gammaproteobacteria class) are similar in at least some life history traits. Representative isolates were cultured from soils (*Dyella*) and wetland sediments (*Dongia*) and were found to be aerobic, Gram-negative, and motile (Baik et al., 2013; Xie and Yokota, 2005). Further, these assemblages also overlapped with green tea-associated microbiomes in the fertilized, dry plots, which were represented by *Legionella spp.* (within class Gammaproteobacteria). Legionellae are found in aquatic and other moist environments with their free-living protozoan hosts (Barker and Brown, 1994). In the wetland soil environment, they can obviously persist but are unlikely optimal for successful growth. Taken together, the litter-associated OTUs unsurprisingly represent distinct taxa across the tree of life. In these nutrient poor wetland soils undergoing nutrient enrichment, the fertilization increased bacterial diversity the potential for increased C losses through decomposition is observed.

In conclusion, this study demonstrates that single species plant litter stoichiometry affects microbial function. If patterns between plant-community wide root C:N ratios can be generalized from species-specific litter type decomposition from this study, then plant nutrient stoichiometry can be used to study differences in soil C efflux at the ecosystem scale. By applying this single species tea result as a model study, future studies can be conducted on a larger scale to disentangle the causal factors of fertilization effects on plant litter quality and subsequent soil bacterial structure-function relationships.

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Tables

Table 1. Summary of Type II Analysis of Variance Table with Kenward-Roger's method comparing soil properties between mowed and mowed/fertilized treatments.. Mean (± standard deviation) of bulk soils of mowed-unfertilized (M) and mowed-fertilized (MF) treatments collected from surface soils.

Soil Factor	Mowed	Mowed/Fertilized	F-value	P-value
Temperature °C	12.90 ± 1.13	12.91 ± 1.10	0.023	0.882
Moisture (%)	31.49 ± 4.55	33.66 ± 4.12	**7.402**	**0.009**
pH	3.99 ± 0.21	4.12 ± 0.16	**6.539**	**0.014**
Total C (%)	4.06 ± 0.98	4.68 ± 1.10	**7.156**	**0.011**
Total N (%)	0.22 ± 0.04	0.26 ± 0.05	**9.618**	**0.003**
Soil C:N (wt:wt)	18.52 ± 1.6	18.26 ± 1.34	0.402	0.530
NH_4^+-N (µg/g dry soil)	0.28 ± 0.18	0.28 ± 0.14	0.006	0.940
NO_3^--N (µg/g dry soil)	0.08 ± 0.10	0.12 ± 0.24	1.280	0.264
Total PO_4^{3-}-P (µg/g soil)	123.54 ± 22.01	286.45 ± 62.89	**157.97**	**<0.0001**
Water ext. PO_4^{3-}-P (µg/g soil)	0.11 ± 0.14	1.98 ± 1.51	**48.747**	**<0.0001**

Table 2. Summary of Type II Analysis of Variance Table with Kenward-Roger's method comparing decomposition rates due to fertilization treatment and tea type in plots close to (dry plots) or far from (wet plots) the drainage ditch.

Fixed Effect	SumSq	MeanSq	NumDF	DenDF	F value	Pr(>F)
source	3489.5	3489.5	1	15.426	204.863	**<0.0001**
treatment	380.3	380.3	1	15.426	22.329	**<0.0001**
ditch	79.3	79.3	1	5.919	4.658	0.075
source:treatment	81.3	81.3	1	15.247	4.776	**0.045**
source:ditch	1.9	1.9	1	15.452	0.110	0.744
treatment:ditch	12.6	12.6	1	15.452	0.740	0.403
source:treatment:ditch	5.2	5.2	1	15.248	0.304	0.589

Table 3. Summary PERMANOVA comparing bacterial communities due to source (green tea, rooibos tea), treatment (fertilized, unfertilized), and proximity to ditch (wet, dry).

Factor	df	SumsOfSqs	MeanSqs	F.Model	R^2	Pr(>F)
source	1	0.710	0.710	4.361	0.125	**0.001**
treatment	1	0.377	0.377	2.318	0.067	**0.003**
ditch	1	0.594	0.594	3.647	0.105	**0.001**
source:treatment	1	0.198	0.198	1.216	0.035	0.214
source:ditch	1	0.340	0.340	2.086	0.060	**0.015**
treatment:ditch	1	0.197	0.197	1.211	0.035	0.210
source:treatment:ditch	1	0.162	0.162	0.992	0.028	0.410
Residuals	19	3.093	0.163	0.545		
Total	26	5.670	1.000			

Table 4. Summary of Type II Analysis of Variance Table with Kenward-Roger's method comparing alpha diversity metrics (OTU richness, Shannon Diversity Index H′, and Simpson's evenness) associated with source (bulk soil, green tea, rooibos tea), treatment (fertilized, unfertilized), and proximity to ditch (wet, dry).

(A) OTU richness

Fixed Effect	SumSq	MeanSq	NumDF	DenDF	F value	Pr(>F)
source	27269829	13634915	2	25.585	92.599	**<0.0001**
treatment	1189815	1189815	1	25.469	8.081	**0.009**
ditch	47449	47449	1	5.823	0.322	0.591
source:treatment	202762	101381	2	25.720	0.689	0.511
source:ditch	302781	151391	2	25.687	1.028	0.372
treatment:ditch	41789	41789	1	25.465	0.284	0.599

(B) Shannon diversity

Fixed Effect	SumSq	MeanSq	NumDF	DenDF	F value	Pr(>F)
source	8.554	4.277	2	25.760	35.117	**<0.0001**
treatment	2.349	2.349	1	25.621	19.289	**<0.0001**
ditch	0.553	0.553	1	5.762	4.539	0.079
source:treatment	0.556	0.278	2	25.936	2.284	0.122
source:ditch	0.395	0.198	2	25.889	1.623	0.217
treatment:ditch	0.035	0.035	1	25.616	0.287	0.597
source:treatment:ditch	0.161	0.080	2	26.007	0.660	0.526

(C) Simpson's evenness

Fixed Effect	SumSq	MeanSq	NumDF	DenDF	F value	Pr(>F)
source	0.0023	0.0011	2	25.698	3.929	**0.032**
treatment	0.0026	0.0026	1	25.566	8.958	**0.006**
ditch	0.0006	0.0006	1	5.784	2.124	0.197
source:treatment	0.0007	0.0003	2	25.859	1.184	0.322
source:ditch	0.0011	0.0005	2	25.817	1.885	0.172
treatment:ditch	0.0001	0.0001	1	25.561	0.394	0.536
source:treatment:ditch	0.0003	0.0002	2	25.925	0.552	0.582

Table 4. Summary of distance-based partial least squares regression (dbplsr) representing how much variation in decomposition rate is explained (%) by each component (Comp) derived from a tea-associated bacterial community Bray-Curtis distance matrix.

	Comp 1	Comp 2	Comp 3	Comp 4	Comp 5	Comp 6
R^2	64.96	88.08	94.65	98.12	99.32	99.77
adjusted R^2	63.56	87.09	93.96	97.78	99.16	99.70
gvar	31.50	41.62	50.10	56.93	61.21	66.39
crit	2.47	0.91	0.44	0.17	0.07	0.03

gvar = total weighted geometric variability; crit = value of criterion defined in method

Figures

Figure 1

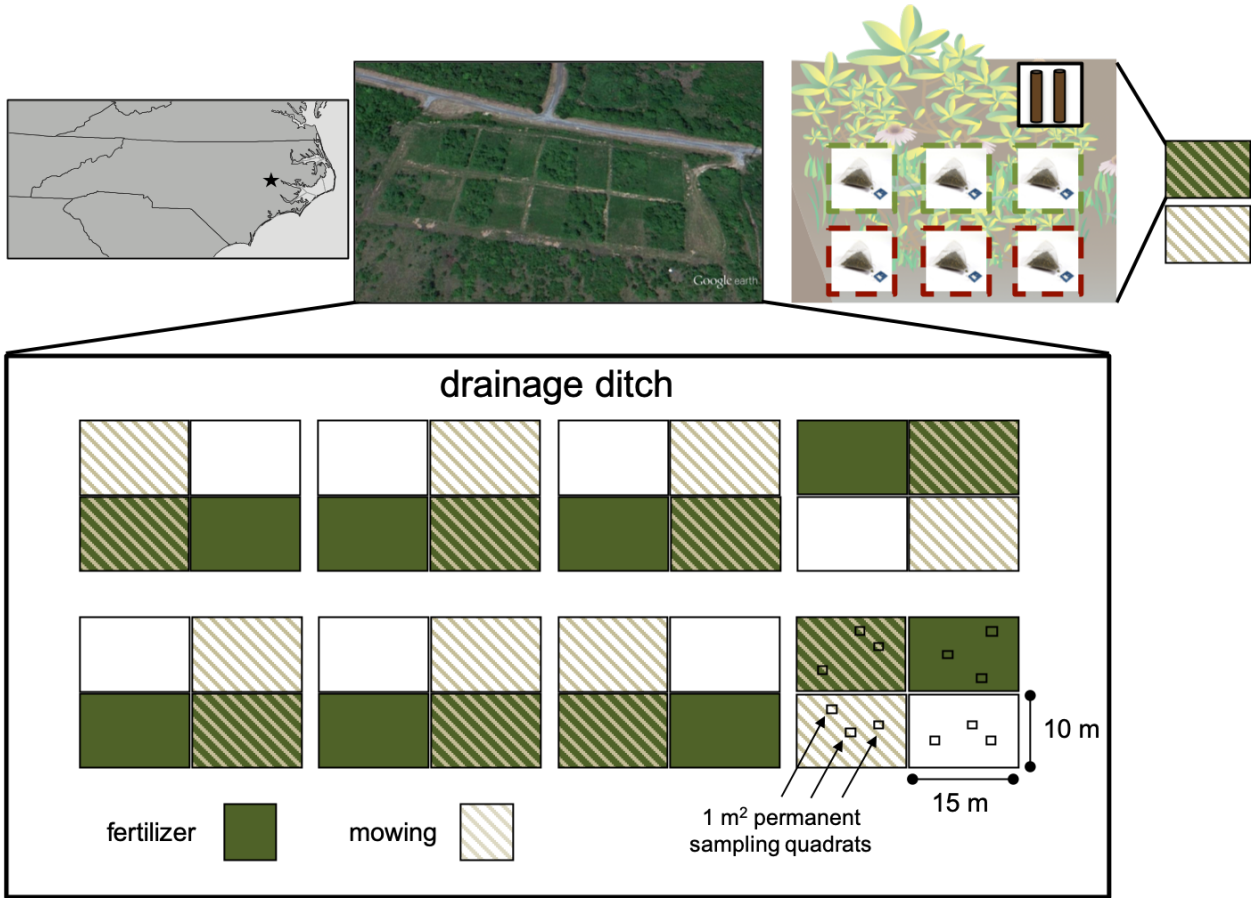

Figure 1. Experimental design of a long-term ecological experiment to test the effects of fertilizer and disturbance by mowing on plant and microbial communities at East Carolina University's West Research Campus (WRC), Greenville, NC, USA. The decomposition protocol for this experiment was adapted from the Tea Bag Index Protocol (Keuskamp et al., 2013) and applied within the eight-replicate mowed/fertilized and mowed/unfertilized plots of the WRC. In one quadrat per replicate plot, three bags of Lipton™ green tea and three bags of Lipton™ rooibos tea were buried and retrieved after 111 days. Bulk soil was sample as a composite sample representing two soil cores from three permanent quadrats per plot.

Figure 2.

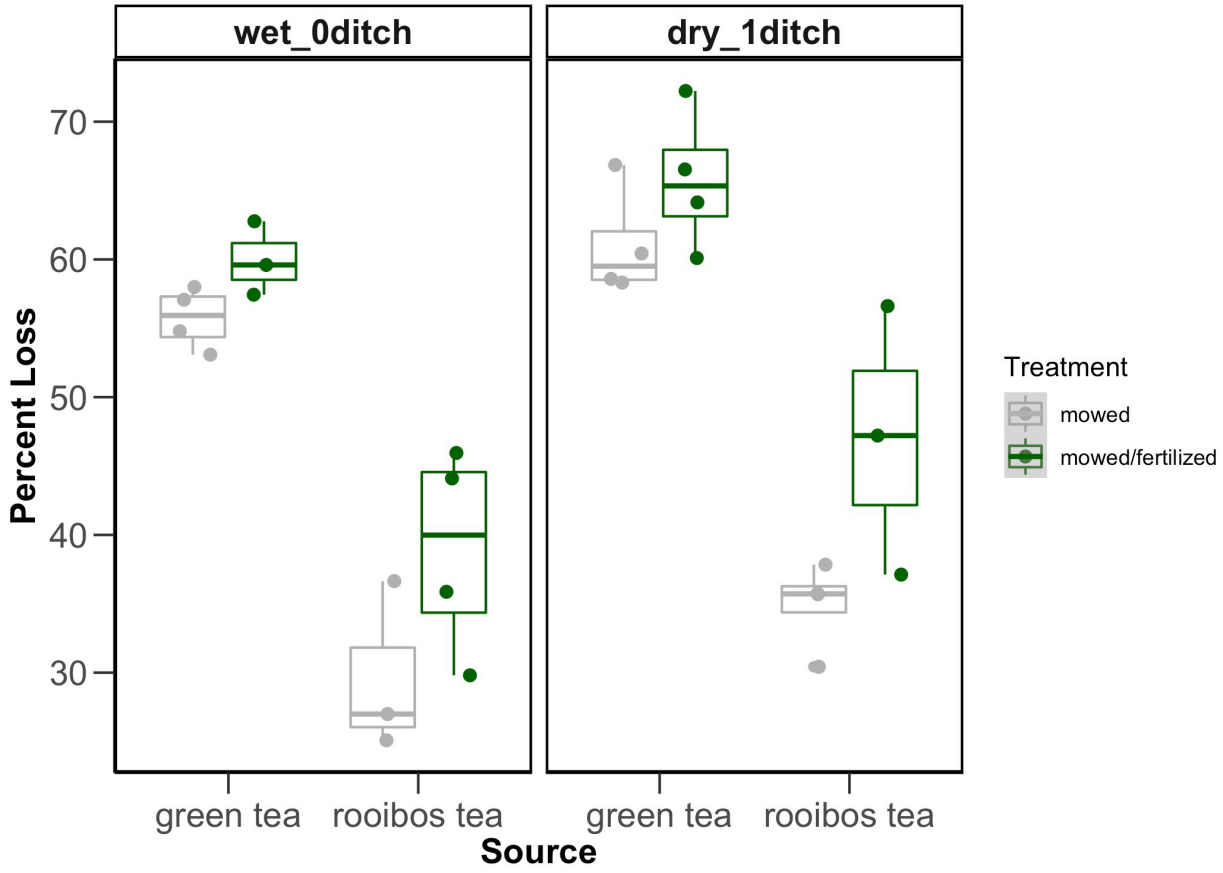

Figure 2. Boxplots representing cumulative mass loss of green and rooibos tea in fertilized (green) and unfertilized (gray) plots. Symbols represent individual data points.

Figure 3

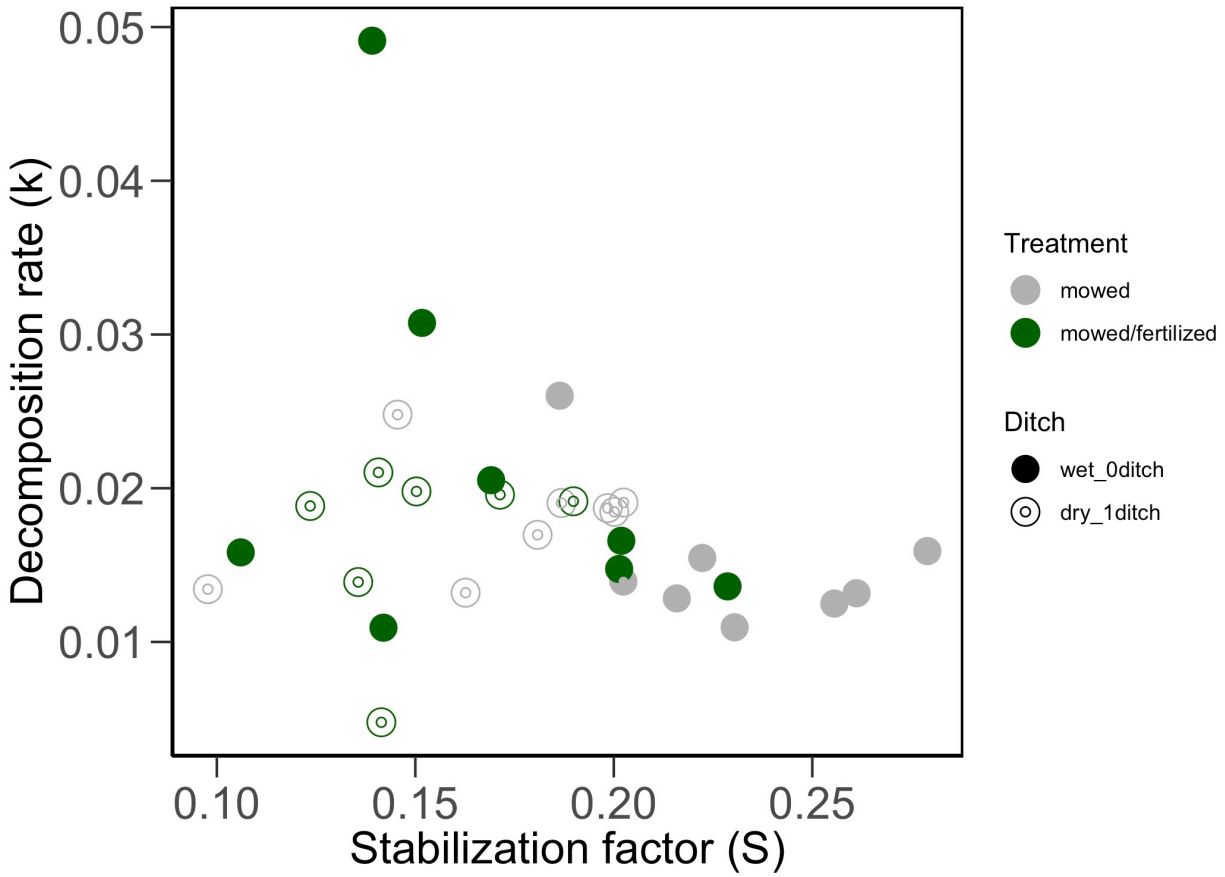

Figure 3. Initial decomposition rate *k* and stabilization factor *S* for different tea bags within the long-term fertilization experiment. Tea bags from mowed/unfertilized plots are indicated in grey, while tea bags from mowed/fertilized plots are indicated in green. Open symbols represented drier plots that were closer to the drainage ditch, while closed symbols represented wetter plots that were further from the ditch.

Figure 4

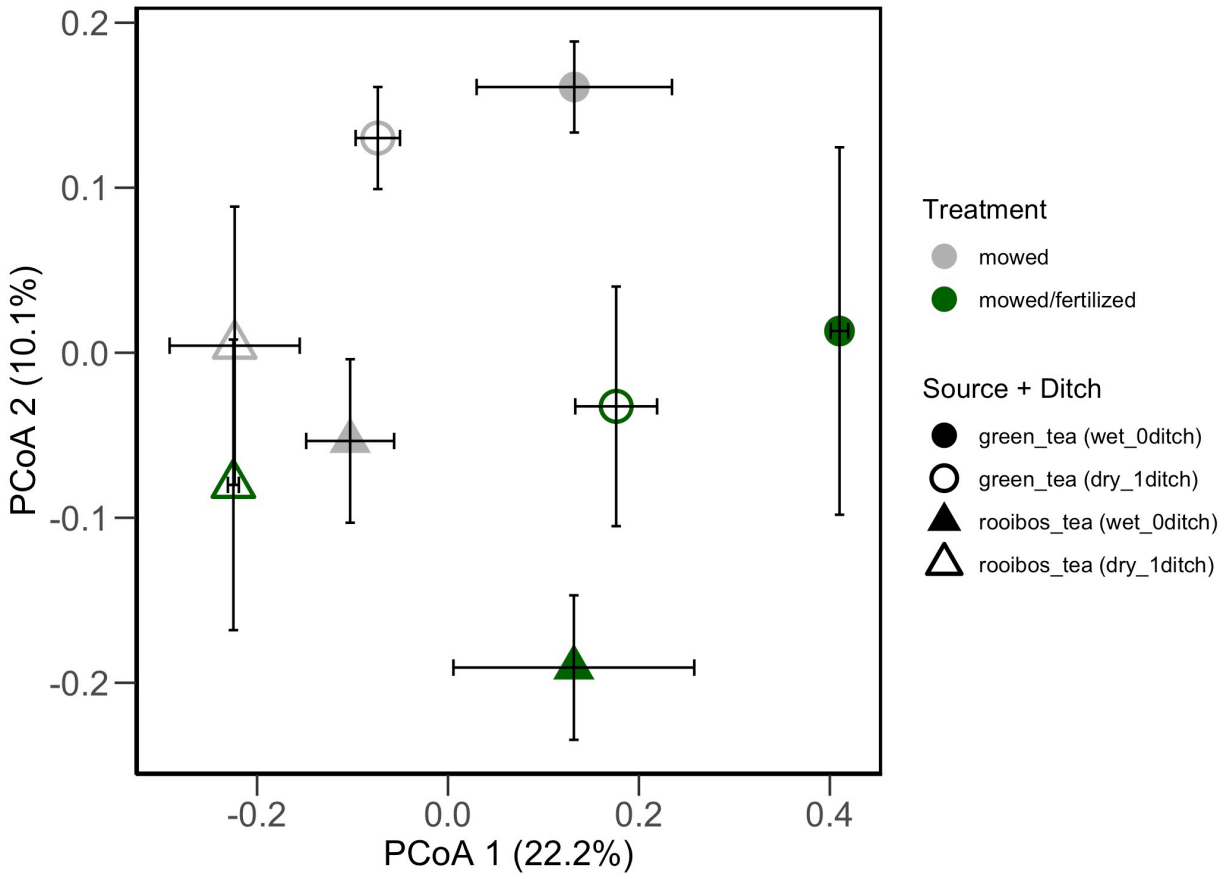

Figure 4. Ordination based on a Principal Coordinates Analysis depicting bacterial community composition according to tea type. Symbols are colored according to fertilization treatment (gray = control, black = fertilized) and tea source (circles = green tea, triangles = rooibos tea) at drier mowed plots situated close to the drainage ditch (open symbols) compared to wetter mowed plots (closed symbols).

Figure 5

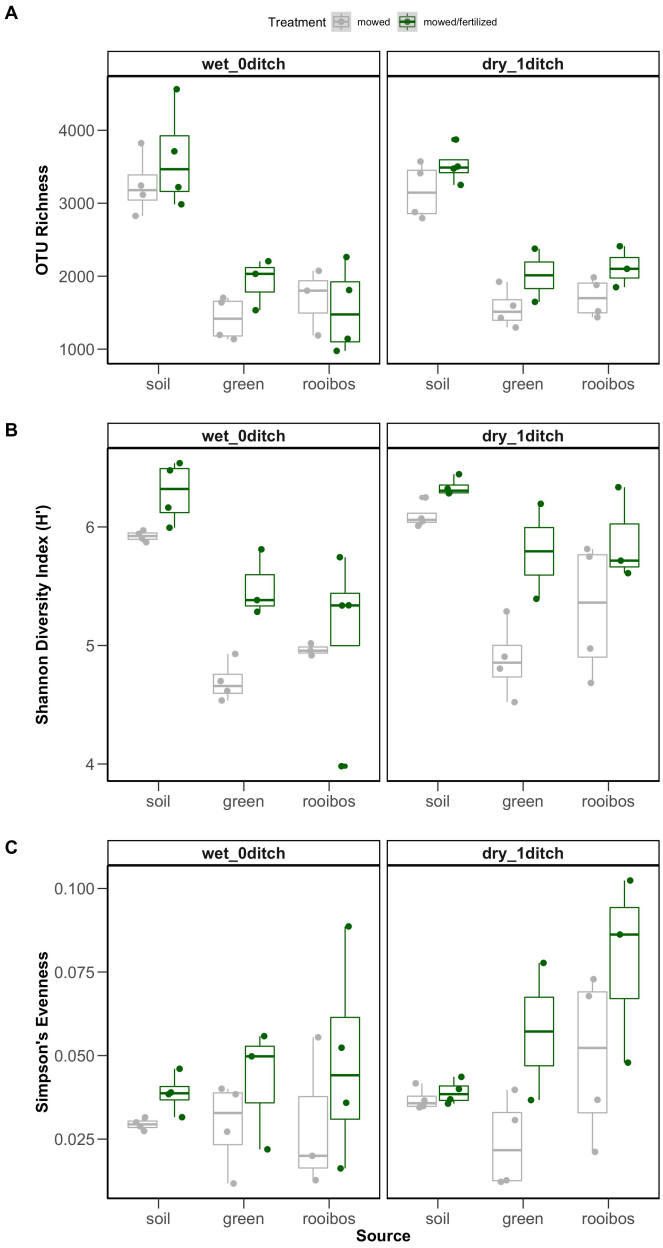

Figure 5. Boxplots depicting of alpha diversity metrics (OTU richness, Shannon Diversity Index H′, and Simpson's Evenness) associated with source (bulk soil, green tea, rooibos tea). Boxplots and symbols are colored according to fertilization treatment (gray = control, green = fertilized) at drier mowed plots situated close to the drainage ditch compared to wetter mowed plots. Symbols represent data points for individual plot samples.