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Distinct microbial communities alter litter decomposition rates in a fertilized coastal plain wetland

Megan E. Koceja, Regina B. Bledsoe, Carol Goodwillie, and Ariane L. Peralta †

Department of Biology, East Carolina University, Howell Science Complex, Mail Stop 551, Greenville, North Carolina 27858 USA

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Abstract. Human activities have led to increased deposition of nitrogen (N) and phosphorus (P) into soils. Nutrient enrichment of soils is known to increase plant biomass and rates of microbial litter decomposition. However, interacting effects of hydrologic position and associated changes to soil moisture can constrain microbial activity and lead to unexpected nutrient feedbacks on microbial community structure–function relationships. Examining feedbacks of nutrient enrichment on decomposition rates is essential for predicting microbial contributions to carbon (C) cycling as atmospheric deposition of nutrients persists. This study explored how long-term nutrient addition and contrasting litter chemical composition influenced soil bacterial community structure and function. We hypothesized that long-term nutrient enrichment of low fertility soils alters bacterial community structure and leads to higher rates of litter decomposition especially for low C:N litter, but low-nutrient and dry conditions limit microbial decomposition of high C:N ratio litter. We leveraged a long-term fertilization experiment to test how nutrient enrichment and hydrologic manipulation (due to ditches) affected decomposition and soil bacterial community structure in a nutrient-poor coastal plain wetland. We conducted a litter bag experiment and characterized litter-associated and bulk soil microbiomes using 16S rRNA bacterial sequencing and quantified litter mass losses and soil physicochemical properties. Results revealed that distinct bacterial communities were involved in decomposing higher C:N ratio litter more quickly in fertilized compared to unfertilized soils especially under drier soil conditions, while decomposition rates of lower C:N ratio litter 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. However, community composition associated with high C:N litter was similar in wetter plots with available nitrate detected, regardless of fertilization treatment. This study provides insight into long-term fertilization effects on soil bacterial diversity and composition, decomposition, and the increased potential for soil C loss as nutrient enrichment and hydrology interact to affect historically low-nutrient ecosystems.

Key words: biodiversity-ecosystem function; carbon cycling; decomposition; microbiomes; tea bag index; wetlands.

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† **E-mail:** peraltaa@ecu.edu

Introduction

Humans modify their landscapes through fossil fuel burning, deforestation, and intense agricultural activity (Vitousek et al. 2010, Fowler et al. 2013). These anthropogenic disturbances have led to increased atmospheric deposition of nitrogen (N) and phosphorus (P) and can be

particularly disruptive to historically nutrient-limited ecosystems (Guignard et al. 2017). This increased nutrient deposition can cause a fertilization effect on plant–microbial interactions which results in increased biomass and shifts in community structure of both plants and microbes (Cherif and Loreau 2009, Leff et al. 2015, Harpole et al. 2016). Fertilization effects can increase plant biomass carbon (C), which fuels heterotrophic microbial growth and leads to increased carbon dioxide (CO_2) respiration rates (Hoosbeek et al. 2004, Kuzyakov 2010). The extent to which nutrient enrichment predicts decomposition rates is also determined by the interaction of soil microorganisms with the plant inputs (Hobbie 2005), the stage of litter decomposition (Fog 1988), and the abiotic soil environment (Fog 1988). Fertilization effects can augment decomposition rates, but N additions have also been observed to inhibit decomposition rates (Fog 1988, Chen et al. 2018). In previous studies, fertilization decreased soil pH, which led to diminished microbial activity (Tian and Niu 2015, Chen et al. 2018). While human alteration of the environment and its effect on C storage and release is relatively well-documented in terrestrial ecosystems, the mechanisms governing the interactive effects of nutrient enrichment on plant–soil–microbial relationships at terrestrial–aquatic interfaces are relatively understudied. The availability of terminal electron acceptors in hydrologically dynamic ecosystems are known to shape the resident microbial community and to control biogeochemical functions (Bernhardt et al. 2017). This makes it challenging to predict microbial responses to environmental change in ecosystem models.

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. Altered bacterial composition, in particular, can give rise to shifts in taxa that are capable (or not) of producing enzymes needed to decompose litter of different chemical compositions (Strickland et al. 2009, Argiroff et al. 2019, Gołębiewski et al. 2019). Prior studies revealed that long-term nutrient addition enhanced grassland plant biomass and increased C inputs into soils (Fornara and Tilman 2012, Harpole et al. 2016, Borer et al. 2017). These additional plant inputs also increased CO_2 outputs via microbial respiration (Hoosbeek et al. 2004, Lange et al. 2015). If microbes are not limited by N and P, the increased plant-derived organic C inputs are more quickly metabolized and soil CO_2 fluxes increase (Peralta and Wander 2008, Cotrufo et al. 2013, Castellano et al. 2015). Plant litter chemical composition and soil nutrient availability affect microbial nutrient use and respiration rates. Based on a meta-analysis of N fertilization studies, N enrichment was associated with decreased lignin-modifying enzymes but no change in cellulase activity. In response to N fertilization, fine root chemical composition increased in lignin-derived substrates and decreased decomposition rates (Argiroff et al. 2019). Inhibition of lignin-modifying enzymes needed for microbial decomposition could contribute to soil C storage (Berg 2014, Chen et al. 2018).

Nuanced interactions between litter chemical composition, abiotic environment, and resident microbial composition can manifest in similar decomposition patterns (i.e., increased rates due to increased nutrient availability). However, the degree to which microbial structure contributes to the magnitude of litter mass loss is challenging to pinpoint since interactions among abiotic (temperature, nutrient availability, moisture) and biotic (community structure) factors determine decomposition rates (Kuzyakov and Blagodatskaya 2015, Meier et al. 2017, Deveau et al. 2018). Microbial community composition and function relationships vary in the strength of relationship and can be measured in different ways (Allison and Martiny 2008, Levine et al. 2011, Rocca et al. 2015, Hall et al. 2018). Both univariate and multivariate approaches can be used to measure associations between bacterial community structure and decomposition function. Instead of a set of predictors, a distance matrix is used to predict the numerical response, in this case mass loss (Boj et al. 2007). Distance matrices can be based on all taxa or based on alpha diversity metrics.

In this study, we examined the extent that litter-associated microbiomes are distinct depending on historical nutrient enrichment and how this community composition contributes to decomposition of litter of varying chemical quality (i.e., C:N ratio). We measured the extent of microbial decay by comparing the mass loss of

chemically distinct plant litter types that were incubated in field conditions for about three months. We used the Tea Bag Index approach since the tea is considered a standardized, well-characterized litter type that is representative of native plant litters (Duddigan et al. 2020). The objectives of this study were to (1) quantify decomposition rates of high and low C:N ratio litters (rooibos and green teas), (2) compare bacterial diversity and composition in bulk soil and tea litter-associated microbial communities, and (3) measure the relationship between bacterial community composition and diversity and decomposition function in response to fertilization and moisture gradient in a coastal plain wetland ecosystem. The >15-yr fertilization treatment modified bacterial community composition. This change in the bulk soil bacterial community also changed the reservoir of microbes capable of accessing and decomposing belowground plant litter (Bledsoe et al. 2020). We hypothesized that long-term nutrient enrichment of low fertility soils alters bacterial community structure and leads to higher decomposition rates of low C:N ratio litter, but low-nutrient and dry conditions constrain microbial decomposition of high C:N ratio litter. We focused on characterizing the bacterial community response since the incubation time of the tea bag decomposition experiment was relatively short (111 d). The soil bacterial response was expected to be greater during the initial phases of decomposition compared to fungal response as evidenced in a recent study (Pioli et al. 2020). Therefore, we characterized bacterial community composition (using targeted amplicon sequencing of the 16S rRNA gene) of the bulk soil and the litter-associated bacteria of two differing litter types occurring in wetland soils exposed to fertilization or not. We hypothesized that the extent that community composition matters to decomposition rate depends on biotic and abiotic factors.

Materials and Methods

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; 35.6298° N, −77.4836° W) 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 are 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 (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture 2019). The annual mean temperature at this site 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 for each nutrient. Plots are mowed by bush-hog annually to simulate fire disturbance (Goodwillie et al. 2020). Within each plot, annual soil and plant sampling takes place at three randomly placed quadrats (Fig. 1). Perennial forbs in two major genera of the Asteraceae—*Eupatorium* and *Solidago*—and graminoid species dominate the wetland plant community in the mowed plots. The relative abundance of major grass species differs between unfertilized/mowed and fertilized/mowed plots, with switchcane (*Arundinaria tecta*) most dominant in fertilized/mowed plots and broomsedge (*Andropogon virginicus*) found almost exclusively in unfertilized/mowed 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. 2020). 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. However, this hydrologic gradient has not yet been characterized by modeling flow according to water levels over time. The ditch was not intentionally manipulated as part of our experimental design,

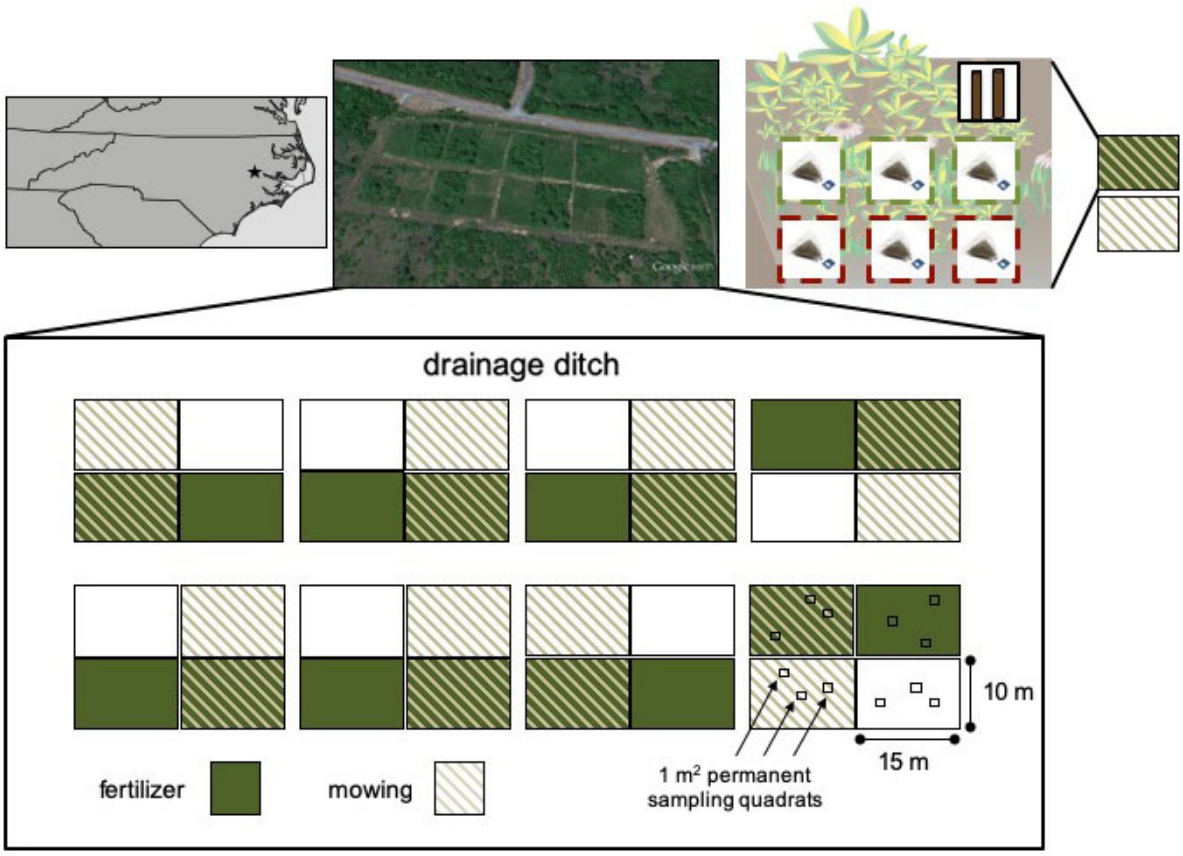

Fig. 1. Experimental design of a long-term ecological experiment to test the effects of fertilizer and disturbance by mowing on plant and microbial communities at East Carolina University's West Research Campus (WRC), Greenville, North Carolina, USA. The decomposition protocol for this experiment was adapted from the Tea Bag Index Protocol (Keuskamp et al. 2013) and applied within the eight-replicate unfertilized/mowed and fertilized/mowed 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 d. Bulk soils were sample as a composite sample representing two soil cores from three permanent quadrats per plot.

but it contributes an important ecological variable to the study (Goodwillie et al. 2020). For this study, we focused the tea litter decomposition experiments in the mowed plots only.

Soil sampling

We collected composite soil samples from all unfertilized/mowed and fertilized/mowed plots, 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 2018 about three months after the tea litter bags were collected from the field.

Soil physicochemical 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 mass. 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 North Carolina State University). Extractable inorganic N for each soil sample was measured on approximately 5 g of field moist soil. We added 45 mL of 2 mol/L KCl to soil, shook the sample for about 1 h, and gravity filtered. Total phosphate (PO_4^{3-}) was extracted by combining 0.1 g dried soil (ground and passed through a 500 μm sieve) with 0.5 mL of 50% w/v $Mg(NO_3)$ and ashing for 2 h at 550°C. Samples were hydrated with 10 mL of 1 mol/L HCl, shaken for 16 h at 250 RPM, and then filtered (22 μm filter). Water extractable PO_4^{3-} was determined by combining 1 g dried soil (ground and passed through a 500 μm sieve) with deionized water, shaken for 1 h, and filtered (22 μm filter). Ammonium (NH_4^+), nitrate (NO_3^-), and PO_4^{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.

Field experimental methods

The field protocol for this experiment was adapted from the Tea Bag Index (TBI) Protocol (Keuskamp et al. 2013) and applied within the eight-replicate unfertilized/mowed and fertilized/mowed 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). The green tea was sourced from *Camellia sinensis* leaves, and rooibos tea was sourced from *Aspalathus linearis* leaves (Duddigan et al. 2020). Duddigan et al. (2020) measured C functional groups alkyl C, O-alkyl C, aromatic C, carbonyl C in the green and rooibos tea litter. For both the alkyl C and O-alkyl C fractions, peaks in this region significantly declined after the 91-d incubation for both green and rooibos teas (Duddigan et al. 2020). Green tea has a measured mean C:N ratio of 12.229 while rooibos tea has a measured mean C:N ratio of 42.870 (Keuskamp et al. 2013). The alkyl C fraction was higher in green tea compared to rooibos tea at the start and after the 91-d incubation (Duddigan et al. 2020). The alkyl C measures amino acid side chains that represent cutans and suberins, aliphatic compounds, lipids, and waxes (Parfitt and Newman 2000, Webster et al. 2000). The O-alkyl C fraction was higher in the rooibos compared to the green tea at the beginning and the end of the incubation period (Duddigan et al. 2020). The O-alkyl C measures alcohols and ethers in cellulose, hemicelluloses, and other polysaccharides, carbohydrates, and lignin propyl side chains (Parfitt and Newman 2000, Webster et al. 2000). The aromatic C and carbonyl C fractions were similar at the start and end of the 91-d incubation for both the green and rooibos teas (Duddigan et al. 2020). The aromatic C groups represent tannins, hydrolysable tannins, and phenyl-propylene subunits of lignin, and the carbonyl C represents peptides, carboxylic C in hydrolysable tannins and resins, secondary amide C in proteins (Parfitt and Newman 2000, Webster et al. 2000). While the TBI approach does not use a native plant litter, this method does provide a robust way to measure microbial community composition differences and decomposition function using standardized, well-characterized litter types (Duddigan et al. 2020).

Prior to field deployment, we pre-labeled and weighed air-dried green and rooibos tea bags. In each treatment plot, we prepared six holes about 20 cm apart and 8 cm deep by removing surface soil using a hand trowel. We placed one tea bag into each hole, and soil was lightly packed around the tea bags, while keeping the labels visible above the soil. Tea bags were recovered after 111 d (21 May–09 September 2018) using hand trowels to loosen soil adjacent to tea bag locations. Based on previous studies, incubation time of about 90 d represented the initial stages of decomposition. In this study, the 111 d, especially with regards to the dry and nutrient-limited conditions, are assumed to be within the early decomposition stages as the TBI method represents (Keuskamp et al. 2013, Duddigan et al. 2020). 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 litter-associated bacterial community analysis. The rooibos tea was processed following the same procedure. Upon returning to the laboratory, 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 h. To quantify mass loss, the tea bags (tea bag + string + label + remaining litter) were reweighed following drying and compared to their initial mass prior to soil incubation.

Soil and litter-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 unfertilized or fertilized treatment, DNA was extracted from green tea (15 total), rooibos tea (14 total), and bulk soil samples (16 total). Three litter samples could not be located and 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 (Caporaso et al. 2012, Apprill et al. 2015, Parada et al. 2016). For each sample, three 50 μL PCR libraries were prepared by combining 35.75 μL molecular grade water, 5 μL Amplitaq Gold 360 10× buffer, 5 μL $MgCl_2$ (25 mmol/L), 1 μL dNTPs (40 mmol/L total, 10 mmol/L each), 0.25 μL Amplitaq Gold polymerase, 1 μL 515 forward barcoded primer (10 μmol/L), 1 μL 806 reverse primer (10 μmol/L), and 1 μL DNA template (10 ng/μL). Thermocycler conditions for PCR reactions were as follows: initial denaturation (94°C, 3 min): 30 cycles of 94°C for 45 s, 50°C for 30 s, and 72°C for 90 s; final elongation (72°C, 10 min). Triplicate PCR reactions were combined for each sample and cleaned according to the Axygen AxyPrep Magnetic Bead Purification Kits protocol (Corning Life Sciences). Following cleaning, PCR product were quantified using Quant-iT dsDNA BR (broad-range) assay (Thermo Scientific, Waltham, Massachusetts, USA). Libraries were pooled in equimolar concentration of 5 ng/μL after being diluted to a concentration of 10 ng/μL. 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, we processed raw sequences using a standard mothur pipeline (v1.40.1; Schloss et al. 2009, 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 binned into OTUs with a 97% sequence similarity level. Operational taxonomic units were classified using the SILVA rRNA database (v128; Yilmaz et al. 2014, Glöckner et al. 2017).

Statistical analyses

All statistical calculations were completed in the R environment (R v3.6.3, R Core Team 2020). Decomposition was measured through mass loss of buried tea bags. Initial and final tea bag masses were used to determine percent of loss, and we averaged two tea bag masses to represent each experimental plot. 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) and the pbkrtest package (Halekoh and Højsgaard 2014). For the mass loss response variable, a linear mixed model was fit by REML and produced type II analysis of variances tables (ANOVA) tables based on the Kenward-Roger's denominator degrees of freedom method using the anova() function. Calculations of decomposition rate (k) and stabilization factor (S) for each tea bag were accomplished according to formulas found within the TBI protocol (Keuskamp et al. 2013). Decomposition rate, k, is a parameter that represents short-term dynamics of new input and was calculated based on the following equation:

$$W(t) = ae^{-kt} + (1 - a), \quad (1)$$

where $W(t)$ is the fraction (by mass) remaining of rooibos tea, a is the predicted labile fraction (by mass) of rooibos tea, and t is the incubation time. Stabilization factor, S, is a parameter that

represents long-term carbon storage and was calculated according to the equation:

$$S = 1 - \frac{a_g}{H_g}, \quad (2)$$

where a_g is the green tea litter fraction that is decomposable (from field data) and H_g is the green tea litter fraction that is hydrolysable (from Keuskamp et al. 2013). We compared k and S values calculated for the WRC wetland ecosystem to those of global ecosystems submitted to the Tea Bag Index protocol (Keuskamp et al. 2013). We plotted calculated values, and locations of samples were compared to those of the larger multi-ecosystem index (Keuskamp et al. 2013).

We ran a series of linear mixed models for the bulk soil factors measured and the computed diversity (OTU richness, Shannon Diversity, Simpson's Evenness) metrics for soil and tea litter-associated bacterial communities. We ran linear mixed effects models with source, treatment and ditch 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) and the pbkrtest package (Halekoh and Højsgaard 2014). Statistical inferences for fixed effects were calculated from type II ANOVA tables and the Kenward-Roger's degrees of freedom method using the anova() 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 but focused on the specific tea litter-associated microbiome patterns. We ran an indicator species analysis to identify which bacterial species were most representative of each litter type. 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() function 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 litter-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 bacterial diversity (mass loss ~ OTU richness + Shannon Diversity + Simpson's Evenness). In addition, we conducted a distance-based partial least squares regression (dbplsr) to measure how much variation in mass loss of litter (function) was explained by the tea litter-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).

Results

Long-term fertilization strongly influenced abiotic and biotic factors within this nutrient-limited wetland environment. Both fertilization and ditch effects influenced soil pH, soil C:N ratio, extractable nitrate concentrations, and water extractable phosphate (Appendix S1: Tables S1A, S2B). A subset of soil factors was distinct between fertilized and unfertilized soils in the mowed plots. Total and water extractable phosphate concentrations, extractable nitrate concentrations, soil C and N content, moisture, and pH were higher in fertilized compared to unfertilized soils (Appendix S1: Table S1B). In addition, soil moisture, C:N ratio, and water extractable phosphate were higher in wetter soils away from the ditch compared to drier soils adjacent to the ditch (Appendix S1: Table S1C). Finally, nitrate concentrations were detectable only in wetter soils away from the ditch with higher concentrations in fertilized soils compared to unfertilized soils while nitrate was below detection limits in all soils near the ditch (Appendix S1: Table S1A). These differences in fertilized compared to unfertilized soils and soil moisture due to ditch proximity related to litter decomposition rates and altered bacterial community structure.

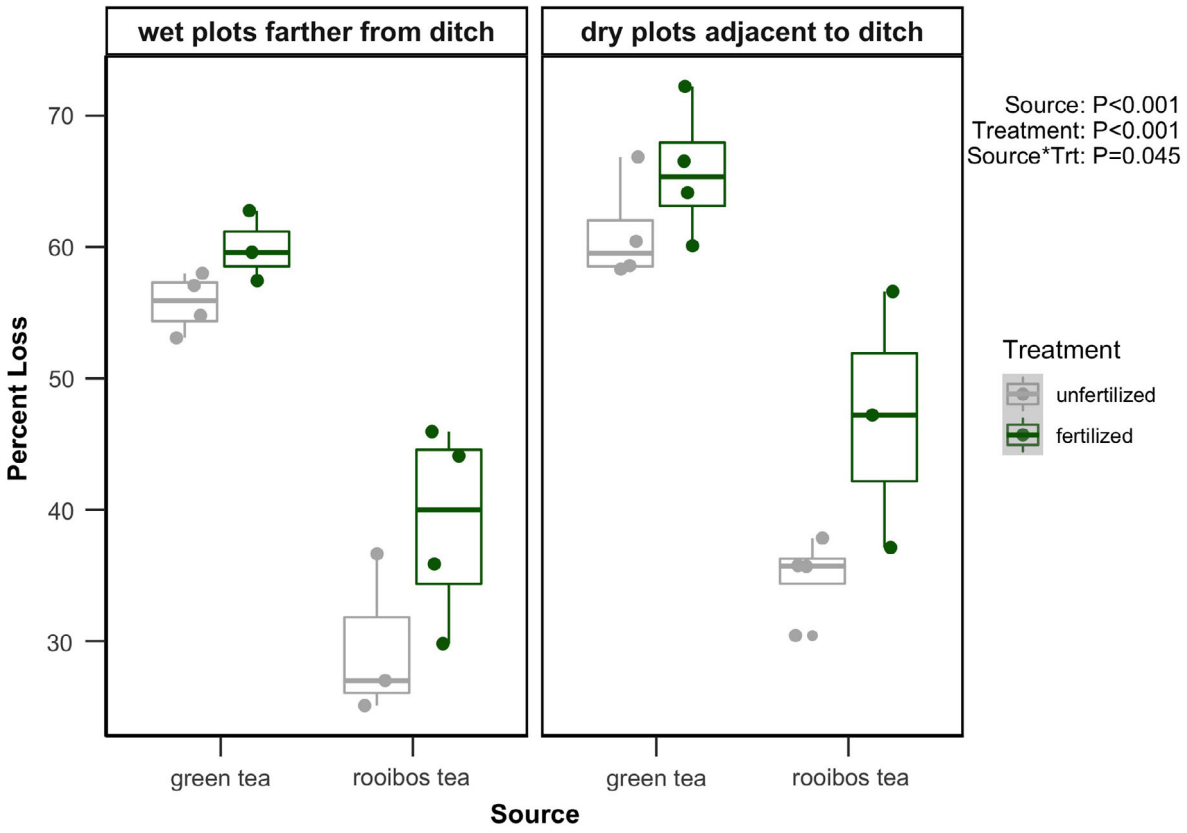

Fig. 2. Boxplots representing cumulative mass loss of green (low C:N ratio litter) and rooibos (high C:N ratio litter) tea in fertilized (green) and unfertilized (gray) plots at wetter mowed plots situated away from the drainage ditch (left) compared to drier mowed plots situated close to the drainage ditch (right). The boxplot is a visual representation of 5 key summary statistics: the median, the 25% and 75% percentiles, and the whiskers which represent the feasible range of the data as determined by $\pm 1.5 \times$ the interquartile range. Symbols represent individual data points. Summary of statistical output in Appendix S1: Table S2A.

To examine how litter type and nutrient addition influenced decomposition rates, we measured mass loss of green (low C:N litter) and rooibos (high C:N litter) tea bags. Following an 111-d incubation, there was a significant litter type (source) × fertilization treatment interaction ($P < 0.05$; Fig. 2, Appendix S1: Table S2). The mass loss of green tea litter was ~24% higher than the overall mass loss of rooibos tea litter (Fig. 2, Appendix S1: Table S2), averaged across fertilized, and unfertilized soils. Both fertilization and proximity to drainage ditch also increased the rate of mass loss (Fig. 2, Appendix S1: Table S2). Across all tea types and drainage ditch proximities, fertilized soils showed an average mass loss of ~7.5% more than that of unfertilized soils. Tea bags buried in closer proximity to the drainage ditch 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). Within this coastal plain wetland system, decomposition rates in fertilized plots were similar to those measured in grassland and forested ecosystems, while decomposition rates in unfertilized plots were similar to those measured in peatlands (Fig. 3). The most variable S and k values were measured in the wetter, fertilized plots (Fig. 3). The unfertilized compared to fertilized samples were generally higher along the stabilization

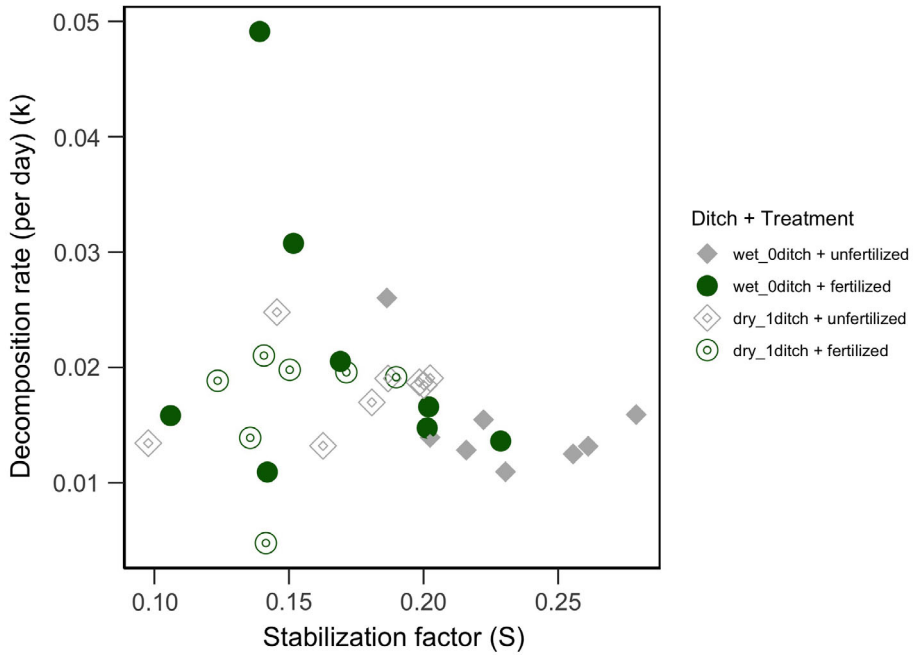

Fig. 3. Initial decomposition rate k and stabilization factor S for different tea bags within the long-term fertilization experiment. Tea bags from unfertilized plots are indicated in gray diamonds, while tea bags from fertilized plots are indicated in green circles. Open symbols represented drier plots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

factor (S) axis. Litter 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 factors (S; Fig. 3).

Fertilization effects on soil physicochemical properties 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 ($R^2 = 0.130$, $P = 0.001$) influenced bacterial community composition the most, while the interaction of tea type and proximity to ditch affected bacterial community patterns to a lesser degree ($R^2 = 0.060$, $P = 0.002$; Fig. 4, Appendix S1: Table S3; see circle vs. triangle). The proximity to ditch (proxy for hydrology) also altered bacterial community composition ($R^2 = 0.110$, $P = 0.001$; Fig. 4, Appendix S1: Table S3; see opened vs. filled symbols). Within tea type, fertilization treatment influenced bacterial community composition ($R^2 = 0.070$, $P = 0.003$; Fig. 4, Appendix S1: Table S3; see green vs. gray). For the drier, ditched plots, bacterial communities associated with high C:N ratio litter (rooibos) overlapped despite fertilization treatment, while bacterial communities were distinct between fertilization treatments in the wetter plots (Fig. 4). When the bulk soil and tea litter-associated bacterial communities were analyzed together, bulk soil or tea most strongly influenced bacterial community composition (source: $R^2 = 0.490$, $P = 0.001$; Appendix S1: Fig. S1, Table S4). In addition to these observed patterns, OTU diversity was generally higher in fertilized compared to unfertilized plots, especially in the drier plots associated with the drainage ditch (Fig. 5, Appendix S1: Table S5). Specifically, OTU richness values were highest in bulk soil compared to tea types (Fig. 5A, Appendix S1: Table S5A), and Shannon diversity values were highest in bulk soil compared to tea type in fertilized plots (Fig. 5B, Appendix S1: Table S5B). Lastly, bacterial evenness was similar across fertilization treatment,

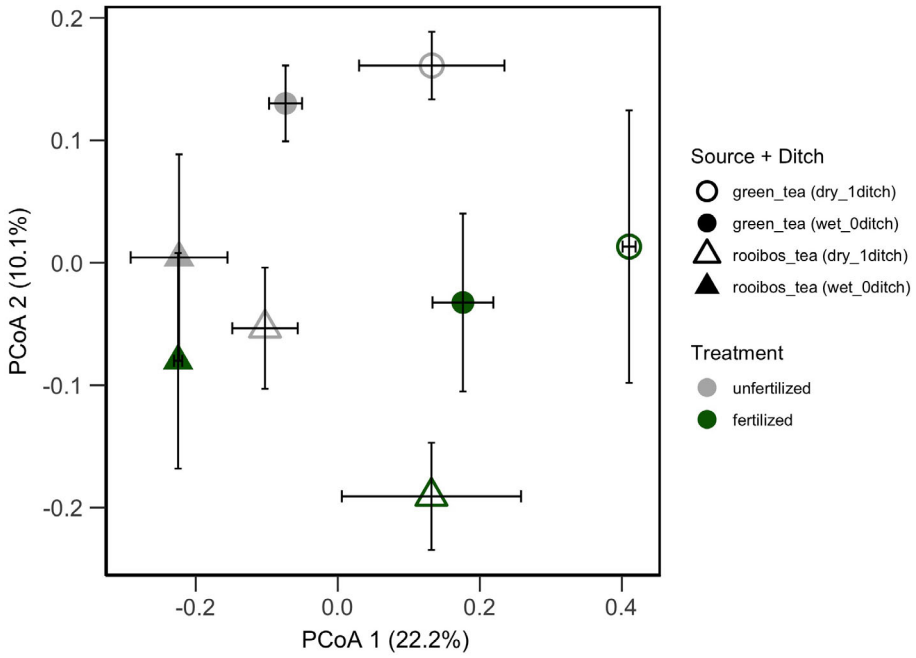

Fig. 4. Ordination based on a principal coordinates analysis depicting bacterial community composition according to tea type. Symbols are colored according to fertilization treatment (gray = unfertilized, green = fertilized) and tea source (circles = low C:N ratio green tea, triangles = high C:N ratio rooibos tea) at drier mowed plots situated close to the drainage ditch (open symbols) compared to wetter mowed plots (closed symbols). Summary of statistical output in Appendix S1: Table S3.

source type, and proximity to ditch (Fig. 5C, Appendix S1: Table S5C).

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 litter-associated microbiomes. The unclassified OTU within the class Spartobacteria and another OTU within the order Acidobacteria Gp1 represented the unfertilized bulk soil bacterial community, while an unclassified OTU within the order Solirubrobacterales represented bulk soils in the fertilized, dry plots. In wetter plots, an unclassified OTU within the order Rhodospirillales represented unfertilized plots while unclassified taxa within the orders Acidobacteria Gp1, Gp2, and Gp6 signified fertilized bulk soils (Appendix S1: Tables S6, S7). In addition, the unclassified OTU within the Acidobacteria Gp3 and *Dongia* spp. represented the green tea litter-associated (low C:N litter) bacterial communities in the unfertilized, dry plots while *Conexibacter* spp. were represented in the fertilized, dry plots. In the wetter plots, *Phenylobacterium* spp. represented green tea litter-associated bacterial communities in unfertilized plots and *Legionella* spp. in fertilized plots. The OTUs *Acidisoma* spp. in unfertilized plots and *Dyella* spp. in fertilized characterized rooibos tea litter-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 litter-associated communities in the wet plots (Appendix S1: Tables S6, S7).

When examined together, the link between bacterial community structure and decomposition function was relatively weak. No relationship between litter mass loss and bacterial diversity was detected ($R^2_{adj} = -0.038$, $P = 0.574$). However, when the total bacterial community was considered, the tea litter-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 1).

Discussion

Litter composition, fertilization, and ditch effects on soil moisture influenced bacterial community structure–function relationships in unexpected ways. In this study, increases in litter mass loss were greater in fertilized compared to unfertilized soils, especially in drier vs. wetter plots. Distinct bacterial communities associated with different litter chemical qualities. Litter mass loss following the field incubation indicated that similar bacterial communities were capable of decomposing higher C:N ratio litter (rooibos tea) more quickly in fertilized compared to unfertilized plots, particularly in drier compared to wetter plots. However, lower C:N ratio litter (green tea) decomposition rates were similar among fertilized and unfertilized plots, although slightly higher in drier compared to wetter plots but were represented by distinct bacterial communities. As expected, the low C:N ratio litter provided an N source to microbes regardless of external soil nutrient conditions, whereas the high C:N ratio litter was reliant on N from the fertilized soil in order to support increased rates of litter decomposition (Duddigan et al. 2020). These results suggested that shifts in bacterial community composition were partly due to differences in litter chemical composition and soil nutrient availability. The tea litter types represented a range of C functional groups along with differences in C:N ratio that would decompose at different rates. In a previous study, during the decomposition of both tea litter types, the O-alkyl C functional group decreased during the incubation. This indicated that simple sugars and cellulose were quickly lost; a subset of this labile carbon was not decomposed but instead was stabilized over time (Duddigan et al. 2020). These persistent compounds were represented by the alkyl C chemical components (Cepáková and Frouz 2015). Microbes need to make enzymes, which requires additional resources and nutrients, to breakdown more resistant compounds such as waxes and lipids (Burns et al. 2013).

In this study, we focused on the early stages of decomposition. There are different explanations for N fertilization decreasing rates during the later stage of decomposition (Berg 2014, Hobbie 2015). Nitrogen fertilization can increase microbial biomass and production of microbial residues that are resistant to breakdown (Cotrufo et al. 2013) or increased carbon-use efficiency that could enhance soil organic matter stabilization due to accumulation of microbial products (e.g., phospholipids, polysaccharides; Manzoni et al. 2010). The comparative results from the TBI-based decomposition experiment indicated that the tea litter bags buried in the unfertilized treatment had decomposition rates (k) and C stabilization factor (S) values similar to that of peatland ecosystems (Keuskamp et al. 2013). Upon comparison with other ecosystems, a large number of fertilized treatment samples grouped near the grassland-ambient ecosystem in Iceland and the forest ecosystem in the Netherlands (Keuskamp et al. 2013). However, the unfertilized treatment samples grouped near the peat-disturbed and peat-undisturbed ecosystems of Iceland and the peat ecosystem of the Netherlands (Keuskamp et al. 2013). Lower decomposition rates occurred in unfertilized plots (i.e., the ambient state of this nutrient-poor habitat). When looking specifically at high C:N ratio litter, less mass was lost to decomposition. This provided some evidence that C storage potential within these plots resembled that of peatlands (Hill et al. 2018). Wetland ecosystems that are disconnected and isolated from pulses of nutrients due to natural processes or agricultural or urban runoff are more commonly 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 add organic C 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 long-term C storage in soils because of high plant biodiversity leading to organic C inputs and balanced with slower decomposition rates (Hooper et al. 2005, Kleber et al. 2011). Results from this coastal plain wetland study provided some evidence that wetland environments store a disproportionate amount of C compared to other ecosystems (Nahlik and Fennessy 2016, Sutfin et al. 2016). However, this

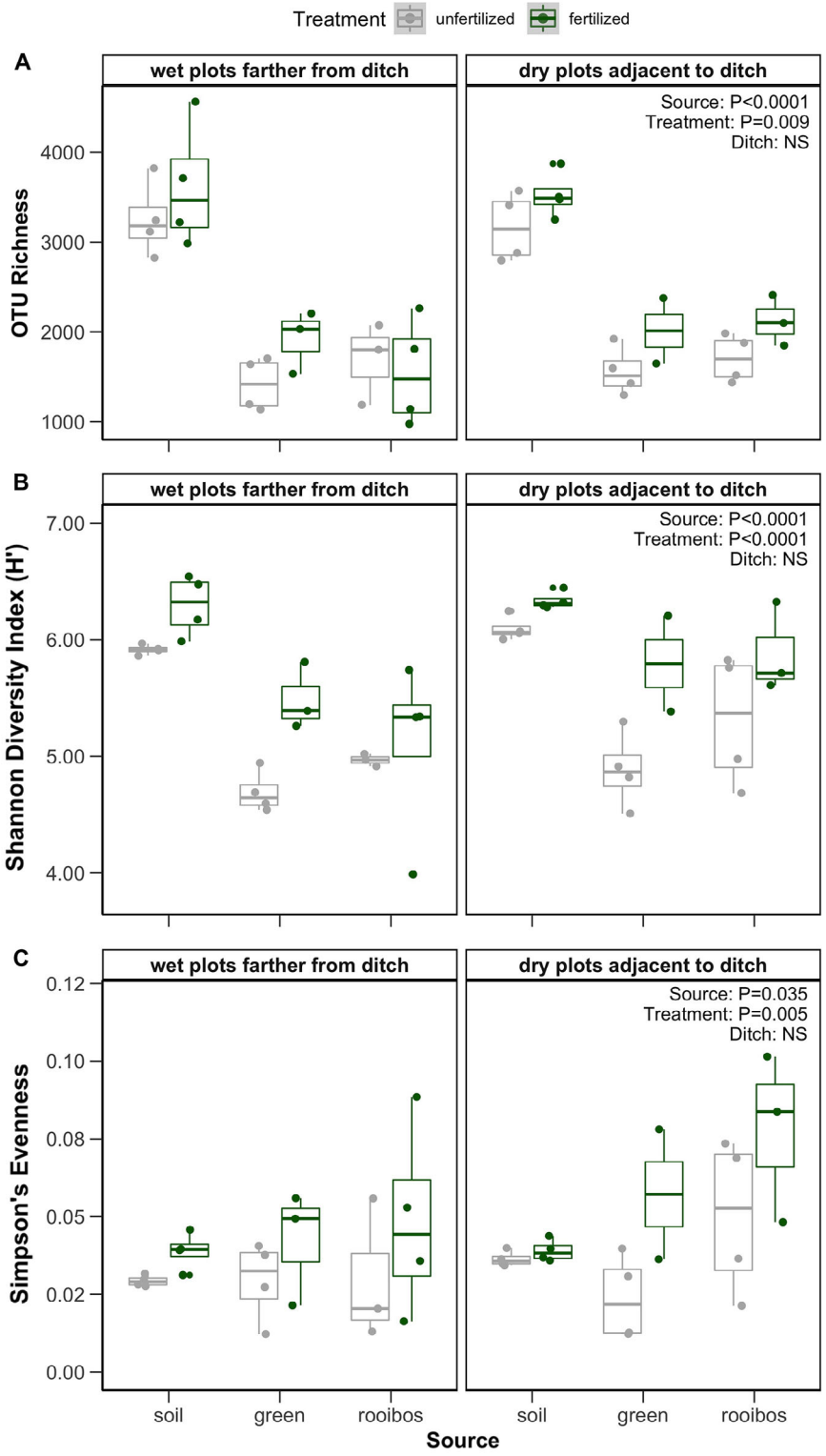

Fig. 5. Boxplots depicting bacterial diversity metrics (OTU richness, Shannon Diversity Index *H′*, and

(Fig. 5. *Continued*)
Simpson's Evenness) associated with source (bulk soil, low C:N ratio green tea, high C:N ratio rooibos tea). Boxplots and symbols are colored according to fertilization treatment (gray = unfertilized, green = fertilized) at wetter mowed plots situated away from the drainage ditch (left) compared to drier mowed plots situated close to the drainage ditch (right). The boxplot is a visual representation of 5 key summary statistics: the median, the 25% and 75% percentiles, and the whiskers which represent the feasible range of the data as determined by $\pm 1.5 \times$ the interquartile range. Symbols represent data points for individual plot samples. Summary of statistical output in Appendix S1: Table S5.

Table 1. 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 litter-associated bacterial community Bray-Curtis distance matrix.

Components	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

Note: Abbreviations are gvar, total weighted geometric variability; crit, value of criterion defined in method.

C storage capacity can be disrupted by nutrient enrichment. The fertilized plots had *k* and *S* values that grouped closer toward grassland and forest ecosystems (Riggs et al. 2015), which have lower C storage potential because more available nutrients and oxic environments support aerobic respiration and higher decomposition rates. Results from this study provided support that nutrient enrichment can have a lasting influence on plant–soil–microbe interactions that affect C storage potential of wetland ecosystems (Lambers et al. 2009, Allison et al. 2014, Hartman et al. 2017).

Patterns in bacterial community composition but not the combined OTU richness, Shannon diversity, and Simpson's evenness diversity metrics, significantly explained decomposition rates. Further, litter composition followed by the fertilization and ditch effects determined bacterial community composition. A trend of higher decomposition rates was associated with high bacterial diversity, especially in the fertilized compared to unfertilized plots. In this study, the fertilized plots supported a more active and diverse bacterial community. The additional nutrients are fueling the increased decomposition rates. Evidence from another study conducted during this time indicated that metabolic diversity of carbon substrates (based on Biolog Ecoplates) revealed higher functional diversity in fertilized compared to unfertilized plots (Bledsoe 2020). 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 C losses from historically low-nutrient ecosystems that experience atmospheric nutrient deposition (Sardans and Peñuelas 2012, Wieder et al. 2015).

When bulk and tea litter-associated microbiomes were considered together, the bulk soil bacterial communities were very distinct from the tea litter-associated bacterial communities. The bulk soil reservoir provided a distinct source community that colonized the litter types. Bulk soil bacterial communities were distinct across fertilized and unfertilized dry plots; indicator taxa were phylogenetically distinct at the phylum level (Proteobacteria vs. Acidobacteria). At the wet plots, bulk soil bacterial composition in the fertilized and unfertilized were similar; the indicator taxa represented distinct phyla (unfertilized: Verrucomicrobia + Acidobacteria, fertilized: Actinobacteria). In contrast, the indicator taxa for tea litter-associated bacterial communities that overlapped in composition were similar at the phylum level. Specifically, similar bacterial assemblages of rooibos tea litter-associated (high

C:N litter) microbiomes in the fertilized and unfertilized, dry plots were associated with Proteobacteria. Indicator bacterial taxa representing high C:N ratio litter-associated microbiomes in fertilized, dry plots were *Dokdonella* spp. (Proteobacteria phylum) and unclassified Microbacteriaceae (Actinobacteria phylum). Nutrient availability is important to N transformations and pathogenicity. 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, Young 2008, Lory 2014). Indicator taxa within the order Alphaproteobacteria represented similar bacterial communities of the high C:N litter-associated microbiomes in unfertilized (indicator OTU *Acidisoma* spp.), wet plots and low C:N litter-associated microbiomes in unfertilized, dry plots (indicator OTU *Phenylobacterium* spp.). In wetter conditions, low C:N litter-associated microbiomes in unfertilized and high C:N litter-associated microbiomes in fertilized 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. In past studies, isolates were cultured from soils (*Dyella*) and wetland sediments (*Dongia*) and were found to be aerobic, Gram-negative, and motile (Xie and Yokota 2005, Baik et al. 2013). Further, these assemblages also overlapped with green tea litter-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 persist but are unlikely active under optimal conditions.

The interaction between source (litter type) and fertilization treatment influenced mass loss of litter but hydrologic setting and soil nitrate altered litter-associated microbiomes to varying degrees. The ditch-derived hydrologic differences at this wetland resulted in different structure–function relationships for high C:N ratio litter-associated microbiomes only. Under the relatively dry plots (i.e., near drainage ditch), microbiomes associated with high C:N ratio litter were similar, but mass loss of litter was higher in fertilized compared to unfertilized plots. In all other instances, distinct, fertilized microbiomes were associated with higher decomposition rates compared to unfertilized plots. In the case that community structure was the same and decomposition rates increased, this pattern provided some evidence of more severe N limitation in drier compared to wetter plots, where soil nitrate concentrations were below detection limits (Appendix S1: Table S1B). This result was only observed under high C:N ratio litter since the low C:N litter provided much needed N to litter-associated microbes. While soil ammonium levels are similar in this case, it is possible that N mineralization is occurring without limitation. However, microbial transformation of ammonium to nitrate via nitrification processes could be slowed due to low soil pH, resulting in differences in extractable soil nitrate but not ammonium concentrations (Hinckley et al. 2019).

In this study, nutrient enrichment and hydrologic status resulted in distinct bacterial communities associated with litter and bulk soils. Drier conditions and nutrient enrichment increased decomposition rates until N limitation constrained microbial community structure. This study provided the opportunity to compare the extent that community composition matters to decomposition rate—using model litter. In these nutrient-poor wetland soils undergoing nutrient enrichment, the fertilization increased bacterial diversity and litter decomposition. Soil organic C losses are expected to increase as wetlands are drained and fertilized.

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conceived and designed the research; MEK, RBB, and ALP collected and analyzed the data; MEK and ALP wrote the manuscript; all authors performed fieldwork and edited the manuscript. All code and data used in this study are located in a public GitHub repository (https://github.com/PeraltaLab/WRC18_RhizoTeaDecomp) and NCBI SRA (ID PRJNA635951).

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3619/full