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Fungal endophyte and tall fescue cultivar interact to differentially effect bulk and rhizosphere soil processes governing C and N cycling

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ABSTRACT

Tall fescue (*Lolium arundinaceum* (Schreb.)) is a cool-season perennial grass within which can live a fungal endophyte (*Epichloë coenophiala*) thought to provide enhanced edaphic and climactic stress tolerance to the host compared to non-infected individuals. Our prior research demonstrated that a variety of root exudate compounds released from tall fescue were differentially affected by tall fescue cultivar, endophyte genotype and their interaction. Changes in root exudates and associated microbial communities could influence soil processes, including carbon and nitrogen cycling, but these effects may differ depending on fescue and endophyte genetics. To test this, we collected rhizosphere and bulk soil samples from six year old field plots located in Lexington, KY planted with two different tall fescue cultivars (PDF and 97TF1), each containing four endophyte treatments [endophyte-free (E−) or infected with one of three strains of *E. coenophiala* (common toxic, novel AR542E+ and novel AR584E+)]. The influence of fescue cultivar, endophyte strain, and soil sample location (rhizosphere vs. bulk) were assessed for soil organic carbon, soil organic nitrogen, particulate and non-particulate organic matter (POM and n-POM, respectively) - C and – N, and dissolved organic carbon and nitrogen (DOC, DON) pools. Soil functional aspects were evaluated by measuring soil respiration and the activity of seven different soil enzymes related to C, N and P cycling. We found that rhizosphere soils had greater microbial biomass, potential enzyme activity, and oxygen utilization, but lesser POM-N concentrations than bulk soils. In rhizosphere soils, tall fescue cultivar had the greatest influence on soil microbial community structure, while endophyte genotype had a stronger influence on soil C fractions (notably POM-C). Changes in root system architecture, biomass, and tissue composition, together with root exudate chemistry, which we have shown in previous studies to be affected by endophyte infection and fescue cultivar, likely explain these findings. There was greater POM-N in bulk soils which was influenced by the interaction of endophyte and cultivar potentially due to differences in the chemical composition of the tissues brought about by this interaction. Our results support current observations that tall fescue cultivar and fungal endophyte strain influence soil C and N cycling and, by analyzing bulk and rhizosphere soils separately, go further to show the level of influence cultivar and endophyte have within each of these compartments.

1. Introduction

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh. = *Schedonorus arundinaceus* (Schreb.) Dumort., formerly *Festuca arundinacea* Schreb. var. *arundinacea* Schreb.] (Gibson and Newman, 2001), is a perennial, cool season bunchgrass thought to have come to the United States in the 1800's from Europe. 'Kentucky 31' is a well-known variety of tall fescue and is often infected by a fungal endophyte, *Epichloë coenophiala* (formerly *Neotyphodium coenophialum*). *E. coenophiala* is an asexual species (heteroploid) persisting from generation to generation by infecting the host plant seeds in a process called 'vertical transmission' (Young et al., 2014).

Secondary metabolites, such as ergot and loline alkaloids and phenolic compounds, produced by the endophyte (Luu et al., 1982; Arechavaleta et al., 1992; Bush et al., 1993) are thought to improve the plants resistance to biotic stresses from mammalian herbivores (Clay et al., 2005), phytophagous insects (Johnson et al., 1985; Clay and Cheplick, 1989), and pathogens (Clement et al., 1990; Chu-chou et al., 1992; Timper et al., 2005; Finkes et al., 2006; Bacetty et al., 2009), as well as improving tolerance to abiotic stresses like water deficit (Elmi et al., 2000; Makino et al., 2007; Bayat et al., 2009) and nutrient deficiency (Lyons et al., 1986; Malinowski et al., 1998b; Najeeb et al., 2011). Aside from the positive aspects of hosting *E. coenophiala* for the plant, there are negative aspects for animals that feed on these infected tissues. Namely, ergot alkaloids are thought responsible for weight loss and reduced fecundity in economically important ruminants (Hurley et al., 1980; Hemken et al., 1981; Schmidt and Osborn, 1993; Paterson et al., 1995; Beck et al., 2008; Sabzalian and Mirlohi, 2010). As a result of losses in animal productivity, there has been substantial effort to replace the 'common toxic endophyte,' which produces ergot alkaloids, with 'novel', non-mammal-toxic endophyte strains that retain many of the advantages of common toxic endophyte infection, but are not toxic to grazing ruminants (Phillips and Aiken, 2009).

In addition to losses in animal productivity, research has shown that the above ground endophytic association also influences below ground biogeochemical processes controlling nutrient cycling and soil organic matter turnover. These processes are important factors in

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the productivity and function of both natural and agro-ecosystems. The first of these studies appeared in 1999 where the authors found that a pasture in Georgia dominated by common toxic endophyte infected (CTE+) tall fescue had higher soil organic carbon (C) and nitrogen (N) concentrations and potentially lower microbial activity than soils from adjacent stands of endophyte free (E−) tall fescue (Franzluebbers et al., 1999). Similarly, particulate organic C and non-particulate organic C were found to be higher in soils dominated by CTE+ tall fescue (Franzluebbers and Stuedemann, 2002). In another study conducted at multiple sites across the Southeastern U.S., endophyte infection (CTE+) significantly increased soil C pools compared to E− stands (Iqbal et al., 2012). However, a 60 day short-term study found no detectable changes in C and N fractions related to endophyte infection status (Franzluebbers, 2006). Similarly, total C and particulate C were also not affected by endophyte infection after four years of observation in another recent study, and opposite to what has been observed, microbial biomass carbon (MBC) was higher under CTE+ than E− stands (Handayani et al., 2011). Given the diversity of responses observed to date, further evaluation of the mechanisms underlying the tall fescue-endophyte symbiosis and how it influences soil nutrient pools is warranted.

Several factors could account for endophyte-related alterations to soil processes, including higher biomass production by CTE+ tall fescue, differences in litter decomposition rates, leaching of toxic alkaloids into the soil, or alterations in rhizodeposits. Rhizodeposits are composed of compounds such as sugars, amino acids, phenolics, organic acids, mucilage and root boarder cells and represent a primary input (roughly, 11–40% of net fixed C) of plant C below ground. Rhizodeposits provide a direct link between the plant and soil microorganisms which interact with the surrounding soil, giving rise to the rhizosphere (Malinowski et al., 1998a; Jones et al., 2004; Schmidtke, 2005; Van Hecke et al., 2005; Broeckling et al., 2008). In our recent research (Guo et al., 2015) we found that tall fescue cultivar, endophyte presence and strain [E−, CTE+, or novel endophyte infected] and their interaction influenced the chemical composition of root exudates. Since rhizodeposits are a primary energy source for microbial communities which in turn control nutrient cycling in soils, the observed changes in root exudate composition led us to question whether endophyte strain, tall fescue cultivar or their interaction differentially influence soil biogeochemical processes.

Several studies have found that endophyte presence alters the soil microbial community. For example (Jenkins et al., 2006), found suppression of the high G+C gram-positive bacterial community in bulk clay loam soil and the Δ-proteobacterial community in rhizosphere clay loam soil associated with CTE+ tall fescue in a 60-week mesocosm study. Interestingly, Buyer et al. (2011) found subtle effects of endophyte infection on soil microbial parameters, that varied over time and soil type during a year-long mesocosm experiment (Buyer et al., 2011). Given that soil microorganisms, supported by C from rhizodeposits, are key factors in regulating organic matter decomposition (Rodriguezkabana et al., 1978; Robinson et al., 1989; Frederick and Klein, 1994), a better understanding of how endophyte infection alters soil microbial communities may improve our ability to predict effects on soil organic matter pools.

Most research to date has focused on how common toxic endophyte infection influences soil biogeochemical processes with much less research on the influence of the newer novel endophyte strains. Furthermore, the majority of existing work has not explored whether the effect of endophyte on below ground processes varies across plant genetic background (i.e., cultivar). To address these unknowns we used research plots containing stands supporting combinations of two tall fescue cultivars (97TF1 and PDF) with four *E. coenophiala* statuses: endophyte-free (E−), common toxic endophyte infected (CTE+), or infected with one of two different novel endophytes (AR542E+ and AR584E+). Because endophyte and cultivar effects may exert stronger influence at the plant-root-soil interface, we sampled two discrete soil regions: rhizosphere soils and bulk soils taken from the root zone but not tightly bound to roots. We examined how soil microbial community structure, function, and C & N cycling differed between and within these two soil compartments, and how they were affected by fescue cultivar, endophyte strain, and their interaction.

2. Materials and methods

2.1. Site description and soil sampling

Plots used in this study were established in 2006 as part of a grazing preference trial at the University of Kentucky Spindletop research farm in Lexington KY, USA (38° 08′ 03″ N, 84° 29′ 56″ W). The experimental design was a randomized complete block with a factorial arrangement of tall fescue cultivar and endophyte combinations. Nitrogen fertilization (56 kg ha^{-1}) was applied in October every year from 2006 to 2011 with periodic mowing 3–4 times per year. The whole experiment was grazed for two, one day intervals with either four (2008) or five (2009), 15 month old beef steers. No cattle grazing occurred after 2009. The mean annual temperature at the site is 13 °C, with 1124 mm mean annual rainfall. Included in each block were the tall fescue cultivars PDF and 97TF1 (The Samuel Roberts Noble Foundation, Ardmore, OK) each without *E. coenophiala* infection (E−), infected with common toxic endophyte (CTE+), or infected with one of two novel endophyte genotypes (AR542E+ and AR584E+; AR = AgResearch; Hamilton, New Zealand). In 2008, endophyte infection rates were ≥82% in E+ plots (CTE+ and novel endophytes) and ≤14% in the E− plots. Bluegrass-Maury silt loam (fine mixed, active, mesic Typic Argiudolls) soils were collected under each tall fescue cultivar and endophyte combination at the height of the plant growing season in mid-April 2012 from seven blocks. Bulk soil, defined here as soil not directly attached to tall fescue roots, was collected by removing several whole plants from each plot and gently shaking the plants to release the soil. Rhizosphere soil, defined here as the soil tightly adhering to the root surface, was then collected by physically brushing the soil from the root surface with a sterile soft-bristled paintbrush. Soil texture (silt loam) was uniform across the site and pH (1:10, soil:water slurry) was not significantly different (~pH 5.9) between soil regions, or due to endophyte, cultivar or their interaction (Table 1, Tables S1 and S2). Samples were immediately passed through a 2 mm sieve removing any obvious root debris in the process, and then placed in pre-labeled plastic bags and stored on dry ice for transport back to the lab.

2.2. Analysis of carbon and nitrogen fractions

Carbon and nitrogen associated with particulate and non-particulate organic matter (POM and n-POM, respectively) was determined following the procedure of Iqbal et al. (2012). Briefly, 30 g of air dried soil was combined with 90 ml 5% sodium hexametaphosphate (NaHMP), and placed on a shaker for 18 h. The dispersed samples were then slowly poured onto a 53 μm screen and washed with 2 L DI water. The material retained on the screen (>53 μm) is considered POM the mass of which was determined after transferring to a pre-washed tin and oven drying at 105 °C. A 130 ml subsample of the <53 μm fraction, considered n-POM, was collected in a Nalgene bot-

Table 1
ANOVA test results for the fixed effects of tall fescue cultivar (PDF and 97TF1), endophyte strain (E−, CTE+, AR584E+ and AR542E+) and their interaction on different soil properties within bulk and rhizosphere soils. Bold values emphasize statistically significant differences ($P \leq 0.05$).

	Tall fescue cultivar				Endophyte strain				Interaction			
	Bulk		Rhizo		Bulk		Rhizo		Bulk		Rhizo	
	F	P	F	P	F	P	F	P	F	P	F	P
Soil property												
TOC	3.12	0.08	0.15	0.71	1.31	0.28	2.54	0.07	0.76	0.52	1.43	0.25
TON	4.12	**0.05**	0.00	0.97	0.65	0.59	2.72	0.06	1.73	0.17	0.38	0.77
TC:TN	0.21	0.65	0.78	0.38	1.34	0.27	0.21	0.89	1.58	0.21	2.77	**0.05**
DOC	3.11	0.09	0.01	0.92	1.20	0.32	0.11	0.96	0.20	0.90	0.18	0.91
DON	0.17	0.68	0.79	0.38	2.22	0.10	0.19	0.90	1.32	0.28	0.40	0.75
POM-N	0.26	0.62	0.01	0.93	1.06	0.38	2.40	0.08	3.25	**0.03**	1.33	0.28
POM-C	0.02	0.89	0.00	0.83	0.66	0.58	3.84	**0.02**	2.69	0.06	1.40	0.26
POM C/N	0.69	0.41	0.25	0.62	0.75	0.53	1.28	0.29	1.05	0.38	0.88	0.46
n-POM-N	0.51	0.48	0.25	0.62	0.05	0.99	0.10	0.96	1.03	0.39	0.35	0.79
n-POM-C	0.22	0.64	0.77	0.39	0.26	0.86	0.21	0.89	0.73	0.54	0.30	0.82
n-POM C/N	0.30	0.59	1.87	0.18	1.58	0.21	1.27	0.30	0.62	0.61	0.43	0.74
pH	0.03	0.86	0.01	0.93	0.25	0.86	0.19	0.90	0.31	0.82	0.04	0.99
Oxygen utilization												
BOU	3.90	**0.05**	1.42	0.24	0.58	0.63	2.81	**0.04**	0.75	0.52	4.19	**0.01**
SIOU	0.11	0.74	0.30	0.58	0.77	0.51	1.58	0.20	1.66	0.18	2.90	**0.04**

TOC = Total Organic Carbon; TON = Total Organic Nitrogen; DOC = Dissolved Organic Carbon; DON = Dissolved Organic Nitrogen; POM-N = Particulate Organic Matter Nitrogen; POM-C = Particulate Organic Matter Carbon; n-POM-N = Nonparticulate Organic Matter Nitrogen; n-POM-C = Nonparticulate Organic Matter Carbon; BOU = Basal Oxygen Utilization; SIOU = Substrate Induced Oxygen Utilization.

tle, freeze dried, and then weighed. All samples were then ground in a ball mill, tested for the presence of significant amounts of inorganic C using 1 N HCl (no inorganic C was observed), and analyzed for total C and N on a Flash Elemental Analyzer 1112 (Thermo Fisher Scientific Inc., Waltham, MA).

Dissolved organic carbon and nitrogen (DOC and DON, respectively) were determined by first preparing a 1:5 (soil: DDI water) soil suspension in 50 ml falcon tubes, shaking for 1 h, and then centrifuging at 8000 rpm for 10 min, after which the supernatant was filtered through 0.45 μm membrane and stored at −20 °C until analysis. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were analyzed using a TOC-V_{CPN} total organic carbon analyzer (Shimadzu, Japan). Sub-samples from the filtered supernatant were taken for NO_3^--N and NH_4^+-N analysis using an automated colorimetric technique (Crutchfield and Grove, 2011). Dissolved organic nitrogen (DON) was calculated as the difference between the sum of NO_3^--N and NH_4^+-N and TDN (Jones and Willett, 2006).

Total organic carbon (TOC) and total nitrogen (TN) concentrations were determined by drying 2 g of already air dried soil at 55 °C for 48 h, then grinding the sample in a ball mill and analyzing on a Flash Elemental Analyzer 1112 NC (Thermo Fisher Scientific Inc., Waltham, MA). Prior to analysis samples were tested using 1 N HCL to determine if they had a significant amount of inorganic C (no reaction was observed).

2.3. Potential enzyme activity

Potential enzyme activity (PEA) of β-Glucosidase (BG), β-Xylosidase (BX), Cellobiohyrolase (CB), β-*N*-acetylglucosaminidase (NAG), Leucine aminopeptidase (LAP), and Acid Phosphatase (AP) (Weintraub et al., 2007) were measured in all rhizosphere and bulk soil samples using a 96 well microtiter plate method described by DeForest (2009) based on Saiya-Cork et al. (2002) with optimization of the incubation times (Saiya-Cork et al., 2002; DeForest, 2009). Briefly, 1 g of soil was added to 60 ml of 50 mM sodium acetate buffer (pH = 6) in a Nalgene bottle, a stir bar was added, the sample vortexed for 1 min and then allowed to stir at 800 rpm for 2.5 min. The soil slurry was then poured into a flat bottom 250 ml, 90 × 50 mm Pyrex® crystallizing dish, the bottles rinsed three times with 25, 20 and 20 ml of buffer, respectively, and then the slurry set to stir at 100 rpm for 3 min. After 3 min, 200 μl of the continuously stirring soil slurry was transferred into black 96 well plates using an eight channel pipet.

Plates were set up for the analysis of three soils per plate as described by DeForest (2009) and included blank, reference standard, quench and negative control wells, as well as sample and sample control wells. Blank wells contained 250 μl of sodium acetate buffer only. Reference standard and quench wells contained 200 μl sodium acetate buffer or 200 μl soil slurry, respectively, and 50 μl of 10 μM 4-Methyllumbelliferyl (MUB) for all enzymes except LAP which used 100 μM aminomethylcoumarin (AMC). Negative control wells contained 200 μl sodium acetate buffer and 50 μl of a 200 μM MUB-linked substrate. The sample control wells contained 200 μl soil slurry and 50 μl sodium acetate buffer. And finally, the assay wells contained 200 μl soil slurry and 50 μl of a 200 μM MUB-linked substrate solution. The incubation began with the addition of the substrate at which point the plates were covered and incubated at room temperature in a dark drawer for 30 min (NAG, AP), 2 h (BG, BX, CB, PER), or 24 h (LAP). After the incubation, 10 μl of 0.5 M NaOH was added into each well to enhance the fluorescence and to stop the reaction. Exactly one minute after adding the NaOH, fluorescence was measured on a Wallac 1420 Victor^2 multilabel fluorescence reader (Perkin-Elmer Inc.) using λ = 355 nm excitation and λ = 450 nm emission filters.

Assays for potential peroxidase (PEROX) activity were conducted in 300 μl clear microtiter plates by adding 50 μl of 25 mM L-dihydroxyphenylalanine (L-DOPA) and 10 μl of 0.3 wt % hydrogen peroxide as the substrate and loaded on the plate to react with soil slurries for photometric measurement at 480 nm. Activity for all enzymes was calculated as shown in DeForest et al. (2009). Data are presented as difference in potential enzyme activity under each endophyte infected treatment relative to the control (E− soil for each cultivar) and

were calculated as follows: relative PEA = endophyte infected (CTE+, AR584E+, or AR542E+) PEA - endophyte free PEA.

2.4. Microbial community structure – phospholipid fatty acid analysis (PLFA)

Phospholipids were extracted from soil samples using the high-throughput methodology described in Buyer and Sasser (2012). Briefly, phospholipids were extracted from freeze dried soil samples in Bligh-Dyer extractant containing internal 19:0 (1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine) standard for 2 h by rotating end-over-end. Samples were then centrifuged for 10 min, the liquid phase transferred to 13 × 100 mm test tubes and 1.0 ml chloroform and deionized water were added. After vortexing for 10 s, the samples were centrifuged for 10 min, the top phase removed, and the lower phase containing the phospholipids evaporated to dryness. Lipid separation was achieved by solid phase extraction (SPE) using a 96 well SPE plate (Phenomenex, Torrance, CA, USA). The dried samples were dissolved in 1 ml hexane and loaded onto the SPE column followed by two 1 ml additions of chloroform and 1 ml of acetone. Phospholipids were then eluted from the column into new vials using 0.5 ml of a 5:5:1 methanol:chloroform:H_2O mixture. The transesterification reagent (0.2 ml) was then added and the samples incubated at 37 °C for 15 min. After incubation acetic acid (0.075 M) and chloroform (0.4 ml) were added, the sample was quickly vortexed and then allowed to separate, after which the bottom phase was removed and evaporated to dryness. The extract was then dissolved in 0.7 μl of hexane and the fatty acid methyl esters (FAME) detected on an Agilent (Agilent Technologies, Wilmington, DE, USA) 7890 gas chromatograph (GC) equipped with automatic sampler, an Agilent 7693 Ultra 2 column, and a flame ionization detector. The carrier gas was ultra-high-purity hydrogen gas with a column split ratio of 30:1. The oven temperature was increased from 190 °C to 285 °C and then to 310°Cat a rate of 10 °C/min and 60 °C/min, respectively. FAME identities and relative percentages were automatically calculated using MIDI methods (Sherlock Microbial Identification System version 6.2, MIDI Inc., Newark, DE) described by Buyer and Sasser (2012).

2.5. Microbial respiration via oxygen consumption

Recent studies using a fluorescence based microplate platform for quantifying oxygen consumption have proven to be a rapid tool for assessing basal respiration and carbon source utilization by the soil microbial community (Garland et al., 2003, 2012; Zabaloy et al., 2008). In this study we used 96 well plates with a fluorescent oxygen sensor built into the bottom of each well (Oxoplate™, PreSens, Regensburg, Germany) to measure both basal and substrate induced oxygen utilization. Per the manufactures recommendation, the fluorescent oxygen indicators at the bottom of the Oxoplates™ were pre-equilibrate prior to the assay by adding 100 μl (basal oxygen utilization) or 50 μl (substrate induced respiration) DI water to each well and allowing them to sit for 1 h. To begin the assay, just prior to completion of the 1 h equilibration period, two grams of either bulk or rhizosphere soil was mixed with 20 ml deionized water (1:10, soil:water slurry) in a 50 ml falcon tube, vortexed for 1 min to break up any large aggregates, and then transferred to a flat-bottomed crystallization dish and continuously stirred at 200 rpm for 1 min. Then, 100 μl of the soil slurry was transferred to eight replicate wells of the pre-equilibrated Oxoplate™ using a multichannel pipette. The wells used for substrate induced respiration received another 50 μl of a 10 g L^{-1} glucose solution to reach a final glucose concentration of 2.5 g L^{-1} and a final volume for both basal and substrate-induced assays of 200 μl. Two rows of 8 replicate wells were reserved for oxygen-free and oxygen-saturated water, respectively, which are used for calibration to determine percent oxygen consumption per the manufacturer's directions. A transparent film dressing (DermaRite Industries LLC, Paterson, NJ) was then applied to seal the Oxoplate™. Fluorescence intensity of the phosphorescent indicator on the bottom of the plate was measured every 10 min during a 360 min incubation on a Wallac 1420 Victor^2 multilabel plate reader (Perkin-Elmer Inc.) using λ = 540 nm excitation and λ = 650 nm emission filters for the indicator ($I_{indicator}$), and λ = 540 nm excitation and λ = 590 nm emission filters for the reference dye ($I_{reference}$). Oxygen partial pressure (pO_2) in % air saturation for each measurement point was calculated using $pO_2 = 100* (k_0/I_R-1)/(k_0/k_{100}-1)$. I_R is the referenced signal of each well calculated via $I_{indicator}/I_{reference}$, k_0 was calculated by taking the average of the signals I_R of the wells containing oxygen-free water; k_{100} was calculated by taking the average of the signals I_R of the wells containing air-saturated water. Oxygen consumption curves were created and total O_2 consumption determined using Sigma Plot Version 12.3 (SPSS Inc., Chicago, IL) by calculating the area under each curve using the trapezoid rule.

2.6. Statistical analysis

Analysis of variance (ANOVA) was conducted on soil C and N fractions and enzyme activity data using a general linear model in JMP Version 10.0 (SAS Institute, Cary, NC) testing first for the effect of soil region (bulk and rhizosphere) and secondly for the effects of endophyte, cultivar and endophyte by cultivar interactions within each soil region. Data not normally distributed were log transformed prior to analysis. Soil region, endophyte status and tall fescue cultivar were treated as fixed effects and block as a random effect. For statistical analysis of the PLFA data, the concentration of individual fatty acids were summed into the following biomarker groups: gram-positive bacteria (G+; iso and anteiso branched), gram-negative bacteria (G-; monounsaturated, cyclopropyl 17:0 and 19:0), actinobacteria (10-methyl fatty acids), general fungi (18:2 ω6c), arbuscular mycorrhizae (16:1 ω5c), and protists (20:3 ω6c and 20:4 ω6c). The proportional abundance of each of the biomarker groups was also calculated by taking the concentration of the biomarker group and dividing by the total concentration of all biomarker groups in that sample. Stress (e.g. heat, desiccation, nutrient deprivation) has been shown to increase microbial membrane fluidity, and changes in membrane PLFA composition in response to the stress is seen as a tolerate mechanism (Kaur et al., 2005). To estimate the level of stress, the total saturated fatty acid to total monounsaturated fatty acid ratio (sat/mono), which increases with stress, was calculated and used in this study (Kieft et al., 1994). Concentrations and proportions of the microbe biomarker groups were treated as continuous response variables and analyzed using a generalized linear mixed model (PROC GLIMMIX, SAS v.9.3), which does not require that the original data be normally distributed (Stroup, 2014). Lognormal and beta distributions with identity and logit link functions, respectively, were fitted to the microbial biomarker group concentration and proportion data, respectively, using the GLIMMIX procedure of SAS. Where the influence of soil region, cultivar, endophyte status and cultivar by endophyte interactions on the environmental variables were found significant, differences between the means were determined using the Student's t-test at a probability level of $P < 0.05$.

To evaluate how soil region, tall fescue cultivar, endophyte status, and their interaction influence microbial community structure, micro-

bial biomarker group concentrations were first Hellinger transformed (Ramette, 2007) before creating a non-metric multidimensional scaling (NMDS) ordination with Sorensen (Bray-Curtis) distances using the slow and thorough settings of the autopilot mode in PC-ORD (version 6.0, MjM Software, Gleneden Beach, OR). A multi-response permutation procedure (MRPP) in PC-ORD was then used to determine if microbial community composition differed significantly between soil region (rhizosphere vs. bulk), tall fescue cultivar (97TF1 vs. PDF), endophyte status (E−, CTE+, AR542E+, AR584E+), or the cultivar and endophyte pairs within each soil region with the null hypothesis being that these groups will not be different. A Bonferroni's correction was applied to the p-values to correct for multiple comparisons. In MRPP, a small p-value indicates that the predefined grouping variables (soil region, cultivar, endophyte or cultivar and endophyte pairs) are more different than expected by chance. The effect size is reflected in the A-value, the chance corrected within-group agreement, which indicates the similarity of samples within a group. A = 1 if the samples in a group are identical, and A is closer to zero if their heterogeneity is higher than expected by chance. An A value > 0.3 for ecological data is considered high.

NMDS was also used to determine relationships between environmental variables and PLFA microbial biomarker groups by correlating soil physicochemical parameters with axis scores in the NMDS ordination. Those parameters that had an associated r^2 value of 0.300 or greater were overlaid on the NMDS ordination as a biplot. The direction and length of the biplot vectors indicate the direction (positive or negative) and strength of the correlation while the angle between vectors indicates the correlation between environmental variables (small angles = higher correlation).

3. Results

Values for all soil parameters were significantly greater in the rhizosphere compared to bulk soils with the exception of POM-N and the nPOM C:N (Tables S1 and S2). Potential enzyme activities for NAG, BG, BX and CB were significantly greater in rhizosphere compared to bulk soils (Table S2) and, while not significant, enzymes AP and LAP followed the same trend ($p = 0.07$ and 0.08, respectively). The only exception to this trend was the potential enzyme activity for PER which was significantly greater in bulk soils. Basal oxygen and substrate induced oxygen utilization were both significantly greater in rhizosphere soils.

Within bulk soils, significant cultivar effects were observed for TN and basal oxygen utilization (BOU) (Table 1). Cultivar PDF had ~3% greater TN and BOU than cultivar 97TF1 (Figs. 1a and 2a). Although only significant at a $p = 0.08$ and 0.09, respectively, TOC was ~3% greater and DOC was 17% lesser in bulk soils associated with PDF vs. 97TF1 (Table 1 and Table S1). There was no significant endophyte main effect observed in bulk soils, but the interaction of cultivar with endophyte influenced POM-N (Table 1). For PDF, infection with CTE+ and AR542E+ resulted in significantly greater (~18%) bulk soil POM-N concentrations than AR584E+, with E− in between. While in 97TF1, E− had on average 17% more POM-N than all endophyte infected material (Fig. 1b). POM-C concentrations in bulk soils followed the same trend as POM-N, however the differences were only significant at $P = 0.06$ (Table 1 and Table S1).

Within rhizosphere soils, no significant cultivar main effects were identified, but an endophyte effect was observed for POM-C (Table 1). CTE+ infected tall fescue had significantly greater POM-C than E− and AR542E+ infected combinations (Fig. 1c). The concentration of POM-C in rhizosphere soils under AR542E+ infected tall fescue

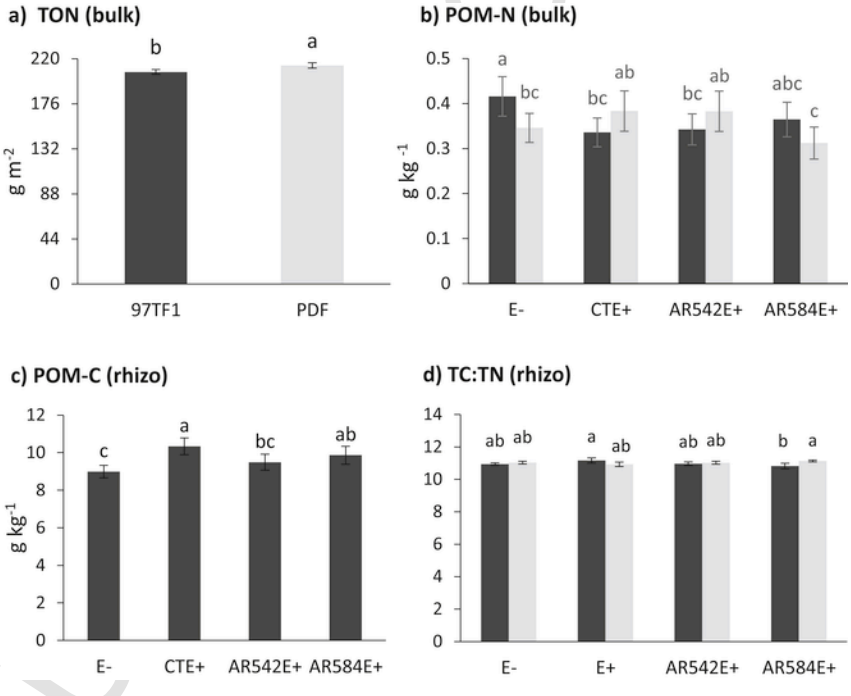

Fig. 1. Total nitrogen in bulk soil a) as affected by tall fescue cultivar, particulate organic matter nitrogen (POM-N) in bulk soils b) as influenced by the interaction of cultivar and endophyte, particulate organic carbon (POM-C) in rhizosphere soil c) as influenced by endophyte status averaged across cultivars, and the TC:TN ratio in rhizosphere soils influenced by the interaction of cultivar and endophyte. Cultivars are indicated in panels (b) and (d) following the colors in panel (a). Error bars indicate one S.E. of the mean. Different letters indicate significant differences among tall fescue cultivar or endophyte status ($P < 0.05$).

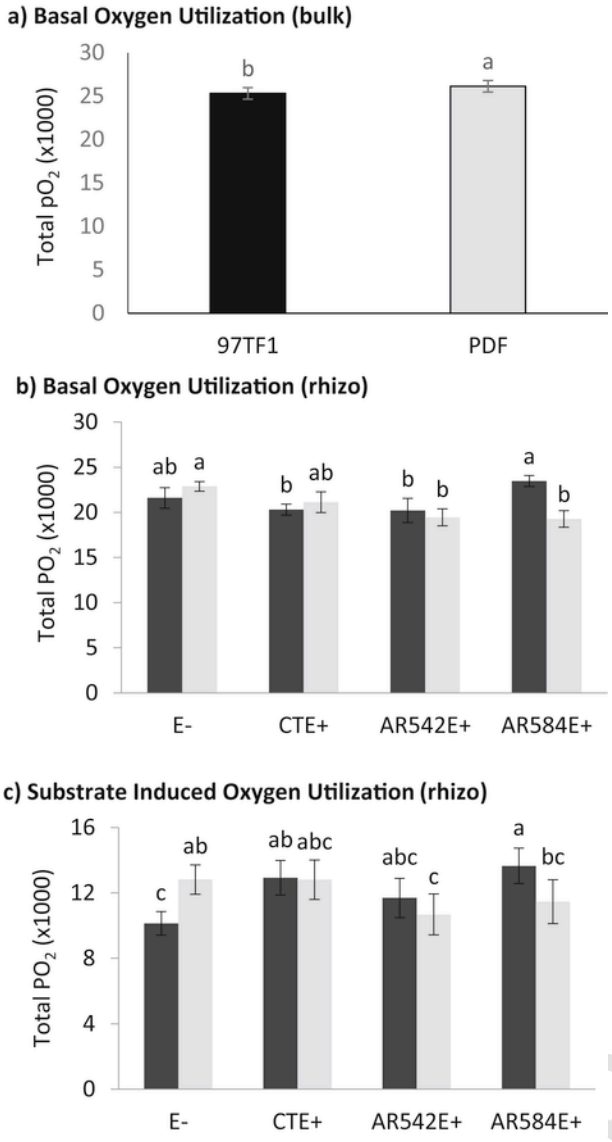

Fig. 2. Comparison of a) mean oxygen utilization in bulk soils significantly affected by cultivar, b) mean basal oxygen utilization and c) mean substrate induced oxygen utilization (±S.E.) in rhizosphere soils under different endophyte and tall fescue cultivar combinations. The bars represent the area under the oxygen utilization curve measured over a 360 min incubation. Note that larger bars indicate lower oxygen utilization. Different letters indicate significant differences among tall fescue cultivar and endophyte status combinations ($P < 0.05$). Black bars are cultivar 97TF1 while gray bars are cultivar PDF.

was intermediate to that observed in soils under CTE+ and AR584E+ infected tall fescue. Values for TN, TC and POM-N in rhizosphere soils followed the same trend as POM-C (Table S1), however the differences were only significant at $P = 0.07$, 0.06 and 0.08, respectively (Table 1). The interaction of tall fescue cultivar and endophyte status had a significant effect on basal oxygen utilization, substrate induced oxygen utilization and TC:TN (Table 1, Fig. 1d). Rhizosphere soils under PDF/E− had significantly lower oxygen utilization than those associated with either novel endophyte infected PDF; however for 97TF1, soils under plants infected with AR584E+ had significantly lower oxygen utilization than those under CTE+ and AR542E+ infected plants, with E− in between (Fig. 2b). For substrate induced oxygen utilization, soils under 97TF1 E− plants had greater oxygen utilization than those from soils under CTE+ and AR584E+, while soils under PDF E− had the lowest rates and differed significantly only from those under PDF infected with AR542E+ (Fig. 2c). Differences in TC:TN across cultivar and endophyte treatments were small (Fig. 1d), with the only significant difference between endophyte strains occurring in 97TF1 material (CTE+ > AR584E+) and the only cultivar difference observed in AR584E+ material (PDF > 97TF1). For the soil enzyme data, only one significant main effect was identified in rhizosphere soils for LAP activity (Table S3). The mean difference in potential LAP activity in rhizosphere soils under endophyte infected relative to endophyte free 97TF1 was significantly lower than similar soils under PDF, indicating that for 97TF1 endophyte infection (irrespective of strain) lowered potential LAP activity, whereas for PDF, endophyte infection increased potential LAP activity.

The NMDS ordination for bulk and rhizosphere soil microbial biomarker groups produced a 2 dimensional solution with a final stress of 8.46 after 68 iterations (Fig. 3). In the orientation shown, axis 1 explained 87.5%, and axis 2 explained 9.8% of the variation for a total of 97.3%. Microbial communities in the rhizosphere and bulk soils separated along axis 1 and 2 of the NMDS plot and were significantly different (MRPP A = 0.4275, $p < 0.001$). Rhizosphere microbial communities were correlated with greater TC (axis 1 $r^2 = 0.334$, axis 2 $r^2 = 0.347$), TN (axis 1 $r^2 = 0.303$, axis 2 $r^2 = 0.332$), POM-C (axis 1 $r^2 = 0.430$, axis 2 $r^2 = 0.344$), n-POM N (axis 1 $r^2 = 0.249$, axis 2 $r^2 = 0.182$), and POM-C/N (axis 1 $r^2 = 0.635$, axis 2 $r^2 = 0.448$), while the bulk soils were correlated with greater POM-N (axis 1 $r^2 = 0.269$, axis 2 $r^2 = 0.180$). While microbial communities were different between bulk and rhizosphere samples (Table S4), within each of these compartments (Fig. 3a and b) there were no significant cultivar (MRPP A < −0.0085, p = 0.4921), endophyte (MRPP A < 0.028, p > 0.104), or interaction (MRPP A < 0.056, p > 0.0686) effects on microbial community composition.

Tall fescue cultivar, endophyte, or their interaction had no effect on the relative proportion of microbial biomarker groups within bulk or rhizosphere soils; the only exception being protists. There was a significant cultivar by endophyte interaction that resulted in greater relative abundance of protists under CTE+ infected 97TF1; although this was largely due to two of the seven replicates (seen in the upper left and middle right of Fig. 3a) having a greater proportion of protists (Table S5). While the relative abundances were mostly unaffected, analysis of concentrations (Table S5) showed that total PLFA, total bacteria, G+ and G- bacterial biomarkers in rhizosphere soils were significantly greater under cultivar PDF than 97TF1 (Table 2). There was also a significant cultivar by endophyte interaction on the total saturated/total monounsaturated fatty acid ratio (Table S5) used as an indicator of stress in both rhizosphere and bulk soils (Fig. S1). In bulk soils, the stress ratio under cultivar 97TF1 infected with AR542E+ was significantly greater than the other endophyte statuses; while in PDF, the stress ratio in soils under plants infected with AR542E+ was similar to CTE+ and E−, with AR584E+ having the lowest ratio. In rhizosphere soils, CTE+ infection in 97TF1 resulted in the highest stress ratio, while in PDF, soils under plants infected with AR542E+ had the highest stress ratio.

4. Discussion

We observed significant endophyte and cultivar effects on exudate chemistry and root biomass in our previous study (Guo et al., 2015), and, therefore, expected cultivar and endophyte effects to be most evident in the rhizosphere soils of this study. Overall, rhizosphere soils

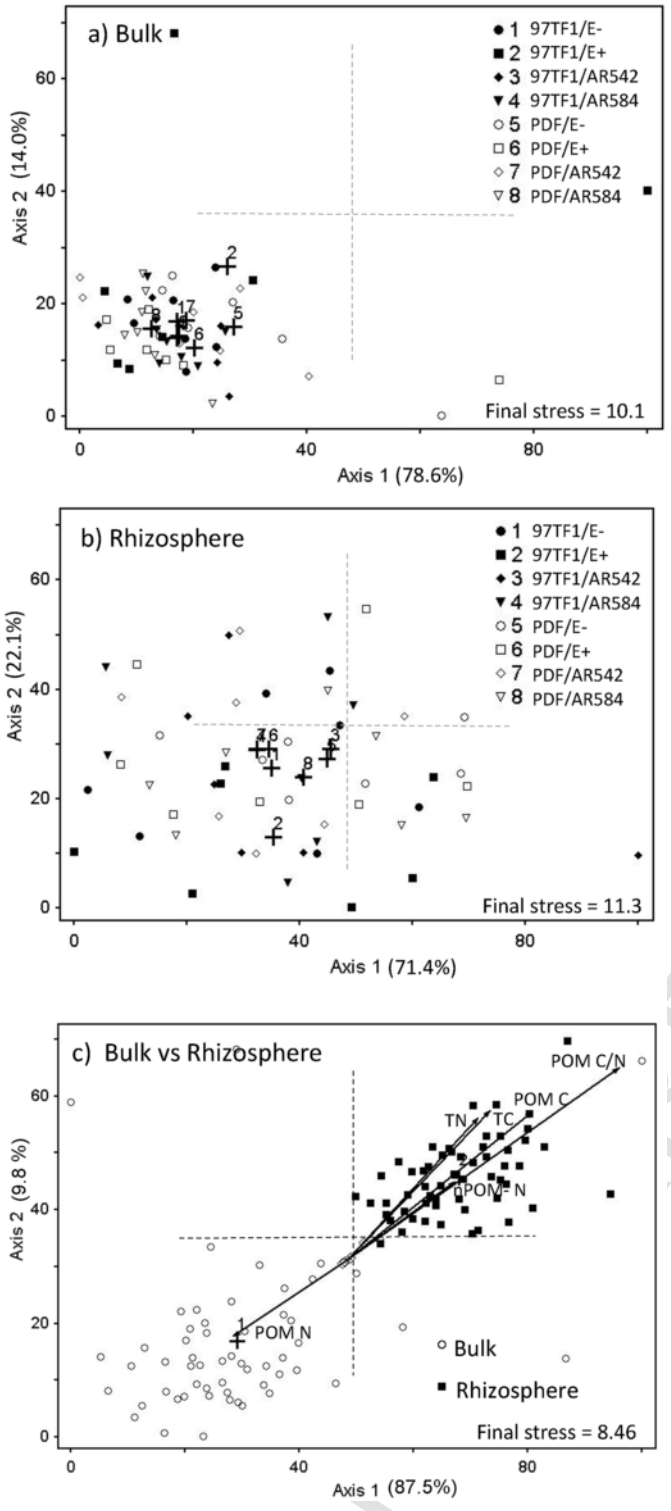

Fig. 3. NMDS ordinations of grouped microbial PLFAs from a) bulk and b) rhizosphere soils under cultivars 97TF1 and PDF uninfected (E−) or infected with novel (AR542E+, AR584E+) or common toxic (CTE+) fungal endophytes. Panel c) shows an NMDS ordination of grouped microbial PLFAs comparing bulk vs. rhizosphere soils where environmental variables with an $r^2 > 0.300$ between the variable and the axis score are displayed as vectors (scaled to 150%) indicating the strength and direction of the relationship. Numbered plus signs (+) in all panels indicate the centroid of each combination as indicated in the figure legend. TC = total carbon; TN = total nitrogen, nPOM-N = non-particulate organic matter nitrogen, POM-N = Particulate organic matter nitrogen, POM C = particulate organic matter carbon, POM C/N = ratio of POMC to POM N.

Table 2
Mean (±s.d.) PLFA microbial biomarker concentrations (nmol g^{-1}) in rhizosphere soils of tall fescue cultivars 97TF1 and PDF. Different letters represent a significant difference between 97TF1 and PDF ($P < 0.05$). Bold values indicate microbial biomarker group concentrations that are significantly different between 97TF1 and PDF.

Biomarker group	97TF1	PDF
Gram +	**72.72 ± 0.97**[b]	**75.77 ± 0.83**[a]
Gram −	**139.56 ± 1.59**[b]	**144.28 ± 1.51**[a]
Actinobacteria	39.21 ± 0.63[a]	40.08 ± 0.56 [a]
General fungi	10.23 ± 0.47[a]	10.92 ± 0.43 [a]
AM fungi	17.19 ± 0.30[a]	17.89 ± 0.26[a]
Protists	4.41 ± 0.12[a]	4.45 ± 0.09[a]
Total fungi	27.41 ± 0.58[a]	28.81 ± 0.56[a]
Total bacteria	**251.50 ± 2.80**[b]	**260.13 ± 2.52**[a]
F:B ratio	0.11 ± 0.00[a]	0.11 ± 0.00[a]
Total PLFA	**335.98 ± 3.77**[b]	**346.83 ± 3.49**[a]

contained significantly greater amounts of most C and N fractions compared to bulk soils (Tables S1 and S2). Within the rhizosphere, there were endophyte effects on several C and N fractions. Notably, POM-C concentrations were significantly greater under CTE+ and AR584E+ infected tall fescue together with a similar trend in TOC, TN, and POM-N. Particulate organic matter C is a slow and stable organic carbon fraction and has been used as a potential indicator of root proliferation (Cambardella and Elliott, 1992). Endophyte infection in tall fescue often results in greater plant biomass (Arachevaleta et al., 1989; Debattista et al., 1990), and in our previous studies, we found plants infected with CTE+ and AR584E+ produced significantly more root biomass than E− plants (Guo et al., 2015). Production of greater root biomass is therefore likely to be a significant factor explaining greater C and N concentrations in soils under tall fescue infected with these endophytes. However, differences in root tissue and exudate composition, and importantly root architecture, could also play a role, but little if any research has gone into studying these aspects (Cheng et al., 2013) and we were unable to quantify them in this field study.

Within the rhizosphere we detected no endophyte effects on total microbial biomass or microbial community composition using the proportional abundance of PLFA biomarker groups (Table S5 and Fig. 3b). However rhizosphere soils under cultivar PDF had significantly greater (Table 2) total bacteria (G+ and G−) and overall microbial biomass. Further, potential soil LAP activity was significantly greater under cultivar PDF when endophyte infected (regardless of strain) compared to 97TF1. Leucine amino peptidase is an enzyme involved in N cycling, so it may be that endophyte infection in PDF changes the secretion level or type of N containing rhizodeposits, stimulating LAP production in the resident rhizosphere microbiome. Potential soil LAP activity has been shown to increase with N additions in some soils (Stursova et al., 2006). In our previous study characterizing root exudate composition from these same cultivar-endophyte combinations (Guo et al., 2015), we found a significant interaction between endophyte and cultivar on the concentration of amines and other rhizodeposits which might provide some explanation as well. Within the rhizosphere, cultivar effects appear to be stronger than those associated with endophyte infection at influencing the concentration of certain groups within the microbial community. On the other hand, endophyte infection exerted a greater influence on total and POM C and N fractions which might be due to endophyte-induced changes in root biomass and exudate chemistry.

In bulk soils, the POM-N concentration was 38% greater relative to rhizosphere soils, and was significantly influenced by the interaction of cultivar with endophyte. There is a gradient in soil biological activity and chemical composition moving out from the rhizosphere into the bulk soils where the direct influence from root inputs is expected to decrease. As distance from the root increases, a greater proportion of C and N inputs likely comes from leaf litter, in which case the concentration of C and N in bulk soils could arguably be influenced more by the amount and composition of shoot biomass produced than roots. In our previous study, we showed that cultivar 97TF1 produced significantly more shoot biomass compared to cultivar PDF (Guo et al., 2015) which is supported by historical data from the research plots used in this study (Owens III, 2011) and in field trials throughout the South-Central United States (Hopkins et al., 2010). Tissue chemistry and structural composition have been shown to influence decomposition, and hence, soil nutrient (notably C and N) cycling (Horner et al., 1988; Walela et al., 2014). In tall fescue, differences in type and amount of alkaloids in endophyte-free vs. endophyte-infected stands have been cited as one possible reason for differences in soil C and N concentrations (Omacini et al., 2004; Lemons et al., 2005). However (Siegrist et al., 2010), in a study comparing CTE+ and E− tall fescue, postulated that changes in tissue chemistry (other than alkaloids) as well as structural composition may be of equal or more importance. Their hypothesis is supported in a study by Soto-Barajas et al. (2015) who reported that endophyte infection in *Lolium perenne* (perennial ryegrass) decreased P, Ca, S, B, neutral detergent fiber and lignin contents which they conclude may influence rates of decomposition. Lignin and cellulose tend to degrade slower, Ca has been linked to increased soil microbial activity, and the N:P ratio in plant tissues has been shown to increase the amount of bacteria (high ratio) or fungi (low ratio) (Gusewell and Gessner, 2009; Berg and McClaugherty, 2013). Our companion study found that endophyte infection had a greater effect on soil fungal community structure than bacteria which may be related to the aforementioned differences in tissue N:P ratios (Rojas et al., 2016). A few studies have demonstrated that endophyte infection can alter the mineral content and structural composition of tall fescue biomass (Vazquez-de-Aldana et al., 1999; Rahman and Saiga, 2005; Belesky et al., 2009); however, assessing whether these alterations influence rates of tissue decomposition and fungal community composition have yet to be performed.

Microbes are the primary drivers of tissue decay and therefore anything that perturbs their function will have an impact on the rate of C and nutrient cycling from dead plant tissues (Walela et al., 2014). PLFA showed no cultivar effects on microbial community structure within bulk soils; however in our companion study using high-throughput DNA sequencing (Rojas et al., 2016) bulk soils under cultivar PDF had significantly lower relative abundance of bacteria from the Firmicutes and α-Proteobacteria phyla, and the fungal phyla Ascomytoca, but greater abundance of fungi from the Chytridiomycota phyla. We hypothesize that these changes in the microbial community structure are likely due to tall fescue cultivar-influenced changes in tissue composition and may be reflective of possible differences in rates of decomposition that gave rise to the different POM-N concentrations we observed in soils under each cultivar. The fact that rhizosphere and bulk soil parameters were differentially impacted by cultivar and endophyte infection may, in part, help explain the often contradictory findings of previous work on this subject (e.g., Iqbal et al., 2012; Handayani et al., 2011) since most existing papers have not differentiated between soil zones. Indeed if our data are averaged across the two sampled soil regions, most of the endophyte and cultivar significant effects disappear. It seems likely that *E. coenophiala* and plant host genetics influence soil processes via different mechanisms and are, therefore, observed at different scales (e.g., rhizo- vs. bulk). Future studies should consider this possibility when sampling. More frequent sampling should also be considered as recent studies have shown that the microbial community changes throughout the growing season (Shi et al., 2015) which may impact some of the more ephemeral soil properties measured in this study.

5. Conclusions

This study examined how tall fescue cultivar, endophyte strain or their interaction effected soil microbial community structure, function, and subsequent carbon and nitrogen cycling in bulk and rhizosphere soils from tall fescue stands in central KY. We found that rhizosphere soils had greater microbial biomass, potential enzyme activity, and basal oxygen utilization, but lesser POM-N concentrations; the most easily accessible N fraction of soil organic matter. In rhizosphere soils, tall fescue cultivar had the greatest influence on soil microbial community structure, while endophyte genotype had a stronger influence on soil C fractions (notably increased POM-C). Root system architecture, biomass, and tissue composition, together with root exudate chemistry, which we have shown in previous studies to be affected by endophyte genotype and fescue cultivar, likely contributed to explain these findings. There was greater POM-N in bulk soils which was influenced by the interaction of endophyte and cultivar potentially due to differences in the chemical composition of shoot tissues brought about by this interaction. Our results support current observations that tall fescue cultivar and fungal endophyte strain influence pasture soil C and N cycling and, by analyzing bulk and rhizosphere soils separately, goes further to show the level of influence cultivar and endophyte have within each of these unique soil compartments.

Role of the funding source/Authors and contributions

DHMJ and RLM conceptualized and designed the experiments, and helped in the analysis and interpretation of the data. TP provided the field plots. JG acquired, analyzed and helped in the interpretation of the data and drafted the manuscript. DHMJ and RLM contributed to critical revision of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.07.014.

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