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Soil bacterial structure and function associated with the invasive grass Microsteguim vimineum and two native grasses

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SOIL BACTERIAL STRUCTURE AND FUNCTION ASSOCIATED WITH THE INVASIVE GRASS *MICROSTEGUIM VIMINEUM* AND TWO NATIVE GRASSES

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ABSTRACT

Soil microorganisms play vital roles in biogeochemical cycles and are necessary for maintaining soil health. An invasive plant that alters the structure and function of the soil microbial assemblage could gain an advantage over native plants, enhancing its ability to invade new habitats, alter ecosystem function, and hinder efforts to reestablish native populations. Assays of enzyme activity and next generation sequencing of bacterial 16S rRNA genes were used to assess the soil microbial function and community structure associated with the invasive grass, *Microstegium vimineum* and two co-occurring native grasses (*Dichanthelium boscii* and *Chasmanthium laxum*). Significantly lower enzyme activity was found for soil associated with *M. vimineum* compared to the other plant species, and that each plant had a distinct bacterial community associated with it. Differences in bacterial community structure were more apparent on the root surface or in the rhizosphere, and may not extend into the surrounding bulk soil and were seen throughout the sampling season. Site chemistry differences affected enzyme activity but not bacterial community composition, suggesting that bacterial community structure is influence more by plant species than soil chemistry. *Microstegium vimineum* also significantly increased soil pH. Long-term invasion of *M. vimineum* will cause local changes in nutrient cycling, changes in soil pH and alter local bacterial community composition.

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INTRODUCTION

An invasive species is a species that reproduces and spreads rapidly to become a dominant species in a different ecosystem (Valéry et al. 2008). There are tens of thousands of invasive species in North America (Pimentel 2004) and they have become common, with an estimated 100 million acres in the USA inhabited with invasive species. The economic impact of these species is significant, and invasive species cause over $130 billion in economic damage and loss annually (Pejchar and Mooney 2009). Worryingly, this impact is predicted to increase with global climate change (Vilá et al. 2011). In particular, the presence of invasive plant species presents a substantial burden on an ecosystem, and can result in changes that extend beyond those of plant community composition.

Any change in plant species composition will change the quantity and quality of root exudates to the soil (Ehrenfeld 2003, Van der Putten et al. 2007), so that invasive plants would be expected to influence soil properties. Invasive plants have been shown to alter rates of nutrient cycling (Mack and D'Antonio 1998, Mack et al. 2002, Fraterrigo et al. 2010) as well as to change inputs of organic matter to the soil (Ehrenfeld 2003). Despite an increasing knowledge of how invasive plants change these soil properties, little is known about how invasive plants influence soil microbial community structure and function (van der Putten et al. 2007). However, changes in the chemical and physical properties of soil, as well as in litter and exudate inputs, are almost certain to impact microbial community composition, potentially changing the functioning of the soil ecosystem.

Plants interact with soil microorganisms in a number of ways. At an immediate level, there are interactions on the surface of the root (i.e. direct physical and chemical interaction); slightly more distant are interactions in the rhizosphere (e.g. through the release of root exudates); at a broader level, there may be interactions away from the plant in the bulk soil (e.g. through litter inputs) (Kulmatiski et al. 2008). Bacterial communities in each of these regions are likely to differ in composition, at least in part because of interactions with specific plant species (Kourtev et al. 2003). Soil microbial communities also vary seasonally, and plant specific differences in soil microbial community structure may increase over successive growing seasons (Smalla et al. 2001, Kourtev et al. 2002, Kulmatiski et al. 2008). Invasive plant species are therefore likely to select for a different soil microbial community than native vegetation, although this phenomenon has been understudied and only minimally addressed using next generation sequencing technologies that facilitate the necessary depth of microbial community characterization.

In this study, I used next generation sequencing (dual indexed Illumina 16S rRNA gene amplification) to determine the structure of the soil bacterial community associated with two native plants of the southeastern USA, *Dichanthelium boscii,*(Bosc's panicgrass) and *Chasmanthium laxum* (slender wood oats), and one invasive plant, *Microstegium vimineum* (Japanese stilt grass). Sampling was conducted at multiple spatial scales in relation to each plant: the root surface, the rhizosphere, and the surrounding bulk soil. Soil function was assessed by assaying the activity of six extracellular enzymes related to C, N, and P cycling in the soil around each plant species. In order to evaluate the impact of seasonality on the influence of plant species on bacterial community composition, sampling was conducted at multiple time points (late

spring green-up, summer peak biomass, and fall senescence) throughout the 2014 growing season.

METHODS

Study Sites and Species:

Sampling occurred at four sites spread over two locations, approximately 60 km apart, in northern Mississippi, USA. Sites 1 and 2 were situated in Strawberry Plains Audubon Center in the Holly Springs National Forest. This area is hilly, and has mesic silt and sandy loamy soils. Both sites were located in upland oak dominated woodlands, and were approximately 1 km apart. Site 1 (34°49'60"N, 89°28'32"W) was located in an area with no history of anthropogenic use, while areas around Site 2 (34°49'52"N, 89°27'17"W) were used for the cultivation of cotton and cattle grazing until 2000. Both sites have been undergoing ecological restoration to restore open oak forest since 2004, with fire and thinning occurring every two years (Maynard and Brewer 2013). In terms of tree coverage, the sites are now dominated by southern red oak (*Quercus falcata*), white oak (*Quercus alba*), post oak (*Quercus stellata*) and hickory (*Carya ovalis* and *Carya tomentosa*). The understory at Site 1 is dominated by *D. boscii, Lonicera japonica, Vitis rotundifolia* and *Parthenocissus quinquefolia*, while the understory at Site 2 is dominated by *D. boscii, C. laxum, Lonicera japonica,* and *Vitis rotundifolia* (Brewer et al. in press).

Sites 3 and 4 were located at the Whirlpool Trails, a wooded area maintained by the University of Mississippi, in Oxford, Mississippi, USA. Site 3 (34°35'26"N, 89°54'49"W) was located in a young (<20 years) stand of trees dominated by sweetgum (*Liquidambar styraciflua)* and sycamore (*Platanus occidentalis*), and was characterized by sandy loam soils. The understory vegetation at this site is dominated by *C. laxum.* Site 4 (34°34'64"N, 89°54'91"W) was located about

km away from Site 3 in an older stand of trees (>60 years), with dominant tree species being sweetgum, white oak (*Quercus alba*), and winged elm (*Ulmus alata*), and having silt loam soils with the understory being largely *M. vimineum*, *Toxicodendron radicans*, and *Podophyllum peltatum* (Brewer 2011).

All four sites have been invaded with *M. vimineum*, and the two native species examined in this study (*C. laxum* and *D. boscii*) also occur at all sites. Both of these native species are perennial C3 grasses, while *M. vimineum* is an annual C4 grass of Asian origin, which was first detected in the USA in Tennessee in 1919 and is now widespread in areas east of the Mississippi Rivers (Gibson et al. 2002). *M. vimineum* produces many inflorescences that seed prolifically and creates a persistent seed bank. The species colonizes stream banks and recent disturbances, yet is remarkably shade tolerant, and can produce dense stands (>400 stems per m^2), which, along with the persistent seed bank, allow it to maintain patches from year to year (Brewer 2011). *M. vimineum* grows throughout the normal growing season and reaches peak biomass late in the season as it approaches leaf senescence.

Sample Collection:

Samples were collected three times throughout the 2014 growing season, in June (late spring green-up), September (summer peak biomass), and November (early fall senescence). On each sampling event, five individuals of each plant species were collected from each site (i.e. 20 individual plants for each of three species on each collection date). Samples were collected using alcohol-sterilized spatulas while wearing latex gloves. Belowground material and soil associated with each plant sample was split into three fractions representing the root (and tightly adhered soil), rhizosphere soil (defined as any soil on the roots that could be easily removed), and bulk soil (defined as the soil directly underneath the plant but not attached to the roots). All samples

were placed in sterile 50 ml centrifuge tubes. Root and rhizosphere samples were taken to the laboratory and immediately frozen at -20°C. The bulk soil sample was split into subsamples for determination of soil chemical characteristics and extracellular enzyme activity, and the remaining material frozen at -20°C.

Soil Characteristics:

One subsample (5 g) of the bulk soil was weighed, dried (48 h, 70 °C) and reweighed to determine gravimetric soil moisture. The oven-dried material was then ground and a 20 mg subsample used for determination of soil C and N concentrations using a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA). A second subsample (2 g) of bulk soil was suspended in 20 ml H_2O and soil pH determined (Miller and Kissel 2010).

Extracellular Enzyme Activity

A subsample of bulk soil (5 g) was used for determination of extracellular enzyme activity. Soil was assayed for the activity of phosphatase, N-acetyl-β-D-glucosaminidase (NAGase), β-glucosidase, cellobiohydrolase (CBH), phenol oxidase, and peroxidase. Activity was assayed using the colorimetric protocols demonstrated by Jackson et al. (2013). Soil was added to 25 ml of 50 mM sodium acetate buffer (5.0 pH) and shaken until mixed. Assays were conducted using 150 µL of this suspension in 96-well deep-well microplates with three replicate assays per sample per enzyme. Activity of phenol oxidase and peroxidase was determined using the substrate 3,4-dihydroxyl-L-phenylalanine (L-DOPA), while *p*-nitrophenyl (*p*NP)-linked substrates were used for the remaining enzymes. In each case, assays received 150 µL of

substrate solution at 5 mM (phosphatase, β-glucosidase, phenol oxidase, peroxidase) or 2 mM (NAGase, CBH), and 15 µL of 0.3% H_2O_2 was added to the peroxidase assay.

Assays were incubated at room temperature for 1 (β-glucosidase, phosphatase), 2 (phenol oxidase, peroxidase) or 3 (NAGase, CBH) h. Following incubation, microplates were centrifuged (4,000 x g, 10 min), and 100 µL of the supernatant removed and transferred to a clear 96-well reading microplate. 10 µL of 1M NaOH and 190 µL H_2O were added to the reading plates of assays using *p*N*P*-linked substrates, while 200 µL of water was added to the reading plates of assays using L-DOPA as a substrate. Absorbance of the colored end products was determined at 410nm (*p*NP-linked substrates) or 460nm (L-DOPA), using a BioTek Synergy HT (Winooski, VT). All assays included duplicate substrate and sample controls, and final activity was expressed as µmoles substrate consumed h^{-1} g dry $weight^{-1}$ of sample.

Bacterial community composition:

DNA was extracted from frozen subsamples (0.25 g soil, 0.10 g root) using PowerSoil DNA Isolation kits (Mo Bio Laboratories, Inc., Carlsbad, CA). Approximately 250 bp of the V4 region of the bacterial 16S rRNA gene was amplified from extracted DNA using an indexed primer set optimized for Illumina MiSeq sequencing (Kozich et al. 2013). Concentration of amplicons was normalized using SequalPrep Plates (Life Technologies, Grand Island, NY) and amplicons were pooled into a single library. The library was then sequenced at the Molecular and Genomics Core Facility of the University of Mississippi Medical Center (UMMC) using an Illumina MiSeq system.

Data Analysis

Differences in enzyme activity and soil characteristics (pH, moisture content, C and N concentrations) were tested using three-way analysis of variance (ANOVA) followed by Tukey's HSD test to determine specific differences in plant species, sampling date and site. Initially, all factors (plant species, sampling date, site) were incorporated into the three-way ANOVA, however there were few consistent significant interaction terms for almost all measurements of enzyme activity, so patterns in the activity of each enzyme were examined within each sample date individually, specifically looking for the effects of plant species, and treating site differences as additional variation in the error term.

Raw sequence data (FASTQ files) were analyzed using the bioinformatics software mothur, following the procedures recommended by Schloss et al. (2011) and Kozich et al. (2013). Contigs were formed, ambiguous bases removed, and sequences trimmed to 250 bp. Sequences were aligned to the SILVA rRNA database (Pruesse et al. 2007) and potential chimeras screened using uchime (Edgar et al. 2011) and removed. The remaining sequences were then grouped into operational taxonomic units (OTUs) using a 97% sequence similarity criterion, and classified according to the Greengenes (DeSantis et al. 2006) database. Sequences that classified as mitochondria, chloroplasts, archaea, eukaryotes, or of unknown origin were removed from the dataset prior to further analyses.

For community analyses, samples with <10,000 remaining valid reads were removed, so that comparisons weren't skewed by few samples with lower numbers of reads. All remaining samples were normalized by subsampling to the equivalent read number of remaining sample with the lowest number of reads (10,250 valid reads). This subsampling was done over 1,000 iterations, with the mean outcome used for each analysis. Beta-diversity (comparisons between samples) was determined using the Jaccard index of similarity, which is based solely on the

presence or absence of each OTU in a sample, as well as the theta index, which incorporates relative abundance of each OTU. Similarity matrices were ordinated using nonmetric multidimensional scaling (NMDS), and analysis of molecular variance (AMOVA, Anderson 2001) used to determine differences in bacterial community structure arising from plant species type, sample type, site, and sampling date. To reduce the impact of rare (and potentially erroneous) OTUs, all community analyses were performed following the removal of OTUs that accounted for <10 reads across all samples, as well as on the full dataset.

RESULTS

Soil Chemistry

Soil pH was significantly ($p<0.001$) higher in bulk soil samples associated with *M. vimineum* than either *C. laxum* or *D. boscii* (Table 1), and species was the only factor related to pH variation. Soils associated with *D. boscii* consistently had lower soil moisture than the other plant species, but this was only significant ($p<0.05$) for the summer sampling date (Table 1). Soils associated with *C. laxum* and *M. vimineum* had higher C content than those associated with *D. boscii* for the spring sampling date ($p<0.05$) but there were no significant differences for later sampling dates, and no consistent effect of species on soil C or N content. C and N content were both strongly influenced by site, with soils at Sites 1 and 2 having significantly lower C and N content than soils Sites 3 and 4 (Table 1; $p<0.001$).

Soil Extracellular Enzyme Activity

Plant species, sample date, and site all had significant ($p<0.001$) effects on phosphatase activity, and there was a significant species x date interaction ($p<0.05$). When each date was analyzed separately, the effect of plant species on phosphatase activity was significant across all dates ($p<0.001$) with soils around *M. vimineum* consistently having lower activity than the other plant species (Fig. 1a-c). Site also had a significant effect on phosphatase activity on each date, although the significance of this varied from very high ($p<0.001$) on the spring sample date, to lower ($p<0.05$) for the summer and fall sampling dates, although in each case samples from Sites

3 and 4 had higher phosphatase activity than Sites 1 and 2. Plant species and sample date also had significant effects on bulk soil NAGase activity ($p<0.001$; Fig. 1d-f), with soils associated with *M. vimineum* having lower NAGase activity than the other two plant species. Analyzing each date separately revealed that this species effect was limited to the first and third sampling dates (Fig. 1d- f), and, in general, activity increased with each successive sample date. However, an exception to this pattern was that soils around *D. boscii* had the highest NAGase activity during the second sampling date (Fig. 1e).

Sample date was the only variable to show a significant ($p<0.001$) effect on soil β-glucosidase activity as a whole, with activity being higher on later sample dates (Fig. 2a-c). However, other variables had significant effects on β-glucosidase activity within each individual sampling date. On the first sampling date, there was a significant ($p<0.01$) effect of site, with Site 3 having the highest activity (Fig. 2a). Plant species was not a significant factor on β-glucosidase activity on this date, but there was a suggestion (p=0.07) of soils around *M. vimineum* having lower β-glucosidase activity than both of the native species. The effect of site remained significant ($p<0.01$) for the following two sampling dates, with Site 3 consistently having the highest β-glucosidase activity, but any suggestion of a plant species effect disappeared (Fig. 2b, c). Species did show a significant effect on soil CBH activity ($p<0.001$), although this was limited to the first and last sample dates (Fig. 2d-f), when soils around *M. vimineum* had lower CBH activity than those around *D. boscii* or *C. laxum*. Date was also significantly ($p<0.001$) related to CBH activity, with increased activity on the later sample dates, as was site ($p<0.05$), with soil from Sites 3 and 4 consistently having higher CBH activity than soil from Sites 1 and 2 (Fig. 2d-f).

Plant species, site, and date, as well as plant x date and site x date interactions were significantly related to bulk soil peroxidase activity (Fig. 3a-c). When each sampling date was analyzed individually, plant species was significant on all sampling dates ($p<0.001$, Fig. 3a-c), with soils associated with *M. vimineum* having lower activity than those around *D. boscii* or *C. laxum*. For the final sampling date, the activity in soils around all plant species were all significantly different from one another ($p<0.001$; Fig. 3c). Site, and a plant x site interaction were also significant on the final sampling date (Fig. 3c, $p<0.001$), with, as with other enzymes, soils at Sites 3 and 4 having higher peroxidase activity than Sites 1 and 2. Site also had a significant ($p<0.001$) effect on bulk soil phenol oxidase activity (Fig. 3d-f), although plant species only impacted soil phenol oxidase on the final sample date ($p<0.01$, Fig. 3f).

Activities of the hydrolytic enzymes (CBH, β-glucosidase, NAGase, and phosphatase) were generally positively correlated (R=0.40-0.80) with correlations generally being the strongest at summer peak biomass and weakest on the spring green-up sampling date. Activity of the oxidative enzymes (phenol oxidase, peroxidase) tended to be less strongly correlated with the activity of the hydrolytic enzymes or with each other, although these correlations increased on the final sampling date (when pairwise correlations ranged from R=0.49-0.73). The relationship between peroxidase and phosphatase was an exception, as activities of these two enzymes were strongly positively correlated on both the second (R=0.82) and final sampling date (R=0.73).

Bacterial Community Structure

A total of 17,587,740 sequences from 394 samples were obtained following Illumina 16S rRNA gene sequencing, with an average sequence length of 253 bp. 184,456 sequences were identified as potential chimeras and were removed, and a further 272,274 sequences were

identified as mitochondria, chloroplasts, eukaryotic or archaea sequences and also removed. The remaining 17,131,010 bacterial sequence reads grouped into 380,410 OTUs. Many of these OTUs were rare, and after removing these rare OTUs (OTUs represented by ≤10 reads across all samples) 53,006 OTUs remained. 17 samples were removed because they had <10,000 valid bacterial reads, leaving 377 samples in the final community analyses.

Overall bacterial community composition significantly differed based on plant species, sample type (root, rhizosphere or bulk soil), site, and sampling date (AMOVA; $p<0.001$ for all). Other than minor differences, this was consistent regardless of whether the theta or Jaccard index was used for community comparisons, or for whether analyses were run on the entire dataset or following the removal of rare OTUs. As with patterns in soil enzyme activity, in order to focus on the effects of plant species or sample type, samples were separated by sample date, with each date analyzed separately. These date-specific community analyses relied upon multiple pairwise comparisons between different samples following AMOVA, so the Bonferroni correction was used, giving a critical p-value of 0.00153 in each case (the value used to determine significance in reported analyses).

Bacterial communities tended to separate by both species and sample type. However, there was a substantial amount of variation in community structure and differences in patterns between sample dates. NMDS ordinations suggested community patterns, although stress values were high (Fig. 4). On the first sampling date, bacterial communities associated with the root surface of *D. boscii* significantly differed from those on the roots of both *M. vimineum* and *C. laxum* (AMOVA, $p<0.001$; Fig. 4a), while communities in the rhizosphere of *D. boscii* differed from those in the rhizosphere of *M. vimineum* (AMOVA, $p<0.001$; Fig. 4a). Bacterial communities associated with the root or rhizosphere of *C. laxum* on this sample date did not

significantly differ from those in the equivalent sample type from *M. vimineum* when compared using the theta index, but there was a suggestion of differences (AMOVA, p=0.007 and 0.004, respectively). However, the root and rhizosphere communities of *M. vimineum* and *C. laxum* were significantly different (AMOVA, p<0.001) when analyzed using the Jaccard index. Interestingly, there were no plant species related differences in the bacterial communities in the bulk soil samples on this date, and more so than the root or rhizosphere samples, the bulk soil samples tended to group together in NMDS ordinations (Fig. 4a). Within individual plant species, the *M. vimineum* root-associated community differed from that in the bulk soil (AMOVA, p<0.001), but neither of those communities differed from the *M. vimineum* rhizosphere. *D. boscii* root and rhizosphere communities differed from those in the bulk soil around that plant (AMOVA, p<0.001), whereas the root, rhizosphere, or bulk soil communities of *C. laxum* were not significantly different from each other on this date.

For the late summer sampling date, during peak plant growth, the root bacterial communities on *C. laxum* were significantly different from those on *M. vimineum* (Fig.4b; AMOVA, p<0.001) regardless of the similarity index used, and significantly different from those on *D. boscii* when the Jaccard index was used for community comparisons (AMOVA, p<0.001). The rhizosphere community associated with *C. laxum* was also significantly different from the rhizosphere of *M. vimineum* (AMOVA, p<0.001) on this date, but only when the Jaccard index was used. These differences between *C. laxum* and *M. vimineum* root and rhizosphere associated communities could be seen on the NMDS ordinations (Fig. 4b), with noticeable separation of samples from these two species. As with the late spring sample date, bacterial communities in the bulk soil samples did not significantly differ from one another in the summer, and within each plant species there were fewer differences between bacterial communities in the root,

rhizosphere, or bulk soil samples on this sampling date. Root and rhizosphere communities associated with *D. boscii* were significantly different (AMOVA, $p<0.001$), but no other significant sample type differences were observed.

For the third sampling date, as plants entered senescence, both the root and rhizosphere bacterial communities of *C. laxum* and *M. vimineum* were significantly different (Fig. 4c; AMOVA, $p<0.001$), but the bacterial communities associated with *D. boscii* did not differ from those associated with either of these two species. As with the other sample dates, bacterial communities in the bulk soil samples were not significantly different, and there were also no significant differences in overall community composition between root, rhizosphere, or bulk soil samples within individual plant species on this sample date.

Across the whole dataset, Proteobacteria (26.8% of sequence reads) were the dominant bacterial phylum, followed by Acidobacteria (20.0% of all reads), Planctomycetes (15.2%) and Verrucomicrobia (10.4%). Proportions of phyla were generally consistent across sample dates and sample types (Fig. 5), but showed some differences that might relate to the overall community patterns reported above. On the first sampling date, the root surface of *M. vimineum* was enriched in Proteobacteria (accounting for a mean of 34% of all sequences in this sample type, but ranging from 26% to 40%) compared to *C. laxum* (mean 28%, range 18.7-36.3%) and *D. boscii* (mean 32.4%, range 24-39.6%). The proportion of sequences classified as members of the Bacteroidetes was also greater in root samples taken from *M. vimineum* on the spring sample date (mean 9.3%; range 5.1-14.9%) compared to those from other species (mean 5.9% (range 2.6-11.4%) for *C. laxum*, and mean 5.71% (range 3.3-8.9%) for *D. boscii*). and depleted in Acidobacteria (mean 15.9% (range 12.2-21%), compared to 21.7% (12.8-28.5%)) and Chloroflexi (3.6% (2-4.65) and 5.8% (3-11%) compared to the roots of *C. laxum*, while the

rhizosphere of *M. vimineum* was slightly enriched in Bacteroidetes (mean 7.5% (3.6-10.3%) and mean 6% (2.8-7.7%)) and depleted in Actinobacteria (mean 7.7% (4.1-18.9%) and mean 9.9% (5.8-14%)) compared to that of *D. boscii.*

On the summer sampling date, the roots of *M. vimineum* had appreciably more Bacteroidetes (mean 6.5%, range 1.8-9.2%), and fewer Actinobacteria (mean 11.8%, range 3.7-17.8%), than the roots of *C. laxum* (mean 3.82%, range 1.6-6.9% for Bacteroidetes; mean 14.2%, range 8.5-21.2 for Actinobacteria). The roots of *M. vimineum* and *D. boscii* were not found to be significantly different, however, *M. vimineum* did have slightly more Proteobacteria (mean 28.9%, range 23.0-38.9%) than the roots of *D. boscii* (mean 26.5%, range 9.5-36.1%). The rhizosphere of *M. vimineum* was enriched in Proteobacteria (mean 27.8%, range 19.4-32.3%) but depleted in Acidobacteria (mean 21.6%, range 13.8-25.8%) and Actinobacteria (mean 8.1%, range 4.6-16.1%) relative to the rhizosphere of *C. laxum* (Proteobacteria mean 25%, range 15.8-29.7, Acidobacteria mean 25.2%, range 16.4-34.3%, Actinobacteria mean 11.5%, range 3.4-15.3%). While not significantly different, the rhizosphere of *M. vimineum* had fewer Actinobacteria than the rhizosphere of *D. boscii* (which had a mean of 10.9%, and a range of 3.8-11.5%).

For the final sampling date, the root surface of *M. vimineum* had a greater representation of Proteobacteria (mean 30.3%, range 18.5-40.2%) than *C. laxum* (mean 26.5%, range 20.7-29.4%) but fewer Acidobacteria (mean 18.2% range 12.1-23.0%; compared *C. laxum* mean 22.0%, range, 4.1-25.0%). The proportions of dominant phyla for the root of *D. boscii* were similar to both species. The proportions of Bacteroidetes in the bacterial community exemplified this, with *M. vimineum* having the most (mean 7.8%, range 3.6-11.7%), *D. boscii* falling in the middle (mean 5.4%, range 1.8-8.7%) and *C. laxum* having the fewest (mean 4.3%, range 3.0-

5.0%). *M. vimineum* also had proportionally more Bacteroidetes (mean 7.6 %, range 3.3-12.8%) in its rhizosphere compared to *C. laxum* (mean 4.6%, range 1.5-5.4%) and *D. boscii* (mean 5.2%, range 2.2-6.4%) , but fewer Planctomycetes (mean 17.9%, range 12.7-22.6%; compared to mean 20%, range 11.5-24.7% for *C. laxum*, and mean 16.5%, range 11.6-20.6%, for *D. boscii.*

There were minimal site effects on community structure associated with the root surface (Table 2), with the only significant differences occurring on the last sampling date, where there was a significant difference in the bacterial community on the *D. boscii* root surface between Sites 2 and 3 (Table 2; AMOVA, $p<0.0083$, the significance value given the Bonferroni correction in these analyses), and between Sites 3 and 4 (AMOVA, $p<0.0083$). On this date, the root community associated with *M. vimineum* was also significantly different between Sites 2 and 4 (Table 2; AMOVA, $p<0.083$). Similarly, rhizosphere bacterial communities were only minimally affected by site, and the only significant site-related differences in those communities were found on the peak biomass sampling date (Table 2). On that date, rhizosphere communities of *C. laxum* were significantly different between Sites 2 and 4 (AMOVA, $p<0.0083$), while those of *D. boscii* were different between Sites 1 and 4 (AMOVA, $p<0.0083$), and those of *M. vimineum* were different between Sites 2 and 3 (AMOVA, $p<0.0083$). Bulk soil bacterial communities at the different sites were only significantly different for *C. laxum*, with those at Sites 3 and 4 being different on the first sampling date (Table 2; AMOVA, $p<0.0083$), and those at Sites 2 and 4 being different on the second sampling date (AMOVA, $p<0.0083$). These few significant site differences are minimal compared to the overall number of site comparisons (162; Table 2), suggesting that any site-related differences are minimal for either the root-associated, rhizosphere, or bulk soil communities of these three plant species.

The 20 most abundant OTUs, together, comprised only 10.7% of the total number of reads (1,831,896 reads) with no single OTU comprising more than 1.7% (283,304 reads) of the total. Four of the 20 most abundant OTUs (OTUs 6, 34, 253 and 284) where unclassified Actinobacteria, together comprising 372,857 reads or 2.2% of the total. Five of the most abundant OTUs (OTUs 85, 100, 320, 7911, and 50636) were classified as Chthoniobacteraceae, a family in the Verrucomicrobia. Together these five OTUs make up 499,431 or 2.9% of all valid reads.

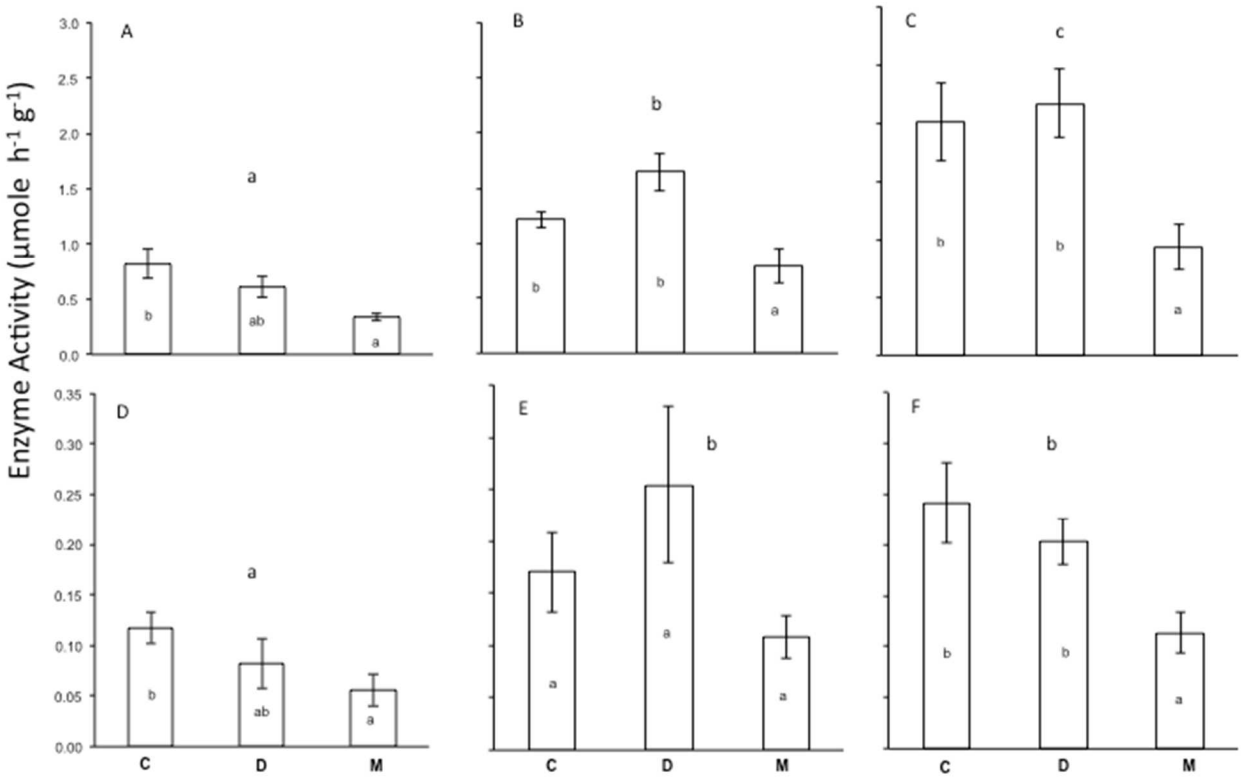

Figure 1. Mean (+/- SE) activity of soil phosphatase (A-C) and NAGase (D-F) associated with the plant species *Chasmanthium laxum* (C), *Dichanthelium boscii* (D), and *Microstegium vimineum* (M) across three sampling dates, corresponding to late spring green-up (A, D), summer peak biomass (B,E), and fall senescence (C,F). Within each sampling date, significant differences in enzyme activity in soils around different plant species are indicated by different lower case letters within each bar. Significant differences between sampling date are indicated by different lower case letters above the bars. n=16 for each mean.

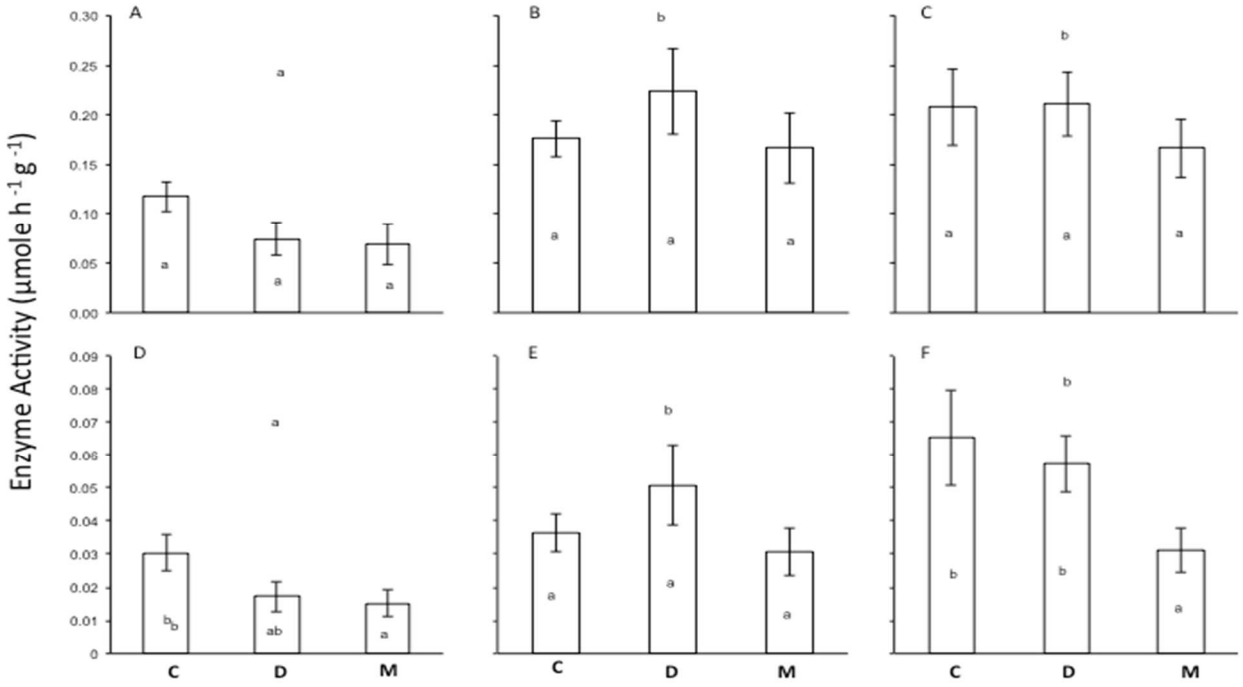

Figure 2. Mean (+/- SE) activity of soil β-glucosidase (A-C) and CBH (D-F) associated with the plant species *Chasmanthium laxum* (C), *Dichanthelium boscii* (D), and *Microstegium vimineum* (M) across three sampling dates, corresponding to late spring green-up (A, D), summer peak biomass (B,E), and fall senescence (C,F). Within each sampling date, significant differences in enzyme activity in soils around different plant species are indicated by different lower case letters within each bar. Significant differences between sampling date are indicated by different lower case letters above the bars. n=16 for each mean.

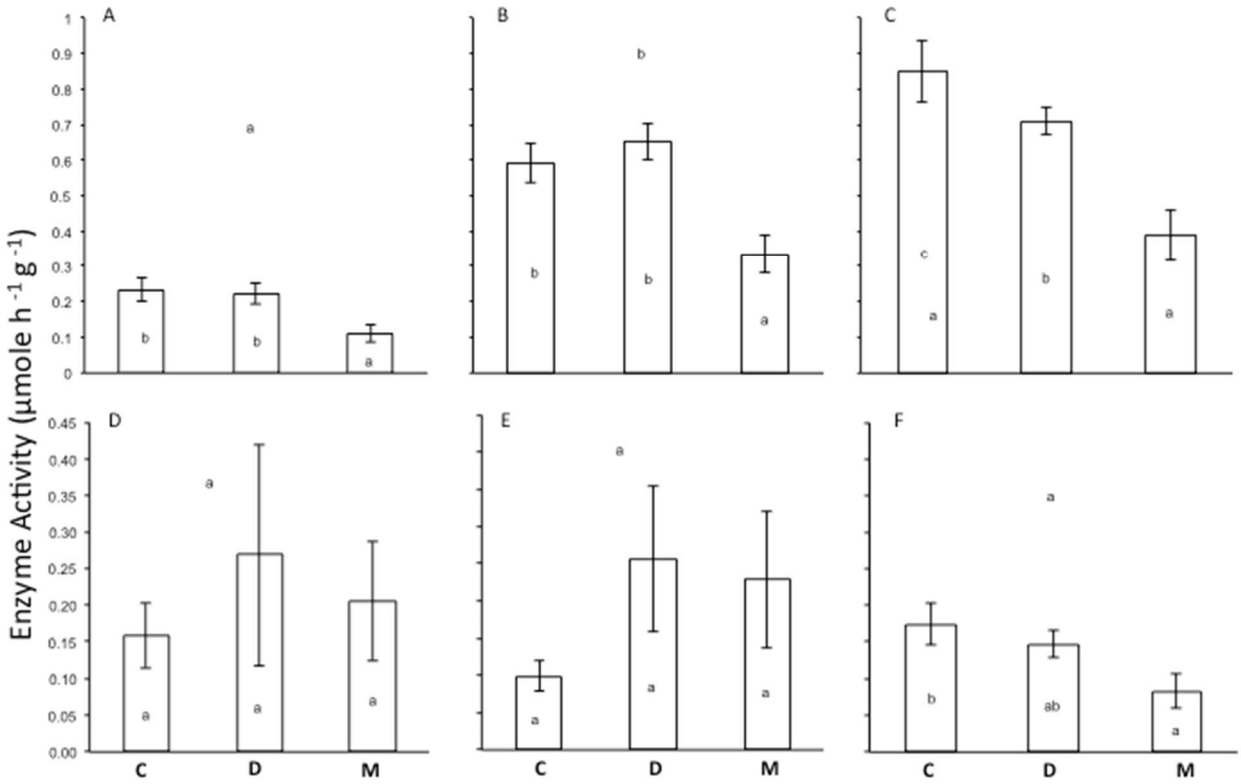

Figure 3. Mean (+/- SE) activity of soil peroxidase (A-C) and phenol oxidase (D-F) associated with the plant species *Chasmanthium laxum* (C), *Dichanthelium boscii* (D), and *Microstegium vimineum* (M) across three sampling dates, corresponding to late spring green-up (A, D), summer peak biomass (B,E), and fall senescence (C,F). Within each sampling date, significant differences in enzyme activity in soils around different plant species are indicated by different lower case letters within each bar. Significant differences between sampling date are indicated by different lower case letters above the bars .n=16 for each mean.

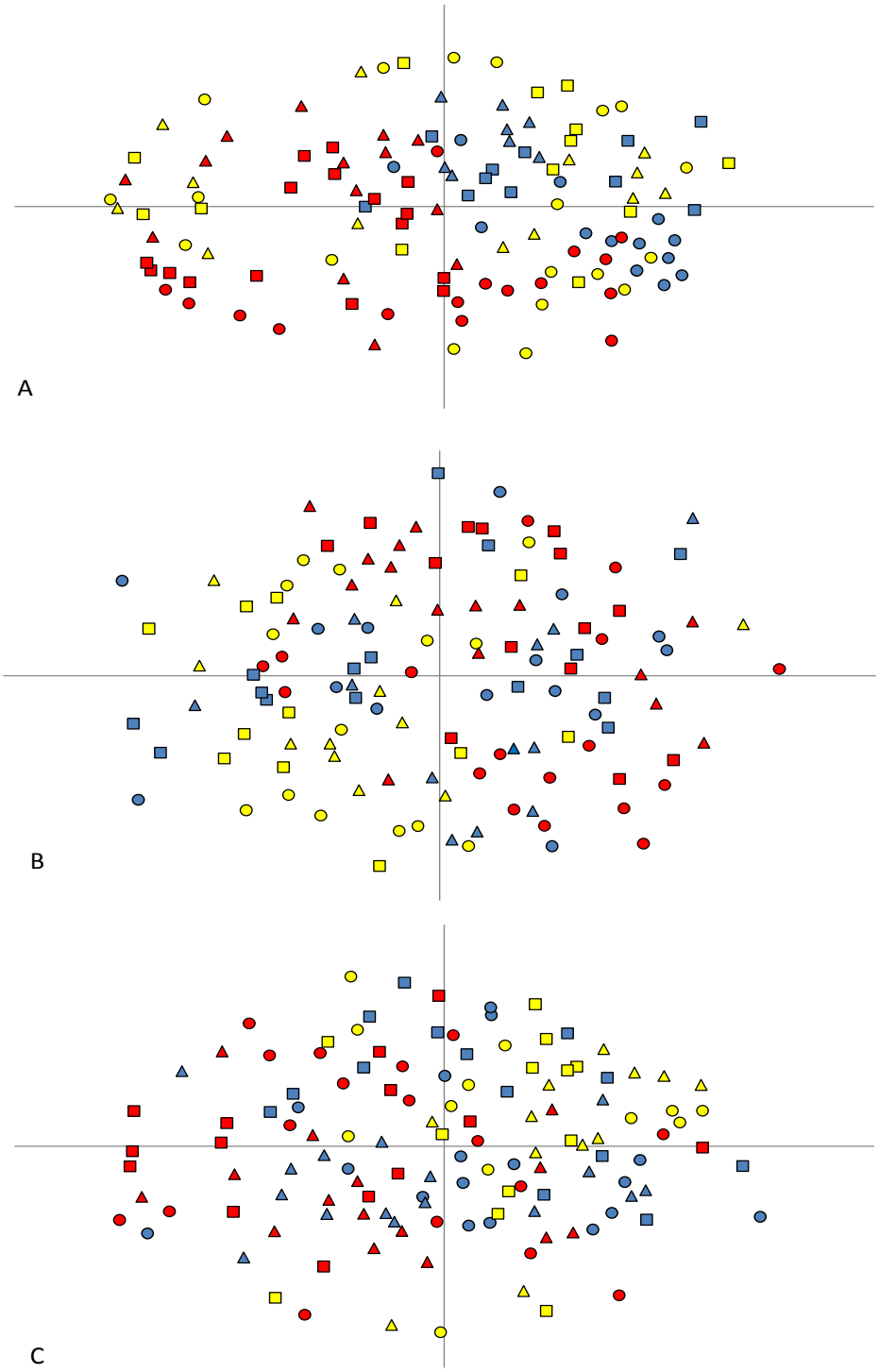
A
B
C

Figure 4. NMDS ordinations showing similarities (theta index) in bacterial communities on the roots (squares) and in the rhizosphere (triangles) and bulk soil (circles) associated with three plant species: *Chasmanthium laxum* (yellow), *Dichanthelium boscii* (blue), and *Microstegium vimineum* (red). Samples were divided into three sample dates corresponding to late spring green-up (a; stress=0.31), summer peak biomass (b; stress=0.32), and fall senescence (c; stress=0.32).

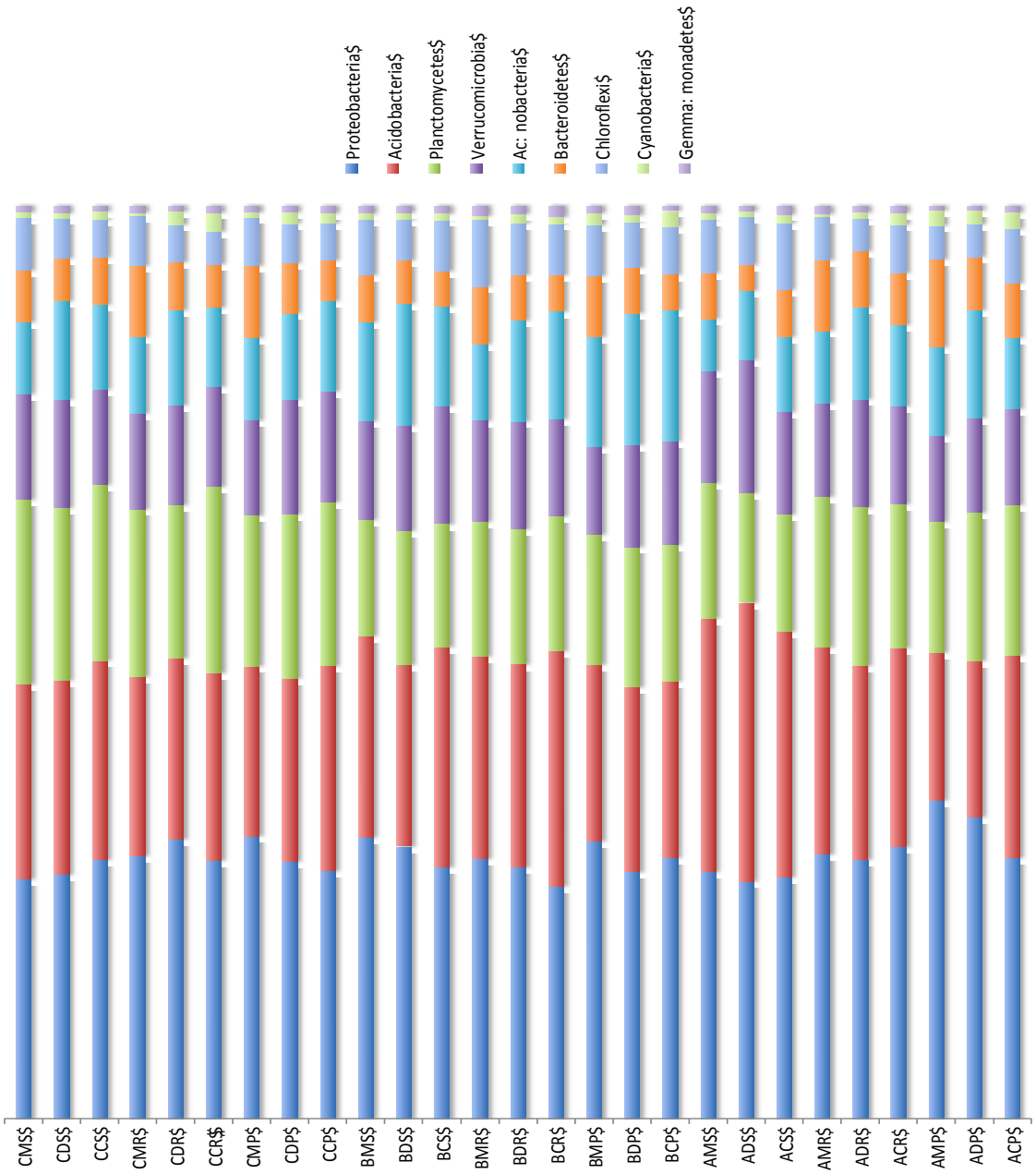

Figure 5. Relative abundance of dominant bacterial phyla (as determined from Ilumina 16S rRNA sequencing) associated with the roots, rhizosphere, or bulk soil around three plant species,

as determined over three seasons. Samples are indicated by three letter codes corresponding to sample date (A=spring green-up, B=summer peak biomass, C=fall senescence), plant species (C=*Chasmanthium laxum*, D=*Dichanthelium boscii*, M=*Microstegium vimineum*), and sample type (P=root surface, R=rhizosphere, S=bulk soil). Each bar represents the mean of 16 samples, and percentages are determined from a total of 377 samples-10,205-135,560 total sequences per sample.

DISCUSSION

In this study, I examined the structure and function of the soil bacterial community associated with the invasive grass *M. vimineum* (Japanese stilt grass) and two native grasses, *C. laxum* (slender wood oats) and *D. boscii* (Bosc's panicgrass), found in the same habitat. Overall, my results suggest that each plant has a different bacterial community associated with it, and that there are differences in soil functioning around these plants. At both a structural and functional level, the microbial assemblage associated with *M. vimineum* appears to be distinct from that associated with *C. laxum*, although that associated with *D. boscii* shows similarities to both *C. laxum* and *M. vimineum*. My results also show that differences in bacterial community structure are more apparent on the root surface or in the rhizosphere, and may not extend into the surrounding bulk soil, even though the surrounding soil is where variation in soil enzyme activity existed among the different plant species.

Previous studies have shown that *M. vimineum* preferentially takes up NH_4^+ and NO_3^- over organic sources, has a higher C:N shoot ratio, and allocates more N to aboveground biomass than native species (Fraterrigo et al. 2011). Areas invaded by *M. vimineum* have been found to increase soil nitrogen cycling, increase soil pH, and reduce rates of litter decomposition (Ehrenfeld et al. 2001; Kourtev et al. 2002, 2003). I found slightly higher soil pH around *M. vimineum* compared to the other plant species examined, but more importantly, soil enzyme activity was lower in the soil around *M. vimineum* than the native species, with significantly lower activities of phosphatase, NAGase, CBH, and peroxidase on the first and last sample dates.

These lower activities of soil enzymes in areas associated with *M. vimineum* are consistent with previous findings that *M. vimineum* lowers soil enzyme activity relative to native species (Ehrenfeld et al. 2001, Kourtev et al. 2003). While those prior studies compared *M. vimineum* to very different plant species than those in this study (to *Vaccinium pallidum,* an invasive shrub, and *Berberis thunbergii*, a native shrub), the consistent finding of lowered enzyme activity around *M. vimineum* does suggest potentially lower rates of nutrient cycling in *M. vimineum* invaded soils. *M. vimineum* is an annual, a life history that could select for slower nutrient cycling since the plant is not relying on the recycling and storage of nutrients from year to year. In contrast, the native species compared in this and prior studies were perennial species (all were chosen because of co-occurrence with *M. vimineum*), which could show different nutrient cycling patterns. *M. vimineum* also has a shallower rooting system than the native grasses sampled in this study, which could limit the spread of exudates into the soil, lessening any selection pressure for soil microbial communities that could facilitate faster nutrient cycling (Wardle et al. 2004).

While there were generally differences in bacterial community structure between plants, these differences were minimized on the final sample date, when the plants were beginning to senesce. I initially expected that differences in bacterial community structure would increase throughout the growing season, so this result was unexpected. Senescence likely corresponds to a reduction in root exudates, which may have resulted in less selection for species-specific bacterial communities on this date. However, this implies that any selective effect of species-specific root exudates is transitory, as these exudates would presumably have been produced throughout the growing season. Interestingly, species-related differences in bacterial community

structure were greatest at the beginning of the growing season, which, while unexpected, has been reported in other systems (Smalla et al. 2001).

Site had only minimal effects on bacterial community composition, whether in the soil, the rhizosphere, or on the root surface. This somewhat validates the decision to treat site as part of the error term for a number of analyses, and the sites therefore appear to be valid replicates. More interesting, however, is that there were site differences in soil chemistry, with Sites 3 and 4 having significantly higher levels of soil C and N than Sites 1 and 2 (which were approximately 50 km further north and in a different system). Thus, regardless of these significant differences in soil chemistry, bacterial communities in the same specific locations (root, rhizosphere, bulk soil) at all sites were generally similar, and plant species or sample type was more important in determining bacterial community structure than site location or soil chemistry. However, there was a site effect on soil enzyme activity with soils from Sites 3 and 4 consistently showing higher enzymatic activity than soils from Sites 1 and 2. This suggests that while bacterial community structure may be similar across all sites, microbial activity (which likely includes activity from non-bacterial microorganisms) is not, and this activity may relate to chemistry differences between the different sites. Higher enzymatic activity is generally related to increased nutrient availability (Sinsabaugh et al. 2014), and the higher activity at Sites 3 and 4 may relate to higher soil C and N at those sites.

The relative abundances of the major phyla of bacteria were similar across all sample types (root, rhizosphere, soil), with no sample type having clear differences at that taxonomic level. This contrasts with previous studies that have shown broad taxonomic differences between the bulk soil and the rhizosphere (Smalla et al. 2001, Kourtev et al. 2002, Fierer et al. 2007, Lundberg et al. 2012). Several of these studies took place before the advent of next generation

sequencing methods (Smalla et al. 2001, Kourtev et al. 2002) which could mean that the sampling depth achieved was not adequate to detect finer taxonomic levels. The studies that have used next generation sequencing methods (Fierer et al. 2007, Lundberg et al. 2012) both had fewer overall sequence reads, while having more overall samples than my study. This suggests that these previous lack sufficient sequencing depth which could imply taxonomic differences where there are none.

There were significant differences in bacterial community composition between plant species. Bacterial communities associated with *C. laxum* and *M. vimineum* were consistently different, on all three sampling dates, while that associated with *D. boscii* was only significantly different from the other two species on the first sampling date. *D. boscii* plants generally occurred much closer to the *M. vimineum* stands than C. laxum, which might account for greater similarity of *D. boscii* and *M. vimineum* bacterial communities. At Site 3, the only *D. boscii* plants that were found were within a dense *M vimineum* stand, while at the other sites *D. boscii* was sampled close to *C. laxum*.

This study suggests that bacterial community structure and soil enzyme activity associated with *M. vimineum* are different from that associated with native grasses, and that plant species has more of an influence on community composition than soil chemistry. However, while site differences in soil chemistry had little influence on bacterial community composition, they were important for enzyme activity, with sites with higher C and N content having consistently higher activity. At a species level, soils associated with *M. vimineum* had consistently lower activity of phosphatase, NAGase, peroxidase and CBH, compared to the native species, suggesting lower rates of nutrient cycling around the invasive. At a structural level, root and rhizosphere bacterial communities of *M. vimineum* were significantly different

from those of *C. laxum* across all sampling dates, while those of *D. boscii* showed similarity to both other species. While bulk soil samples showed differences in enzyme activity with species, they did not differ in terms of bacterial community composition. Thus, the invasive plant *M. vimineum* appears to select for a distinct root and rhizosphere bacterial community, which may be different from those associated with native grasses. While the effects of the plant on community composition may not extend beyond the rhizosphere, soils around *M. vimineum* can still show reduced enzymatic activity and chemical changes. Long-term invasion of *M. vimineum* will cause local changes in nutrient cycling, changes in soil pH and alter local bacterial community composition.

Table 1. Results from three-way ANOVAs (using type II sums of squares) examining differences in the activity of phosphatase, NAGase, cellobiohydrolase (CBH), β-glucosidase, peroxidase, or phenol oxidase in soils vegetated with one of three plant species: *Chasmanthium laxum*, *Dichanthelium boscii*, and *Microstegium vimineum*. Samples were collected from four different sites, each on three sampling dates (spring green-up, summer peak biomass, and fall senescence). Degrees of freedom for each level of analysis are in parentheses and *p* values indicate significant effects with ns indicating $p>0.05$.

	Enzyme Activity					
Effect	Phosphatase	NAGase	CBH	β-glucosidase	Peroxidase	Phenol Oxidase
Plant (2)	$p<0.001$	$p<0.01$	$p<0.001$	$p<0.01$	$p<0.001$	ns
Date (2)	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	ns
Site (3)	$p<0.001$	ns	$p<0.001$	$p<0.001$	$p<0.01$	$p<0.001$
Plant x Date (4)	$p<0.05$	ns	ns	ns	$p<0.001$	ns
Date x Site (6)	ns	ns	ns	ns	$p<0.001$	$p<0.05$
Plant x Site (9)	ns	ns	ns	ns	ns	ns
Plant x Date x Site (4)	ns	ns	ns	ns	$p<0.05$	ns

Table 2. Characteristics of bulk soil associated with three plant species: *Chasmanthium laxum* (C), *Dichanthelium boscii* (D), and *Microstegium vimineum* (M). Soils were sampled at three time points corresponding to different seasons (spring green-up, summer peak biomass, fall senescence) across four sites in northern Mississippi, USA. Values represent the mean (SE) of four plants.

	Soil Characteristics*			
	Soil Moisture (%)	Soil C (mg g^{-1})	Soil N (mg g^{-1})	Soil pH
Spring Green-up				
Site 1				
C	22.8 (2.2)	1.26 (0.17)	0.10 (0.01)	5.7 (0.0)
D	19.4 (0.4)	0.82 (0.08)	0.07 (0.01)	5.6 (0.0)
M	23.1 (1)	1.31 (0.13)	0.10 (0.01)	6.2 (0.3)
Site 2				
C	15.2 (0.5)	0.89 (0.12)	0.07 (0.01)	5.7 (0.0)
D	11.7 (0.6)	0.76 (0.05)	0.06 (0.00)	5.89 (0.1)
M	13.9 (0.7)	1.16 (0.20)	0.09 (0.00)	6.1 (0.1)
Site 3				
C	19.2 (2.8)	2.48 (0.65)	0.10 (0.65)	7.3 (0.3)
M	22.8 (2.2)	2.91 (0.27)	0.15 (0.01)	7.2 (0.1)
Site 4				
C	29.3 (1.2)	2.47 (0.28)	0.20 (0.02)	5.7 (0.1)
D	23.5 (1.6)	1.82 (0.22)	0.10 (0.01)	5.6 (0.1)
M	28.2 (1.3)	2.24 (0.12)	0.18 (0.01)	6.1 (0.0)

Summer Peak Biomass				
Site 1				
C	22.2 (1.7)	1.73 (0.28)	0.11 (0.01)	6.6 (0.2)
D	14.2 (1)	1.13 (0.06)	0.08 (0.01)	5.8 (0.1)
M	26.0 (1.8)	1.80 (0.28)	0.13 (0.01)	6.3 (0.1)
Site 2				
C	16.0 (2.1)	1.18 (0.19)	0.09 (0.01)	6.4 (0.1)
D	13.6 (0.4)	0.72 (0.11)	0.06 (0.01)	6.2 (0.1)
M	22.0 (5.3)	2.09 (0.42)	0.15 (0.02)	6.33 (0.1)
Site 3				
D	15.6 (0.4)	2.60 (0.18)	0.17 (0.01)	6.0 (0.1)
M	20.7 (2.3)	2.73 (0.32)	0.15 (0.02)	7.1 (0.1)
Site 4				
C	15.6 (1.9)	1.78 (0.27)	0.13 (0.02)	5.6 (0.0)
D	20.7 (3.3)	1.84 (0.52)	0.12 (0.04)	6.1 (0.2)
M	24.9 (2.7)	1.76 (0.25)	0.14 (0.02)	6.2 (0.0)
Fall Senescence				
Site 1				
C	25.1 (4.1)	2.05 (0.27)	0.13 (0.01)	5.7 (0.1)
D	19.9 (1.1)	2.04 (0.24)	0.14 (0.01)	5.7 (0.1)
M	25.1 (1.3)	2.12 (0.10)	0.14 (0.01)	6.4 (0.2)

Site 2				
C	17.5 (1.6)	1.85 (0.22)	0.14 (0.02)	6.5 (0.1)
D	16.8 (3.9)	1.74 (0.60)	0.12 (0.04)	6.4 (0.3)
M	17.5 (1.8)	1.6 (0.20)	0.12 (0.01)	6.5 (0.1)
Site 3				
D	23.0 (1.0)	2.75 (0.13)	0.19 (0.01)	5.9 (0.2)
M	24.5 (2.9)	2.16 (0.35)	0.16 (0.02)	6.7 (0.1)
Site 4				
C	25.9 (2.1)	2.4 (0.30)	0.17 (0.03)	5.7 (0.0)
D	15.5 (1.0)	2.11 (0.58)	0.14 (0.03)	6.1 (0.0)
M	25.9 (2.0)	2.16 (0.32)	0.18 (0.03)	5.9 (0.1)

* Soil pH was significantly higher ($p<0.001$) for soils around *M. vimineum* on all sample dates. Soil N and soil moisture were significantly lower ($p<0.05$) for soils around *D. boscii* on the first sample date. Soil C and N content were significantly higher at Sites 3 and 4 compared to Sites 1 and 2 on all sample dates ($p<0.001$). Missing plant species on some dates is because not all plant species were found at all sites on all sampling dates.

Table 3. Pairwise comparisons of bacterial community structure associated with three plant species at four sites over three different seasons in northern Mississippi, USA. Sites were labeled as 1-4, with Sites 1 and 2 (1 km apart) being approximately 50 km north of Sites 3 and 4(0.5 km apart). Samples are designated by a three letter code indicating sample date (A=spring green-up, B=summer peak biomass, C=fall senescence), plant species (C=*Chasmanthium laxum*, D=*Dichanthelium boscii*, M=*Microstegium vimineum*), and sample type (P=root surface, R=rhizosphere, S=bulk soil). Significant differences in community structure by site are denoted by *, while ns indicates no significant effect according to AMOVA.

Site Comparisons Sample	1 to 2	1 to 3	1 to 4	2 to 3	2 to 4	3 to 4
ACP	ns	ns	ns	ns	ns	ns
ADP	ns	ns	ns	ns	ns	ns
AMP	ns	ns	ns	ns	ns	ns
BCP	ns	ns	ns	ns	ns	ns
BDP	ns	ns	ns	ns	ns	ns
BMP	ns	ns	ns	ns	ns	ns
CCP	ns	ns	ns	ns	ns	ns
CDP	ns	ns	ns	*	ns	*
CMP	ns	ns	ns	ns	*	ns
ACR	ns	ns	ns	ns	ns	ns
ADR	ns	ns	ns	ns	ns	ns
AMR	ns	ns	ns	ns	ns	ns
BCR	ns	ns	ns	ns	*	ns
BDR	ns	ns	*	ns	ns	ns
BMR	ns	ns	ns	*	ns	ns
CCR	ns	ns	ns	ns	ns	ns
CDR	ns	ns	ns	ns	ns	ns

CMR	ns	ns	ns	ns	ns	ns
ACS	ns	ns	ns	ns	ns	*
ADS	ns	ns	ns	ns	ns	ns
AMS	ns	ns	ns	ns	ns	ns
BCS	ns	ns	ns	ns	*	ns
BDS	ns	ns	ns	ns	ns	ns
BMS	ns	ns	ns	ns	ns	ns
CCS	ns	ns	ns	ns	ns	ns
CDS	ns	ns	ns	ns	ns	ns
CMS	ns	ns	ns	ns	ns	ns

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PEER REVIEWED PUBLICATIONS

Tucker, C., **Bell J.**, K. Ogle, E. Pendall. "Does carbon use efficiency explain thermal acclimation of soil respiration?" *(Global Change Biology 2013)*

Nie, M., Meng, L., **Bell, J.**, Raut, S., Pendall, E., "Plant root traits as sensors and regulators of elevated CO_2 in terrestrial ecosystems" *(Global Ecology and Biogeography 2013*