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Plant nitrogen uptake drives rhizosphere bacterial community assembly during plant growth

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ABSTRACT

When plants establish in novel environments, they can modify soil microbial community structure and functional properties in ways that enhance their own success. Although soil microbial communities are influenced by abiotic environmental variability, rhizosphere microbial communities may also be affected by plant activities such as nutrient uptake during the growing season. We predicted that during the growing season, plant N uptake would explain much of the variation in rhizosphere microbial community assembly and functional traits. We grew the invasive C_3 grass *Bromus tectorum* and three commonly co-occurring native C_3 grasses in a controlled greenhouse environment, and examined rhizosphere bacterial community structural and functional characteristics at three different plant growth stages. We found that soil N availability and plant tissue N levels strongly correlated with shifts in rhizosphere bacterial community structure. It also appeared that the rapid drawdown of soil nutrients in the rhizosphere during the plant growing season triggered a selection event whereby only those microbes able to tolerate the changing nutrient conditions were able to persist. Plant N uptake rates inversely corresponded to microbial biomass N levels during periods of peak plant growth. Mechanisms which enable plants to influence rhizosphere bacterial community structure and function are likely to affect their competitive ability and fitness. Our study suggests that plants can alter their rhizosphere microbiomes through influencing nutrient availability. The ways in which plants establish their rhizosphere bacterial communities may now be viewed as a selection trait related to intrinsic plant species nutrient demands.

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1. Introduction

When plants establish in soils, they immediately interact with soil microbial communities and begin to profoundly alter the habitat within the rhizosphere-the rooting zone where soil microbes quickly assimilate plant-derived carbon and compete with plants for available nutrients (Schimel and Bennett, 2004; Mendes et al., 2013; Philippot et al., 2013). Plants affect the physical and chemical conditions within the rhizosphere in several ways, such as altering the soil environment through root growth (Bever et al., 2010; Padilla et al., 2013) increasing organic carbon availability through root exudation (Nannipieri et al., 2008; Kuzyakov, 2010; Bird et al., 2011) and decreasing water and nutrient availability through uptake (Jackson et al., 1989; Herman et al., 2006; Marschner et al., 2011). Clearly, these myriad changes can drive rapid and fundamental shifts in the rhizosphere microbiome. Not surprisingly, rhizosphere microbial communities differ in structure and function from bare soils and even bulk soils within any environment (Knelman et al., 2012; Bell et al., 2014a; Ciccazzo et al., 2014). But which of these many plant-mediated changes are most important in structuring microbial communities?

While soil microbial communities often differ among plant species (Grayston et al., 1998; Donn et al., 2014) and even among plant genotypes (Zancarini et al., 2012; Mariotte et al., 2013), in some cases plant species do not explain the variation among rhizosphere microbiomes (Arenz et al., 2014; Bell et al., 2014a). This

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raises the question of whether plant species differ in their ability to modify their rhizosphere microbiome, and if so, what are the mechanisms underlying these differences? One challenge is that observational studies of mature field plants offer little insight into the mechanisms by which plants affect rhizosphere microbiomes, as any differences could be due to establishment in favorable microsites. Mounting evidence that rhizosphere bacterial community composition changes throughout plant developmental stages (Houlden et al., 2008; Micallef et al., 2009; Ziegler et al., 2013) suggests that plants do have an important role in structuring soil bacterial communities throughout the growing season (Kourtev et al., 2002; Marschner et al., 2004; Fierer et al., 2013). But again, we are left to wonder how plant species may differentially influence their rhizosphere microbiomes.

Plant – microbe – soil feedbacks play a fundamental role in plant community establishment and success (Reynolds et al., 2003). When plant species establish in novel environments, they appear to shift rhizosphere microbial community structural and functional associations in ways that enhance their success (Ehrenfeld et al., 2005; Turan et al., 2012; Wang et al., 2012). By establishing positive soil feedbacks (Levine et al., 2006; Diez et al., 2010; Bever et al., 2012) plants are sometimes able to stimulate increased nutrient mineralization which may improve their ability to compete with other plant species. For example, when the exotic C_3 annual grass *Bromus tectorum* invades habitats in western North America upon disturbance, it appears to support soil bacterial communities within its rhizosphere that are capable of higher N mineralization rates relative to rhizosphere communities associated with native plants (Hawkes et al., 2005; Schaeffer et al., 2011). This positive plant-soil feedback contributes to *B. tectorum's* ability to outcompete native plants for available nutrients (Sperry et al., 2006; Vasquez et al., 2008).

Plant nutrient uptake can stimulate soil bacterial N mineralization (Bever et al., 2010, 2012). Any reduction in soil nutrient availability increases C:N stoichiometry within the rhizosphere, which can initiate microbial extracellular enzyme production to oxidize or hydrolyze soil organic matter to release otherwise unavailable nutrients (Allison et al., 2007; Kuzyakov, 2010; Drake et al., 2013). Although it is clear that plant nutrient uptake can influence changes in soil N availability by inducing soil microbial enzyme production, we do not yet know if plant nutrient uptake also influences rhizosphere bacterial community composition.

Due to the relatively narrow stoichiometric flexibility of soil bacteria (Anderson et al., 2005; Cherif and Loreau, 2013), rapid shifts in N availability within the rhizosphere resulting from plant nutrient uptake could strongly alter soil bacterial community structure (Hessen et al., 2004; Ramirez et al., 2012; Sardans et al., 2012). Plant N uptake coupled with root C inputs (via exudation or root sloughing) during the growing season (Fig. 1) could impose strong N limitation within the rhizosphere (Phillips et al., 2011; Perveen et al., 2014), favoring only those microbes that can tolerate the altered nutrient conditions (Sinsabaugh et al., 2009; Sistla and Schimel, 2012; Fanin et al., 2013). Although plant and soil microbial communities often appear to be closely linked (Bell et al., 2014a; Courty et al., 2014; Schmidt et al., 2014), few studies have directly observed plant influences on bacterial community assembly and function at multiple points throughout the growing season within the rhizosphere.

If plant N uptake and subsequent declines in available N within the rhizosphere influence bacterial community composition, then this may be a key mechanism by which plants affect bacterial community structure and function throughout the plant growing season. The main objective of this research was to study how plant nutrient uptake and soil C and N dynamics within the rhizosphere and in unvegetated soils influence bacterial community structure during different plant growth stages. We grew plants in a controlled greenhouse environment, and examined bacterial community composition and biomass within the plant rhizosphere as well as plant N uptake and soil N availability at three time points throughout the growing season to represent early, mid, and peak plant growth. We predicted that plant N uptake would explain much of the variation in rhizosphere bacterial community assembly and functional traits among plant species, directly corresponding to timing and nature of plant growth. We also predicted that soil and microbial biomass C:N would inversely correlate with their respective enzyme activities. More specifically, since the invasive grass *B. tectorum* exhibits higher N uptake rates compared to native grass species (Miller et al., 2006; Sperry et al., 2006; Perkins et al., 2011), we predicted that soil enzyme activities and soil nutrient availability would increase more quickly in the rhizosphere of *B. tectorum* during the early stages of the growing season compared to the other native grass species. We also predicted that *B. tectorum* would alter bacterial community structure within the rhizosphere more quickly than the other native grasses.

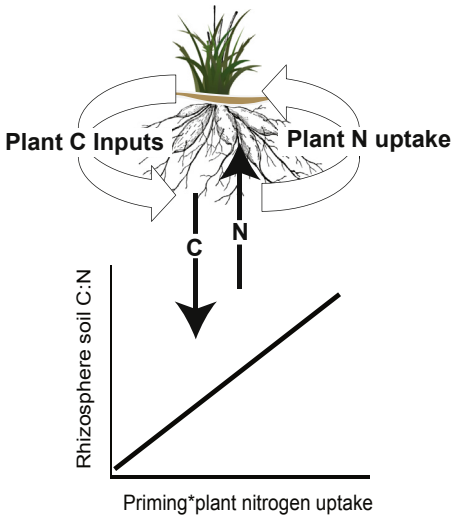

Fig. 1. During periods of rapid plant growth, high nutrient uptake may impose nutrient constraints within the rhizosphere zone that can act as a filter on microbial community characteristics.

2. Materials and methods

2.1. Experimental design

This study focused on elucidating the influence of actively growing plants on soil nutrient availability and bacterial community structure by characterizing species-specific rhizosphere microbe traits across a growing season. We selected four C_3 grass species, including: *B. tectorum* L. (Cheatgrass), *Koeleria macrantha* (prairie Junegrass; (Ledeb.) Schult.), *Pascopyrum smithii* (western wheatgrass (Rydb.) Á. Löve), and *Vulpia octoflora* (Sixweeks fescue; (Walter) Rydb.) to observe at three different stages throughout the growing season. These plant species use the same photosynthetic pathway, but differ in life history (phenology) and root structural traits, which could influence rhizosphere bacterial associations. For example, *B. tectorum* is an invasive winter annual grass introduced to North America with a fibrous rooting pattern. *K. macrantha* is a native perennial bunchgrass with a fine fibrous rooting pattern. *P. smithii* is a native perennial with a rhizomatous rooting pattern. Lastly, *V. octoflora* is a winter annual native to North America also with a fibrous rooting pattern. Seeds for all three native grass

species were purchased from Granite Seed CO (Lehi, UT). Seed for *B. tectorum* was collected within the city limits of Fort Collins, CO.

The soil used for plant growth was collected to a depth of 10 cm (on February 2012) at a 130-hectare property managed by Colorado State University located north of Fort Collins, Colorado (40°42′54.45″N, 105° 5′53.78″W; 1584 m.a.s.l.; 1–3 % slopes). The dominant vegetation at this site previously consisted of *Agropyron cristatum* L. Gaertn. (crested wheatgrass) and *Ericameria nauseosus* (Pall. ex Pursh) G.L. Nesom & Baird (rubber rabbit brush), which was removed by tilling in October 2010. These alkaline soils (field soil pH mean ± SE = 8.3 ± 0.05) are widely found in agro-ecosystems, and broadly classified in the Alfisols soil order, and more specifically characterized as Stoneham loam (fine-loamy, mixed, Haplustalfs) (NRCS, 2012).

Once collected, soils were sieved and mixed at a 1:1 ratio with sterile sand to maximize soil porosity while maintaining a reasonable amount of field soil for its nutrient and bacterial properties. Before mixing, soils were sieved using a 4 mm sieve to remove large objects (rocks, roots, etc.) and then using a 2 mm sieve to better homogenize aggregate size (Boone et al., 1999). The sand (Quikrete; Atlanta, GA) was first sterilized in an autoclave for 30 min at 121 °C and 17 PSI, and was then saturated with tap water (on three separate occasions for ~ 30 min intervals) until a pH of ~8.2 was achieved to match the *in situ* field soil pH conditions (prior to mixing, sand pH mean ± SE = 10.6 ± 0.01). The sieved field soil – sand mix was stored in covered plastic totes for 5 weeks in a low humidity dark area to stabilize before use.

The soil mixture was then used to fill rectangular planting boxes (24-cm × 2.5-cm, 40-cm tall) with Plexiglas doors (custom designed by Fort Collins Plastics; Fort Collins, CO), hereafter referred to as root boxes. Each root box was first filled with a 2.5 cm layer of washed and autoclaved pea gravel (Quikrete; Atlanta, GA) then 35 cm of the field soil - sand mix. The Plexiglas doors of the root boxes were secured with Velcro straps and covered with opaque vinyl in order to exclude light from the soil profile. Soils were then saturated and allowed to settle in the root boxes for two weeks before planting.

Our goal was to establish enough individual plants per root box so that the majority of the soil profile would eventually be influenced directly by roots. All plant species were grown in monoculture, with approximately 6 plants per each root box. The unvegetated soil controls were not sown, but were watered the same as the seeded root boxes. To ensure that we would be able to achieve the desired sample size in case of any plant death across the study, twenty–two replicate root boxes were sown with the seeds of each grass species on 16 March, 2012 at the Plant Growth Facilities greenhouse on the Colorado State University campus (Fort Collins, Colorado). The greenhouse maintained a temperature of 23 °C for daytime and 17 °C at night, with a 16 h photoperiod. To allow for optimal access to the rhizosphere during sampling, the root boxes were angled at approximately 75 degrees (within the plastic crates) to promote root growth via gravity toward the Plexiglas hinged door. The top 10 cm of soil was re-wetted daily until seeds germinated and plants were established. Germination rates varied somewhat among plant species, but all plant species' seed germinated within two weeks of planting. Watering was then reduced to approximately every other day to keep the soil profile consistently moist so water was not a limiting factor for plant growth throughout the study. Caution was taken not to 'over-water' the soils to avoid leaching of water and soil nutrients through the bottom of the root boxes. Unvegetated (bare) soil controls were watered less frequently (as needed) to remain moist, but ultimately demonstrated significantly higher soil moisture levels in the latter stages of the study due to the absence of evapotranspiration associated with the grasses (Table 1). Plants were sampled at three different stages throughout the growing season, representing early growth, mature growth and peak biomass/senescence. The sampling periods were at days 28, 76, and 152 days from germination.

Table 1
Soil N, and edaphic characteristics among four – C_3 plant species rhizospheres across a growing season at days 28, 76, and 152 from plant seed germination.

		Day 28	Day 76	Day 152
DV	Species	$P = 0.65$	$\mathbf{P = 0.03}$	$\mathbf{P = 0.01}$
NH_4	bare	0.54 ± 0.03	**0.52 ± 0.11^{bc}**	**0.66 ± 0.06^{b}**
	BRTE	0.68 ± 0.11	**0.51 ± 0.07^{bc}**	**1.03 ± 0.24^{a}**
	KOMA	0.54 ± 0.05	**0.4 ± 0.09^{c}**	**0.39 ± 0.04^{bc}**
	PASM	0.52 ± 0.03	**1.44 ± 0.37^{a}**	**0.59 ± 0.04^{b}**
	VUOC	0.66 ± 0.13	**1.23 ± 0.38^{ab}**	**0.37 ± 0.02^{c}**
		$P = 0.4$	$\mathbf{P < 0.001}$	$\mathbf{P = < 0.001}$
NO_3	bare	19.11 ± 1.63	**34 ± 3.45^{a}**	**12.6 ± 1.18^{a}**
	BRTE	18.49 ± 3.52	**1.33 ± 0.04^{d}**	**1.14 ± 0.53^{c}**
	KOMA	13.85 ± 2.86	**9.25 ± 1.84^{b}**	**0.83 ± 0.27^{c}**
	PASM	15.19 ± 2.91	**9.8 ± 2.39^{b}**	**0.25 ± 0.03^{d}**
	VUOC	12.7 ± 5.04	**3.67 ± 0.89^{c}**	**3.94 ± 1.93^{b}**
		$P = 0.32$	$\mathbf{P = 0.001}$	$\mathbf{P < 0.001}$
SM%	bare	9.68 ± 1.5	**12.05 ± 1.18^{a}**	**17.23 ± 0.51^{a}**
	BRTE	9.15 ± 1.5	**6.2 ± 0.22^{c}**	**8.66 ± 0.35^{c}**
	KOMA	8.81 ± 1.04	**8.49 ± 0.73^{b}**	**8.37 ± 0.32^{c}**
	PASM	6.32 ± 0.79	**7.34 ± 0.25^{bc}**	**8.69 ± 0.2^{c}**
	VUOC	7.58 ± 1.27	**7.55 ± 0.73^{bc}**	**11.73 ± 0.93^{b}**
		$P = 0.06$	$\mathbf{P < 0.001}$	$\mathbf{P = 0.004}$
pH	bare	8.02 ± 0.02	**8.02 ± 0.04^{b}**	**8.17 ± 0.01^{b}**
	BRTE	8.09 ± 0.04	**8.27 ± 0.03^{a}**	**8.3 ± 0.06^{a}**
	KOMA	8.11 ± 0.02	**8.33 ± 0.02^{a}**	**8.31 ± 0.01^{a}**
	PASM	8.06 ± 0.03	**8.28 ± 0.05^{a}**	**8.18 ± 0.01^{b}**
	VUOC	8.13 ± 0.02	**8.3 ± 0.04^{a}**	**8.35 ± 0.04^{a}**

Descriptive statistics table presented as mean ± S.E.; N = 4. Letters next to the mean ± S.E. indicate significant differences at $p \leq 0.05$ using Tukey post hoc tests following ANOVA. Rhizosphere associated dependent variables (DV) include: extractable soil ammonium (NH_4), extractable soil nitrate (NO_3), soil pH, and % soil moisture content (SM). All soil N parameters are expressed as $\mu g^{-1} g^{-1}$soil. Significant differences at $p \leq 0.05$ are indicated in **bold**.

All plants demonstrated relatively similar growth across the study, which gave us high confidence that plants were maintaining similar phenological characteristics. However, *V. octoflora* (unlike the other three grass species) produced seed by the day 76 sample period and above ground plant tissue appeared almost fully senesced by day 152. Furthermore, *V. octoflora* also exhibited substantial root degradation by this time period which was not observed in the other plant species, which contributed to higher soil moisture levels associated with *V. octoflora* by day 152 (Table 1). During plant harvesting, aboveground biomass and seed material were collected and ground together to account for any plant tissue nutrient characteristics.

2.2. Sampling and processing

At each of the three sampling periods, day 28 (19 April 2012), day 76 (06 June 2012) and day 152 (22 August 2012) from germination, plant – rhizosphere replicates (along with unvegetated soil control) were randomly sampled for bacterial and nutrient analysis. We destructively harvested plants and rhizosphere soils as single, intact plant/soil complexes. The sample size for each plant species and unvegetated soil was N = 4 for the 28 and 76 day sampling periods; and N = 6 for the 152 day sample period.

We defined rhizosphere soils as any soil ≤0.5 cm of any root structure (*i.e.* fine or coarse) that remained attached to the root zone after excavating the plant/soil complexes followed by lateral shaking and moderate pressure applied by hand to the soil aggregates still clinging to the roots after shaking (Bell et al., 2014a). The rhizosphere soil was carefully removed from the roots using a 2 mm sieve in the greenhouse immediately upon excavation. Soils from

the unvegetated replicates were collected for analysis just below the soil surface from ~ 2 to 7 cm depths and sieved (2 mm) in the greenhouse. After sieving, ~ 1 g soil subsamples intended for bacterial community molecular analysis were collected in sterile 2 mL micro centrifuge tubes and stored immediately at −80 °C. The remaining soils were stored at 4 °C for bacterial and nutrient analysis which was initiated within 24 h of sample collection.

2.3. Soil nutrient and abiotic properties

Inorganic N in the forms of ammonium (NH_4^+) and nitrate (NO_3^-) was assessed via soil KCl extracts (10 g ± 0.02 g of soil in 50 mL of 2 M KCl) that were subsequently analyzed on an Analytical Flow Solution IV instrument (OI Analytical, College Station, TX 77842-9010). We combined extractable NO_3— N and NH_4— N to represent the total available N pools (as $\mu g^{-1} g^{-1}$ soil). Soil pH was measured using an ion-specific probe using a 2:1 soil:DI H_2O ratio (Robertson et al., 1999). Soil moisture was measured by drying soils in an oven at 60 °C for 48 h (Jarrell et al., 1999).

2.4. Plant measurements

Plant material was sorted into aboveground shoot biomass by clipping the plants at the soil surface, pooled for each root box, and placed in a paper bag. Belowground root biomass was washed free of soil (with DI H_2O) using a 2 mm sieve to capture fine roots and bagged. All biomass was then dried (60 °C) for 48 h and weighed. The dried plant tissues were ground to a fine powder for chemical analyses. %C and %N of roots and aboveground production were measured separately on a Finnigan Delta^{Plus} XP connected to a Carlo Erba NC-2500 elemental analyzer via a Finnigan ConFlo III open-split interface. Units for plant root and shoot tissue C and N are expressed as % dry weight.

Plant N uptake was estimated for each sampling period using a mass balance approach. Mean plant N uptake (g N/root box/day) was calculated as:

$$\frac{\overline{W_2 * NF_2} - \overline{W_1 * NF_1}}{t_2 - t_1}$$

where W_t is the dry weight of the plants in the root box, NF_t is the nitrogen fractions. To assess how efficiently plants use nitrogen to produce new biomass, plant nutrient productivity (g dry mass/g N/day) was calculated as:

$$\frac{\bar{r}}{\overline{NF_2}}$$

where $\bar{r}$ is the mean whole plant relative growth rate of time period t_1 to t_2 calculated following Hoffmann and Poorter (2002), as:

$$\frac{\overline{\ln(W_2)} - \overline{\ln(W_1)}}{t_2 - t_1}.$$

2.5. Microbial biomass

Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) was assessed among all rhizosphere and unvegetated soils across all three sample periods using the chloroform fumigation extraction method followed by K_2SO_4 soil extractions (10 g ± 0.02 g of soil in 50 mL of 0.5 M K_2SO_4) (Vance et al., 1987). Fumigated and unfumigated extracts were immediately frozen upon extraction and analyzed together at the end of the final sampling period for total extractable organic C and total extractable N on a Total Organic Carbon analyzer with an N measuring unit (Shimadzu TOC-V_{CPN}; Shimadzu Scientific Instruments, Wood Dale, IL, USA). MBC and MBN were calculated using published correction coefficients: k_{EC} and k_{EN} = 0.30 (Sparling and Zhu, 1993).

2.6. Microbial enzyme activities

We measured the potential activity of seven hydrolytic soil enzymes that degrade a range of substrates that are common constituents of organic matter. These enzymes were selected to represent the degradation of C-rich substrates (β −1,4-glucosidase, β-D-cellubiosidase, α-Glucosidase, and β-Xylosidase), N-rich substrates (β −1,4-N-acetylglucosaminidase and leucine aminopeptidase) and one P-rich substrate (phosphatase) (Sinsabaugh et al., 2009). Enzyme assays were conducted using standard fluorimetric techniques (Saiya-Cork et al., 2002; Wallenstein and Weintraub, 2008; Bell et al., 2013). In brief, assays were conducted by homogenizing 2.75 g of soil in 91 mL of 50 mm sodium acetate buffer (pH 6.8) in a Waring blender for 1 min. The soil slurries were then added to a 96-deep-well (2 mL) microplate using an 8-channel repeat pipettor. Additional quench control replicates of soil slurry and 4-methylumbellfferone (MUB) or 7-amino-4-methylcoumarin MUC standard curves (0–100 μM concentrations) were included with each sample. Soil slurries with fluorometric substrates were incubated for 3 h at 25 °C. After the incubation period, plates were centrifuged for 3 min at 2900 *g*, after which 250 μL of soil slurry was transferred from each well into black Greiner flat-bottomed 96-well plate (into corresponding wells) and then scanned on a TECAN Infinite M200 microplate reader using excitation at 365 nm and emission at 450 nm. Units for all enzyme nutrient acquisition activities are expressed as (nmol activity g dry soil $^{-1}$ h $^{-1}$).

2.7. Bacterial community structure

Bacterial community composition was assessed among all rhizosphere and unvegetated soils across all three sample periods via high throughput 454 pyrosequencing (Margulies et al., 2005; Kuczynski et al., 2010). DNA was extracted with MO BIO PowerSoil DNA isolation kits (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The targeted 16S small-subunit ribosomal genes were amplified using universal 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R GGACTACVSGGGTATCTAAT primers (Caporaso et al., 2011) via polymerase chain reaction (PCR) with a 12 bp unique Golay barcoded primer for each sample (Lauber et al., 2008). The sample amplicons were subsequently cleaned using MO BIO UltraClean-htp 96 Well PCR Clean-Up Kits (MO BIO Laboratories, Carlsbad, CA, USA), quantified using the Quant-iT PicoGreen dsDNA Reagent and Kits (Life Technologies Corporation, Grand Island, NY, USA) and pooled for sequencing. Sequencing data was processed using the Quantitative Insights Into Microbial Ecology (QIIME 1.7.0) pipeline (Caporaso et al., 2010) for determining operational taxonomic unit and taxonomy assignment. In brief, bacterial operational taxonomic units were picked using the 'pick_de_novo_otus.py' work flow script, which assigns sequences to OTUs at 97% similarity by default using the Greengenes database (version 12_10). Beta diversity followed by UniFrac analyses with Principle Coordinates Analysis was also accomplished using QIIME commands (Lozupone and Knight, 2005; Lauber et al., 2009). Weighted Unifrac coordinates were chosen to take operational taxonomic unit (OTU) abundance into consideration when calculating principle coordinate distances between bacterial communities among plant species.

2.8. Statistical methods

Differences in rhizosphere bacterial community structure among plant species and unvegetated soils was assessed using UniFrac (phylogenetic distance) dissimilarity measures followed by Principle Coordinates Analysis (Lozupone and Knight, 2005; Lauber et al., 2009) within the Quantitative Insights Into Microbial Ecology (QIIME 1.7.0) pipeline (Caporaso et al., 2010). To explore other differences in rhizosphere bacterial functional traits across the growing season at days 28, 78, and 152, we used distance-based redundancy analysis (dbRDA) using the R: vegan package (Oksanen et al., 2013). The rhizosphere components we used in this dbRDA model included seven discrete enzyme activities: four C cycling enzymes (BG = β-Glucosidase; CB = β-D-cellubiosidase; XYL = β-Xylosidase; AG = α-Glucosidase); two N cycling enzymes (LAP = Leucine aminopeptidase; NAG = N-acetyl-β-Glucosaminidase) and one P cycling enzyme (PHOS- = Phosphatase). We chose dbRDA over other multivariate statistical approaches because it has non-linear distance-metric options with robust multi-dimensional resolution to assess categorical variables, which is a well acknowledged approach for ecological studies (Legendre and Anderson, 1999). Distance based RDA is a 3-step ordination technique that tests the effects of response parameters (*i.e.* rhizosphere bacterial and soil nutrient characteristics) on defined ecological groups (*i.e.* plant species). First, a dissimilarity or distance matrix is calculated for the community. We selected the Bray–Curtis dissimilarity (non-linear) measure to model the species matrix as suggested by Legendre and Anderson (1999). In steps two and three of the dbRDA, a principal coordinate analysis (PCoA) is calculated based on the distance matrix, from which the eigenvalues (obtained in the PCoA) were applied to a redundancy analysis (RDA).

One-way analysis of variance (ANOVA) was used to determine differences in univariate C:N components of plant traits (*i.e.* enzyme C:N nutrient activities, microbial biomass C:N, soil C:N, plant leaf C:N, and plant root C:N ratios) among species rhizosphere and bare soil samples using SPSS v 20.0 (SPSS: IBM Corp). Non-ratio univariate parameters, including: plant biomass; plant tissue C and N; total C enzyme activities; total N enzyme activities; MBC and MBN; C cycling enzymes: BG, CB, XYL, AG; two N cycling enzymes: LAP and NAG; one P cycling enzyme: PHOS; extractable soil ammonium (NH_4); extractable soil nitrate (NO_3); total extractable inorganic N ($\sum NH_4 + NO_3$); soil pH and % soil moisture content (SM) were analyzed using ANOVA to assess significant belowground differences among plant species and bare soil samples. Tukey post-hoc comparisons were used for all univariate analysis using SPSS v 20.0 (SPSS: IBM Corp). Pearson correlations were calculated to determine relationships among plant, microbial and soil components over time using SPSS v 20.0 (SPSS: IBM Corp).

Stepwise multiple regression analysis was performed to identify the different abiotic and biotic drivers that strongly influence microbial community structural dynamics within soils and plant rhizospheres, using SPSS v 20.0 (SPSS: IBM Corp). The stepwise method was chosen because this technique provides as the first step a single-variable model that accounts for the most variation, and then calculates successively more complex models (Field, 2005). This approach allows for an examination of the relative contributions of each variable to the most parsimonious descriptive model. First, soil moisture, soil pH, soil C and soil N data was modeled as a function of abiotic environmental parameters to assess bacterial community structural shifts in response to environmental conditions among unvegetated and rhizosphere soils. Secondly, plant parameters were also included in to the model (*i.e.* total plant C, total plant N, total plant C:N, total plant biomass and plant N uptake) along with the abiotic parameters (listed above) and modeled as a function of abiotic + plant influences to assess bacterial community structural shifts in response to environmental and/or plant influences strictly among rhizosphere soils. Every R^2 statistic reported in this experiment is from the final significant model ($P \leq 0.01$) provided by the stepwise process. The stepping-method criteria entered any variable with the probability of $F \leq 0.005$ and removed any variable from the model $F > 0.01$.

The Shapiro–Wilk test of normality and Levene's test of equal variances was performed to assess if any univariate parameter distributions significantly deviated from normal using SPSS v 20.0 (SPSS: IBM Corp). We selected natural-log data transformations (as needed) to improve the assumption of normality and homoscedasticity for all subsequent statistical analyses. Means and standard errors were calculated for all parameters assayed using SPSS v 20.0 (SPSS: IBM Corp).

3. Results

3.1. Soil nutrient properties among plant rhizospheres and unvegetated soils

At day 28, soil nitrogen levels (both NH_4 and NO_3) were similar among plant species rhizospheres and unvegetated soils. However, soil NO_3 steadily declined among all plant rhizospheres throughout the remainder of the study (Table 1; $P \leq 0.03$). Among plant species, *B. tectorum* had the lowest soil NO_3 compared to all other plants ($F \geq 18.47$; $P < 0.001$; Table 1); while soil NH_4 was higher in *P. smithii* compared to *B. tectorum* and *K. macrantha* ($F = 3.62$; $P = 0.03$; Table 1) at day 76. By day 152, *B. tectorum* demonstrated the highest NH_4 compared to all other plants and unvegetated soils ($F = 4.18$; $P = 0.01$; Table 1).

Across the growing season, wider soil C:N levels corresponded with higher plant N uptake (Fig. 2a, b; $r = 0.57$; $P \leq 0.025$). By day 78, *B. tectorum* had the highest root N uptake rates ($P = 0.014$; Table 2) which influenced the wider rhizosphere soil C:N ($F = 20.84$; $P < 0.001$; Fig. 3b) due to relatively lower NO_3 and NH_4 availability ($P \leq 0.03$; Table 1). By day 152, soil C:N among all plant rhizosphres was similar, and higher than unvegetated soils (Fig. 3c; $F = 12.51$; $P < 0.001$). At day 152, total plant biomass ($P = 0.004$; Table S1) and total N uptake ($P = 0.026$; Table 2) was similar among *B. tectorum*, *K. macrantha* and *P. smithii*. However, due to earlier senescence, *V. octoflora demonstrated lower* N uptake ($P < 0.001$; Table 2) and lower *biomass* ($P \leq 0.004$; Table S1).

3.2. Soil abiotic properties among plant rhizospheres and unvegetated soils

Soil moisture was similar among plants and unvegetated soils at day 28 (Table 1). However, at day 76 and 152, soil moisture was highest in the unvegetated soils compared to all rhizospheres, likely due to lower evapotranspiration rates in the unvegetated soils ($P \leq 0.001$; Table 1). Among plant species, soil moisture trended lowest in the rhizosphere of *B. tectorum* at day 76, but differences were only significant when compared to *K. macrantha* ($P = 0.05$; Table 1). By day 152, *V. octoflora* exhibited significantly higher soil moisture compared to other rhizosphere soils ($P < 0.001$; Table 1), which was likely due to the substantial root senescence that occurred, which decreased soil moisture uptake. Soil pH was similar among plants and unvegetated soils at day 28 (Table 1). By day 76, soil pH was significantly higher in all rhizosphere soils compared to unvegetated soils ($P \leq 0.001$; Table 1). This pattern persisted for the remainder of the study ($P = 0.004$; Table 1).

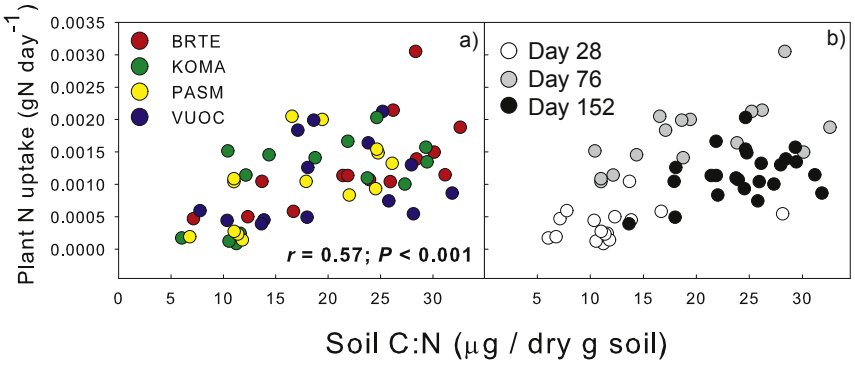

Fig. 2. Plant N uptake positively correlated with increased rhizosphere soil C:N across the growing season. Footnote: Temporal shifts in plant N uptake in response to rhizosphere soil C:N among the four − C3 plant species. Significant correlations (at $P \leq 0.05$) are indicated in bold. The sample size for all plant species is N = 4 for days 28 and 76, and N = 6 for day 152. Plant abbreviations and color codes for the four − C3 grass species include: *Bromus tectorum* (BRTE); *Koeleria macrantha* (KOMA); *Pascopyrum smithii* (PASM); *Vulpia octoflora* (VUOC). Soil C:N = soil extractable organic C (SOC)/total soil N (TN).

Table 2
Plant N uptake rates among four − C_3 plant species rhizospheres across a growing season at days 28, 76 and 152 from plant seed germination. Significant differences at $p \leq 0.05$ are indicated in **bold**. Letters next to the mean ± S.E. indicate significant differences at $p \leq 0.05$ using Tukey post hoc tests following ANOVA.

		Day 28	Day 76	Day 152
DV	Species	***P* = 0.025**	***P* = 0.014**	**P < 0.001**
Root N uptake (g N/day)	BRTE	**6.19E-05 ± 2.14E-05^{ab}**	**4.25E-04 ± 1.14E-04^{a}**	**1.72E-04 ± 2.74E-05^{b}**
	KOMA	**2.07E-05 ± 3.35E-06^{c}**	**1.21E-04 ± 1.56E-05^{b}**	**2.96E-04 ± 3.98E-05^{a}**
	PASM	**2.23E-05 ± 6.27E-06^{bc}**	**1.91E-04 ± 4.01E-05^{b}**	**3.48E-04 ± 5.25E-05^{a}**
	VUOC	**7.60E-05 ± 1.41E-05^{a}**	**1.20E-04 ± 2.46E-05^{b}**	**6.45E-05 ± 1.39E-05^{c}**
		***P* = 0.001**	P = 0.13	**P = 0.026**
Total Plant N Uptake (g N/day)	BRTE	**6.50E-04 ± 1.35E-04^{a}**	2.14E-03 ± 3.31E-04	**1.15E-03 ± 5.06E-05^{ab}**
	KOMA	**1.56E-04 ± 3.41E-05^{b}**	1.38E-03 ± 8.19E-05	**1.45E-03 ± 1.56E-04^{a}**
	PASM	**2.11E-04 ± 2.78E-05^{b}**	1.54E-03 ± 2.78E-04	**1.19E-03 ± 1.21E-04^{ab}**
	VUOC	**5.09E-04 ± 3.70E-05^{a}**	1.90E-03 ± 1.04E-04	**8.42E-04 ± 1.55E-04^{b}**
		***P* < 0.001**	***P* = 0.01**	P = 0.7
Plant Nutrient Productivity (g dry mass/g N/day)	BRTE	**2.62 ± 0.08^{b}**	**1.78 ± 0.02^{b}**	1.81 ± 0.26
	KOMA	**2.2 ± 0.06^{c}**	**2.4 ± 0.18^{a}**	1.76 ± 0.26
	PASM	**2.06 ± 0.13^{c}**	**2.27 ± 0.03^{a}**	1.83 ± 0.22
	VUOC	**3.34 ± 0.08^{a}**	**1.86 ± 0.18^{b}**	0.99 ± 0.23

3.3. Soil microbial biomass C and N among plant rhizospheres and unvegetated soils

MBC and MBN levels varied over time among the different plant rhizosphere soils and unvegetated soils. For example, MBC and MBN levels were relatively lower within the rhizosphere of *V. octoflora* and in the unvegetated soils compared to the other plants rhizospheres at day 28 (Table 3). Although MBC and MBN trended higher in *B. tectorum*, *K. macrantha* and *P. smithii* at day 28 (F = 13.95; $P < 0.001$; Table 3); these levels plummeted from day 28 to day 76 (Table 3); coinciding with the period that exhibited the highest plant N uptake rates (Table 2). On the contrary, MBC and MBN steadily increased within the rhizosphere of *V. octoflora* and in the unvegetated soils throughout the growing season. Lower MBC:N was observed in the rhizosphere of *B. tectorum* at day 76 (F = 3.38; $P = 0.04$; Fig. S1e) due to the sharp declines in MBC (Table 3); which was also observed to a lesser degree with *K. macrantha* and *P. smithii* (Table 3). The MBC

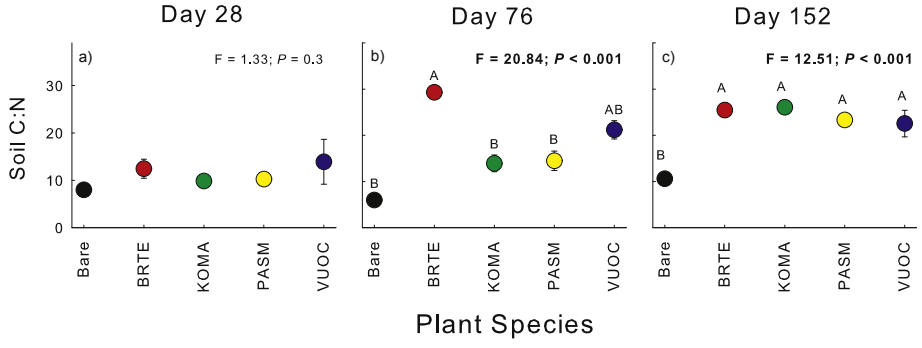

Fig. 3. Soil C:N increased among plant species within the rhizosphere across the growing season. Footnote: Differences in rhizosphere soil C:N characteristics were considered significant at $p \leq 0.05$ using Tukey post hoc tests. The sample size for all plant species and unvegetated (bare) soils is N = 4 for days 28 and 76, and N = 6 for day 152. Plant abbreviations for the four − C3 grass species and unvegetated soils include: *Bromus tectorum* (BRTE); *Koeleria macrantha* (KOMA); *Pascopyrum smithii* (PASM); *Vulpia octoflora* (VUOC), and unvegetated soil (Bare; white). Nutrient ratios (DV = dependent variables) include soil C:N = soil extractable organic C (SOC)/total soil N (TN).

Table 3
Microbial biomass and total C and N enzyme activities among four − C_3 plant species rhizospheres across a growing season at days (a) 28, (b) 76, and (c) 152 from plant seed germination.

		Day 28	Day 76	Day 152
DV	Species	***P* < 0.001**	***P* = 0.09**	*P* = 0.36
MBC	bare	**38.47 ± 8.53**^{c}	**85.61 ± 27**^{ab}	238.78 ± 69.84
	BRTE	**268.57 ± 17.4**^{a}	**47.4 ± 17.42**^{b}	171.15 ± 52.44
	KOMA	**226.88 ± 27.63**^{a}	**89.6 ± 14.63**^{ab}	228.15 ± 114.27
	PASM	**151.36 ± 39.1**^{b}	**104.03 ± 25.82**^{ab}	124.99 ± 7.4
	VUOC	**66.05 ± 21.79**^{c}	**140.74 ± 20.29**^{a}	313.12 ± 55.48
		***P* = 0.12**	*P* = 0.73	*P* = 0.83
MBN	bare	**22.7 ± 3.22**^{b}	23.97 ± 7.59	49.76 ± 13.61
	BRTE	**50.15 ± 12.56**^{a}	14.08 ± 3.34	41.12 ± 8.08
	KOMA	**35.53 ± 9.7**^{ab}	19.29 ± 2.51	55.5 ± 21.45
	PASM	**32.35 ± 8.13**^{ab}	21.43 ± 6.59	62.08 ± 17.93
	VUOC	**20.84 ± 3.43**^{b}	19.62 ± 3.13	44.57 ± 2.93
		P = 0.28	***P* = 0.03**	*P* = 0.38
Enzyme C	bare	219.55 ± 18.35	**218.25 ± 10.94**^{b}	69.66 ± 3.81
	BRTE	267.67 ± 53.12	**276.76 ± 21**^{a}	75.37 ± 5.76
	KOMA	197.46 ± 19.99	**307.3 ± 27.98**^{a}	70.83 ± 4.55
	PASM	192.41 ± 17.17	**256.15 ± 9.87**^{ab}	83.09 ± 4.23
	VUOC	241.43 ± 9.86	**269.3 ± 14.08**^{a}	78.79 ± 6.51
		***P* = 0.02**	*P* = 0.17	*P* = 0.12
Enzyme N	bare	**320.9 ± 12.59**^{a}	238.93 ± 72.64	186.25 ± 4.97
	BRTE	**262.04 ± 17.83**^{b}	154.4 ± 25.65	213.22 ± 5.31
	KOMA	**255.28 ± 22.12**^{b}	162.78 ± 26.41	197.32 ± 7.98
	PASM	**311.17 ± 6.78**^{ab}	395.79 ± 159.94	201.56 ± 6.91
	VUOC	**269.44 ± 7.83**^{b}	141.01 ± 32.56	203.01 ± 8.15

Descriptive statistics table presented as mean ± S.E.; N = 4. Letters next to the mean ± S.E. indicate significant differences at p ≤ 0.05 using Tukey post hoc tests following ANOVA. Rhizosphere associated dependent variables (DV) include: Enzyme C = ∑C cycling enzymes: [(BG = β-Glucosidase) + (CB = β-D-cellubiosidase) + (XYL = β-Xylosidase) + (AG = α-Glucosidase)], Enzyme N = ∑N cycling enzymes = [(LAP = Leucine aminopeptidase) + (NAG = N-acetyl-β-Glucosaminidase)], MBC = microbial biomass carbon, MBN = microbial biomass nitrogen. Units for all enzyme nutrient acquisition activities are expressed as (nmol activity g dry soil $^{-1}$ h^{-1}). Units for MBC and MBN are expressed as $\mu g^{-1} g^{-1}$soil. Differences at p ≤ 0.1 are indicated in **bold**.

and MBN levels in the rhizosphere *B. tectorum*, *K. macrantha* and *P. smithii* recovered after day 76; and by the end of the growing season, MBC and MBN was similar among all plants and unvegetated control soils (Table 3).

3.4. Soil microbial enzyme activities among plant rhizospheres and unvegetated soils

PCoA analysis showed that rhizosphere enzyme activities differed from unvegetated soils at all growth stages ($P \leq 0.02$; Fig. 4). These differences early in the study (at day 28) were due to higher total N enzyme activity ($P = 0.02$; Table 3) in the unvegetated soils, driven mostly by higher leucine aminopeptidase (LAP) activity (F = 4.73; P = 0.01; Table S2). Among plant species, *B. tectorum* demonstrated relatively wider enzyme C:N acquisition ratios at day 28, but these observations were only significant when compared to *P. smithii* (F = 4.75; $P = 0.01$; Fig. S1a). The higher C:N enzyme activities, primarily influenced by higher C degrading enzyme activities ($P \leq 0.03$; Table S2; Table 3) and lower LAP activities (Table S2), persisted in *B. tectorum* at day 76 along with *K. macrantha and V. octoflora* ($F = 4.16$; $P = 0.018$; Fig. S1b). At day 152, enzyme activities among all plant rhizospheres were similar; continuing to differ from unvegetated soils; but now due to the relatively lower peptidase (LAP) activities (F = 5.34; $P = 0.003$; Table S2; Fig. 4c) and higher chitinase (NAG) activities (F = 10.58; $P < 0.001$; Table S2; Fig. 4c, f) associated with unvegetated soils. These enzyme activity patterns that were observed across the study suggest that the soil N resource availability is substantially different between rhizosphere and unvegetated soils.

3.5. Soil bacterial community structural shifts among plant rhizospheres and unvegetated soils

Similar to enzyme activities, rhizosphere bacterial relative abundances among plant species rhizospheres were overall similar ($P \leq 0.046$; Fig. 5a–c), but strongly differed between the rhizosphere and unvegetated soils across the study (Fig. 5; Fig. 6). For example, Actinobacteria relative abundances were consistently higher in rhizosphere soils compared to unvegetated soils at day 76 and 152 ($P \leq 0.038$; Fig. 6a). Furthermore, Chloroflexi relative abundances were also higher in rhizosphere soils compared to unvegetated soils by day 152 ($P = 0.001$; Fig. 6g). At day 76 and 152, Proteobacteria, Gemmatimonadetes and OD1 relative abundances were higher in the unvegetated soils compared to rhizosphere soils ($P \leq 0.042$; Fig. 6b, d, i). Acidobacteria relative abundances were also higher in the unvegetated soils compared to most rhizosphere soils (with the exception of *P. smithii*; $P = 0.003$; Fig. 6c) at day 152. Specifically among plant rhizospheres, at day 28 the relative abundances of the AD3 bacterial phylum was higher under *B. tectorum*, *K. macrantha*, *and V. octoflora* compared to *P. smithii* ($P = 0.001$; Fig. 6f); and Firmicutes relative abundances were significantly higher in the rhizosphere of *B. tectorum* compared to all other grasses (F = 3.90; $P = 0.03$; Fig. 6h). By the end of the study, *B. tectorum* exhibited lower bacterial diversity (*i.e.* Shannon's Diversity; $P = 0.05$) and evenness (Simpsons inverse; $P = 0.005$) compared to *P. smithii* and unvegetated soils (Table 4).

3.6. Biotic and abiotic drivers influencing microbial community structural characteristics

Overall, plant N uptake appeared to strongly shape bacterial community structure. Bacterial diversity (*i.e.* Shannon's Diversity) declined among all plant species rhizospheres compared to unvegetated soils between day 28–76 (P ≤ 0.01; Table 4), which was the plant growth period that was associated with the highest plant N uptake rates (Table 2). Microbial nutrient levels were also influenced, as MBN decreased with increased plant N uptake among all plant species across the growing season (r = - 0.51; $P < 0.001$; Fig. 7a). Furthermore, shifts in bacterial community structure strongly correlated with plant growth over time ($r = -0.56$; P < 0.001; Fig. 7b). We used stepwise regression to assess how biotic plant parameters (total plant C, total plant N, total plant C:N, total plant biomass and plant N uptake) and abiotic parameters (*i.e.* soil moisture, soil pH, soil C and soil N) influenced bacterial community composition specifically within the plant rhizosphere across the growing season. In the stepwise model output, total plant N was identified as the only significant predictor of bacterial community composition (R^2 = 0.47; $P < 0.001$, Table 5).

We again used stepwise regression to assess how only abiotic and nutrient parameters (*i.e.* soil moisture, soil pH, soil C and soil N) influenced bacterial community structure across the growing season using both rhizosphere and unvegetated soil samples. This time, the stepwise model identified soil moisture as the single strongest predictor influencing bacterial community composition (R^2 = 0.54; $P < 0.001$); while total soil N levels improved the strength of the relationship (R^2 = 0.61; $P < 0.001$; Table 5). Overall, these findings suggest that bacteria phyla differ in abundance between rhizosphere and unvegetated soils. These results also suggest that bacterial groups within the rhizosphere are less responsive to abiotic conditions such as soil moisture variability, but tightly constrained by plant mediated soil N dynamics.

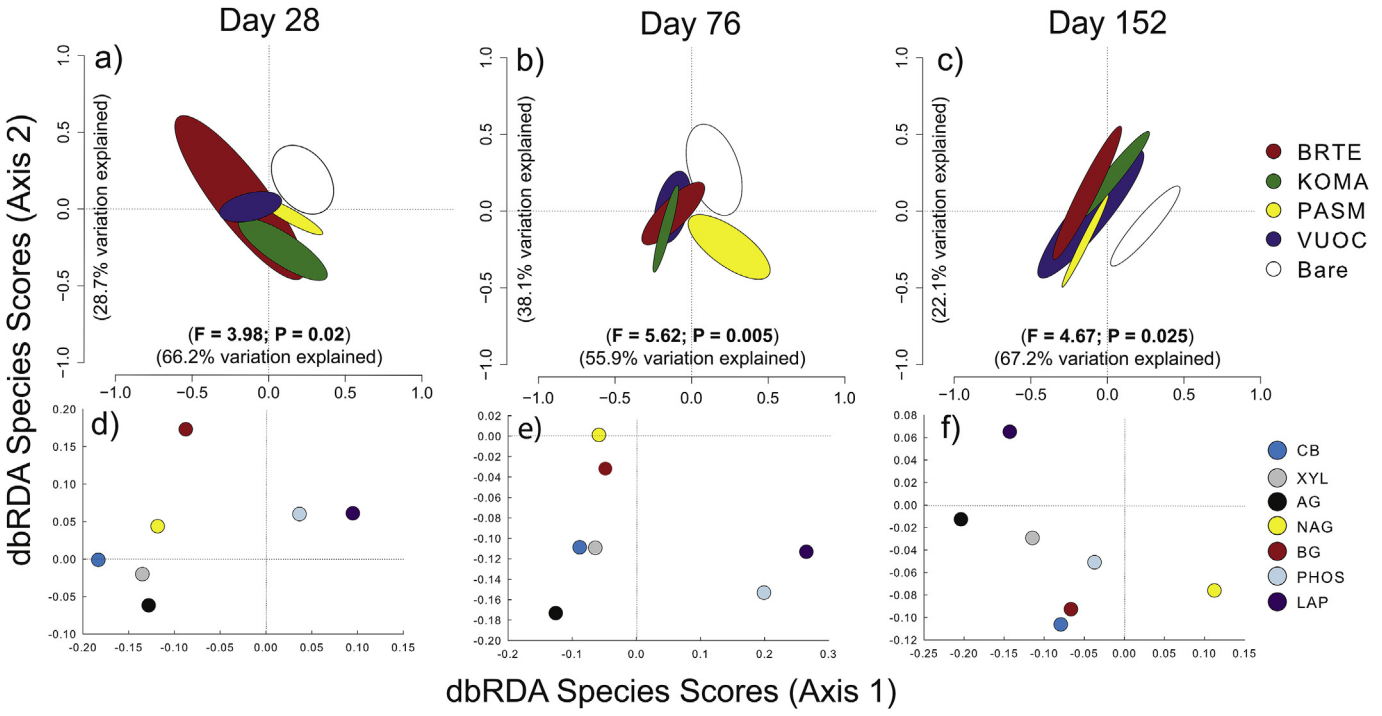

Fig. 4. Overall shifts in select C, N or P enzyme acquisition activities among four − C_3 plant species rhizospheres across a growing season at days (a, d) 28, (b, e) 76, and (c, f) 152 from plant seed germination demonstrated by distance based Redundancy Analysis (dbRDA). Footnote: Overall (multivariate) differences in rhizosphere nutrient characteristics were considered significant at $p \leq 0.05$. The sample size for all plant species and unvegetated (bare) soils is N = 4 for days 28 and 76, and N = 6 for day 152. Plant abbreviations for the four − C3 grass species and unvegetated soils include: *Bromus tectorum* (BRTE); *Koeleria macrantha* (KOMA); *Pascopyrum smithii* (PASM); *Vulpia octoflora* (VUOC), and unvegetated soil (Bare; white). Enzyme activities included in the model: four C cycling enzymes (BG = β-Glucosidase; CB = β-D-cellubiosidase; XYL = β-Xylosidase; AG = α-Glucosidase); two N cycling enzymes (LAP = Leucine aminopeptidase; NAG = N-acetyl-β-Glucosaminidase) and one P cycling enzyme (PHOS = Phosphatase). The scatter plots (Fig. e–g) represent species scores (*i.e.* coordinates for enzyme parameters included in model) corresponding to the above ordination plots. These scores can be interpreted as the strength of influence each enzyme variable has in separating plant species along Axis 1 (left − right) and/or along Axis 2 (top − bottom). Species scores (*i.e.* coordinates) further from one another indicate differences in specific enzyme activities among plant species rhizospheres.

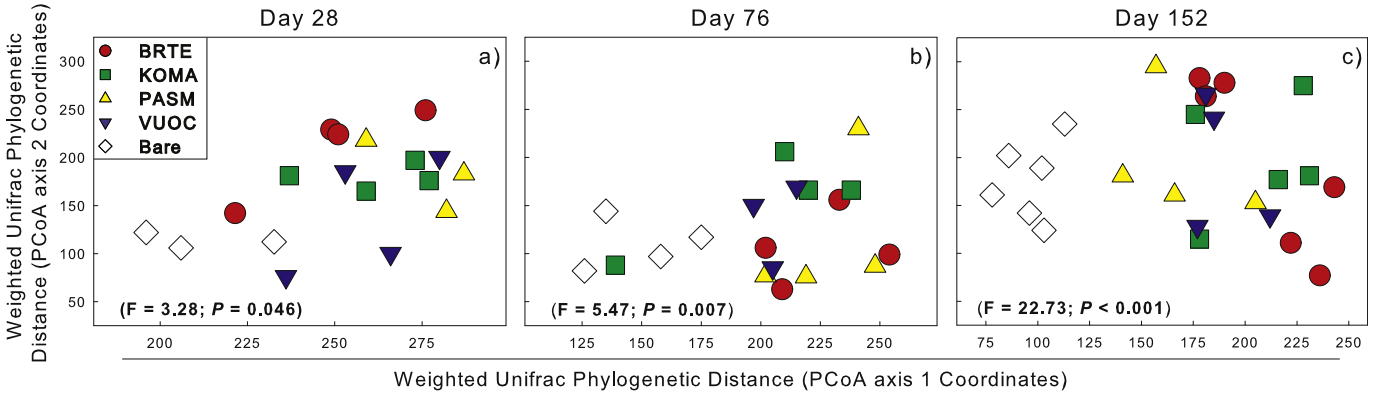

Fig. 5. Scatter plots of rhizosphere bacterial community structure as assessed using UniFrac phylogenetic distances among the four − C_3 plant species at days a) 28, b) 76 and c) 152 from plant seed germination. Footnote: Overall multivariate differences in rhizosphere bacterial genetic composition were considered significant at $p \leq 0.05$. The sample size for all plant species and unvegetated (bare) soils is N = 4 for days 28 and 76, and N = 6 for day 152. Plant abbreviations for the four − C3 grass species and unvegetated soils include: *Bromus tectorum* (BRTE); *Koeleria macrantha* (KOMA); *Pascopyrum smithii* (PASM); *Vulpia octoflora* (VUOC); and unvegetated soil (Bare). The scatter plots represent the multivariate weighted UniFrac distances for each sample. The spacing of the sample scores along Axis 1 (left − right) and/or along Axis 2 (top − bottom) can be interpreted as the relative relationship among samples with respect to bacterial phylogenetic properties among discrete plant rhizospheres.

4. Discussion

Across the growing season, plants can stimulate rhizosphere bacteria to increase soil nutrient availability for plant nutrient uptake (Reynolds et al., 2003; Levine et al., 2006; Bever et al., 2012), ultimately benefiting plant productivity (Ehrenfeld et al., 2005; Turan et al., 2012; Wang et al., 2012). Plant nutrient uptake decreases N availability for bacteria, causing them to allocate resources to produce enzymes to degrade N-rich substrates. Ultimately, this might enhance soil bacterial N mineralization rates (Bever et al., 2010, 2012). Our findings demonstrated that higher rates of plant N uptake during the early and mid-stages of plant growth corresponded to declines in MBN, consistent with N limitation to bacterial growth. As plant tissue N levels increased across the growing season, the corresponding decrease in soil N also led to shifts in rhizosphere bacterial community structure.

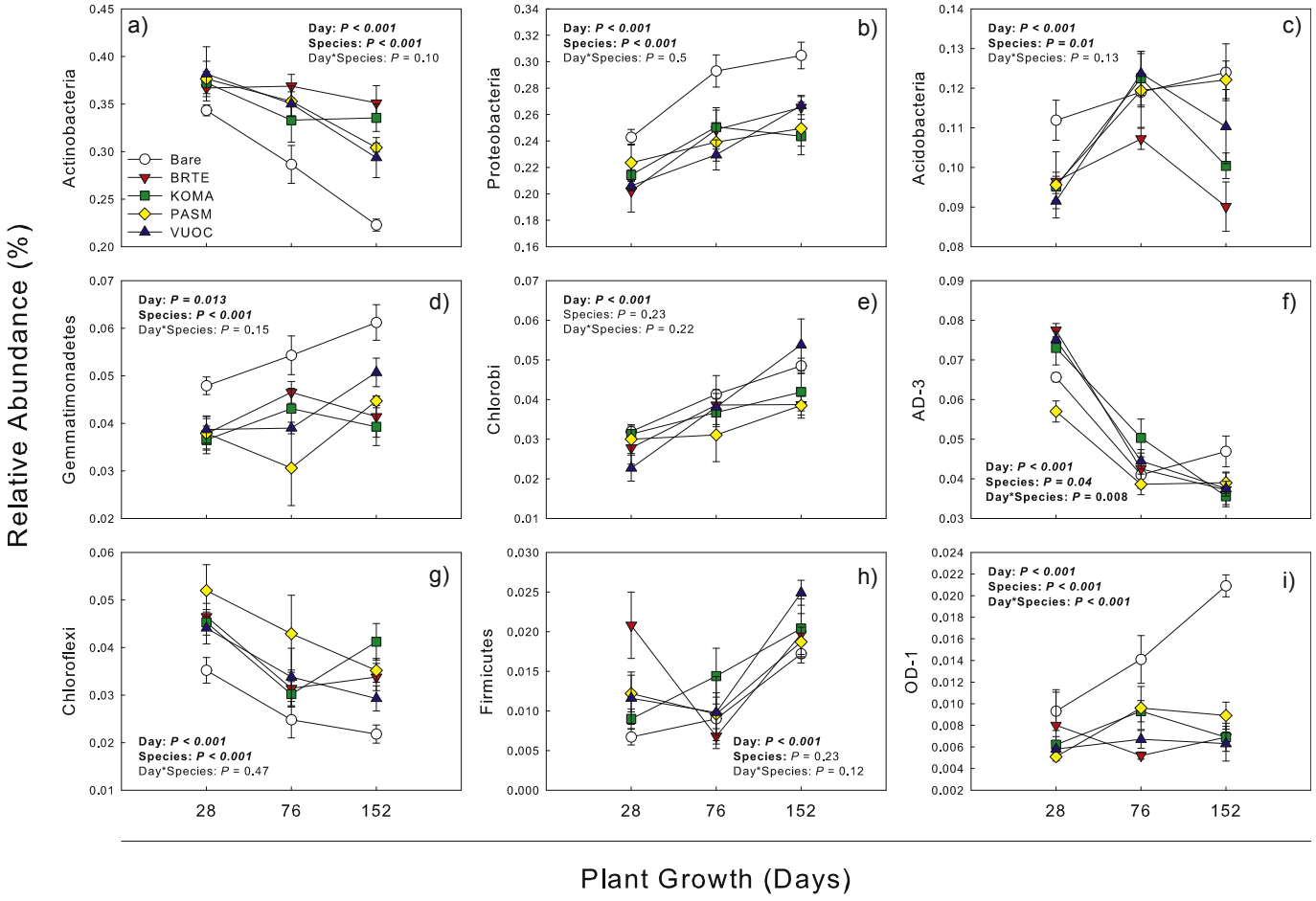

Fig. 6. Bacterial phylum shifts among plant species rhizospheres and unvegetated soils across a growing season at days 28, 76, and 152 from plant seed germination. Footnote: The 9 phyla above represent the majority of bacterial relative abundances detected at each sample period across the study. Overall, the phyla featured above represent ~88% of the rhizosphere bacterial phylum detected at days 28, 76 and 152 (88.6%, 88.5% and 87.1%, respectively). Bacterial relative abundance values are presented as mean ± S.E. Significant differences were considered at $p \leq 0.05$ using Tukey post hoc tests. Plant abbreviations for the four − C3 grass species and unvegetated soils include: *Bromus tectorum* (BRTE); *Koeleria macrantha* (KOMA); *Pascopyrum smithii* (PASM); *Vulpia octoflora* (VUOC), and unvegetated soil (Bare; white). The sample size for all plant species and unvegetated (bare) soils is N = 4 for days 28 and 76, and N = 6 for day 152. Significant differences were considered at $p \leq 0.05$ using Tukey post hoc tests are indicated in bold.

Even after plant growth slowed and soil N availability recovered, rhizosphere bacterial community composition never returned to previous conditions. We hypothesize that only the bacterial communities that were able to tolerate the lower N availability during the early and mid-stages of plant growth were able to persist throughout the late plant growth stages and into senescence. Thus, severe N limitation resulting from rapid plant N uptake appears to leave a legacy in the structure of rhizosphere bacterial communities.

Previous studies have also shown that soil nutrient availability affects bacterial community composition (Grandy et al., 2009; Ramirez et al., 2012; Bell et al., 2014a). In one of these studies, N additions consistently increased the relative abundance of Actinobacteria and Firmicutes, while Acidobacteria relative abundances declined with increased N availability (Ramirez et al., 2012). Consistent with these results, we observed that Actinobacteria significantly declined with lower soil N availability throughout the growing season due to plant N uptake. In another study, Fierer et al. (2012) showed that the relative abundance of some members of the Proteobacteria phylum (classified as a copiotrophic taxa) increased with N fertilization, while Acidobacteria phylum (an oligotrophic taxa) exhibited the opposite pattern. In our study, Proteobacteria greatly increased with the higher soil N levels in the unvegetated plots, but only slightly increased over time within the rhizosphere. Our findings suggest that plant N uptake may influence bacterial community structure by mediating the timing and magnitude of soil N availability.

Plant-mediated shifts in rhizosphere soil bacterial community structure and function can also be driven by changes in other abiotic parameters such as soil pH (Fierer and Jackson, 2006; Lauber et al., 2009) and soil moisture availability (Bell et al., 2008, 2009, 2014b). Overall, soil pH increased among all plant rhizospheres across the growing season, and was higher than the unvegetated soils throughout the study. The higher soil pH observed within the plant rhizospheres could have been influenced by plant N uptake, as plants used NO_3 and released HCO_3^- to maintain electrical balance (Smiley, 1974; Nye, 1981; Šimek and Cooper, 2002). There are many potential mechanisms that could influence soil pH which can also influence soil nutrient availability, microbial community structure and plant nutrient uptake (Eckersten and Jansson, 1991; Kourtev et al., 2003; Canbolat et al., 2006).

Soil moisture is another important environmental variable that can strongly influence soil bacterial community structure (Fierer et al., 2003; Brockett et al., 2012; Bell et al., 2014b) and which cannot be easily decoupled from plant nutrient uptake (Gebauer and Ehleringer, 2000). Although we minimized soil moisture

Table 4
Microbial community α-diversidy indicies among four − C_3 plant species rhizospheres across a growing season at days 28, 76 and 152 from plant seed germination. Significant differences at $p \leq 0.05$ are indicated in **bold**. Letters next to the mean ± S.E. indicate significant differences at $p \leq 0.05$ using Tukey post hoc tests following ANOVA.

		Day 28	Day 76	Day 152
DV	Species	$P = 0.71$	$P = 0.25$	$\mathbf{P = 0.05}$
Shannon	Bare	8.41 ± 0.03	8.4 ± 0.04	**8.44 ± 0.03^{a}**
	BRTE	8.47 ± 0.03	8.38 ± 0.03	**8.29 ± 0.05^{b}**
	KOMA	8.43 ± 0.04	8.32 ± 0.05	**8.36 ± 0.03 ^{ab}**
	PASM	8.37 ± 0.06	8.28 ± 0.02	**8.4 ± 0.05^{a}**
	VUOC	8.41 ± 0.07	8.29 ± 0.05	**8.38 ± 0.02^{ab}**
		$P = 0.89$	$P = 0.97$	$\mathbf{P = 0.005}$
Simpson Evenness (inverse)	Bare	214.93 ± 16.27	235.63 ± 8.8	**235.77 ± 6.96^{a}**
	BRTE	198.46 ± 15.59	201.65 ± 4.06	**199.57 ± 6.44^{c}**
	KOMA	201.01 ± 18.3	210.8 ± 15.78	**214.03 ± 6.06^{bc}**
	PASM	192.83 ± 18.7	198.36 ± 5.79	**234.63 ± 12.15^{ab}**
	VUOC	191.15 ± 6.8	195.66 ± 11.63	**232 ± 4.11 ^{ab}**
		$P = 0.44$	$P = 0.39$	$\mathbf{P = 0.04}$
PD (whole tree)	Bare	60.25 ± 0.9	59.31 ± 1.62	**60.96 ± 0.82^{a}**
	BRTE	60.59 ± 1.21	56.06 ± 0.89	**56.54 ± 1.25^{b}**
	KOMA	59.64 ± 1.89	58.7 ± 2.06	**56.83 ± 0.7^{b}**
	PASM	57.29 ± 0.99	56.96 ± 2.08	**57.85 ± 1.66^{b}**
	VUOC	58.83 ± 1.26	55.49 ± 1.35	**57.66 ± 0.78^{b}**

variability by maintaining a consistent watering schedule, we still lack a basis to completely disentangle the interactions of abiotic soil moisture availability from plant N uptake. However, when we included plant and soil nutrient properties as well as abiotic variables (soil pH and moisture) into a regression model, total plant N was the only significant predictor of bacterial community structure. This finding may suggest that once established within the rhizosphere, bacterial communities may be relatively buffered from the effects of abiotic (pH and soil moisture) stress − and more influenced by plant root interactions.

Plant-microbe interactions undoubtedly influence soil stoichiometry (via nutrient mineralization and immobilization) throughout the growing season (Sterner and Elser, 2002; Hessen et al., 2004; Frost et al., 2005). In this study, plant N uptake strongly correlated with wider soil C:N within the rhizosphere driven by declines in soil N availability. We predicted that variations in soil C and N availability would inversely correlate with their respective enzyme C:N activities. On the contrary, wider soil C:N closely mirrored the wider soil C:N patterns within plant rhizospheres. However, soil microbial biomass C:N inversely corresponded with the wider enzyme C:N and soil C:N patterns across the first two sampling time points in the growing season. Shifts in bacterial community assembly also corresponded with enzyme C:N acquisition activities. This may reveal that these soil bacterial communities exhibited non-homeostatic relationships within the rhizosphere, and are thus constrained by the environmental nutrient conditions imposed by plants (McGroddy et al., 2004; Cleveland and Liptzin, 2007; Bell et al., 2014a).

The widespread success of *B. tectorum* that has displaced many native C_3 grasses across the western United States has been largely attributed to higher soil N uptake (Miller et al., 2006; Sperry et al., 2006; Perkins et al., 2011) and its ability to facilitate higher soil N mineralization through unique rhizosphere microbiome associations (Hawkes et al., 2005; Schaeffer et al., 2011; Rout and Callaway, 2012). The early emergence and faster seedling growth of *B. tectorum* certainly represents a very effective competitive fitness strategy that is dependent on soil resources (DiTomaso, 2000; Warembourg and Estelrich, 2001). In this study, *B. tectorum* was associated with higher early season enzyme C:N acquisition activities, plant N uptake rates and root biomass along with lower soil N availability and MBC:N within its rhizosphere. The lower MBC:N observed during peak plant growth may suggest that the rhizosphere microbes associated with *B. tectorum* are capable of assimilating soil N more efficiently than some native species.

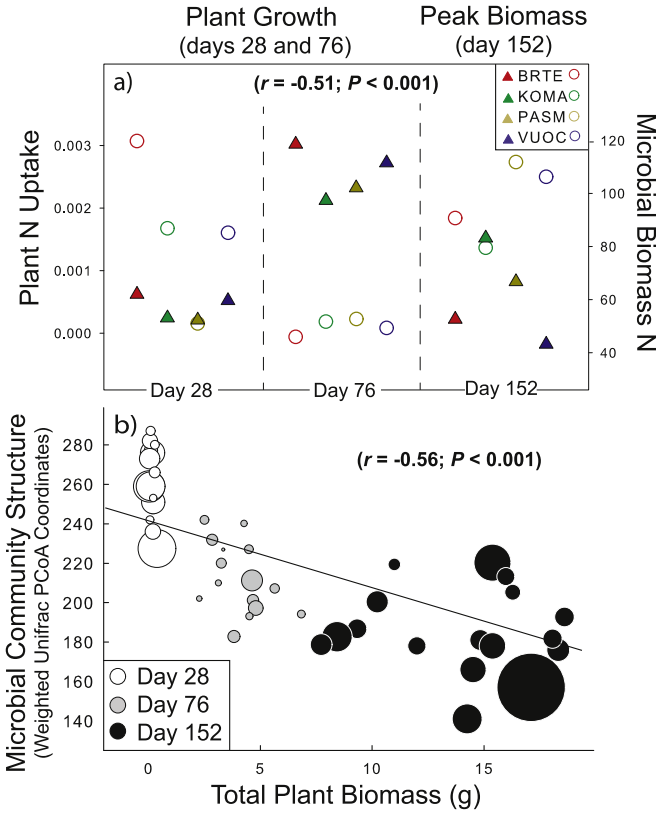

Fig. 7. Scatter plots demonstrating negative correlations between microbial biomass N and plant N uptake among the four − C_3 grass rhizospheres across the plant growing season. Footnote: a) Plant N uptake negatively correlated with microbial biomass across the growing season. (Note: For visual clarity, data is presented as mean values. Filled triangles = Plant N uptake corresponding to the different plant species; and open circles = Microbial Biomass N levels corresponding to the different plant species. Different plant species are indicated by color). b) The scatter plot demonstrates bacterial phylogenetic shifts among all plant rhizospheres in response to increased plant biomass across the growing season. The scatter plot symbols represent discrete plant rhizosphere samples, and the size of the symbol is scaled MBN levels; demonstrating how shifts in microbial biomass N corresponds with microbial − plant relationships across the growing season. (Note, total plant biomass strongly correlated with plant N uptake across the first two harvest periods (*i.e.* Day 28−76; $r = 0.94$; $P < 0.001$)). The sample size for all plant species is N = 4 for days 28 and 76, and N = 6 for day 152. Plant abbreviations for the four − C3 grass species and unvegetated soils include: *Bromus tectorum* (BRTE); *Koeleria macrantha* (KOMA); *Pascopyrum smithii* (PASM); and *Vulpia octoflora* (VUOC).

Table 5
Soil and rhizosphere bacterial communtiy structural responses to abiotic and biotic influences across the growing season.

Model	r	R^2	Model predictors
Rhizosphere only soils			
1	0.69	0.47	Total Plant N
Rhizosphere and Unvegetated soils			
1	0.73	0.54	SM
2	0.78	0.61	SM, TN

Multiple regression was used to assess the influence of abiotic soil parameters (soil moisture, soil pH, soil C and soil N) and plant related parameters (i.e. total plant C, total plant N, total plant C:N, total plant biomass and plant N uptake) on shaping microbial communtiy structure. Every R^2 statistic reported in this experiment is from the final significant model ($P \leq 0.01$) provided by the stepwise process. The stepping-method criteria entered any variable with the probability of $F \leq 0.005$ and removed any variable from the model $F > 0.01$.

B. tectorum appeared to trigger bacterial community shifts more quickly within its rhizosphere compared to the other native grasses. However, *B. tectorum's* ability to uniquely alter its rhizosphere microbiome is less clear, as *B. tectorum*, *K. macrantha* and *V. octoflora* demonstrated many similarities across the study. However, the rhizosphere bacterial community structure associated with *B. tectorum* exhibited relatively lower evenness (*i.e.* the relative abundance of species), which may be evidence of its ability to alter rhizosphere bacterial community structure. When establishing in novel environments, if *B. tectorum* favors a less even community, it may drive the microbial community structure and soil ecosystem properties further from its 'natural' state (de Vries et al., 2012; Wittebolle et al., 2009) to outcompete native plants. This feedback may represent a key control imposed by *B. tectorum* to influence soil nutrient availability.

The timing of the onset of plant nitrogen uptake may be an important and previously overlooked plant trait that could vary among different plant species. We studied four different grass species at multiple time points representing different plant growth stages to improve our ability to observe how temporal differences among plant traits influence rhizosphere bacterial community structure and nutrient properties (Fujita et al., 2010; Isbell et al., 2011). Although our findings are strongly suggestive of plants' abilities to influence their rhizosphere bacterial community assemblages, more research is needed to better understand how these interactions affect plant competitive fitness. *Nonetheless*, the ability of plants to establish beneficial rhizosphere bacterial communities may be an important aspect of plant fitness and should be viewed as an intrinsic plant trait that may be under selection (Schweitzer et al., 2014; Lennon and Lau, 2014).

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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.2015.03.006.

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