

Nitrate leaching as a function of plant community richness and composition, and the scaling of soil nutrients, in a restored temperate grassland

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Abstract Two, two-factor experiments manipulated species and functional form plant richness and the spatial scaling of either nitrogen (N) or phosphorous (P) in restored tallgrass prairie in North Dakota, USA. Nitrate (NO_3^-) leaching was measured in these plots and analyzed for its response to the treatment factors and measured plant community parameters. Nitrate extracted from anion exchange resin was regressed against the first principal component of species and functional form richness, the spatial scaling of N or P, the measured biomass of the functional forms used and the plot values for plant parameters based on weighted averages by species biomass. The treatments applied in the N and P experiments were 1, 2, 5, 10, or 20 plant species taxa, and the application of fertilizer in a random fractal pattern with either fine-scale or coarse-scale heterogeneity. Nitrate leaching decreased with plant diversity and increased by a factor of two going from fine-scale to coarse-scale N.

It was also related to a number of plant functional parameters, and was positively correlated with the biomass of late successional C_3 grasses (*Koeleria cristata* (Lam.) Beauv., *Poa pratensis* L., *Stipa comata* Trin. & Rupr., and *Stipa viridula* Trin.), which are known from previous studies to have negative mycorrhizal responsiveness and are characterized by high root lateral spread per unit of root biomass. Our results show that while plant diversity has a highly significant influence on plant community uptake of NO_3^- , this effect is mediated by the scaling of soil N and the functional traits of the species comprising the plant assemblage.

Keywords Nutrient cycling · Plant community composition · Functional form diversity · Northern tallgrass prairie restoration · Species diversity · Temperate grassland

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Introduction

As highlighted by Tilman et al. (1996), Darwin (1859) proposed that more diverse plant communities would have higher productivity as well as lower nutrient losses and more sustainable soils due to differences in physiology, morphology, nutrient requirements, and life histories of various plant species. The effects of diversity on nutrient cycling are most easily observed with nitrogen (N), as it is required in large amounts by all organisms and is

most readily lost from the soil via leaching of anions (Bardgett 2005). As alpha diversity in grasslands is being depleted globally, N is also depleted from these systems (Hooper et al. 2005). Reduced diversity should result in reduced uptake of N from the soil, leading to greater mineralization followed by nitrification and leaching of N (Matson et al. 2002). In soil water, soluble N occurs primarily in the form of ammonium (NH_4^+) and nitrate (NO_3^-) ions, but most NH_4^+ that is not taken up by plants becomes fixed by soil colloids, primarily clays and organic matter; hence, the vast majority of soluble N that leaches out of the soil is in the form of NO_3^- (Bardgett 2005). Increases in soil N levels can result in an increase in the rate of conversion of organic N to mineral N (mineralization) by soil microbes, and thus a positive feedback leading to increased NO_3^- leaching (Stevenson and Cole 1999). Therefore, plant productivity and nutrient utilization are critical factors influencing the quantity of NO_3^- leached from the soil (Chapin 1980). The greater the plant productivity, and the greater the reliance on soil N by plants, the greater the total uptake of N by plants (Lea and Azevedo 2006). As the proportion of soil nutrients that fall within the mycorrhizosphere increases concurrently with plant growth rates, the ratio of N uptake to leaching increases (Hamel et al. 1991).

On average, with higher plant diversity, net primary production (NPP) should increase as a result of more complete nutrient utilization across space, chemical structure, and time due to variation in root morphology, compositional uptake, life histories, and resource ratio acquisition of plants, as well as associated increases in arbuscular mycorrhizal (AM) hyphal infiltration (Miller and Jastrow 1992a, 1992b; Tilman et al. 1996; Bingham and Biondini 2009). This increased NPP and utilization of soil nutrients should include higher NO_3^- uptake, resulting in reduced NO_3^- leaching, increased stability associated with higher minimum annual production, and reduced invasion by ruderal species as N availability is diminished (Biondini 2007). In our study, an attempt was made to connect N cycling directly with species and functional form richness, and link it to other results from the same system, namely, AM fungal production (Bingham and Biondini 2009), productivity, and stability (Biondini 2007), and nutrient acquisition parameters of the plant species present (Levang-Brilz and Biondini 2003), as it would be

beneficial to know what combinations of native and naturalized plants would minimize the entry of NO_3^- into lakes and streams and, in turn, increase plant community production and stability due to more efficient N utilization. The importance of nutrient retention by the plant community to nutrient cycling has been studied in a number of herbaceous plant communities (Hooper et al. 2005; Cardinale et al. 2007; van der Hieijden et al. 2008), but few studies have assessed the interaction between plant diversity and the spatial distribution of soil nutrients (nutrient scaling) in their effects on N cycling (e.g., Maestre et al. 2007). Plants differ in the scaling properties of their root systems and this is likely to interact with the scaling of soil nutrients to affect species coexistence and the uptake of those nutrients (Biondini and Grygiel 1994). Artificial seeding and fertilizing are commonly utilized for grassland restoration and grazing land, and both can result in low plant diversity and N contamination of water, depending on the administration of the treatments. It would be beneficial to know how the scaling of fertilizer affects these outcomes. Tilman et al. (1996) found that NO_3^- in the rooting zone decreased asymptotically as species richness increased in tallgrass prairie plots. They ran a multiple linear regression of NO_3^- in the rooting zone on species richness, plant total cover, and belowground biomass to find that species richness was the only significant factor, suggesting that complementary resource partitioning was responsible for the increase in NO_3^- uptake. We expected to also find a decline in NO_3^- leaching with species richness; thus, an additional objective for this study was to determine the effects of nutrient scaling and parse out relationships with plant functional form parameters (Johnson and Biondini 2001; Levang-Brilz and Biondini 2003).

Nitrogen cycling needs to be understood for proper grassland management and restoration because (1) it is a driver and a consequence of plant community composition, production, stability, and carbon (C) sequestration; and (2) an incomplete utilization of soil N related to plant community levels of diversity, composition, or length of EAM hyphae can result in NO_3^- leaching and invasion by ruderals. The specific objectives in this study were to (1) determine if increased plant species and functional form richness (possibly leading to increased plant and fungal productivity) interact with the spatial distribution of

N and phosphorus (P) to reduce NO_3^- leaching; and (2) relate this back to the functional composition of the plant community derived by Levang-Brilz and Biondini (2003). The working hypothesis for this study was that plant species and/or functional form richness should lead to a greater utilization of N in soil solution and, hence, less NO_3^- being leached.

Methods

Experimental design

The study site was located at the North Dakota State University (NDSU) Albert Ekre Grassland Preserve in southeastern North Dakota and was conducted in a subset of a large long term experiment described in Biondini (2007). The soils belong to the Embden-Tiffany fine sandy loam soil map unit (coarse-loamy, mixed, superactive, frigid Pachic Hapludolls, and frigid Typic Endoaquolls): moderate to low fertility ($\approx 2.2\%$ organic matter), 0–3% slopes, moderately well drained, and combined A&B horizon of approximately 69 cm. Prior to seeding, the site had been cultivated for over 100 years, most recently with corn and soybeans. It was disked in 1997 and 1998 to reduce the seed bank and treated with Roundup (Monsanto; 18% Glyphosate, 0.73% Diquat, 0.3% Imazapic) prior to planting to minimize plant regrowth. The plots were planted in the fall of 1998 and spring of 1999, then sampled for the first time in 2000 to allow establishment of the seeded species and prevent uprooting of young plants. Sierra[®] slow-release fertilizer prills (Pursell Technologies, Inc.) were used to produce the desired N and P supply rates and spatial patterns. Fertilizer was applied in early spring, just after the snow had melted (Appendix A: Online Resource 1 in Supplementary Material).

We used a total of 100 plots, which were 3×3 m with a 1-m buffer zone. Of the 100 plots measured, 50 were applied with N and 50 were applied with P, which were analyzed separately so as not to confound more subtle effects of other variables. The mean supply rates for the nutrient chosen were 20 g m^{-2} per year for the N treatment and 4 g m^{-2} per year for the P treatment.

This study was organized as two experiments (N and P), each consisting of a completely randomized design with a 5×2 factorial arrangement with

five replications. For our analyses, factor 1 represented species taxa diversity and consisted of treatments with 1, 2, 5, 10, or 20 plant species taxa, each replication consisting of the given number of species drawn randomly, without replacement, from the list in Appendix B: Online Resource 2 in Supplementary Material. Factor 2 consisted of two distinct stationary random fractal spatial patterns of the fertilizer (N or P), applied with a specially built fertilizer machine, and generated by stationary fractal models (Biondini and Grygiel 1994) using scaling constants of either $H = 0.2$ (fine-scale, high spatial variability at short scales and low spatial variability at large scales) or $H = 0.8$ (coarse-scale, low spatial variability at short scales and high spatial variability at large scales) (see Appendix C for details in Supplementary Material).

Plant biomass sampling

Aboveground (shoot) biomass was sampled at peak biomass in 2000, 2001, and 2002 by clipping individual species using four 0.25-m^2 quadrats per plot at the end of July or August (depending on timing of peak biomass). The clipped biomass was separated by species and the harvest processed following protocol described by Biondini (2007). The quadrats were randomly located in separate and non-overlapping sections of the plot, avoiding portions that had been clipped in previous years. Species and functional form richness were determined by a yearly complete inventory of presence/absence in the entire 9-m^2 area of each plot. The number of seeded species present in each plot largely stabilized during the 2000 growing season. Belowground (root) biomass for each species in a plot was calculated by multiplying the aboveground biomass by the root to shoot ratio (R:S). The R:S data for each species were derived from Levang-Brilz and Biondini (2003), data from an ongoing field experiment, and detailed analysis of the root architecture of 1759 plants from 77 herbaceous species (Biondini 2008). We acknowledge that these data were derived from models and measurements of plants in the absence of interspecific competition and harvested at a younger phase of development than would be eventually reached in the field, and that R:S are affected by interspecific competition (Wilson and Tilman 1995; Gersani et al. 2001) and ontogeny (McConnaughay and Coleman 1999), but it is exceedingly difficult to

measure root traits in mixed communities, and in our plots it would have required destructive sampling, which was precluded by the ongoing nature of the experiment. Thus, we consider the values used in our analyses to be a best available estimate, and note that they were derived from the same ecosystem. Total (whole plant) biomass was the sum of belowground biomass and aboveground biomass. Aboveground biomass, belowground biomass, and total biomass for each species in a plot were summed for a plot total value or a total functional form plot value. Parameters used by Levang-Brilz and Biondini (2003) to characterize functional form groups are shown in Appendix B: Online Resource 2 in Supplementary Material.

Detection of soil nitrates

We used anion exchange resin (AER) bags to detect soil NO_3^- at the base of the rooting zone, which gave us an index for NO_3^- leaching. The advantage of AER bags is a cumulative recovery of NO_3^- ions passively from gravitational water (Wyland and Jackson 1993). They were easily retrievable with a minimum of soil disturbance. Two holes were bored in each plot using a soil auger to a depth of slightly over 1 m. In each hole, a 1-m long tube consisting of polyvinyl chloride (PVC) pipe was inserted (Appendix A: Online Resource 1 in Supplementary Material). Anion exchange resin bags, similar to those described by Binkley et al. (1986), were created using 10 g of AER beads (Bio Rad AG[®] 1-X8 Resin, 20–50 mesh chloride form) stuffed in nylon pouches that were tied shut using fishing line and dropped to the bottom of each hole. They were pressed into the bed of the hole, which was ~ 5 cm below the end of the pipe, to maximize surface area contact with the surrounding soil. The tubes were capped to prevent the entry of precipitation and surface runoff into the tubes. The end of the fishing line opposite the AER bag was attached to the cap to provide easy retrieval of the bags. The bags were deposited immediately after the ground had thawed in spring of 2002, retrieved shortly before the ground froze in the fall of 2002, and analyzed using methods similar to those described by Binkley et al. (1986). Parts per million NO_3^- were extracted in 100 ml 2 M potassium chloride (KCl) for 24 h and analyzed using the standard steam distillation method (Bremner 1965). Subsamples were totaled for each plot. Since the total

NO_3^- leached per plot cannot be quantified using this method, it is effectively an index comparing the plots and treatments.

Statistical analysis

The overall difference between the N and P applications was assessed on raw data using the Wilcoxon–Mann–Whitney test to validate the field and lab technique. Differences among fertilizer scaling and diversity treatments in total ppm NO_3^- extracted per plot (NO_3^- captured) were analyzed parametrically, after reciprocal transformation for N plots and logarithmic transformation for P plots to conform to normality and homogeneity of variance.

Species and functional form richness were treated as in Bingham and Biondini (2009), following Naeem (2002), after square root transforming the two variables to increase linearity. The first principal component (PC1) passed the broken stick test (McCune and Grace 2002), and therefore was used in place of species and functional form richness in all subsequent analyses to capture their combined effects. The effects of species and functional form richness, represented by PC1, on soil NO_3^- under N and P application were tested via multiple regression analysis (Tabachnick and Fidell 2001). Variables used in the regression runs included fertilizer scaling and its interaction with PC1, various partitions of total plot biomass (root, aboveground, and combined; 2002 and cumulative 2000–2002), and the same partitions of the various functional forms growing in the plots. For each plot we also had physiological, morphological, and growth parameters (Appendix B: Online Resource 2 in Supplementary Material) of each species present standardized (0–1 to make slopes comparable among one another), weighted by plot based on species measured aboveground biomass (see previous section), and summed among species for a plot total. The original parameters were further used to calculate root lateral spread (RLS), root length (RL), and root surface area (RSA) for each plot based on species' inferred root biomass (RB) (Appendix B: Online Resource 2 in Supplementary Material). Biomass, RLS, RSA, and RL were logarithmically transformed ($\ln [x + 1]$) to increase linearity, and were included with the plant parameters as variables in the regression runs to minimize confounding variance in NO_3^- related to plant

community composition, and to parse what combination of variables had the greatest influence on soil NO_3^- beyond diversity effects. This gave us an indirect assessment of what functional forms were having proportionately greater effects on soil NO_3^- , independent of richness effects. Principal component 1 and significant variables were regressed with soil NO_3^- after adjusting each observation of NO_3^- captured for other continuous variables in the regression model. This was accomplished by adding the product of each variable's residual and slope from the multiple model (Milliken and Johnson 2002). Statistical differences in regression slopes and elevations were tested between fine-scale and coarse-scale for all regressions (Snedecor and Cochran 1976).

Results

The reliability of the NO_3^- capture method was supported by a significant difference in NO_3^- recovered from the N and P treatments, with NO_3^- captured from the N treatment being close to double that of the P treatment ($P < 0.0001$ based on Wilcoxon-Mann-Whitney test): mean and standard error (SE) values for NO_3^- captured were 26.5 (± 4.61) for the N treatment and 12.6 (± 0.907) for the P treatment. Nutrient scaling was found to be a significant factor in the N plots (Table 1), with NO_3^- recovered from coarse-scale treatments being close to double that recovered from fine-scale treatments: mean and SE values were 15.5 (± 1.11) under fine-scale and 37.4 (± 8.65) under coarse-scale. The scaling factor, H, did not affect NO_3^- captured under P application.

Ninety-seven percent of the total variance in the principal components analysis was explained by PC1 ($\lambda = 1.93 > \text{broken stick } \lambda = 1.5$), the only significant axis. The square roots of both species and functional form richness were positively correlated with PC1 ($R^2 = 0.97$, $P < 0.0001$). Principal component 1 is used in place of species and functional form richness in all subsequent analyses.

Nitrate leaching under nitrogen application

Principal component 1 was positively correlated with the reciprocal of NO_3^- captured under N (1/[ppm]), and thus species and functional form richness negatively affected NO_3^- leaching (Fig. 1, Table 1).

Table 1 Multiple regression testing for response of the reciprocal of NO_3^- recovered from anion exchange resin at 1-m depth under N application (NO_3^- captured under N (1/[ppm])) to species and functional form richness (principal component 1 [PC1]) and spatial scaling factor H (see text for details), after adjustment for plant parameters associated with various species compositions

Multiple regression: AIC = - 183.3			
Variable	Coefficient	Standard error	P-value
PC1	0.0103	0.00257	0.0003
H	N/A	0.00509	0.0024
Ln (G1C4 biomass)	-0.00466	0.00206	0.0301
Ln (RLS)	-0.0107	0.00366	0.0062
I_{\max} -P	-0.0944	0.0291	0.0026

Ln (G1C4 biomass) is the natural log of the cumulative biomass (2000–2002 in g m^{-2}) of late successional C_4 grasses *Andropogon gerardii* Vitman, *Panicum virgatum* L., *Schizachyrium scoparium* (Michx.) Nash-Gould and *Sorghastrum nutans* L. All other parameters are derived from Johnson and Biondini (2001), Levang-Brilz and Biondini (2003), and Biondini (2007), where Ln (RLS) is the logarithm of root lateral spread ($\ln [m + 1]$) calculated based on cumulative 2000–2002 root biomass (RB[g]) ($\text{RLS}[m] = \alpha * \text{RB}^\beta$), and I_{\max} -P is the maximum influx rate per unit of root surface area for P ($\text{gP m}^{-2} \text{d}^{-1}$) (see Appendix B: Online Resource 2 in Supplementary Material for further explanation). Note the coefficient values are only given for continuous variables, and should be multiplied by -1 for the true direction of the relationship with NO_3^- captured under N

While PC1 did not interact significantly with H, the y-intercept for the regression was higher under fine-scale than coarse-scale (Fig. 1), consistent with the *t*-test confirming overall lower leaching in fine-scale N application. This was true for all the continuous variables included in this model. While leaching decreased with PC1, all of the plant parameters included in this model were associated with an increase in leaching, as exhibited by their negative relationships with the reciprocal of NO_3^- captured. Of the plant species variables that were calculated based on root biomass, RLS, calculated based on cumulative 2000–2002 RB [g] ($\text{RLS}[m] = \alpha * \text{RB}^\beta$) (Appendix D: Online Resource 5 in Supplementary Material, Appendix A in Supplementary Material) Tables 1, 2, accounted for the greatest variance beyond that accounted for by PC1. Root lateral spread, and combined root and aboveground cumulative biomass (2000–2002 in g m^{-2}) of late successional C_4 grasses *Andropogon gerardii* Vitman, *Panicum virgatum* L., *Schizachyrium scoparium*

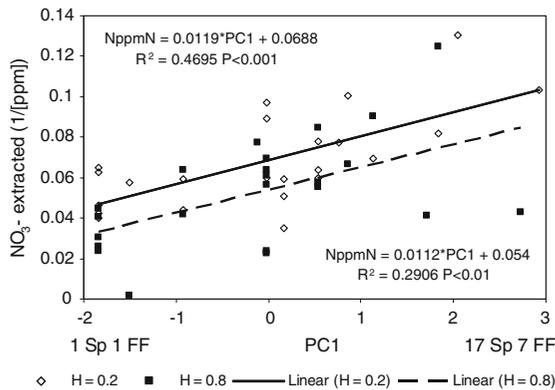


Fig. 1 Relationship between the reciprocal of NO_3^- recovered from anion exchange resin at 1-m depth under N (NppmN (1/[ppm])) and species and functional form richness (principal component 1 [PC1]) after adjusting for Ln (C_4 biomass), Ln (RLS), and $I_{\text{max}}\text{-P}$ (see Table 1 for regression model). For details on variables, see statistical section. The elevations of the regressions for fine-scale and coarse-scale are statistically different ($P < 0.05$). *Sp* species, *FF* functional forms

[Michx.) Nash-Gould, and *Sorghastrum nutans* L. (Appendix D: Online Resource 5 and 4 in Supplementary Material, Table 1) were both negatively correlated with the reciprocal of NO_3^- captured under N, suggesting that these four species, as well as species associated with high root lateral spread, were not as effective as other species at rapidly capturing N on a per area basis. As with PC1, the elevation of the regression was higher for fine-scale than coarse-scale; however, the regression for coarse-scale was not significantly different from zero (RLS; $R^2 = 0.139$, $P = 0.0802$) (C_4 biomass; $R^2 = 0.1117$, $P = 0.119$). Maximum P influx rate per unit of RSA ($I_{\text{max}}\text{-P}$ [$\text{g m}^{-2} \text{d}^{-1}$]) exhibited a similar pattern, but decreasing with the reciprocal of NO_3^- captured under N under coarse-scale, while exhibiting no response under fine-scale ($R^2 = 0.0666$, $P = 0.235$) (Appendix D: Online Resource 7 in Supplementary Material, Table 2).

Nitrate leaching under phosphorus application

In the P plots, PC1 decreased NO_3^- leaching, as shown by the negative relationship with the logarithm of NO_3^- captured (NO_3^- captured under P (ln [ppm])) (Fig. 2, Table 2). Of the RB-derived species variables, the greatest confounding variable was the combined root and aboveground cumulative biomass (2000–2002 in g m^{-2}) of late successional C_3 grasses

Table 2 Multiple regression testing for response of the natural log of NO_3^- recovered from anion exchange resin at 1-m depth under P application (NO_3^- captured under P in parts per million) to species and functional form richness (principal component 1 [PC1]) and spatial scaling factor H (see text for details), after adjustment for plant parameters associated with various species compositions

Multiple regression: AIC = 51.3

Variable	Coefficient	Standard error	P-value
PC1	-0.190	0.0543	0.0012
Ln (G2C3 biomass)	0.104	0.0309	0.0017
$I_{\text{max}}\text{-N}$	5.23	2.14	0.0193
$I_{\text{max}}\text{-P}$	-4.42	1.68	0.0122
τ	1.15	0.416	0.0088

Ln (G2C3 biomass) is the natural log of the cumulative biomass (2000–2002 in g m^{-2}) of late successional C_3 grasses *Koeleria cristata* (Lam.) Beauv., *Poa pratensis* L., *Stipa comata* Trin. & Rupr., and *Stipa viridula* Trin. All other parameters are derived from Johnson and Biondini (2001), Levang-Brilz and Biondini (2003), and Biondini (2007), where $I_{\text{max}}\text{-N}$ is the maximum influx rate per unit of root surface area for N ($\text{gN m}^{-2} \text{d}^{-1}$), $I_{\text{max}}\text{-P}$ is the maximum influx rate per unit of root surface area for P ($\text{gP m}^{-2} \text{d}^{-1}$), and τ is a scaling index for root plasticity ($\tau = 0$ is no plasticity) (see Appendix B: Online Resource 2 in Supplementary Material for further explanation)

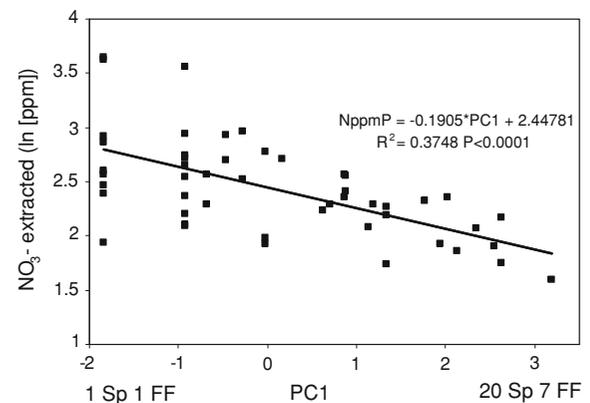


Fig. 2 Relationship between the logarithm of NO_3^- recovered from anion exchange resin at 1-m depth under P (NppmP (ln [ppm])) and species and functional form richness (principal component 1 [PC1]) after adjusting for Ln (C_3 biomass), $I_{\text{max}}\text{-N}$, $I_{\text{max}}\text{-P}$, and τ (see Table 2 for regression model). For details on variables, see statistical section. *Sp* species, *FF* functional forms

Koeleria cristata (Lam.) Beauv., *Poa pratensis* L., *Stipa comata* Trin. & Rupr., and *Stipa viridula* Trin (Appendix D: Online Resource 8 in Supplementary Material, Table 2). Maximum N and P influx rates

per unit of RSA ($I_{\max\text{-N}}$ [$\text{g m}^{-2} \text{d}^{-1}$] and $I_{\max\text{-P}}$), and the scaling parameter, τ , for root plasticity ($\tau = 0$ no plasticity), also showed significant relationships with NO_3^- captured under P (Appendix D: Online Resource 8–10 in Supplementary Material; Table 2). Coefficients were positive for both $I_{\max\text{-N}}$ and τ , negative for $I_{\max\text{-P}}$.

Discussion

As hypothesized, plant diversity reduced NO_3^- leaching in both the N and P plots; however, this reduction was mediated by the functional traits of the plants involved and varied substantially between the N and P plots, as well as between fine-scale and coarse-scale heterogeneity of N. While the scaling of P application was unimportant, NO_3^- leaching under coarse-scale N application was double that of NO_3^- leaching under fine-scale N. It is interesting to note that Biondini and Grygiel (1994) simulated the response of the percentage of patches with adult plants for monocultures of plants with high RLS and plants with low RLS under N scaling of fine-scale and coarse-scale using ALLOCATE. They found that, going from fine-scale to coarse-scale, the percentage of patches with adult plants increased for the species with high RLS and decreased for the species with low RLS. The increase in NO_3^- leaching when going from fine-scale to coarse-scale under N appears to be consistent with what Biondini and Grygiel (1994) found. Species having high RLS are associated with low RSA, low R:S, and low $I_{\max\text{-N}}$, while high EAM hyphal length is associated with species having low RLS (Levang-Brilz and Biondini 2003; Bingham and Biondini 2009). This is further supported by our finding that NO_3^- leaching increased with RLS. Furthermore, addition of N is known to increase N mineralization and addition of P is known to increase N immobilization, and thus changes in mineralization and immobilization of N are likely to partially account for the differences between the N and P experiments (Phoenix et al. 2003; Moore et al. 2006). However, we don't think increases in N mineralization are responsible for increased NO_3^- leaching under coarse-scale N addition, as we would predict greater total N mineralization under the fine-scale treatment where N is dispersed such that there are no large gaps in soil organic matter or the soil microbial

community that are distantly isolated from N patches (Moore et al. 2006; Robertson and Groffman 2007).

Previous results from the same experimental plots (Biondini 2007; Bingham and Biondini 2009) found that (1) plant species and functional form richness increased aboveground biomass and decreased inter-annual variability, resulting from increases in minimum biomass; (2) these increases in production and stability were positively correlated with relative growth rate, root density, RSA per unit of root biomass, uptake rates of N or P per unit of RSA, and N-use efficiency of the plot; (3) plant species and functional form richness decreased invasion by non-seeded species; and (4) plant species and functional form richness increase extraradical hyphal length (hyphal density) of AM fungi. Corresponding decreases in NO_3^- leaching found in this study on a subset of the same experiment is evidence that more complete and consistent uptake of N from the soil by the plant community was a mechanism for the increases in production, stability, and resistance to invasion, and that increases of mycorrhizal hyphal surface area in the soil may have been partially responsible for the increased utilization of soil NO_3^- . While we cannot rule out effects on N mineralization/immobilization as playing a role in diversity effects on NO_3^- leaching, the increases we've found in productivity and uptakes rates of N and P in these same plots are evidence that uptake of N by the plant community was the primary driver of observed decreases in NO_3^- leaching. This inference is partly supported by results from a number of other studies (van der Hiejden et al. 1998; Fargione et al. 2007; Cardinale et al. 2007). Conclusions reached in these studies included (1) plant community productivity increases with plant diversity; (2) nutrient (especially NO_3^-) capture increases with plant diversity; (3) mycorrhizal hyphal length increases concurrently with plant diversity, plant community biomass, and nutrient capture; (4) plant diversity increases input and retention of N by the plant community; (5) when the ratio of intergroup to intragroup competition is low, the coefficient of variation of community nutrient acquisition, and therefore community biomass, should decrease with diversity when diversity is low (i.e., stability increases); (6) plant community composition affects nutrient acquisition independent of diversity; and (7) plant diversity reduces plant community invasibility via competition for nutrients

and other resources. Increased NO_3^- capture by the plant community, therefore, has the potential to (1) decrease NO_3^- leaching; (2) increase productivity; (3) increase stability; and (4) decrease invasibility.

As with other results from this large-scale experiment (Biondini 2007; Bingham and Biondini 2009), and other studies (Scherer-Lorenzen et al. 2003; Hooper et al. 2005), functional variation among species was great enough within groupings that the assigned functional forms were no more important in their numbers than the number of species; however, the functional composition of the plots mattered (Appendix B: Online Resource 2 in Supplementary Material).

While NO_3^- leaching was reduced under fine-scale N heterogeneity as compared to coarse-scale N heterogeneity, under coarse-scale N, regression analysis revealed a positive relationship with $I_{\text{max}}\text{-P}$, suggesting that some of the variation in NO_3^- leaching was related to effects of N/P ratios on mineralization/immobilization. In the plots with applied P, there was a positive overall relationship of NO_3^- leaching with $I_{\text{max}}\text{-N}$ and the scaling index for root plasticity ($\tau = 0$ is no plasticity) (Online Resource 8–10), and a negative correlation with $I_{\text{max}}\text{-P}$. This discrepancy was not expected given the positive relationship that Levang-Brilz and Biondini (2003) found between RGR and both $I_{\text{max}}\text{-N}$ and $I_{\text{max}}\text{-P}$.

Nitrate leaching was also positively correlated with RLS under fine-scale heterogeneity in the N plots (Appendix D: Online Resource 5 in Supplementary Material). In Bingham and Biondini (2009), a positive relationship was found between the biomass contribution of late successional C_4 grasses (*Andropogon gerardii*, *Panicum virgatum*, *Schizachyrium scoparium*, and *Sorghastrum nutans*) and EAM hyphal length, and this functional group has characteristically low parameters (α and β , Appendix B: Online Resource 2 in Supplementary Material) relating RB to RLS, so it is plausible that the lower surface area of hyphae associated with species having higher RLS is resulting in reduced N uptake, and thus increased mineralization and leaching. Consistent with this, NO_3^- leaching under P application increased with the cumulative biomass (2000–2002 in g m^{-2}) of late successional C_3 grasses *Koeleria cristata* (Lam.) Beauv., *Poa pratensis* L., *Stipa comata* Trin. & Rupr., and *Stipa viridula* Trin., a functional group that has characteristically high

α and β . But, contradicting this, under fine-scale heterogeneity in the N plots late successional C_4 grasses were associated with an increase in NO_3^- leaching (Appendix D: Online Resource 6 in Supplementary Material). While late successional C_4 grasses are obligatory mycorrhizal, C_3 grasses tend to have relatively low, or even negative, mycorrhizal responsiveness (Wilson and Hartnett 1998). This was shown by Wilson and Hartnett (1998) to be related to lower root colonization by C_3 grasses relative to forbs and C_4 grasses. Of the plants belonging to these two groups in our plots that were tested by Wilson and Hartnett (1998), the C_4 grasses (*Andropogon gerardii*, *Panicum virgatum* and *Sorghastrum nutans*) averaged 98.9% in mycorrhizal responsiveness and the C_3 grasses (*Koeleria cristata* and *Poa pratensis*) averaged -18% . Why the late successional C_4 grasses were associated with a decrease in N uptake and/or increase in N mineralization in the N plots is unclear. Members of this functional group do exhibit substantially reduced R:S under high N (see Levang-Brilz and Biondini 2003), and N levels may have been high enough in the N plots to have an inordinately large effect on these species. Johnson et al. (2003) also showed that N application reduces EAM hyphal length when N is limiting among five Long Term Ecological Research (LTER) grassland sites, and the site that was most N-limited was the Cedar Creek LTER, the site closest to ours in distance and climate. In either case, this functional group of grasses is K-selected relative to most of the other species utilized in our study, and thus has a strategy tending toward lower rates of growth, resource acquisition, and reproduction, even when resource availability is high, but the ability to achieve large biomass over long periods of time while successfully acquiring and retaining resources in $\text{c}I_{\text{max}}$ communities where nutrient cycling is tight.

If N uptake and/or N immobilization is increasing with plant diversity in part due to increased EAM hyphal length (Bingham and Biondini 2009), then maintaining a grassland community with high plant diversity and obligately mycorrhizal species should be favorable for sequestering large pools of both N and C. This is likely to be maximized in the most diverse and persistent late successional communities (Treseder and Allen 2000; Fargione et al. 2007). As carbon dioxide (CO_2) concentration rises, productivity and thus sequestration of C is likely to increase

(Treseder and Allen 2000; Gill et al. 2002) the most in plant communities where soil nutrients are most completely utilized, concurrently increasing plant N sequestration (Niklaus et al. 2001; Dijkstra et al. 2007). A reduction of N inputs into nearby water bodies should follow (Scherer-Lorenzen et al. 2003).

Nitrogen and carbon cycling in grasslands are affected by plant community diversity and composition via changes in productivity, stability, density of mycorrhizal hyphae in the soil, N uptake and probably N mineralization/immobilization; thus, managers need to consider plant diversity and composition in restored and native grasslands. The major conclusions from this study are that to maximize N retention in tallgrass prairie plant communities: (1) high levels of plant species and functional form richness should be achieved and maintained; (2) insure that C₄ grasses (e.g., *Andropogon gerardii*, *Panicum virgatum*, and *Sorghastrum nutans*) are present to avoid over-dominance by C₃ grasses (e.g., *Koeleria cristata* and *Poa pratensis*); (3) include species in seed mixtures having low root lateral spread per unit of root biomass, low root plasticity, high root surface area per unit of root biomass, and high root to shoot ratio; and (4) if N fertilizer is used, any spatial heterogeneity in the application should be at the finest scale. While caution should be taken in generalizing all of these details to every temperate grassland, it is likely that the diversity and nutrient scaling effects observed in this study, and affiliated research, hold true for many C₄-dominated temperate grassland ecosystems, barring introduction of invasive exotic plant species.

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