

## PLANT DIVERSITY, CO<sub>2</sub>, AND N INFLUENCE INORGANIC AND ORGANIC N LEACHING IN GRASSLANDS

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**Abstract.** In nitrogen (N)-limited systems, the potential to sequester carbon depends on the balance between N inputs and losses as well as on how efficiently N is used, yet little is known about responses of these processes to changes in plant species richness, atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]), and N deposition. We examined how plant species richness (1 or 16 species), elevated [CO<sub>2</sub>] (ambient or 560 ppm), and inorganic N addition (0 or 4 g·m<sup>-2</sup>·yr<sup>-1</sup>) affected ecosystem N losses, specifically leaching of dissolved inorganic N (DIN) and organic N (DON) in a grassland field experiment in Minnesota, USA. We observed greater DIN leaching below 60 cm soil depth in the monoculture plots (on average 1.8 and 3.1 g N·m<sup>-2</sup>·yr<sup>-1</sup> for ambient N and N-fertilized plots respectively) than in the 16-species plots (0.2 g N·m<sup>-2</sup>·yr<sup>-1</sup> for both ambient N and N-fertilized plots), particularly when inorganic N was added. Most likely, loss of complementary resource use and reduced biological N demand in the monoculture plots caused the increase in DIN leaching relative to the high-diversity plots. Elevated [CO<sub>2</sub>] reduced DIN concentrations under conditions when DIN concentrations were high (i.e., in N-fertilized and monoculture plots). Contrary to the results for DIN, DON leaching was greater in the 16-species plots than in the monoculture plots (on average 0.4 g N·m<sup>-2</sup>·yr<sup>-1</sup> in 16-species plots and 0.2 g N·m<sup>-2</sup>·yr<sup>-1</sup> in monoculture plots). In fact, DON dominated N leaching in the 16-species plots (64% of total N leaching as DON), suggesting that, even with high biological demand for N, substantial amounts of N can be lost as DON. We found no significant main effects of elevated [CO<sub>2</sub>] on DIN or DON leaching; however, elevated [CO<sub>2</sub>] reduced the positive effect of inorganic N addition on DON leaching, especially during the second year of observation. Our results suggest that plant species richness, elevated [CO<sub>2</sub>], and N deposition alter DIN loss primarily through changes in biological N demand. DON losses can be as large as DIN loss but are more sensitive to organic matter production and turnover.

**Key words:** dissolved inorganic nitrogen; dissolved organic nitrogen; drainage; elevated CO<sub>2</sub>; grassland; leaching; lysimeter; nitrogen deposition; nitrogen loss; plant species richness.

### INTRODUCTION

For many terrestrial ecosystems, leaching is an important pathway of N loss (Chapin et al. 2002) that can be very sensitive to biotic and abiotic change. Because net primary productivity in most terrestrial ecosystems is nitrogen (N) limited (Vitousek and Howarth 1991), factors that influence N loss also affect their potential to sequester carbon (C; Vitousek and Reiners 1975, Rastetter et al. 2005). Although the magnitude of annual N losses through leaching are typically only a small fraction of total ecosystem N pools (Chapin et al. 2002, Van Breemen et al. 2002), persistent N leaching losses can, in the long-term, significantly reduce accumulation of N and C in plant biomass and soil organic matter (Vitousek et al. 1998,

Rastetter et al. 2005). Even when biotic demand for N is high, N can still leak from ecosystems with drainage water in forms that are not directly available for plants, or that are in low demand by microorganisms (i.e., recalcitrant dissolved organic N or DON; Hedin et al. 1995, Perakis and Hedin 2002, Neff et al. 2003). Despite its importance for C sequestration and other ecosystem functions, very little is known about the interactive biotic and environmental effects on inorganic and organic N loss through leaching. This lack of understanding is increasingly important to resolve in the context of multiple, human-induced changes to terrestrial ecosystems. Here we explore the interactive effects of variation in plant species richness (one or 16 species), elevated atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) (560 ppm), and inorganic N addition (4 g N·m<sup>-2</sup>·yr<sup>-1</sup>) on N leaching in both inorganic and organic N forms, using a long-term grassland field experiment in Minnesota (Reich et al. 2001a, b, 2006).

In N-limited ecosystems, demand for N by plants and soil microorganisms exerts a large control over inorganic N leaching losses (NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> or dissolved

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inorganic N, DIN), and as long as biological demand for N is high, DIN losses from terrestrial ecosystems generally remain small (Vitousek and Reiners 1975, Hedin et al. 1995, Vitousek et al. 1998). More diverse plant communities (greater number of species or functional groups) generally reduce DIN losses compared to less diverse communities (Tilman et al. 1996, 1997, Hooper and Vitousek 1997, Niklaus et al. 2001). Species-rich communities tend to be more effective at taking up available soil N than species-poor communities because of complementary resource use (e.g., in space and/or time; Tilman et al. 1996, 1997) and because high N demanding plants are more likely to be present (Hooper and Vitousek 1997). However, DIN losses do not always decrease with increased plant diversity, but can also depend on plant species composition. For example, plots with legumes lost significantly more nitrate than plots without them in a mid-European grassland ecosystem (Scherer-Lorenzen et al. 2003).

Elevated atmospheric  $[\text{CO}_2]$  can reduce DIN loss because of increased plant N uptake and microbial N immobilization (Johnson et al. 2001, 2004, Niklaus et al. 2001, Hagedorn et al. 2005). Plant species richness and atmospheric  $[\text{CO}_2]$  effects on DIN loss may, in turn, depend on the level of N inputs. Nitrogen inputs may alleviate ecosystem N limitation, thereby causing greater DIN leaching. This alleviation may be more likely to occur in species-poor plant communities and under ambient atmospheric  $[\text{CO}_2]$  where plant N uptake and microbial N immobilization are low compared to species-rich plant communities and under elevated atmospheric  $[\text{CO}_2]$  conditions. We therefore hypothesized that inorganic N inputs will increase DIN leaching, with the greatest increases occurring under conditions of low plant species richness and ambient atmospheric  $[\text{CO}_2]$ .

While most research on N leaching losses has focused on inorganic N, more recent work has shown that leaching losses of dissolved organic N (DON) can be relatively large, even in the presence of high biotic demand for N (Hedin et al. 1995, Lajtha et al. 1995, Perakis and Hedin 2002). Because DON losses often occur despite N limitation of plant productivity, it is assumed that DON that is lost through leaching is recalcitrant and not directly available for plant uptake (Neff et al. 2003). DON losses do not appear to be controlled by seasonal biological controls (Campbell et al. 2000, Goodale et al. 2000, Cairns and Lajtha 2005, Dijkstra et al. 2006), but may be more affected by soil texture and hydrologic seasonality (Lajtha et al. 1995, Campbell et al. 2000) and organic matter content (Neff et al. 2000, but see Aitkenhead-Peterson et al. 2002). Because of the apparent minor effect that plant N demand has on DON loss, we hypothesized that increased species richness, elevated  $[\text{CO}_2]$ , and N addition will not affect DON leaching. However, DON leaching should become relatively more important (compared with DIN leaching) as a pathway for N loss

with increased species richness and elevated  $[\text{CO}_2]$ , especially under low N availability.

## MATERIALS AND METHODS

### *Study area and experimental design*

The location of our study was the Long-Term Ecological Research site at the Cedar Creek Natural History Area (CCNHA), Minnesota, USA (45° N, 93° W). The site is situated on a nutrient-poor sandy outwash plain (Typic Udipsamments). Mean annual precipitation is 660 mm with mean monthly temperatures of  $-11^\circ\text{C}$  in January and  $22^\circ\text{C}$  in July. The BioCON (Biodiversity,  $\text{CO}_2$ , and N) grassland field experiment was established in 1997 in an abandoned agricultural field. In total, 354 plots ( $2 \times 2$  m), evenly distributed over six circular areas (or rings, 20 m diameter), were planted with either 1, 4, 9, or 16 grassland species after the natural vegetation was removed and the soil treated with methyl bromide to eradicate the seed bank. The 16 species that were used for this experiment are the  $\text{C}_4$  grasses *Andropogon gerardii* Vitman, *Bouteloua gracilis*, *Schizachyrium scoparium* (Michaux) Nash, and *Sorghastrum nutans* (L.) Nash, the  $\text{C}_3$  grasses *Agropyron repens* (L.) Beauv., *Bromus inermis* Leysser, *Koeleria cristata* Pers, and *Poa pratensis* L., the forbs *Achillea millefolium* L., *Anemone cylindrica* A. Gray, *Asclepias tuberosa* L., and *Solidago rigida* L., and the legumes *Amorpha canescens* Pursh, *Lespedeza capitata* Michaux, *Lupinus perennis* L., and *Petalostemum villosum* Nutt. (= *Dalea villosa* (Nutt.) Spreng.). In this study, we report N losses from 176 plots, including the monoculture plots (eight plots for each species; 2  $\text{CO}_2$  treatments  $\times$  2 N treatments  $\times$  2 replicates) and 16-species plots (48 plots; 2  $\text{CO}_2$  treatments  $\times$  2 N treatments  $\times$  12 replicates). Three rings were treated with elevated atmospheric  $[\text{CO}_2]$  (560 ppm) during daylight hours and during the growing season (April–November), starting in 1998 using free-air  $\text{CO}_2$  enrichment technology. The equivalent of 4 g  $\text{N}\cdot\text{m}^{-2}\cdot\text{yr}^{-1}$  ( $\text{NH}_4\text{NO}_3$ ) was added to half of the plots, applied in three doses during the growing season (in May, June, and July). The experiment was set up as a split-plot design with the  $\text{CO}_2$  treatment nested within ring.

### *Sampling and analyses*

In the spring of 2003, we installed tension lysimeters (Rhizon SMS, Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands), one in each of the 128 (16 species  $\times$  8 plots) monoculture and 48 16-species plots at 60 cm soil depth ( $\pm 5$  cm), 75 cm from the edge of the plot. Lysimeters, 10 cm long with a diameter of 0.5 cm, consisted of a hydrophilic porous polymer sheath fitted around a stainless steel wire and connected to PVC tubing. We attached a 1-m stainless steel rod (0.1 cm diameter) alongside the lysimeter that enabled us to insert the lysimeter into the soil after a hole was made with a metal rod (0.7 cm diameter) at a  $30^\circ$  angle. While

we inserted the lysimeter, we also injected a silica-water slurry into the hole to improve contact between the lysimeter and the soil. The installation of our small lysimeters did not cause major disturbance to the soil. We obtained soil solutions by connecting vacuumed bottles (50 kPa) to the PVC tubes for 24 h. We collected soil solutions on 27 June, 14 September, and 5 November in 2003, 6 April, 10 May, 2 June, and 26 August in 2004, and on 13 April in 2005. Sampling always occurred within 48 h after rainfall events. We were unable to sample during the winter period because of frozen soils. After collection, we immediately transported solution samples to the laboratory where they were split into two subsamples and frozen until analyses for dissolved inorganic N (DIN,  $\text{NO}_3^- + \text{NH}_4^+$ ) on an Alpkem auto-analyzer (Pulse Instrumentation, Saskatoon, Saskatchewan, Canada) and total dissolved N (TDN) on a Shimadzu TOC-V<sub>CPN</sub> (Shimadzu Scientific Instruments, Wood Dale, Illinois, USA). We calculated dissolved organic N concentration (DON) as the difference between the TDN and DIN concentration.

We estimated daily water fluxes via leaching below 60 cm soil depth based on the hydrological model BROOK90 (Federer 1995) that uses daily values of precipitation, maximum and minimum air temperature, and monthly values of leaf area index (LAI) as input. Daily values of precipitation and maximum and minimum air temperature came from the Cedar Creek weather station (NOAA-National Climatic Data Center). We used LAI values measured at monthly intervals from May until October in 2003 and 2004 in each plot with a linear irradiance sensor array (AccuPar LP-80, Decagon Devices, Pullman, Washington, USA). Because of the large variation in measured LAI among replicated plots, we used average monthly LAI values for plots with identical treatments. That is, for the 16-species plots we used the average monthly LAI value for each of the  $\text{CO}_2$  and N treatments. For the monoculture plots we used species-specific monthly LAI values for each of the  $\text{CO}_2$  and N treatments. We reduced the stomatal conductance in the model by 24% for the elevated  $[\text{CO}_2]$  plots (Lee et al. 2001). We modeled water fluxes below 60 cm soil depth in the 16-species plots for each of the  $\text{CO}_2$  and N treatments separately (pooled across all 12 replicates), and within the monoculture plots also for each plant species (pooled across the two replicates). We had previously calibrated this model by comparing modeled soil moisture contents with soil moisture contents measured at different soil depths in a savanna site located within CCNHA (Dijkstra et al. 2006). The calibrated model performed well in predicting soil moisture contents at different soil depths measured in each plot of the BioCON experiment during the growing seasons of 2003 and 2004. We calculated daily DIN and DON concentrations using linear interpolation of DIN and DON concentrations measured between successive sampling events. We then calculated annual DIN and

DON leaching below 60 cm soil depth by multiplying daily DIN and DON concentrations with daily water fluxes. Although there were gaps in DIN and DON concentration measurements during the winter period (no measurements from November until March), N leaching losses during that period were very small because of the virtual absence of water flow in the frozen soil (data not shown, but see Dijkstra et al. 2006). We calculated annual DIN and DON leaching for the periods from 1 May 2003 to 30 April 2004, and from 1 May 2004 to 30 April 2005, so that each period included four sampling events. We will refer to these two periods subsequently as the "first year" and "second year," respectively. With the limited number of sampling events, our calculated estimates of annual DIN and DON leaching may have considerable uncertainty, but they should provide a good indication of the relative differences in leaching losses among treatments.

We used analyses of variance (ANOVA) to test for main effects ( $\text{CO}_2$ , N, and number of species) and their interactions on soil solution N concentrations and N losses. The effect of  $\text{CO}_2$  was tested against the random effect of ring nested within  $\text{CO}_2$ . We used repeated measures multivariate analyses of variance (MANOVA) to test for main effects ( $\text{CO}_2$ , N, and number of species) and their interactions with time (eight measurement dates) on soil solution N concentrations. We used orthogonal regression (with univariate variance estimates) to relate DIN concentrations at ambient  $[\text{CO}_2]$  to DIN concentrations at elevated  $[\text{CO}_2]$  averaged by number of species and N treatment for each date. Where needed, data were log-transformed to meet the assumptions of ANOVA. All statistical analyses were done with JMP (version 4.0.4; SAS Institute, Cary, North Carolina, USA).

## RESULTS

The 16-species plots had significantly lower average DIN concentrations at 60 cm soil depth than the monoculture plots at all times measured (Tables 1 and 2). Nitrate dominated DIN concentrations (more than 95% of DIN was in the form of  $\text{NO}_3^-$ , data not shown). In the monoculture plots, DIN concentrations were, on average, between three (on 6 April 2004) and 20 times (on 5 November 2003 and 26 August 2004) higher than in the 16-species plots. As expected, N addition increased DIN concentration at all times, but the increase was only significant on four out of eight measurement dates (between 104% on 14 September 2004 and 370% on 27 June 2003). The increase in DIN concentration with N addition was always larger in the monoculture plots than in the 16-species plots, sometimes causing a significant number of species  $\times$  N interaction term (Tables 1 and 2, Fig. 1A). Significant N addition and number of species  $\times$  N interaction effects on DIN concentration occurred especially during periods when plants were growing fast (June–October), but disappeared in the winter and early growing season

TABLE 1. Mean (and SE) dissolved organic nitrogen (DIN) concentrations (mg/L) in soil solutions at 60 cm soil depth for different dates in monoculture plots and 16-species plots.

CO <sub>2</sub> level	N level	27 Jun 2003	14 Sep 2003	5 Nov 2003	4 Apr 2004	10 May 2004	2 Jun 2004	26 Aug 2004	13 Apr 2005
Monoculture plots									
Ambient	ambient	0.9 (0.4)	7.1 (1.8)	6.1 (1.4)	2.0 (0.7)	6.8 (1.6)	0.6 (0.2)	5.2 (1.6)	2.3 (0.6)
Ambient	elevated	9.0 (2.0)	16.7 (3.5)	13.4 (3.0)	2.6 (0.9)	7.6 (2.1)	1.8 (0.5)	10.7 (2.6)	4.0 (0.8)
Elevated	ambient	1.2 (0.4)	5.8 (1.8)	5.6 (1.8)	1.0 (0.2)	5.0 (1.3)	0.7 (0.2)	4.0 (1.5)	3.5 (1.0)
Elevated	elevated	4.5 (1.1)	9.2 (2.5)	11.2 (3.2)	1.4 (0.5)	5.1 (1.6)	1.2 (0.3)	5.6 (1.8)	3.9 (1.0)
16-species plots									
Ambient	ambient	0.3 (0.1)	1.0 (0.3)	0.7 (0.2)	0.6 (0.2)	0.9 (0.1)	0.2 (0.1)	0.5 (0.3)	0.7 (0.2)
Ambient	elevated	0.5 (0.2)	0.6 (0.1)	0.4 (0.1)	0.4 (0.1)	0.5 (0.2)	0.1 (0.0)	0.5 (0.3)	0.4 (0.1)
Elevated	ambient	1.2 (0.7)	0.4 (0.1)	0.3 (0.1)	0.4 (0.1)	0.3 (0.1)	0.1 (0.1)	0.2 (0.1)	0.4 (0.2)
Elevated	elevated	0.4 (0.2)	0.5 (0.1)	0.3 (0.1)	0.3 (0.1)	0.8 (0.2)	0.1 (0.0)	0.2 (0.0)	0.3 (0.1)

when plant aboveground biomass was absent or sparse and when plants were inactive (November–May). When we included all eight measurement dates in one single statistical test (repeated-measures MANOVA), we observed significant number of species ( $P = 0.01$ ) and N-addition effects ( $P = 0.05$ ), but no significant number of species  $\times$  N interaction. We observed no significant main CO<sub>2</sub> or interaction effects on DIN concentration. However, DIN concentrations were often smaller at elevated [CO<sub>2</sub>] than at ambient [CO<sub>2</sub>], particularly at times when DIN concentrations were high (e.g., in the monoculture plots with N addition). In fact, when we relate DIN concentrations at ambient [CO<sub>2</sub>] to DIN concentrations at elevated [CO<sub>2</sub>] averaged by number of species and N treatment for each date, using an orthogonal regression (i.e., standardized or reduced major axis), then the slope (0.67) of the regression is significantly lower than 1 (upper 95% confidence limit, 0.75; Fig. 2).

In contrast to DIN, average DON concentrations at 60 cm soil depth were significantly larger in the 16-species plots than in the monoculture plots (between 51% on 27 June 2003 and 229% on 10 June 2004), except on the last three measurement dates (Tables 3 and 4). Nitrogen addition significantly increased DON concentrations on five of the eight measurement dates (between 74% on 6 April 2004 and 660% on 26 August 2004), although this time we found no clear seasonal pattern. The increase in DON concentration with N addition was especially large in the ambient CO<sub>2</sub> plots causing

sometimes significant CO<sub>2</sub>  $\times$  N interaction terms (Tables 3 and 4, Fig. 1B). We did not observe any significant main CO<sub>2</sub>, number of species  $\times$  N, or number of species  $\times$  CO<sub>2</sub> interaction effects on DON concentration. A repeated-measures MANOVA including all eight measurement dates showed that only species number ( $P = 0.0002$ ) and N-addition effects ( $P = 0.04$ ) were significant.

The average annual modeled water flux below 60 cm soil depth was higher in the monoculture plots than in the 16-species plots, both in the first (by 11%) and second year (by 14%; Table 5). The reduced water fluxes in the 16-species plots and N-addition plots were caused by greater LAI and transpiration rates in these plots (data not shown). Nitrogen addition only slightly reduced the annual water flux in both years (by 2% and 3%, respectively). Despite a reduced stomatal conductance, water fluxes were only slightly higher in the elevated CO<sub>2</sub> plots during the first year (by 2%) and did not change in the second year because the reduced stomatal conductance was offset by higher LAI in the elevated CO<sub>2</sub> plots with little net effect on total transpiration (data not shown). Annual water fluxes below 60 cm soil depth were higher during the second year than during the first year because of higher annual precipitation (731 mm during 1 May 2003–3 April 2004 and 897 mm during 1 May 2004–30 April 2005).

Annual DIN leaching below 60 cm soil depth was significantly higher in the monoculture plots than in the 16-species plots in both years (by 1800 and 1200%

TABLE 2.  $P$  values from ANOVA for the effects of number of species (1 vs. 16), CO<sub>2</sub> level (ambient or elevated), and N level (ambient or elevated) on DIN concentrations in soil solutions at 60 cm soil depth for different dates.

Factor	27 Jun 2003	14 Sep 2003	5 Nov 2003	4 Apr 2004	10 May 2004	2 Jun 2004	26 Aug 2004	13 Apr 2005
No. species	<b>0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.0003</b>	<b>&lt;0.0001</b>	<b>0.0005</b>	<b>0.004</b>	<b>0.0005</b>
CO <sub>2</sub> level	0.76	0.26	0.72	0.67	0.54	0.53	0.17	0.67
N level	<b>&lt;0.0001</b>	<b>0.01</b>	<b>0.01</b>	0.49	0.39	<b>0.007</b>	<i>0.07</i>	0.27
No. species $\times$ CO <sub>2</sub> level	0.86	0.36	0.85	0.97	0.72	0.72	0.49	0.69
No. species $\times$ N level	<b>0.01</b>	<b>0.05</b>	<b>0.04</b>	0.18	0.39	<i>0.09</i>	<b>0.05</b>	0.47
CO <sub>2</sub> level $\times$ N level	0.32	0.23	0.76	0.93	0.53	0.34	0.33	0.50
No. species $\times$ CO <sub>2</sub> level $\times$ N level	0.91	0.43	0.81	0.43	0.24	0.50	0.64	0.61

Note:  $P$  values smaller than 0.05 are shown in boldface type, and those smaller than 0.1 are shown in italic type.

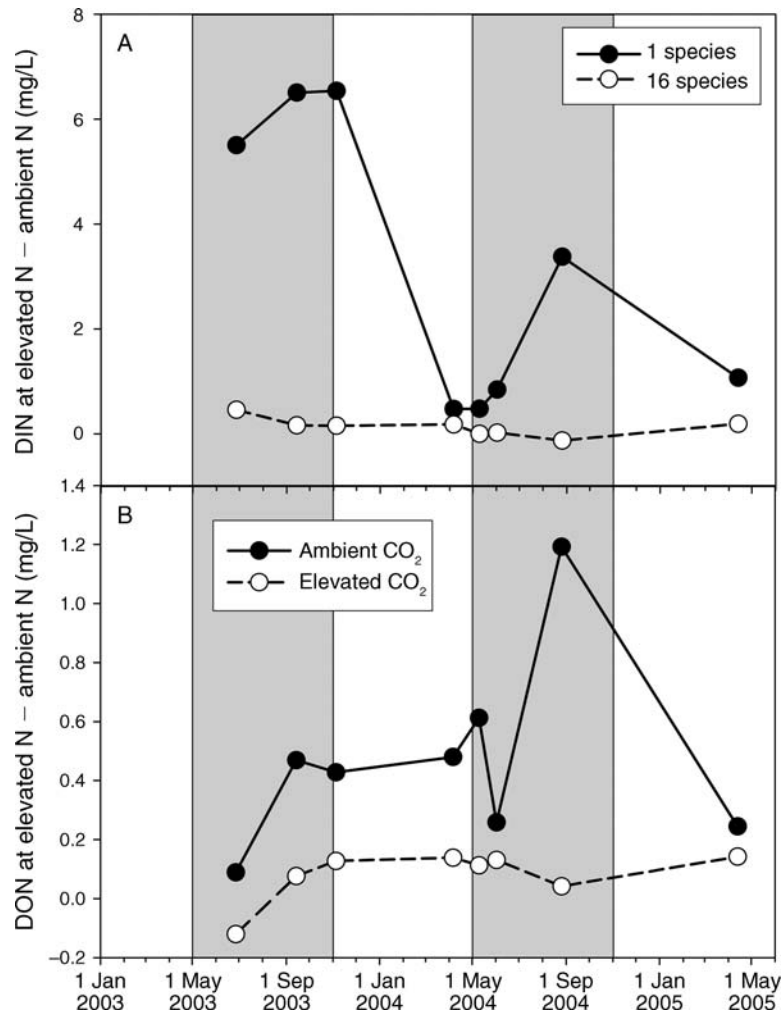


FIG. 1. (A) The difference in the mean dissolved inorganic nitrogen (DIN) concentrations in the N-fertilized (elevated N, pooled across  $\text{CO}_2$  treatments) and non-fertilized plots (ambient N, pooled across  $\text{CO}_2$  treatments) for the monoculture and 16-species plots for each date of measurement. (B) The difference in the mean dissolved organic nitrogen (DON) concentrations in the N-fertilized (elevated N, pooled across number-of-species treatments) and non-fertilized plots (ambient N, pooled across number-of-species treatments) for the ambient and elevated  $\text{CO}_2$  plots for each date of measurement. Shaded areas represent the growing season.

respectively; Fig. 3). During the first year, N addition significantly increased annual DIN leaching, but only in the monoculture plots (by 126%, significant number of species  $\times$  N interaction). Although not significant, in the second year N main treatment and number of species  $\times$  N interaction effects showed a similar pattern as in the first year. We observed no significant  $\text{CO}_2$  main treatment or interaction effects on DIN leaching.

Annual DON leaching below 60 cm soil depth was significantly higher in the 16-species plots than in the monoculture plots in both years (by 73% and 45%, respectively; Fig. 3). In fact, DON was the dominant form of N leaching in the 16-species plots (on average 64% of total N leaching), while in the monoculture plots, annual DON leaching was an order of magnitude lower than annual DIN leaching. Annual DON leaching increased with N addition, particularly in the monocul-

ture plots during the second year (significant N and marginally significant number of species  $\times$  N interaction effect; Fig. 3). In both years, N addition increased annual DON leaching with ambient  $[\text{CO}_2]$ , but not with elevated  $[\text{CO}_2]$ .

Average annual DIN and DON leaching varied substantially among the 16 species grown as monocultures (Fig. 4). Annual DIN leaching was particularly high for the legumes and forbs (with the exception of *S. rigida*) in both years. Annual DON leaching in the *S. scoparium*, *P. pratensis*, and *S. rigida* monoculture plots during the first year and in the *B. gracilis*, *A. repens*, *B. inermis*, *P. pratensis*, and *S. rigida* monoculture plots during the second year were as high as or higher than in the 16-species plots.

The total amount of N (DIN + DON) leached below 60 cm soil depth was significantly higher in the

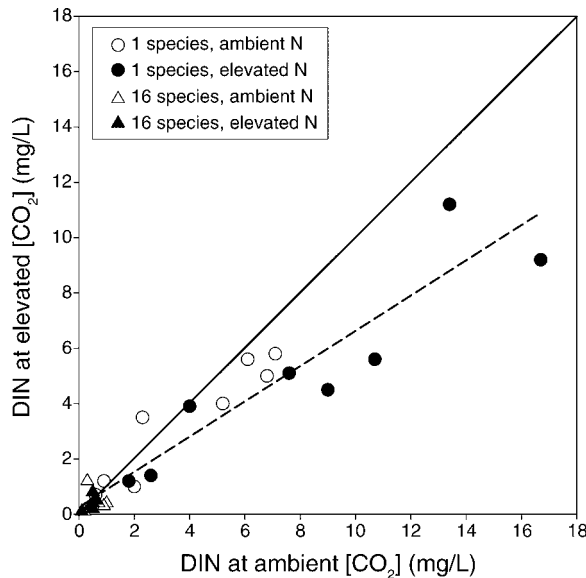


FIG. 2. Relationship of DIN concentrations between ambient and elevated CO<sub>2</sub> treatments averaged by number of species and N treatment for each date of measurement. The dashed line represents orthogonal regression (with univariate variance estimates) for all data points (slope, 0.67; upper 95% CI, 0.75).

monoculture plots than in the 16-species plots in both years (by 40% in both years; Tables 5 and 6). Nitrogen addition increased total N leaching as expected, especially in the first year (by 93%), but interactions with CO<sub>2</sub> or number of species that were significant for annual DIN and DON leaching were not found for total N leaching. The total N leached below 60 cm soil depth in the fertilized monoculture plots approached ~75% on average of the amount of N added as fertilizer (4 g·m<sup>-2</sup>·yr<sup>-1</sup>). We observed no significant main CO<sub>2</sub> or number of species × CO<sub>2</sub> interaction effects.

DISCUSSION

As we hypothesized, the increase in plant species richness from 1 to 16 grassland species greatly reduced DIN leaching below 60 cm soil depth. Reduced DIN

leaching most likely resulted from greater overall N uptake by plants in more diverse plots, caused by complementary resource use (e.g., temporal and/or spatial differentiation in N uptake among species [Tilman et al. 1996, 1997, Niklaus et al. 2001]) or by a greater likelihood of the presence of plants with high N demand (Hooper and Vitousek 1997). The 16-species plots also had greater root biomass and total plant N (Reich et al. 2001a, 2004) than the monoculture plots indicating increased inorganic N uptake by plants. The 16-species plots had greater organic matter inputs, which resulted in higher gross N mineralization rates but lower net N mineralization rates than the monoculture plots, implying increased N immobilization rates with greater diversity (Reich et al. 2006, West et al. 2006; P. B. Reich, S. E. Hobbie, D. Tilman, J. M. H. Knops, S. Naeem, and D. Wedin, unpublished data). This may also have contributed to lower DIN leaching in the 16-species plots than in the monoculture plots. Even with increased inorganic N inputs, DIN leaching was low in the 16-species plots, indicating that these plots maintained low inorganic N concentrations in the rooting zone and remained strongly N limited after seven years of 4 g/m<sup>2</sup> inorganic N addition. There is evidence that plant composition can play a greater role than plant species richness in reducing DIN leaching (Hooper and Vitousek 1997, 1998, Scherer-Lorenzen et al. 2003). For instance, the presence of N-fixing legumes significantly increased DIN and nitrate leaching in grassland systems in California and Germany (Hooper and Vitousek 1997, Scherer-Lorenzen et al. 2003), obscuring plant richness effects at the German site (Scherer-Lorenzen et al. 2003). With our experimental design we were unable to separate species richness effects from plant composition effects on DIN leaching in mixed assemblages. However, DIN concentrations and leaching were often higher in the legume monoculture plots than in the non-legume monoculture plots (Fig. 4), likely because of N-fixation (West et al. 2005) that increased inorganic N availability in the soil. Nevertheless, despite the considerable abundance of legumes in all of the 16-species plots (48% and 38% of aboveground biomass in June and August of 2003, and 52% and 41% in June and August

TABLE 3. Mean (and SE) dissolved organic nitrogen (DON) concentrations (mg/L) in soil solutions at 60 cm soil depth for different dates in monoculture plots and 16-species plots.

CO <sub>2</sub> level	N level	27 Jun 2003	14 Sep 2003	5 Nov 2003	4 Apr 2004	10 May 2004	2 Jun 2004	26 Aug 2004	13 Apr 2005
Monoculture plots									
Ambient	ambient	0.52 (0.08)	0.26 (0.06)	0.42 (0.06)	0.33 (0.08)	0.01 (0.14)	0.36 (0.04)	0.00 (0.31)	0.56 (0.09)
Ambient	elevated	0.57 (0.13)	0.69 (0.22)	0.58 (0.13)	0.72 (0.11)	0.53 (0.20)	0.62 (0.23)	0.58 (0.36)	0.87 (0.14)
Elevated	ambient	0.58 (0.07)	0.49 (0.09)	0.47 (0.08)	0.42 (0.06)	0.22 (0.13)	0.30 (0.05)	0.41 (0.17)	0.62 (0.09)
Elevated	elevated	0.37 (0.07)	0.57 (0.09)	0.58 (0.16)	0.58 (0.13)	0.39 (0.11)	0.46 (0.07)	0.52 (0.23)	0.78 (0.15)
16-species plots									
Ambient	ambient	0.66 (0.04)	1.88 (0.56)	0.91 (0.17)	0.50 (0.05)	0.71 (0.10)	0.33 (0.05)	0.67 (0.09)	0.63 (0.16)
Ambient	elevated	0.75 (0.09)	1.90 (0.50)	1.68 (0.33)	1.13 (0.20)	1.11 (0.17)	0.61 (0.10)	0.72 (0.22)	0.49 (0.12)
Elevated	ambient	0.87 (0.18)	1.09 (0.24)	1.03 (0.17)	0.69 (0.10)	0.98 (0.14)	0.48 (0.16)	1.15 (0.15)	0.56 (0.12)
Elevated	elevated	0.81 (0.10)	0.95 (0.16)	1.05 (0.08)	0.67 (0.09)	0.74 (0.07)	0.45 (0.06)	0.58 (0.11)	0.67 (0.12)

TABLE 4. *P* values from ANOVA for the effects of number of species (1 vs. 16), CO<sub>2</sub> level (ambient or elevated), and N level (ambient or elevated) on DON concentrations in soil solutions at 60 cm soil depth for different dates.

Factor	27 Jun 2003	14 Sep 2003	5 Nov 2003	4 Apr 2004	10 May 2004	2 Jun 2004	26 Aug 2004	13 Apr 2005
No. species	<b>0.001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.03</b>	<b>&lt;0.0001</b>	0.46	0.12	0.37
CO <sub>2</sub> level	0.92	0.95	0.61	0.48	0.60	0.31	0.12	0.81
N level	0.43	<b>0.01</b>	<i>0.06</i>	<b>0.007</b>	<b>0.006</b>	<b>0.007</b>	<b>0.03</b>	0.11
No. species × CO <sub>2</sub> level	0.2	<i>0.07</i>	0.21	0.6	0.63	0.38	0.64	0.58
No. species × N level	0.50	<i>0.09</i>	0.38	0.90	0.28	0.63	0.20	0.32
CO <sub>2</sub> level × N level	0.12	0.13	<i>0.10</i>	<b>0.04</b>	<b>0.04</b>	<b>0.01</b>	<b>0.03</b>	0.71
No. species × CO <sub>2</sub> level × N level	0.72	0.54	0.13	0.30	0.80	0.79	0.73	0.49

Note: *P* values smaller than 0.05 are shown in boldface type, and those smaller than 0.1 are shown in italic type.

of 2004; P. B. Reich, S. E. Hobbie, D. Tilman, J. M. H. Knops, S. Naeem, and D. Wedin, *unpublished data*), DIN leaching remained low, suggesting that the fixed N by the legumes subsidized the non-legume plants with N, as demonstrated by Lee et al. (2003).

Elevated [CO<sub>2</sub>] did not significantly reduce annual DIN leaching, nor did we find a significant interaction with N on annual DIN leaching. However, for specific measurement dates, DIN concentrations in soil solutions did decrease with elevated [CO<sub>2</sub>], particularly when DIN concentrations were high under ambient [CO<sub>2</sub>]. This suggests that, at least under conditions when soil DIN concentrations are high (e.g., at low plant diversity and at high inorganic N input), elevated [CO<sub>2</sub>] is able to reduce DIN leaching. Several studies have shown reduced DIN leaching and increased N retention in soils with elevated [CO<sub>2</sub>] (Niklaus et al. 2001, Johnson et al. 2004, Hagedorn et al. 2005), suggesting that these ecosystems under elevated [CO<sub>2</sub>] were able to more fully exploit and immobilize soil inorganic N.

In contrast to DIN leaching losses, DON losses increased with increased species number. Loss of DON also increased with inorganic N addition as has been observed by others (McDowell et al. 2004, Pregitzer et al. 2004). It has been argued that much of the DON leached below the rooting zone is recalcitrant and largely unavailable for plants and soil microorganisms (Neff et al. 2003). Therefore, changes in biological demand for N caused by increased species number or inorganic N addition should not affect DON leaching.

Increased species number and inorganic N addition may have increased DON leaching because of increased DON production. DON production can be proportional to the production of organic N pools, e.g., because of physical dissolution of litter or SOM (Aitkenhead-Peterson et al. 2002). DON production can also be proportional to the turnover of detrital pools or microbial activity, because DON is produced through decomposition processes (Seely and Lajtha 1997, Trasar-Cepeda et al. 2000). At our site, plant productivity increased significantly with increased plant species richness and with N addition (Reich et al. 2001a, 2004), which may have increased organic N pools in the soil and DON leaching. Gross N mineralization rates also increased with increased species number and with N addition (West et al. 2006), suggesting greater microbial activity and potentially greater DON production, and consequently greater DON leaching. However, when we related annual DON leaching to plant biomass or gross N mineralization rates that have been measured in these plots, we observed only weak positive relationships (with *R*<sup>2</sup> values always smaller than 0.1, data not shown). Other soil or plant characteristics such as root-free soil respiration (West et al. 2006) or plant tissue N concentration (Reich et al. 2006) also showed only weak relationships with DON leaching, suggesting that a combination of factors influenced DON leaching.

The increase in DON leaching with increased species number may partly have stemmed from plant composition effects and differences among species in their

TABLE 5. Mean (and SE) annual water fluxes below 60 cm soil depth and N losses through leaching from 1 May 2003 to 30 April 2004 and from 1 May 2004 to 30 April 2005 in monoculture plots and 16-species plots.

CO <sub>2</sub> level	N level	1 May 2003–30 April 2004		1 May 2004–30 April 2005	
		Water flux (mm)	N leached (g·m <sup>-2</sup> ·yr <sup>-1</sup> )	Water flux (mm)	N leached (g·m <sup>-2</sup> ·yr <sup>-1</sup> )
Monoculture plots					
Ambient	ambient	416 (9)	1.52 (0.39)	570 (14)	2.55 (0.52)
Ambient	elevated	408 (9)	3.97 (0.98)	550 (15)	3.44 (0.59)
Elevated	ambient	426 (10)	1.70 (0.57)	572 (11)	2.36 (0.51)
Elevated	elevated	409 (8)	2.97 (0.82)	545 (13)	2.61 (0.56)
16-species plots					
Ambient	ambient	366	0.51 (0.19)	490	0.65 (0.09)
Ambient	elevated	368	0.64 (0.12)	484	0.60 (0.09)
Elevated	ambient	377	0.38 (0.05)	492	0.51 (0.05)
Elevated	elevated	384	0.46 (0.07)	488	0.47 (0.07)

TABLE 6. ANOVA for the effects of number of species (1 vs. 16), CO<sub>2</sub> level (ambient or elevated), and N level (ambient or elevated) on N losses through leaching from 1 May 2003 to 30 April 2004 and from 1 May 2004 to 30 April 2005.

Factor	1 May 2003–30 April 2004	1 May 2004–30 April 2005
No. species	<b>0.005</b>	<b>0.0001</b>
CO <sub>2</sub> level	0.94	0.38
N level	<b>0.01</b>	0.32
No. species × CO <sub>2</sub> level	0.66	0.73
No. species × N level	0.16	0.57
CO <sub>2</sub> level × N level	0.32	0.57
No. species × CO <sub>2</sub> level × N level	0.86	0.77

Note: P values smaller than 0.05 are shown in boldface.

effects on DON leaching. For instance, the relatively high abundance of *P. pratensis* in the 16-species plots (15% and 19% of aboveground biomass in June and August of 2003 and 22% and 31% in June and August of 2004; P. B. Reich, S. E. Hobbie, D. Tilman, J. M. H. Knops, S. Naeem, and D. Wedin, unpublished data) may have increased DON leaching in the 16-species plots. As

a monoculture, *P. pratensis* caused among the highest DON leaching (Fig. 4), although it was not among the most productive species (Reich et al. 2001b).

Associations between dissolved organic matter and NO<sub>3</sub><sup>-</sup> can form DON (Davidson et al. 2003), potentially increasing DON production and loss in the enriched N plots. This mechanism could potentially increase the ratio between DON and dissolved organic carbon (DOC). However, the DON:DOC ratio was not affected by N fertilization, or by the other treatments in our study.

Regardless of the mechanisms responsible for the increase in DON loss with increased species number and inorganic N addition, our results (particularly in the species-rich plots) show that DON loss can be a significant part of total N loss through leaching. The 16-species plots had DIN leaching losses that were much smaller than the monoculture plots. However, total N leaching in the 16-species plots was dominated by DON, indicating that DON is an important pathway of N leakage in ecosystems where inputs of inorganic N through atmospheric deposition are low and biological demand for N remains high (Hedin et al. 1995, Perakis

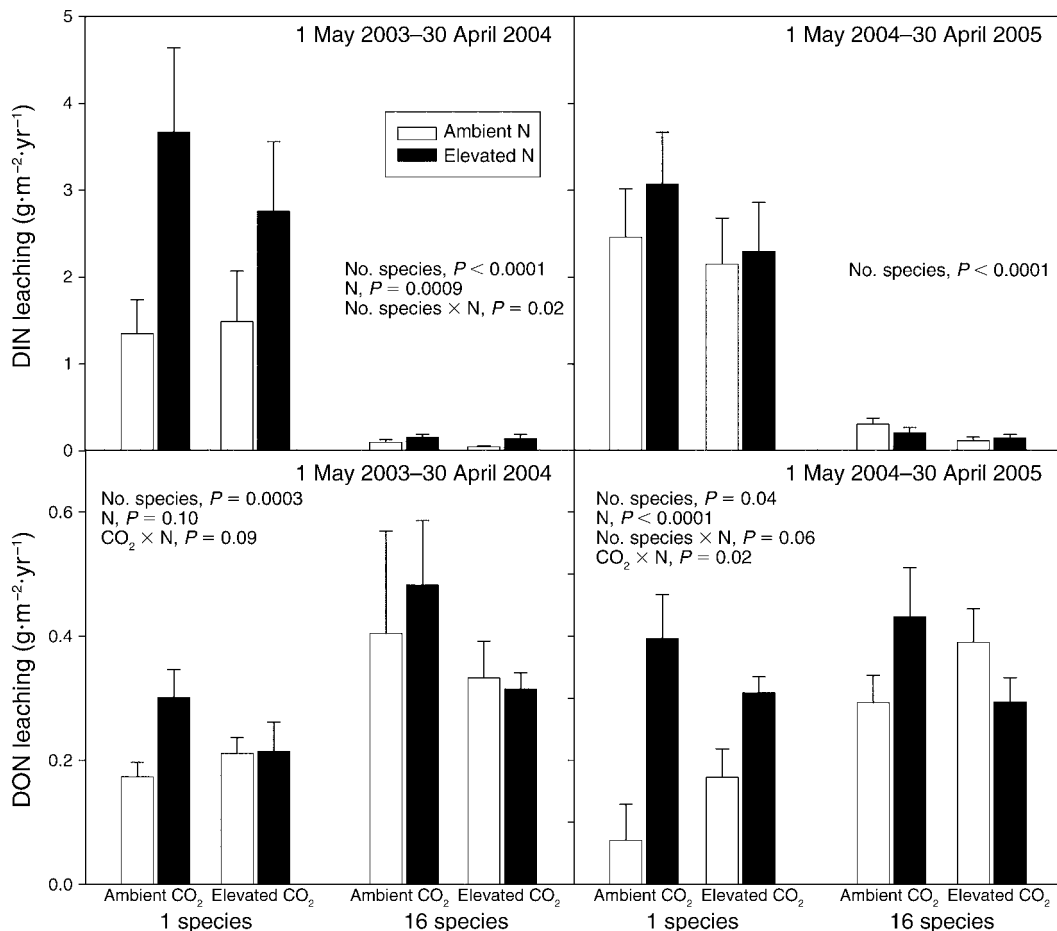


FIG. 3. Annual DIN and DON leaching (mean + SE) during two years of observation.



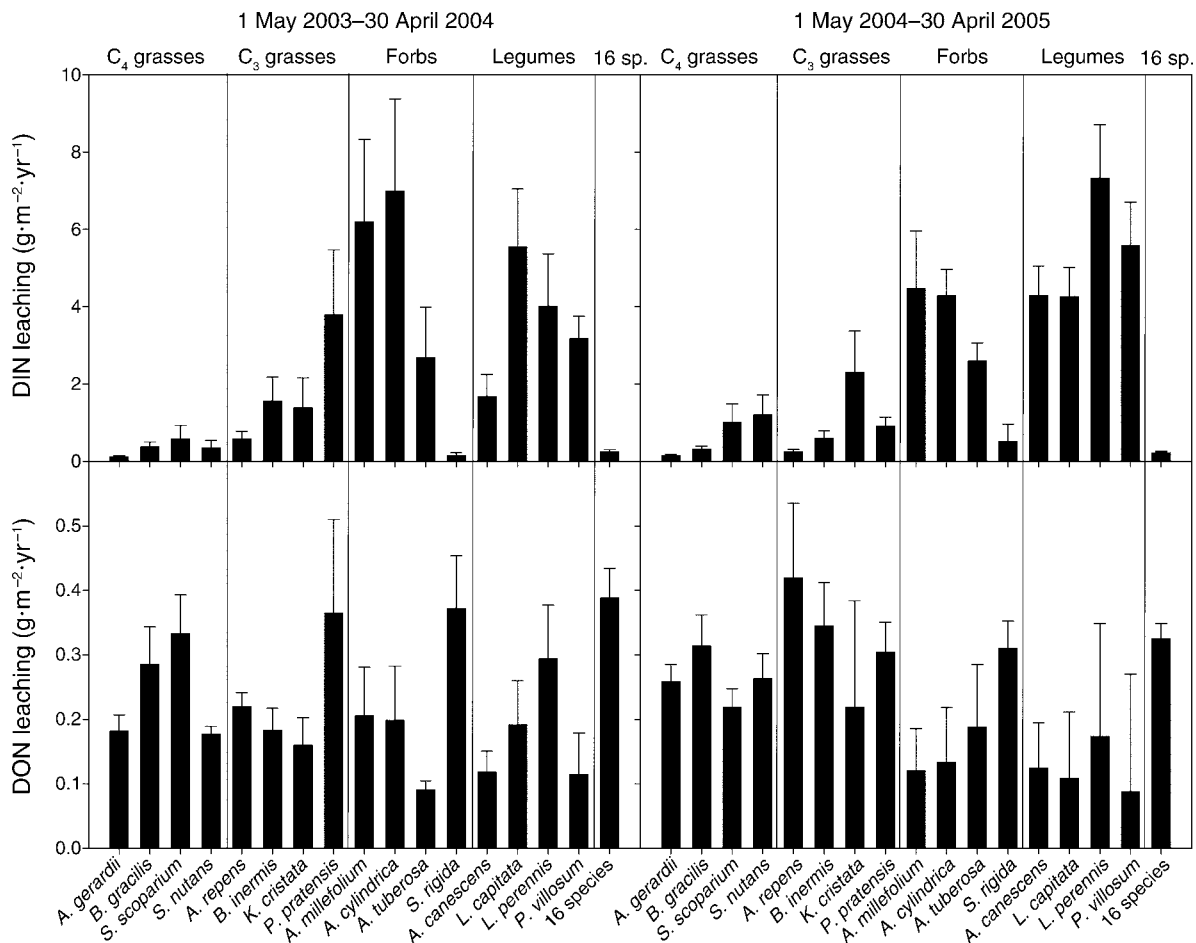


FIG. 4. Annual DIN and DON leaching (mean + SE, pooled across  $\text{CO}_2$  and N treatment) for the 16 grassland species grown as monocultures and for the 16 species grown together. Species are *Andropogon gerardii*, *Bouteloua gracilis*, *Schizachyrium scoparium*, *Sorghastrum nutans*, *Agropyron repens*, *Bromus inermis*, *Koeleria cristata*, *Poa pratensis*, *Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa*, *Solidago rigida*, *Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis*, and *Petalostemum villosum*.

and Hedin 2002). While it has been shown that increased plant species richness enhances inorganic N retention and plant productivity in these grassland systems (Tilman et al. 1996, 1997), our results suggest that in the long-term DON leaching could pose a constraint on plant productivity in species rich communities. The sandy soil (93% sand) at our site may have limited capacity to adsorb DON (e.g., Lajtha et al. 1995), thus promoting high rates of DON leaching. In more sorptive soils N leaching may therefore be less dominated by DON.

DON leaching showed large increases with N addition in the ambient  $\text{CO}_2$  plots, but small or virtually no change with added N in the elevated  $\text{CO}_2$  plots, especially during the second year of observation. These results are hard to explain based on presumed variation among treatments in DON production. Since 2002, the increase in plant productivity with elevated  $[\text{CO}_2]$  was much less without N addition than with N addition among all species-richness treatments (Reich et al. 2006).

Gross N mineralization measured during the summer of 2003 showed no significant  $\text{CO}_2 \times \text{N}$  interaction effect, although gross N mineralization in the 16-species plots did increase with N addition in the ambient  $\text{CO}_2$ , but not in the elevated  $\text{CO}_2$  plots (West et al. 2006). Taken together, these results suggest that at least for the 16-species plots, DON production was more controlled by microbial turnover than by organic matter production. Factors other than organic matter processing or litter production may affect DON production. For instance, changes in chemical characteristics of litter inputs and in microbial communities caused by N addition and elevated  $[\text{CO}_2]$  could potentially alter DON production and loss (Park et al. 2002, Smolander and Kitunen 2002, Yano et al. 2005), although very little is known about these potential effects. Nevertheless, the persistent  $\text{CO}_2 \times \text{N}$  interaction on DON concentrations and leaching suggests that greater losses of DON in this grassland system in response to atmospheric N deposition inputs are tempered by increasing atmospheric  $[\text{CO}_2]$ .

Treatment effects on N loss through leaching were largely caused by their effects on N concentrations in the soil solution rather than by their effects on water drainage. Relative differences in water drainage below 60 cm soil depth were much smaller (up to 14% difference between monoculture and 16-species plots) than for soil solution N concentrations among treatments. Changes in plant productivity and water use efficiency caused by increased plant species richness, N addition, and elevated [CO<sub>2</sub>] have been shown to significantly alter soil moisture content and water drainage (Hungate et al. 2002, Schafer et al. 2002, Scherer-Lorenzen et al. 2003). However, the soil at our site is very sandy (93% sand) with low water-holding capacity, which strongly reduced biotic effects on water drainage.

#### CONCLUSIONS

We observed much greater DIN leaching in monoculture than in diverse plots, particularly with inorganic N addition. Therefore, in grassland systems such as the one studied here, loss of plant species richness could lead to large increases in DIN leaching, especially under elevated N deposition, which both increases N availability and often reduces plant diversity (Bobbink et al. 1998, Stevens et al. 2004). Thus, declines in diversity with N deposition could accelerate the process of N saturation in grassland systems. Elevated [CO<sub>2</sub>] only influenced DIN leaching when such losses were high, suggesting that this could be important in conditions and situations where N losses are high. We have shown that DON leaching increased with increased plant species richness, and more so under elevated N deposition, likely because of greater litter production and microbial processing of soil organic N. Consequently, DON became the dominant form of N leaching in the more plant diverse plots. We further found that the increase in DON leaching with inorganic N addition decreased with elevated [CO<sub>2</sub>] for reasons that are unclear. These relatively large changes in annual DIN and DON leaching caused by plant species richness, N addition and elevated [CO<sub>2</sub>] are small compared to total soil N pools (~370 g N/m<sup>2</sup> in the top 60 cm of the soil), but we argue that they could significantly alter long-term N retention and ecosystem C sequestration.

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