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Ecological mechanisms associated with the positive diversity–productivity relationship in an N-limited grassland

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Abstract. In a 13-year grassland biodiversity experiment in Minnesota, USA, we addressed two main questions: What set of ecological mechanisms caused aboveground productivity to become ~340% greater in highly diverse plant mixtures than in the average monoculture? Why did the effect of diversity on productivity become so much stronger through time? Because our grassland system is N limited, we simultaneously measured critical variables associated with the storage and cycling of this element, such as plant and soil N pools, soil N availability, soil N mineralization rates, and plant N-use efficiency, as well as the initial soil N concentration of each diversity plot when the experiment was established in 1994. We used linear and multiple regression analyses to test for potential effects of these variables on aboveground productivity and to address whether and how such variables were in turn affected by plant species diversity and functional composition across years and also at different time intervals within the same year.

We found that seven variables simultaneously controlled productivity: (1) initial total soil nitrogen (N) of each plot, (2) diversity-dependent increases in total soil N through time, (3) soil N mineralization rates, (4) soil nitrate (NO_3^-) utilization, (5) increases in plant N-use efficiency at greater plant diversity, (6) legume presence, and (7) higher species numbers. The surprising continued significance of higher plant diversity may occur because of its effects on seasonal capture of soil NO_3^- and moisture and on the accumulation of root-N pools, all of which may have also increased productivity through time at higher species numbers.

Key words: C_3 grasses; C_4 grasses; facilitation effects; fine roots; forbs; functional complementarity; functional groups; legumes; selection effects; soil nitrogen mineralization.

INTRODUCTION

Biodiversity research over the past decade has shown that primary productivity increases with plant species number in many experimental grassland systems (Tilman et al. 1996, Hector et al. 1999, Loreau et al. 2001, Hooper et al. 2005, Spehn et al. 2005, van Ruijven and Berendse 2005) and that this effect of diversity becomes stronger through time (Cardinale et al. 2007, Fargione et al. 2007). There is increasing evidence that complementarity between plant species and/or functional groups may be a cause of these diversity effects (Tilman et al. 2001, Spehn et al. 2005, van Ruijven and Berendse 2005, Cardinale et al. 2007, Fargione et al. 2007) but that selection and facilitation effects may also contribute (Huston 1997, Wardle 1999, Roscher et al. 2005, Cardinale et al. 2006). Here we focus on a related issue that has received much less attention—the proximate ecological processes and mechanisms associated with both (1) the positive effect of plant diversity on

productivity and (2) the long-term increase in the magnitude of this effect.

Aboveground productivity in many grassland systems is influenced by different environmental variables such as climate, soil type, and disturbance regimes (e.g., fire, grazing, trampling, and so forth) which interact to affect the number and type of resources available (e.g., nitrogen, phosphorus, water, and others) for plant growth. Because soil N availability limits productivity in many terrestrial systems (Vitousek and Howarth 1991) and because the system in which we work is N limited, we focus on this limiting resource. Four mechanisms associated with the use and retention of N have been proposed so far. The first mechanism hypothesizes that more diverse plant assemblages are more productive because they use available forms of soil N (i.e., NH_4^+ and NO_3^-) more completely than less diverse assemblages (Tilman et al. 1996, Hooper and Vitousek 1997, Hector et al. 2002, Hooper et al. 2005, Spehn et al. 2005). A signature of this effect is that growing-season average concentrations in the rooting zone of soil NH_4^+ and NO_3^- should be lower at higher plant diversity. This could occur if different plant species or functional groups have different rooting depths, different phenologies of nutrient uptake, different abilities to exploit pulses of nutrients, and so on

(Hooper and Vitousek 1997). It is not clear, however, how many functional groups may significantly contribute to reduce NO_3^- concentrations and how they affect N supply/use seasonally.

The second mechanism proposes that higher diversity indirectly increases rates of ecosystem N cycling through positive effects on the microbial community (Zak et al. 2003). The authors used ^{15}N isotope-pool dilution techniques to show that higher numbers of species enhanced the microbial release of NH_4^+ from soil organic matter and caused greater gross N-mineralization rates which, surprisingly, were not significantly associated with the presence of particular plant functional groups. The lack of a plant functional effect may have different explanations, including the fact that pool dilution techniques may not fully be able to assess the actual rate at which NH_4^+ ions become available for plant–microbe competition (see Schimel and Bennett 2004). Also, because NH_4^+ readily adsorbs to the negatively charged surfaces of soil minerals and organic matter (Chapin et al. 2002), it is likely that estimates of bioavailable NH_4^+ are strongly influenced by the size of the soil-organic-matter pool (Booth et al. 2005), which is greater at higher species diversity (Fornara and Tilman 2008). In this study we measured net soil N-mineralization rates, being aware that this represents an *indirect* index related to the spatial/temporal availability of N for plant uptake (Schimel and Bennett 2004). However, because the presence of legume species has strong positive effects on nitrification (i.e., the conversion of NH_4^+ to NO_3^-; Scherer-Lorenzen et al. 2003), we expect that soil NO_3^- accumulation rates in plant assemblages containing legumes will have significant effects on the N uptake by non-N-fixing plants and in turn on aboveground productivity.

The third mechanism suggests that some plant species may increase their N-use efficiency at higher diversity by producing biomass with higher C:N ratios than those produced when growing in monocultures (van Ruijven and Berendse 2005, Fargione et al. 2007). Such a mechanism may operate in response to stronger competition for light in highly diverse communities and force some species to invest in height (Spehn et al. 2000) by producing higher C:N ratio stem tissues versus low C:N ratio leaf tissues (van Ruijven and Berendse 2005).

The fourth mechanism hypothesizes that higher productivity at higher diversity results from greater plant accumulation of tissue N pools (Hector et al. 1999, Spehn et al. 2005, Fargione et al. 2007) at higher diversity. In particular because roots are the perenniating organs of herbaceous perennial plant species, the ability of roots to acquire and retain N year after year is likely to be associated with primary productivity, but some other mechanisms must be invoked to explain why such N stores would increase with diversity (Mulder et al. 2002).

These four and other potential mechanisms that affect the supply, use, and storage of N are probably associated with complementary, selection, and facilitation effects but have never been simultaneously addressed before. Our understanding of potential diversity–productivity relationships could improve if we simultaneously address multiple mechanisms associated with the storage and cycling of key limiting resources (e.g., nitrogen), including spatial and temporal variation in resource supply and use along gradients of plant diversity. We therefore ask (1) What set of ecological mechanisms associated with complementarity, selection, and/or facilitation is responsible for greater productivity at higher species numbers? And (2) how do these mechanisms contribute to enhance productivity through time at higher species diversity? To address these questions, we used data spanning the 13 years of the biodiversity study and also measured other parameters and processes associated with belowground N dynamics including the initial total soil N in 1994 (from archived soils) and its change through time.

METHODS

We collected data from a large biodiversity experiment established in 1994 at the Cedar Creek Ecosystem Science Reserve (formerly Natural History Area), Minnesota, USA (Tilman et al. 1997, 2001). Climate is continental with long, cold winters and warm, humid summers. Mean annual temperature is ~6°C; total annual precipitation averages 650–680 mm. The soil is a well-drained Zimmerman fine sand poor in nitrogen that developed from a glacial outwash deposited ~14 000 years ago (Grigal et al. 1974). Agriculture that began in the late 1800s to early 1900s was often abandoned as soils became less fertile, and such fields have been colonized by native prairie species, some of which were used in the biodiversity experiment. We utilized the 152 experimental plots dominated by herbaceous perennials (Tilman et al. 2006). Each plot was 9×9 m and seeded to contain 1, 2, 4, 8, or 16 grassland and savanna species. Plant species were randomly chosen from a pool of 18 species including four C_4 grasses (*Panicum virgatum*, *Andropogon gerardi*, *Schizachyrium scoparium*, *Sorghastrum nutans*), four C_3 grasses (*Poa pratensis*, *Agropyron smithii*, *Elymus canadensis*, *Koeleria cristata*), four legumes (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis*, *Petalostimum purpureum*), four non-legume forbs (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis*, *Petalostimum purpureum*), and two woody-savanna species (*Quercus ellipsoidalis*, *Quercus macrocarpa*). This resulted in more than 20 replicates at each level of species diversity. Plot composition was maintained by weeding (three or four times annually) and by burning each year in spring before growth began. Of the original 168 plots in this experiment, some have never carried fire, especially those planted with just woody species, just forbs, or with low diversity mixtures of woody species

and forbs. Because of this, these plots are not included in our analyses, and we analyzed data of 152 plots in total.

Plots were sampled for annual production of aboveground biomass by clipping, drying, and weighing four parallel and evenly spaced 0.1 × 3.0 m vegetation strips per plot in 1996, 1997, 1998, and four 0.1 × 6.0 m strips in 2002, 2003, 2004, 2005, and 2006. Aboveground living plant biomass was collected in mid-August for all years and was considered a good estimate of annual primary productivity because spring burning left bare soil each year. Plots were also sampled for belowground biomass using twelve soil cores per plot, 5 cm diameter each, 0–30 cm deep in 1998, 2002, 2004, and 2006, collected in mid-August from the same vegetation strips. In 2006, roots were also collected between the 30–60 cm soil depth increment. All belowground samples were dried and weighed after any soil residual had been removed. Aboveground and belowground plant samples were ground and analyzed for total C and N following standard methods on a 1500 NA Carlo-Erba element analyzer (CE Elantech, Lakewood, New Jersey, USA).

Soil C and N samples were collected in 1994 and 2006 at 0–20 and 20–40 and 40–60 cm soil depth increments for each of nine sites per plot. Soil was analyzed for total C and N by combustion and gas chromatography (COSTECH Analytical ECS 4010 instrument; COS-TECH, Valencia, California, USA). An additional set of archived soil samples collected before planting in 1994 between 0 and 20 cm soil depth was also analyzed in 2006 in a similar way to be used as independent estimate of initial soil N and C concentrations (see *Results*).

Net soil N-mineralization rates were estimated in July 2007 with an aerobic lab incubation. Soil samples were collected to 20 cm soil depth from three sites within each of the 152 plots. Samples from a plot were mixed. A subsample was extracted with 1 mol/L KCl, shaken for 0.5 h, settled overnight at 4°C, and analyzed for NH_4^+-N and NO_3^--N with a Bran-Luebbe AA3 auto analyzer (Mequon, Wisconsin, USA). An additional 25-g sub-sample for each plot was incubated for 30 days in a dark room at 22°C after roots were sieved out and water was added to each sample to reach field moisture capacity (9%). Samples were checked after two weeks of incubation and water was added if necessary to keep moisture constant. After 30 days, soil samples were extracted and analyzed for NH_4^+-N and NO_3^--N in the same way. To determine net N mineralization rates, initial extractable concentrations of NH_4^+ and NO_3^- were subtracted from final extractable concentrations at the end of the incubation (i.e., net mineralization rates = net ammonification + net nitrification).

To determine the levels to which plants reduced available soil N, soil NO_3^- concentrations to 20 cm soil depth were measured in July 2007 by collecting an additional set of soil samples (three samples per plot) one week earlier than the soil sampling for the lab incubation.

To estimate seasonal variation in soil NO_3^- concentrations and soil water content (i.e., soil moisture) as a function of plant diversity, we collected soil samples between 0 and 10 cm, 10 and 20 cm, and 20 and 40 cm soil depth from May to October 2001 (samples were collected from each plot twice every month). Soil NO_3^- concentrations were measured as previously described, and soil water content was estimated from wet and dry soil subsamples which were oven dried at 55°C for three days.

Finally, root production was measured in 152 plots during 2007 by using ingrowth soil cores. We removed roots from a soil volume of 251.2 cm^3 which was collected at three different soil depths (0–20, 20–40, and 40–60 cm deep) in two sites per plot by using a metallic cylinder corer. Soil cores were extracted mid-May 2007 and roots were sieved and removed from the soil samples. A hardware mesh wire (1 cm diameter) was shaped to fit into the hole until reaching a soil depth of 30 cm, then the root-free soil was returned to the hole from which it was collected. After two months (mid-July 2007) soil samples were extracted in the same place by coring within the mesh wire. New ingrown roots were sieved, dried, and weighed. Root values for 2007 were averaged with root values obtained from a previous root ingrowth measurement carried out in the same plots in 2006 (Fornara and Tilman 2008) to better include potential seasonal variations in root production.

DATA ANALYSIS

Linear and multiple regression analyses were performed to address potential relationships between aboveground productivity and plant diversity. Plant diversity was expressed either as number of species seeded in each plot or as the presence/absence of four plant functional groups among C_3 grasses, C_4 grasses, legumes, and non-leguminous forbs in each plot. To quantify how the productivity of each plot changed through time during this experiment, we calculated the annual rate of change in aboveground biomass (r). To do this, we used the mean biomass at the start of the experiment (plot means across 1996, 1997, and 1998) and for the last three years of the experiment (plot means across 2004, 2005, and 2006), with r being defined as follows:

$$r = \log_e(\text{mean biomass}_{end}/\text{mean biomass}_{start})/t$$

where t, time, is 8 years. This gives, for each plot, the rate of change in its aboveground biomass with units of yr^{-1}. The effects of plant diversity and different variables related to belowground N dynamics (e.g., net soil N mineralization, soil NO_3^- concentrations, changes in soil and root N concentration over years, initial soil N, and so on) on aboveground biomass production and rates of change of biomass through time were tested either individually or simultaneously in type III multiple regressions, which are highly conservative. We also performed multivariate analysis of variance (MANO-

VA) to test for the effects of plant diversity and functional composition on seasonal variations in soil nitrate availability and in soil moisture. Some soil variables included in our analyses were not measured within the same soil-depth interval across years. This is partly because in early stages of the biodiversity experiment, sampling was done only in shallower soils and also because root and soil samples were collected following different protocols (i.e., metal soil cores with different diameters were used for root or soil sampling and at different soil depths). However, for each variable in our analyses, we only used data that were comparably collected within the same soil-depth interval across seasons or years. Data were analyzed using JMP v. 6.0.2 (SAS Institute 2006).

<center>RESULTS</center>

Plant diversity effects on aboveground productivity

We found that mean aboveground biomass production for the last three years of this experiment (2004, 2005, and 2006) and the annual instantaneous rate of change in aboveground biomass through years both were positively and significantly dependent on species number (Fig. 1a, b). The 16-species plots were 341% (328 ± 11.5 g/m^2; mean $\pm$ SE) more productive on average than the monoculture plots (96.8 ± 14.7 g/m^2; mean $\pm$ SE). A multiple regression including the presence/absence of the four plant functional groups showed that the presence of legumes, forbs, and C_4 grasses had significant positive effects on total aboveground biomass averaged for 2004, 2005, and 2006 (Table 1). We found that the annual instantaneous rate of change in aboveground biomass was significantly and positively affected by the presence of legumes and forbs and negatively affected by the presence of C_4 grasses (Table 1). However, we found that a legume × C_4 grass interaction positively affected the annual instantaneous rate of change in aboveground biomass ($F_{1,147} = 11.2$, $P = 0.001$).

Belowground N dynamics and aboveground productivity

Because belowground N dynamics are likely to affect community productivity in our N-limited system (Tilman et al. 1996, Wedin and Tilman 1996), we first addressed the effects of N supply, plant N uptake, and changes in soil/root N through time on aboveground productivity. We found that net soil N mineralization rates as measured in the laboratory incubation had a significant positive effect on aboveground productivity (Fig. 2a). A multiple regression with the presence/absence of four plant functional groups (C_3 grasses, C_4 grasses, legumes, and forbs) showed that net N-mineralization rates were strongly positively affected by the presence of legumes and strongly negatively affected by the presence of C_4 grasses (Table 1). We found that species number had a significant positive effect on net soil N-mineralization rates ($F_{1,142} = 5.17$, P
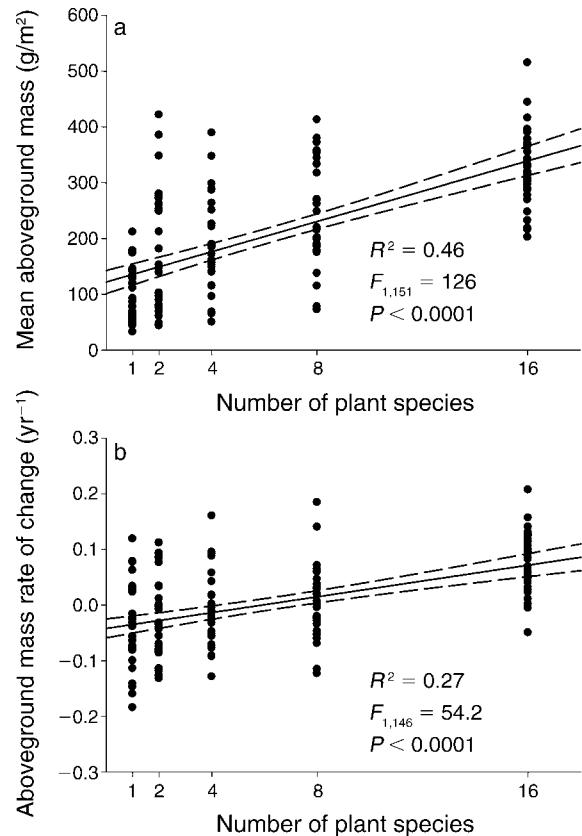

FIG. 1. Dependence of (a) aboveground biomass production as averaged for three recent years (2004, 2005, and 2006) and (b) the instantaneous rate of change in aboveground biomass on the number of species planted in each plot within the 13-year-long grassland biodiversity experiment. The solid line shows the mean, while the dotted lines show the 95% confidence interval.

$= 0.024$) only when seven legume monocultures were not included in our analyses.

A separate linear regression showed that mean aboveground biomass (averaged for 2004, 2005, and 2006) was positively affected by net change in total soil percentage N concentration after 12 years (1994–2006) to 40 cm soil depth (Fig. 2b). A multiple regression showed that net change in total soil percentage N over 12 years was positively affected by the presence of legumes (Table 1). A linear regression also showed that the number of species had a significant effect on net change in total soil percentage N over 12 years ($F_{1,151} = 13.8$, $R^2 = 0.09$, $P = 0.0003$).

In a separate linear regression we found that net change in root percentage N over 8 years (1998–2006) had a positive significant effect on aboveground biomass production (Fig. 2c). A multiple regression showed that only legume presence and C_3 grasses contributed to significantly increase root percentage N over 8 years (Table 1). In a separate linear regression we also found that the number of species had a positive significant

TABLE 1. Dependence of different ecosystem variables on the presence or absence of legumes, C_3 grasses, C_4 grasses, and forbs in each plot as determined by eight separate multiple regressions (one for each response variable).

| Response variable | Regression parameters for presence of each functional group | | | | | Overall R^2 | Overall F value (df = 4, 151) |
	Intercept	Legumes	C_3 grasses	C_4 grasses	Forbs		
Mean aboveground mass (g/m^2)	166****	75.6****	3.49^{NS}	16.9**	30****	0.55	44.2****
Aboveground mass rate of change (yr^{-1})	0.0009*	0.025****	0.0008^{NS}	−0.01*	0.025****	0.37	20.6****
Net soil N-mineralization rates (mg/kg)	2.18****	0.65****	0.03^{NS}	−0.72****	0.037^{NS}	0.42	26.8****
Net change in total soil N for 12 years (%)	0.001**	0.002**	0.0007^{NS}	0.001^{NS}	0.00008^{NS}	0.10	3.85**
Net change in total root N for 8 years (%)	0.45****	0.12****	0.07**	0.03^{NS}	0.0005^{NS}	0.26	13.1****
Soil NO_3^- concentrations (mg/kg)	0.21****	0.024*	−0.025*	−0.07****	−0.028*	0.30	16****
Total root-N pool after 12 years (g/m^2)	9.41****	4.68****	1.16**	1.96****	0.72^{NS}	0.54	43.1****
Fine-root production for two years (g/m^2)	135****	36.1****	7.4^{NS}	23.8**	25.2**	0.27	13.5****

Notes: Mean aboveground biomass production was averaged for three recent years (2004, 2005, and 2006); net soil N mineralization rates were measured between 0 and 20 cm soil depth in July 2007 in a lab incubation; net change in total soil percentage N to 40 cm soil depth was calculated between 1994 and 2006; net change in total root percentage N was measured between 1998 and 2006 on roots collected between 0 and 30 cm soil depth; net soil NO_3^- concentrations were measured between 0 and 20 cm soil depth in the field during July 2007; initial soil percentage N concentrations to 20 cm soil depth in 1994 were measured on a separate set of archived soil samples; total root-N pool was calculated in 2006 to 60 cm soil depth; fine root production was measured between August–October 2006 and May–July 2007 to 60 cm soil depth.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; NS = not significant.
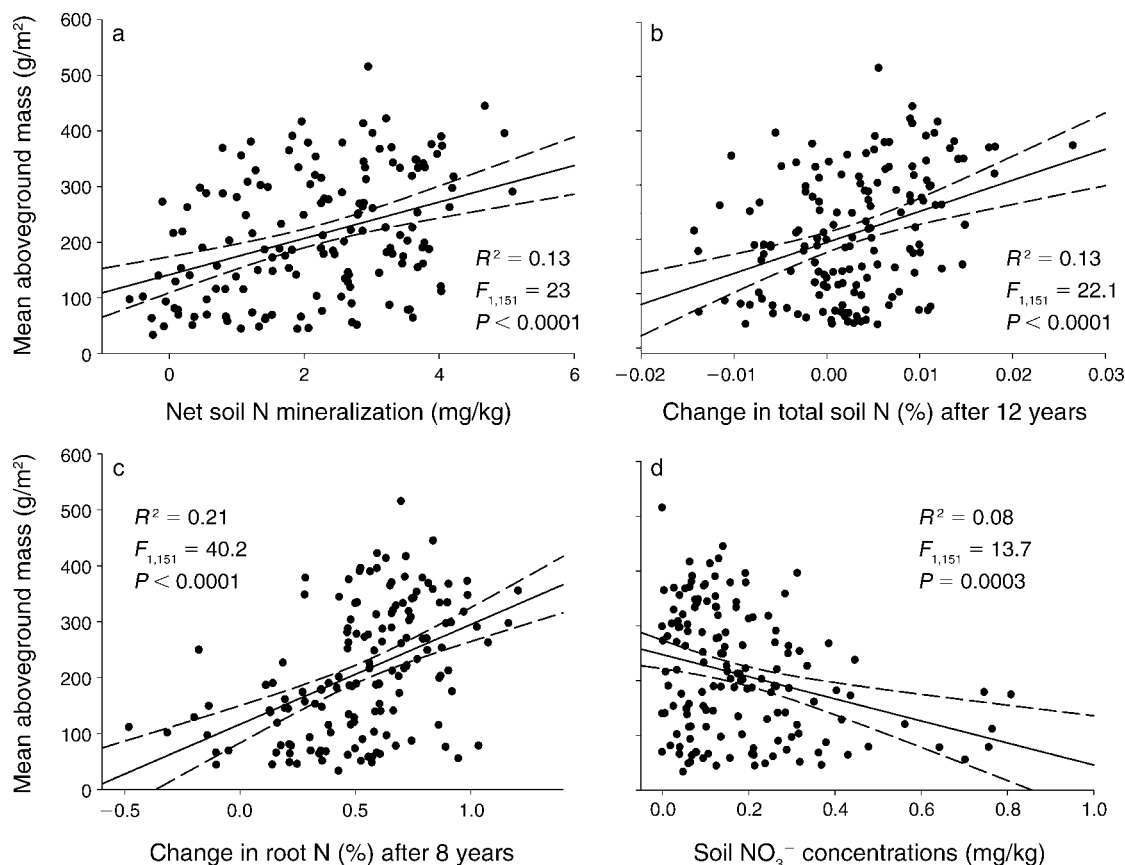

FIG. 2. Dependence of aboveground biomass production (a) on net soil-N-mineralization rate as measured between 0 and 20 cm soil depth in July 2007 in lab incubation, (b) on net change in total soil percentage N to 40 cm soil depth after 12 years, (c) on net change in root percentage N between 0–30 cm soil depth after 8 years, and (d) on soil NO_3^- concentrations between 0 and 20 cm soil depth as measured in the field in July 2007. The solid line shows the mean, while the dotted lines show the 95% confidence interval.

TABLE 2. Results of a multiple regression analysis showing the dependence of mean aboveground biomass production for three recent years (2004, 2005, and 2006).

Regression parameters	Response variable: mean aboveground biomass for 3 years (g/m²)†			
	Estimate	SE	$F_{1,151}$	P
Net soil N mineralization rates (0–20 cm soil depth; mg/kg)	6.5	4.58	33.5	<0.0001
Net change (%) in total soil N for 12 years (0–40 cm soil depth)	2107	712	8.75	0.0036
Soil NO_3^- concentrations (0–20 cm soil depth; mg/kg)	−139.7	37.8	13.6	0.0003
Initial soil N (%) in 1994 (0–20 cm soil depth)	1882	467	16.2	<0.0001
Mean aboveground C:N ratio for 3 years	3.07	0.74	16.8	<0.0001
Legume presence or absence	67.4	8.18	67.9	<0.0001
Number of plant species	6.02	1.03	33.8	<0.0001

Notes: The regression tested for effects of (1) net soil N mineralization rates measured between 0 and 20 cm soil depth in July 2007 in a lab incubation, (2) net change in total soil percentage N to 40 cm soil depth after 12 years, (3) soil NO_3^- concentrations between 0 and 20 cm soil depth as measured in the field in July 2007, (4) initial soil percentage N concentrations to 20 cm soil depth in 1994 as measured on a separate set of archived soil samples, (5) mean aboveground mass C:N ratio as averaged for three years 2004, 2005, and 2006, (6) the presence of legume species in each plot, and (7) the number of species in each plot. The multiple regression results refer to the final accepted model which only included the effects of the significant variables.

† Intercept estimate = −91.12, SE = 39.8, t ratio = −2.28, P = 0.0238. Total $F_{7,151}$ = 62.7, R^2 = 0.78, P < 0.0001.

effect on net root percentage N change across years ($F_{1,151} = 35.2$, $R^2 = 0.19$, $P < 0.0001$).

A further linear regression showed that aboveground biomass productivity was greater in plots in which soil NO_3^-, measured between 0 and 20 cm soil depth in 2007 during the peak season of plant growth (i.e., July), had been reduced to lower levels (estimate = −199, $F_{1,151} = 13.7$, $R^2 = 0.08$, $P < 0.0001$; Fig. 2d). We found that the presence of C_4 grasses, C_3 grasses, and forbs, but not the presence of legumes, all contributed to decrease soil NO_3^- concentrations (Table 1). Two interactions such as C_4 grasses × forbs ($P < 0.0001$) and C_4 × C_3 grasses ($P = 0.0022$) also decreased soil NO_3^- concentrations. Finally, we found that the number of species seeded in each plot significantly contributed to decrease available soil NO_3^- concentrations ($F_{1,151} = 17.4$, $R^2 = 0.10$, $P < 0.0001$).

Simultaneous effects of belowground N dynamics and diversity on aboveground productivity

We performed a multiple regression analysis including as independent variables the (1) net rate of soil N mineralization (from the lab incubation), (2) net change in total soil percentage N concentration after 12 years, (3) net change in root percentage N after 8 years, (4) mean soil NO_3^- concentrations as measured in July 2007, and (5) the initial soil percentage N concentration to 20 cm soil depth as measured from the archived soil samples collected in 1994 (this was to test the potential effects of the history of the site where the large biodiversity experiment was established on productivity). We found that each of these variables significantly contributed to higher aboveground biomass productivity ($P < 0.0001$ for net N mineralization, $P = 0.014$ for net change in total soil N, $P < 0.0001$ for net change in root N, $P < 0.0001$ for soil NO_3^- concentrations, and $P = 0.01$ for initial soil N), explaining 53% of the overall variability ($F_{5,150} = 32.4$, $R^2 = 0.53$, $P < 0.0001$).

We then asked whether other additional variables hypothesized to be of importance might also significantly explain aboveground productivity when added to the previous five independent variables in a multiple regression. We added the C:N ratio values of aboveground biomass because the third mechanism invoked to explain aboveground productivity (see *Introduction*) suggests that plant species may increase plant structural parts (e.g., plant stems) in order to compete for light at higher species diversity, therefore increasing the C:N ratio of aboveground biomass. We also added the number of species and presence/absence of particular plant functional groups to address whether these components of diversity might still explain some variability in aboveground productivity after we controlled for belowground N dynamics and aboveground C:N ratio. We found that net root percentage N change (estimate = 14.9%, $F_{11,151} = 0.71$, $P = 0.39$), the presence of C_4 grasses (estimate = 2.09, $F_{11,151} = 0.04$, $P = 0.83$), of C_3 grasses (estimate = 12.04, $F_{11,151} = 1.83$, $P = 0.17$), and of forbs (estimate = 18.5, $F_{11,151} = 3.77$, $P = 0.06$) were not significant in a multiple regression that also included other components of diversity and of belowground N dynamics (Table 2). However, each of the other seven variables included in a type III multiple regression had significant effects on aboveground biomass production (Table 2), and all together explained 78% of the overall variability associated with aboveground productivity (Table 2). This suggests that the number of species in each plot and the presence of legumes (but not the presence of C_3 grasses, C_4 grasses, and forbs) have additional effects other than those summarized by their effects on the overall N supply, on N use, and on changes in soil and root N through time.

One of these effects associated with species number may involve the seasonal availability of NO_3^- as well as changes in soil moisture, which may in turn affect belowground N dynamics. Indeed, results from a multivariate analysis of variance (MANOVA) show a
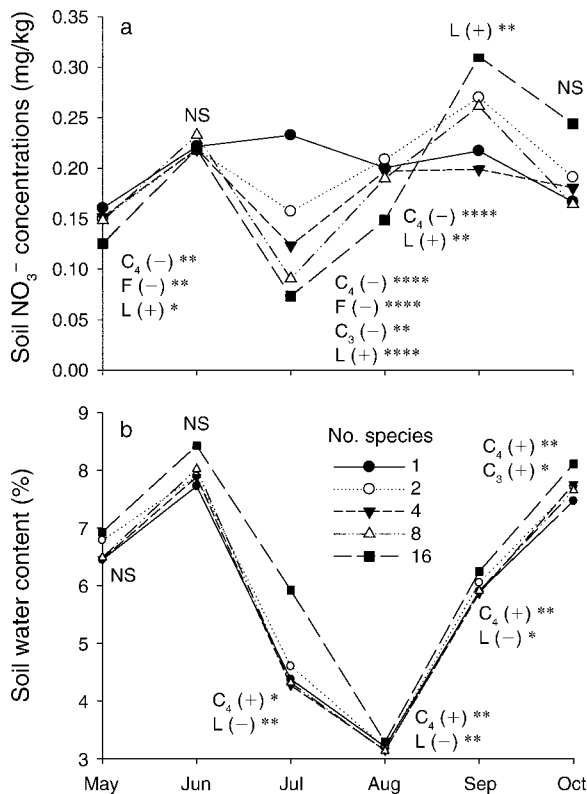
FIG. 3. (a) Dependence of seasonal soil NO_3^- concentrations and (b) soil water content measured between 0 and 40 cm soil depth throughout year 2001 on the number of species. Functional-group effects due to the presence/absence of legume (L), C_3 grass, C_4 grass, and forb (F) species were determined by six separate multiple regressions, one for each monthly measurement. Positive and negative signs (+ and −) indicate significant positive and negative effects of each plant functional group.

* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; NS, not significant.

significant species number × time interaction ($P < 0.0001$; see Fig. 3a) on the seasonal variation in soil NO_3^- concentrations in the rooting zone to 40 cm soil depth across the year 2001. Moreover, we found that the presence of different functional groups had both positive (legumes) and negative effects (C_4 grasses, C_3 grasses, and forbs) on soil NO_3^- concentrations throughout 2001 (see Fig. 3a). Similarly, we found a significant species number × time interaction on the seasonal changes in soil water content ($P < 0.0031$; see Fig. 3b) with the high-diversity plots having on average a greater soil moisture than lower diversity plots during most of the year. Finally we found that legumes had significant negative effects on soil moisture whereas C_4 grasses (and C_3 grasses) had significant positive effects on soil moisture measured between 0 and 40 cm soil depth (Fig. 3b).

We found that soil moisture as measured in July 2007 to 20 cm soil depth and soil NO_3^- concentrations were negatively related ($P = 0.004$), which suggests that increased soil water content may facilitate plant NO_3^- uptake. We also found that soil moisture was strongly

positively affected by total soil percentage C to 20 cm soil depth as measured in 2006 ($F_{1, 151} = 183.3$, $R^2 = 0.55$, $P < 0.0001$), which in turn was positively affected by the number of species ($P = 0.002$).

The number of species or the presence of legumes may also have strong effects on the accumulation of root N which may then represent an important nutrient pool to sustain productivity through time. In separate linear regressions we found that either species number or the presence of legumes had significant positive effects on total root-N pool ($P < 0.0001$ for both analyses). In a multiple regression with the presence/absence of the four plant functional groups, we found that C_4 grasses, C_3 grasses, and legumes all contributed to increase root-N pool after 12 years (Table 1). Total root-N pool had significant positive effects on the instantaneous rate of change in biomass over years (Fig. 4a), a faster increase in aboveground productivity was associated with greater root-N pools. Moreover the root-N pool increased more significantly at higher than lower species number throughout years (Fig. 4b), which suggests a greater ability to store and retain nutrients in plant below-ground tissues at higher species diversity. In fact, in a multiple regression, which included the presence/absence of legume species and the number of species in each plot, we found that both variables significantly and positively enhanced root-N pool across years ($P < 0.0001$ for both predictor variables).

Finally, a linear regression showed that mean fine-root production as measured between 2006 and 2007 was positively affected by the number of species ($F_{1, 151} = 49.3$, $R^2 = 0.25$, $P < 0.0001$) while a multiple regression with the presence/absence of the four functional groups showed that fine-root production was positively affected by the presence of C_4 grasses, forbs, and especially legumes (Table 1).

DISCUSSION

Thirteen years of data from a grassland biodiversity experiment shows that both mean aboveground productivity and its annual rate of change (increase) were positively affected by species number (Fig. 1a, b). This was caused by the positive effects of the presence of several different plant functional groups on below-ground N dynamics, on seasonal changes in soil nitrate and water, and on root N storage throughout the experiment. We also found that mean aboveground productivity measured in three recent years of the diversity experiment (2004, 2005, and 2006) was still positively affected by initial soil total N in 1994. This shows that there are long-term effects of initial differences in soil fertility, likely because of slow rates of N mineralization from the recalcitrant forms of organic matter in soil.

As suggested by previous studies (Tilman et al. 1996, Hooper and Vitousek 1997, Hector 2002, Hooper et al. 2005, Spehn et al. 2005), we found that overall soil NO_3^- concentrations in the rooting zone were significantly
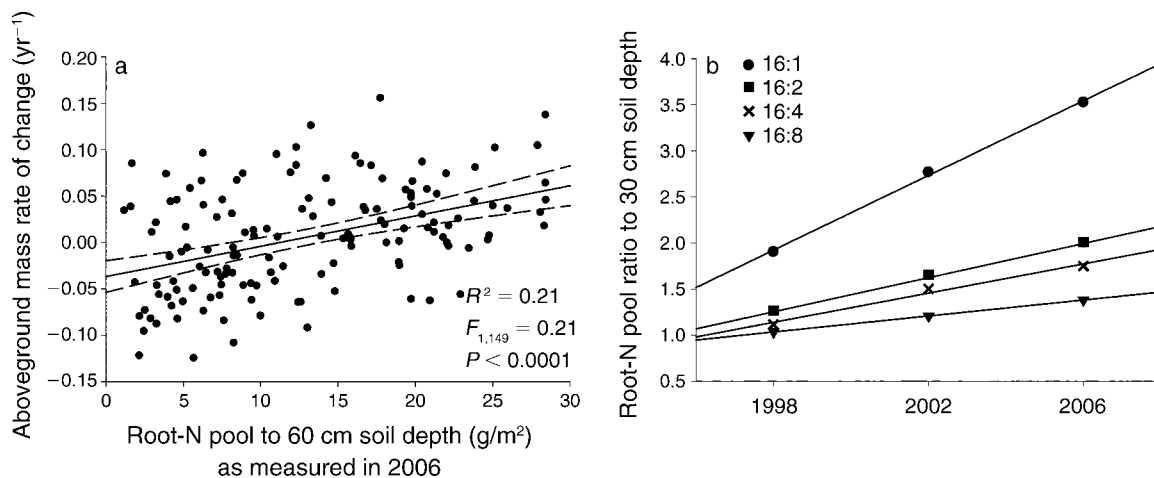
FIG. 4. (a) Dependence of the instantaneous rate of change in aboveground biomass on total root-N pool to 60 cm soil depth as measured in 2006 and (b) ratio of mean root-N pool measured to 30 cm soil depth in 1998, 2002, and 2006 to means of root-N pool of each lower diversity treatment. In panel (a) the solid line shows the mean, while the dotted lines show 95% confidence interval.

lower at greater species number, and this was caused by the presence of C_4 grasses, C_3 grasses, and forbs, and by their interactions. This suggests that the presence of these functional groups, in total, may have enhanced overall plant N uptake, thus reducing soil NO_3^- concentrations in early spring and during the peak of growing season (i.e., July, Fig. 3a). Although we do not have direct data on plant N uptake in the winter months, it could be that the presence of "cool-season" C_3 grasses may play a role during these periods since C_3 grasses are metabolically active then (Wedin and Tilman 1996).

Our results show that net soil N mineralization rates positively affected aboveground productivity (Fig. 2a, Table 2) and that rates of N mineralization were enhanced by the presence of legumes. We found that species number had significant positive effects on net N mineralization rates only when seven legume monocultures were not included in our analyses. This suggests that overall net N mineralization rates are relatively high at greater species numbers because of the increased probability of including more legume species ($P <$ 0.0001; see Scherer-Lorenzen et al. 2003). It also suggests that increased N-supply rates are not sufficient, on their own, to explain high aboveground productivity at high diversity. The seven legume monocultures, despite having high soil N mineralization rates, produced less than half (126 ± 13 g/m^2 [mean $\pm$ SE]) the aboveground biomass produced by the 16-species plots (328 ± 11 g/m^2) on average for the three most recent years (2004, 2005, and 2006). Indeed, if the dominance of legume species has indirect positive effects on N supply rates by contributing to increase litter decomposition (Spehn et al. 2005), it is likely that such positive feedbacks will only be transient because of the limitation of microbial activity by C availability (Chapin et al. 2002, Booth et al. 2005). Moreover, because NO_3^- ions are highly mobile in the soil matrix, they are easily leached if not

taken up by plant roots (Scherer-Lorenzen et al. 2003), especially in coarse-textured, sandy soils such as those of our grassland system at Cedar Creek. C_3 grass, C_4 grass, and forb monocultures were not very productive either. Their aboveground biomass, as averaged for three most recent years, was 74.1 ± 22.8, 68 ± 12.5, and 109 ± 13.2 g/m^2 [mean $\pm$ SE], respectively, likely because of low soil N availability in the absence of legumes.

Thus highly diverse plant assemblages benefit from legume-driven N mineralization rates in two ways: (1) by increasing plant NO_3^- uptake and (2) by contributing to increase the formation of soil organic matter across years with consequent storage and retention of soil C, N (Fornara and Tilman 2008), and water. The presence of C_3 grasses, forbs, and C_4 grasses is crucial to sustain these two processes. For example, C_3 grasses, such as *Poa pratensis* and *Koeleria cristata*, and C_4 grasses, such as *Andropogon gerardi* and *Schizachyrium scoparium*, have high root-length density (Fargione and Tilman 2006), which creates dense root systems (sod-forming species) that contribute to enhanced N uptake and nutrient retention. Many forb species may uptake N at deeper soil depths when coexisting with grasses in plant mixtures (Jumpponen et al. 2002, Fargione and Tilman 2005). Such rooting spatial patterns in resource supply and use (Berendse 1982, McKane et al. 1990) might also contribute to enhance aboveground productivity in mixed plant assemblages. Total soil C (grams/square meter) and total soil N (grams/square meter) measured in 2006 to 20 cm soil depth had strong positive effects (in separate linear regressions) on soil water content between 0 and 20 cm as measured in July 2007 ($P <$ 0.0006 for both analyses) or on soil water content as averaged for June, July, and August 2001 ($P <$ 0.0001 for both analyses). This means that the accumulation of soil organic matter at higher species diversity may increase water retention which may then allow plants to

maximize uptake of readily available NH_4^+ or NO_3^- ions from the soil solution. Further studies need to address the linkages between plant species identity, mycorrhizal fungi, and N-fixing bacteria to understand whether plant uptake of different forms of inorganic and organic N (Schimel and Bennett 2004, van der Heijden et al. 2008) increases at higher species diversity and how they can affect aboveground productivity.

Another key cause of the increased aboveground biomass at greater plant diversity was the increase, for the higher diversity plots, in total soil N to 40 cm soil depth from 1994 to 2006. Interestingly, this 12 year change in total soil N did not significantly explain soil-incubation N mineralization ($P = 0.161$), perhaps because much of the accumulated N may still be "locked" in a recalcitrant form not readily accessible to the microbial community (Sollins et al. 2007). If so, soil N supply rates for plant uptake may depend more on the seasonal availability of readily decomposable fractions of legume leaf and root litter. Although leaf litter biomass has not been seasonally quantified in our experiment, we observed that leaf litter mass from cool-season legume species (e.g., *Lupinus perennis*) produced after burning the plots in early spring is greatly reduced by early autumn. The quick decomposition of this litter mass possibly stimulates microbial activity and increases available forms of N in the topsoil. Multiple seasonal measurements of soil N availability, soil N mineralization rates, plant N adsorption, and so on within each plot may greatly reduce the observed variability of their effects on productivity (Figs. 1 and 2).

We found that fine-root production was higher at greater species diversity, which may suggest higher root-turnover rates. In support of high root-turnover rates, we found that soil NH_4^+ concentrations (before and after the lab incubation) in the top 20 cm soil depth were strongly positively related with fine root production ($P < 0.0001$). This suggests that fine roots may be an important source of readily decomposable, labile organic fractions which enter the soil and may partly explain the results of Zak et al. (2003), which showed that higher numbers of species enhanced the microbial release of NH_4^+ from labile organic matter and caused greater gross N mineralization rates at greater diversity. Because fine roots may account for 90% of total grassland litter production (Steinaker and Wilson 2005), have rapid turnover (Gill et al. 2002, Ruess et al. 2003), and are highly responsive to resource availability (Fitter 1986, Crick and Grime 1987, Tibbett 2000) it is likely that fine-root production is responsible for important positive feedbacks that sustain high aboveground productivity.

Our results seem to suggest that aboveground biomass production is also determined by greater N use efficiency of some grassland species and/or functional groups at higher species richness. We found that the C:N ratio of aboveground mass was positively related to above-ground biomass production (Table 1). This agrees with the findings of a biodiversity experiment set up in The Netherlands in which no legumes were planted (van Ruijven and Berendse 2005), and in which higher productivity at higher diversity resulted from grass and dicot species having higher C:N ratios at greater plant diversity. In our experiment, the increased C:N ratio of aboveground biomass at higher species diversity was mainly determined by the presence of C_4 grasses ($P < 0.0001$).

If greater belowground N dynamics supports higher productivity at higher species diversity, our results also suggest that the rate of change of aboveground biomass through time is strongly affected by total root-N pool to 60 cm soil depth as measured in 2006 (Fig. 4a). The root-N pool significantly increased throughout years more at higher species diversity than in lower diversity assemblages (Fig. 4b) and is positively affected by the presence of legumes, C_4 grasses, and C_3 grasses (Table 1). Our evidence is that total root-N pool contributes to sustain high aboveground productivity across years at least through its positive effects on net N mineralization rates ($P < 0.0001$), root production ($P < 0.0001$), and positive changes in total soil N over time ($P < 0.0001$). The size of the root-N pool is likely dependent on positive feedbacks between belowground and aboveground plant biomass production, and although it may not be a primary cause of enhanced aboveground productivity, its increase over years likely has positive effects on soil fertility, soil N supply, and indirectly on plant N uptake rates by positive feedbacks with fine-root production.

CONCLUSION

We found that seven variables simultaneously controlled aboveground productivity in a grassland biodiversity experiment: (1) initial total soil N of each plot, (2) diversity-dependent increases in soil total N over years, (3) soil N mineralization rates, (4) soil nitrate utilization, (5) increases in plant N use efficiency at greater plant diversity, (6) legume presence, and (7) higher species numbers. Surprisingly, higher plant diversity still contributed to increase productivity even after we controlled for six key variables associated with N dynamics. This is perhaps because the increased presence of C_4 grasses, C_3 grasses, legumes, and forbs at higher diversity, in turn or simultaneously, affected seasonal N supply and use, seasonal soil moisture, root production, and the total root-N pool to maximize aboveground productivity. Thus the four mechanisms proposed so far for explaining the positive diversity-productivity relationship (i.e., increased N supply, N uptake, N-use efficiency, and plant-N pool at greater species diversity) may simultaneously contribute to enhance aboveground production in plant mixtures because of the presence of different functional groups/traits. Further studies are needed to test whether and to what extent (1) a similar set of ecological mechanisms may influence productivity in more naturally constructed communities where a positive diversity–productivity relationship was (Flombaum and Sala 2008) or was not

(Grace et al. 2007) observed; (2) the same mechanisms are still important in grasslands where climate, soil type, and disturbance regimes affect the availability of other key limiting resources; and (3) the use/exploitation of other limiting resources (e.g., phosphorous, water) could simultaneously affect productivity (Harpole and Tilman 2007).

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