

C.P.H. Mulder · A. Jumpponen · P. Högberg  
K. Huss-Danell

## How plant diversity and legumes affect nitrogen dynamics in experimental grassland communities

Received: 18 September 2001 / Accepted: 12 June 2002 / Published online: 5 September 2002  
© Springer-Verlag 2002

**Abstract** Positive relationships between species richness and ecosystem processes such as productivity or nitrogen cycling can be the result of a number of mechanisms. We examined how species richness, biomass, and legume presence, diversity, and abundance explained nitrogen dynamics in experimental grassland plots in northern Sweden. Nitrogen concentrations and  $\delta^{15}\text{N}$  values were measured in plants grown in 28 mixtures (58 plots) including 1, 2, 4, 8 or 12 local grassland species over four years. Values for  $\delta^{15}\text{N}$  declined over time for all three functional groups (grasses, legumes, and non-leguminous forbs), suggesting greater reliance on N fixed by legumes over time by all species. Above ground percent nitrogen (%N) also declined over time but root %N and total N did not. Path analysis of above ground data suggested that two main factors affected %N and the size of the N pool. First, higher plant diversity (species richness) increased total N through increased biomass in the plot. Although in the first two years of the experiment this was the result of a greater probability of inclusion of at least one legume, in the last two years diversity had a significant effect on biomass beyond this effect. Second, percent legumes planted in the plots had a strong effect on above ground %N and  $\delta^{15}\text{N}$ , but a

much smaller effect on above ground biomass. In contrast, greater plant diversity affected N in roots both by increasing biomass and by decreasing %N (after controlling for effects mediated by root biomass and legume biomass). Increased legume biomass resulted in higher %N and lower  $\delta^{15}\text{N}$  for both non-legume forbs and grasses in the first year, but only for grasses in the third year. We conclude that a sampling effect (greater probability of including a legume) contributed towards greater biomass and total N in high-diversity communities early on in the experiment, but that over time this effect weakened and other positive effects of diversity became more important.

**Keywords** Nitrogen fixer · Sampling effect · Species richness · Stable isotopes

### Introduction

In recent years the number of studies experimentally evaluating the effect of plant species diversity on ecosystem functioning has increased dramatically (e.g. Naeem et al. 1996; Tilman et al 1996; Hooper and Vitousek 1998; Hector et al 1999; see review in Schmid et al. 2001). Most have focused on how changes in plant diversity (primarily species richness) affect productivity. Many studies also report on some aspects of soil nutrient status (e.g. Wardle et al. 1997; Hooper and Vitousek 1998; Symstad et al. 1998), but nutrient dynamics of plants have received little attention in these studies. A Europe-wide study of the relationships between species richness and ecosystem functioning in experimental grasslands (BIODEPTH) found a positive relationship at many of the sites, including our site in northern Sweden where productivity increased approximately linearly with increased species richness (Hector et al. 1999). This study expands on that work by examining plot-level patterns in nitrogen dynamics at the Swedish site over the course of four years. Nitrogen dynamics in plant communities (particularly experimental ones) are likely to

C.P.H. Mulder (✉) · A. Jumpponen · P. Högberg  
Soil Science Section, Department of Forest Ecology,  
Swedish University of Agricultural Sciences, 90183 Umeå,  
Sweden

C.P.H. Mulder · A. Jumpponen · K. Huss-Danell  
Crop Science Section,  
Department of Agricultural Research for Northern Sweden,  
Swedish University of Agricultural Sciences, Box 4097,  
90403 Umeå, Sweden

*Present address:*

C.P.H. Mulder, Institute of Arctic Biology  
and Department of Biology and Wildlife,  
University of Alaska Fairbanks, Fairbanks,  
AK 99775, USA

*Present address:*

A. Jumpponen, Division of Biology, Ackert Hall,  
Kansas State University, Manhattan, KS 66506, USA

vary over time, as plant and microbial communities change. We examined patterns during four growth seasons that allowed us to distinguish between initial effects, transient effects, and longer-term trends.

We focus on the effects of species richness, the presence and number of legumes, and legume abundance (percent of total biomass) on productivity and nitrogen dynamics (%N, total N, and  $\delta^{15}\text{N}$ ). This allows us to distinguish between several mechanisms that have been proposed to link species richness, productivity, and nitrogen dynamics. For example, the increased biomass at higher diversities seen in some studies (Naeem et al. 1996; Tilman et al. 1996; Hector et al. 1999) may represent a "sampling effect" (Aarssen 1997; Huston 1997; Wardle 1999): an increased probability of including species with  $\text{N}_2$ -fixing symbionts (" $\text{N}_2$  fixers") such as legumes. Legumes not only provide much of their own nitrogen through  $\text{N}_2$  fixation, thereby reducing competition with other species, but may also increase soil nitrogen for other species through leaching or decomposition. Alternatively, increased diversity could lead to greater niche complementarity, which in turn could result in a greater total N supply to the plant community. Different species may have different rooting depths (Berendse 1979, 1981, 1983), vary in their ability to exploit high-nutrient patches (Farley and Fitter 1999) or in their relative uptake rates of different types of N (Nordin et al. 2001), or show temporal separation in nutrient uptake (Van den Bergh and de Wit 1960; Berendse 1983; Hooper and Vitousek 1998). A third possibility is that greater species richness results in greater microbial diversity and

higher decomposition rates, although there is currently little experimental support for this hypothesis (Finlay et al. 1997; Wardle et al. 1997; Hector et al. 2000).

The extent to which growth form (e.g. herbaceous vs graminoid, or annual vs perennial) and relative dominance of non- $\text{N}_2$ -fixing species (hereafter "non-fixers") affect their ability to benefit from the presence of  $\text{N}_2$  fixers will determine how relationships between species richness and productivity are altered by the inclusion of  $\text{N}_2$  fixers. For example, if inclusion of an  $\text{N}_2$  fixer primarily increases growth rates of large dominant species, then the relationship between species richness and productivity will be positive as a result of a double sampling effect: increased probability of including an N fixer and increased probability of including a large dominant species that benefits from additional N availability. In other words, in multi-species plant communities, species traits (such as ability to respond to higher nutrient levels) as well as competitive interactions may determine the mechanisms by which  $\text{N}_2$  fixers affect relationships between diversity and productivity, and the extent to which they do so. We examined effects on individual species and their dominance levels to evaluate this possibility.

## Materials and methods

### Site description and experimental design

The field site was located at the Swedish University of Agricultural Sciences in Umeå, Sweden (63°45'N, 20°17'E, 12 m a.s.l.). The soil was a fine silty sand with little clay (4.1% clay, 57.9% silt,

**Table 1** Experimental design.

Two plots were planted with each combination listed, except for the 12-species plot of which four replicates were planted. Species abbreviations are as follows: *Am* = *Achillea millefolium*, *Dg* = *Dactylis glomerata*, *Fo* = *Festuca ovina*, *Lv* = *Leucanthemum vulgare*, *Lc* = *Lotus corniculatus*, *Pa* = *Phalaris arundinacea*, *Pp* = *Phleum pratense*, *Th* = *Trifolium hybridum*, *Tp* = *Trifolium pratense*, *Tr* = *Trifolium repens*, *Raa* = *Ranunculus acris*, *Rua* = *Rumex acetosa*. Note that the mean for each functional group at each diversity level = 33% and that each species is represented equally at each diversity level

Diversity	Grasses	Legumes	Forbs	Percent legumes
1	Dg	–	–	0
1	Fo	–	–	0
1	Pa	–	–	0
1	Pp	–	–	0
1	–	Lc	–	100
1	–	Th	–	100
1	–	Tp	–	100
1	–	Tr	–	100
1	–	–	Am	0
1	–	–	Lv	0
1	–	–	Raa	0
1	–	–	Rua	0
2	Da Pa	–	–	0
2	Pp	Tp	–	50
2	–	Lc	Lv	50
2	Fo	–	Am	0
2	–	–	Raa Rua	0
2	–	Th Tr	–	100
4	Pa Pp	Lc Tp	–	50
4	–	Th Tr	Lv Raa	50
4	Dg Fo	–	Am Rua	0
4	Dg Pa	Lc	Lv	25
4	Pp	Tp	Raa Rua	25
4	Fo	Th Tr	Am	50
8	Dg Fo Pa Pp	Th Tr	Raa Lv	25
8	Dg Fo	Lc Th Tp Tr	Am Rua	50
8	Pa Pp	Lc Tp	Am Lv Raa Rua	25
12	Dg Fo Pa Pp	Lc Th Tp Tr	Am Lv Raa Rua	33

38.0% fine sand) with a pH of 6.0. In the three years prior to the experiment the field had been used for potato and barley cultivation. It had been fertilized every year up to and including 1995 with an application of 400 kg ha<sup>-1</sup> N-P-K (11-5-18). During the summer of 1995, 7.5 g ha<sup>-1</sup> of herbicide (Expresspreparat, DuPont Agro, Malmö, Sweden) was applied to reduce weed growth. The last barley crop was harvested in the fall of 1995, and the site was ploughed without removal of straw. In the spring of 1996 the site was repeatedly harrowed. No fertilizer was added after 1995.

In June 1996, prior to the start of the experiment, 34 soil cores were collected in a grid pattern from across the experimental field, and analyzed for percent nitrogen (%N) and  $\delta^{15}\text{N}$  using the same methods as for plant samples (see below). Most of the cores (28 in total) were 4 cm wide and 15 cm deep but every fifth core (6 in total) was taken using a 1-cm diameter corer to a depth of 50 cm, and split into 0–10 cm, 10–30 cm, and 30–50 cm sections. These soil samples contained  $0.14 \pm 0.03\%$  N (mean  $\pm$  SD) in the first 15 cm, and %N dropped sharply below topsoil (0–10 cm:  $0.15 \pm 0.02$ ; 10–30 cm:  $0.15 \pm 0.07$ ; 30–50 cm:  $0.05 \pm 0.02$ ). The  $\delta^{15}\text{N}$  was  $4.75 \pm 0.74$  for the first 15 cm, and also decreased with depth, especially below topsoil (0–10 cm:  $4.58 \pm 0.85$ ; 10–30 cm:  $4.25 \pm 0.29$ ; 30–50 cm:  $2.64 \pm 0.55$ ).

In early June 1996, 72 plots measuring 2.2  $\times$  5 m were established. Plots were planted with 1, 2, 4, 8, or 12 species (Table 1) from three "functional groups": grasses, legumes, and non-leguminous forbs. Six plots were left bare and another 8 were used in a different experiment and will not be discussed here; the remaining 58 plots formed the basic experiment. They were planted at a total density of 2,000 seed m<sup>-2</sup>, and hand-weeded to prevent invasion of unwanted species. Borders with a width of 1.5 m were established between the plots and sown with *Phleum pratense*; these were mowed regularly and clippings were removed.

Plots were planted with 28 unique species mixtures (Table 1). There were two replicates of each mixture, except for the 12-species mixture, for which there were four replicates. Species were randomly allocated to mixtures under the following constraints: (1) all species were equally represented at all levels of diversity (e.g. at each level of diversity 1/12th of seeds sown were *Trifolium repens*); (2) the relative contribution of the three functional groups was varied so that the same relative contributions occurred at multiple levels of diversity; and (3) half of the 4-species mixes consisted of combinations of the 2-species mixes, while 8-species mixes consisted of the 4-species mixes plus an additional four species. Thus legume seeds represented on average one third of all seeds planted at each level of diversity, but there was variation within all diversity levels except for the highest level (Table 1). However, because all high-diversity plots (8 and 12 spp.) contained at least some legumes, this design allowed for only partial distinction between the effects of diversity and those of including at least some legumes.

## Measurements

Plants were sampled in 1996, 1997, 1998 and 1999. In mid-August of each year, biomass above 5 cm in height was removed from a centrally located area of 0.2  $\times$  0.5 m in each plot, sorted by species, and dried at 60°C for 24 h. The entire plot was then mowed to 5 cm and clippings were removed; the same central plot was sampled in each year. An additional biomass sample from 1–5 cm in height was collected from a separate 15  $\times$  20 cm plot to give a better estimate of biomass of low-growing species and was treated in the same manner; a different plot was used for this estimate in each year. All samples were sorted to species, but in 1996 a number of species had not yet flowered, and some specimens of *Phleum pratense* could not be distinguished from *Phalaris arundinacea*, and some specimens of *Trifolium repens* could not be distinguished from *T. hybridum*. Large samples (>1 g) were homogenized with a blade mill, followed by grinding of a subsample in a ball mill; smaller samples (<1 g) were ground in their entirety in a ball mill. Samples <0.1 g were not analyzed.

Root biomass in each plot was estimated by removing four cores per plot (depth 20 cm, diameter 4 cm) from random loca-

tions within each quadrant of the plot in late August. Roots were carefully hand-washed on a screen (sieve size 100  $\mu\text{m}$ ), dried at 60°C for 24 h, and weighed. Roots from different species could not be distinguished. Samples were ground in a ball mill and analyzed for total N and  $\delta^{15}\text{N}$  (see below).

The use of stable isotopes has greatly facilitated the study of nitrogen dynamics in natural communities (e.g. Handley and Scrimgeour 1997; Högberg 1997). During biological nitrogen fixation, N is obtained from atmospheric N<sub>2</sub>, a process which results in  $\delta^{15}\text{N}$  values close to 0‰ (see review in Högberg 1997). In contrast, the acquisition of N from soil may result in  $\delta^{15}\text{N}$  values distinctly different from 0‰ (Högberg 1997). Plant species which form symbioses with nitrogen fixing bacteria, such as legumes, therefore commonly have  $\delta^{15}\text{N}$  values closer to 0‰ than non-N<sub>2</sub> fixers, and approach this value as the dependence on N<sub>2</sub> fixation increases. In agricultural settings, non-fixers are usually well above 0‰, while in forests and many other natural ecosystems non-fixers may be above or below 0‰ (Högberg 1997). Above ground samples of each plant species (1996, 1998) or pooled plant communities (1997, 1999) were analyzed for %N and for <sup>15</sup>N abundance using an online CN analyzer (Europa Scientific ANCA-NT) coupled to an isotope ratio mass spectrometer (Europa Scientific Europa 20–20; Barrie and Lemley 1989; Ohlsson and Wallmark 1999). Results for <sup>15</sup>N abundance are expressed as  $\delta^{15}\text{N}$  in parts per thousand (‰) relative to the international standard (atmospheric N<sub>2</sub>, at 0.3663 atom %; Junk and Svec 1958; Mariotti 1983):

$$\delta^{15}\text{N} = \left[ \left( R_{\text{sample}} / R_{\text{standard}} \right) - 1 \right] \times 1,000 \quad (1)$$

where  $R$  = molar ratio of <sup>15</sup>N / <sup>14</sup>N.

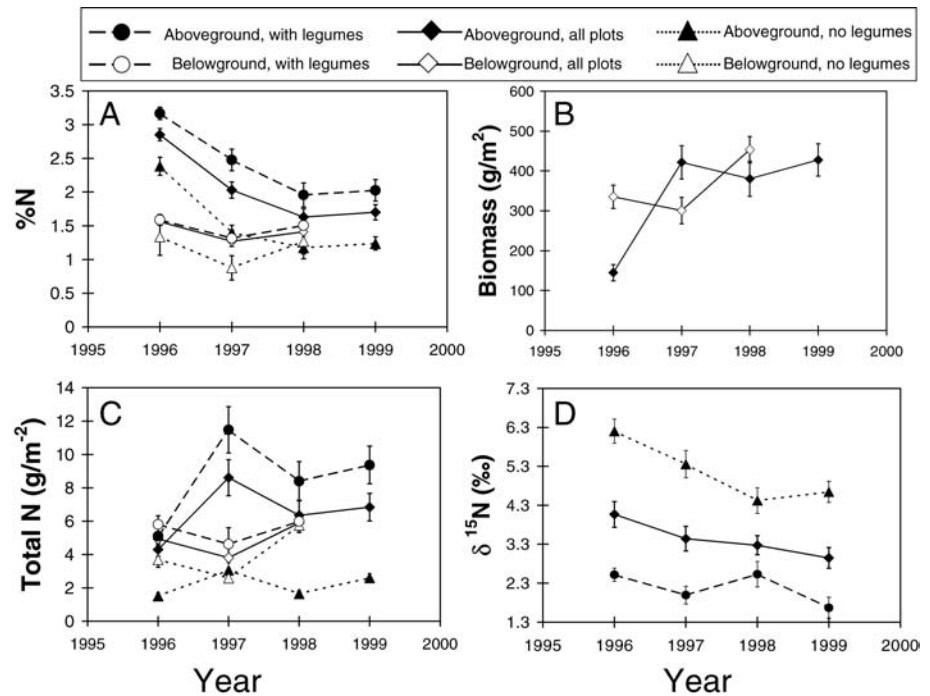
Pooled above ground samples for each plot were analyzed for %N and  $\delta^{15}\text{N}$  in all four years, but samples were analyzed by species in 1996 and 1998 only. Roots (pooled per plot) were analyzed for %N in 1996, 1997 and 1998 and  $\delta^{15}\text{N}$  in 1997 and 1998 only.

## Data analysis

Data were analyzed using SAS (version 6.12, SAS 1999). Means for all cores per plot were used for roots. Differences between years were analyzed using one-way ANOVA. Analyses of relationships between variables across all years were made using repeated measures analysis of variance (PROC GLM in SAS) with the Huynh-Feldt epsilon correction for lack of sphericity. We treated plots as independent datapoints since the more conservative approach with respect to detecting effects of diversity (using means per mixture) did not allow us to include variables that differed between mixtures (e.g. percent of biomass in legumes). When comparing functional group responses we used means per plot for each group as species within a plot are not independent of each other. Because of the small sample sizes we did not statistically analyze patterns for individual species.

Since explanatory variables affecting nitrogen pools and other aspects of N dynamics are likely to be correlated (e.g. percent biomass in legumes and biomass of all species), we used path analysis (Wright 1934) to identify potential causal effects. Path analysis allows one to construct a causal model which includes several independent and dependent variables, and to decompose the correlations which exist between variables into components assumed to be causal and non-causal (Schemske and Horvitz 1988). Causality is assumed rather than demonstrated, since additional unmeasured variables may be the true cause of correlations. The magnitude of the path coefficient (standardized regression coefficient) indicates the strength of the direct effect of an independent variable on a dependent variable (see Schemske and Horvitz 1988 and Mitchell 1993 for good explanations of path analysis in an ecological context). Path diagrams were built similarly for above ground and below ground variables; in both cases the percent of above ground biomass represented by legumes ("percent legume biomass") had to be used since roots could not be sorted by species. In the path analyses, percent legume biomass could be affected by percent of planted species that were legumes, and number of species planted

**Fig. 1A–D** Nitrogen dynamics between 1996 and 1999. Values are means across the 58 plots and error bars are standard errors. *Filled symbols* represent above ground data; *open symbols* represent below ground data. *Diamonds* represent all plots, *circles* plots with at least some legumes, and *triangles* plots without legumes. **A** %N (g N per 100 g d wt) in above ground and below ground vegetation. **B** Above ground biomass ( $\text{g m}^{-2}$ ) and below ground biomass ( $\text{g m}^{-2}$  to 20 cm depth). **C** Total nitrogen above ground ( $\text{g m}^{-2}$ ) and below ground ( $\text{g m}^{-2}$  to 20 cm depth). **D** Values for  $\delta^{15}\text{N}$  in above ground vegetation



(diversity), while plot biomass was affected by diversity and percent of biomass in legumes. In our models N concentration (%N) was potentially affected by percent legume biomass and by diversity, while total N was potentially affected by %N and plot biomass (this does not explain all variation in total N because in this model effects are additive rather than multiplicative).

## Results

### N dynamics over time

For above ground biomass %N decreased from 1996 to 1998 and then levelled off [ $F_{(3, 165)}=18.99$ ,  $P<0.0001$ ; Fig. 1 A]. Percent N in root biomass differed between years [ $F_{(1, 52)}=4.66$ ,  $P=0.014$ ] but did not show a consistent pattern over time (Fig. 1A). The ratio of %N above ground to %N below ground decreased over time [ $F_{(2, 51)}=15.71$ ,  $P<0.001$ ], and by 1998 was only approximately half that in 1996. For both above ground and root %N, plots with legumes had higher values than plots without legumes but patterns over time were similar (Fig. 1A).

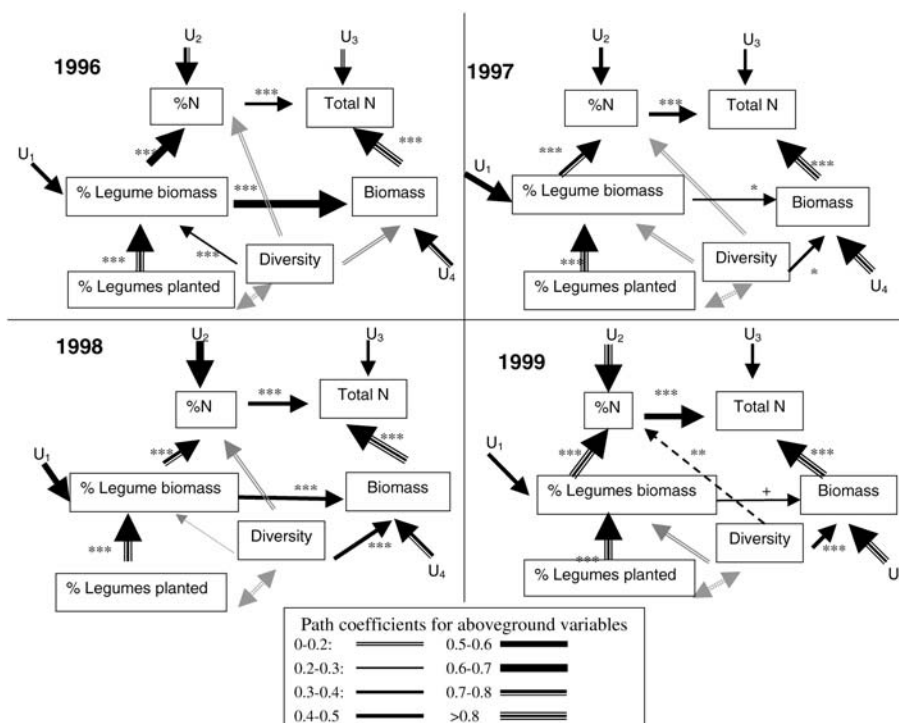
Annual variation in biomass was large but showed no consistent pattern over time (Fig. 1B). As a result there were significant differences between years (but no pattern over time) in above ground total N [ $F_{(3, 53)}=4.20$ ,  $P=0.008$ ], but not in root total N [ $F_{(2, 47)}=1.29$ ,  $P=0.29$ ; Fig. 1C]. Neither % biomass in roots nor % biomass in legumes differed between years ( $P>0.1$ ). As for %N, plots with legumes had a greater total N than plots without legumes both above ground and below ground, but the pattern over time was the same for the two groups. Values for  $\delta^{15}\text{N}$  in above ground biomass across all plots decreased linearly over time [Fig. 1D;  $F_{(3, 165)}=10.57$ ,  $P<0.0001$ ], and there was a strong negative correlation between per-

cent N and  $\delta^{15}\text{N}$  in all years (Pearson correlation coefficients ranged from  $-0.58$  to  $-0.72$ ,  $P<0.001$  for all). Most of the decline, however, was due to plots without legumes (Fig. 1D). Values for root  $\delta^{15}\text{N}$  decreased only marginally between 1997 and 1998 [paired  $t$ -test,  $T_{(55)}=1.76$ ,  $P=0.084$ ; no values available for 1996 or 1999] and in both years were negatively correlated with %N (correlation coefficients  $=-0.41$  and  $-0.55$  respectively).

### Whole-community N dynamics above ground

The path diagrams in Fig. 2 summarize whole-plot N dynamics above ground for each year. The amount of variation in above ground biomass explained by diversity (number of species planted) as opposed to percent legume biomass (the percent of above ground biomass attributable to legumes) increased over time: in 1996 percent legume biomass explained a large proportion of the variation (path coefficient  $=0.62$ ), while by 1999 diversity explained more than percent legume biomass (path coefficients were 0.49 and 0.23 respectively). In contrast, diversity explained significant variation in percent legume biomass in 1996 only. The only direct effect of diversity on %N was a negative one in 1999. In all years, plot biomass explained more of the variability in total N than did mean %N. These results show that above ground effects of diversity on plot-level N dynamics were mediated almost entirely through changes in total above ground biomass, and that this effect strengthened over time. Diversity effects were not mediated through changes in legume biomass or direct effects on %N. In contrast, percent legume biomass effects on %N did increase in strength over time.

**Fig. 2** Path diagrams for the above ground relationships between diversity (number of species planted), percent legumes planted, legumes as the actual percentage of the above ground biomass (percent legume biomass), total above ground biomass, and total above ground nitrogen for all four years. Thickness of the arrow indicates the strength of the path coefficient. Arrows in gray indicate no significant relationship; *P* values for other arrows are indicated as follows: + 0.5–0.1; \* 0.01–0.5; \*\*\*<0.001. Solid lines indicate a positive relationship while dashed lines indicate a negative relationship. *U* is the proportion of unexplained variation in the variable, and was calculated as  $(1-R^2)^{1/2}$ .



**Table 2** Testing the sampling hypothesis: the relative contribution of diversity versus legume presence on above ground, below ground, and total biomass over time. Models are stepwise multiple

regressions (*P*-to-enter =0.15, *P*-to-stay =0.05) with diversity and legume presence as explanatory variables. *NR* Not retained in model; *NA* not available. *neg* indicates a negative relationship

Year	Diversity partial <i>R</i> <sup>2</sup>			Legume presence partial <i>R</i> <sup>2</sup>		
	Above	Below	Total	Above	Below	Total
1996	NR	0.10*	0.10*	0.28****	NR	NR
1997	NR	NR	NR	0.18***	NR	0.9*
1998	0.07*	0.21***	0.32***	0.23***	0.15*** (neg)	NR
1999	0.24****	NA	NA	NR	NA	NA

Values for *P*: \*=<0.05, \*\*\*<0.001, \*\*\*\*<0.0001

The sampling effect refers to diversity effects that result from an increased probability of including at least one legume (“legume presence”), a variable not included in the path analyses. We examined whether legume presence explained the increase in above ground biomass better than diversity by including both variables in a stepwise multiple regression (Table 2). Although initially legume presence explained most of the variation in above ground biomass, over time the strength of this effect decreased (so that by 1999 it was insignificant) while that of diversity increased (and by 1999 was highly significant; Table 2).

Mean above ground  $\delta^{15}\text{N}$  values for the whole plot indicate the contribution of  $\text{N}_2$  fixation to the available soil N pool for communities containing legumes. There was a significant negative relationship between  $\delta^{15}\text{N}$  and diversity when all four years were examined simultaneously [ $F_{(3, 25)}=4.07, P=0.0175$ ], but for individual years this relationship was significant in 1996 only [ $F_{(1, 54)}=4.08, P=0.048$ ]. Furthermore, when percent legume biomass and diversity were included in a stepwise regression, val-

**Table 3** Effects of diversity and percent legumes on  $\delta^{15}\text{N}$  values for above ground biomass. Models are stepwise multiple regressions (*P*-to-enter =0.15, *P*-to-stay =0.01). Denominator degrees of freedom are 55 for 1996 and 56 for all other years. All significant relationships are negative. *NR* Not retained in the model

Year	Diversity		Percent legumes	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
1996	NR	–	524.36	<0.001****
1997	NR	–	76.17	<0.001****
1998	NR	–	132.22	<0.001****
1999	8.72	0.0046**	103.73	<0.001****

Values for *P*: \*\*<0.01; \*\*\*\*<0.0001

ues for  $\delta^{15}\text{N}$  decreased with increased percent legume biomass in all years, and diversity explained significant additional variation only in 1999 (Table 3).

The path analyses included percent legume biomass as the primary legume-related variable affecting biomass and N dynamics, but this is potentially confounded with legume presence and the number of legume species

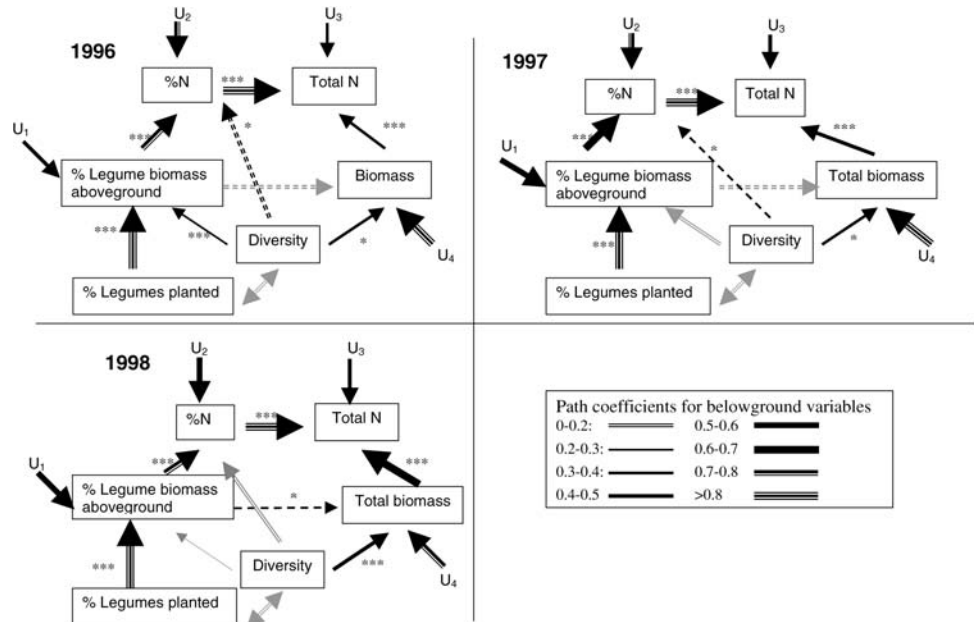
**Table 4** Contributions of the presence of legumes, percent legume biomass above ground and number of legume species to overall legume effects on %N,  $\delta^{15}\text{N}$ , and biomass. Models are stepwise

multiple regressions ( $P$ -to-enter =0.15,  $P$ -to-stay =0.01). *Dir* Direction of the relationship, *NR* not retained in the model

Response	Year	Legume presence			Percent legumes			No. legume species		
		<i>F</i>	<i>P</i>	<i>Dir</i>	<i>F</i>	<i>P</i>	<i>Dir</i>	<i>F</i>	<i>P</i>	<i>Dir</i>
Above ground:										
%N	1996	NR			50.61	****	+	NR		
	1997	NR			77.30	****	+	NR		
	1998	NR			56.45	****	+	NR		
	1999	NR			120.89	****	+	NR		
$\delta^{15}\text{N}$	1996	NR			120.64	****	-	NR		
	1997	12.45	***	-	30.75	****	-	NR		
	1998	NR			12.69	***	-	NR		
	1999	NR			120.89	****	-	NR		
Biomass	1996	NR			34.82	****	+	NR		
	1997	12.69	****	+	NR			NR		
	1998	16.81	****	+	NR			NR		
	1999	NR			NR			21.92	****	
Below ground:										
%N	1996	18.53	****	-	70.47	****	+	NR		
	1997	NR			47.05	****	+	NR		
	1998	NR			101.48	****	+	13.36	***	-
Biomass	1996	NR			NR			NR		
	1997	NR			NR			NR		
	1998	NR			NR			NR		

Values for *P*: \*\*\*<0.001; \*\*\*\*<0.0001

**Fig. 3** Path diagrams for the above ground relationships between diversity (number of species planted), percent legumes planted, legumes as the actual percentage of the above ground biomass (percent legume biomass), total above ground biomass, and total above ground nitrogen for all four years. Thickness of the arrow indicates the strength of the path coefficient. Arrows in gray indicate no significant relationship; *P* values for other arrows are indicated as follows: + 0.5–0.1; \* 0.01–0.5; \*\*\*<0.001. Solid lines indicate a positive relationship while dashed lines indicate a negative relationship



planted (not included in the path analyses). We used all three variables in a stepwise multiple regression (Table 4) and found that only percent legumes explained above ground %N and (with the exception of 1997)  $\delta^{15}\text{N}$ , while the number of legume species did not explain additional variation in any year. However, for biomass percent legumes was the best explanatory variable in the first year, with legume presence emerging as

the best explanatory variable in 1997 and 1998, and number of legume species in 1999. It thus appears that %N and  $\delta^{15}\text{N}$  can be explained primarily by percent biomass in legumes, not the presence of any legumes or legume diversity. In contrast, the presence of any legumes (regardless of abundance) appears to result in increased above ground biomass.

**Table 5** Nitrogen data in 1996 and 1998 for individual species. Values are mean±SE. Numbers after species names refer to number of plots from which samples were analyzed in 1996 and 1998 respectively. In 1996, *Phalaris arundinacea* and *Phleum pratense*

could not be separated, nor could *Trifolium hybridum* and *T. repens*, so the same values are shown for both species. Total above ground N and percent of all N were calculated across the entire experiment

Species	$\delta^{15}\text{N}$ (‰)		%N		Total above ground N (g)		Percent of all above ground N	
	1996	1998	1996	1998	1996	1998	1996	1998
Non-fixers: grasses								
<i>Dactylis glomerata</i> (17, 13)	5.93±0.33	3.79±0.21	2.07±0.11	1.48±0.07	288.53	124.63	11.76	3.00
<i>Festuca ovina</i> (14, 6)	6.02±0.30	3.25±0.33	3.05±0.23	1.56±0.31	40.59	73.26	1.65	1.76
<i>Phalaris arundinacea</i> (17, 15)	6.17±0.32	5.18±0.38	2.70±0.11	0.92±0.09	195.80	263.12	7.98	6.32
<i>Phleum pratense</i> (14, 17)	6.17±0.32	4.72±0.24	2.70±0.11	0.87±0.06	195.80	450.67	7.98	10.83
Non-fixers: forbs								
<i>Achillea millefolium</i> (7, 9)	7.01±0.57	4.14±0.13	3.31±0.19	1.54±0.10	16.83	237.49	0.69	5.71
<i>Leucanthemum vulgare</i> (4, 7)	8.02±0.87	3.87±0.20	2.44±0.92	1.74±0.17	28.71	95.26	1.17	2.29
<i>Ranunculus acris</i> (12, 9)	4.55±0.75	3.42±0.23	2.49±0.19	1.16±0.10	17.82	137.83	0.72	3.31
<i>Rumex acetosa</i> (13, 10)	6.93±0.52	4.12±0.45	2.92±0.18	1.63±0.26	45.10	83.82	1.84	2.01
Legumes								
<i>Lotus corniculatus</i> (17, 3)	4.70±0.51	0.02±10.91	2.64±0.11	4.21±0.34	130.13	11.55	5.30	0.28
<i>Trifolium hybridum</i> (17, 15)	0.56±0.31	-0.01±0.08	3.49±0.14	2.82±0.21	478.92	1,080.53	19.51	25.99
<i>Trifolium pratense</i> (17, 17)	1.20±1.30	-0.22±0.07	3.38±0.10	2.82±0.15	537.35	930.38	21.89	22.38
<i>Trifolium repens</i> (17, 16)	0.56±0.31	0.16±0.10	3.49±0.14	3.18±0.09	478.92	669.24	19.51	16.10

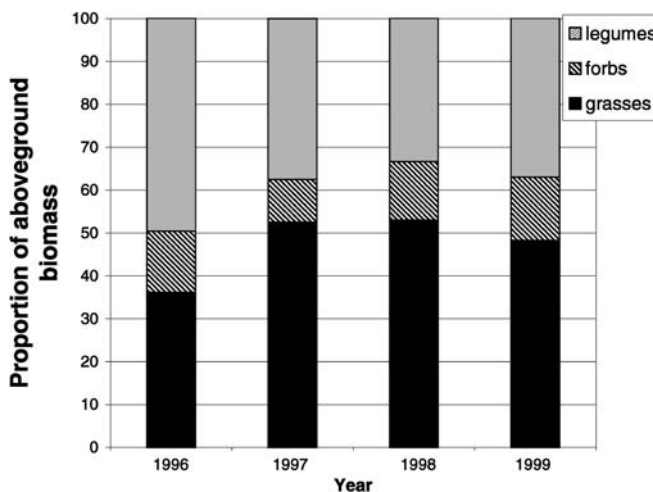
#### Whole-community N dynamics below ground

The path diagrams in Fig. 3 summarize whole-plot N dynamics below ground for 1996–1998 (data were not available for 1999). There was a positive relationship between diversity and total root biomass in all three years, while root %N was negatively related to diversity in the first two years. Somewhat surprisingly, percent legume biomass above ground, which had a very strong effect on above ground biomass, did not affect total root biomass. Furthermore, variation in %N rather than root biomass explained most of the variability in total root N. Step-wise multiple regressions of diversity and legume presence on root biomass showed a significant positive effect of diversity in two years (1996 and 1998) and a negative effect of legume presence in one year (1998; Table 2). It thus appears that diversity affects N dynamics in roots both by affecting biomass (positively) and by affecting %N directly (negatively).

As for above ground data, we examined the relative contributions of legume presence, percent legume biomass (above ground), and number of legume species to below ground %N and biomass (Table 4). Although percent legume biomass again best explained variation in %N in all years, the number of legume species explained additional variation in 1998. However, this relationship was negative. None of these variables explained significant variation in root biomass in any year.

#### Effects on individual species and functional groups

The relative abundance of functional groups (grasses, legumes and forbs) across all plots in the experiment shift-



**Fig. 4** Contributions to total above ground biomass by grasses, forbs and legumes summed across all plots

ed over time. In the first year, legumes accounted for approximately half of the biomass above ground. In later years grasses had the most biomass and legume biomass was reduced to approximately one-third of the total (Fig. 4). To evaluate which species were benefiting from any additional nitrogen made available by N fixation, we examined nitrogen variables (%N, total N and  $\delta^{15}\text{N}$ ) for individual species (Table 5). Percent N was slightly greater in legumes than in non-fixers in 1996 [ $F_{(1, 10)}=5.00$ ,  $P=0.049$ ] and much greater in 1998 [ $F_{(1, 10)}=46.18$ ,  $P<0.001$ ]. However, when comparing functional groups within the same plot, there was no difference in %N above ground between legumes and non-fixers in either year [1996:  $F_{(1, 88)}=1.42$ ,  $P=0.24$ ; 1998:  $F_{(1, 88)}=0.88$ ,

$P=0.35$ ]. Despite the substantial differences between years in %N for individual species between 1996 and 1998, and the large decline in legume biomass as a proportion of the total, the proportion of all N that was in legumes was almost identical (66.2% in 1996, 64.8% in 1998). Values for  $\delta^{15}\text{N}$  were lower in 1996 than in 1998 for all species, and in both years mean values for legumes were significantly lower than for non-legumes [1996:  $F_{(1, 88)}=3.97$ ,  $P=0.49$ ; 1998:  $F_{(1, 88)}=639.45$ ,  $P<0.0001$ ]. In both years,  $\delta^{15}\text{N}$  values for the three *Trifolium* species were not significantly different from zero, while those for all non-leguminous species were significantly greater than zero (Table 4). For *Lotus corniculatus*,  $\delta^{15}\text{N}$  values were  $>0$  in 1996 but not in 1998.

Next we examined whether an increase in legume abundance had similar effects on N availability for legumes and non-fixers (forbs and grasses). In 1996, %N in above ground vegetation increased with percent legume biomass for non-fixers [ $F_{(1, 49)}=19.83$ ,  $P<0.0001$ ] but for legumes there was no relationship [ $F_{(1, 22)}=2.55$ ,  $P=0.12$ ]. Furthermore, for non-fixers the relationship between  $\delta^{15}\text{N}$  and percent legume biomass was negative [ $F_{(1, 49)}=29.63$ ,  $P<0.001$ ], and examination of individual species suggested this was due primarily to forbs rather than grasses. There was, however, no relationship for legumes [ $F_{(1, 22)}=1.09$ ,  $P=0.31$ ], which had consistently low values. By 1998 %N was not affected by percent legume biomass in either non-fixers [ $F_{(1, 46)}=0.00$ ,  $P=0.95$ ] or legumes [ $F_{(1, 24)}=1.19$ ,  $P=0.29$ ]. There was still a negative relationship between  $\delta^{15}\text{N}$  values and percent legume biomass in non-fixers [ $F_{(1, 46)}=9.62$ ,  $P=0.0033$ ], but for legumes there was a significant positive relationship [ $F_{(1, 24)}=6.23$ ,  $P=0.020$ ].

Next we examined whether plants in different functional groups were affected similarly by changes in diversity. In 1996, there was no significant relationship between %N and diversity for either legumes [ $F_{(1, 22)}=0.01$ ,  $P=0.92$ ] or non-fixers [ $F_{(1, 49)}=1.08$ ,  $P=0.31$ ], and including percent legume biomass in the model did not alter this result. However, this was due to opposite relationships for the non-fixing functional groups: forbs and legumes (except for *L. corniculatus*) generally showed negative relationships while grasses generally showed positive relationships. In 1998 %N and diversity were unrelated for both groups ( $P=0.23$  and  $P=0.36$  for non-fixers and legumes respectively). For legumes  $\delta^{15}\text{N}$  decreased with increased diversity in both 1996 and 1998 [ $F_{(1, 22)}=29.63$ ,  $P<0.001$ , and  $F_{(1, 23)}=14.78$ ,  $P=0.0008$  respectively] while there was no significant relationship for non-fixers [ $F_{(1, 49)}=1.1$ ,  $P=0.31$  and  $F_{(1, 46)}=0.26$ ,  $P=0.61$  respectively]. These relationships remained unchanged when percent legume biomass in the plot was added to the model first.

Finally we asked whether the effect of legumes on N availability was greater for plant species that were dominant in mixture. We calculated the mean biomass per 2,000 seeds planted for each species in polyculture and in monoculture and used the ratio of the two values as a measure of dominance in mixture. As a measure of the

strength of the effect of legumes on N availability we calculated the slope of the regression between  $\delta^{15}\text{N}$  and percent legumes biomass in the mix for each species. There was no relationship between our measure of dominance in mixture and the strength of the legume effect ( $P>0.1$  for all species together and for non-fixers only). Among the species that showed a clear decrease in  $\delta^{15}\text{N}$  values as the proportion of legumes increased were two species that were dominant in polyculture (*Phleum pratense* and *Phalaris arundinacea*) as well as two species that showed a decreased biomass in polyculture (*F. ovina* and *R. acetosa*).

## Discussion

### Whole-community dynamics: changes over time

In our experimental plots most of the above ground biomass was removed annually, and no fertilizer was added. Over time one would therefore expect soil nutrient availability to decline and the root : shoot ratio of plants to increase as nutrients become more limiting (e.g. Chapin 1980; Givnish 1983; Bloom et al. 1985). As expected, overall %N in above ground tissues declined between 1996 and 1998. However, root %N did not decline between 1996 and 1998. Similarly, total N did not show a consistent pattern over time above ground or below ground, primarily as a result of large fluctuations between years in biomass. Furthermore, the root to shoot ratio did not change consistently between 1996 and 1998. There is therefore only limited evidence that total N availability to plants decreased over time.

One explanation for the lack of a clear decline is that additional nitrogen was made available by nitrogen fixers. Legumes have been shown to increase soil nitrate concentrations following the release of symbiotically fixed N through root and shoot turnover in intercropping experiments (Mallarino and Wedin 1990; Ranells and Wagger 1997). Such an increase in reliance on fixed N is supported by three lines of evidence: (1) the consistent decline in  $\delta^{15}\text{N}$  values across years for plots with legumes but not those without legumes; and (2) the decline in  $\delta^{15}\text{N}$  between 1996 and 1998 for all species in all functional groups, and (3) the strong negative relationships between mean %N and  $\delta^{15}\text{N}$  values in all years. One should be cautious in interpreting these changes in  $\delta^{15}\text{N}$  as being due entirely to changes in relative use of recently fixed N versus soil N, as a decrease in soil N availability may result in a decrease in nitrification and hence in less plant uptake of  $^{15}\text{N}$  enriched ammonium, which would also alter  $\delta^{15}\text{N}$  values in the same direction (Garten and van Miegrot 1994; Högberg 1997). However, additional data support the former hypothesis. First, although the pattern of decline in %N above ground over time was the same for plots with and without legumes, starting values were lower for plots without legumes and this therefore represents a greater proportional drop in %N for this group. The decrease in  $\delta^{15}\text{N}$  for plots with



legumes (but not those without legumes) suggests this difference in the rates of declines is due to the presence of legumes. Consistent with this hypothesis, when N concentrations of legumes and non-fixers within the same plot were compared there was no difference, even though on a per-species basis the N concentrations of legumes were much higher. Percent legume biomass had a strong effect on above ground total biomass but not root total biomass, suggesting that plants were increasing their allocation to shoots when N became more available. Unfortunately we could not separate roots by species and therefore could not test whether all species showed such a shift. In general, then, it appears that total N availability declined initially, but that the communities relied increasingly on N fixed by legumes to compensate for the decline in soil N. Additionally, legumes may have increased activity of soil microbes involved in mineralization (Spehn et al. 2000), thereby increasing N availability to all plants.

#### Whole-community dynamics: effects of diversity

Increased diversity could result in increased N in the community through the sampling effect (a higher probability of including at least some nitrogen-fixers in the community, leading to greater dominance by these relatively high-N species and/or to greater N availability for all plants in the community) or through greater use of soil N due to niche complementarity. The strength of these effects should increase as soil N becomes more limiting. Our path diagrams indicate that the primary pathway through which increased plant diversity resulted in increased total nitrogen was through greater plant biomass, and the strength of this effect increased over time both above ground and below ground (the significant effect of diversity on percent legume biomass is probably simply a reflection of their rapid initial establishment and their presence in all high-diversity plots). Furthermore, when we included both diversity and the presence of legumes in a stepwise multiple regression, we found that over time the importance of diversity increased: in the first two years only the presence of legumes contributed to biomass, while in 1998 both were important, and in 1999 only diversity explained a significant amount of the variation in above ground biomass. There was no evidence that dominance of legumes increased over time in high diversity plots or in the experiment as a whole; their biomass as a proportion of the total was fairly constant after 1997. Furthermore, once legume presence was controlled for, percent legume biomass explained biomass (whether above ground or below ground) only in the first year. We therefore conclude that although a sampling effect did contribute to greater plant biomass (and therefore a greater N pool), diversity explained variation in biomass beyond that explained by the inclusion of legumes.

There are two possible explanations for the positive effect of high diversity on biomass but not %N: (1) higher diversity leads to greater N availability or capture,

which in turn results in greater growth but no change in N concentration due to a dilution effect; or (2) higher diversity leads to greater capture of another different limiting resource (e.g. light, water, or other nutrients), which results in greater growth while greater root biomass allows N capture to keep pace with overall growth. These two explanations are not mutually exclusive, and our experiment could not distinguish between them. Hooper and Vitousek (1998) found evidence for greater resource use (including inorganic nitrogen, phosphorus, and soil water) in communities with more functional groups, but no decrease in nitrogen loss from leaching. That experiment used species selected specifically for their differences in traits relevant to nutrient cycling, so a stronger relationship between diversity and nutrient capture might be expected than was observed in our experiment. To clearly distinguish between these possible mechanisms requires an experiment in which (1) legumes are both absent and present at all levels of diversity, (2) multiple resources are manipulated, and (3) multiple nutrients in soil and in plants are intensively monitored. Improved sampling of roots, preferably through removal of entire plants, would also be desirable.

#### Individual species and functional group dynamics

Values for  $\delta^{15}\text{N}$  indicate that all three *Trifolium* species functioned as nitrogen fixers starting in 1996. *Lotus corniculatus* appears to have been dependent on soil N in 1996, but by 1998 its  $\delta^{15}\text{N}$  values were not different from zero, indicating that it too was a functional nitrogen fixer. The additional N provided by these  $\text{N}_2$ -fixing species appears to have been made available to all non-fixing species as indicated by the decrease in  $\delta^{15}\text{N}$  values over time for all species and negative relationships between  $\delta^{15}\text{N}$  and increased biomass of legumes for non-fixers as a group.

Furthermore, the relatively high  $\delta^{15}\text{N}$  values for *Trifolium* species at high percent legume biomass and low diversity suggest that in monoculture these species may obtain more of their N from the soil than in plots with low percent legume biomass, where competition with other species may force greater reliance on fixed N. Although species differed in their ability to take advantage of increased N from  $\text{N}_2$  fixation, there was no evidence to suggest that dominant species were the primary beneficiaries: for both dominant and subordinate species %N increased and  $\delta^{15}\text{N}$  decreased with increased proportion of legumes in the plot.

#### Conclusions

Our data emphasize the need for multi-year studies of responses of ecosystem processes to changes in diversity since the importance of species richness for the maintenance of high biomass became more important over time. Species richness influenced N dynamics primarily

by increasing biomass, suggesting that other ecosystem processes (such as carbon fixation) were more affected than N uptake per se. Clearly, the percent legumes in plots was the single most important factor controlling %N, total N and  $\delta^{15}\text{N}$ . Furthermore, the additional N provided by the presence of N-fixing legumes appeared to increase N availability to all species, whether dominant or subordinate. However, these legumes are not long-lived, and the continued presence of legumes (other than *T. repens*) in our plots is dependent on regeneration from seed. Future studies should focus on the mechanisms through which diversity affects the N pool and the role that species richness plays in maintaining nitrogen fixers in a community over time.

**Acknowledgements** We thank H. Wallmark, K. Johanson, A-S Hahlin, and L. Ericson for technical assistance, and S. Forsberg, H. Gustafsson, K. Östlund, and I. Söderbergh, and the volunteers who helped with harvests and weeding. Svalöf-Weibull AB (Umeå) kindly provided seeds for five species. The manuscript was improved by the comments of two anonymous reviewers. Funding for this project was provided by the European Commission (Framework IV, contract ENV4-CT95-0008).

## References

- Aarssen LW (1997) High productivity in grassland ecosystems: affected by species diversity or productive species? *Oikos* 80:183–184
- Barrie A, Lemley M (1989) Automated N-15 / C-13 analysis of biological materials. *Int Lab Tech* 19:82–91
- Berendse F (1979) Competition between plant populations with different rooting depths. I. Theoretical considerations. *Oecologia* 43:19–26
- Berendse F (1981) Competition between plant populations with different rooting depths. II. Pot experiments. *Oecologia* 48:334–341
- Berendse F (1983) Interspecific competition and niche differentiation between *Plantago lanceolata* and *Anthoxanthum odoratum* in a natural hayfield. *J Ecol* 71:379–390
- Bloom AJ, Chapin FS III, Mooney HA (1985) Resource limitation in plants – an economic analogy. *Annu Rev Ecol System* 16:363–392
- Chapin FS III (1980) The mineral nutrition of wild plants. *Annu Rev Ecol Syst* 11:233–260
- Farley RA, Fitter AH (1999) The responses of seven co-occurring woodland herbaceous perennials to localized nutrient-rich patches. *J Ecol* 87:849–859
- Finlay BJ, Maberly SC, Cooper JI (1997) Microbial diversity and ecosystem function. *Oikos* 80:209–213
- Garten CT, van Miegrot H (1994) Relationships between soil nitrogen dynamics and natural  $^{15}\text{N}$  abundance in plant foliage from the Great Smoky Mountain National Park. *Can J For Res* 24:1636–1645
- Givnish TJ (1983) Optimal stomatal conductance, allocation of energy between leaves and roots, and the marginal cost of transpiration. In: Givnish TJ (ed) *On the economy of plant form and function*. Cambridge University Press, New York, pp 171–213
- Handley LL, Scrimgeour CM (1997) Terrestrial plant ecology and  $^{15}\text{N}$  natural abundance: the present limits to interpretation of uncultivated systems with original data from a Scottish old field. *Adv Ecol Res* 27:133–212
- Hector A, Schmid B, Beierkuhnlein C, Caldeira MC, Diemer M, Dimitrakopoulos PG, Finn J, Freitas H, Giller PS, Good J, Harris R, Höglberg P, Huss-Danell K, Joshi J, Jumpponen A, Körner C, Leadley PW, Loreau M, Minns A, Mulder CPH, O'Donovan G, Otway SJ, Pereira JS, Prinz A, Read DJ, Scherer-Lorenzen M, Schulze E-D, Siamantziouras A-S D, Spehn E, Terry AC, Troumbis AY, Woodward FI, Yachi S, Lawton JH (1999) Plant diversity and productivity in European grasslands. *Science* 286:1223–1127
- Hector A, Beale AJ, Minns A, Otway SJ, Lawton JH (2000) Consequences of the reduction of plant diversity for litter decomposition: effects through litter quality and microenvironment. *Oikos* 90:357–371
- Högberg P (1997)  $^{15}\text{N}$  natural abundance in soil-plant systems. *New Phytol* 137:179–203
- Hooper DU, Vitousek PM (1998) Effects of plant composition and diversity on nutrient cycling. *Ecol Monogr* 68:121–149
- Huston MA (1997) Hidden treatments in ecological experiments: re-evaluating the ecosystem function of biodiversity. *Oecologia* 110:449–460
- Junk G, Svec HV (1958) The absolute abundance of the nitrogen isotopes in the atmosphere and compressed gas from various sources. *Geochim Cosmochim Acta* 14:234–243
- Mallarino AP, Wedin WF (1990) Seasonal distribution of topsoil ammonium and nitrate under legume-grass and grass swards. *Plant Soil* 124:137–140
- Mariotti A (1983) Atmospheric nitrogen is a reliable standard for natural  $\delta^{15}\text{N}$  abundance measurements. *Nature* 303:685–687
- Mitchell R J (1993) Path analysis: pollination. In: Scheiner SM, Gurevitch J (eds) *Design and analysis of ecological experiments*. Chapman and Hall, New York, pp. 211–231
- Naeem S, Håkansson K, Lawton JH, Crawley MJ, Thompson LJ (1996) Biodiversity and plant productivity in a model assemblage of plant species. *Oikos* 76:259–264
- Nordin A, Höglberg P, Näsholm T (2001) Soil N form availability and plant N uptake along a boreal forest productivity gradient. *Oecologia* 129:125–132
- Ohlsson KEA, Wallmark PH (1999) Novel calibration with correction for drift and non-linear response for continuous flow isotope ratio mass spectrometry applied to the determination of  $\delta^{15}\text{N}$ , total nitrogen,  $\delta^{13}\text{C}$ , and total carbon in biological material. *Analyst* 124:571–577
- Ranells, NN, Waggoner MG (1997) Grass-legume bicultures as winter annual cover crops. *Agron J* 89: 659–665
- SAS (1999) SAS/STAT user's guide. Release 6.12 edn. SAS Institute, Cary, N.C. USA
- Schemske DW, Horvitz CC (1988) Plant-animal interactions and fruit production in a neotropical herb: a path analysis. *Ecology* 69:1128–1137
- Schmid BJ, Joshi J, Schläpfer F (2001) Empirical evidence for biodiversity – ecosystem functioning relationships. In: Kinzig A, Tilman D, Pacala P (eds) *Functional consequences of biodiversity: experimental progress and theoretical extensions*. Princeton University Press, Princeton
- Spehn EM, Joshi J, Schmid B, Alpehi, J, Körner C (2000) Plant diversity effects on soil heterotrophic activity in experimental grassland ecosystems. *Plant Soil* 224:217–230
- Symstad AJ, Tilman D, Willson J, Knops JMH (1998) Species loss and ecosystem functioning: effects of species identity and community composition. *Oikos* 81:389–397
- Tilman D, Wedin D, Knops J (1996) Productivity and sustainability influenced by biodiversity in grassland ecosystems. *Nature* 379:718–720
- Van den Bergh JP, de Wit CT (1960) Concurrentie tussen timothee (*Phleum pratense* L.) en reuk-grass (*Anthoxanthum odoratum* L.). *Jaarboek Instituut voor Biologisch en Scheikundig Onderzoek van Landbouwgewassen*, pp 155–166
- Wardle D (1999) Is “sampling effect” a problem for experiments investigating biodiversity-ecosystem function relationships? *Oikos* 87:403–407
- Wardle DA, Bonner KI, Nicholson KS (1997) Biodiversity and plant litter: experimental evidence which does not support the view that enhanced species richness improves ecosystem function. *Oikos* 79:297–258
- Wright S (1934) The method of path coefficients. *Ann Math Stat* 5:161–215