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The role of legumes as a component of biodiversity in a cross-European study of grassland biomass nitrogen

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To investigate how plant diversity loss affects nitrogen accumulation in above-ground plant biomass and how consistent patterns are across sites of different climatic and soil conditions, we varied the number of plant species and functional groups (grasses, herbs and legumes) in experimental grassland communities across seven European experimental sites (Switzerland, Germany, Ireland, United Kingdom (Silwood Park), Portugal, Sweden and Greece). Nitrogen pools were significantly affected by both plant diversity and community composition. Two years after sowing, nitrogen pools in Germany and Switzerland strongly increased in the presence of legumes. Legume effects on nitrogen pools were less pronounced at the Swedish, Irish and Portuguese site. In Greece and UK there were no legume effects. Nitrogen concentration in total above-ground biomass was quite invariable at 1.66 ± 0.03% across all sites and diversity treatments. Thus, the presence of legumes had a positive effect on nitrogen pools by significantly increasing above-ground biomass, i.e. by increases in vegetation quantity rather than quality. At the German site with the strongest legume effect on nitrogen pools and biomass, nitrogen that was fixed symbiotically by legumes was transferred to the other plant functional groups (grasses and herbs) but varied depending on the particular legume species fixing N and the non-legume species taking it up. Nitrogen-fixation by legumes therefore appeared to be one of the major functional traits of species that influenced nitrogen accumulation and biomass production, although effects varied among sites and legume species. This study demonstrates that the consequences of species loss on the nitrogen budget of plant communities may be more severe if legume species are lost. However, our data indicate that legume species differ in their N_2 fixation. Therefore, loss of an efficient N_2-fixer (*Trifolium* in our study) may have a greater influence on the ecosystem function than loss of a less efficient species (*Lotus* in our study). Furthermore, there is indication that P availability in the soil facilitates the legume effect on biomass production and biomass nitrogen accumulation.

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The relationship between biodiversity and ecosystem processes has emerged as a central issue in ecology during the last decade. Recent experimental work, manipulating plant species and/or plant functional group diversity in randomly assembled grassland communities, mainly has found relationships between increasing plant diversity and biomass production that range from positive to null (Tilman et al. 1997a, Hector et al. 1999, Tilman et al. 2001) or resource uptake (Tilman et al. 1996, Hooper and Vitousek 1998) in experimental grassland communities. The interpretation of these results led to many debates and controversies (Loreau et al. 2001). Central to the difficulties in interpreting this positive diversity-productivity relationship in experimental studies is to disentangle the relative contributions of species within a diverse community. On the one hand, it is possible that a very small percentage of plant species, even in the most diverse communities, governs ecosystem processes such as growth or nutrient use. On the other hand, it is possible that all species contribute relatively equally when averaged over space and time. In general, the effect of species richness on ecosystem processes depends on ecological differences among the species within a community (Schmid et al. 2001). Teasing apart the many facets of biodiversity, taxonomic, functional, and identity, is difficult for species that are similar, but legumes represent a distinct, unique functional group in grasslands due to their ability to directly use atmospheric N_2 as an additional nitrogen source via symbiotic root bacteria. The role of the legume association in fixing nitrogen, and its effects on parameters such as soil fertility or litter quality, is well studied, but its role in biodiversity experiments is poorly understood. In general, legumes may be complementary in nitrogen usage with non-fixing species and can also interact positively with other species (Harper 1977) by increasing soil nitrogen pools and nitrogen supply rates for other plants. Agricultural intercropping experiments, mainly focussed on the influence of nitrogen-fixers in low-diversity sytems, have shown that nitrogen fixers commonly increase the nitrogen availability for their non-legume neighbors (Vandermeer 1989, 1990). Nitrogen-fixing organisms have a large effect on nitrogen availability in many natural and agricultural ecosystems, since nitrogen is the nutrient in the soil that most likely limits plant productivity (Chapin et al. 1986, Vitousek and Howarth 1991, Marschner 1995). In the long run, the activity of nitrogen fixers might even alleviate growth limitation by nitrogen at the ecosystem level provided P-availability is sufficient to support the high P demand of N-fixation (Vitousek and Howarth 1991). Generally, the amount of nitrogen symbiotically fixed by grassland legumes ranges from 15–680 kg N ha^{-1} yr^{-1}, depending on legume species and genotypes, bacterial strains, and abiotic factors (Ledgard and Giller 1995, Marschner 1995). Legumes might therefore constitute a key component of biodiversity affecting the relationship between diversity and ecosystem processes that are influenced by nitrogen (Vitousek and Howarth 1991, Chapin et al. 1996) and one or a few keystone legume species may dominate nutrient uptake in more diverse communities (Tilman and Wedin 1991). However, effects of plant diversity on ecosystem processes not only arise from differences between, but also within functional groups.

Plant diversity might increase total nitrogen uptake through complementarity in resource use. If nutrients are acquired from different portions of the available pool (in space or time, or in different chemical forms) through effective niche differentiation, total plant nitrogen-uptake may be greater and losses to leaching may be lower as diversity increases (Trenbath 1974, Bazzaz 1987, Vandermeer 1990, Hooper and Vitousek 1997). For example, a decline in soil nitrate concentration has been observed as plant species richness increased in experimental manipulations ranging from 1 to >20 species (Tilman et al. 1996, Niklaus et al. 2001). In the long run, different requirements of plant species for nitrogen and/or other nutrients (e.g. phosphorus) may also contribute to diversity and compositional effects on the nitrogen cycle. Slow-growing species usually respond less to nitrogen addition than fast-growing ones, due to a lower demand (Andrew and Johansen 1978). Species-specific differences in nitrogen concentration in vegetation commonly translate into different litter quality and subsequent decomposition rates, which in turn feed back on nitrogen availability and thus species composition and nitrogen-cycling (Swift et al. 1979, Hobbie 1992, Wedin and Pastor 1993). Wedin and Tilman (1990) demonstrated that annual net nitrogen mineralization diverged up to 10-fold under different perennial grass species grown on initially identical soils. This divergence corresponded to differences in tissue nitrogen concentrations and belowground biomass investments of species. In soils with low levels of nitrogen, nutrient mineralization limits nutrient uptake and therefore biomass production (Flanagan and Vancleve 1983), whereas in nitrogen-rich agricultural soils such as old-fields and productive grassland, plant vigor, i.e. sink strength, controls plant nutrient uptake.

We examined the relative contributions of the different components of biodiversity on ecosystem functioning by varying taxonomical, functional and identity components in a large-scale manipulation of plant biodiversity in experimental grassland plots. The study was part of the BIODEPTH project (BIODiversity and Ecological Processes in Terrestrial Herbaceous ecosystems, a pan-European study on the relationship between biodiversity and ecosystem processes). This experimental system provides a unique opportunity to test biodiversity hypotheses. In this part of the overall research program, we focused on legumes explicitely to test three hypotheses related to the role of biodiversity components in ecosystem nitrogen:

(1) Plant diversity (number of species and functional groups) increases ecosystem nitrogen pools and biomass production in different grassland ecosystems.

(2) Legumes are keystone species in generating this relationship (in the sense that their impact on ecosystem processes is disproportionate to their contribution to the overall biomass) irrespective of their specific identity, and local conditions (soil types and climatic conditions etc.).

(3) Non-fixing plant species benefit from symbiotically fixed nitrogen by legumes in multispecies assemblages.

Methods

Study sites

This study was carried out at seven of the eight European experimental sites: Switzerland (CH, 47°N, 08°E, 439 m a.s.l.), Germany (G, 50°N, 12°E, 350 m a.s.l.), Ireland (IR, 52°N, 08°W, 75 m a.s.l.), Great Britain (UK, Silwood Park, 51°N, 01°W, 50 m a.s.l.), Portugal (P, 39°N, 09°W, 25 m a.s.l.), Sweden (S, 64°N, 20°E, 12 m a.s.l.) and Greece (GR, 39°N, 27°E, 30 m a.s.l.). Sites differed widely in climate and other major environmental factors. For details on climate, previous land use, method of site preparation, number of biomass harvests, and mean above-ground biomass production see Hector et al. (1999). For more detailed information on single sites see Diemer et al. (1997), Mulder et al. (1999), Scherer-Lorenzen (1999), Hector et al. (2000a) and Troumbis et al. (2000).

Establishment of the experimental communities

The field experiments were established in spring 1995 in Switzerland, autumn 1996 in Portugal and spring 1996 at all other sites. Plots of at least 2 × 2 m (CH: 2 × 8 m, S: 2.2 × 5.2 m) were seeded with 2000 viable seeds m^{-2} divided equally between the number of species in each plant assemblage. Seeds were locally collected as far as possible, or otherwise purchased from national commercial sources avoiding agricultural cultivars. Prior to sowing, the existing vegetation was removed and the soil seed bank was eliminated by continuous weeding (CH, S), steam sterilization (G), heat (soil was covered with black plastic for 2.5 months, P) or methyl bromide application (UK, IR, GR). To reduce post-application effects of methyl bromide on legumes, an inoculum of Rhizobium was applied. Plots were regularly weeded to remove unwanted species emerging from the remaining seedbank or invading from outside. Plots were separated by 1.5 m wide borders sown with non-clonal grass species (CH, G, IR, UK, S, P) or were not seperated (GR). The plots were not fertilized during the experimental period.

Experimental design

We established five levels of species richness, ranging from monocultures to higher diversity mixtures. The highest diversities approximately matched background levels of diversity in comparable semi-natural grasslands at each site (Table 1). In addition, we varied the number of functional groups-graminoids (grasses), nitrogen-fixing legumes and other herbaceous species (herbs) within the different levels of species richness. At low levels (one or two species, respectively) only one or two functional groups could be included, whereas at high levels it was unrealistic that all species belonged to only one or two functional groups. As a result, if the factorial combinations of species richness and number of functional groups are considered, the design unavoidably contains empty cells. Further, we constrained our random selection of species from the local pool of

Table 1. The experimental design at each location, showing numbers of plots (assemblages containing legumes/all assemblages) per species richness level and for each functional group richness. Plant assemblages (where an assemblage is a particular species or a mixture of species) were replicated in two plots at each site, with the same mixture sometimes occurring at more than one site.

	Species richness										
	1	2	3	4	8	11	12	14	16	18	32
Germany	4/20	8/14		6/10	6/10				6/6		
Ireland	4/20	8/16	4/4	12/20	6/10						
Great Britain	4/22	4/12		6/12	6/10	6/10					
Switzerland	4/20	4/14		8/16	6/10						4/4
Portugal	6/28	2/10		10/10	4/4			4/4			
Sweden	8/24	6/12		10/12	6/6		4/4				
Greece	2/14	6/12		6/10	8/8					8/8	
Functional richness											
1	32/148	2/30		0/14	0/6	0/2					
2		28/60		18/40	10/18	2/4					
3			4/4	36/36	34/34	4/4	4/4	4/4	6 /6	8/8	4/4

grassland species such that all assemblages included the functional group of grasses. Each particular combination of species richness and number of functional groups, hereafter called a diversity level, was replicated with several different species compositions, hereafter called an assemblage, at each site to avoid confounding diversity effects per se with effects of species identity (Givnish 1994, Tilman et al. 1997b, Allison 1999). The number of replicates within diversity levels was reduced with increasing species richness because it was expected that the variability of ecosystem processes should be lower at high diversity as species overlap inevitably increases if the total species pool is fixed (Schmid et al. 2001). All assemblages were randomly allocated within two replicated blocks (except P with fully randomized plots). In total, the experiment comprised 7 sites, 426 plots and 182 different plant assemblages, with the same assemblage sometimes occurring at more than one site.

Biomass

Above-ground biomass of plants was determined by harvesting standing crop above 5 cm in one or two sampling areas of 20 × 50 cm per plot once (UK, P, S, GR) or twice (G, CH, IR) a year. Then the entire plots were mowed to 5 cm, following the harvesting regime used in agriculture of most areas. Plant samples were dried at 80°C for 24 hours before they were weighed. Diversity effects on plant biomass two years after sowing are reported in Hector et al. (1999). Total above-ground pools of nitrogen were calculated from all biomass harvests of the second year.

Nitrogen analysis

To study plot-scale levels of nitrogen in the vegetation, biomass samples were ground and analysed for nitrogen content. In Germany, Switzerland, Greece, UK and Sweden, % nitrogen of dry weight was measured by dry combustion using an automated C–H–N analyser (CH and GR: LECO CHN-900, LECO Corporation, St. Joseph, Mich., USA; G: C/N analyser, CARLO ERBA NA 1500, Mailand, Italy; S: Europa Scientific ANCA-NT). In P, UK and IR the samples were digested and analysed with a semi-automatic Kjeldahl procedure (Tecator Inc., Herndon, Virginia, USA).

At the German and Portuguese site nitrogen inputs from biological fixation and transfer of symbiotically fixed nitrogen to neighboring non-fixing plants were estimated with the ^{15}N natural abundance method (Lajtha and Marshall 1994, Högberg 1997). At the German site, leaf tissue from all plant species per plot were decomposed in an elemental analyser (NA 1110 CN; CE Instruments, Rodano, Italy) and analysed (online) in an isotope ratio mass spectrometer (Delta plus XL; Finnigan MAT, Bremen, Germany) via a modified ConFlo III open split interface (Werner et al. 1999). At the Portuguese site, leaf tissue of five species (2 grasses: *Holcus lanatus* and *Dactylis glomerata*; 2 legumes: *Trifolium subterraneum* and *Ornithopus compressus*; and 1 herb: *Plantago lanceolata*) were sampled in monoculture and in plots with highest diversity (14 sown species). Analyses were performed on an Europa Scientific ANCA-SL Stable Isotope Analyses System (Europa Scientific Ltd, Crewe, UK).

The $\delta^{15}N$ values were calculated relative to the atmospheric nitrogen isotope ratio:

$$\delta^{15}N\ [\text{Högberg 1997}] = (R_{sample}/R_{atmosphere} - 1) \times 1000,$$

where R represents the ratio $^{15}N/^{14}N$

The $\delta^{15}N$ signal of actively nitrogen fixing plants is similar to the atmospheric $\delta^{15}N$ signal and ranges from 0.998 and 1.002, because fractionation of nitrogen isotopes in symbiotic N_2-fixation is generally very small (Högberg 1997). In contrast, plants depending on soil nitrogen generally show higher $\delta^{15}N$ values, because microbes discriminate against compounds with the heavy isotope. Thus, the heavier isotope accumulates in the soil N-pool, from which non-legumes are supplied. This method roughly distinguishes actively nitrogen fixing plants from non-fixing ones and non-fixing plants with no or partial access to legume derived nitrogen (Högberg 1997), but it does not allow quantification of the amount of nitrogen fixed. Different mycorrhizal associations, differences in rooting depth and different humus age can interfere with $\delta^{15}N$ signals.

Deviation from expected nitrogen yield

We compared observed and expected nitrogen accumulation in biomass by the proportional index D_T (Wardle et al. 1997, Loreau 1998) and calculated as follows: $D_T = (O_T - E_T)/E_T$ where O_T is the observed nitrogen yield and E_T the expected nitrogen yield based on monoculture nitrogen yield of each species grown in the mixture ($E_T = (\Sigma_i\ E_i)/n_i$, where E_i is the monoculture nitrogen yield of a species and n_i the number of species in the mixture). We used 171 mixtures (of 426 total), containing only species which were grown in monoculture (Table 3).

Statistical analysis

The data were analysed with analysis of variance (ANOVA) using general linear model approaches (Neter and Wasserman 1974) implemented in Genstat 5 (Payne et al. 1993). The sequential analysis determined

Table 2a. Analyses of variance for above-ground nitrogen pool and biomass, all sites (locality) combined; effects of legumes tested before species richness.
Nitrogen pool

ANOVA of all sites Source of variation	d.f.	s.s.	%s.s.	m.s.	F	P
Locality	6	3335.557	23.1	555.926	39.854	0.0001
Locality × Block	6	83.694	0.6	13.949	2.037	0.0623
Legume presence	1	3606.513	25.0	3606.513	183.304	6.4×10^{-27}
Species richness	10	580.69	4.0	68.299	3.471	0.0004
Funct. Group richness	2	74.608	0.5	74.608	3.792	0.0250
Legume presence × Locality	6	2168.314	15.0	361.386	23.635	1.5×10^{-8}
Species richn. × Locality	18	274.044	1.9	29.787	1.947	0.0692
Fct. Group richn. × Locality	11	122.538	0.9	21.86	1.43	0.2283
Assemblage	135	2656.168	18.3	19.675	1.287	0.2511
Assemblage × Locality	22	336.376	2.3	15.29	2.233	0.0019
Residual	203	1390.022	8.4	6.847		
Total	415	14473.64	100	34.876		

Above-ground biomass

ANOVA of all sites Source of variation	d.f.	s.s.	%s.s.	m.s.	F	P
Locality	6	13830378	31.5	2305063	56.499	0.0001
Locality × Block	6	244786	0.5	40798	1.988	0.0689
Legume presence	1	5843988	13.3	5843988	79.501	2.7×10^{-15}
Species richness	10	2630883	6.0	1262922	17.181	7.1×10^{-20}
Funct. Group richness	2	326333	0.7	326333	4.439	0.0136
Legume presence × Locality	6	3718290	8.5	619715	10.130	2.1×10^{-5}
Species richn. × Locality	18	1455867	3.3	164214	2.684	0.0147
Fct. Group richn. × Locality	11	679857	1.5	117253	1.917	0.0934
Assemblage	137	10070627	23.0	73508	1.202	0.3182
Assemblage × Locality	22	1345879	3.1	61176	2.982	2.6×10^{-5}
Residual	205	4288290	8.6	20518		
Total	423	43848725	100.0	103661		

by our design and a priori hypotheses included the effects of locality (LO), species richness (S), number of functional groups (FG), the presence of legumes (L) and identity of species assemblage (C). According to the hierarchical structure of the experimental design the following terms served as error strata (Payne et al. 1993): block (within locality), assemblage (within diversity level), locality-by-assemblage interaction and plot (within all previous terms). To specifically test if the effects of diversity levels might be explained in part by effects of species or functional group identities, we also carried out analyses in which the a priori sequence of treatment terms was reversed (Fig. 1c). In particular, we were interested in the effects of legumes per se, i.e. ignoring their inevitable contribution towards increasing the numbers of species and functional groups (Fig. 1d). However, because of the unavoidable imbalance and co-linearity involved in biodiversity designs discussed above and elsewhere (Allison 1999) it is impossible to unequivocally separate the effects of legumes from other diversity effects with our design.

Results

We focus on nitrogen pools (Fig. 2), and consider nitrogen concentration and biomass as the two component variables that make up the integrated variable (Fig. 3). After presenting the analyses for the diversity-level and composition effects, we investigate the effects of legumes per se (Fig. 4, 5) and the transfer of symbiotically fixed nitrogen from legumes to the other plant functional groups (Fig. 6).

Above-ground nitrogen pools

When all sites were analysed together, above-ground nitrogen pools differed significantly among sites ($P < 0.001$, Fig. 2). Above-ground nitrogen pools in Greece and Portugal were especially low compared to other sites. Plant species richness ($P < 0.001$) and functional group richness ($P < 0.001$) on average increased above-ground nitrogen pools over all sites, but not to the same extent at each site (mean-squares for interactions with site smaller than for main effects but significant ($P < 0.001$)). The assemblage term (composition) had the largest effect on above-ground nitrogen pools ($P < 0.001$). While richness explained 22% of the total sum of squares (SS), species composition explained 44%, and a third of this variation (14% of SS) could be attributed to the presence or absence of one functional group, the nitrogen-fixing legumes (Fig. 2). However, the effect of legumes explained more (25% of total SS)

Table 2b. Analyses of variance for above-ground nitrogen pool and biomass, sites separated; effects of legumes tested before speceis richness.
Nitrogen

Single sites	Sweden		Ireland		GB		Germany		Switzerland		Portugal		Greece	
Source of var.	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*
Legume presence	26.7	0.0033	18.3	0.0013	8.6	0.0620	84.1	7.0×10^{-11}	70.0	3.2×10^{-9}	14.8	0.0545	2.8	0.3388
Species richness	5.1	0.6963	8.5	0.2157	9.1	0.4196	2.8	0.2696	1.7	0.6950	1.8	0.9704	11.3	0.4377
Spec. richn. × Leg.	2.1	0.6413	13.3	0.0393	7.8	0.4904	0.8	0.6583	1.0	0.7045	6.7	0.1851	2.8	0.6148
Func. gr. Richness	4.4	0.4036	0.8	0.7530	0.9	0.8245	0.8	0.4542	0.26	0.8375	0.8	0.6389	2.2	0.6820
Assemblage	39.2	0.0036	28.1	0.1774	46.5	0.0069	9.5	4.5×10^{-7}	15.6	0.0307	63	5.1×10^{-6}	45.3	0.0489
Residual	22.5		31.0		27.1		1.9		11.4		12.9		35.6	

Above-ground biomass

Single sites	Sweden		Ireland		GB		Germany		Switzerland		Portugal		Greece	
Source of var.	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*	%S.S.	*P*
Legume presence	13.26	1.5×10^{-4}	4.3	0.0024	1.6	0.1712	72.3	5.4×10^{-22}	56.3	4.1×10^{-13}	17.7	1.9×10^{-5}	0.2	0.7347
Species richness	6.5	0.6881	31.1	0.0116	21.1	0.0465	4.6	0.2819	2.8	0.6918	9.6	0.5006	6.6	0.5723
Spec. richn. × Leg.	2.6	0.6418	5	0.4634	6.6	0.4743	3.7	0.2516	1.3	0.7904	0	0.9071	2.4	0.5912
Func. gr. Richness	4.1	0.5019	2.8	0.2368	4.9	0.1171	0.4	0.4893	0.4	0.5968	0.3	0.7306	1.3	0.4524
Assemblage	48.6	0.0004	42.8	3.3×10^{-5}	39.3	0.0182	15.9	3.1×10^{-7}	26.5	0.0018	52.6	3.5×10^{-4}	45.8	0.2152
Residual	24.9		14		26.5		3.1		12.7		19.8		43.7	

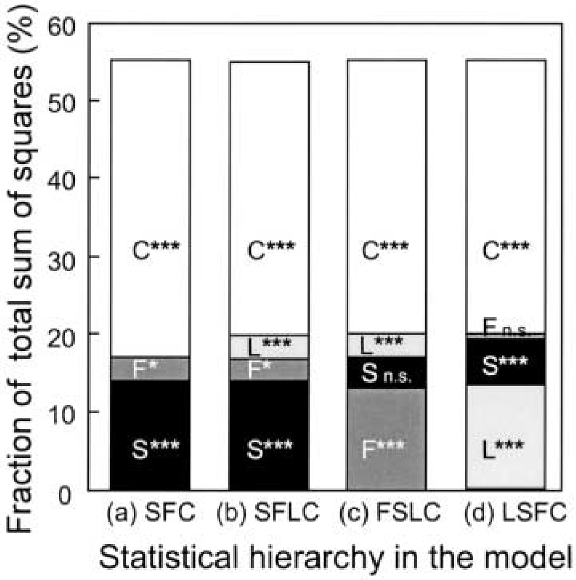

Fig. 1. Different components of diversity (S = species richness, F = functional group richness, L = legume presence/absence, C = remaining composition effect) explain different percentages of total variation (SS) depending on hierarchy of the statistical model. Note that (a) and (b) are the only adequate models for testing the given experimental design, whereas (c) and (d) are exploratory analyses, revealing that F and L explain as much variance as S if tested first, because all three factors (S, F and L) show some degree of co-linearity.

when entered first into the a posteriori ANOVA-model and differed strongly between sites ($P < 0.001$, Table 2a). In Germany and Switzerland, nitrogen pools then appeared to be driven mainly by legumes (84% and 70% of SS, $P < 0.001$), in Sweden, Ireland and Portugal legumes were less important ($P = 0.05$, 15–27% of SS), and in Greece and UK they had no significant effects (see Table 2b). With this reverse fitting sequence the remaining part of the total SS explained by the richness terms explained less variation (4.5% of SS), showing that the diversity effect on above-ground nitrogen pools in the a priori model was confounded with part of the legume effect (see Fig. 2).

Nitrogen concentration

Nitrogen (N) concentration in the above-ground biomass ranged from about 0.5–4% with an average of 1.66% and standard error (SE) of 0.03%. N concentration varied significantly among sites ($P < 0.001$, Fig. 3), and also site-by-diversity interactions were significant, for both species richness ($P < 0.001$) and number of functional groups ($P < 0.001$). As with the nitrogen pools, the assemblage term had the largest effect on nitrogen concentration, explaining 60% of the total SS ($P < 0.001$). Almost half of this effect of species composition could again be attributed to the presence of

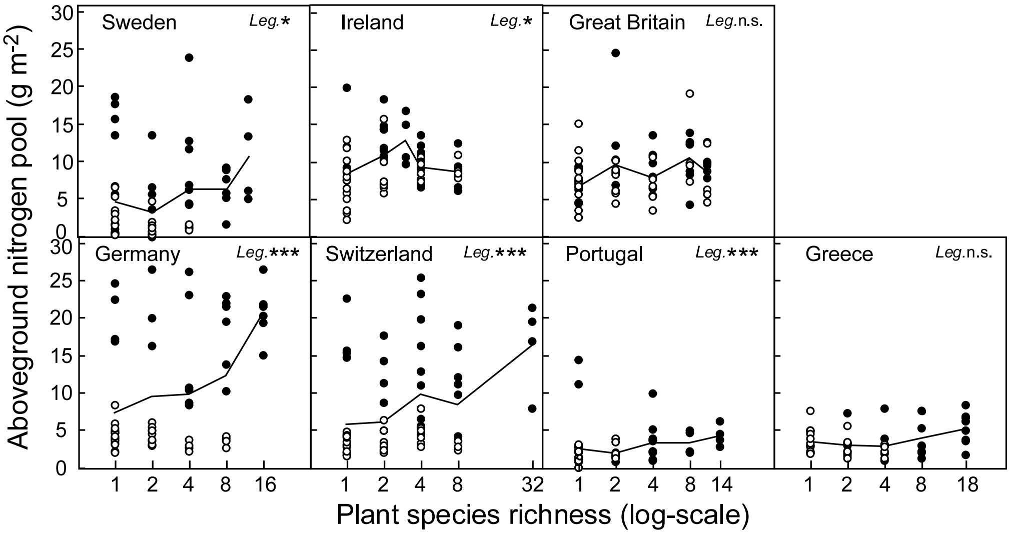

Fig. 2. Species richness effects on above-ground nitrogen pool at different sites. Closed symbols for assemblages with legumes, open symbols without legumes. Each symbol represents a plot. The line links arithmetic means for all plots per diversity level.

legumes in the assemblages, leading to higher nitrogen concentration ($P < 0.001$, 25% of total SS). If effects of legumes on nitrogen concentration per se were investigated, by fitting biomass fraction of legumes as a covariate before the diversity terms (sequence of model (d) in Fig. 1), they explained 41% of total SS ($P < 0.001$). A posteriori individual species contrasts explained little of the overall composition effect. Only four species (of a total of 100 species) showed significant effects on the nitrogen concentration of the community in which they were present (*Trifolium repens*, 5.9% of SS, $P < 0.001$, *Trifolium hybridum*, 4.2% of SS, $P = 0.002$; *Trifolium pratense*, 1.8% of SS, $P = 0.04$; *Plantago lanceolata* (the only non-leguminous forb), 1.7% of SS, $P < 0.05$). Note that contrasts differed in balance and power depending on species occurence.

Above-ground biomass

The size of the biomass nitrogen pool was largely determined by the amount of biomass itself, which was more variable than the nitrogen concentration of the plant tissue (Fig. 3). Our analysis of peak season above-

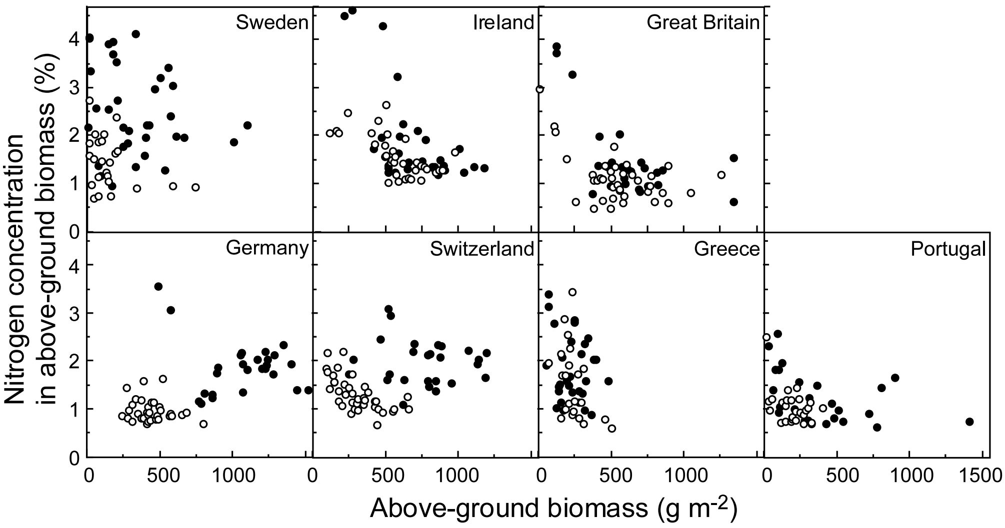

Fig. 3. Legume effects on the relationship between the two variables that determine nitrogen above-ground pools, above-ground biomass (g m^{-2}) and tissue nitrogen concentration (%). Closed symbols represent assemblages with legumes, open symbols assemblages without legumes.

Table 3. Differences in yield (D_T) of total above-ground nitrogen pool (mean ± S.E.). $D_T > 0$ indicates more nitrogen in mixtures than in the average of component species monocultures ("overyielding").

Sites	Legumes in mixtures	D_T of nitrogen yield
Sweden	−	−0.09 ± 0.31 (n = 9)
	+	0.32 ± 0.20 (n = 33)
Ireland	−	0.20 ± 0.08 (n = 20)
	+	0.09 ± 0.06 (n = 26)
Great Britain	−	0.22 ± 0.18 (n = 14)
	+	0.39 ± 0.11 (n = 16)
Germany	−	−0.08 ± 0.06 (n = 10)
	+	0.63 ± 0.16 (n = 6)
Switzerland	−	0.12 ± 0.12 (n = 10)
	+	0.43 ± 0.25 (n = 4)
Portugal	−	0.12 ± 0.09 (n = 8)
	+	0.60 ± 0.17 (n = 20)
Greece	−	−0.04 ± 0.21 (n = 4)
	+	−0.05 ± 0.31 (n = 2)

ground biomass across all sites (Hector et al. 1999) showed a log-linear decrease with declining species richness and number of functional groups. This analysis was addressing the diversity effects, as implied in the experimental design. If analysed separately, at three sites the general trend was not the best fit to the data. In Ireland and UK, the decrease was monotonic and in Greece no trend could be detected. In the across-sites analysis, the assemblage term explained 39% of the total SS and only 3% of SS could be attributed to the presence/absence of legumes (Hector et al. 1999). One particular species, the legume *Trifolium pratense*, had a particularly marked effect and its omission reduced productivity by approximately 360 g m^{-2} on average and across sites (Hector et al. 1999). In this analysis (i.e. at seven of the eight BIODEPTH sites), when the fitting sequence was reversed and the presence of legumes entered first into the a posteriori ANOVA model (Table 2a), legume presence explained 9.5% of the total SS (Table 2a). As for the nitrogen pools, with this reverse fitting sequence the remaining part of the total SS explained by the diversity terms was much smaller, though still significant for species richness (Table 2a). Thus, the diversity effect on above-ground biomass in the a priori model was largely confounded with almost all (68%) of the legume effect (Fig. 1). However, with the fitting sequence of the a posteriori ANOVA model, the effect of legumes varied significantly among sites (Table 2b). While in Germany and Switzerland the effect of legumes explained more than half of the total variation in above-ground biomass (72% and 55% of SS), there were no significant legume effects in UK and Greece (1.6% and 0.2%), and at other sites (Ireland, Sweden and Portugal) the effect was significant, but not very strong (8–16% of SS, Table 2b). The presence or absence of legumes explained more of the variation in above-ground biomass than an alternative a posteriori model, in which the relative contribution of legumes to the above-ground biomass was used as a covariate instead. We performed a series of Box-Cox transformations of the covariate y = "Biomass fraction of legumes" with $y' = y^{\lambda}$, where λ was decreased from 1 to 0.001, thereby increasingly transforming the biomass fraction of legumes to a legume presence/absence variable, where presence/absence now refers to the situation at harvest rather than sowing. These transformed covariates explained more variation in the statistical analysis of all sites, the smaller λ was in the transformation, therefore the more the legume biomass covariates were similar to presence/absence data (3.3% of SS for $\lambda = 1$ (= untransformed), 7% of SS for $\lambda = 0.001$). The first reported presence of legumes contrast, corresponding to a sowing presence/absence covariate at the beginning of the experiment, explained even more of the total SS (10%) indicating that the legume effects were accumulating or integrating over time (i.e. the above-ground biomass two years after sowing was not only related to the occurrence of legumes in the communities at harvest, but rather to their presence at the different times throughout the experiment).

Complentarity in nitrogen yield

Nitrogen yield in mixtures should be greater than in monocultures, if nitrogen limits production and plants are complementary in their nitrogen use. To test for complementary nitrogen use, we calculated the difference (D_T) between mixture and monoculture treatments for nitrogen yield in above-ground biomass (Table 3). Complementarity in nitrogen use would give $D_T > 0$, whereas competition for nitrogen would either give $D_T \approx 0$, if there are compensatory trade-offs among species or even $D_T < 0$, if species interfere or species, which are less efficient in capturing nitrogen, dominate the community (Hooper 1998). We found positive means of D_T at all sites except Greece and significant increases of D_T with legume presence ($P = 0.02$) and species richness ($P = 0.017$). In Sweden and Germany, mixtures containing no legumes showed no complementarity (mean $D_T < 0$, Table 3).

Success of legumes

To test if legumes would benefit from the overall N limitation, we compared relative abundance of legumes to non-nitrogen fixing plants at the beginning of the experiment (sown fraction of legumes) with the biomass fraction of legumes in the second year (Fig. 4). We expected that legume species would increase provided that other nutrients such as P permit. In Germany and Switzerland the fraction of legumes two years after sowing was higher than the sown fraction ($P < 0.001$), in Sweden and Portugal the biomass fraction was the same as the sown fraction ($P = 0.17$ and 0.09 respecitvely) and in Ireland, UK and Greece the legume biomass fractions were significantly lower than expected from seed fraction ($P < 0.001$).

Transfer of symbiotically fixed nitrogen to non-fixing plants

At the German site with the strongest and the Portuguese site with an intermediate legume effect on above-ground biomass and nitrogen accumulation, differences in ^{15}N natural abundance and leaf nitrogen concentrations demonstrate that legumes in this experiment did fix nitrogen symbiotically (Fig. 5). At the German site, three different groups were clearly identified: (1) non-legume species – i.e. all grasses and herbs, (2) the legumes *Lotus corniculatus*, *Vicia cracca* and *Vicia sepium*, and (3) the legumes *Trifolium pratense* and *Trifolium repens* (Fig. 5a). Both measures differed significantly between non-legumes and legumes (%N: $P < 0.001$; ^{15}N: $P < 0.001$; two-tailed Mann-Whitney U-test). Within legumes, leaf nitrogen concentrations differed significantly between the genera *Lotus*/*Vicia* and *Trifolium* ($P < 0.001$), but $\delta^{15}N$ values did not ($P = 0.371$). The Portuguese site also showed significant differences in ^{15}N ($P < 0.001$) and leaf nitrogen concentration ($P = 0.01$) between legumes (*Ornithopus compressus* and *Trifolium subterraneum*) and non-legumes (*Dactylis glomerata*, *Holcus lanatus* and *Plantago lanceolata*). We have data from the German site indicating that this symbiotically fixed nitrogen is transferred to non-nitrogen fixers (7 grasses, 2 non-leguminous forbs; Fig. 6). If grown in plots with legumes, most species had significantly higher nitrogen concentrations and significantly lower $\delta^{15}N$ values than when grown in monoculture or in a non-leguminous environment (Fig. 6). The high variability of leaf nitrogen and of the ^{15}N natural abundance in non-legume species was therefore not only caused by species-specific differences, but also by the nitrogen input through legume neighbors.

Discussion

Two years after sowing, nitrogen-fixation by legumes appeared to be the primary functional trait of species that influenced nitrogen accumulation and biomass production, although effects varied greatly among sites and not all legume species had similar impacts.

Nitrogen accumulation in the standing biomass primarily reflected species composition. Changing species

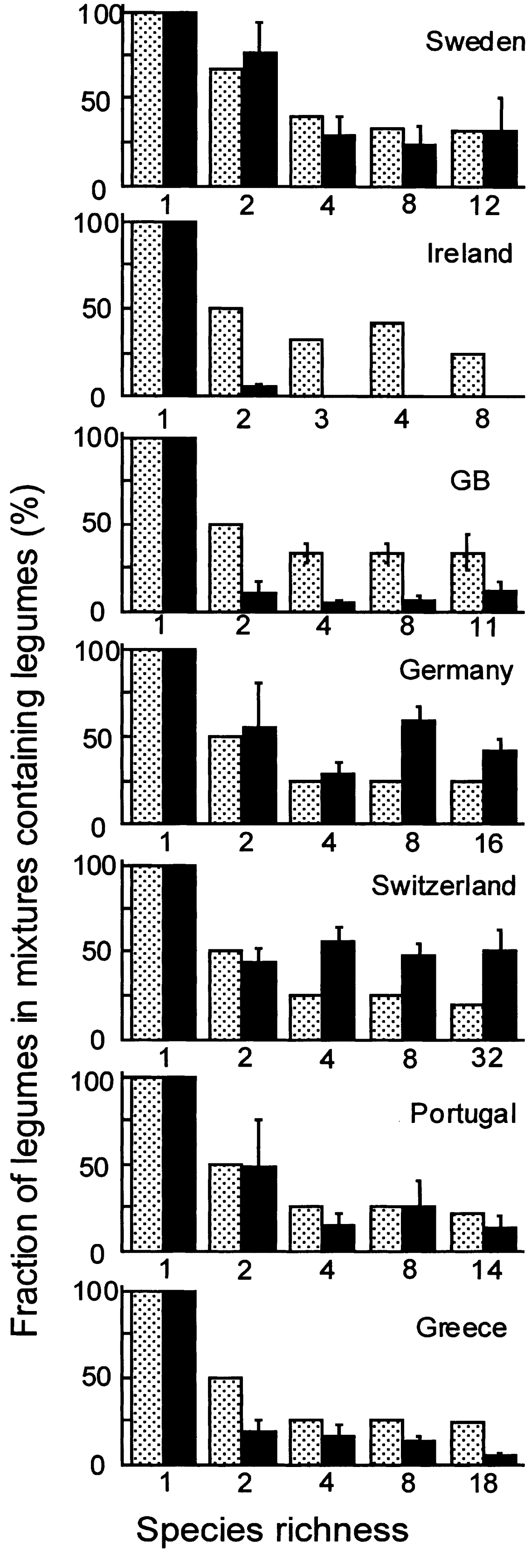

Fig. 4. Effect of plant species richness on legume biomass, expressed as a fraction (±SE) of both the total peak season biomass of year two (dark bars) and the sown seed fraction of legumes per diversity level (light bars) for each site.

abundance over time may thus alter nitrogen content of the communities (Swift and Anderson 1993, Huston and DeAngelis 1994). Several studies have shown that nutrient dynamics in natural or semi-natural ecosystems are influenced by the diversity of the plant community (Wedin and Tilman 1990, Ewel et al. 1991, Naeem et al. 1995, Tilman et al. 1997a, Hooper 1998, Hooper and Vitousek 1998). Many of these studies focused on soil nutrient depletion, but two of them analysed the effects of plant diversity on the total amount of nitrogen stored in the plant biomass (Tilman et al. 1997a, Hooper 1998), a valuable integrative measure of nitrogen incorporation over the whole growing period of a plant. Tilman et al. (1997a) found that nitrogen in above-ground vegetation significantly increased with increasing functional diversity, which paralleled an increase in biomass; this finding is supported by the present study. Hooper (1998) also found higher nitrogen yields in those mixtures containing legumes, but little evidence for complementary nitrogen yield among non-legume functional groups due to strong competition during critical parts of the growing season.

At several of our sites, both isotope and biomass data indicate that the presence of legumes enhanced nitrogen availability for co-occurring species, which increased their biomass production and tissue nitrogen concentrations. Nitrogen transfer from legumes to non-legume species had been shown many times, but mostly on agricultural and pasture systems (Henzell and Vallis 1977, Boller and Nösberger 1987, Laidlaw et al. 1996). The amount of nitrogen transferred from legumes to neighboring plants in mixed pastures ranged from 8–39% of the total nitrogen in the non-legume species, or from 3–102 kg N ha^{-1} a^{-1} (Ledgard and Giller 1995). The fixed nitrogen was made available to non-fixing neighboring plants by the mineralisation of legume litter (roots, nodules and shoots) (Peoples and Craswell 1992, Ledgard and Giller 1995), but possibly also by rhizodeposition and direct transfer by interconnected mycorrhizal hyphae, as most legumes have arbuscular mycorrhizal partners (Read 1993). This sort of nitrogen transfer might increase with plant species richness, as we found increased amounts of fine roots at some sites (G: Scherer-Lorenzen 1999 and CH: unpubl. data). Transfer may also increase with increased proximity of roots between legumes and non-legumes (Brophy et al. 1987).

The different facets of biodiversity effects

Biodiversity can affect ecosystem functioning through selection from the species pool, niche complementarity and positive interactions among species (Tilman et al. 1997b, Loreau 2000). Our results partially supported the sampling or selection effect (Aarssen 1997, Huston 1997, Loreau 1998, 2000) in the way that it predicts diverse communities to have a higher probability of including a nitrogen-fixing species (selection of extreme trait values) if species are selected randomly. The selection effect certainly played a role in generating our productivity patterns although, as we were looking at richness and numbers, we were partly looking for probabilistic processes by definition. However, it did not support the prediction of the sampling or selection effect that communities become dominated by these species. Although the fraction of legumes exceeded the sown fraction at two sites, they did not dominate community biomass. At most sites, the sown legume fraction either persisted or even decreased. Additionally, the sown legume fraction within a community decreased with increasing species richness at most sites, in accordance with our experimental design. The results also did not support the "mass-ratio hypothesis" (Grime 1998), which suggests that ecosystem properties should be determined to a large extent by the characteristics of the species dominating the biomass of the

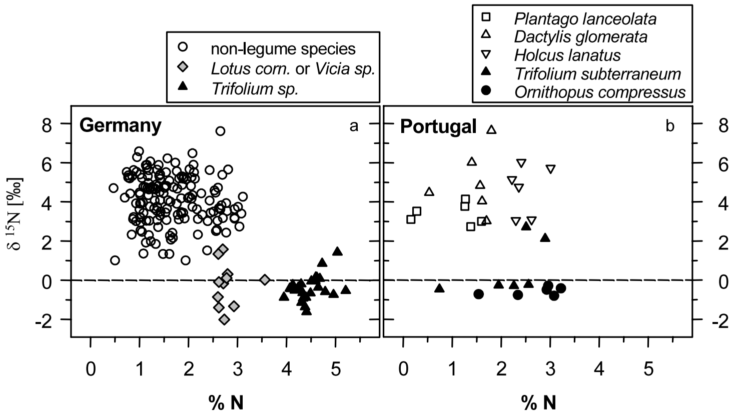

Fig. 5. Relationship between leaf nitrogen concentration and leaf δ^{15}N of individual species in all plots of the German (a) and Portuguese (b) site, measured at the first biomass harvest in the second year of the experiment. Closed symbols represent legume species, open symbols represent non-legume species.

community. The strong effects of legumes were independent of their total biomass in the community, although one could expect that legumes might have a greater chance to increase their fraction of total biomass than non-nitrogen fixers, if nitrogen is limiting. Effects of legumes were even stronger if their presence/absence rather than relative biomass abundance was tested in the statistical analysis. Nitrogen-fixers may therefore be better defined as keystone plant species, as their impact on ecosystem processes is disproportionate to their contribution to the overall biomass (Bond 1993).

Separating positive species interactions and niche complementarity is difficult (Hector et al. 1999). The influence of legumes was arguably both positive (adding resources) and complementary (reduction of competition/resource partitioning). This is particularly true given our experimental design since the presence and number of legume species was often positively correlated with number of non-nitrogen fixing species. Loreau and Hector (2001), by demonstrating a new approach of seperating selection effects from complementarity effects in biodiversity studies, showed that increased complementarity in species-rich mixtures involved not only complementarity between legumes and other plant types, but also complementarity between species within each of these groups. However, in Germany there was no evidence that species richness had a positive effect on the productivity of assemblages without legumes. For this site, at least in the short term, the original null hypothesis (ecosystem functions are insensitive to species deletions or additions, Lawton 1994) seemed to hold, as we found no dominance of larger species and no complementarity for assemblages without legumes (Schmid et al. 2001) at least over the length of the experiment. Identification of complementary interactions between species that are not due to nitrogen-fixers may require additional experiments with designed contrasts to separate complementary and positive legume effects. For example, one approach would be to have a highly replicated experiment within levels of species richness where community composition is determined by a fully random draw of species from the full species pool as done by Tilman and colleagues (1997a), but with all species grown in monoculture. Alternatively, the same assemblage of non-nitrogen fixing species could be grown with and without legumes, or even with different numbers and types of legumes to test their effects separately.

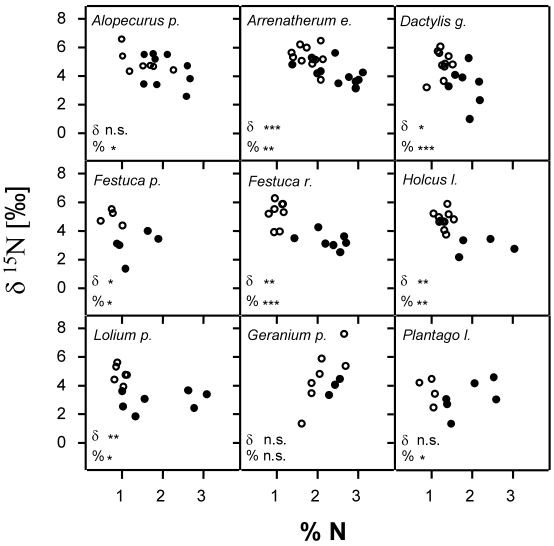

Fig. 6. Leaf nitrogen concentration and leaf $\delta^{15}N$ of selected species of the German site, at the first biomass harvest of the second growing season of the experiment. Closed symbols for grown with legumes, open symbols for grown without legumes. Grasses: *Alopecurus pratensis*, *Arrhenatherum elatius*, *Dactylis glomerata*, *Festuca pratensis*, *Festuca rubra*, *Holcus lanatus* and *Lolium perenne*. Herbs: *Geranium pratense* and *Plantago lanceolata*.

Differences in plant composition may have large effects on ecosystem processes if the traits of one or a few species dominate. In principle, all effects on ecosystem processes within a site can be attributed to two factors (Hector et al. 2000b): (1) traits of individual species, (2) interactions between species in assemblages (including interactions with other trophic levels). Species richness effects are in fact a subset of compositional effects, i.e. those where compositional effects show a trend with species richness (Schmid et al. 2001). The hierarchy of treatment factors in our statistical model is therefore crucial for interpretation. If the presence of legumes was fitted first, residual diversity effects sometimes remain significant (biomass Table 2a; complementary effect, Loreau and Hector 2001), but sometimes do not (nitrogen pools, Table 2a). The legume effect on nitrogen accumulation therefore explains most of the diversity effect (Fig. 1) and can be considered as the main underlying mechanism. However, the BIODEPTH experiment was primarily designed to manipulate species richness, not to test for legume presence. The factors species richness, functional group richness and legume presence were confounded by design, but also by theory, e.g. with increasing species numbers there is an increasing number of functional groups and an increasing number of assemblages with legumes. Therefore, our design cannot fully separate the effects of diversity between and within functional groups.

Differences in legume effects between sites

One explanation of site-specific differences in legume response may be P availability (Stöcklin and Körner 1999). In Greece very low levels of P availability in the soil (A. Troumbis, unpubl. data) might have limited legumes, because legume growth requires adequate phosphorus for development of nodules (Bordelau and

Prévost 1994). Nitrogen fixation is often limited by other nutrients like phosphorus, molybdenum or iron, thus representing the ultimate factors limiting biomass production most of the time (Vitousek and Howarth 1991, Crews 1993, Badarneh 1995). Legumes might have also suffered from low levels of phosphorous due to soil acidity ($\mathrm{pH} = 4.5$) in Ireland, explaining their poor growth and contribution to assemblages in terms of biomass (Fig. 4). Additionally, environmental factors reducing the amount of available carbon such as high summer temperatures and low water supply (Sprent 1972, Pate 1976) could have limited nitrogen fixation in the southern European sites, especially in Greece and Portugal. For example, *Trifolium subterraneum* did not fix nitrogen in monocultures at the Portuguese site (indicated by two datapoints not differing from non-legumes in Fig. 5), because it was suffering from water stress in monocultures, as ^{13}C data showed (Caldeira et al. 2002).

Differences between legume species

Not all legumes had similar impacts on nitrogen accumulation and biomass production. Species from one genus in particular, *Trifolium*, appeared to have particularly marked effects and may form a keystone genus within European grasslands. *Trifolium repens* is usually the most effective nitrogen fixing species in mid-European pastures (Rieder 1983). N_2-fixation efficiency even increased in mixed stands compared to legume monocultures. Legume monocultures generally had higher levels of mineralised nitrogen in the soil, which could inhibit the nitrogenase activity in the legume nodules (Marschner 1995). In addition to N_2 fixation, *T. pratense* and *T. repens* had a pronounced effect on the nitrogen pool in above-ground biomass, because they also have a high capacity for biomass production. At three sites *T. pratense* was the most productive species in monoculture. However, *T. pratense* commonly cannot be grown for several years in agricultural monocultures due to the occurrence of root-rot, mainly caused by *Fusarium* spp. among others (Taylor and Quesenberry 1996), therefore this high monoculture biomass may not persist. Thus the effects of loss of diversity on productivity and N accumulation in biomass depend on which functional groups and species are lost. In our case, effects will be stronger where legumes are lost and, in particular, where *Trifolium* species are lost. Conversely, where legumes (and particularly *Trifolium* spp.) remain, the effects of reduced diversity may be weaker, at least on above-ground nitrogen and carbon pools and in the short term. There is evidence from other grassland studies that legume effects on biomass strongly depended on the legume species, and could therefore not be generalized (Grünzweig and Körner 2001).

Conclusions

The results clearly showed that the specific composition of the communities and the presence of one particular functional group, legumes, had major effects on nitrogen accumulation in the community and biomass. At some sites, legumes played a key role, supporting the keystone species hypothesis. Whether or not legumes contributed to a biodiversity-productivity effect depended on both the legume species present and the site, especially the availability of key nutrients such as P. Changes in diversity clearly affected the nitrogen budget of plant communities but the exact consequences depended strongly on the identity and functional traits of the species lost.

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