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Additional carbon sequestration benefits of grassland diversity restoration

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Summary

1. In Europe, grassland agriculture is one of the dominant land uses. A major aim of European agri-environment policy is the management of grassland for botanical diversity conservation and restoration, together with the delivery of ecosystem services including soil carbon (C) sequestration.
2. To test whether management for biodiversity restoration has additional benefits for soil C sequestration, we investigated C and nitrogen (N) accumulation rates in soil and C and N pools in vegetation in a long-term field experiment (16 years) in which fertilizer application and plant seeding were manipulated. In addition, the abundance of the legume *Trifolium pratense* was manipulated for the last 2 years. To unravel the mechanisms underlying changes in soil C and N pools, we also tested for effects of diversity restoration management on soil structure, ecosystem respiration and soil enzyme activities.
3. We show that the long-term biodiversity restoration practices increased soil C and N storage especially when these treatments were combined with the recent promotion of the legume *Trifolium pratense*, sequestering 317 g C and 35 g N m^{-2} $year^{-1}$ in the most successful management treatment. These high rates of C and N accumulation were associated with reduced ecosystem respiration, increased soil organic matter content and improved soil structure. Cessation of fertilizer use, however, reduced the amount of C and N contained in vegetation.
4. *Synthesis and applications.* Our findings show that long-term diversity restoration practices can yield significant benefits for soil C storage when they are combined with increased abundance of a single, sub-ordinate legume species. Moreover, we show that these management practices deliver additional ecosystem benefits such as N storage in soil and improved soil structure.

Key-words: biodiversity, carbon sequestration, C:N ratio, ecosystem functioning, ecosystem respiration, global change, nitrogen sequestration, SOC, soil enzymes, soil structure

Introduction

Globally, soils are the largest terrestrial carbon (C) reservoir, but there is compelling evidence that over the last few decades large amounts of C have been lost from soils of natural and agricultural ecosystems through erosion, leaching and accelerated soil respiration (Lal 2004; Bellamy *et al.* 2005; Davidson & Janssens 2006; Quinton *et al.* 2010). Recently, the management of farmed ecosystems to deliver ecosystem services such as C storage and enhanced biodiversity has become an important aim of European agri-environment schemes (Sutherland 2004; Hopkins & Wilkins 2006; Jackson, Pascual & Hodgkin 2007). However, empirical studies that couple management for plant diversity with ecosystem services such as C storage are scarce (Hopkins & Wilkins 2006; Jackson, Pascual & Hodgkin 2007; Woodward *et al.* 2009). This is surprising given the evidence that diverse grassland communities may deliver such

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ecosystem services through altering the amount and quality of plant inputs to the soil (Tilman, Hill & Lehman 2006; De Deyn, Cornelissen & Bardgett 2008; Fornara & Tilman 2008; De Deyn *et al*. 2009).

Here, we investigated the effects of long-term management for the restoration of plant diversity combined with the promotion of a N-fixing plant species, the legume *Trifolium pratense*, on soil C storage. We used a multi-factorial experiment established in 1990 on plant species-poor fertilized grassland in northern England (Smith *et al*. 2003, 2008a). We focussed on two management treatments (and their controls) which have successfully increased plant species diversity between 1990 and 2004, namely the cessation of NPK fertilizer application, which increased species richness from 19·3 to 21·4 per 4 m^2, and the addition of seed mixtures which promoted species richness from 18·7 to 22·0 per 4 m^2 (Smith *et al*. 2003, 2008a). Nitrogen-fixing legumes, such as *T. pratense* which is typically associated with unfertilized species-rich grasslands (Rodwell 1992; Smith *et al*. 2003, 2008a), are widely recognized as keystone grassland plant species that influence both soil N availability and overall plant community production (Rochon *et al*. 2004; Hopkins & Wilkins 2006; Van der Heijden, Bardgett & van Straalen 2008). However, their role in grassland soil C storage is less clear, although it has been suggested that they have the potential to promote soil C sequestration (Soussana *et al*. 2004; De Deyn, Cornelissen & Bardgett 2008; Fornara & Tilman 2008; De Deyn *et al*. 2009). We hypothesized that biodiversity restoration management treatments promote soil C sequestration by reducing the quality of plant litter inputs to soil, and that the promotion of *T. pratense* stimulates C sequestration by increasing the amount of C and nitrogen (N) entering soil.

To test our hypotheses we quantified the effects of the two long-term management treatments of fertilizer application and the addition of seed mixtures, and of the short-term promotion of *T. pratense*, on total C and N stocks in vegetation, soil organic matter fractions and rates of soil C and N accumulation. In order to explore the mechanisms underlying the responses, we also investigated treatment effects on the soil microbial community, ecosystem respiration, soil enzyme activities that underlie the decomposition and hence loss of soil C (Fenner, Freeman & Reynolds 2005; De Deyn, Cornelissen & Bardgett 2008), and soil organic matter content and soil structure because soil aggregation can promote the physical protection of C and N in the soil (Rochon *et al*. 2004; Holtham, Matthews & Scholefield 2007).

Materials and methods

EXPERIMENTAL SYSTEM

The study was conducted at Colt Park meadows, located in the UK Ingleborough National Nature Reserve (latitude 54°12′N, longitude 2°21′W) on *Lolium perenne-Cynosorus cristatus* grassland, northern England. The soil is a shallow brown-earth (average depth 28 cm) over limestone of moderate-high residual fertility (15 mg P_2O_2 L^{-1}). Measurements were made on 36 plots of 3 × 3 m comprising two management treatments (and their controls) in a long-term multi-factorial grassland restoration experiment which have successfully increased plant species diversity, namely the cessation of NPK fertilizer application and the addition of seed mixtures (of *R. bulbosus*, *L. corniculatus*, *Briza media* and *Geranium sylvaticum*) (Fig. 1; Smith *et al*. 2003, 2008a). The long-term management treatments were distributed over three main blocks containing plots with the same historic grazing management (autumn and spring grazing) and no application of farmyard manure (Smith *et al*. 2000, 2003, 2008a). Each main block was divided into three sub-blocks historically with different hay-cut dates from 1990 until 1998 but all cut on 21 July since 1999 (Smith *et al*. 2008a). In 2004, these sub-blocks were used to install a new *Trifolium pratense* treatment, but in such a way that each different historic date of hay cut was represented at each level of the *T. pratense* seeding treatment so that both factors were not confounded.

Each sub-block contained one of four long-term treatment combinations: cessation of or continued use of mineral fertilizer, with or without addition of seed mixtures. Recent seeding treatments of *T. pratense* comprised no seed addition ('none' in Fig. 1), or addition of 5·2 g m^{-2} *T. pratense* seeds of one of two different batches in September 2004 and August 2005, with one batch yielding increase in *T. pratense* abundance ('Establishment' in Fig. 1) and the other one not ('No establishment' in Fig. 1). The identity and % cover of vascular plant species in the central 2 × 2 m of each plot was determined in June 2004 and 2006 according to Stace (1991). Initial vegetation recordings made in June 2004 showed no difference in cover of *T. pratense* ($F_{2,4}$ = 0·50, P = 0·64) between the plots, whereas in 2006 one of the *T. pratense* seed addition treatments had resulted in a significant fourfold increase in *T. pratense* cover ($F_{2,4}$ = 9·8, P = 0·028). Average cover significantly increased from 0·4% cover in the control ('none' in Fig. 1 or 'no *T. pratense*' in Figs 2–4) to 1·6% (P < 0·05) with the good *T. pratense* batch ('Establishment' in Fig. 1 or 'with *T. pratense*' in Figs 2–4) and changed non-significantly to 0·6% (P > 0·05; 'No establishment' in Fig. 1) in the seeding treatment with poorly establishing seeds, with no significant difference in all measured response variables (data not shown in Figs 2–4 as similar to 'no *T. pratense*'). The long-term seeding treatment, started in 1990, also included the legume *Lotus corniculatus* but this species was present in only very low density in the plots with *T. pratense* (Fig. S1, Supporting Information).

SOIL, VEGETATION AND MICROBIAL C AND N STOCKS

In July 2006, immediately before the hay cut, aboveground vegetation biomass was determined in each plot from a randomly positioned 25 × 25 cm quadrat, clipped 2 cm above the soil surface. At the same time, mass of soil, roots, litter and moss were determined from five randomly sampled soil cores per plot (34 mm diameter, 10 cm deep), collected immediately after clipping; these five cores were pooled to form a composite sample for each treatment plot. Total mass of soil and roots in these cores was obtained by hand-sorting, sieving (2·8 mm mesh) and weighing roots and soil. We used a standardized wet sieving method, modified from Puget, Chenu & Balesdent (2000), to separate the soil particle size fractions. This involved sieving fresh soil (10 g dw equivalent) in de-mineralized water using a series of sieves (2·8 mm, 200 μm and 50 μm meshes) and a membrane filter of 0·45 μm to separate the finest soil fraction from dissolved organic C. The mass of the coarse (0·2–2·8 mm) and fine (50–200 μm) soil fractions were obtained by weighing and for the very fine fraction (50–0·45 μm) by sub-traction of the weighed soil fractions from the initial soil mass before fractioning.

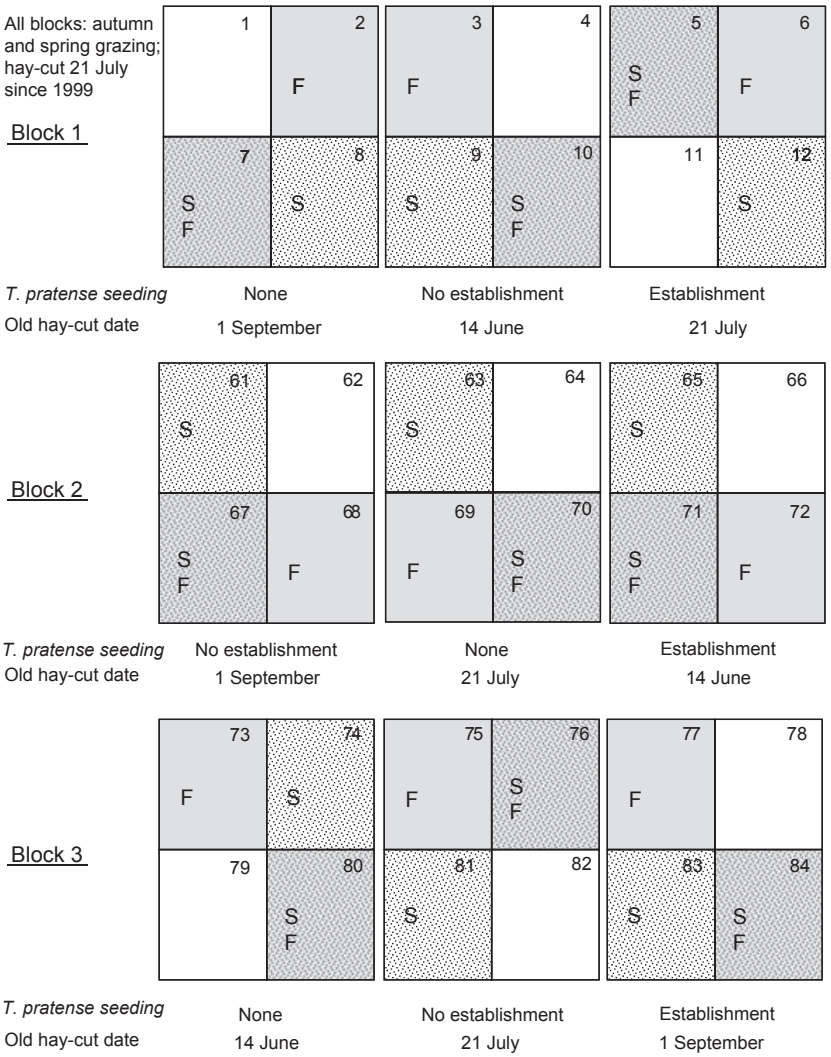

Fig. 1. Experimental design of the field experiment. S = long-term seed addition (dotted), F = long-term fertilizer addition (grey) and SF = long-term seed and fertilizer addition (grey with dots). All blocks were grazed in autumn and spring, hay cut date was 21 July since 1999. Seeding treatments of *T. pratense* were added in 2004 comprising no addition: None (or No *T. pratense*), successful seeds: Establishment (or with *T. pratense*) and non-successful seeds: No establishment. Numbers indicate plot number as in Smith *et al.* (2000).

Aboveground vegetation, litter, moss and roots were dried at 70°C, bulk soil at 105°C and soil fractions at 40°C to constant weight before recording the weight and grinding in a ball mill. Concentrations of C and N in plant tissue and soil were analysed on an Elementar Vario EL elemental analyzer (Hanau, Germany) using a subsample of 5 mg ground material, oven dried at 70°C (plant fractions) or 105°C (bulk soil and soil fractions). While drying soils at 105°C might have led to some loss of C and N, we do not think that this will have been significant because degradation of lignin and hemicellulose (which are major constitutes of soil organic matter) generally starts at 130°C and volatilization of organic matter and N at 200°C (Knicker 2007). Moreover, the higher drying temperature of 105 versus 40 to 70°C will have resulted in faster soil drying and hence a shorter time for organic matter decomposition by soil microbes. Soil C and N content per m^2 were determined by multiplication of %C and %N in root free soil by the dry weight of root free soil per m^2. Soil bulk density of root free soil was determined from the dry weight of the soil cores minus the dry weight of the roots contained in them.

Microbial biomass C and N were determined using the chloroform-fumigation technique according to the method described in Gordon, Haygarth & Bardgett (2008). Microbial community structure, and specifically the biomass of fungi and bacteria, were determined by phospholipids fatty acid (PLFA) analysis of soil using PLFA 18:2ω6 as an indicator for abundance of saprophytic fungi and PLFAs i15:0, a15:0, 15:0, i17:0, 17:0, cy17:0 and cy19:0 for abundance of bacteria. The ratio of fungal PLFA 18:2ω6 to the sum of bacterial PLFAs was used to asses the fungal to bacterial ratio (F:B) in soil, as also described by Bardgett, Hobbs & Frostegård (1996) and Gordon, Haygarth & Bardgett (2008). Organic matter content in root free soil was determined through mass loss on ignition (%LOI) at 550°C for 6 h. Soil samples were also taken from all treatments in 2004 immediately prior to the establishment of the *T. pratense* seeding treatments. Soil C and N content of these samples was analysed as above, showing that plots contained on average 4·63 ± 0·08 kg soil C per m^2 and 0·44 ± 0·01 kg N per m^2 in 2004. The exact values per plot in 2004 were used to calculate soil C and N accumulation rates per plot from the difference in soil C and N content between 2006 and 2004. Total soil C proved to be indicative for organic soil C given the very strong correlation between soil %C and %LOI ($r = 0{\cdot}96$, $P < 0{\cdot}001$).

COMPUTED TOMOGRAPHY (CT) SCANS

Intact soil cores (64 mm diameter × 100 mm deep) from each long-term treatment plot at two levels of the *T. pratense* seeding treatment (0·4 and 1·6% cover of *T. pratense*; i.e. 'None' and 'Establishment' in Fig. 1) were collected ($n = 24$) at the end of January 2007. The soil cores were freeze-dried and scanned using X-ray tomography (Bird *et al.* 2008) at 145 kV, 65 μA, at 1 frame per second and 1169 angular positions to make a 3D dataset. A standard area of soil of 366 × 414 pixels (width × height) of 45·3 μm in 100 successive slices down the

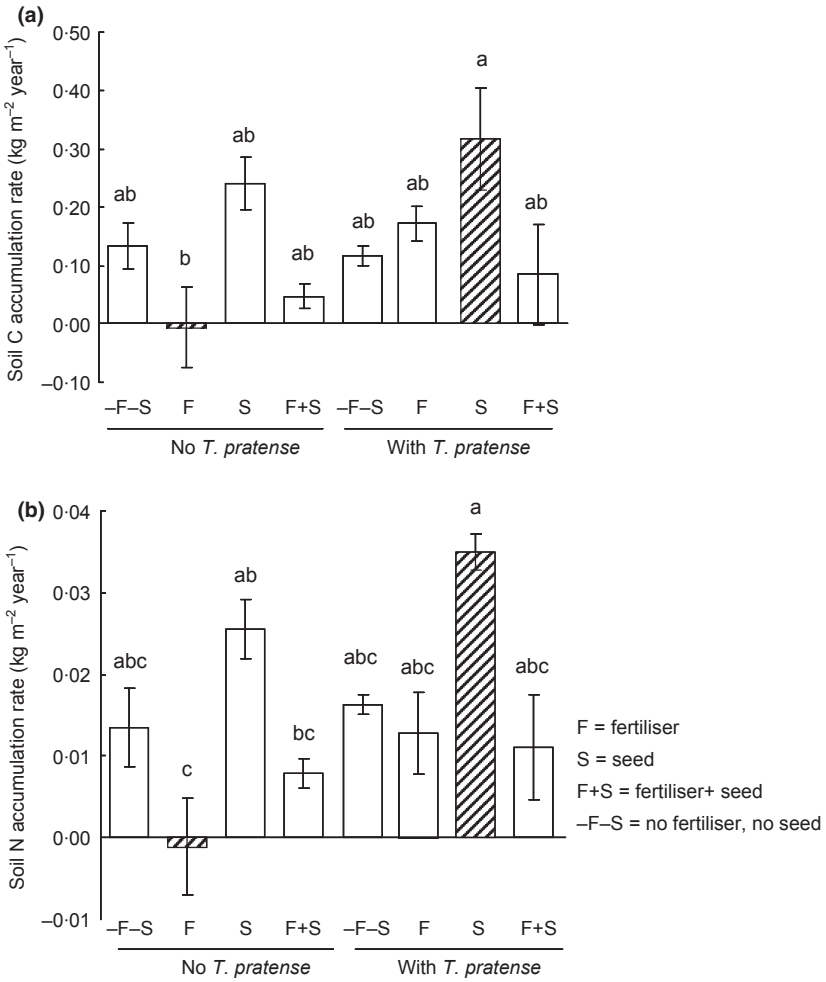

Fig. 2. Soil C (a) and N (b) accumulation rates as affected by new (No *T. pretense* = 0·4%, with *T. pretense* = 1·6% cover) and old (mineral fertilizer use: with = +F, without = -F; sowing of seed mixtures: with = +S, without = -S) combined restoration treatments. Bars represent mean values ± 1 SE (n = 3 but n = 2 for the treatment +S with *T. pratense* due to a missing value), shaded bars illustrate the extreme treatments and effects. Bars not sharing the same letter are statistically different at $P < 0{\cdot}05$.

soil profile was selected for the analysis of soil porosity, pore connectivity, pore surface area and fractals using an in-house software package SCAMP (Bird *et al.* 2008), written as a plug-in for ImageJ (Rasband 2008).

FIELD BASED C-FLUXES

Measurements of *in situ* CO_2-flux rates were determined in all plots every 2 weeks between January and December 2007 at midday using a portable infra red gas analyzer (IRGA EGM-4 with SRC-1 soil respiration chamber; PP-Systems), fitted with a 155 mm extension hood (total volume = 3764 cm^3) to cover the vegetation with minimal disturbance. To measure gross ecosystem CO_2 respiration, we used an opaque PVC extension hood, while for net ecosystem CO_2 uptake we used a transparent extension hood which transmitted 95% of ambient PAR.

ENZYME ACTIVITIES

Activities of phenol oxidase, β-*N*-acetylglucosaminidase (NAGase) and β-glucosidase enzymes were determined on fresh, sieved soil (2 mm mesh) using the methodology described by Fenner, Freeman & Reynolds (2005). Phenol oxidase was determined as the rate of dicq (2,3-dihydroindole-5,6-quinone-2-carboxylate) formation (nanomole dicq min^{-1} g^{-1} soil dry weight) in a 1 ml subsample of soil solution (1 cm^3 soil homogenized in 4 ml ultra pure water) to which 1 ml of 10 mM L-DOPA (dihydroxy phenylalanine) was added. This soil solution was left to react for 1 or 3 min at 15°C and was then centrifuged (72 000 *g* for 5 min.) and filtered. The absorbance of the filtrate was measured at 460 nm and the difference in absorbance of soil with different incubation time was used to calculate product formation. Hydrolase activities (NAGase and β-glucosidase) were determined using Methylumbelliferyl (MUF) formation rates in 1 ml subsamples of soil slurry (1 cm^3 soil homogenized in 1 ml ultra pure water) to which 3·5 ml substrate (MUF-N-acetyl-β-D-glucosaminide or MUF-β-D-glucoside) was added and which were incubated for 1 h at 15°C and then centrifuged at 72,000*g* for 5 min.. The fluorescence of 0·5 ml supernatant in 2·5 ml ultra pure water and 0·5 ml pH 6·6 buffer solution was measured at 450 nm emission and 330 nm excitation.

DATA ANALYSIS

We used ANOVAS for split-split plot designs with long-term seed additions and fertilizer treatments as subplot factors within the recent *T. pratense* seeding treatments to test effects of field management (*T. pratense* seeding treatments, fertilizer, long-term seed addition and their combinations) on the response variables (soil C and N accumulation rates, plant and microbial C and N pools, C fluxes, PCA projection scores of plant species abundances, F:B ratio, soil organic matter content, soil enzyme activities and soil structure parameters). The relationship between the accumulation rate of soil C and N and the C:N ratio of roots and moss, plant species richness and of the abundance cover of *T. pratense* was tested using Pearson correlation analysis. Tests on *in situ* C-flux rates were performed on the average

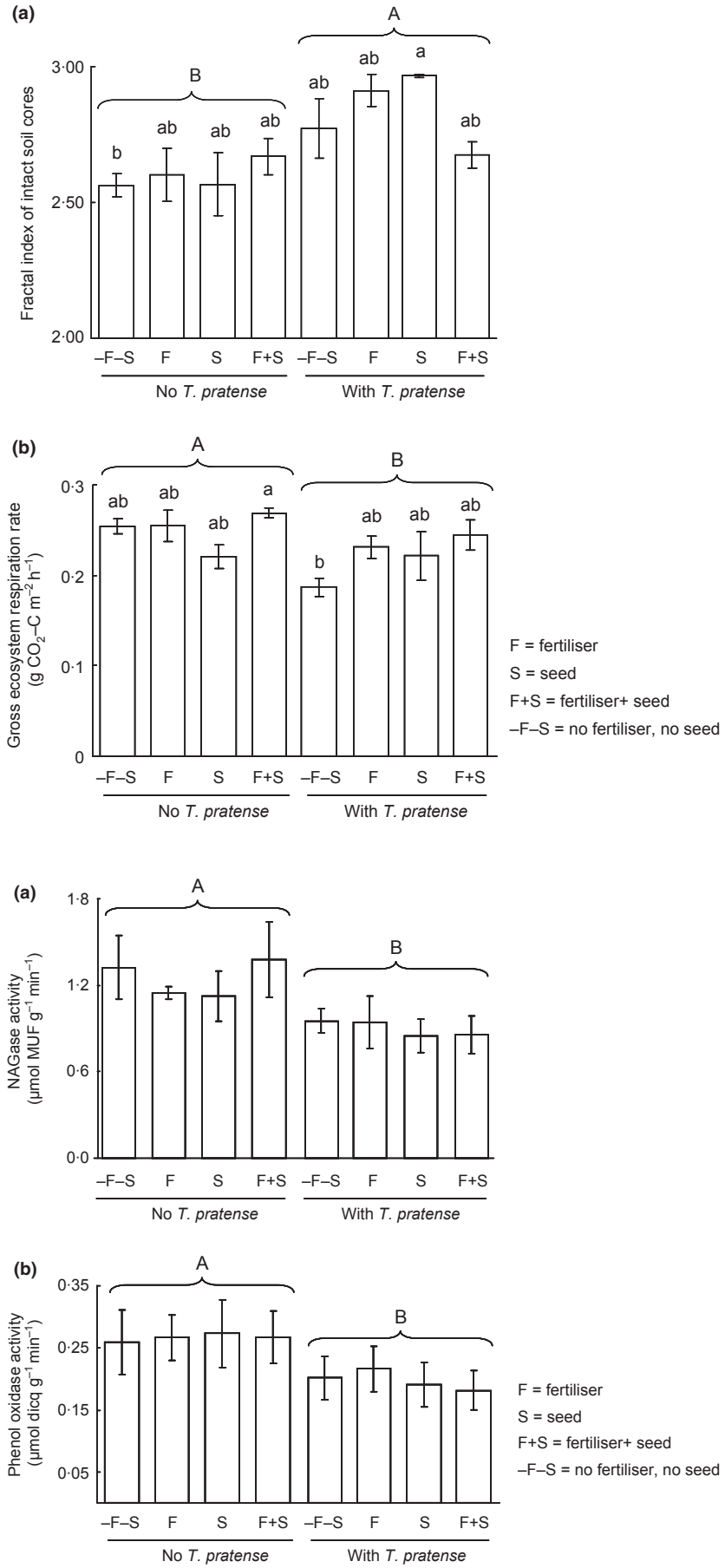

Fig. 3. Soil structure parameter fractal index (a) and ecosystem CO_2–C respiration (b) as affected by new and old combined restoration treatments (treatment legend as in Fig. 2). Bars represent mean values ±1 SE (n = 3 but n = 2 for treatments +S no *T. pratense* and +S with *T. pratense* in figure due to missing values). Bars not sharing the same letter are statistically different at $P < 0{\cdot}05$; capital letters: main effect of the *T. pratense* treatment.

Fig. 4. Soil enzyme activity: NAGase (a) and phenol oxidase (b) as affected by new and old combined restoration treatments (treatment legend as in Fig. 2). Bars represent mean values ±1 SE (n = 3). Bars not sharing the same letter are statistically different at $P < 0{\cdot}05$.

across all sampling dates. For the soil C and N pools there was one missing value (long-term seed addition with increased *T. pratense* cover) and for the soil structural data (fractal index) there were two missing values (long-term seed addition without *T. pratense* seeding or with increased *T. pratense* cover), these values were estimated via the method of Anderson (1946) before statistical analysis of treatment effects; but note that means and standard errors in graphs and text are based on measured values only. Normality of the data was verified by Shapiro–Wilks test. The residuals from the analysis of variance were tested for normality with the Anderson–Darling test and for the fractal index it was necessary to transform, using square root, to improve the fit of the residuals. Differences between treatments were further tested with Tukey HSD tests. PCA (principal component analysis) and RDA (redundancy analysis) of the plant community composition in June 2006 was performed in CANOCO using management treatments in the RDA (Ter Braak & Smilauer 2002).

Results

Both long-term treatments, i.e. seeding of seed mixtures and cessation of fertilization, have significantly enhanced plant species richness over the years (Smith *et al*. 2003, 2008a). In 2006, long-term seed addition had promoted species richness from 18·8 to 23·1 (± 0·8) per 4 m^2, while cessation of fertilizer application increased species richness from 20·0 (± 0·8) to 21·9 (± 1·1), and the addition of *T. pratense* from 20·0 ± 1·2 without (i.e. 'None' in Fig. 1) to 22·4 ± 1·0 with *T. pratense* (i.e. 'Establishment' in Fig. 1) and 20·5 ± 1·3 in unsuccessful *T. pratense* seeding (i.e. 'No establishment' in Fig. 1). At the time of our study in 2006, plant biomass and C and N stocks in vegetation were only affected by the cessation of fertilizer use. Shoot ($F_{1,6}$ = 51·9, P = 0·0004) and root ($F_{1,6}$ = 41·75, P = 0·0007) biomass were both reduced by abandonment of mineral fertilizers, whereas moss biomass increased ($F_{1,6}$ = 24·68, P = 0·0026) (Table 1). Consistent with this, the total amount of C ($F_{1,6}$ = 16·09, P = 0·007) and N ($F_{1,6}$ = 22·43, P = 0·0033) stored in plants and litter declined after long-term cessation of fertilizer application. Cessation of fertilizer use also reduced the quality of plant inputs to soil, as reflected by a significant increase in the C:N ratio of roots ($F_{1,6}$ = 17·05, P = 0·0062) and moss tissue ($F_{1,6}$ = 174·09, P = 0·0001), but it did not alter the soil organic matter content (% LOI, Table 1). Despite reduced production of plant biomass, the cessation of fertilizer use caused a significant increase in soil accumulation of C ($F_{1,6}$ = 6·87, P = 0·039) and N ($F_{1,6}$ = 10·72, P = 0·016) between 2004 and 2006 (Table S1, Supporting information). However, there was no relationship between the C:N ratio of roots and the accumulation rate of C (r = 0·14, P = 0·42) and N (r = 0·10, P = 0·56) in soil. The C:N ratio of moss, however, showed a positive trend with the accumulation rate of soil C (r = 0·29, P = 0·09) and N (r = 0·31, P = 0·08).

There was also no relationship between plant species richness and the accumulation rate of soil C (r = 0·04, P = 0·82) or N (r = 0·10, P = 0·58). However, the combination of long-term treatments of seed additions and cessation of fertilizer use, which was the most successful long-term practice for increasing plant species diversity (Smith *et al*. 2003, 2008a), and the seeding of *T. pratense* (with *T. pratense* + S in Fig. 2) resulted in a high soil C accumulation rate of 317 g C m^{-2} $year^{-1}$ (Table S1: Tp × F × S interaction between long-term seed addition and cessation of fertilizer use and recent seeding of *T. pratense*: $F_{2,11}$ = 6·73, P = 0·012; Fig. 2a) and N accumulation rate of 35 g N m^{-2} $year^{-1}$ ($F_{2,11}$ = 4·59, P = 0·035; Fig. 2b). In contrast, there was a net loss of 8 g C m^{-2} $year^{-1}$ and 1 g N m^{-2} $year^{-1}$ when *T. pratense* was not introduced in treatments with fertilizer application and without long-term seed additions (no *T. pratense* + F in Fig. 2); these treatments also had the lowest overall plant species richness (Smith *et al*. 2003, 2008a). Moreover, cover of *T. pratense* in 2006 across all treatments related positively to the rate of soil C (r = 0·52, P < 0·01) and N (r = 0·46, P < 0·01) accumulation between 2004 and 2006. With respect to the physical position in space, in the treatments with *T. pratense* we found significant increases in the fine (50–200 μm) soil size fraction (i.e. bound to soil mineral particles) for both C ($F_{2,4}$ = 7·91, P = 0·040) and N ($F_{2,4}$ = 6·78, P = 0·051) (Fig. S1, Supporting information). In addition, in intact soil cores we found a reduction in pore space clustering, as evidenced by an increase in the fractal dimension index in the plots with *T. pratense* added especially when combined with long-term restoration treatments (Tp ×

Table 1. Vegetation biomass (kg dw m^{-2}), vegetation C:N ratio and soil organic matter content (%LOI) as affected by cessation of fertilizer use (+F = N:P:K 2:1:1 with N 25 kg ha^{-1} $year^{-1}$) and its combination with long-term seed addition (S) and *T. pratense* seeding (–*Tp* = 0·4%; +*Tp* = 1·6% cover; data for *T. pratense* = 0·6% cover not shown as these are similar to 0·4% cover).

		+ Fertilizer, n = 18	-Fertilizer, n = 18		-*Tp* + F-S, n = 3	+ *Tp*-F + S, n = 2	
Biomass	Shoot	0·32 ± 0·02	0·22 ± 0·01	***	0·28 ± 0·03	0·27 ± 0·06	ns
	Root	0·53 ± 0·04	0·40 ± 0·03	***	0·64 ± 0·12	0·29 ± 0·02	*
	Moss	0·12 ± 0·01	0·21 ± 0·01	**	0·14 ± 0·05	0·20 ± 0·02	ns
	Litter	0·52 ± 0·04	0·50 ± 0·03	ns	0·51 ± 0·07	0·47 ± 0·01	ns
C:N	Shoot	28·8 ± 0·5	26·2 ± 0·7	*	28·96 ± 1·47	26·11 ± 0·02	ns
	Root	38·3 ± 0·8	43·5 ± 0·7	**	34·9 ± 0·9	41·9 ± 4·3	ns
	Moss	27·2 ± 0·5	37·2 ± 0·9	***	18·4 ± 9·2	35·4 ± 3·0	ns
	Litter	30·7 ± 0·6	32·2 ± 0·3	ns	29·9 ± 1·1	32·0 ± 0·4	ns
%LOI		19·4 ± 0·5	19·8 ± 0·5	ns	18·3 ± 1·3	21·6 ± 0·6	*

Values are mean ± 1 SE, significance: *P < 0·05, **P < 0·01, ***P < 0·001; ns, not significant.

F × S interactive effect: $F_{1,6} = 5{\cdot}92$, $P = 0{\cdot}05$; with *T. pratense* + S in Fig. 3a) (Table S1).

Soil organic matter content was also enhanced by the successful addition of *T. pratense* ($F_{2,4} = 11{\cdot}81$, $P = 0{\cdot}021$), especially in the treatments that did not receive long-term seeding treatments (interaction between recent *T. pratense* seeding and long-term seed additions: $F_{2,11} = 4{\cdot}16$, $P = 0{\cdot}045$). Plant community tissue quality, measured as C:N ratio of root, shoot, litter and moss did not differ significantly between the management treatments that differed most in accumulation rate of soil C and N (Table 1 last two columns).

Overall, plant community composition, as determined by the projection scores on the main PCA axis after ordination analysis of the cover of all plant species, was only significantly affected by the fertilizer treatment (fertilizer main effect: $F_{1,6} = 14{\cdot}47$, $P = 0{\cdot}002$) and not by the long-term or recent seeding treatments. Abundances of *Festuca rubra*, *Cardamine pratensis*, *Rumex acetosa* and *Veronica chamaedrys* declined and those of *Trifolium repens*, *Ranunculus repens* and *Ranunculus ficaria* increased with cessation of fertilizer use (Fig. S2). However, the loss of soil C through ecosystem respiration *in situ*, measured by CO_2 exchange rates of soil and vegetation, was reduced in the plots with enhanced cover of *T. pratense* (Table S1: *T. pratense* seeding main effect: $F_{2,4} = 5{\cdot}43$, $P = 0{\cdot}07$; Fig. 3b and Fig. S3), especially in treatments without long-term seed additions. In the long-term seed addition without *T. pratense* (no *T. pratense* + S) treatment respiration rates were also low (Table S1: interaction between recent *T. pratense* seeding and long-term seed additions: $F_{2,11} = 8{\cdot}99$, $P = 0{\cdot}005$; Fig. 3b).

We found a significant increase in the amount of N stored in soil microbial biomass in the *T. pratense* seeding treatments when combined with long-term cessation of fertilizer use (interaction between recent *T. pratense* seeding and long-term fertilizer use: $F_{2,6} = 6{\cdot}11$, $P = 0{\cdot}035$; microbial N increased from 91 to 101 ± 4 g per m^2 without *T. pratense* + F versus with *T. pratense* –F). Yet there were no such treatment effects of *T. pratense* on soil microbial community composition in terms of relative abundances of saprophytic fungi and bacteria as determined by PLFA analysis (F:B ratio 0·048 ± 0·004 in no *T. pratense* + F and 0·047 ± 0·003 in with *T. pratense* –F treatments). Soil microbial enzyme activities, however, showed a trend of reduced activity in the *T. pratense* seeding treatments. For instance, with higher *T. pratense* cover we found lower activities of NAG-ase ($F_{2,4} = 6{\cdot}49$, $P = 0{\cdot}055$) and of phenol oxidase ($F_{2,4} = 4{\cdot}03$, $P = 0{\cdot}12$) (Fig. 4), while activity of the enzyme β-glucosidase, which acts on common substrates, was not altered ($F_{2,4} = 0{\cdot}56$, $P = 0{\cdot}61$).

Discussion

The cessation of fertilizer use increased plant species diversity in this long-term biodiversity restoration experiment, and increased the rate of soil C and N accumulation, despite a reduction in plant community biomass aboveground and belowground. In contrast, the restoration treatment of long-term seed addition had no effect on soil C accumulation, although this treatment increased plant species richness most, and stimulated N accumulation. However, the rate of soil C and N sequestration was promoted to the greatest extent when cessation of fertilizer use was combined with long-term seed addition, and recent addition of *T. pratense*. These findings indicate that changes in the presence of key species in the plant community, rather than plant species richness or standing plant biomass, drive soil C and N accumulation. The relationship between plant diversity and ecosystem functioning has been heavily debated, and while mechanisms exist which can contribute to higher levels of ecosystem functioning in more species rich communities (Hooper *et al*. 2005), we found no relationship between plant species richness, or plant biomass, and the ecosystem functions of soil C and N sequestration. Rather, our findings clearly indicate that the addition of a subordinate plant species, *T. pratense*, into restoration grassland, rather than increased diversity *per se*, can have very strong effects on ecosystem functions. These responses are probably due to a combined effect of *T. pratense* on soil nutrient cycling and soil physical properties which together enhance the retention of newly fixed and residing C and N in soil.

The C accumulation rate observed with increased *T. pratense* cover within long-term diversity restoration treatments was over five times greater than the average C sink estimated for European grasslands (i.e. 60 g m^{-2} $year^{-1}$ Janssens *et al*. 2005), and six times greater than expected with conversion of arable cropland to permanent grassland (i.e. 50 g m^{-2} $year^{-1}$ Soussana *et al*. 2004). However, this rate of soil C accumulation is comparable to that associated with conversion of agriculturally degraded cropland to plant species-rich grassland communities (Tilman, Hill & Lehman 2006) and is within the range of annual potential soil C input by roots (between 56 and 400 g C $year^{-1}$) in species rich temperate grassland (Steinbeiss *et al*. 2008). Moreover, the soil in our field site is relatively shallow (Smith *et al*. 2008a), which may also have contributed to the high rates of soil C and N accumulation in the topsoil (Steinbeiss *et al*. 2008). We recognize that our results related to the *T. pratense* treatments are based on short-term observations with limited number of replicates. The long-term benefits will depend on the new equilibrium level between soil C input and output and on the continuation of the management practices (Soussana *et al*. 2004). Nevertheless, the strong responses we found demonstrate the need and scope for more (multi-site) and longer-term investigations of management practices that can deliver combined benefits for the restoration of biodiversity and soil based ecosystem functions.

Several mechanisms underlying soil C and N sequestration have been proposed in recent studies. Previous studies have attributed increases in soil C storage in croplands (Smith *et al*. 2008b) and grassland (Tilman, Hill & Lehman 2006; Fornara & Tilman 2008) to increases in aboveground and belowground plant production, but given the reduction in biomass we observed this mechanism is not likely in our study. However, we observed increased soil organic matter content (%LOI) in the treatments that stimulated soil C and N sequestration

most. This suggests that changes in quality (e.g. C:N ratio) rather then quantity of plant community belowground inputs via root exudates, roots and their rapid turnover especially in plant communities with *T. pratense* (Rochon *et al.* 2004; Ayres *et al.* 2007), were primary drivers of the rapid increase in soil C and N accumulation rates. These changes in availability of high quality soil inputs (low C:N ratio) probably occurred at finer spatial scales (e.g. very fine roots and exudates) than captured in community level shoot, litter and root biomass quality, as C:N ratios tended to increase slowing down their decomposition. The combination of local availability of high and low quality resources may have resulted in sustained availability of resources for soil microbes and plants, and a reduced need for microbes to decompose new recalcitrant and resident soil organic matter for nutrients, thereby reducing soil C and N loss. Furthermore, the high soil N accumulation rate (350 kg ha^{-1} $year^{-1}$) observed with increased *T. pratense* cover can be partly attributed to biological N_2 fixation. This accumulation rate is comparable to published estimates of N_2 fixation rates (i.e. 373 kg ha^{-1} $year^{-1}$) observed in forage grasslands with high *T. pratense* cover (Carlsson & Huss-Danell 2003).

Year-round *in situ* ecosystem respiratory CO_2 flux rates of vegetation and soil suggest that the loss of C from soil through microbial and root respiration was indeed reduced when there was a greater *T. pratense* cover in the sward, or with the long-term seed addition treatments. Given that effluxes of soil C and N are closely related (Reich, Hungate & Luo 2006), reduced C loss is likely to confer reduced N loss too, contributing to greater retention of plant C and N inputs to soil. Although increased inputs of C and N rich root exudates from legumes typically stimulate the growth of soil microbes (Mawdsley & Bardgett 1997; Denton *et al.* 1999), our measures of the abundances of different groups of soil microbial PLFA biomarkers indicate that changes in gross respiration were not associated with alteration of microbial biomass or community structure, at least in terms of relative abundances of fungi and bacteria. Analysis of soil PLFA may not be the most sensitive method to evaluate shifts in soil microbial composition, but it does provide an indication of broad-scale shifts in soil microbes that are typically related to changes in the functioning of soil in terms of C and N cycling (Wardle, Walker & Bardgett 2004; Bardgett *et al.* 2006; De Deyn, Quirk & Bardgett 2010). However, the results of soil exo-enzyme assays revealed significant changes in microbial activity with a trend towards reduced activity for the decomposition of recalcitrant polyphenols and chitin containing compounds, contributing to reduced soil C and N mineralization and CO_2 loss by respiration.

As a final mechanism, we propose that changes in soil physical structure could also have contributed to the rapid increase in soil C and N sequestration. The soil structural changes of increased fractal index are indicative of improved soil aggregation and the physical protection of soil organic matter (Young & Crawford 2004; Holtham, Matthews & Scholefield 2007). They are likely to have influenced the function of the soil pore space, water permeability and gas exchange rates. Moreover, the transfer of C and N to finer soil fractions, as we observed, is known to increase the stability and residence times due to physical protection from microbial and enzymatic decay (Puget, Chenu & Balesdent 2000; Neff *et al.* 2002; Young & Crawford 2004).

Conclusions

Our findings offer an insight into the potential impacts of restoration practices to increase plant diversity on soil C sequestration and the role of N-fixing plant species. Specifically, we show that long-term biodiversity restoration practices increased soil C and N storage especially when these treatments were combined with the recent promotion of the legume *T. pratense*. Our results come from a long-term grassland study with management treatments that are commonly used in grassland restoration schemes across Europe to enhance botanical diversity (Smith *et al.* 2003, 2008a). Although our understanding of the mechanisms involved in the enhancement of soil C and N sequestration is incomplete, these results have far reaching implications, especially if these effects are common to other legume species and diversity restoration practices. In conclusion, this work shows that the observed benefits of *T. pratense* for soil C and N storage are compatible with the restoration of grassland biodiversity, a major goal of European agri-environment policy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. ANOVA results of main and interactive (x) effects of the field treatments.

Fig. S1 Soil C and N storage in different soil size fractions.

Fig. S2 RDA diagram of plant species responses to restoration management.

Fig. S3 Seasonal CO_2 efflux of gross and net ecosystem respiration over one year.

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