

Interactive Effects of Time, CO₂, N, and Diversity on Total Belowground Carbon Allocation and Ecosystem Carbon Storage in a Grassland Community

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ABSTRACT

Predicting if ecosystems will mitigate or exacerbate rising CO₂ requires understanding how elevated CO₂ will interact with coincident changes in diversity and nitrogen (N) availability to affect ecosystem carbon (C) storage. Yet achieving such understanding has been hampered by the difficulty of quantifying belowground C pools and fluxes. Thus, we used mass balance calculations to quantify

the effects of diversity, CO₂, and N on both the total amount of C allocated belowground by plants (total belowground C allocation, TBCA) and ecosystem C storage in a periodically burned, 8-year Minnesota grassland biodiversity, CO₂, and N experiment (BioCON). Annual TBCA increased in response to elevated CO₂, enriched N, and increasing diversity. TBCA was positively related to standing root biomass. After removing the influence of root biomass, the effect of elevated CO₂ remained positive, suggesting additional drivers of TBCA apart from those that maintain high root biomass. Removing root biomass effects resulted in the effects of N and diversity becoming neutral or negative (depending on year), suggesting that the positive effects of diversity and N on TBCA were related to treatment-driven differences in root biomass. Greater litter production in high diversity, elevated CO₂, and enhanced N treatments increased annual ecosystem C loss in fire years and C gain in non-fire years, resulting in overall neutral C storage rates. Our results suggest that frequently burned grasslands are unlikely to exhibit enhanced C sequestration with increasing atmospheric CO₂ levels or N deposition.

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INTRODUCTION

Determining if terrestrial ecosystems will buffer or intensify rising atmospheric CO₂ concentrations requires understanding the mechanisms that control the balance between ecosystem carbon (C) inputs and outputs. Because atmospheric CO₂ levels are increasing in conjunction with environmental changes such as in biodiversity and nitrogen (N) availability (Sala and others 2000; Galloway and others 2004), it is necessary to understand how (or if) such changes interact with CO₂ to modify ecosystem C cycling.

The effect of elevated CO₂ on ecosystem C storage is typically quantified by measuring the response of net ecosystem production (NEP) or various ecosystem C pools to elevated CO₂. In the few cases where it has been measured, NEP either increased or was unaffected by elevated CO₂ (Reich and others 2006a). Biomass C pools tend to increase with CO₂ (Reich and others 2006a, b), but its ability to act as a moderately long-term C sink depends on the presence and response of woody biomass, which may store C for decades to centuries because of its long turnover time (Schlesinger 1997; McCarthy and others 2006). However, CO₂ induced increases in forest productivity do not always translate into increased C allocation to wood; in some cases, it may decrease C allocation to wood (McCarthy and others 2006). Thus, the ability of ecosystems to act as truly long-term C sinks will often depend on increasing soil C sequestration, because soils contain about 2 times more C than terrestrial biomass, with turnover times of decades to millennia (Schlesinger 1997; Jackson and others 2002). This is especially true for grasslands, where aboveground biomass C turns over annually (Scurlock and Hall 1998; Knops and Bradley in press). Therefore, a number of experiments have characterized the effects of elevated CO₂ on ecosystem C storage by examining changes in total soil C. These experiments have yielded mixed results (for example, Hungate and others 1997; Gill and others 2002; Heath and others 2005; Jastrow and others 2005; Luo and others 2006), which, in turn, have produced a diversity of hypotheses regarding the response of soil C to elevated CO₂.

In the face of conflicting site-based responses and hypotheses, we addressed how changes in CO₂, N,

and diversity affect ecosystem C storage by investigating the response of inputs to and outputs from belowground C. With the exception of soil respiration, which increases with CO₂, N, and diversity (Craine and others 2001a; Bernhardt and others 2006), belowground pools and fluxes are exceptionally difficult to measure (Cannell and Dewar 1994; Giardina and others 2003). Thus, our understanding of these pools and fluxes remains rudimentary and primarily qualitative.

Yet, determining the size of belowground C inputs quantitatively, or relative to the size of C outputs, is necessary to predict how and why ecosystem C storage will change with variation in CO₂, diversity, and N availability. A potentially useful tool for quantifying C fluxes is total belowground carbon allocation (TBCA): the total amount of C that plants send belowground each year for root respiration, root production, rhizodeposition, exudation, and to support mycorrhizae (Giardina and Ryan 2002). TBCA is calculated using a C mass balance approach and provides a quantitative, in situ estimate of plant C inputs into the belowground system.

There is little information on how CO₂, N, and diversity affect TBCA, and we were unable to find any information on how TBCA responds to environmental variables in non-forested ecosystems. In forested ecosystems, TBCA tends to be larger than aboveground NPP (Law and others 1999), increase with elevated CO₂ (Palmroth and others 2006), and respond variably to high N availability (Raich and Nadelhoffer 1989; Ryan and others 1996; Raich 1998; Giardina and others 2003).

Here, our aims were twofold: first, to determine how TBCA, as the primary source of organic belowground C, responds to changes in CO₂, N, and diversity in a grassland ecosystem; and second, to compare TBCA with other major inputs and outputs of C, to predict how or why ecosystem C storage may change with CO₂, N and diversity. To accomplish these goals we used data from the biodiversity, CO₂, and N (BioCON) Free Air CO₂ Enrichment (FACE) project (Reich and others 2001a, b) to calculate TBCA and examine C fluxes and storage. First, we hypothesized that more diverse systems would have greater TBCA because complementary resource use results in greater C fixation and productivity (Tilman and others 1996; Firestone and others 2007). Second, we hypothe-

sized that because root production, respiration, exudation, and mycorrhizal allocation tend to increase at elevated CO₂, likely due to increased photosynthate availability (Matamala and Schlesinger 2000; Treseder and Allen 2000; Pendall and others 2004; Allen and others 2005; Trueman and Gonzalez-Meler 2005), TBCA would increase at elevated CO₂. However, over time, the CO₂ fertilization effect may create feedbacks that reduce N availability and thus also reduce the stimulation of biomass by elevated CO₂ (progressive N limitation, PNL; Reich and others 2006b). Our site has extremely N-limited soils (Tilman and others 1996) and adding N increased the positive effect of CO₂ on total biomass over time in BioCON, suggestive of PNL (Reich and others 2006a, b). We therefore hypothesized that TBCA would respond more strongly to elevated CO₂ with added N, and that this effect would increase over time.

We were also interested in comparing the size of concurrent C inputs and outputs with respect to variation in diversity, N, and CO₂. Data syntheses have suggested that total soil C is insensitive to elevated CO₂ unless N is added, when soil C increases (DeGraaff and others 2006; Luo and others 2006; Reich and others 2006a). This suggests that when N is limiting, decomposers may respond to increased C inputs (caused by elevated CO₂) by decomposing soil organic matter to gain access to N (Cheng 1999; Kuzakov and others 2000). Alternatively, when N is sufficient, decomposition rates remain lower and soil C accumulates. Thus, we predicted that the increased C inputs caused by elevated CO₂ would be matched by C outputs at ambient N but would outpace C outputs at elevated N, thereby only increasing ecosystem C storage at elevated N. Because diversity tends to increase the effect of CO₂ and N on plant productivity (Reich and others 2001a), we expected that diversity would also increase the size of the CO₂ by N interaction.

METHODS

BioCON

The BioCON experiment is located in an old-field grassland on a nutrient poor, sandy outwash plain in the Long-Term Ecological Research site at the Cedar Creek Ecosystem Science Reserve (CCESR) in Minnesota, USA (latitude 45°N, longitude 93°W). In 1997, vegetation from six 20-m diameter circular areas (rings) was removed and the soil was tilled and fumigated with methyl bromide to eliminate the soil seed bank. Soils were re-inoculated with microbes using water extracts from

surrounding soil. By 2000, soil respiration and AM fungi communities had recovered to levels similar to the surrounding areas (Wolf and others 2003; unpublished data). Plots were seeded with 1, 4, 9, or 16 species randomly chosen from 16 grassland species in four functional groups (C₃ and C₄ perennial grasses, forbs, and legumes), at a rate of 12 g m⁻² (divided equally among species in a plot). Since 1998, three rings have been treated with 560 ppm CO₂ during each day throughout the growing season, and half of all plots have received 4 g N m⁻² y⁻¹, an N deposition rate comparable to highly industrialized areas (Vitousek 1994). All plots were burned in the early spring in roughly half of the years of this study period (2000, 2002, 2003, and 2005) to mimic pre-settlement fire frequencies in tall grass prairies.

The main BioCON experiment consists of 296 individual 4 m⁻² plots evenly distributed among the six rings and is a split-plot arrangement of treatments in a completely randomized design. CO₂ treatment is the whole-plot factor. The subplot diversity and N treatments were randomly distributed and replicated in individual plots among the six rings. Across all rings for each of the four CO₂ and N treatments, there were 32 replicates for the 1-species plots (2 per species), 15 for the 4-species plots, 15 for 9-species plots, and 12 for 16-species plots (Reich and others 2001a).

Calculation of TBCA

We calculated annual TBCA from 1999 to 2006 using a C mass balance approach, which assumes that change in total belowground C storage is equal to C inputs into minus C outputs from the belowground system (Giardina and Ryan 2002). In grasslands, the belowground system consists of C in the litter layer, plant roots, and soil, and is nearly equivalent to ecosystem C storage, because significant C does not accrue in aboveground biomass. Annual C inputs consist of aboveground plant production (F_A), which becomes litter at the end of each year, and TBCA. Outputs include C loss through export (leaching, F_E), combustion (fire, F_F), and soil respiration (F_S , root respiration and decomposition of soil organic matter and litter). Carbon inputs minus outputs are set equal to changes in ecosystem (belowground system) C, measured as changes in the amount of C in the litter layer (ΔC_L), mineral soil (ΔC_S), and root biomass (ΔC_R) over a given time period (Δt):

$$[F_A + \text{TBCA}] - [F_S + F_F + F_E] = \Delta[C_S + C_L + C_R]/\Delta t \quad (1)$$

Thus, through conservation of mass, TBCA can be estimated ($\Delta t = 1$ year) by rearranging equation (1):

$$\text{TBCA} = [F_S + F_F + F_E] - F_A + \Delta[C_S + C_L + C_R] \quad (2)$$

Aboveground, root, and litter biomass were measured each year in June and August. Litter and live biomass in a 0.1 m² strip within each plot was collected, dried, and weighed. In the same strip, roots were sampled (0–20 cm) using three 5 cm diameter cores, washed, dried, and weighed. We estimated F_A as the mean of June and August aboveground biomass, converted to g C m⁻² using annual plot-level aboveground biomass %C measurements (by combustion; Costech ECS4010 element analyzer, Valencia, California). As a check of this estimate of aboveground net primary productivity (ANPP), we also estimated ANPP and TBCA using the peak biomass method (ANPP_{peak}; this method is equivalent to the positive biomass increment method, because there were two annual biomass measurements) and the positive biomass increment method by species (ANPP_{spp}; Scurlock and others 2002). ANPP_{peak} was estimated for all years, whereas ANPP_{spp} was estimated for years when biomass data were recorded by species (1998–2004). On average, litter biomass declined from June to August by 8.9 g m⁻², indicating that litter was decomposing. We therefore did not estimate ANPP using the litter plus biomass positive increment method, which requires that we assume negligible decomposition (Scurlock and others 2002). The F_A measures differed by 20% from the ANPP_{peak} and ANPP_{spp} measures, but TBCA estimates using ANPP_{peak} and ANPP_{spp} were only 6% lower than TBCA estimates using F_A . Treatment analysis results were the same for all TBCA estimates. Because (1) the ANPP estimation method did not affect TBCA estimates or our results and (2) ANPP_{peak} and ANPP_{spp} required us to assume that biomass measured at any given time truly captured peak or greater biomass rather than the substantial within plot variation present in BioCON plots, we chose to calculate TBCA using F_A .

Annual ΔC_L was estimated by multiplying litter biomass by the %C of aboveground biomass and subtracting litter C from the previous year from that of the current year. We used biomass %C because (1) litter %C was not measured annually, (2) when it was measured, freshly senesced litter %C was not different from that of aboveground biomass (Knops and others 2007), and (3) using the relationships developed from limited litter decomposition data to estimate declines in litter %C after 1 and 2 years did not change ΔC_L , TBCA estimates,

or our treatment analysis results (Supplemental Appendix A). We estimated ΔC_R , by subtracting average belowground biomass multiplied by belowground biomass %C from the previous year from that of the current year.

Total soil C (0–20 cm) was determined in all plots in 1997 and 2002 and for various plot subsets in 2001, 2003, and 2006 (Costech ECS4010 element analyzer, Valencia, California). Analyzing soil C within each year that it was measured revealed no significant treatment effects except for diversity, which increased soil C similarly in all years. This suggests that the CO₂ and N treatments are not changing soil C (or are doing so at rates below our detection ability) and that the effect of diversity is not changing substantially over time. When the soil C data were examined over time by each CO₂, N, and diversity treatment, there were no consistent temporal patterns. For our TBCA estimates, we therefore assumed that soil %C did not change over time ($\Delta C_S = 0$). However, our results were similar if annual ΔC_S from 1999 to 2006 was assumed to be linear and equal to the plot-level annual rate of change between the 1997 and 2002 samplings.

By using 0–20 cm soil and root measurements to calculate TBCA, we assumed that treatment effects on the entire rooting depth profile are similar to treatment effects on the 0–20 cm profile section. This assumption would not bias our assessment of treatment effects unless treatment effects on ΔC_R or ΔC_S for the entire profile were not well-predicted by data from 0 to 20 cm. Root biomass measurements to 100 cm (2000–2002) indicate that this assumption is justified, as 0–20 cm ΔC_R in these 3 years was not significantly different from 0 to 100 cm ΔC_R .

Annual F_S was estimated using a combination of modeled non-growing season soil C flux (SCF) estimates and growing season SCF measurements. From 1998 to 2006, SCF in each plot was measured an average of 8 times per year (samplings) between April and November using a LI-COR 6200 gas exchange system with a LI-COR 6400-09 soil respiration chamber (LI-COR, Lincoln, Nebraska, USA; Craine and others 1998, 2001a, b). Soil temperatures across all SCF samplings ranged between 5.8 and 28.8°C.

For each sampling, SCF for each plot was measured over 2–3 days with consistent weather, between 08:00 and 18:00 local time. SCF increased throughout the day, but the slope of this linear correlation was shallow ($\text{SCF} = 1.27 + 0.000064 \times \text{sampling time in hours}$). We also randomized the order of ring sampling during each SCF sampling,

such that from 1998 to 2006, the average, earliest, and latest sampling times for each plot were from 11:00–13:00, 8:00–9:00, and 16:00–18:00 local time, respectively. We therefore used these measurements to estimate daily rates of SCF. Cumulative growing season SCF was determined by multiplying a plot's average daily SCF for two consecutive measurements by the number of intervening days and adding this value to the previously calculated cumulative SCF.

Because we had SCF measurements only during the growing season, estimating annual F_S required modeling non-growing season SCF as a function of soil temperature (T_s) and moisture. Soil temperature data were obtained from a CCESR weather station or generated using the Boltzmann sigmoidal equation (a function of air temperature; Supplemental Appendix B). Soil moisture data were generated using the BROOK90 model (Supplemental Appendix B). We used these data, combined with the arctangent temperature function, $F_T(T_s)$, and a soil moisture function, $F_W(\text{RWC})$, to estimate SCF during non-measurement periods (Del Grosso and others 2005; Supplemental Appendix B):

$$R_s = F_T(T_s) * F_W(\text{RWC}) \quad (3)$$

$$F_T(T_s) = 11.4 + (29.7 * \arctan[\pi * 0.0309(T_s - 15.7)]) / \pi \quad (4)$$

$$F_W(\text{RWC}) = 5 * (0.287 + [\arctan(\pi * 0.009 * [\text{RWC} - 17.47])]) / \pi \quad (5)$$

$$\text{RWC} = \frac{W - \text{WP}}{\text{FC} - \text{WP}} \quad (6)$$

where R_s = daily SCF (normalized by average daily SCF when T_s is 10–15°C), RWC = relative soil water content, W = soil water content, WP = wilting point, and FC = field capacity. Note that this model produced one estimate per year for all plots (that is, treatments did not influence the model results). Annual F_S was calculated by summing measured and modeled SCF.

In years without prescribed burns, the June litter layer was 61% of the previous August's litter layer plus aboveground biomass (June litter = 0.6062 * [August litter + biomass], $R^2 = 0.278$, $P < 0.0001$). This mass loss rate of 39% is higher than over-winter litter mass loss rates measured in temperate forest systems (up to 33%; Uchida and others 2005; McBrayer and Cromack 1980), likely because it includes fall, over-winter and early spring litter leaching, and decomposition. To estimate fire-

related C losses in burn years (F_F), we subtracted the June post-fire litter layer from 61% of the biomass plus litter layer from the previous August (that is, mass loss differences greater than 39% were attributed to fire losses). This avoided accounting for over-winter litter layer decomposition C loss twice (in F_F and F_S). Mass loss due to fire in each plot was multiplied by the %C of aboveground biomass. In non-fire years, F_F was set equal to zero.

In 2003 and 2004, C export via leaching of dissolved organic carbon (F_E) was estimated using tension lysimeters (Rhizon SMS, Eijkelkamp) in 1- and 16-species plots at 60 cm soil depth (Dijkstra and others 2007). Leaching C losses were very low compared to all other fluxes (Dijkstra, unpublished data), so we assumed that $F_E = 0$.

Using equation (2) and the data described above, we calculated TBCA for each plot from 1999 to 2006. We did not calculate TBCA in 1998 because it was not possible to accurately estimate the change in root biomass or litter layer without measurements from the previous year (1997).

Data Analysis

Calculating TBCA depended on the aggregation of measurements that all included some level of quantifiable error. We quantified error propagation following Bevington (1969). The standard error of U (S_U) can be approximated by the Gaussian error propagation rule:

$$S_U = \sqrt{\sum_{j=1}^n \left(\frac{\partial U}{\partial X_j} S_{X_j} \right)^2 + 2 \left(\sum_{j=1}^n \sum_{k=1}^n \left(\frac{\partial U}{\partial X_j} S_{X_j} \right) \left(\frac{\partial U}{\partial X_k} S_{X_k} \right) r_{X_j X_k} \right)} \quad (7)$$

where S_{X_j} are the sample standard errors of $X_j = 1$ to n variables, $\frac{\partial U}{\partial X_j}$ are the partial derivatives of U with respect to its component X_j variables, and $r_{X_j X_k}$ is the correlation coefficient between X_j and X_k . If U is the product of uncorrelated variables,

$$S_U = \sqrt{\sum_{j=1}^n \left(\frac{S_{X_j}}{\bar{X}_j} \right)^2} \quad (8)$$

where \bar{X}_j is the variable mean. Finally, if U is the product of a quantity, X , and a constant, c :

$$S_U = \sqrt{(c * S_X)^2} \quad (9)$$

To determine the effects of elevated CO₂, diversity, and enhanced N on TBCA, we used repeated measures ANOVA with ring nested within CO₂

treatment and plot nested within diversity, CO₂, and N levels as random effects (N = 296 per year; JMP 5.0.1, SAS Institute, Cary, North Carolina). All treatments were fixed effects. We also calculated CO₂ effects (TBCA in elevated CO₂ plots – TBCA in ambient CO₂ plots) and N effects (TBCA in enhanced N plots – TBCA in ambient N plots) to examine the size and direction of CO₂ and N effects over time.

In BioCON, root biomass increases with CO₂, N, and diversity (Reich and others 2001a; also in unpublished 1999–2006 data). High TBCA may be associated with large belowground biomass because of related high belowground C demand (for example, increasing SCF and root production in BioCON; Craine and others 2001b; Craine and Wedin 2002; unpublished root production and SCF data). To account for between-plot variation in standing belowground biomass C (BGC), we used BGC as a covariate in an ANCOVA. This allowed us to ascertain whether TBCA responds to CO₂, N, and diversity treatments in other ways besides those related to root biomass, or if TBCA is simply a function of the response of belowground biomass to these treatments. To determine if CO₂, N, or diversity treatments influenced the relationship between TBCA and BGC, we included two-way interactions between BGC and each treatment in the ANCOVA. We again calculated the CO₂ and N effects using the least-squares means from the TBCA ANCOVA.

Finally, to test our prediction that ecosystem C storage would increase only in response to elevated CO₂ and enhanced N availability, we performed repeated-measures ANOVA on annual ecosystem C storage rates ($\Delta[C_S + C_L + C_R]$). Because annual ecosystem C storage fluctuated between fire and non-fire years, we repeated the analysis for fire and non-fire years separately.

RESULTS

Annual Soil Carbon Flux

Non-growing season daily soil C flux (SCF) estimated by equations (3)–(6), ranged from 0.26 to 0.42 g C m⁻² day⁻¹ and was within the range of winter daily SCF values found in the literature (Del Grosso and others 2005; Mosier and others 2002). Total modeled SCF averaged 8% of total annual SCF (F_S), which, consistent with F_S measured in other grassland ecosystems, averaged about 850 g C m⁻² y⁻¹ (Table 1; Raich and Potter 1995; Knapp and others 1998; Pendall and others 2003; McCulley and others 2005).

TBCA Error Propagation

Averaged across all years, the error associated with calculating TBCA was only slightly larger than the standard error of TBCA (Table 2). Both terms were substantially smaller than the treatment means and, with one exception (N treatments at ambient CO₂), were also smaller than differences between treatment means. This indicated that in BioCON, the accumulated error associated with calculating TBCA was minimal, and the results of our statistical analyses should not be unduly affected by large amounts of error propagation.

TBCA, CO₂, and N effects ANOVAs

As hypothesized, increased diversity, elevated CO₂, and enhanced N availability all increased TBCA (Figures 1A, 2; Tables 1, 3). TBCA increased slightly but significantly over time (significant year effect with year as a continuous variable, data not shown). All treatments interacted with year, although the N by year interaction was only marginally significant ($P = 0.059$; Table 3). In contrast to our hypothesis, there were no significant interactions among treatments. Year, CO₂, and diversity had the largest effects on TBCA (Tables 1, 3).

Averaged across other treatments and years, the full range of diversity increased TBCA by 87 g C m⁻² y⁻¹. Increasing diversity from 1 to 4 species had the largest, most consistent positive impact on annual TBCA (Figure 1A; Table 1). Monocultures allocated the least C belowground in nearly all years, whereas increasing the number of species from 4 to 16 had variable effects on TBCA depending on year (significant diversity by year effect; Figure 1A and Table 3).

As predicted, elevated CO₂ increased TBCA across all treatments and years (Figure 2A, C) by an average of 150 g C m⁻² y⁻¹. The effect of CO₂ increased (although variably) over time (significant CO₂ by year interaction; Figure 2C). Adding N had the smallest effect on TBCA (40 g C m⁻² y⁻¹; Table 1). In contrast to our prediction based on PNL, the positive effect of N declined over time in both CO₂ treatments (marginally significant N by year effect; Figure 2E).

TBCA ANCOVA (Standardized for Root Biomass)

TBCA increased with BGC in the ANCOVA, but a linear regression of TBCA against BGC (all plots in all years; $P < 0.0001$) found that only 18% of the variation in TBCA was associated with between-plot and treatment differences in BGC, leaving 82%

Table 1. Annual Carbon (C) Inputs, Outputs, and Change in C Storage (Litter and Root C) in BioCON by CO₂, N, and Diversity Treatment

CO ₂ treatment	N treatment	Diversity (# species)	Yearly soil C respiration	Yearly aboveground C	Yearly C loss through fire	Change in root C	Change in litter C	Yearly TBCA
Ambient CO ₂	Ambient N	1	694.57 (9.84)	67.1 (2.93)	27.46 (2.79)	-17.22 (4.49)	4.84 (4.24)	655.72 (11.59)
		4	767.67 (10.14)	106.39 (5.6)	45.24 (5.75)	-28.11 (8.3)	4.28 (7.7)	682.7 (13.83)
		9	801.32 (12.83)	139.38 (5.69)	62.2 (7.47)	-20 (8.98)	11.67 (9.7)	715.81 (16.28)
Ambient CO ₂	Added N	16	809.09 (12.12)	168.01 (6.1)	65.15 (7.58)	-16.57 (10.93)	12.93 (10.73)	702.58 (18.47)
		1	697.03 (9.48)	90.99 (3.49)	42.34 (4.15)	-12.09 (5.87)	7.23 (5.86)	657.14 (12.27)
		4	819.84 (12.7)	134.44 (5.49)	61.34 (7.44)	-17.84 (11.02)	17.48 (12.04)	750.74 (18.35)
Elevated CO ₂	Ambient N	9	823.02 (13.44)	143.28 (4.63)	65.44 (6.97)	-22.83 (9.57)	11.74 (10.31)	728.41 (16.62)
		16	818.16 (14.3)	162.52 (4.97)	73.61 (8.44)	-18.91 (10.3)	10.65 (12.1)	722.45 (18.91)
		1	801.8 (8.44)	71.77 (3.64)	29.54 (3.07)	-14.96 (4.78)	5.27 (4.65)	759.93 (10.1)
Elevated CO ₂	Added N	4	955.37 (13.71)	132.42 (7.55)	57.57 (6.99)	-26.35 (8.09)	10.5 (8.74)	864.67 (18.13)
		9	969.22 (16.32)	167.21 (7.22)	70.49 (7.84)	-27.16 (9.4)	14.6 (10.87)	859.22 (21.58)
		16	953.62 (12.56)	179.45 (6.06)	78.17 (9.31)	-17.29 (10.77)	20.24 (13.69)	855.29 (18.44)
Elevated CO ₂	Added N	1	841.83 (11.8)	111.45 (4.87)	53.93 (4.9)	-13.1 (6.46)	10.86 (6.87)	797.33 (15.22)
		4	1008.06 (14.4)	180.48 (8.47)	86.42 (10.08)	-16.05 (10.99)	24.37 (13.66)	924.14 (21.38)
		9	1015.21 (13.85)	155.34 (5.58)	77.4 (8.26)	-19.57 (10.84)	22.98 (13.67)	945.9 (19.96)
		16	1005.17 (15.99)	168.48 (5.24)	81.06 (9.25)	-19.79 (12.05)	16.46 (13.53)	913.58 (21.23)

Mean (standard error), units are g C m⁻² y⁻¹.
TBCA total belowground C allocation.

Table 2. Mean TBCA, Propagated Error for Calculating TBCA and Standard Error of TBCA for 1999–2006 by CO₂ and N Treatment

CO ₂ treatment	N treatment	Mean TBCA	Propagated error	Standard error
Ambient CO ₂	Ambient N	682.04	24.59	18.86
Ambient CO ₂	Added N	703.25	27.37	21.56
Elevated CO ₂	Ambient N	819.18	24.49	20.64
Elevated CO ₂	Added N	873.54	29.79	26.32

Units are $\text{g C m}^{-2} \text{y}^{-1}$.

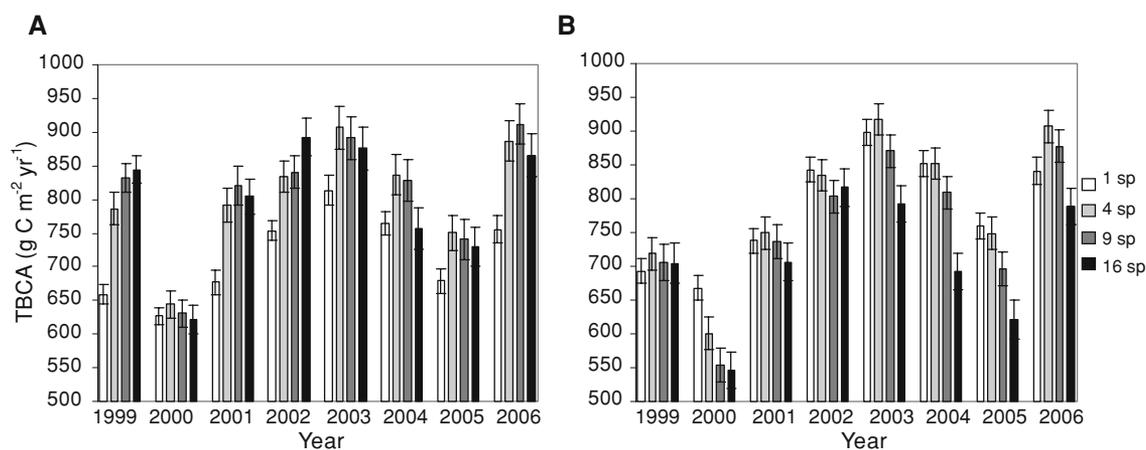


Figure 1. **A** Mean TBCA by year and diversity level and **B** lsmeans for TBCA by year and diversity level from the ANCOVA. Error bars are ± 1 standard error.

of the variation unexplained (Table 3; Figure 3). TBCA per unit BGC decreased from a mean TBCA/BGC ratio of 28 in plots with less than 100 g BGC m⁻² to a mean of 3 in plots with more than 100 g BGC m⁻² (Figure 3). This stark difference may be partly related to a sampling method that results in some unrealistically low BGC estimates in plots with low, patchy biomass. Additionally, root turnover (calculated using root ingrowth core data) was higher at low BGC (0.75 y⁻¹ when BGC is less than 100 g C m⁻²) than at high BGC (0.34 y⁻¹ when BGC is at least 100 g C m⁻²; data not shown), a pattern that would lead to higher TBCA per BGC at low than high BGC.

As predicted, the main effects of CO₂ and diversity remained significant in the ANCOVA indicating that these treatments influenced TBCA through processes unrelated to root biomass (Table 3). However, diversity and N both interacted significantly with year, such that the diversity and N effects were either neutral or negative, depending on year (Figures 1B, 2F). Elevated CO₂ also interacted significantly with year, but it increased TBCA in all

years (Figure 2D). There were no other significant interactions (Table 3).

Accounting for differences in BGC diminished the size and changed the direction of the diversity effect (Figure 1; Table 3). Depending on year, increasing diversity had neutral or negative effects on non-BGC associated TBCA (Figure 1B), indicating that the increase in TBCA between monocultures and diverse plots was associated with parallel differences in standing BGC. Diversity also interacted significantly with year (Table 3), with increasing diversity having essentially no effect on non-root biomass related TBCA in some years (for example, 1999) and decreasing non-root biomass-related TBCA in other years (for example, 2005; Figure 1B).

Even after removing (accounting for) BGC effects on TBCA, the effect of elevated CO₂ remained positive during all years and increased over time (Figure 2B, D). The average size of the non-BGC CO₂ effect, 130 g C m⁻² y⁻¹, was only slightly smaller than when including BGC effects, 150 g C m⁻² y⁻¹, indicating that approximately 87% of the effect of CO₂ on TBCA was unrelated to

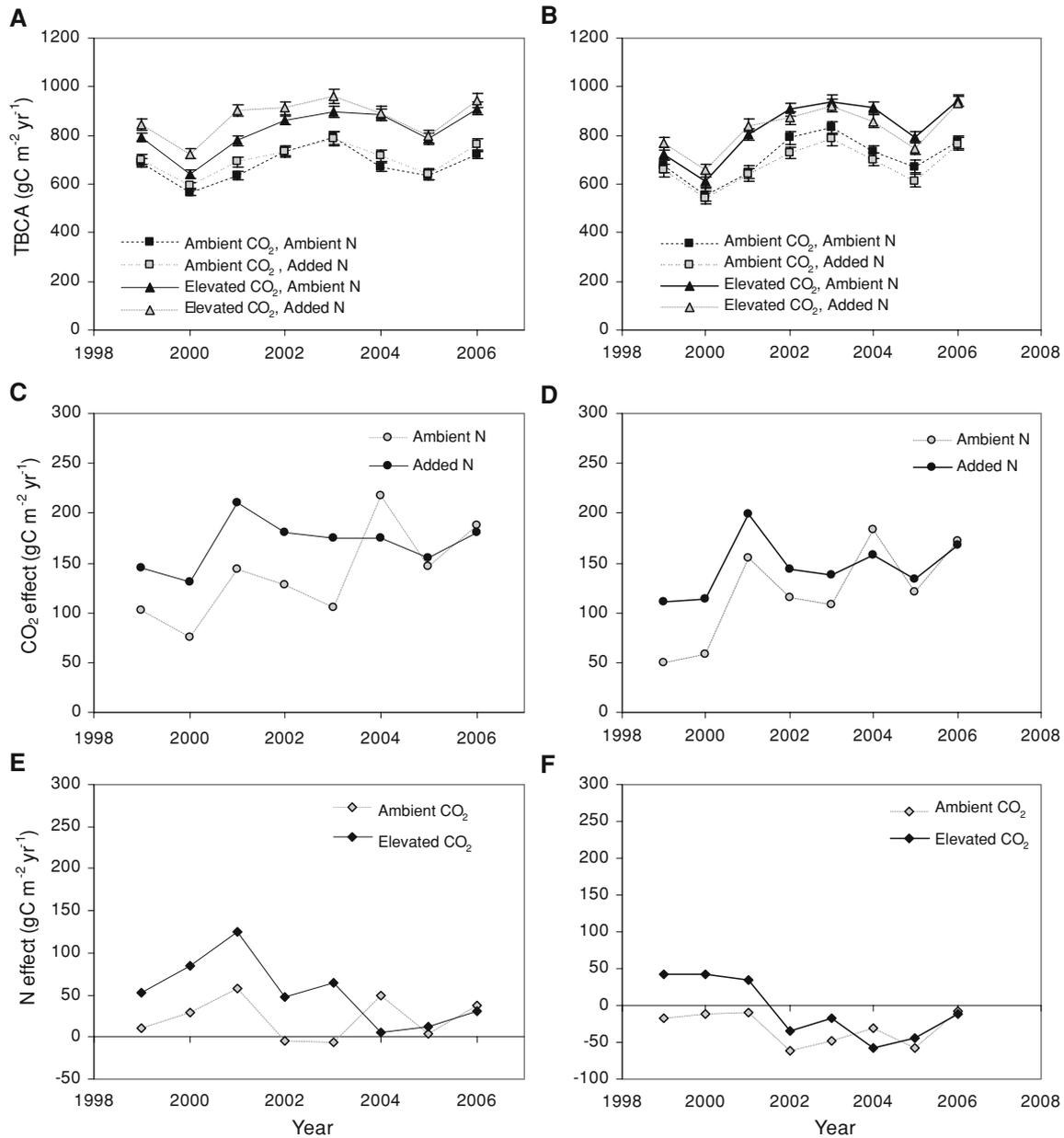


Figure 2. TBCA, CO₂, and N effects on TBCA by year. **A** mean TBCA by CO₂ and N treatment, **C** CO₂ effect by N treatment, and **E** N effect by CO₂ treatment. CO₂ and N effects were calculated by subtracting mean TBCA in ambient CO₂ or N treatments from mean TBCA in elevated CO₂ or N treatments. **B**, **D**, and **F** show lsmeans from the belowground biomass C ANCOVA. **B** TBCA by year, CO₂, and N treatment, **D** CO₂ effect by N treatment, and **F** N effect by CO₂ treatment. Error bars are ± 1 standard error.

BGC. This is a marked contrast to the diversity and N effects, which failed to affect or decreased TBCA in the absence of BGC-related effects. As in the ANOVA, the effect of N on the CO₂ effect appeared to decrease in 2004–2006, but this effect was not significant (Figure 2D). Elevated CO₂ did not change the slope of the relationship between TBCA and BGC (no CO₂ by BGC interaction), but it did increase TBCA per unit BGC (Figure 3A).

In contrast to our hypothesis and the ANOVA results, in the ANCOVA the enriched N treatment had a primarily negative effect on non-BGC associated TBCA. Added N initially increased (elevated CO₂) or had no effect (ambient CO₂) on non-BGC associated TBCA, but the N effect decreased over time and became negative in both CO₂ treatments by 2002 (N by year interaction; Table 3; Figure 2F). Adding N did not change the slope of

Table 3. Total Belowground Carbon Allocation (TBCA)

Source	TBCA ANOVA			TBCA ANCOVA		
	DF	F ratio	P	DF	F ratio	P
R^2	0.5222			0.6087		
CO ₂	1	48.2822	0.0017	1	29.2104	0.0047
N	1	10.1121	0.0016	1	2.2519	0.1345
CO ₂ *N	1	1.3888	0.2397	1	1.1166	0.2916
Diversity	3	20.1624	<0.0001	3	6.9743	0.0001
CO ₂ *diversity	3	1.5403	0.2044	3	1.8623	0.1360
N*diversity	3	0.7730	0.5100	3	1.2080	0.3069
CO ₂ *N*diversity	3	0.4030	0.7510	3	0.2262	0.8781
Year	7	63.8435	<0.0001	7	102.5136	<0.0001
CO ₂ *year	7	4.1076	0.0002	7	4.2898	0.0001
N*year	7	1.9440	0.0592	7	2.5234	0.0139
CO ₂ *N*year	7	1.1147	0.3508	7	0.7036	0.6690
Diversity*year	21	3.5020	<0.0001	21	3.5476	<0.0001
CO ₂ *diversity*year	21	0.9687	0.5002	21	0.8068	0.7140
N*diversity*year	21	0.7598	0.7714	21	0.6575	0.8769
CO ₂ *N*diversity*year	21	1.1491	0.2884	21	1.0856	0.3562
BGC				1	387.9950	<0.0001
CO ₂ *BGC				1	0.6466	0.4215
N*BGC				1	0.2364	0.6269
Diversity*BGC				3	1.2042	0.3068

Results are from the repeated measures ANOVA with main effects of CO₂, diversity, and N, with ring nested within CO₂ treatment, and from the ANCOVA including belowground carbon (BGC) as a covariate. Bold values are statistically significant ($P < 0.05$).

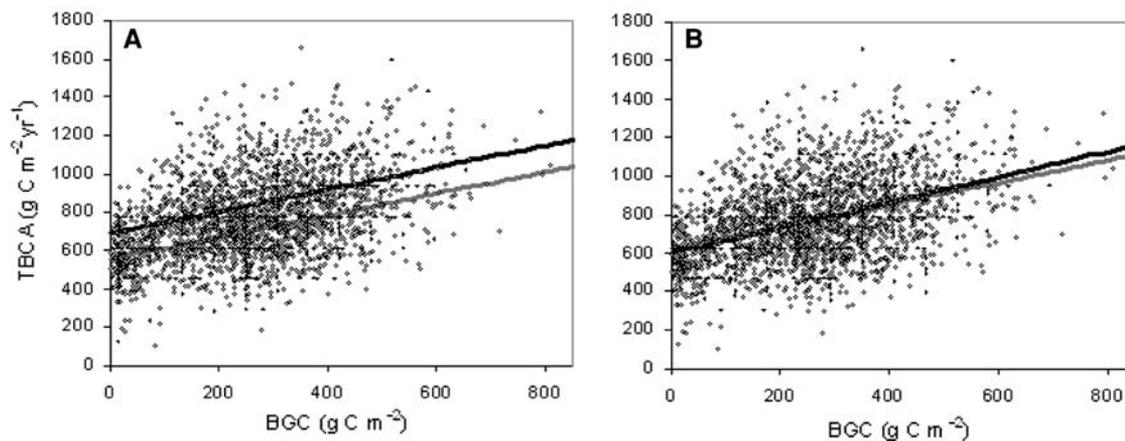


Figure 3. TBCA versus belowground biomass C (BGC). Predicted TBCA for **A** elevated CO₂ (gray line; $TBCA = 689.7 + 0.57 \cdot BGC$, $R^2 = 0.16$, $P < 0.0001$) and ambient CO₂ (black line; $TBCA = 557.1 + 0.57 \cdot BGC$, $R^2 = 0.18$, $P < 0.0001$) versus BGC and **B** added N (gray line; $TBCA = 597.9 + 0.66 \cdot BGC$, $R^2 = 0.20$, $P < 0.0001$) and ambient N (black line; $TBCA = 622.4 + 0.57 \cdot BGC$, $R^2 = 0.12$, $P < 0.0001$) versus belowground biomass C. Open circles are data.

the relationship between TBCA and BGC or TBCA per unit BGC (Figure 3B).

C Fluxes and Storage

Although C inputs and outputs were not always equal among treatments or years, they were on the

same order of magnitude, as there was little C storage in soil or biomass (Table 1). Annual TBCA and F_S were of similar magnitude. Losses due to fire (F_F ; when they occurred) were of similar magnitude as aboveground litter inputs (F_A). However, TBCA and F_S were 6–7 times larger than F_A and F_F (Table 1).

Table 4. Results from the Annual Ecosystem C Storage Repeated Measures ANOVAs for All Years, Fire Years, and Non-Fire Years, with Main Effects of CO₂, Diversity, and N, with Ring Nested Within CO₂ Treatment

Source	All years			Fire years			Non-fire years		
	DF	F ratio	P	DF	F ratio	P	DF	F ratio	P
R ²	0.2363			0.1731			0.3083		
CO ₂	1	4.82	0.0751	1	6.70	0.0487	1	20.81	0.0035
N	1	6.39	0.0121	1	13.44	0.0003	1	21.78	<0.0001
CO ₂ *N	1	0.31	0.5763	1	0.46	0.4974	1	0.01	0.9250
Diversity	3	0.53	0.6637	3	27.75	<0.0001	3	17.59	<0.0001
CO ₂ *diversity	3	0.11	0.9528	3	0.95	0.4172	3	0.76	0.5195
N*diversity	3	3.25	0.0224	3	0.73	0.5321	3	3.26	0.0221
CO ₂ *N*diversity	3	0.74	0.5268	3	1.89	0.1325	3	1.95	0.1211
Year	7	203.79	<0.0001	3	106.85	<0.0001	3	39.61	<0.0001
CO ₂ *year	7	4.77	<0.0001	3	3.94	0.0083	3	2.02	0.1100
N*year	7	6.31	<0.0001	3	0.68	0.5642	3	2.63	0.0488
CO ₂ *N*year	7	1.11	0.3553	3	0.19	0.9059	3	2.52	0.0566
Diversity*year	21	8.70	<0.0001	9	5.83	<0.0001	9	1.32	0.2248
CO ₂ *diversity*year	21	0.92	0.5703	9	1.02	0.4222	9	0.75	0.6650
N*diversity*year	21	1.49	0.0716	9	1.12	0.3476	9	1.39	0.1868
CO ₂ *N*diversity*year	21	1.33	0.1465	9	0.82	0.5964	9	1.32	0.2198

Storage is annual change in litter plus root C, where change in soil C = 0.
 Bold values are statistically significant ($P < 0.05$).

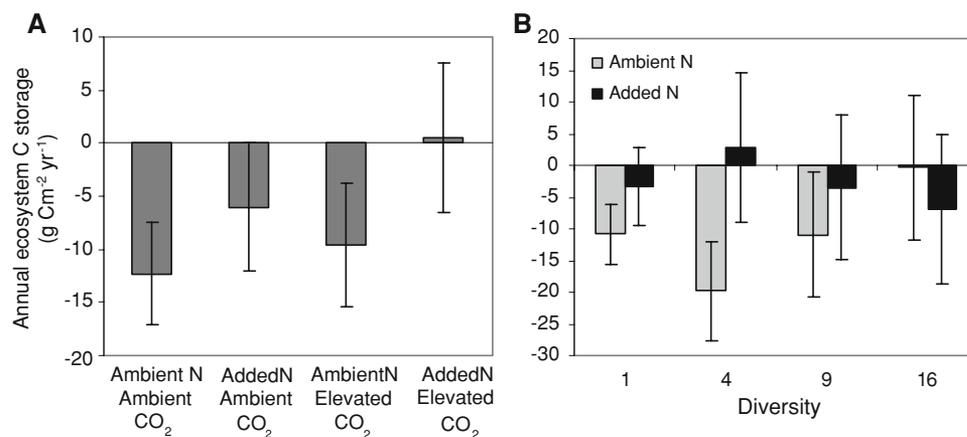


Figure 4. Ecosystem C storage rates by year and **A** CO₂ and N treatments and **B** diversity and N treatments. Error bars are ± 1 standard error.

Across all years, only N consistently altered ecosystem C storage rates by decreasing C loss, but this effect varied slightly with diversity (significant N by diversity effect; Table 4; Figure 4). Diversity, CO₂, and N treatments all interacted with year (Table 4). In years with fires, ecosystem C storage was negative (that is, C loss) and elevated CO₂, added N, and non-monoculture plots had the greatest C losses. In non-fire years, the opposite was true: generally positive C storage rates were greatest in elevated CO₂, added N and non-monoculture plots (Figure 5).

Running the ANOVA for only the fire years indicated that increasing CO₂, N, and diversity all

increased ecosystem C loss (Table 4). Increased CO₂ either increased or had no effect on C loss depending on year, and the magnitude of C loss due to diversity treatments varied between years (significant treatment by year interactions; Table 4; Figure 5B, C). When run for only the non-fire years, the ANOVA indicated similar trends, but in the opposite direction: CO₂, N, and diversity increased ecosystem C storage, and N interacted with both year and diversity as the size of these positive effects varied with year and diversity treatment (Figure 5; Table 4).

Overall rates of annual ecosystem C storage were neutral (zero, or with error bars that encompassed

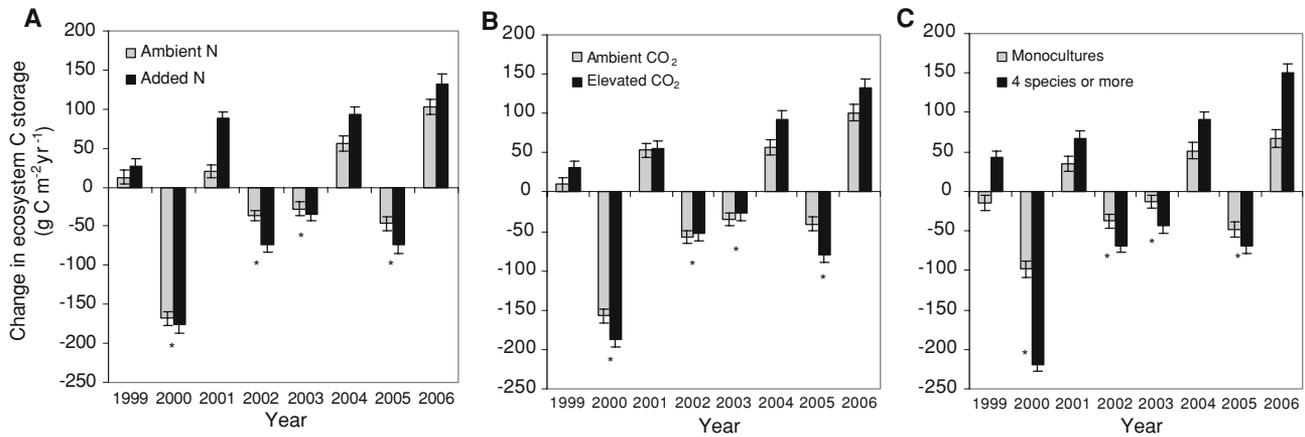


Figure 5. Ecosystem C storage rates by year and **A** N treatment, **B** CO₂ treatment, and **C** diversity treatment. Asterisks indicate fire years. Error bars are ±1 standard error.

zero) or slightly negative (Figure 4). Negative rates of C storage were associated with ambient N and lower diversity treatments (1-, 4-, and 9-species plots; Figure 4). The unresponsiveness of ecosystem C storage rates to elevated CO₂ and relatively small response of C storage rates to added N and diversity treatments indicate that increased C inputs in these treatments were matched by similar increases in C outputs (Figure 4).

DISCUSSION

The total amount of C allocated belowground increased strongly with standing BGC. Although large differences in standing root biomass can result from similar TBCA but different root turnover rates, the strong linkage here between TBCA and BGC suggests that turnover-rate differences among treatments are modest compared to treatment differences in biomass production. Removing the effect of BGC greatly diminished N and diversity effects on TBCA, but did not substantially alter the CO₂ effect on TBCA. This suggests that increasing N and diversity primarily affect the BGC-related portion of TBCA, whereas elevated CO₂ mainly influences non-BGC associated aspects of TBCA. This is consistent with the response of BGC to BioCON treatments over the same period: adding N nearly doubled BGC and increasing diversity increased BGC by 30%, whereas elevated CO₂ increased BGC by only 14% (data not shown). Our results also support the hypothesis that elevated CO₂ accelerates C cycling more than it increases biomass C pools (Hungate and others 1997; Van Kessel and others 2000).

Diversity increased TBCA when root biomass effects were included, but contrary to our expectations, once root biomass effects were removed, diversity had either neutral or negative effects on annual TBCA. This suggests that the positive effect of diversity on TBCA was related to maintaining high BGC, perhaps by increasing root productivity due to more intense competition for belowground resources and/or complementary resource use, which may increase the spatial and temporal distribution of root biomass. Our results are consistent with those of Dijkstra and others (2005, ANOVA only) who found that the positive effect of diverse communities on labile and recalcitrant soil C was primarily associated with high root biomass. Additionally, our results are consistent with analyses that found the fraction of TBCA allocated to root production in BioCON increased with diversity (root ingrowth core data, results not shown).

As we hypothesized, elevated CO₂ increased TBCA whether or not the treatment effects were examined independently of differences in BGC. Indeed, nearly 90% of the increase in TBCA at elevated CO₂ was unrelated to BGC, and elevated CO₂ actually increased TBCA per unit BGC. Elevated CO₂ almost always, including in BioCON, increases rates of photosynthesis, thereby increasing photosynthate availability (Lee and others 2001; Curtis and Wang 1998; Wand and others 1999). As a result, elevated CO₂ tends to increase aboveground production (Reich and others 2004; DeGraaff and others 2006) and many of the components of TBCA (that is, root production, root exudates, rhizodeposition, and allocation to mycorrhizae; Matamala and Schlesinger 2000; Treseder and Allen 2000; Pendall and others 2004;

Allen and others 2005; Trueman and Gonzalez-Meler 2005). In BioCON, elevated CO₂ has increased root production (unpublished root ingrowth data), soil respiration (Craine and others 2001a), and AM fungi abundance and biomass (Wolf and others 2003; Antoninka, unpublished results). However, root productivity in BioCON averages only one-fifth of TBCA, and this fraction did not respond to elevated CO₂ (unpublished root ingrowth data). In conjunction with our results, this suggests that elevated CO₂ increases TBCA by increasing the amount of C allocated to the non-root production components of TBCA per unit BGC.

In line with our prediction of N limitation of photosynthate availability, TBCA increased with N additions, but, in contrast to our prediction of progressive N limitation (and the effects of N and CO₂ on total biomass in BioCON), the N effect declined over time to become largely neutral in both CO₂ treatments. After accounting for variation in BGC, the primary effect of added N became negative, suggesting that the root- and non-root-related portions of TBCA were affected quite differently by N availability. Our results for TBCA including root-related C allocation are consistent with research in forest stands across large scale gradients in productivity, where TBCA increased across fertility gradients (Raich and Nadelhoffer 1989; Raich 1998), and also with results that found the fraction of TBCA allocated to root production in BioCON increased with added N (unpublished root ingrowth data). The effects of N on the other components of TBCA (for example, exudation, rhizodeposition) are not clear (Grayston and others 1996; Henry and others 2005). Our results suggest that these fluxes may decline in high N environments, and are consistent with results indicating that added N decreased the abundance of AM fungi in BioCON (Antoninka, unpublished data).

The onset of negative N effects on non-BGC associated TBCA and reduced N effects on total TBCA after 2001 coincided with the decline and stabilization of the root fraction (root/total biomass) in 2002, and the decline and stabilization of root production after 2000. These temporal trends in TBCA, root production, and root fraction may signify the end of the plant community "establishment" phase and indicate a mature plant community. Thus, the initial increase in TBCA with enhanced N availability may have been due to the relatively greater distribution of biomass to roots than to aboveground biomass early during perennial establishment growth. Additionally, the ratio of aboveground production (F_A) to root produc-

tivity increased over time, further suggesting that plant communities reduced investment in root biomass relative to aboveground biomass over time (data not shown).

Elevated CO₂, N, and diversity increased C inputs into and outputs from soil, resulting in neutral ecosystem C storage rates. Our results are consistent with results finding changes in NEP to be small at elevated CO₂ and relatively insensitive to added N (Reich and others 2006a). They are also consistent with experiments finding elevated CO₂ to increase soil C turnover rates, resulting in little or no change in soil C (Hungate and others 1997; Van Kessel and others 2000; Hagedorn and others 2003; Lichter and others 2005). Our results contrast with those of Fornara and Tilman (2008) who found diversity and N addition to increase ecosystem C storage via increases in total soil C in an annually burned grassland. In BioCON, soil C did not change significantly over time (see *Methods*). These contrasting temporal soil C patterns may be due to the relatively C depleted initial state of the soils in Fornara and Tilman's (2008) experiment (approximately 0.48% versus an average of 0.63% at the beginning of BioCON and 0.60% in the diverse plots at the end of the Fornara and Tilman (2008) experiment).

Diversity, CO₂, and N all increased the amplitude of annual ecosystem C storage rates due to interactions between litter accumulation and fire-induced C losses. In fire years, ecosystem C storage rates were negative, and elevated levels of all treatments resulted in greater rates of C loss. In non-fire years, ecosystem C storage rates were positive, and elevated levels of all treatments increased C storage rates. This effect was driven by greater aboveground productivity, and thus accumulation of litter, in elevated CO₂, added N, and diverse plots. Elevated CO₂, added N, and diversity increased litter layer C by 10–20 g C m⁻² y⁻¹. Thus, in non-fire years, rates of litter accumulation were highest in these plots and in fire years, rates of litter loss were greatest in these plots (rates of change in root C did not vary strongly between fire and non-fire years).

In contrast to our hypothesis, adding N did not result in C sequestration. Annual ecosystem C storage rates varied between neutral and negative, depending on year. However, in plots with less than 16 species, negative C storage rates in ambient N plots were increased to zero by adding N, suggesting that high N availability could contribute to C sequestration by reducing C losses. Rates of C loss in ambient N plots were very small (11 g C m⁻² y⁻¹) compared to total ecosystem C in BioCON

(2130 g C m⁻² y⁻¹, soil C to a depth of 20 cm), suggesting that ecosystem C storage will change very little or that it will take a substantial period of time to affect total ecosystem C storage.

However, our results also suggest that in the absence of fire, these grasslands would store C in litter at an average rate of 66 g C m⁻² y⁻¹. During non-fire years, elevated CO₂, N, and diversity increased storage rates by 25, 40, and 50 g C m⁻² y⁻¹, respectively. Unburned grasslands, therefore, may store more C as atmospheric CO₂ and N deposition increase. These estimates may be high, however, as rates of C accumulation in litter may be at a maximum in years following burns and saturate after multiple years without burning, because the potential for C litter storage in grasslands is low (relative to woody systems). Increased litter inputs in unburned grasslands could translate into increased soil C storage as litter is decomposed and incorporated into soil organic matter (Fornara and Tilman 2008; McLauchlan and others 2006), and perhaps especially so under elevated CO₂. However, unburned grasslands may ultimately undergo conversion to savannah or woodland, thereby further increasing C sequestration in biomass and potentially in soils (Tilman and others 2000; Hibbard and others 2001; McKinley and Blair 2008).

CONCLUSIONS

In this ecosystem, elevated CO₂, increased diversity, and enhanced N availability all increased TBCA, but the effects were complex. Elevated CO₂ strongly increased total TBCA, especially the portion of TBCA unrelated to maintaining high BGC. In contrast, high diversity and N increased total TBCA, but had primarily negative impacts on non-BGC associated TBCA.

Elevated CO₂, N, and diversity increased C gain in non-fire years and C loss in fire years. These gains and losses were balanced such that across all years, ecosystem C losses were very small or zero, resulting in little change in total ecosystem C over time. Our results imply that increasing atmospheric CO₂ or N deposition are unlikely to increase C sequestration in diverse, periodically burned, grassland ecosystems, although both enhance C cycling.

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