

# Effects of plant diversity, N fertilization, and elevated carbon dioxide on grassland soil N cycling in a long-term experiment

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## Abstract

The effects of global environmental changes on soil nitrogen (N) pools and fluxes have consequences for ecosystem functions such as plant productivity and N retention. In a 13-year grassland experiment, we evaluated how elevated atmospheric carbon dioxide (CO<sub>2</sub>), N fertilization, and plant species richness alter soil N cycling. We focused on soil inorganic N pools, including ammonium and nitrate, and two N fluxes, net N mineralization and net nitrification. In contrast with existing hypotheses, such as progressive N limitation, and with observations from other, often shorter, studies, elevated CO<sub>2</sub> had relatively static and small, or insignificant, effects on soil inorganic N pools and fluxes. Nitrogen fertilization had inconsistent effects on soil N transformations, but increased soil nitrate and ammonium concentrations. Plant species richness had increasingly positive effects on soil N transformations over time, likely because in diverse subplots the concentrations of N in roots increased over time. Species richness also had increasingly positive effects on concentrations of ammonium in soil, perhaps because more carbon accumulated in soils of diverse subplots, providing exchange sites for ammonium. By contrast, subplots planted with 16 species had lower soil nitrate concentrations than less diverse subplots, especially when fertilized, probably due to greater N uptake capacity of subplots with 16 species. Monocultures of different plant functional types had distinct effects on N transformations and nitrate concentrations, such that not all monocultures differed from diverse subplots in the same manner. The first few years of data would not have adequately forecast the effects of N fertilization and diversity on soil N cycling in later years; therefore, the dearth of long-term manipulations of plant species richness and N inputs is a hindrance to forecasting the state of the soil N cycle and ecosystem functions in extant plant communities.

**Keywords:** ammonium, monocultures, nitrate, nitrification, nitrogen mineralization, root biomass, root nitrogen, species richness, temporal

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## Introduction

The soil nitrogen (N) cycle impacts key terrestrial ecosystem functions, such as plant productivity and retention of N from atmospheric deposition or fertilization. Aspects of the soil N cycle also can influence the response of these ecosystem functions to environmental change (Polley *et al.*, 2011). In grasslands, for example, the availability of inorganic N in soil can limit the response of plant productivity to both elevated atmospheric carbon dioxide (CO<sub>2</sub>) concentrations and changes in plant diversity (Reich *et al.*, 2001a, 2006; Schneider *et al.*, 2004; Fargione *et al.*, 2007). In addition, the effect of elevated CO<sub>2</sub> and plant diversity on N retention depends not only on how these treatments impact plant N uptake but also on the response of soil

N transformations, including N mineralization and nitrification (Niklaus *et al.*, 2001; Oelmann *et al.*, 2011). However, it remains difficult to predict how environmental change will influence these ecosystem functions; this is partly because the duration of most relevant experiments, typically less than 5 years, is shorter than the timescale of some treatment–plant–soil interactions (Fornara *et al.*, 2009; Eisenhauer *et al.*, 2011; Reich *et al.*, 2012). To address this uncertainty, we measured soil N transformations and dissolved inorganic N concentrations for 13 consecutive years in the ‘BioCON’ grassland experiment, where atmospheric CO<sub>2</sub>, herbaceous plant diversity, and N fertilization treatments were implemented in a factorial design (Reich *et al.*, 2001a).

Several factors can mediate the effects of environmental change on soil N cycling over time, including (i) plant N uptake and storage, (ii) the abundance and composition of organic substrates utilized by soil microbes, such as plant litter, root exudates, and soil

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organic matter (Zak *et al.*, 2000b; Knops *et al.*, 2002; Luo *et al.*, 2004; Parton *et al.*, 2007), and (iii) climatic conditions such as precipitation and temperature (Niklaus & Korner, 2004). Furthermore, the effects of CO<sub>2</sub>, N fertilization, and plant diversity on soil N cycling are likely to be dynamic because some components of the N cycle respond to environmental manipulations over different timescales. For example, plant N uptake and plant tissue N concentrations can respond quickly to elevated CO<sub>2</sub> and plant diversity treatments (Tilman *et al.*, 1997; Reich *et al.*, 2001a; Gill *et al.*, 2002; Niklaus & Korner, 2004; Knops *et al.*, 2007). In contrast, it can take years or decades for these treatments to impact accumulation or destabilization of organic N in soil (Fornara & Tilman, 2008). The effect of environmental perturbations on soil N cycling can also be dependent on the initial state and the trajectory of ecosystem N pools and fluxes. In ecosystems with small soil organic matter pools, the responsiveness of litter N concentrations and litter N mineralization likely has a substantial effect on total N mineralization in soil (Zak *et al.*, 2000a). Conversely, if soil organic matter pools are large or accumulate over time, the effects of environmental change on total N mineralization could be controlled primarily by the response of mineralization/immobilization of N in soil organic matter, not litter (Zak *et al.*, 2000a). Similarly, the effects of CO<sub>2</sub>, N fertilization, and diversity on nitrate leaching and N retention should depend on the initial size, composition, and trajectory of the inorganic N pool; effects on N retention will be larger when nitrification rates are high and nitrate is abundant.

Assumptions or observations about treatment effects on various intermediate factors can be used to generate predictions regarding potential effects on soil N cycles over time. For example, according to the progressive N limitation hypothesis (Luo *et al.*, 2004), if elevated CO<sub>2</sub> or high plant diversity reduces litter nutrient concentrations or increases storage of N in *stable* soil organic matter over time, then net N mineralization could subsequently decline. Regarding N retention, it has been shown that high plant diversity and elevated atmospheric CO<sub>2</sub> can reduce soil nitrate concentrations by stimulating plant N uptake or inhibiting nitrification (Tilman *et al.*, 1996; Niklaus *et al.*, 2001; Oelmann *et al.*, 2011). If the stimulation of plant N uptake increases over time, as observed by Reich *et al.* (2012), the reduction in soil nitrate could become larger. Conversely, if elevated CO<sub>2</sub> or high plant diversity have less negative, or even positive, effects on N mineralization and nitrification rates over time (Oelmann *et al.*, 2011; Reich *et al.*, 2012), soil nitrate levels could increase. Finally, if the activity or quantity of roots in monocultures declines over time, as could occur due to accumulation of belowground pathogens (De Kroon *et al.*, 2012),

monocultures could become increasingly 'leaky' with respect to nitrate as compared with diverse communities. Predictions such as these are infrequently tested because their evaluation requires long-term records of multiple components of the terrestrial N cycle.

To test these predictions and to explore dynamics in the effects of elevated CO<sub>2</sub>, N fertilization, and plant diversity on soil inorganic N pools and fluxes, we evaluate and present 13 years of data from the BioCON experiment. At BioCON, planted species richness ranged from 1 to 16 species drawn from a pool of four N-fixing forbs (legumes), four other forbs, and four each of C3 and C4 grasses (all perennials). CO<sub>2</sub> and N fertilization treatments included ambient and elevated CO<sub>2</sub> (+180 ppm) and unfertilized and fertilized communities (+4 g N m<sup>-2</sup> yr<sup>-1</sup> of ammonium nitrate). For the first 2 years, plant diversity had large negative effects on midsummer net N mineralization rates and inorganic N concentrations in soil, whereas N fertilization substantially increased net N mineralization and inorganic N concentrations. Contrastingly, elevated CO<sub>2</sub> had smaller, and statistically insignificant, effects on these soil N parameters (Reich *et al.*, 2001a). In later analyses, temporal patterns in treatment effects on net N mineralization began to emerge; elevated CO<sub>2</sub> increased net N mineralization but only in fertilized communities and after the 3rd year of the experiment (Reich *et al.*, 2006; Reich & Hobbie, 2012). However, diversity effects on these soil N parameters have not been fully reported (Reich *et al.*, 2012) or compared with effects of elevated CO<sub>2</sub> or N fertilization, since the 2nd year of the experiment. Here, for the first time, we also report net nitrification rates and separately evaluate treatment effects on nitrate and ammonium, which have different behavior and significance in soils. None of our previous efforts focused on the temporal dynamics of treatment effects on the soil N cycle. This focus is essential given the role of inorganic N pools and fluxes in controlling plant productivity (Reich *et al.*, 2001b; Baer *et al.*, 2003; Fornara & Tilman, 2009) and nitrate leaching.

## Materials and methods

### Study site

The BioCON experiment was constructed and seeded with plants in 1997 on sandy glacial outwash soils (>90% sand) that were previously occupied by secondary successional grassland. Nitrogen fertilization and CO<sub>2</sub> treatments were initiated in 1998. Experimental treatments were arranged in a split-plot, randomized, factorial design with CO<sub>2</sub> treatment as the whole-plot factor. Atmospheric CO<sub>2</sub> was elevated by 180 ppm using free-air carbon dioxide enrichment (FACE) technology. The elevated CO<sub>2</sub> treatment was assigned randomly to three

of six total plots, i.e., blocks that are commonly called 'rings' in FACE studies. Factorial combinations of N fertilization and species richness treatments were randomly arranged in 2 m × 2 m subplots located within each large plot (a total of ≈ 60 per plot). Ammonium-nitrate fertilizer was applied once in May, June, and July of each year to achieve a nitrogen (N) enrichment of 4 g m<sup>-2</sup> yr<sup>-1</sup>. The experiment includes four levels of planted species richness: 1, 4, 9, and 16 species. Planted species included four N-fixing forbs (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis*, *Petalostemum villosum*), four other forbs (*Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa*, *Solidago rigida*), four C3 grasses (*Agropyron repens*, *Bromus inermis*, *Koeleria cristata*, *Poa pratensis*), and four C4 grasses (*Andropogon gerardii*, *Bouteloua gracilis*, *Schizachyrium scoparium*, *Sorghastrum nutans*). Subplots were regularly weeded to remove unplanted species.

### Soil N measurements

On one day in late June or early July from 1998 to 2010, four 2 cm diameter soil cores were removed from each subplot to a depth of 20 cm and two PVC tubes (20 cm long, 2 cm diameter, with a beveled edge on the bottom) were placed in each subplot and capped with a rubber stopper. The four removed soil cores were composited, sieved (2 mm), and extracted with 1 M KCl. Nitrate and ammonium concentrations in KCl extracts were then measured on an Alpkem autoanalyzer. After 30 days, the PVC tubes were removed and then composited, sieved, and extracted as described above. Net N mineralization rates were estimated by subtracting the total inorganic N, including ammonium and nitrate, in the four soil cores from that in the field-incubated PVC tubes. Net nitrification rates were calculated similarly, but using only nitrate data.

In a subset of subplots in 2001, net N mineralization was measured monthly between April and September. Across subplots, total net N mineralization during this growing season was positively correlated with net N mineralization during ca. July ( $R^2 = 0.52$ ,  $P < 0.0001$ ,  $n = 166$ ). Also, net N mineralization in July accounted for approximately one-third of total net N mineralization during the growing season (mean = 35%, median = 27%). Thus, in other years, the July measurements likely reflect much of the variation in total net N mineralization during the growing season.

### Statistics

To improve normality for use of linear models, soil nitrate and ammonium concentrations were log<sub>10</sub> transformed and rates of net N mineralization and net nitrification were square-root transformed; for negative values, the square root of the absolute value of the number was multiplied by -1 to retain its original sign. To describe and compare average values of these soil N parameters from different treatments and years, we calculated the mean of transformed values and back transformed the mean; for log-transformed variables, this is equivalent to calculating the geometric mean for log-transformed variables. For example, the average soil nitrate concentration is represented by 10<sup>X</sup>, where X is the mean of log<sub>10</sub>-transformed nitrate concentrations.

Soil nitrate was not detected in some samples. To conduct analyses on the full set of subplots, we assigned these samples a value of zero and 0.01 was added to all values prior to log<sub>10</sub> transformation. The distributions of soil ammonium concentrations and N transformation rates contained outliers, i.e., observations that extended beyond the upper or lower quartile by a value more than 1.5 times the interquartile range, but outliers accounted for less than 5% of observations in each case. The results of our statistical analyses were not substantially influenced by inclusion of subplots without detectable nitrate or by exclusion of outliers for ammonium concentrations and N transformations (Tables S1–S4). Below we report statistics from models that included subplots without detectable nitrate and excluded outliers for the other soil N variables.

Following Reich *et al.* (2006) the significance of experimental treatment effects on soil N parameters was evaluated in nested, repeated-measures ANOVA models. Treatments and their interaction terms were modeled as fixed effects, whereas subplot and FACE ring were modeled as random factors. The CO<sub>2</sub>, N, and species richness terms were nested within subplot and the CO<sub>2</sub> term was nested within ring. We then conducted a variety of additional analyses to assess how treatment effects on soil N cycling were mediated by other factors, including plant functional composition, other belowground properties, and climatic variables.

We evaluated legume effects on soil N parameters because these N-fixing plants can strongly influence soil N cycling. The 16-species subplots were all planted with four legume species, but legume abundance was variable both across subplots and across years. For these species-rich subplots, we assessed the influence of legumes on soil N parameters by adding legume aboveground biomass to the repeated-measures models described above. For monocultures, we evaluated potential differences among legumes, C3 grasses, C4 grasses, and forbs using repeated-measures models with plant functional type, CO<sub>2</sub>, and N fertilization as predictors; all possible interactions among these were also included.

We used additional repeated-measures models to evaluate how soil N pools and fluxes were related to belowground, subplot-level properties that could mediate the treatment effects on soil N cycling, including root biomass, root N concentration, soil C and N concentration, and soil moisture. With one exception, effects of these 'mediating factors' were evaluated using models both with and without experimental treatments, e.g., elevated CO<sub>2</sub>, as covariates. Nitrogen fertilization was included as a factor in all models of soil nitrate and ammonium.

Separate analyses were required to evaluate whether inter-annual climate variability, measured at the site-level only, mediated the effects of experimental treatments on soil N cycling. Specifically, we used linear regression to assess correlations between climatic variables and average treatment effects for each year. We focused on the following climatic variables: (i) average high and low air temperatures for June and July, (ii) total precipitation in June and July, and (iii) gravimetric soil moisture measured at the beginning of the *in situ* incubation for net N mineralization, when soils were also sampled for inorganic N concentrations. Effects of N fertilization

were calculated by subtracting the average of fertilized subplots from that of ambient N subplots for each level of species richness (using only ambient CO<sub>2</sub> subplots). Carbon dioxide effects were calculated by subtracting the average of elevated CO<sub>2</sub> subplots from that of ambient CO<sub>2</sub> subplots for each level of species richness (using only ambient N subplots). Species richness effects were calculated by subtracting the average of 16-species subplots from that of monocultures for all four combinations of N and CO<sub>2</sub> treatments.

For all repeated-measures models, we compared models with two different structures to gain insights regarding the nature of temporal effects on soil N cycling: (i) experiment year coded as a continuous variable, which evaluates the extent to which temporal effects, including dynamic effects of treatments and mediating factors, were linearly cumulative, and (ii) experiment year coded as a discrete variable, which allows temporal effects to be noncumulative because each year is a group that is independent of other years.

When evaluating effects of mediating factors, such as root N and legume abundance, we also used models that excluded experiment year as a covariate, allowing interannual variation in each mediating factor to fully contribute to its explanatory power. When year is included as a continuous variable, variation in mediating factors across years can also contribute to their explanatory power, but only to the extent that temporal changes in each mediating factor are independent of linear changes in the dependent variable over time. When year is included as a discrete variable, the explanatory power of mediating factors is evaluated across subplots within years but not across years. Thus, for example, if the proportion of variance explained by root N declines when year is included in the model, then some variation in N cycling could be associated exclusively with variation in root N *across* years. If the effect of root N is insignificant when year is included as a

discrete parameter, then variation in root N *within* years is not associated with variation in soil N cycling.

In some cases, we used *F*-ratios to compare the explanatory power of different predictors. We only compared *F*-ratios for predictors with the same degrees of freedom; thus, differences between *F*-ratios are proportional to differences in the sums-of-squares, i.e., in the variance explained by each predictor. When comparing two predictors, the *F*-ratio of main effects and interaction terms were summed for each predictor, excluding the interaction terms that included the two parameters being compared.

## Results

### *Linear vs. noncumulative temporal dynamics*

For each soil N parameter, models with noncumulative temporal effects gave better fits than models with linear temporal effects (Tables S1–S4 and Tables 1–3). Below, when describing BioCON treatment effects, we provide statistics only from models with noncumulative temporal effects, i.e., with year as a discrete parameter.

### *N transformations*

In ambient subplots, net N mineralization rates varied from year to year ( $P < 0.0001$  for the year effect; Table S1) but not in a directional, linear manner (Fig. S1). There were not significant main effects of CO<sub>2</sub> or N fertilization treatments on net N mineralization ( $P > 0.2$ ). The effect of N fertilization on net N mineralization did vary over time but not in a directional way ( $P < 0.01$  for

**Table 1** Results from repeated-measures models of net N mineralization rates. Prior to analysis, mineralization rates were square-root transformed and 163 outliers were excluded (see Table S1 and *Methods*). Three model structures are compared, one with year excluded, one with year as a continuous parameter, and one with year as discrete parameter. Subplot was included as a random effect (data not shown)

| Model term                  | Year excluded  |                   | Year continuous |                   | Year discrete  |                   |
|-----------------------------|----------------|-------------------|-----------------|-------------------|----------------|-------------------|
|                             | <i>F</i> ratio | <i>P</i> value    | <i>F</i> ratio  | <i>P</i> value    | <i>F</i> ratio | <i>P</i> value    |
| Year                        | na             | na                | 6               | <b>0.02</b>       | 4              | <b>&lt;0.0001</b> |
| Soil moisture               | 39             | <b>&lt;0.0001</b> | 18              | <b>&lt;0.0001</b> | 3              | 0.11              |
| Root biomass                | 39             | <b>&lt;0.0001</b> | 40              | <b>&lt;0.0001</b> | 20             | <b>&lt;0.0001</b> |
| Root N                      | 70             | <b>&lt;0.0001</b> | 65              | <b>&lt;0.0001</b> | 74             | <b>&lt;0.0001</b> |
| Soil moist. × root biom.    | 25             | <b>&lt;0.0001</b> | 1               | 0.34              | 0              | 0.87              |
| Soil moist. × root N        | 1              | 0.41              | 1               | 0.23              | 1              | 0.45              |
| Root biom. × root N         | 0              | 0.58              | 2               | 0.17              | 4              | <b>0.04</b>       |
| Year × soil moisture        | na             | na                | 13              | <b>0.0004</b>     | 1              | 0.20              |
| Year × root biomass         | na             | na                | 40              | <b>&lt;0.0001</b> | 5              | <b>&lt;0.0001</b> |
| Year × root N               | na             | na                | 4               | <b>0.04</b>       | 1              | 0.33              |
| Model <i>n</i>              | 3279           |                   | 3279            |                   | 3279           |                   |
| Model <i>R</i> <sup>2</sup> | 0.14           |                   | 0.16            |                   | 0.21           |                   |

*P* values less than 0.01 are in bold print, those between 0.01 and 0.05 are in bold, italicized print, those between 0.05 and 0.1 are in italicized print.



**Table 2** Results from repeated-measures models of soil nitrate concentrations ( $\log_{10}$  transformed after adding 0.01). Three model structures are compared, one with year excluded, one with year as a continuous parameter, and one with year as discrete parameter. Subplot was included as a random effect (data not shown)

| Model term                  | Year excluded |             | Year continuous |               | Year discrete |             |
|-----------------------------|---------------|-------------|-----------------|---------------|---------------|-------------|
|                             | F ratio       | P value     | F ratio         | P value       | F ratio       | P value     |
| Year                        | na            | na          | 358             | <0.0001       | 55            | <0.0001     |
| N fertilization             | 447           | <0.0001     | 425             | <0.0001       | 498           | <0.0001     |
| Soil moisture               | 295           | <0.0001     | 63              | <0.0001       | 5             | <b>0.02</b> |
| Nitrification               | 0             | 0.52        | 0               | 0.70          | 2             | 0.19        |
| Root biomass                | 954           | <0.0001     | 714             | <0.0001       | 679           | <0.0001     |
| Fert. × soil moist.         | 77            | <0.0001     | 25              | <0.0001       | 3             | <i>0.07</i> |
| Fert. × nitrification       | 2             | 0.22        | 7               | <b>0.009</b>  | 1             | 0.24        |
| Fert. × root biom.          | 2             | 0.17        | 3               | <i>0.09</i>   | 5             | <b>0.02</b> |
| Soil moist. × nitrif.       | 22            | <0.0001     | 14              | <b>0.0002</b> | 0             | 0.95        |
| Soil moist. × root biom.    | 7             | <b>0.01</b> | 0               | 0.53          | 1             | 0.42        |
| Nitrif. × root biom.        | 6             | <b>0.01</b> | 21              | <0.0001       | 22            | <0.0001     |
| Year × fertilization        | na            | na          | 11              | <b>0.001</b>  | 30            | <0.0001     |
| Year × soil moisture        | na            | na          | 193             | <0.0001       | 2             | 0.11        |
| Year × nitrification        | na            | na          | 2               | 0.19          | 6             | <0.0001     |
| Year × root biomass         | na            | na          | 2               | 0.19          | 22            | <0.0001     |
| Model <i>n</i>              | 3336          |             | 3336            |               | 3336          |             |
| Model <i>R</i> <sup>2</sup> | 0.53          |             | 0.61            |               | 0.75          |             |

*P* values less than 0.01 are in bold print, those between 0.01 and 0.05 are in bold, italicized print, those between 0.05 and 0.1 are in italicized print.

the year by N fertilization interaction, hereafter 'year × fertilization'; Fig. 1a). The effect of CO<sub>2</sub> on net N mineralization did not vary significantly over time ( $P > 0.1$  for CO<sub>2</sub> × year; Fig. 1b). In contrast, the effect of species richness on net N mineralization rates was significant ( $P < 0.0001$ ) and varied in a unidirectional manner over time ( $P < 0.0001$  for year × richness). Initially, net N mineralization rates in 16-species plots were more than 80 percent lower than in monoculture plots, on average (Fig. 1c). Over time this effect was reduced such that near neutral and even positive effects of diversity on net N mineralization were observed after the 10th year of the experiment. The four-way interaction term that included year and all three treatments was significant ( $P < 0.01$ ), but this effect was small relative to overall effects of year and species richness.

Plant functional composition influenced net N mineralization in both monocultures and species-rich subplots. In the first several years of the experiment,

legume monocultures had rates of net N mineralization that were generally more than 2 times higher than monocultures of C4 grasses, C3 grasses, or non-N-fixing forbs ( $P < 0.0001$  for plant functional type and year × functional type; Fig. 2a). In 16-species subplots, net N mineralization rates were significantly positively related to legume aboveground biomass, both across and within years ( $P < 0.05$ , Table S5). Interaction terms showed that the positive effects of legumes on net N mineralization were especially apparent in certain years and in fertilized subplots.

When we compared 16-species subplots separately to subplots of intermediate richness (nine and four species) and to monocultures of different plant functional types, the differences in net N mineralization over time were variable. Sixteen-species subplots had net N mineralization rates that were temporally divergent from monocultures and four-species subplots, but not nine-species subplots (Fig. 2b). The greatest difference in net

**Table 3** Results from repeated-measures models of soil ammonium concentrations ( $\log_{10}$  transformed). Three model structures are compared, one with year excluded, one with year as a continuous parameter, and one with year as discrete parameter. Only experiment years 5 and 10 were used in these analyses due to the availability of soil C data. Subplot was included as a random effect (data not shown)

| Model term                  | Year excluded  |                   | Year continuous |                   | Year discrete  |                   |
|-----------------------------|----------------|-------------------|-----------------|-------------------|----------------|-------------------|
|                             | <i>F</i> ratio | <i>P</i> value    | <i>F</i> ratio  | <i>P</i> value    | <i>F</i> ratio | <i>P</i> value    |
| Year                        | na             | na                | 95              | <b>&lt;0.0001</b> | 95             | <b>&lt;0.0001</b> |
| N fertilization             | 94             | <b>&lt;0.0001</b> | 138             | <b>&lt;0.0001</b> | 146            | <b>&lt;0.0001</b> |
| Soil moisture               | 90             | <b>&lt;0.0001</b> | 12              | <b>0.0006</b>     | 11             | <b>0.001</b>      |
| Soil C                      | 46             | <b>&lt;0.0001</b> | 1               | 0.28              | 2              | 0.20              |
| Fert. × soil moisture       | 24             | <b>&lt;0.0001</b> | 0               | 0.82              | 0              | 0.82              |
| Fert. × soil C              | 10             | <b>0.002</b>      | 24              | <b>&lt;0.0001</b> | 24             | <b>&lt;0.0001</b> |
| Soil moisture × soil C      | 4              | <b>0.05</b>       | 0               | 0.49              | 0              | 0.49              |
| Year × fertilization        | na             | na                | 16              | <b>&lt;0.0001</b> | 16             | <b>&lt;0.0001</b> |
| Year × soil moisture        | na             | na                | 15              | <b>0.0001</b>     | 15             | <b>0.0001</b>     |
| Year × soil C               | na             | na                | 3               | <i>0.08</i>       | 3              | <i>0.08</i>       |
| Model <i>n</i>              | 560            |                   | 560             |                   | 560            |                   |
| Model <i>R</i> <sup>2</sup> | 0.46           |                   | 0.54            |                   | 0.54           |                   |

*P* values less than 0.01 are in bold print, those between 0.01 and 0.05 are in bold, italicized print, those between 0.05 and 0.1 are in italicized print

N mineralization rates and steepest temporal changes occurred between 16-species subplots and legume monocultures (Fig. 2b), mirroring a steep decline with time in net N mineralization in legume monocultures (Fig. 2a). C3 grass monocultures had more stable rates of net N mineralization (Fig. 2a) and differed the least from 16-species subplots (Fig. 2b).

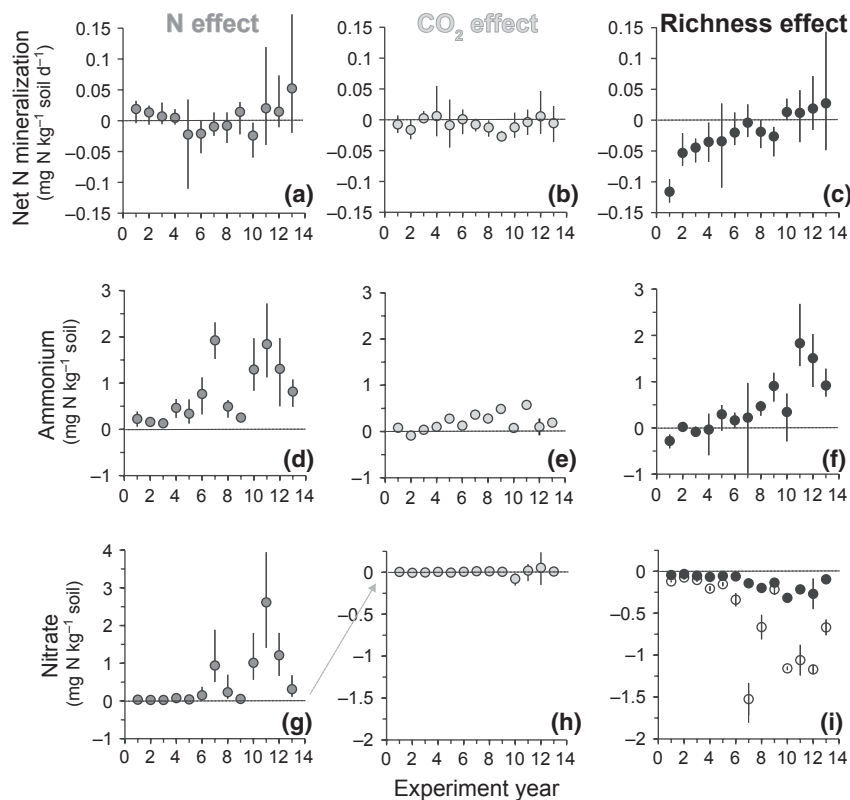
Net N mineralization was dominated by nitrate production; for 71 percent of all observations, net nitrification rates exceeded net ammonification rates by at least 50 percent. Consequently, treatment effects on net nitrification were very similar to those for net N mineralization (Tables S1, S2, and S5).

#### *Inorganic N concentrations*

In ambient subplots, midsummer soil nitrate and ammonium concentrations were temporally variable ( $P < 0.0001$  for the year effect; Tables S3 and S4), exhibiting a large increase from the 6th year of the experiment to the 12th year before declining substantially in the 13th year (Fig. S1). Nitrogen fertilization had positive effects on nitrate and ammonium concentrations, with larger effects during years when inorganic N concentrations were high in ambient subplots ( $P < 0.0001$  for fertilization and year × fertilization; Fig. 1d and g). There were not significant overall effects of CO<sub>2</sub> on soil nitrate or ammonium concentrations ( $P > 0.05$ ), but elevated CO<sub>2</sub> tended to reduce soil nitrate concentrations in fertilized subplots by 19% on average ( $P = 0.1$  for CO<sub>2</sub> × fertilization), and elevated CO<sub>2</sub> increased the

geometric mean of soil ammonium in ambient N subplots by 20% on average across years ( $P < 0.01$  for CO<sub>2</sub> × fertilization; Fig. 1e). Species richness had significant effects on soil nitrate and ammonium concentrations ( $P < 0.0001$ ), with the richness effect dependent on both time ( $P < 0.0001$  for year × richness) and N fertilization ( $P \leq 0.05$  for year × fertilization × richness). Soil nitrate concentrations were lower in 16-species subplots than in less speciose subplots, especially when fertilizer was applied and in later years (Fig. 1i). Contrastingly, plant species richness had increasingly positive effects on soil ammonium concentrations over time (Fig. 1f), especially in unfertilized subplots. During years 11–13, average ammonium concentrations in unfertilized subplots with 16-species were more than two times higher than in unfertilized monocultures.

Plant functional composition also influenced soil nitrate and ammonium concentrations. Legume monocultures had average soil nitrate concentrations that were at least two times higher than monocultures of forbs and grasses, with the difference increasing in later years ( $P < 0.0001$  for plant functional type and year × functional type; Fig. 3). For soil ammonium concentrations, the differences among monocultures were smaller; legumes had average soil ammonium concentrations that were higher than forbs but lower than C3 and C4 grass monocultures ( $P < 0.0001$  for functional type and year × functional type; Fig. 2). In 16-species subplots, variation in legume biomass across years, but not within years, was positively related to soil nitrate and ammonium concentrations ( $P < 0.0001$ ; Table S5).



**Fig. 1** Treatment effects on net N mineralization (top panels), ammonium concentrations (center panels), and nitrate concentrations (lower panels). Positive values indicate positive treatment effects and vice versa. In each year, average values were calculated for each treatment combination as described in the *Materials and Methods*. Nitrogen (N) effects (left panels) were calculated by subtracting the average of fertilized subplots from that of ambient N subplots for each level of species richness (using only ambient  $\text{CO}_2$  subplots). Carbon dioxide ( $\text{CO}_2$ ) effects (center panels) were calculated by subtracting the average of elevated  $\text{CO}_2$  subplots from that of ambient  $\text{CO}_2$  subplots for each level of species richness (using only ambient N subplots). Thus, for N and  $\text{CO}_2$  effects, each point represents the mean of average treatment effects for each species richness level (1, 4, 9, and 16 spp.). For all four combinations of N and  $\text{CO}_2$  treatments, species richness effects (right panels) were calculated by subtracting the average of 16-species subplots from that of monocultures. For species richness effects, with one exception, each point represents the mean of the average richness effects for all combinations of  $\text{CO}_2$  and N treatments. The exception is the bottom right panel, for which richness effects were calculated separately for ambient N (filled circles) and fertilized subplots (open circles). For  $\text{CO}_2$  and N effects, the vertical lines extend to the maximum and minimum effect among the four levels of species richness. For richness effects, the vertical lines extend to the maximum and minimum effect among the four  $\text{CO}_2$  and N treatment combinations. The arrow from panel (g) to (h) shows the shift in the position of zero on the Y-axis.

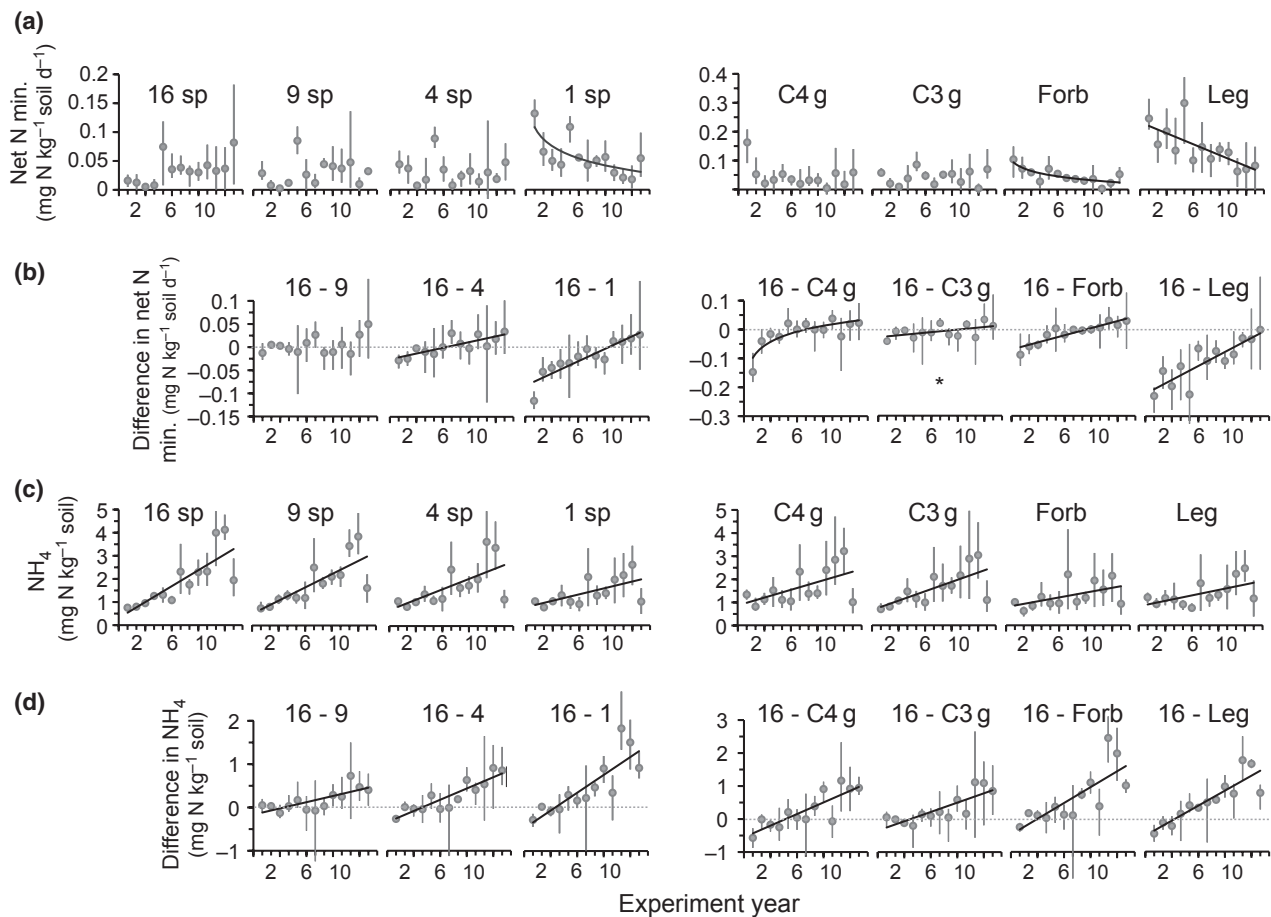
The differences between soil nitrate concentrations in 16-species subplots and less diverse subplots were influenced by functional composition, species richness, and N fertilization (Fig. 3). Across years and in both fertilized and unfertilized subplots, average soil nitrate concentrations in 16-species subplots were more than 95% lower than legume monocultures and more than 80% lower than forb monocultures. By contrast, soil nitrate concentrations in 16-species subplots were only notably lower than in C4 grass monocultures and nine-species subplots when nitrate concentrations were high, as in later experiment years, and especially in fertilized subplots.

By year 13, soil ammonium concentrations were higher in 16-species subplots than in subplots of inter-

mediate richness and in monocultures of all plant functional types (Fig. 2d). For all richness levels and monocultures of all plant types, soil ammonium concentrations during later years were higher than or similar to the beginning of the experiment (Fig. 2c).

#### *Mediating factors*

Net N mineralization and nitrification rates were positively related to the percent of N in roots and negatively related to fine root biomass. Effects of root N and biomass were evident both across years, i.e., when experiment year was excluded from models or included as a continuous parameter, and within years, i.e., when year was included as a discrete parameter



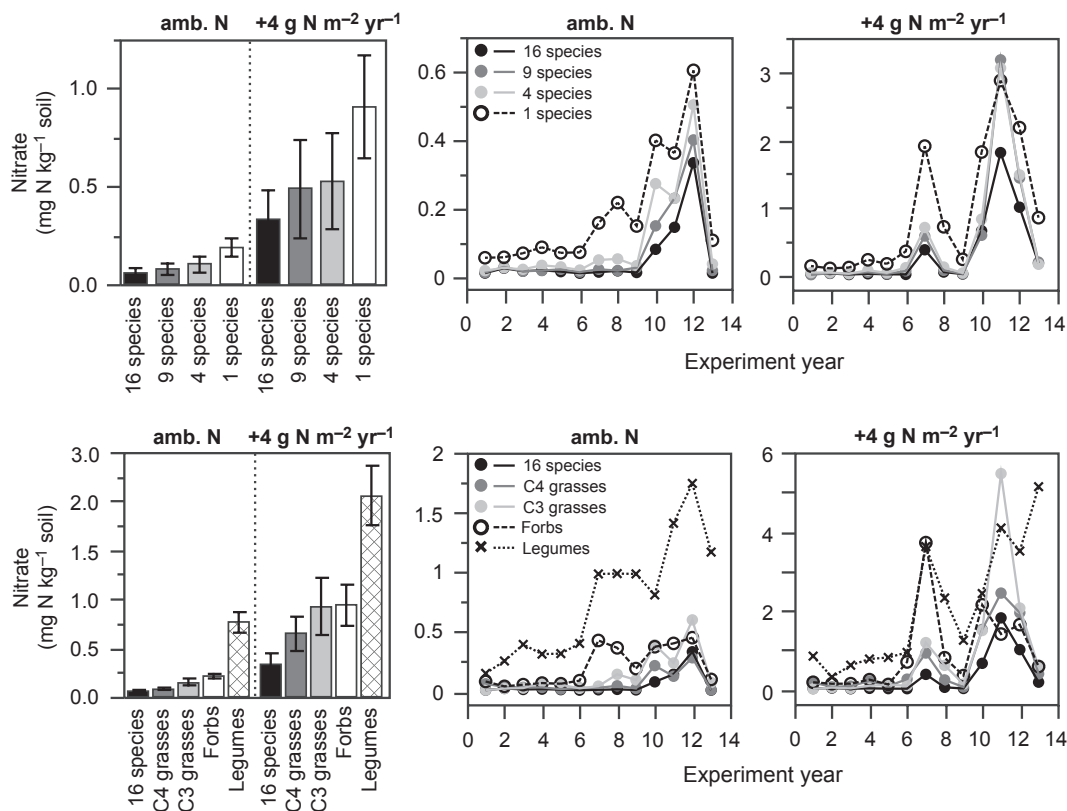
**Fig. 2** Comparing net N mineralization rates and ammonium ( $\text{NH}_4^+$ ) concentrations in the most diverse subplots, planted with 16 species, with lower diversity subplots (nine and four species) and monocultures of different plant functional types (mono = all monocultures combined; C4 g = C4 grass monocultures; C3 g = C3 grass monocultures; Forb = monocultures of non-N-fixing forbs; Leg = monocultures of nonwoody legumes). For each year in panels (a) and (c), four averages were calculated (as described in the caption of Fig. 1), one for each combination of  $\text{CO}_2$  and N treatments, and each point represents the mean of those four averages. In panels (b) and (d), effects of species richness on net N mineralization and ammonium concentrations are shown as the difference between the mean of 16-species subplots and less diverse subplots; each point represents the mean difference of all four combinations of  $\text{CO}_2$  and N treatments. Vertical lines extend from each point to the maximum and minimum treatment averages (a and c) or the maximum and minimum difference between treatment averages (b and d). Positive values in (b) and (d) indicate higher net N mineralization or ammonium concentrations in the highest diversity subplots as compared with the lower diversity subplots. With one exception, lines were fit when significant temporal trends were apparent ( $P < 0.05$  in linear regressions). The exception is noted by an asterisk, where the linear fit had a  $P$  value of 0.07. Logarithmic fits are shown when the coefficient of determination for log functions was higher, by at least 0.1, than for linear functions.

( $P < 0.0001$ ; Table 1). Soil moisture was positively related to N transformations across years ( $P < 0.0001$ ) but not within years ( $P = 0.11$ ). There was some evidence that the effect of these mediating factors, especially root biomass, was variable over time. Specifically, the negative effect of root biomass on N transformations diminished over time ( $P < 0.0001$  for year  $\times$  root biomass). According to  $F$ -ratios, root N explained three times more within-year variation in net N mineralization than root biomass and nearly 20 times more than soil moisture. Data on percent organic C and percent

total N in soil were only available for the 5th and 10th experiment years, but for those years, soil N transformations were unrelated to soil percent C, percent N, or C to N ratios.

Nitrate concentrations in soil were negatively related to fine root biomass, both across years and within years ( $P < 0.001$ ; Table 2). Soil moisture was positively correlated with nitrate concentrations in all models ( $P < 0.05$ ), but the correlation was weak within years, evident in a much lower  $F$ -ratio when experiment year was included as a discrete parameter. Nitrification was





**Fig. 3** Comparing soil nitrate concentrations in the highest diversity subplots, planted with 16 species, with lower diversity subplots (9, 4, 1 spp.; top) and monocultures of different plant functional types (bottom). For the left panels, each bar represents the geometric mean of soil nitrate for each plant community across all 13 years of the experiment. Error bars indicate standard error. For the center and right panels, each point represents the geometric mean of soil nitrate for each plant community type and each year. Results are shown separately for ambient N and added N subplots; within each of these N treatments, ambient and elevated CO<sub>2</sub> subplots were combined and averaged together.

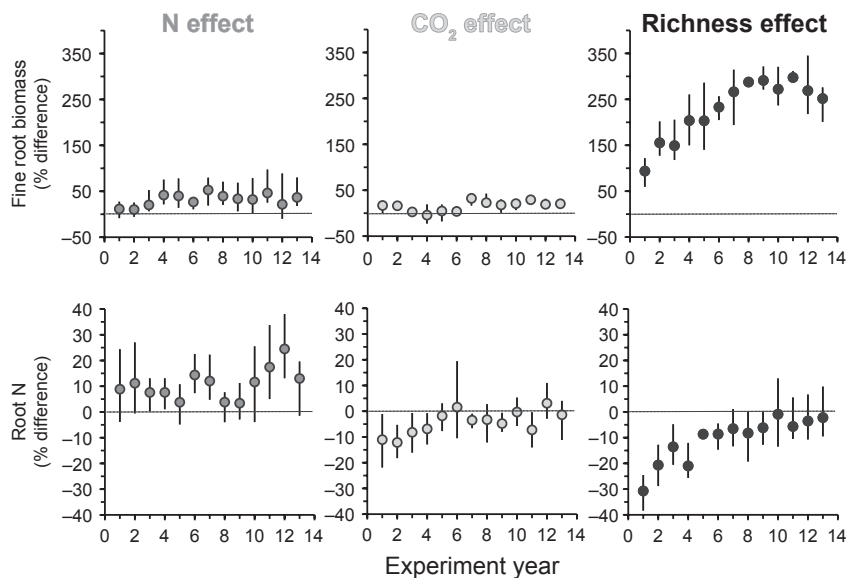
not correlated with nitrate concentrations across years, but had a positive effect in some years ( $P < 0.001$ ). According  $F$ -ratios, root biomass accounted for 50 times more within-year variance in nitrate concentrations than net nitrification and soil moisture.

Soil ammonium concentrations were positively related to soil C concentrations, especially in unfertilized subplots and later in the experiment ( $P < 0.0001$  for fertilization  $\times$  soil C;  $P < 0.1$  for year  $\times$  soil C; Table 3). Soil moisture was also positively correlated with ammonium concentrations, but more so in the 5th experiment year than in the 10th ( $P < 0.0001$  for year  $\times$  soil moisture). Soil moisture and soil C explained similar amounts of variation in soil ammonium concentrations.

For each soil N parameter, when potential mediating factors, such as root N, and experimental treatments were included as predictors in the same model, the mediating factors remained significant predictors. The  $F$ -ratios for treatment effects and interactions were lower when mediating factors were included, especially

for species richness effects, suggesting that treatment effects on soil N cycling were partly controlled by these mediating factors (Tables S1–S4).

We did not observe strong correlations between any climatic variable and annual CO<sub>2</sub> effects on soil N cycling ( $R^2 < 0.05$ ). Some climatic variables, especially soil moisture, were correlated with both annual effects of N fertilization on inorganic N concentrations and annual effects of species richness on each soil N parameter, but the relationships were not very strong ( $R^2 < 0.35$ ). The relationships between climatic variables and richness effects were not significant in regression models that included experiment year as a second predictor because these models accounted for temporal trends in climatic variables. This suggests that the correlations between climate and richness effects on soil N cycling were not causative, but a consequence of climate being correlated with experiment duration, which was also correlated with the actual controls of richness effects on soil N cycling, such as root N concentrations and root biomass.



**Fig. 4** Effects of N fertilization, elevated CO<sub>2</sub>, and planted species richness on root biomass and root N concentrations over time. Here, the difference between treatment levels was calculated as described in the caption of Fig. 1, but then the difference was divided by the average of the baseline treatment, i.e., ambient subplots or monocultures, so that treatment effects are expressed as percentages. For example, the CO<sub>2</sub> effect was calculated for each year by subtracting the average for the elevated CO<sub>2</sub> subplots from that of the ambient CO<sub>2</sub> subplots, dividing that difference by the average of ambient CO<sub>2</sub> subplots, and multiplying the resulting value by 100. A CO<sub>2</sub> treatment effect of 20% for root biomass indicates that elevated CO<sub>2</sub> increased root biomass by 20% relative to ambient CO<sub>2</sub>. For calculating the effect of species richness, the difference between the highest planted species richness (16 species) and monocultures was used.

## Discussion

During the first 13 years of the BioCON experiment, elevated CO<sub>2</sub> had insignificant, or small, and relatively stable effects on soil N parameters. In contrast, planted species richness had substantial and dynamic effects on soil N transformations and concentrations of inorganic N in soil. The temporal trends in effects of diversity and N fertilization, and their interactions, were not linear and depended on nonlinear changes in both ambient and treated subplots. Consequently, the long-term effects of these treatments would have been poorly predicted if the experiment had been limited to a few years. Even a 6-year study, which is uncommonly long, would have failed to predict the treatment effects and interactions during subsequent years. For example, the larger effects of species richness on soil nitrate concentrations during experiment years 7–13, especially in fertilized subplots, were not apparent during the first 6 years. These results highlight the need to evaluate the N cycle using additional, long-term manipulations of biodiversity.

### *N* transformations

The contrasting effects of elevated CO<sub>2</sub> and plant species richness on net N mineralization can be explained, in part, by the effect of these treatments on

the factors that more directly cause variation in net N mineralization. The strongest apparent driver of net N mineralization was root N concentration, which was positively related to N mineralization. The influence of root N on soil N transformations is consistent with both stoichiometric theory (Manzoni *et al.*, 2008) and other observations at this field site (Wedin & Tilman, 1990; Reich *et al.*, 2001b; Dijkstra *et al.*, 2006; Fornara *et al.*, 2009) and elsewhere (Hobbie *et al.*, 2007; Parton *et al.*, 2007). Elevated CO<sub>2</sub> caused only a minor decline in root N concentrations, typically less than 10%, whereas root N concentrations in 16-species subplots increased over time, causing the negative diversity effect on root N to diminish; Fig. 4). Fine root biomass was negatively correlated with net N mineralization but only in the early years of the experiment when CO<sub>2</sub> and diversity effects on root biomass were smaller (Fig. 4). The negative effect of fine root biomass on soil N transformations was perhaps due to N immobilization associated with labile C sources that scale with fine root biomass, such as root exudates or nonstructural C sources in roots (Zak *et al.*, 2000b; Knops *et al.*, 2002). Near the end of the 13-year record, the subplots with the most root biomass had greater root N concentrations than in previous years, which could have minimized the influence of root biomass and N immobilization as drivers of soil N transformation rates.

The dynamic richness effects on net N mineralization were a consequence of declining net N mineralization in dicot monocultures, especially legumes, and increasing net N mineralization in the most diverse subplots. In later years of the experiment, forb and legume monocultures had less than 50 g of root biomass (per m<sup>2</sup>) compared with at least 200 g of root biomass for grass monocultures, such that microbial activity and N mineralization could have been substrate limited. In the most diverse subplots, the absolute and relative abundance of legumes increased over time (data not shown) and likely contributed to the increased N mineralization, perhaps by facilitating higher root N concentrations at the community level.

Prior grassland studies have documented both positive and negative effects of elevated CO<sub>2</sub> on net N mineralization, some of which were mediated by changes in soil moisture (Hungate *et al.*, 1997; Ebersberger *et al.*, 2003) or soil C:N ratios (Gill *et al.*, 2002, 2006). At BioCON, soil C : N ratios (0–20 cm) have not changed in response to elevated CO<sub>2</sub> and were not correlated with net N mineralization (J.M.H. Knops, unpublished data from experiment years 5 and 10). Soil moisture is increased by elevated CO<sub>2</sub> at BioCON, but modestly (Adair *et al.*, 2011), and there was not a strong or consistent correlation between soil moisture and net N mineralization rates. The effects of elevated CO<sub>2</sub> on soil N transformations could also be dampened by factors we did not measure or by the high variability among subplots within the same treatments. This is evident in the moderate explanatory power of our statistical models of soil N transformations (*R*<sup>2</sup> values of best fit models were 0.29 and 0.39; Tables S1 and S2).

During the first 13 years of the BioCON experiment, progressive N limitation was not induced through a negative effect of elevated CO<sub>2</sub> on net N mineralization. Rather, as Reich & Hobbie (2012) showed, elevated CO<sub>2</sub> had a stimulating effect on net N mineralization, but only in fertilized subplots and only after the 3rd year of the experiment. Here, we evaluated net N mineralization rates with greater temporal and compositional resolution than in that recent study, and showed the positive effect of elevated CO<sub>2</sub> in fertilized subplots also depended slightly on plant species richness; specifically, it tended to be strongest in the 16-species subplots (*P* < 0.01 for year × richness × CO<sub>2</sub> × fertilization; Table S1).

#### Nitrate

The negative effects of plant species richness on soil nitrate concentrations (see also Tilman *et al.*, 1996; Niklaus *et al.*, 2001; Scherer-Lorenzen *et al.*, 2003) were largely due to the positive effects of species

richness on fine root biomass (Fig. 4) and, ostensibly, plant N uptake, with a lesser role for diversity effects on net nitrification rates (Table 2). Other studies suggest that nitrification could largely explain the effects of plant diversity on soil nitrate concentrations (Niklaus *et al.*, 2001). In our study, methodology could have limited our ability to detect effects of nitrification on nitrate concentrations; we measured *in situ* nitrification rates *after* sampling for soil nitrate.

We observed that species-rich plant communities reduced soil nitrate concentrations, relative to less speciose communities, by a larger amount over time. These results contrast with results from a grassland diversity experiment in Jena, Germany (Oelmann *et al.*, 2011), where diverse communities had lower soil nitrate concentrations only in the 1st year of the experiment. Those authors suggested that the negative effect of species richness on soil nitrate was neutralized by positive effects of species richness on soil organic matter accumulation and N mineralization. In our study, negative effects of species richness on soil nitrate persisted even as organic matter accumulated in species-rich subplots (J.M.H. Knops unpublished), as richness effects on net N mineralization and nitrification became increasingly positive, and as species richness itself declined (Reich, 2009). Effects of species richness on soil nitrate at the Jena experiment might be smaller or negligible because richness effects on root biomass were not observed (Bessler *et al.*, 2009) and because it is a more fertile site, presumably with higher nitrification rates; pretreatment soil N ranged from 0.1 to 0.27% in the Ap horizon at Jena (Roscher *et al.*, 2004) and from 0.03 to 0.12% in the upper 10 cm at BioCON (J.M.H. Knops unpublished). Also, soils at the Jena experiment were sampled in the spring and fall, such that effects of species richness on soil nitrate concentrations during summer could have been obscured. In contrast, we sampled soil nitrate concentrations during the peak of the growing season: approximately midsummer. By sampling a neighboring diversity experiment with greater frequency, Fornara & Tilman (2009) showed that the greatest effects of richness on soil nitrate occurred in July.

The results of our study suggest that grass monocultures can be strongly retentive of nitrogen, but that diverse plant communities are more likely to reduce N-losses than grass monocultures during periods of high leaching potential, such as would occur with increasing atmospheric N deposition or during 'hot moments' of nitrate abundance that occur seasonally (Dijkstra *et al.*, 2007). Under such circumstances, greater nitrogen retention might be achieved in diverse subplots via complementarity among species in the timing or depth of water and nutrient uptake.

Elevated CO<sub>2</sub> reduced soil nitrate concentrations slightly (see also Niklaus *et al.*, 2001; Carrillo *et al.*, 2012) but only in fertilized subplots ( $0.05 < P < 0.1$ ). This result is consistent with earlier studies at BioCON; Dijkstra *et al.* (2007) showed elevated CO<sub>2</sub> reduced total inorganic N concentrations and inorganic N leaching in fertilized subplots. This pattern might be partially explained by differences in fine root biomass because the increase in root biomass due to elevated CO<sub>2</sub> was larger for fertilized and unfertilized subplots from 2001 to 2010 (Reich & Hobbie, 2012). In addition, the effect of CO<sub>2</sub> on total plant biomass and total plant N pools was greater in fertilized subplots than in unfertilized subplots over the same period (Reich & Hobbie, 2012), such that plants in fertilized subplots could also have been a greater sink for soil nitrate.

### Ammonium

We hypothesize that the increasingly positive effect of plant species richness on soil ammonium concentrations are partly associated with positive effects of species richness on soil C concentrations over time at BioCON (J.M.H. Knops unpublished) because some of the ammonium ions are likely occupying cation exchange sites on soil organic matter. This is consistent with: (i) the positive correlation between soil C concentrations and soil ammonium concentrations, and (ii) the low pretreatment cation exchange capacity due to the sandy, organic-poor nature of the soils. Soil C concentrations were also positively correlated with root biomass (J.M.H. Knops unpublished), mirroring results from a neighboring diversity experiment (Fornara & Tilman, 2008). Thus, richness effects on soil ammonium concentrations are perhaps derived, in part, from the effect of high root biomass on soil C accumulation. This explanation cannot, however, account for the lower soil ammonium concentrations observed in the 13th year of the experiment. Additional measurements are necessary to determine if this was an anomalous observation.

Soil ammonium concentrations were also positively impacted by elevated CO<sub>2</sub>, but the CO<sub>2</sub> effect occurred only in unfertilized subplots and was smaller than the effect of plant species richness. This larger pool of ammonium might help sustain the positive effect of elevated CO<sub>2</sub> on plant productivity in the absence of N fertilization (Reich & Hobbie, 2012). However, the cause of increased ammonium concentrations in elevated CO<sub>2</sub>, unfertilized subplots remains uncertain. Elevated CO<sub>2</sub> did not substantially increase soil C concentrations in unfertilized subplots (J.M.H. Knops unpublished); thus, the response of ammonium to CO<sub>2</sub> does not appear coupled to that of soil C. Elevated CO<sub>2</sub>

did increase soil moisture (Adair *et al.*, 2011), which was positively related to ammonium concentrations in soil.

### Implications

Two broader conclusions can be drawn from our analysis of N fertilization and species richness effects on soil N cycling. First, increases in N availability at high diversity have likely contributed to the growing magnitude of the species richness effect on productivity (Fornara & Tilman, 2009; Reich *et al.*, 2012). Second, because the effects of plant species richness and N fertilization on N cycling have not reached an apparent equilibrium after 13 years, continued observations at BioCON and additional long-term experiments are critically needed to (i) predict how plant diversity and N fertilization will influence the soil N cycle over longer timescales, (ii) identify which factors mediate the effects of species richness and N fertilization at different stages of ecosystem development, and (iii) determine whether such changes will also influence longer term, e.g., 15–30 year, responses of C and N cycling to elevated CO<sub>2</sub> levels.

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### Supporting Information

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

**Table S1.** Results of repeated-measures models for net nitrogen mineralization.

**Table S2.** Results of repeated-measures models for net nitrification.

**Table S3.** Results of repeated-measures models for soil nitrate concentrations.

**Table S4.** Results of repeated-measures models for soil ammonium concentrations.

**Table S5.** Results of repeated-measures models of soil N in the most diverse subplots.

**Figure S1.** Temporal trends for soil inorganic N concentrations and net N transformations in ambient subplots.