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GLOBAL CHANGE ECOLOGY - ORIGINAL RESEARCH

# Plant community change mediates the response of foliar $\delta^{15}N$ to $CO_2$ enrichment in mesic grasslands

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**Abstract** Rising atmospheric $CO_2$ concentration may change the isotopic signature of plant N by altering plant and microbial processes involved in the N cycle. $CO_2$ may increase leaf $\delta^{15}N$ by increasing plant community productivity, C input to soil, and, ultimately, microbial mineralization of old, $^{15}N$-enriched organic matter. We predicted that $CO_2$ would increase aboveground productivity (ANPP; g biomass $m^{-2}$) and foliar $\delta^{15}N$ values of two grassland communities in Texas, USA: (1) a pasture dominated by a $C_4$ exotic grass, and (2) assemblages of tallgrass prairie species, the latter grown on clay, sandy loam, and silty clay soils. Grasslands were exposed in separate experiments to a pre-industrial to elevated $CO_2$ gradient for 4 years. $CO_2$ stimulated ANPP of pasture and of prairie assemblages on each of the three soils, but increased leaf $\delta^{15}N$ only for prairie plants on a silty clay. $\delta^{15}N$ increased linearly as mineral-associated soil C declined on the silty clay. Mineral-associated C declined as ANPP increased. Structural equation modeling indicted that $CO_2$ increased ANPP partly by favoring a tallgrass (*Sorghastrum nutans*) over a mid-grass species (*Bouteloua curtipendula*). $CO_2$ may have increased foliar $\delta^{15}N$ on the silty clay by reducing fractionation during N uptake and assimilation. However, we interpret the soil-specific, $\delta^{15}N$–$CO_2$ response as resulting from increased ANPP that stimulated mineralization from recalcitrant organic matter. By contrast, $CO_2$ favored a forb species (*Solanum dimidiatum*) with higher $\delta^{15}N$ than the dominant grass (*Bothriochloa ischaemum*) in pasture. $CO_2$ enrichment changed grassland $\delta^{15}N$ by shifting species relative abundances.

**Keywords** Isotope · Plant productivity · Soil carbon · Soil type · Tallgrass prairie

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## Introduction

Atmospheric $CO_2$ enrichment commonly leads to changes in the N cycle because of the tight coupling that exists between organic forms of C and N (Luo et al. 2004; De Graaff et al. 2006; Gill et al. 2006). Plant $^{15}N$ natural abundances provide an integrated metric of how N-containing pools in soils and plants are transformed, accessed, and mixed (Robinson 2001) and thus can be used to interpret net effects of $CO_2$ on the N cycle. Plant $\delta^{15}N$ values, reflective of the ratio of $^{15}N$–$^{14}N$, have been found to decrease, increase, or not change at elevated $CO_2$ (Billings et al.

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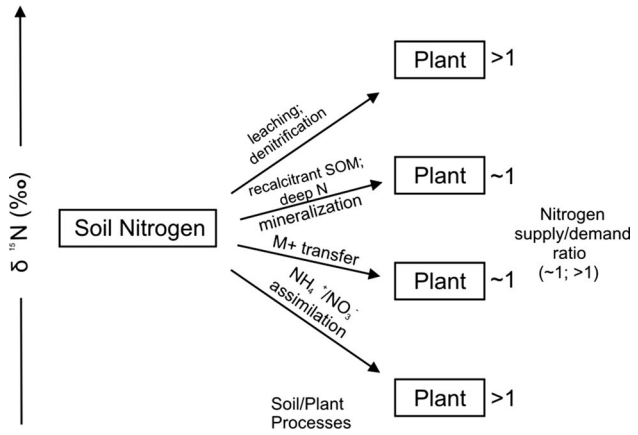


**Fig. 1** Directional impacts of selected soil and plant processes on the $\delta^{15}N$ of plants (*Plant*) relative to an initial $\delta^{15}N$ value of the N pool in soil solution (*Soil Nitrogen*). Fractionating processes associated with denitrification or plant assimilation of $NH_4^+$ or $NO_3^-$ exert their largest effect on plant $\delta^{15}N$ when the ratio of the supply rate or concentration of soil inorganic N is high relative to plant demand for N (ratio > 1). Conversely, $\delta^{15}N$ values are more influenced by plant exploitation of alternative N sources (e.g., mineralization of recalcitrant SOM; increased acquisition of N from deep soil layers) or mechanisms of N uptake (e.g., via mycorrhizae, *M*+) when N supply approaches plant demand (ratio = ~1)

2002; Bassirirad et al. 2003; Stock and Evans 2006; Garten et al. 2011), testament to both the diversity of N cycle processes potentially influenced by $CO_2$ and the range of plant and ecosystem responses to $CO_2$.

$CO_2$-caused changes in plant $\delta^{15}N$ signatures result from changes in soil- or plant-mediated processes that affect or are influenced by isotopic fractionation. $CO_2$ effects on plant $\delta^{15}N$ are expected to vary as a function of the supply rate of inorganic N in soil solution relative to the demand for organic N by plants (Fig. 1; Evans 2001; Robinson 2001; Kalcsits et al. 2014). Fractionation may occur during N transformations in soil and plant uptake, assimilation, and turnover/export of N (Evans 2001; Robinson 2001; Soper et al. 2014). Fractionation is expected to be greatest when N supply exceeds demand. For example, discrimination during nitrate and ammonium assimilation in roots is thought to occur when plant pools of inorganic N are only partially consumed and inorganic N is lost from roots to the surrounding substrate (Evans 2001; Kalcsits et al. 2014). Assimilated plant N will be depleted in $^{15}N$ relative to source N when N supply exceeds plant demand and fractionation is expressed, but organic and inorganic plant N will not differ in $\delta^{15}N$ signature if the entire inorganic pool in plants is assimilated (Kalcsits et al. 2014). An excess in N supply relative to plant demand could also lead to an increase in the $\delta^{15}N$ signature of inorganic N in soil solution, likely without changing the $\delta^{15}N$ signature of the larger N pool in bulk soil. Solution N is enriched in $^{15}N$ by losses of $^{15}N$-depleted N molecules to leaching or denitrification (Robinson and Conroy 1999; Garten et al. 2011).

$CO_2$ is usually assumed to influence plant $\delta^{15}N$ by increasing plant demand for N relative to supply, resulting in a change in the isotopic signature of plant-available soil N. $CO_2$ could change the $\delta^{15}N$ of the plant-available N pool by causing the addition or loss of isotopically distinct N or permitting plants to access a previously underexploited pool of isotopically distinct N (Robinson 2001). The progressive N limitation (PNL) hypothesis holds that $CO_2$ enrichment will intensify N limitation of plant production by increasing C input to soil and N sequestration in soil organic matter (SOM) or long-lived plant tissues (Luo et al. 2004). This tightening of the N cycle is anticipated to reduce N losses. A decline in denitrification would reduce the $\delta^{15}N$ of soil $NO_3^-$ (Robinson and Conroy 1999), for example, whereas increasing denitrification is thought to contribute to the positive correlation that is often observed between $\delta^{15}N$ values and foliar N concentration or soil N availability or mineralization rate (Kahmen et al. 2008; Craine et al. 2009). Conversely, $CO_2$ enrichment could increase plant $\delta^{15}N$ in systems in which N losses are minimal by increasing soil microbial activity (Billings et al. 2002) and microbial immobilization of N or mineralization of old, recalcitrant organic matter (Johnson DW et al. 2000; Gill et al. 2002; Dijkstra et al. 2010), the latter of which is enriched in $^{15}N$ (Tiessen et al. 1984). The PNL theory also holds that effects of a $CO_2$-caused decline in N availability to plants can initially be overcome by a transfer of N from low C:N ratio fractions, such as recalcitrant SOM, to plants with higher C:N ratios. Additionally, $CO_2$ enrichment could alter plant reliance on mycorrhizal fungi for N uptake (Bassirirad et al. 2003). Nitrogen derived from mycorrhizal associates is depleted in $^{15}N$ compared to source N (Evans 2001; Hobbie and Hobbie 2008). Finally, plants may exhibit no change in $\delta^{15}N$ values, particularly when N is strongly limiting. Fractionation is reduced when plant demand approaches N supply rates (Robinson 2001; Billings et al. 2002).

Soil properties mediate several of the processes that determine how N containing pools are transformed, accessed, and mixed, and thus may affect how plant $\delta^{15}N$ values respond to $CO_2$. Soil differences in texture and related hydrological properties potentially influence organic pools of N (Fay et al. 2009) and N mineralization and denitrification rates (Bechtold and Naiman 2006; Gu and Riley 2010), all of which may affect the plant $\delta^{15}N$ response to $CO_2$.

Several of the processes that affect $\delta^{15}N$ values of plants or plant communities may be influenced indirectly by $CO_2$-caused change in the composition or relative abundances of species in plant communities (community change). We consider effects of $CO_2$-caused community change on

abundance-weighted (community) values of leaf $\delta^{15}N$ to be species-specific when the species involved differ in $\delta^{15}N$ values. $CO_2$ could alter community $\delta^{15}N$ by differentially favoring plant species that differ in the form or source of N accessed or within-plant fractionation. Legumes often have leaf $\delta^{15}N$ signatures near zero because of their reliance on symbiotic $N_2$ fixation (Robinson 2001; Hungate et al. 2004). Other species may differ in $\delta^{15}N$ signatures because of differing preferences for $NH_4^+$ compared to $NO_3^-$ (Robinson 2001; Kahmen et al. 2008; Wang and Macko 2011) or rooting depth (Handley and Scrimgeour 1997), for example. Alternatively, increased $CO_2$ could shift the $\delta^{15}N$ signatures of species by a similar magnitude and direction by favoring species that disproportionately influence soil C inputs, microbial activity, and, ultimately, the $\delta^{15}N$ signature of N in soil solution. $CO_2$ effects on community $\delta^{15}N$ values are non-species-specific when the magnitude and direction of change in $\delta^{15}N$ are similar among species in the community.

We use data from two experiments on grasslands to determine how leaf $\delta^{15}N$ values of dominant perennial species responded to a pre-industrial to elevated gradient in $CO_2$ concentration and to assess possible explanations for $\delta^{15}N$ change. The first grassland is a formerly-grazed pasture dominated by an exotic $C_4$ grass. The second grassland consists of assemblages of native tallgrass prairie species grown on each of three soil types. Both grasslands were exposed to the $CO_2$ gradient for 4 years during separate experiments. $CO_2$ enrichment increased ANPP (g biomass $m^{-2}$) and changed community composition in both pasture (Polley et al. 2003) and prairie assemblages (Fay et al. 2012; Polley et al. 2012a). The increase in ANPP and shift in communities was accompanied by increased demand for N in aboveground plant tissues (Gill et al. 2006; Polley et al. 2011). Likely as a consequence of heightened plant demand for N, we observed changes in N mineralization rates, the C:N ratio of SOM, soil fungal communities, and activities of microbially-derived extracellular enzymes involved in C and N cycling in soil, and found evidence for a net transfer of N from recalcitrant SOM with low C:N ratios to plants with larger C:N ratios (Gill et al. 2002, 2006; Kelley et al. 2011; Procter et al. 2014). $CO_2$ enrichment has repeatedly been shown to alter soil microbial dynamics and N cycling and, in some ecosystems, to increase N transfer from soils to plants (King et al. 2004; Dijkstra et al. 2010) apparently by stimulating ANPP and C input to soil. Yet, there remains little direct or even correlative evidence that plant N dynamics are influenced by $CO_2$-caused changes in soil microbial processes. We predicted that $CO_2$ enrichment would increase foliar $\delta^{15}N$ values in pasture and prairie assemblages, consistent with evidence that $CO_2$ stimulated both ANPP (Polley et al. 2003, 2012b; Fay et al. 2012) and mineralization of recalcitrant SOM (Gill et al. 2002). Mineralization of recalcitrant SOM would be expected to increase $\delta^{15}N$ values of plant available N (Johnson DW et al. 2000; Billings et al. 2002). Greater productivity may increase C input to soil and thereby stimulate microbes to decompose relatively old SOM (Gill et al. 2002; Dijkstra et al. 2010). Consequently, we anticipated that foliar $\delta^{15}N$ values would increase with increased ANPP. The magnitude of the ANPP increase potentially is influenced by soil properties (Fay et al. 2012) and community dynamics (Polley et al. 2014). We predicted that $CO_2$ enrichment would shift the $\delta^{15}N$ signatures of species by a similar magnitude and direction by favoring species that augment the ANPP–$CO_2$ response and, ultimately, soil C input and microbial activity. An alternative prediction is that $CO_2$ will shift community $\delta^{15}N$ by altering relative abundances of species that differ in $\delta^{15}N$ values.

## Materials and methods

### Experimental design

We report results from two experiments in which elongated field chambers were used to expose vegetation to a continuous gradient in $CO_2$ spanning pre-industrial to elevated concentrations. In 1997–2000, we studied $CO_2$ effects on previously grazed $C_3/C_4$ grassland (hereafter, pasture) using the Prairie $CO_2$ Gradient (PCG) facility (Johnson HB et al. 2000). In 2006–2009, we evaluated $CO_2$ effects on assemblages of tallgrass prairie species grown in soils of three types using the Lysimeter $CO_2$ gradient (LYCOG) facility (Polley et al. 2008; Fay et al. 2009). Both facilities were located in central Texas USA (31°05′N, 97°20′W) and consisted of two transparent and tunnel-shaped chambers, aligned parallel along a north–south axis. Each chamber was divided into ten consecutive compartments each 5 m long and 1.0 m (PCG) or 1.2 m (LYCOG) wide and tall. Chambered vegetation was enclosed in a transparent polyethylene film. Photosynthesis by enclosed vegetation progressively depleted the $CO_2$ concentration in air as it was moved by blowers toward the air outlet of each chamber to create daytime $CO_2$ gradients of 560 (PGG) or 500 $\mu L\ L^{-1}$ (LYCOG) to 370 $\mu L\ L^{-1}$ (elevated chamber) and 370 $\mu L\ L^{-1}$ to 200 (PCG) or 250 $\mu L\ L^{-1}$ (LYCOG) (subambient chamber). Night-time $CO_2$ concentrations were regulated at 130–150 $\mu L\ L^{-1}$ above daytime values along each chamber. Air temperature and vapor pressure deficit were regulated near ambient values by cooling and dehumidifying air at 5-m intervals along chambers. $CO_2$ treatments were maintained each growing season from March/April through mid-November.

The earlier PCG facility was constructed on grassland dominated by the $C_4$ perennial grass *Bothriochloa*

*ischaemum* (L.) Keng and the $C_3$ perennial forbs *Solanum dimidiatum* Raf., *Ratibida columnaris* (Sims) D. Don, and *Solidago canadensis* L. (hereafter referenced by genus; Polley et al. 2003). The site had been grazed for at least 50 years prior to construction. The soil was a silty clay. Soil beneath chambers was separated from surrounding soil to a depth of 0.9 m with a rubber-coated fabric. The LYCOG facility was constructed atop 5-m-long × 1.2-m -wide × 1.6-m-deep steel containers that were buried to 1.2 m depth. Two intact soil monoliths (each 1 × 1 × 1.5 m deep) of each of two soil types were placed into each of the 20 5-m-long containers. Three soil types of contrasting physical and hydrological properties were represented: (1) silty clay on which the PCG facility was constructed, (2) clay, and (3) sandy loam (Fay et al. 2009). Two monoliths of each of two soil types were randomly placed in each 5-m-long compartment. Four perennial $C_4$ grass species and three perennial $C_3$ forb species, all characteristic of tallgrass prairie in central Texas, were transplanted into 60 of the total of 80 monoliths in June 2003, 3 years prior to $CO_2$ treatment (Polley et al. 2008; Fay et al. 2009). Eventual dominants included the $C_4$ grasses *Bouteloua curtipendula* (Michx.) Torr., and *Sorghastrum nutans* (L.) Nash and the forb species *Solidago canadensis* (hereafter referenced by genus).

Irrigation equivalent to rainfall was applied to grassland in the PCG facility on the day following precipitation (Polley et al. 2002). Each monolith in the later LYCOG facility was irrigated twice weekly during each growing season. Irrigation was applied to simulate the seasonal distribution and average of growing season precipitation in central Texas (560 mm; Polley et al. 2011).

## Vegetation and soil sampling and data analysis

Soils and upper canopy leaves (or leaf blades) were sampled from along the PCG and LYCOG experiments for N isotope measurements. Soil cores (4.2 cm diameter, 53 cm depth) were collected in December 2000 at 1-m distance from both the air entrance and exit of each 5-m compartment of the PCG. Soils from the two cores per compartment were composited over 0–8, 8–23, 23–37, and 37–53 cm depth increments. We collected fully expanded leaves of *Solanum* in June 2000 and of *Bothriochloa* in June 1997 and 2000, the first and fourth years of the PCG experiment, and leaves of the forb *Solidago* and two grasses, *Bouteloua* and *Sorghastrum*, in June 2005 and 2009, the year prior to $CO_2$ treatment and fourth year of the LYCOG experiment. Grasslands in central Texas usually approach maximal physiological activity during June (Mielnick et al. 2001). Leaves were collected from multiple individuals of each of the target species, when possible, and composited by species for each 1-$m^2$ area sampled along the $CO_2$ gradient (PCG) or for each monolith (LYCOG). ANPP of each species was determined by clipping vegetation to 5-cm height at the end of each growing season.

The N isotope composition of soil samples and whole leaves was determined by mass spectrometry and expressed as $\delta^{15}N$, ‰ (parts per thousand) $^{15}N$ relative to air (PCG–Isotope Services, Los Alamos, NM, USA; LYCOG–University of California Davis Stable Isotope Facility, Davis, CA, USA). The N content of samples was measured during isotope analyses and expressed as [N], mg N $g^{-1}$ biomass. We measured the N isotope signature of top-soil samples (8–23 cm depth increment) for each 5-m compartment and of soil from three additional depth increments (0–8, 23–37, and 37–53 cm) for each of three compartments (at 550, 430, 360 μL $L^{-1}$ $CO_2$; PCG). We included an additional five leaf samples in each analysis of plant materials as internal standards. Data from these samples were used to standardize results from multiple measurement sessions for each experiment (PCG, LYCOG). Results for standards from any two measurement sessions were highly correlated ($r^2 > 0.97$). Pre-treatment to post-treatment changes in $\delta^{15}N$ values during each experiment were calculated by subtracting $\delta^{15}N$ measured on leaves collected in 1997 or 2005 from $\delta^{15}N$ of leaves from 2000 or 2009 (PCG and LYCOG experiments, respectively).

LYCOG soils were sampled to 15-cm depth following the fourth season of $CO_2$ treatment (2009) to determine SOM fractions. Samples from the two monoliths of a given soil type in each 5-m chamber compartment were combined for physical fractionation following Gill (2007). SOM was fractionated into three components: coarse particulate organic matter (POM) C (>250 μm), fine POM C (53–250 μm), and mineral-associated C (<53 μm). Coarse POM represents recently-added organic C, whereas mineral-associated C is the most recalcitrant size fraction (Cambardella and Elliott 1992).

Bivariate regression analysis was used to determine relationships between variables measured during the PCG experiment and $CO_2$ concentration. Differences in mean values for the two species from the PCG experiment were analyzed with paired *t* tests. Spearman rank-order correlation analysis was used to compare trends in leaf N concentration, [N], between species pairs from the LYCOG experiment.

Data from the LYCOG experiment were analyzed using a mixed-model analysis of covariance (ANCOVA). Analyses were conducted with SAS (Littell et al. 2002). Soil type and species identity were considered fixed effects. The assignment of soil types to 5-m lengths of chambers was considered a random effect. $CO_2$ treatment and [N] of leaves were treated as covariates in the analysis.

Structural equation modeling (SEM) with observed variables (path modeling) was used to partition the net effect of $CO_2$ on change in $\delta^{15}N$, when significant, into a direct effect

and indirect effects mediated through changes in community ANPP and the fractional contribution of dominant species to community ANPP (Shipley 2000; Grace 2006). Bivariate relationships between modeled variables were linear. The SEM model was fit using IBM SPSS AMOS 21 software. The hypothesized relationship among variables in a SEM is considered to be consistent with data when the probability level of the statistical test (chi-squared statistic) is greater than the significance level ($P = 0.05$; Shipley 2000). Data were standardized by subtracting the mean and dividing by the standard deviation prior to analysis.

## Results

### PCG (pasture) experiment

#### *ANPP*

The ANPP–$CO_2$ response of pasture declined from the first to fourth years of treatment (1997–2000), but averaged 120 g $m^{-2}$ per 100 μL $L^{-1}$ increase in $CO_2$ over the first 3 years of the experiment (Polley et al. 2003). The 1997–2000 change in ANPP of the grass *Bothriochloa* was a negative linear function of $CO_2$ (Fig. 2a). ANPP of *Bothriochloa* increased from the first to fourth years of treatment at $CO_2$ levels <320 μL $L^{-1}$, but declined at higher $CO_2$. The 1997–2000 change in ANPP of the forb *Solanum* was not correlated with $CO_2$ ($P = 0.27$), but was greater on average over elevated than subambient concentrations (increase of 49 and 30 g biomass $m^{-2}$, respectively). The ratio of *Solanum* ANPP to *Bothriochloa* ANPP increased from a mean of 0.05 in 1997 to 0.18 and 0.30 at subambient and elevated $CO_2$, respectively, in 2000. Together, the two species contributed 60 and 30 % of community ANPP in the first and fourth years across $CO_2$ treatments.

#### *$\delta^{15}N$*

The $\delta^{15}N$ of *Bothriochloa* leaves from both early in the initial treatment year of 1997 and final year of 2000 declined linearly at higher $CO_2$ (Fig. 3a). The slopes of $\delta^{15}N$–$CO_2$ regressions did not differ significantly between years for *Bothriochloa* ($P > 0.50$), but $\delta^{15}N$ values were 1.27 ‰ lower (less positive) in 2000 than 1997. Thus, $CO_2$ did not affect the decline in leaf $\delta^{15}N$ between 1997 and 2000. $CO_2$ also had no consistent effect on $\delta^{15}N$ values of leaves of the forb *Solanum* in 2000. Leaf [N] and $\delta^{15}N$ were not correlated for either *Bothriochloa* ($P = 0.21$) or *Solanum* ($P = 0.09$), but both [N] and $\delta^{15}N$ were greater for the forb than the grass in the fourth treatment year (2000). Leaf [N] declined linearly at higher $CO_2$ for *Solanum* in 2000 and was greater on average by a factor of three for the forb

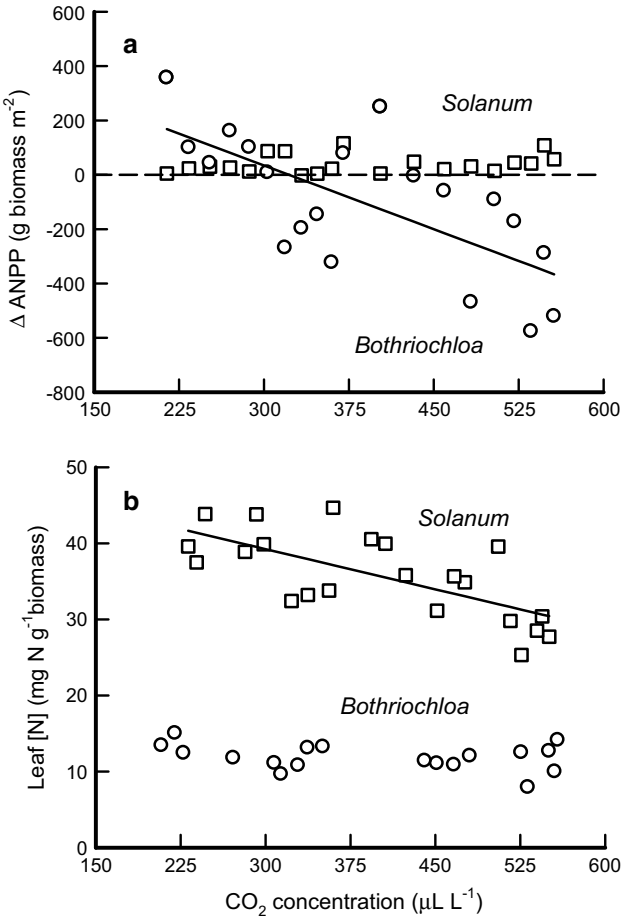


**Fig. 2** Relationships between the change in ANPP (*Δ ANPP*) of the $C_3$ forb *Solanum* (*square*) and $C_4$ grass *Bothriochloa* (*circle*) over 4 years of the PCG experiment on pasture and $CO_2$ concentration (**a**) and between the N concentration, [N], of leaves of the forb and grass from year four of treatment and $CO_2$ (**b**). **a** Δ ANPP for *Bothriochloa* and **b** the [N]–$CO_2$ relationship for *Solanum* were fit with linear regressions (Δ ANPP = 502.61 − 1.56 × $CO_2$, adj. $r^2 = 0.44$, $P = 0.0008$, $n = 20$; [N] = 49.796 − 0.035 × $CO_2$, adj. $r^2 = 0.44$, $P = 0.0004$, $n = 22$). **a** Δ ANPP of *Solanum* and **b** leaf [N] of the *Bothriochloa* were not significantly related to $CO_2$ ($P = 0.27$ and 0.21, respectively)

(35.8 mg N $g^{-1}$ biomass) than grass (11.8 mg N $g^{-1}$ biomass; Fig. 2b). Leaf $\delta^{15}N$ values averaged 5.58 and 1.66 ‰ across $CO_2$ treatments for *Solanum* and *Bothriochloa*, respectively (Fig. 3a). $\delta^{15}N$ values of bulk soil increased with depth at all three positions sampled along the $CO_2$ gradient in 2000 (Fig. 3b).

### LYCOG (tallgrass prairie) experiment

#### *ANPP*

$CO_2$ increased the 4-year average of ANPP of prairie communities by 100–110 g $m^{-2}$ per 100 μL $L^{-1}$ rise (Polley et al.

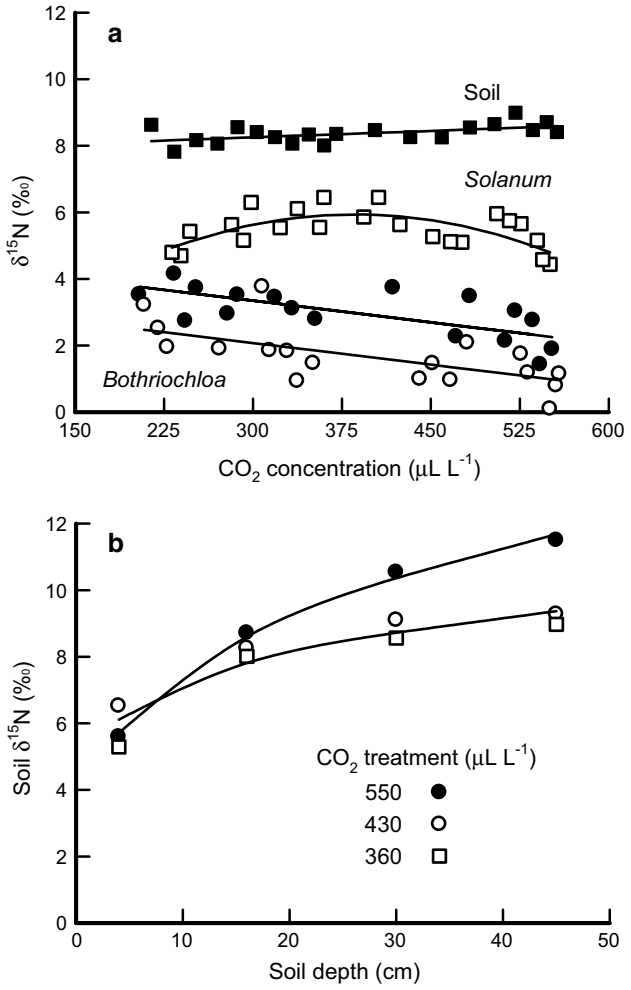


**Fig. 3** Relationships between $\delta^{15}N$ values of bulk soil (as opposed to soil solution) or leaves from pasture and either $CO_2$ concentration (**a**) or soil depth (**b**) in the PCG experiment. **a** Soil (*closed square*) and leaves of the forb *Solanum* (*open square*) were collected in 2000. Leaf blades of the grass *Bothriochloa* were harvested during the first (*closed circle*, 1997) and fourth years of treatment (*open circle*, 2000). $\delta^{15}N$–$CO_2$ relationships for bulk soil (8–23 cm depth increment; adj. $r^2 = 0.25$, $P = 0.02$, $n = 20$) and *Solanum* leaves (adj. $r^2 = 0.40$, $P = 0.003$, $n = 22$) were fit with linear and quadratic regression equations, respectively. Those for *Bothriochloa* from 1997 and 2000 were fit with *parallel lines* (slopes did not differ significantly, $P > 0.50$). **b** Relationships between $\delta^{15}N$ and soil depth (three locations along the $CO_2$ gradient) were fit with exponential equations (550 μL $L^{-1}$ $CO_2$, adj. $r^2 = 0.99$, $P = 0.002$, $n = 4$; 430 and 360 μL $L^{-1}$ $CO_2$ combined; adj. $r^2 = 0.88$, $P = 0.0004$, $n = 8$)

2012b). The ANPP–$CO_2$ response was linear on the silty clay and sandy loam soils, but was strongly non-linear on the clay soil with little change in ANPP at >390 μL $L^{-1}$ $CO_2$.

### *δ15N*

The $\delta^{15}N$ values of leaves collected during the fourth year of the LYCOG experiment (2009) differed among species and plants grown on different soil types (Table 1). Leaf $\delta^{15}N$ was greater for the tallgrass species *Sorghastrum* than mid-grass *Bouteloua* and forb *Solidago* ($n = 46$, 51, and 55, respectively). Leaf $\delta^{15}N$ also was greater for plants grown on the clay than silty clay and sandy loam soils ($n = 52$, 60, and 40, respectively). Species effects on $\delta^{15}N$ did not depend on soil type, but the regression relationship between leaf $\delta^{15}N$ and $CO_2$ differed among soils. Leaf $\delta^{15}N$ was a significant linear function of $CO_2$ for the silty clay only (Fig. 4a). Regression relationships between $\delta^{15}N$ and $CO_2$ did not differ significantly among species (Table 1).

Leaf $\delta^{15}N$ was a significant and positive linear function of leaf [N] for the two clay soils (Table 1; Fig. 5). Leaf $\delta^{15}N$ increased by 3.2–3.9 ‰ for each 10 mg N $g^{-1}$ biomass increase in [N]. Leaf [N] was not, however, correlated with $CO_2$ treatment for any soil type ($P = 0.63$, 0.30, 0.93 for silty clay, sandy loam, and clay soils, respectively). Leaf [N] was a negative function of species' fractional contributions to ANPP on the silty clay soil when analyzed using data for all species combined (not shown; adj. $r^2 = 0.18$, $P = 0.0005$, $n = 60$). There was no relationship between

**Table 1** Results from a mixed-model analysis of covariance of species and soil effects on leaf $\delta^{15}N$ values and of slopes of regression relationships between $\delta^{15}N$ and both $CO_2$ and leaf [N] (LYCOG experiment)

| Variable/unit | Mean/slope | *P* value |
|---|---|---|
| Species/$\delta^{15}N$ (‰) | | <0.0001 |
| *Sorghastrum nutans* | 1.3 (0.2) a | |
| *Bouteloua curtipendula* | 0.0 (0.3) b | |
| *Solidago canadensis* | −0.5 (0.3) b | |
| Soil type | | <0.0001 |
| Clay | 2.9 (0.3) a | |
| Sandy loam | −0.9 (0.3) b | |
| Silty clay | −1.3 (0.3) b | |
| Species X soil type | | 0.74 |
| $CO_2$ (soil type)/‰ change $\delta^{15}N$ per 100 μL $L^{-1}$ increase in $CO_2$ | | 0.001 |
| Clay | – | 0.35 |
| Sandy loam | – | 0.85 |
| Silty clay | 1.9 | <0.0001 |
| [N] (soil type)/‰ change $\delta^{15}N$ per 10 mg N $g^{-1}$ biomass increase in [N] | | <0.0001 |
| Clay | 3.2 | 0.0004 |
| Sandy loam | – | 0.15 |
| Silty clay | 3.9 | <0.0001 |
| $CO_2$ (species) | | 0.44 |
| [N] (species) | | 0.04 |

*P* values indicate the significance of variables or variable interactions on leaf $\delta^{15}N$ and regression slopes. Means (SE) of $\delta^{15}N$ do not differ significantly among species ($n = 46$–55) or soil types ($n = 40$–60) if followed by the same letter

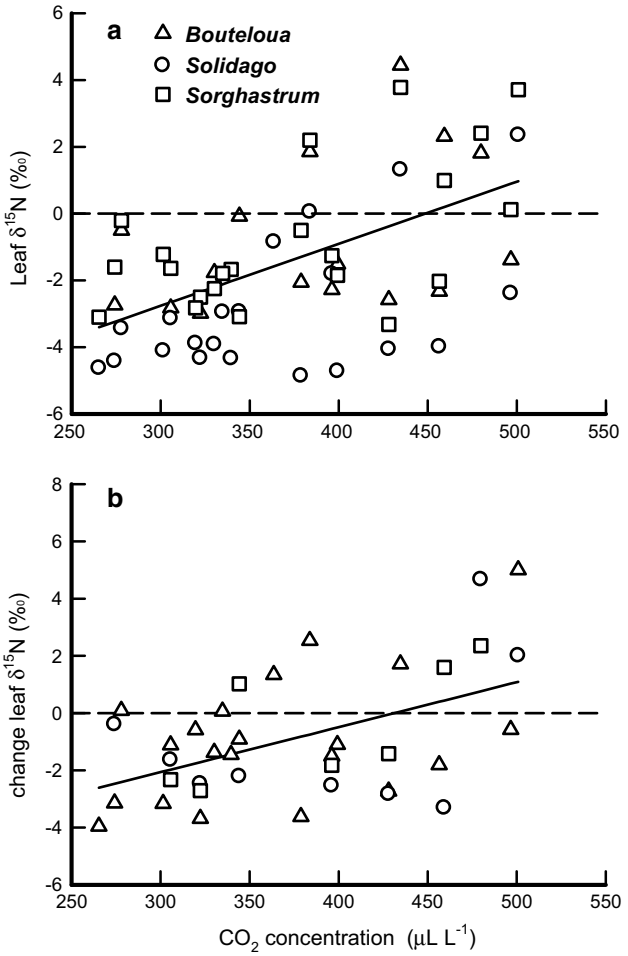


**Fig. 4** Relationships between leaf $\delta^{15}N$ values of perennial prairie species (*Bouteloua*, *Solidago*, *Sorghastrum*) grown on a silty clay soil and $CO_2$ concentration during the LYCOG experiment. Linear regressions between **a** $\delta^{15}N$ values of leaves and $CO_2$ treatment (year 4 of treatment; $\delta^{15}N = -8.346 + 0.019 \times CO_2$, adj. $r^2 = 0.28$, $P < 0.0001$, $n = 60$), and **b** between the 2005 and 2009 change in leaf $\delta^{15}N$ and $CO_2$ were derived from an analysis of covariance (change $\delta^{15}N = -5.845 + 0.014 \times CO_2$, adj. $r^2 = 0.23$, $P = 0.008$, $n = 37$)

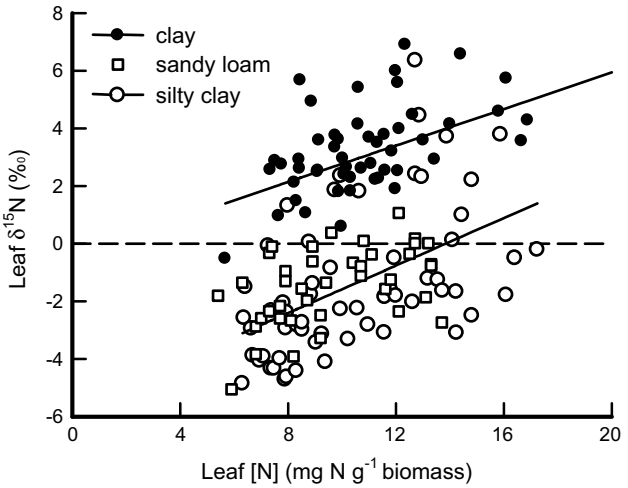


**Fig. 5** Relationships between values of leaf $\delta^{15}N$ and N concentration, [N], for perennial prairie species grown on each of three soil types (clay, sandy loam, silty clay) during the LYCOG experiment. Linear regressions were derived from an analysis of covariance and were significant for the two clay soils (clay, $P = 0.0004$, $n = 52$; silty clay, $P < 0.0001$, $n = 60$)

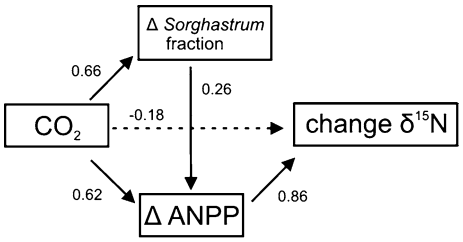


**Fig. 6** Structural equation model describing direct and indirect effects of $CO_2$ on the pre-treatment to post-treatment (4th year) change in leaf $\delta^{15}N$ values (change $\delta^{15}N$) of prairie species (*Bouteloua*, *Solidago*, *Sorghastrum*) grown on a silty clay soil during the LYCOG experiment ($n = 37$). We modeled indirect effects of $CO_2$ through the pre- to post-treatment change in both ANPP (Δ ANPP) and the *Sorghastrum* fraction of community ANPP (Δ *Sorghastrum* fraction) and a direct effect resulting from changes not linked to ANPP. The non-significant pathway is indicated by a *dashed line*. Standardized coefficients are listed beside each path

leaf [N] and species' contributions to ANPP for the sandy loam ($P = 0.61$) or clay soils ($P = 0.50$). Together, $CO_2$ and leaf [N] explained 53 % of the variance in $\delta^{15}N$ values for the silty clay soil [$\delta^{15}N = -6.027 + 0.439 \times$ [N] $+ 0.018 \times$ r_$CO_2$, where r_$CO_2$ = variance in $CO_2$ concentration ($\mu L\ L^{-1}$) not explained by correlation with [N] (mg N $g^{-1}$ biomass); $P < 0.0001$].

### *Change in $\delta^{15}N$*

The pre-treatment to post-treatment (2005–2009) change in leaf $\delta^{15}N$ values generally was negative for prairie vegetation, indicating that $\delta^{15}N$ values declined during $CO_2$ treatment as observed for the PCG experiment (Fig. 4b). Analysis of covariance indicated that the temporal change in leaf $\delta^{15}N$ did not differ among species ($P = 0.25$) or soil types ($P = 0.14$). Slopes of regression relationships between the change in leaf $\delta^{15}$ N and both $CO_2$ and leaf [N] did not differ among species ($P = 0.45$ and 0.36, respectively). Regressions between change in leaf $\delta^{15}N$ and $CO_2$ did differ among soil types ($P = 0.02$).

The change in $\delta^{15}N$ was a significant linear function of $CO_2$ for the silty clay soil only (Fig. 4b). Leaf $\delta^{15}N$ declined from 2005 to 2009 at subambient $CO_2$, but increased slightly at >450 $\mu L\ L^{-1}$ $CO_2$. The positive effect of $CO_2$ on the change in $\delta^{15}N$ was mediated entirely through an increase in ANPP in a SEM that included possible $CO_2$

effects via mechanisms not linked to ANPP (Fig. 6). The more that ANPP increased from 2005 to 2009, the more the values of $\delta^{15}N$ increased. $CO_2$ enrichment increased ANPP both via a direct pathway and, indirectly, via community change associated with an increase in the *Sorghastrum* fraction. For the silty clay soil, community change increased $\delta^{15}N$ by increasing ANPP. We consider this impact of community change on foliar $\delta^{15}N$ to be non-species-specific, as it was expressed similarly among species (Fig. 4b).

Soil C fractions were strongly correlated with the 4-year change in ANPP along the $CO_2$ gradient in the silty clay soil (Fig. 7). Pools of the readily decomposable coarse POM fraction increased, whereas pools of recalcitrant, mineral-associated C decreased linearly as community ANPP increased. High values of change in leaf $\delta^{15}N$ were associated with reduced levels of mineral-associated C.

Would the quantity of N released during decomposition of mineral-associated SOM have been sufficient to supply N demands of the observed increase in ANPP at elevated $CO_2$? As estimated from regression (Fig. 7b), an increase in ANPP of 400 g biomass $m^{-2}$ was associated with an approximately 130 g C $m^{-2}$ decline in the mineral-bound organic fraction in the silty clay soil (from 420 to 290 g C $m^{-2}$). Assuming a C:N ratio of 13–25 with extremes in C:N reflecting the measured ratio of total organic C to organic N (13) and the C:N of microaggregate POM in the silty clay (25; Gill et al. 2006), decomposition of 130 g $m^{-2}$ of mineral-associated C may have released a maximum of 5–10 g N $m^{-2}$. $CO_2$ did not affect the [N] in aboveground tissues of dominant grass species harvested in June 2009, but reduced the [N] of grass assemblages by 6 % (from 9.3 to 8.7 mg N $g^{-1}$ biomass) by increasing the relative abundance of *Sorghastrum*, the grass with the lowest [N] (Polley et al. 2011). At these plant N concentrations, a maximum of 3.5–3.7 g N $m^{-2}$ of additional N would have been required to fully supply demands of the 400 g biomass $m^{-2}$ increase in ANPP at elevated $CO_2$. This additional N requirement represents approximately 35–75 % of the N potentially released during decomposition of mineral-associated SOM at elevated $CO_2$.

The $\delta^{15}N$ value for newly-mineralized N can be estimated by assuming that mineralization of recalcitrant SOM fully supplied the N demands of increased ANPP. As much as 60 % of total ANPP at elevated $CO_2$ resulted from $CO_2$ stimulation of biomass production above the pre-treatment mean of 250 g biomass $m^{-2}$. The 2005–2009 change in leaf $\delta^{15}N$ was approximately −2 ‰ at subambient $CO_2$ (Fig. 4b). Temporal change in leaf $\delta^{15}N$ was approximately 0 ‰ in prairie species grown at 450 μL $L^{-1}$ $CO_2$. $CO_2$ stimulated ANPP by as much as 400 g biomass $m^{-2}$. Using a simple two-member isotope mixing model, we estimate that a change in $\delta^{15}N$ of approximately 1.3 ‰ from pre-treatment values would be required in the 400 g $m^{-2}$ biomass added at elevated $CO_2$ to effect a net change in plant $\delta^{15}N$ of 0 ‰ as observed at 450 μL $L^{-1}$ $CO_2$ (Fig. 4b).

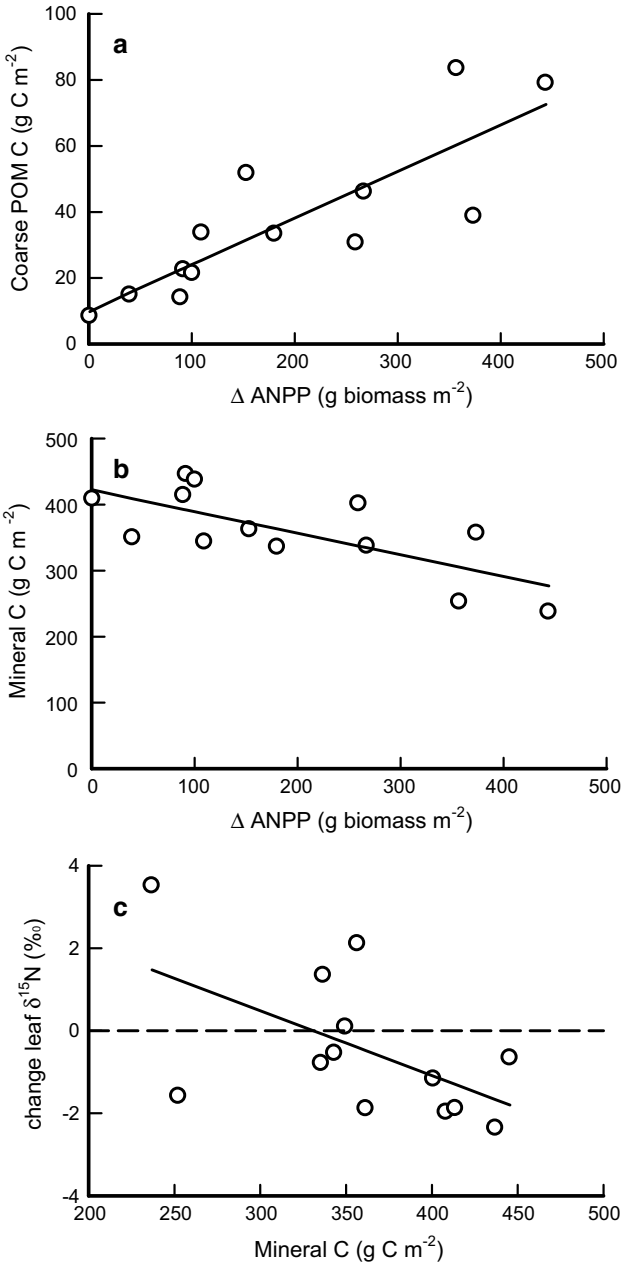


**Fig. 7** Relationships between organic C fractions in soil (coarse POM, **a**, or mineral-associated C, **b**) and the pre-treatment to post-$CO_2$ treatment change in ANPP of prairie vegetation (Δ ANPP) and between the pre- to post-treatment change in leaf $\delta^{15}N$ values (change leaf $\delta^{15}N$) of prairie species and C in the mineral-associated fraction (**c**). Data were derived from monoliths of the silty clay soil from along the $CO_2$ gradient in the LYCOG experiment. Data points represent mean values (Δ ANPP, change leaf $\delta^{15}N$) or results from composited samples (C fractions) from the two monoliths of silty clay soil per 5-m-long compartment along $CO_2$ chambers. Relationships were fit using linear regression (adj. $r^2 = 0.67, 0.47, 0.25$; $P = 0.0004, 0.006, 0.05$ for data in **a**, **b**, and **c**, respectively; $n = 13$)

## Discussion

We predicted that foliar $\delta^{15}N$ values would (1) vary as a positive function of the amount by which $CO_2$ increased community ANPP, and (2) respond similarly among species. Our predictions were only partially supported. $CO_2$ enrichment increased ANPP of both pasture (PCG experiment; Polley et al. 2003) and prairie vegetation (LYCOG experiment; Fay et al. 2012; Polley et al. 2012b), but increased leaf $\delta^{15}N$ values to a similar extent for the three prairie species, and then only on a silty clay soil. The inconsistent response of $\delta^{15}N$ to $CO_2$ among soils resulted partly because $CO_2$ effects on prairie ANPP and species abundances differed among soil types. The silty clay soil on which $CO_2$ increased leaf $\delta^{15}N$ values was the least productive of the three soils on which prairie vegetation was grown, but displayed the most consistent ANPP–$CO_2$ responses among years (Fay et al. 2012; Polley et al. 2012b). Change in community composition, as reflected in a shift in dominance from a mid-grass to tallgrass species at elevated $CO_2$, was the main driver of the large increase in ANPP on the silty clay. Increased ANPP, in turn, was linked with a decline in mineral-associated C in soil that may have contributed to greater plant reliance on $^{15}N$-rich N derived from microbial decomposition of this recalcitrant pool of SOM. $CO_2$ enrichment did not consistently influence $\delta^{15}N$ values of either the dominant grass or forb in the PCG experiment, but increased the relative abundance of the forb species with higher $\delta^{15}N$ values than the dominant $C_4$ grass. Our results highlight a role of plant species (community) change in determining $CO_2$ effects on $\delta^{15}N$ values of grassland vegetation.

$CO_2$ altered community $\delta^{15}N$ values by favoring some species over others, but the mechanism involved in the feedback of community change on $\delta^{15}N$ differed between experiments. $CO_2$ effects were species-specific in the PCG experiment, as they resulted from change in the relative abundances of species that differed in $\delta^{15}N$ values. $CO_2$ enrichment favored a perennial forb with high leaf $\delta^{15}N$ values over the dominant grass with lower $\delta^{15}N$. Conversely, $CO_2$ effects on $\delta^{15}N$ were non-species-specific in the LYCOG experiment. A shift in dominance from a mid-grass to tallgrass species enhanced the ANPP–$CO_2$ response on the silty clay soil (Fay et al. 2012; Polley et al. 2012b), thereby increasing soil pools of readily decomposable SOM, decomposition rates, and microbial biomass (Procter et al. 2014), and apparently stimulating mineralization of older SOM. Whether responding to an accompanying change in the $\delta^{15}N$ of plant-available N or to fractionation from other N transformations in soil or plants, $\delta^{15}N$ values of three prairie species increased by a similar magnitude at elevated $CO_2$.

Leaf $\delta^{15}N$ responded to $CO_2$ in only one of two 4-year experiments and then only on a silty clay soil on which leaf $\delta^{15}N$ values declined at subambient $CO_2$ and increased slightly at >450 $\mu L\ L^{-1}$ $CO_2$. The $CO_2$-caused increase in leaf $\delta^{15}N$ on the silty clay soil could imply a lesser decline or even an increase in losses of $^{15}N$-depleted molecules to leaching or denitrification at elevated $CO_2$ (Garten et al. 2011), but this interpretation is not compatible with the large and consistently positive ANPP–$CO_2$ response on the silty clay soil (Polley et al. 2012b) nor with the absence of $CO_2$ effects on leaf [N]. Alternatively or in addition, $CO_2$ may have increased foliar $\delta^{15}N$ on the silty clay soil by reducing fractionation during plant uptake, assimilation, and turnover/export of N (Evans 2001). Change in plant fractionation alone seems an unlikely explanation for $\delta^{15}N$ trends. In order to have explained results, fractionation must have responded similarly to $CO_2$ in a forb and two grasses on the silty clay, but not responded to $CO_2$ on clay and sandy loam soils. Rather, we suggest that $CO_2$ increased leaf $\delta^{15}N$ values on the silty clay soil by favoring uptake of N that differed in isotopic signature. Uptake of N differing in isotopic signature may have occurred because $CO_2$ (1) favored N acquisition from deeper versus shallower soil layers, or (2) changed the $\delta^{15}N$ signature of source N by increasing microbial immobilization (Billings et al. 2002; Dijkstra et al. 2010) or mineralization of recalcitrant SOM (Gill et al. 2002; Fig. 7). Soil $\delta^{15}N$ increased with depth in the pasture that we studied, a trend observed in other ecosystems (e.g., Nadelhoffer and Fry 1988). Microbes discriminate against the heavier $^{15}N$ isotope (Robinson 2001). Microbial fractionation thus enriches the N in recalcitrant forms of SOM (Tiessen et al. 1984). In the LYCOG experiment, $CO_2$ enrichment increased pools of coarse POM-C by four-fold in the two clay soils, but reduced pools of mineral-associated organic C, generally considered to be recalcitrant to decomposition, on the silty clay soil only (Procter et al., submitted). $CO_2$ thus may have increased leaf $\delta^{15}N$ values by increasing mineralization of older, $^{15}N$-enriched SOM. Such reallocation of N from soil to plants is consistent with one prediction of the PNL hypothesis (Luo et al. 2004), that $CO_2$-caused N limitation may initially be overcome by the transfer of N from SOM with low C:N ratio to plants with higher C:N ratio (Gill et al. 2006). $CO_2$ enrichment increased $\delta^{15}N$ values in desert plants (Billings et al. 2002) and the dominant grass of tallgrass prairie (Williams et al. 2006). Billings et al. (2002) attributed greater $\delta^{15}N$ of desert plants to greater fractionation in soil as a result of increased soil microbial activity at elevated $CO_2$. Johnson DW et al. (2000) attributed an increase in foliar $\delta^{15}N$ of ponderosa pine (*Pinus ponderosa* Dougl.) to greater uptake of N derived from recalcitrant SOM at elevated $CO_2$.

Tightening the N cycle could reduce plant $\delta^{15}N$ by reducing fractionation during N uptake and assimilation by plants (Evans 2001), increasing plant reliance on mycorrhizae for N uptake (Bassirirad et al. 2003; Hobbie and

Hobbie 2008), decreasing the recycling of N through fractionating processes in the soil/plant system, or reducing losses of $^{15}$N-depleted N molecules to leaching or denitrification (Garten et al. 2011). Indeed, foliar $\delta^{15}$N values declined over the 4 years of both the PCG and LYCOG experiments. This decline in $\delta^{15}$N is consistent with the view that fractionation resulting from ecosystem N losses, plant assimilation of N, or other processes declined, or plants exhibited increased reliance on mycorrhizal fungi during community development, regardless of $CO_2$ treatment (Evans 2001).

On the other hand, our finding that $CO_2$ did not consistently affect plant $\delta^{15}$N is seemingly at variance with results from some $CO_2$ experiments (Billings et al. 2002; Bassirirad et al. 2003; Garten et al. 2011) and studies in which $CO_2$ effects on N cycling have been inferred from decadal changes in plant $\delta^{15}$N values (Peñuelas and Estiarte 1997; McLauchlan et al. 2010). The absence of a consistent $\delta^{15}$N–$CO_2$ response could imply either that N strongly limited plant growth across experiments and soils or that N cycling and plant N assimilation were relatively insensitive to $CO_2$ despite apparent tightening of the N cycle during each 4-year experiment. Fractionation of soil N and discrimination during the assimilation of inorganic to organic N in plants are reduced when N is strongly limiting and plant demand approaches N supply rates (Robinson 2001; Billings et al. 2002; Kolb and Evans 2003; Kalcsits et al. 2014). Losses of $^{15}$N-depleted N molecules should also be minimal in strongly N-limited systems. Our data on $CO_2$ effects, however, are most consistent with the interpretation that N cycling and plant fractionation were relatively insensitive to $CO_2$ during the first 4 years of treatment. Resin-available soil N ($NH_4^+$, $NO_3^-$) decreased by only about 15 % from 280 to 480 μL $L^{-1}CO_2$ in the LYCOG experiment and was not correlated with ANPP on any soil type (Fay et al. 2012). Similarly, $CO_2$ did not consistently affect the [N] in aboveground tissues of dominant species from the LYCOG experiment (Polley et al. 2011) and had inconsistent effects on the [N] of dominants from the PCG experiment (Polley et al. 2003). The changes in grassland $\delta^{15}$N that did occur along the $CO_2$ gradient involved changes in species relative abundances, highlighting the underappreciated role of community change in $CO_2$–ecosystem interactions.

**Author contribution statement** All authors contributed to idea formulation, sampling design, and data collection. HWP analyzed data and wrote the manuscript; other authors provided editorial advice.

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