Contrasting effects of elevated CO_2 and warming on nitrogen cycling in a semiarid grassland

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Summary

- Simulation models indicate that the nitrogen (N) cycle plays a key role in how other ecosystem processes such as plant productivity and carbon (C) sequestration respond to elevated CO_2 and warming. However, combined effects of elevated CO_2 and warming on N cycling have rarely been tested in the field.
- Here, we studied N cycling under ambient and elevated CO_2 concentrations (600 μmol mol^{-1}), and ambient and elevated temperature (1.5 : 3.0°C warmer day:night) in a full factorial semiarid grassland field experiment in Wyoming, USA. We measured soil inorganic N, plant and microbial N pool sizes and NO_3^- uptake (using a ^{15}N tracer).
- Soil inorganic N significantly decreased under elevated CO_2, probably because of increased microbial N immobilization, while soil inorganic N and plant N pool sizes significantly increased with warming, probably because of increased N supply. We observed no $CO_2 \times$ warming interaction effects on soil inorganic N, N pool sizes or NO_3^- uptake in plants and microbes.
- Our results indicate a more closed N cycle under elevated CO_2 and a more open N cycle with warming, which could affect long-term N retention, plant productivity, and C sequestration in this semiarid grassland.

Introduction

Combined effects of elevated atmospheric CO_2 and warming have only rarely been investigated in grassland field experiments (e.g. Dukes *et al.*, 2005; Dermody *et al.*, 2007; Wan *et al.*, 2007; Garten *et al.*, 2008; Hovenden *et al.*, 2008) and most information comes from simulation models. Simulation models predict strong responses of semiarid grasslands to elevated CO_2 and warming, but differ regarding the direction and magnitude of those responses (Melillo *et al.*, 1993; Coughenour & Chen, 1997; Pepper *et al.*, 2005; Parton *et al.*, 2007). Melillo *et al.* (1993) predicted a large increase in plant productivity in response to elevated CO_2 but only a small increase in response to warming, while Coughenour & Chen (1997) predicted a decrease in plant productivity with warming under ambient CO_2, but an increase with warming under elevated CO_2. Pepper *et al.* (2005) and Parton *et al.* (2007) predicted an increase in plant productivity with both elevated CO_2 and warming, with the greatest responses occurring when both factors were combined. These simulated plant productivity responses strongly depended on how elevated CO_2 and warming affected nitrogen (N) cycling, which in turn was mediated by changes in soil water availability. Understanding soil moisture and N cycling responses to elevated CO_2 and warming is therefore critical to predicting how semiarid grasslands respond to these global change factors.

Because soil moisture constrains biological activity in dry systems, changes in soil moisture caused by elevated CO_2 and warming could result in strong N cycling responses that differ from responses in mesic ecosystems. In a meta-analysis of CO_2 enrichment studies conducted in a wide range of ecosystems, elevated CO_2 did not affect gross and net N mineralization, but significantly increased microbial N immobilization and plant biomass (De Graaff *et al.*, 2006). Increased retention of N in long-lived plant biomass and soil organic matter could reduce soil N availability and eventually constrain plant growth under elevated CO_2 (a concept also referred to as 'progressive nitrogen limitation' (PNL); Luo *et al.*, 2004; Reich *et al.*, 2006). However, 5 yr of elevated CO_2 increased rates of N mineralization and plant N uptake in a semiarid grassland in Colorado (Dijkstra *et al.*, 2008). It was suggested that PNL had not yet occurred in this system because of an increase in soil moisture induced by elevated CO_2 that may have stimulated N mineralization and plant N uptake. Further, both field and modeling results indicated decreased nitrogen oxide (NOx) gas loss with CO_2 enrichment in this semiarid grassland (Mosier *et al.*, 2002; Parton *et al.*, 2007), suggesting a more closed N cycle. In the long term, lower N losses could contribute to the increased N mineralization under elevated CO_2 (Parton *et al.*, 2007).

As with elevated CO_2, warming may influence N cycling differently in semiarid ecosystems than in systems where biological activity is less constrained by soil moisture. In various field studies, experimental warming increased net N mineralization and N loss (Rustad *et al.*, 2001; Pendall *et al.*, 2004; Schmidt *et al.*, 2004; Bijoor *et al.*, 2008). Some have suggested that warming has a greater effect on net N mineralization than on plant N uptake, thereby increasing the potential for N loss (Lükewille & Wright, 1997; Rustad *et al.*, 2001; Verburg, 2005). However, none of these experimental warming studies was performed in moisture-limited ecosystems. By contrast, soil organic matter decomposition and potential N mineralization decreased with an increasing natural gradient in mean annual temperature in the Central Great Plains of the USA (Epstein *et al.*, 2002; Burke *et al.*, 2008). While the direct effect of warming can enhance biological activity, resulting in increased rates of net N mineralization, reduced soil water availability with experimental warming (Harte *et al.*, 1995; Dermody *et al.*, 2007) could reduce net N mineralization, thereby offsetting the positive temperature effects. Indeed, recently it was suggested that the lack of a clear warming effect on soil inorganic N availability in a tallgrass prairie was attributable to a reduction in soil moisture with warming (Verburg *et al.*, 2009).

We studied the effects of elevated CO_2, warming, and elevated CO_2 plus warming on soil inorganic N pool sizes, plant N concentrations and pool sizes, and plant and microbial nitrate (NO_3^-) uptake in a semiarid grassland field experiment in Wyoming, USA. We also studied the direct effects of soil water availability with a separate irrigation treatment. We measured the soil inorganic N pool size in soil cores and used plant root simulator (PRS) probes (Western Ag Innovations, Saskatoon, SK, Canada) to obtain an integrative measure of the inorganic N present in the soil during the period for which probes were in the soil ('PRS-available N'). We measured N concentration and pool sizes in shoot and root biomass. We further conducted a short-term ^{15}N tracer study to measure plant and microbial uptake of NO_3^-.

A novel aspect of this study is that model predictions were produced before any field results were obtained. Using the DAYCENT model, Parton *et al.* (2007) predicted that elevated CO_2 would increase soil water content, plant production, soil respiration, and N mineralization at our site. They further predicted that warming would decrease soil water content, but increase N mineralization, soil respiration, and plant production. They predicted that combined effects of elevated CO_2 and warming on N cycling would be additive rather than interactive. Based on these predictions, we tested the following hypotheses.

- Elevated CO_2 (enrichment to 600 μmol mol^{-1}) increases the plant N pool size, and plant and microbial uptake of NO_3^- (as a result of alleviation of moisture limitation on growth and activity), and as a result, decreases the soil inorganic N pool size and PRS-available N (suggesting that the uptake of N by plants and microbes increases more than the net release of N through mineralization).
- Despite increased water stress with warming (1.5 : 3.0°C warmer during the day:night), the plant N pool size, and plant and microbial uptake of NO_3^- increase. Warming increases the soil inorganic N pool size and PRS-available N (suggesting a greater difference between net release of N through mineralization and uptake of N by plants).
- Effects of elevated CO_2 and warming on N cycling are additive (i.e. no $CO_2 \times$ warming interactions).

Materials and Methods

Study site

The Prairie Heating and CO_2 Enrichment (PHACE) experiment is located at the US Department of Agriculture Agricultural Research Service (USDA-ARS) High Plains Grasslands Research Station, Wyoming, USA (latitude 41°11′N, longitude 104°54′W). The ecosystem is a northern mixed-grass prairie (NMP) where plant productivity is limited by N (Blumenthal, 2009). The vegetation is dominated by the cool-season C_3 grass *Pascopyrum smithii* (Rydb.) A. Love and the warm-season C_4 grass *Bouteloua gracilis* (H.B.K) Lag. (these two species comprise *c.* 50% of the total aboveground biomass). Other species include the C_3 grass *Hesperostipa comata* Trin and Rupr., the sedge *Carex eleocharis* L. Bailey, the sub-shrub *Artemisia frigida* Willd., and other grasses and forbs. There are no N-fixing plants in our plots. The site was grazed up until 2004. Mean annual precipitation is 384 mm and mean air temperatures are 17.5°C in July and −2.5°C in January. The soil is a fine-loamy, mixed, mesic Aridic Argiustoll with pH of 7.9. Five replicates of each treatment (see the 'Experimental design' section) were established, two on the north side of the site, where the soil was of the Ascalon series, and three on the south side, on the Altvan series. The soil of the

Altvan series contains a gravel layer starting at 70 cm soil depth that is absent in the soil of the Ascalon series.

Experimental design

In 2005, 30 circular plots (diameter 3.4 m) were established. Each plot was surrounded by a 3.7-m-diameter plastic flange that was buried to 60 cm soil depth. The plots were split in half with a 25-cm-deep steel flange. One half of each plot was used to study invasive plants, while the other half was native NMP. The research presented here was conducted on the native NMP side of the plots. Twenty of the 30 plots were used for the CO_2 and warming treatments ('core plots'), while the other 10 plots were used for irrigation treatments. Two concentrations of CO_2 (ambient and 600 μmol mol^{-1}) and two levels of warming (no warming and 1.5°C : 3°C warming of the canopy above ambient during the day:night) were established in a full factorial design with five replicates of each of the four combinations (ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature).

We used free-air CO_2 enrichment (FACE) technology (Miglietta *et al.*, 2001) to increase the atmospheric CO_2 concentration to 600 μmol mol^{-1} (± 40 μmol mol^{-1}) in the elevated CO_2 plots. Pure CO_2 was injected into the plots from a plastic pipe, 3.4 m in diameter, perforated with 300-μm laser-drilled holes, surrounding the plot. The CO_2 treatment started in April 2006. The CO_2 was only injected during daylight hours and during the growing season (April–November). We used ceramic infrared heaters (1000 W; Mor Electric Heating Assoc., Inc., Comstock Park, MI, USA (Trade and company names are given for the reader's benefit and do not imply endorsement or preferential treatment of any product by the USDA)) controlled by a proportional-integral-derivative feed-back loop (Kimball *et al.*, 2008) to warm the canopy of the heated plots to 1.5°C above ambient temperature during the day and 3°C above ambient temperature during the night. Each heated plot had six infrared heaters attached to a triangular frame, 1.5 m above the ground. The warming treatment began in April 2007. Five of the 10 plots that were not used for the CO_2 and warming treatments were irrigated (ct-i) with 20 mm five times in 2007 (7 June, 20 June, 11 July, 21 September, and 15 November; total of 100 mm) and three times in 2008 (26 June, 18 July, and 19 September; total of 60 mm). The amount and timing of the water additions were designed to maintain soil moisture conditions in the ct-i plots close to the Ct plots during the growing season. The other five plots were irrigated in the spring and fall, and were not used for this study. As with the warming treatment, there was no irrigation treatment in 2006. However, because 2006 was a very dry year, all 30 plots received eight 20-mm irrigations during the course of the season (five in June, two in July, and one in August; Fig. 1) to facilitate establishment in the adjacent invasive species experiment. These irrigations increased total annual precipitation in 2006 to 382 mm (increase of 72% of annual total).

Soil moisture and temperature

In each plot, volumetric soil moisture content was monitored at 10, 20, 40, 60 and 80 cm soil depth (EnviroSMART probe; Sentek Sensor Technologies, Stepney, Australia), and soil temperature was monitored at 3 and 10 cm soil depth using thermocouples, starting in July 2006. Soil moisture and temperature data were logged every hour (CR10X data loggers; Campbell Scientific, Logan, UT, USA).

Soil inorganic N pool size and PRS-available N

We measured the soil inorganic N pool size (NH_4^+ + NO_3^-) at 0–5 and 5–15 cm soil depth in August 2007, April 2008, and August 2008. In each plot, three soil cores (diameter 3 cm) were taken to 30 cm soil depth, separated into 0–5, 5–15, and 15–30 cm soil depth samples, and then pooled for each depth. In April 2008, soils were only taken to 15 cm soil depth in the 20 core plots and separated into 0–5 and 5–15 cm samples. The August 2007 and August 2008 soil samples were also used for root biomass sampling (see the 'Shoot and root N concentrations and pool sizes' section). The 0–5 and 5–15 cm soil samples were extracted with 0.05 M K_2SO_4 and analyzed for NH_4^+ and NO_3^- on a flow injection analyzer (QuickChem FIA+; Lachat Instruments, Milwaukee, WI, USA). Because soil inorganic N pool sizes can be highly variable in time as a result of temporal fluctuations in rates of plant uptake and mineralization, we also used Plant Root Simulator (PRS™) resin probes (Western Ag Innovations) which provide an integrative measure of the inorganic N pool in the soil during the period for which the probes are in the soil (PRS-available N; Johnson *et al.*, 2007). Each probe contained a single 17.5-cm^2 resin membrane, which was placed vertically, between 2 and 7.6 cm below the soil surface. To minimize effects of small-scale spatial variation in PRS-available N, four pairs of probes, each comprised of one cation and one anion probe, were inserted into each plot, at least 25 cm inside the edge of the treated area. In 2006, probes were inserted in May and removed in September, while in 2007, probes were inserted in May and removed in October (at this site, probes do not become saturated within a single growing season; Blumenthal, 2009). To examine seasonal differences in PRS-available N, two 1-month-long insertion periods were used in 2008, the first in March–April and the second in July. However, because the soil was very dry in July, probes did not have good contact with the soil and

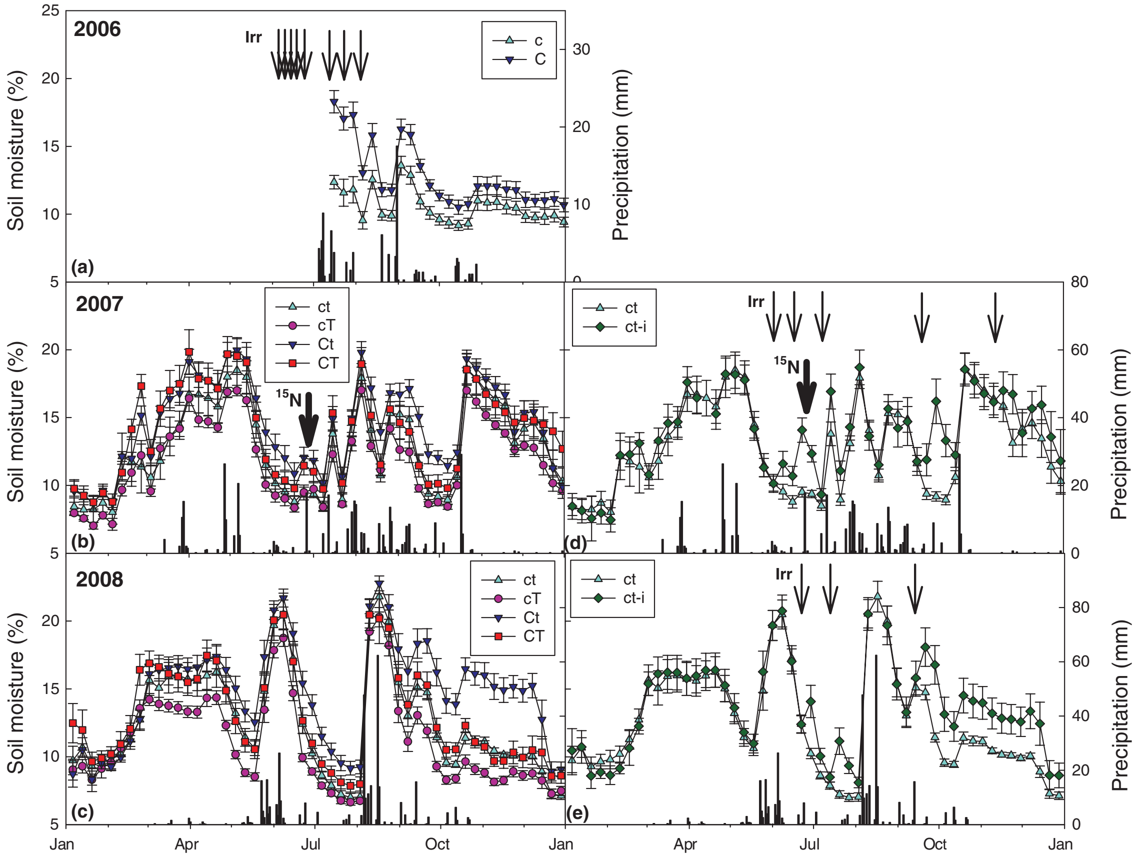

Fig. 1 Soil moisture at 10 cm soil depth in 2006 averaged by elevated CO_2 treatment (a; c, ambient CO_2; C, elevated CO_2) and in 2007 and 2008 averaged by elevated CO_2 and warming treatment (b, c; ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature) and by irrigation treatment (d, e; ct-i, ambient CO_2 and ambient temperature, but irrigated). Each data point is a weekly average of the hourly logged data. Bars in each panel show daily precipitation. Arrows indicate the time when 20-mm water events occurred (Irr), and when the ^{15}N tracer study (^{15}N) was performed in 2007. Error bars indicate ± 1 SE.

NO_3^- and NH_4^+ absorption to the probes was extremely low. We therefore do not present data from this set of probes. Probes were cleaned with deionized water immediately after removal from the soil, and shipped to Western Ag Innovations for analysis. At Western Ag Innovations, probes were eluted with 17.5 ml of 0.5 M HCL for 1 h, and inorganic N (NH_4^+-N and NO_3^--N) was determined colorimetrically, using a Technicon Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, NY, USA, Hangs *et al.*, 2004). The PRS-available N was expressed in μg N 10 cm^{-2}, where the area unit reflects the area of the resin membrane, not the soil area. Thus, the PRS probes provide only an index of the inorganic N present in the soil.

Shoot and root N concentrations and pool sizes

During the last week of July in 2006, 2007 and 2008, shoot biomass was clipped. A 1 × 1.5 m metal wire grid was made containing 24 quadrats (each 25 × 25 cm; total grid area of 1.5 m^2). This grid was placed over each plot and the shoot biomass in every other quadrat was clipped to 1 cm above the soil surface (12 quadrats or a total of 0.75 m^2). Clipped quadrats inside the harvest grid area alternated among years. This clipping protocol resembles a moderate intensity of grazing by cattle (Milchunas *et al.*, 1988). The August 2007 and 2008 soil samples (to 30 cm soil depth; see the ‘Soil inorganic N pool size and PRS-available N’ section) were used for root biomass sampling. Roots were sampled by sieving the soils (2 mm) and by hand-picking roots that fell through the sieve. Shoot and root biomass was dried (60°C) and weighed, and then analyzed for N on an elemental analyzer connected to a mass spectrometer (20-20 Stable Isotope Analyzer; Europa Scientific, Cheshire, UK).

^{15}N labeling experiment

In May 2007, we pounded polyvinyl chloride (PVC) collars (height 25 cm, diameter 10 cm) 22 cm into the ground, one collar in each plot. We also pounded five extra collars into the ground outside of the plots that were used to obtain background values of N and ^{15}N content in plant and soil (off-plot collars). Plant species inside the collars were

P. smithii and *B. gracilis*, the two dominant species. On 23 June 2007, when all plants were still in the vegetative stage, we injected 0.4 g N m^{-2} of 98 atom% KNO_3 in the soil inside each collar of the 30 plots using 18-gauge Quincke spinal needles (Becton Dickinson, Franklin Lakes, NJ, USA). We decided not to use NH_4^+ as a ^{15}N tracer, because of the high pH (7.9) and strong spatial heterogeneity of carbonates present in the surface soil, which would cause large variation in ammonia volatilization. Use of NH_4^+ as a ^{15}N tracer would then also create large variation in ^{15}N recovery in plant and soil, making it difficult to detect treatment effects. To increase homogenous distribution of the labeled N, we injected the labeled N as a solution to three soil depths (2.5, 7.5 and 12.5 cm), with three injections at each depth (total of nine injections per collar). We injected a total of 27 ml of solution in each collar (3 ml per injection), thereby increasing the soil moisture content inside the collars by 2.3% volumetrically or 1.9% gravimetrically. Forty-eight hours after injection, we harvested the 30 collars and the five off-plot collars outside the plots which were not labeled with ^{15}N. We clipped the aboveground biomass and divided soil within the collar into 0–5 and 5–15 cm depth portions. The soils were immediately sieved (2 mm) in the field to separate roots from soil. Sieved soils were picked for roots that fell through the sieve. Soils were transported on ice and stored in a refrigerator until the next day for processing.

Soil subsamples were dried at 60°C. Crowns from the 0–5 cm soil sample were separated from the roots. Roots from both soil depths and crowns were washed, and together with aboveground biomass dried (60°C) and weighed. The plant and soil samples were then ground and analyzed for total N and ^{15}N on a mass spectrometer (20-20 Stable Isotope Analyzer; Europa Scientific).

We measured microbial biomass N and ^{15}N using fumigation-extraction (Bruulsema & Duxbury, 1996). After thoroughly homogenizing the sample we added a 25-g subsample to 60 ml of 0.05 M K_2SO_4. Another 25-g subsample was fumigated with chloroform for 5 d in a vacuum dessicator and then also added to 60 ml of 0.05 M K_2SO_4. Samples were shaken for 1 h and filtered through pre-leached Whatman No. 1 filter paper. We analyzed aliquots of the extracts for total organic carbon (C) and total N on a Total Organic Carbon (TOC) analyzer with an N measuring unit attached (Shimadzu TOC-V_{CPN}; Shimadzu Scientific Instruments, Wood Dale, IL, USA). Another aliquot of 6 ml was freeze-dried and analyzed for ^{15}N on a mass spectrometer.

We calculated microbial N as the difference between N in the fumigated and nonfumigated samples divided by 0.54 (Brookes *et al.*, 1985). We calculated the ^{15}N atom% in microbial biomass ($^{15}N_{mic}$) using:

$$^{15}N_{mic} = (^{15}N_f \times N_f - ^{15}N_e \times N_e)/(N_f - N_e) \qquad \text{Eqn 1}$$

($^{15}N_f$ and N_f, the ^{15}N atom% and total amount of N in the fumigated extracts; $^{15}N_e$ and N_e, the ^{15}N atom% and total amount of N in the nonfumigated extracts.) We calculated ^{15}N recovery in the microbial N pool in the ^{15}N labeled collars ($^{15}N_{rec,\ mic}$) using:

$$^{15}N_{rec,mic} = N_{mic,l} \times (^{15}N_{mic,l} - ^{15}N_{mic,n}) / (^{15}N_{label} - ^{15}N_{mic,n}) \qquad \text{Eqn 2}$$

($N_{mic,\ l}$ and $^{15}N_{mic,\ l}$, the total amount of N and ^{15}N atom% in the microbial biomass labeled with ^{15}N; $^{15}N_{mic,\ n}$, the average ^{15}N atom% in the microbial biomass not labeled with ^{15}N (average of the five off-plot collars); $^{15}N_{label}$, the ^{15}N atom% of the label.) We calculated ^{15}N recovery in the plant N pools and in the total soil N pool in a similar way. We calculated total ^{15}N recovery by summing the ^{15}N recovery in plants and soil.

Statistical analyses

We used repeated measures ANOVA to test for main effects of CO_2 (ambient or elevated) and date (weekly averages), and $CO_2 \times$ date interactions on soil moisture in 2006. For the 20 core plots we used repeated measures ANOVA to test for main effects of CO_2, warming (no warming or warming), date (weekly averages from 1 May 2007 to 31 December 2008), and their interactions on soil moisture and temperature. For the 20 core plots we used ANOVA to test for main effects of CO_2, warming, and $CO_2 \times$ warming interactions on soil inorganic N pool sizes, and plant and microbial N and ^{15}N recovery. For the ct (ambient CO_2 and ambient temperature) and ct-i plots (ambient CO_2 and ambient temperature with irrigation) we used ANOVA to test for irrigation effects. We included the random effect of soil type (north or south) in all ANOVAs. For root biomass, microbial N and ^{15}N recovery we included soil depth (0–5 or 5–15 cm) as a main factor, and its interactions with CO_2 and warming in the ANOVA. Although soil depth was sometimes significant, we observed no significant interactions with CO_2, warming, or irrigation for root biomass and microbial ^{15}N recovery. We therefore reported total root biomass and microbial ^{15}N recovery at 0–15 cm soil depth and removed soil depth and its interactions from the ANOVA. In some cases, data were log-transformed to improve assumptions of normality and homoscedasticity. All statistical analyses were performed with JMP (version 4.0.4; SAS Institute, Cary, NC, USA).

Results

As expected, elevated CO_2 significantly increased (P = 0.007) and warming significantly decreased soil moisture at 10 cm soil depth between 1 May 2007 and 31

December 2008 (P = 0.04; Fig. 1a–c; note that the warming treatment started in mid April 2007). In 2006, soil moisture (monitoring started in July of that year) was always higher in the elevated CO_2 plots (C plots, elevated CO_2, on average by 2.36% v/v; P < 0.0001). Warming caused a similar reduction in soil moisture under ambient and elevated CO_2 (on average warming reduced soil moisture by 1.3% v/v in the ambient CO_2 plots, and by 1.4% v/v in the elevated CO_2 plots between 1 May 2007 and 31 December 2008), and we observed no significant $CO_2 \times$ warming interaction (P = 0.92; repeated measures ANOVA). Irrigation events caused spikes in soil moisture relative to the ambient plots that were short in duration (Fig. 1d,e). Soil temperature increased in the warming treatment on average by 2.45 (P = 0.008) and 1.81°C (P = 0.02) at 3 and 10 cm soil depth, respectively (from 1 May 2007, when the treatment started, to 31 December 2008, averaged across CO_2 treatments; Supporting Information Fig. S1). Warming had similar effects on soil temperature in the ambient and elevated CO_2 plots.

Elevated CO_2 decreased the soil inorganic N pool size at all three dates and both soil depths (Table 1). The decrease was more significant in mid-summer 2007 and 2008 (decreases of 25% and 38%, respectively, averaged across warming treatments and soil depths) than in April 2008 (decrease of 17%), and greater at 0–5 than at 5–15 cm soil depth (34% and 25% decreases, respectively, averaged across years). By contrast, warming significantly increased the soil inorganic pool size in mid-summer of 2007 and 2008 at 0–5 cm soil depth (by 31% and 63%, respectively, averaged across CO_2 treatments), and in mid-summer 2008 at 5–15 cm soil depth (by 42%). In April 2008 warming only marginally increased the soil inorganic N pool size at 0–5 cm soil depth (by 17%). The PRS probes showed similar results. Elevated CO_2 significantly decreased the PRS-available N in 2006 (by 54%; P = 0.0003), 2007 (by 53%; P = 0.0009), and 2008 (by 36%; P = 0.02), while warming significantly increased PRS-available N in 2007 (by 100%; P = 0.009), but had no effect in early spring of 2008 (Fig. 2). There were no interactions between elevated CO_2 and warming for measurements of inorganic N in soil cores or on PRS probes. Irrigation significantly decreased the soil inorganic N pool size measured in the soil cores at 0–5 cm soil depth in August 2008 (by 43%), but not at other times (Table 1), while irrigation significantly decreased PRS-available N in 2007 (by 61%; P = 0.02), but not in early spring of 2008 (Fig. 2). Most of the inorganic N measured in the soil cores and on the PRS probes was in the form of NO_3^- (on average 72% and 93% of total inorganic N, respectively).

Shoot N pool sizes from the harvest grid were not affected and shoot N percentage was decreased by elevated CO_2 in all three years (Fig. 3). These results coincided with a significant increase in shoot biomass in 2007 (Morgan *et al.*, 2008) and 2008 (J.A. Morgan *et al.*, unpublished) under elevated CO_2. Warming marginally increased the shoot N pool size in 2007 (on average by 19%), but had no effect on shoot N percentage in that year. However, in 2008 the shoot N pool size and shoot N percentage were significantly higher in the warming treatment (by 22% and 16%; P = 0.02 and 0.0009, respectively). There were no significant interactions between elevated CO_2 and warming in terms of effects on shoot N pool sizes or shoot N percentage. We observed no significant main effects of CO_2 on

Table 1 Soil inorganic nitrogen (N) pool sizes (NH_4^+ + NO_3^-) at 0–5 and 5–15 cm soil depth averaged by the CO_2 and warming treatments

	Soil inorganic N (g m^{-2})					
	August 2007		April 2008		August 2008	
Treatment	0–5 cm	5–15 cm	0–5 cm	5–15 cm	0–5 cm	5–15 cm
ct	0.30 ± 0.04	0.27 ± 0.05	0.12 ± 0.01	0.24 ± 0.03	0.28 ± 0.04	0.34 ± 0.03
cT	0.38 ± 0.05	0.30 ± 0.03	0.15 ± 0.01	0.24 ± 0.02	0.46 ± 0.11	0.47 ± 0.06
Ct	0.21 ± 0.02	0.21 ± 0.02	0.11 ± 0.01	0.20 ± 0.03	0.15 ± 0.01	0.23 ± 0.02
CT	0.29 ± 0.01	0.23 ± 0.01	0.12 ± 0.01	0.19 ± 0.03	0.24 ± 0.04	0.34 ± 0.07
ct-i	0.24 ± 0.05	0.26 ± 0.05	nd[1]	nd	0.16 ± 0.02	0.28 ± 0.02
ANOVA *P*-values						
CO_2	**0.02**	**0.05**	**0.05**	0.12	**0.008**	**0.02**
T	**0.04**	0.36	*0.08*	0.99	**0.04**	**0.02**
$CO_2 \times$ T	0.88	0.84	0.27	0.79	0.45	0.75
Water[2]	0.33	0.89	nd	nd	**0.03**	0.14

ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature; and ambient CO_2 and ambient temperature, but irrigated (ct-i). T, temperature.
P-values are in bold when P < 0.05 and in italics when P < 0.1.
[1]Not determined.
[2]ANOVA *P*-values for the water treatment were obtained for the ct and ct-i plots only.

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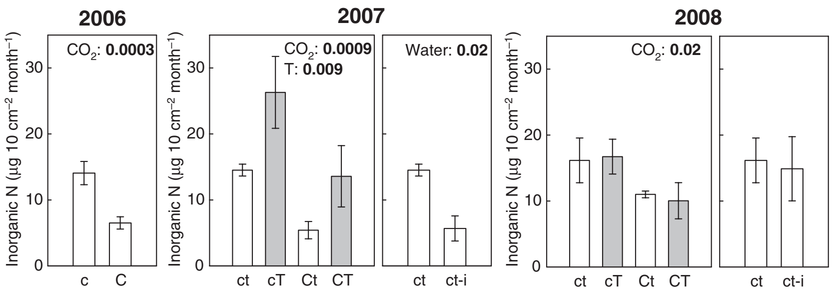

Fig. 2 Soil inorganic nitrogen (N) availability ($NH_4^+ + NO_3^-$) at 2–7.6 cm soil depth in 2006 averaged by CO_2, and in 2007 and 2008 averaged by CO_2, warming, and irrigation treatments. Treatments: c, ambient CO_2; C, elevated CO_2; ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature; ct-i, ambient CO_2 and ambient temperature, but irrigated. T, temperature. Soil inorganic N availability is expressed in µg N 10 cm^{-2} resin membrane area of the plant root simulator (PRS) probes per month incubation time. Error bars indicate ± 1 SE. ANOVA *P*-values are reported when $P < 0.05$ (in bold) or $P < 0.1$ (in italics).

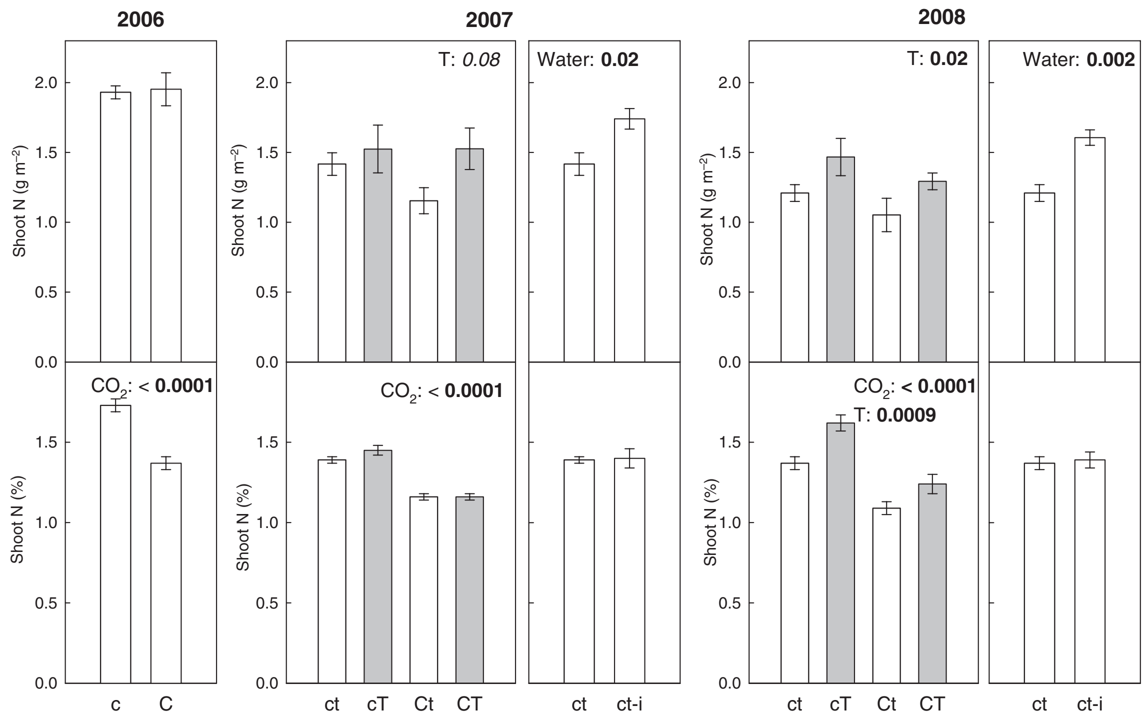

Fig. 3 Shoot nitrogen (N) pool sizes and N concentrations from the harvest grid in 2006 averaged by CO_2, and in 2007 and 2008 averaged by CO_2, warming, and irrigation treatments. Treatments: c, ambient CO_2; C, elevated CO_2; ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature; ct-i, ambient CO_2 and ambient temperature, but irrigated. T, temperature. Error bars indicate ± 1 SE. ANOVA *P*-values are reported when $P < 0.05$ (in bold) or $P < 0.1$ (in italics).

root N pool sizes measured in 2007 and 2008, while warming marginally increased the root N pool size in 2008 (Table 2). However, elevated CO_2 significantly reduced root N percentage in 2008 ($P = 0.005$), coincident with a significant increase in root biomass (by 33%; $P = 0.04$). Warming marginally increased root N percentage in 2007, but only in the elevated CO_2 plots (marginally significant CO_2 × warming interaction; $P = 0.09$), but warming had no effect on root biomass. Irrigation significantly increased the shoot N pool size in 2007 (by 23%; $P = 0.02$) and 2008 (by 33%; $P = 0.002$), but marginally decreased the root N pool size in 2008 ($P = 0.07$). Irrigation had no effect on shoot or root N percentage, or on root biomass.

There were no significant treatment effects on plant N pool sizes from the ^{15}N labeling study with the exception of crown N, which was significantly lower under elevated CO_2 (Table 3). Plant N concentrations were not affected by elevated CO_2, warming or irrigation, except for shoot N concentration, which was significantly lower under elevated CO_2 (1.77 ± 0.07% and 1.43 ± 0.06% (mean ± SE) for

Table 2 Root biomass, nitrogen (N) pool sizes and N concentrations at 0–30 cm soil depth from the harvest grid averaged by the CO_2 and warming treatments

	Root biomass and N pool sizes (g m^{-2})				Root N%	
	2007		2008			
Treatment	Biomass	N	Biomass	N	2007	2008
ct	376 ± 30	3.68 ± 0.46	417 ± 51	4.17 ± 0.41	0.97 ± 0.04	1.01 ± 0.05
cT	329 ± 42	3.22 ± 0.47	412 ± 38	4.28 ± 0.44	0.97 ± 0.03	1.04 ± 0.03
Ct	404 ± 57	3.38 ± 0.46	488 ± 48	4.17 ± 0.42	0.84 ± 0.03	0.86 ± 0.04
CT	399 ± 22	3.99 ± 0.38	597 ± 76	5.55 ± 0.64	0.99 ± 0.06	0.94 ± 0.02
ct-i	338 ± 28	3.17 ± 0.26	325 ± 14	3.01 ± 0.24	0.94 ± 0.05	0.93 ± 0.04
ANOVA *P*-values						
CO_2	0.23	0.60	**0.04**	0.20	0.22	**0.005**
T	0.52	0.87	0.19	*0.06*	*0.07*	0.15
$CO_2 \times T$	0.61	0.24	0.31	0.20	*0.09*	0.48
Water[1]	0.38	0.29	0.16	*0.07*	0.74	0.24

ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature; and ambient CO_2 and ambient temperature, but irrigated (ct-i). T, temperature.
P-values are in bold when $P < 0.05$ and in italics when $P < 0.1$.
[1]ANOVA *P*-values for the water treatment were obtained for the ct and ct-i plots only.

Table 3 Plant and microbial nitrogen (N) pool sizes (g m^{-2}) from the ^{15}N tracer study in June 2007 averaged by the CO_2 and warming treatments

	Plant N				Microbial N	
Treatment	Shoots	Crowns	Roots	Total	0–5 cm	5–15 cm
ct	1.1 ± 0.1	4.7 ± 0.4	4.9 ± 0.8	9.2 ± 0.4	6.2 ± 0.5	6.5 ± 0.3
cT	0.8 ± 0.2	6.2 ± 0.7	4.6 ± 1.0	10.7 ± 1.0	6.8 ± 0.6	6.8 ± 0.5
Ct	0.9 ± 0.2	3.9 ± 0.6	3.6 ± 0.3	8.5 ± 0.3	8.1 ± 0.9	7.4 ± 0.7
CT	0.8 ± 0.1	4.0 ± 0.6	4.5 ± 0.5	8.8 ± 0.9	7.8 ± 0.8	6.8 ± 0.8
ct-i	1.3 ± 0.2	5.2 ± 0.9	3.3 ± 0.4	9.5 ± 1.2	7.1 ± 0.6	7.5 ± 0.8
ANOVA *P*-values						
CO_2	0.52	**0.02**	0.47	0.14	**0.05**	0.47
T	0.18	0.39	0.67	0.29	0.99	0.79
$CO_2 \times T$	0.67	0.48	0.38	0.48	0.30	0.43
Water[1]	0.52	0.74	0.16	0.80	0.38	0.50

ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature; and ambient CO_2 and ambient temperature, but irrigated (ct-i). T, temperature.
P-values are in bold when $P < 0.05$ and in italics when $P < 0.1$.
[1]ANOVA *P*-values for the water treatment were obtained for the ct and ct-i plots only.

ambient and elevated CO_2 plots, respectively). Microbial N was significantly higher (by 23%) under elevated CO_2 at 0–5 cm soil depth, but showed no treatment effects at 5–15 cm soil depth.

Elevated CO_2 significantly increased ^{15}N recovery in roots by 57% (P = 0.02) and warming increased ^{15}N recovery in roots by 40% (P = 0.07). Despite a marginally greater shoot N pool size, warming reduced ^{15}N recovery in shoots on average by 28% (P = 0.07; Fig. 4). Possibly, transport of ^{15}N from roots to shoots was delayed with the warming treatment. We observed no significant CO_2 or warming effects on total plant ^{15}N recovery and no significant $CO_2 \times$ warming interactions. We also observed no significant irrigation effects on plant ^{15}N recovery.

Elevated CO_2 significantly increased ^{15}N recovery in microbial biomass by 186% (P = 0.0009; Fig. 5). Warming did not affect ^{15}N recovery in microbial biomass. Irrigation marginally increased ^{15}N recovery in microbial biomass by 119% (P = 0.09). None of the treatments showed significant effects on total (plant + soil) ^{15}N recovery. Total recovery of the ^{15}N label added to the soil was on average 90%.

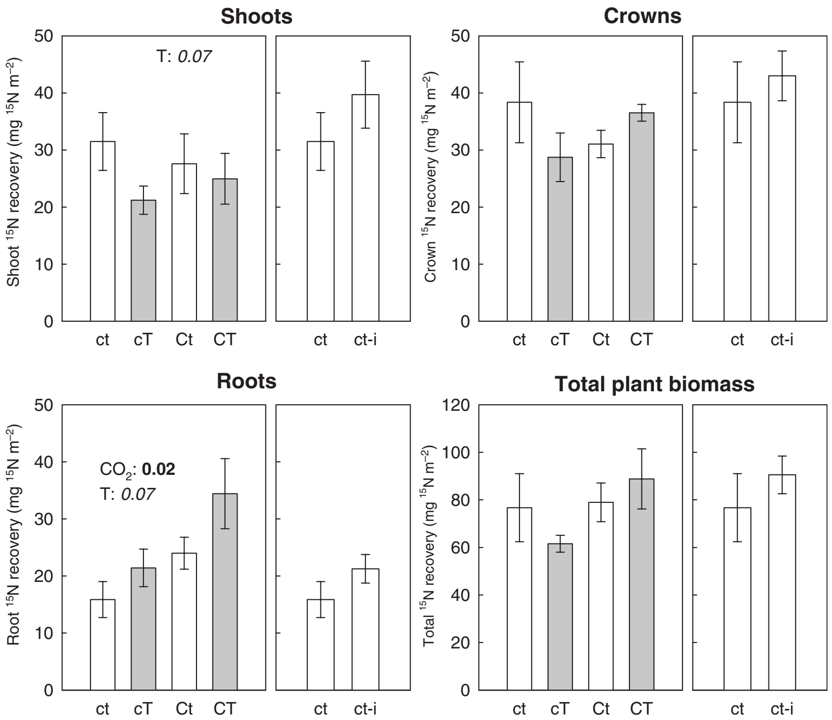

Fig. 4 ^{15}N recovery in shoots, crowns, roots, and total plant biomass averaged by CO_2, warming, and irrigation treatments. Treatments: ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature; ct-i, ambient CO_2 and ambient temperature, but irrigated. T, temperature. Error bars indicate ± 1 SE. ANOVA *P*-values are reported when $P < 0.05$ (in bold) or $P < 0.1$ (in italics).

Discussion

Elevated CO_2 effects

In support of our first hypothesis, elevated CO_2 reduced the soil inorganic N pool size and PRS-available N. In contrast to our hypothesis, this reduction in soil inorganic N was probably not mediated by plant N uptake, as elevated CO_2 did not affect plant N pool sizes or total plant NO_3^- uptake. Rather, we observed a significant and large increase in microbial NO_3^- uptake and in the microbial N pool size at 0–5 cm soil depth under elevated CO_2, suggesting that increased microbial N immobilization reduced soil inorganic N under elevated CO_2. Reduced soil inorganic N under elevated CO_2 as a result of increased loss of N through volatilization and/or leaching seems unlikely. Loss of N through volatilization is very small in semiarid grasslands (Mosier *et al.*, 2008; F. A. Dijkstra, unpublished) and is only important for long-term N cycling (Parton *et al.*, 2007). Also, elevated CO_2 tends to decrease, not increase, N leaching in other grassland systems (Niklaus *et al.*, 2001; Dijkstra *et al.*, 2007). Thus, elevated CO_2 made the N cycle more closed in this semiarid grassland, mostly because of increased microbial N immobilization.

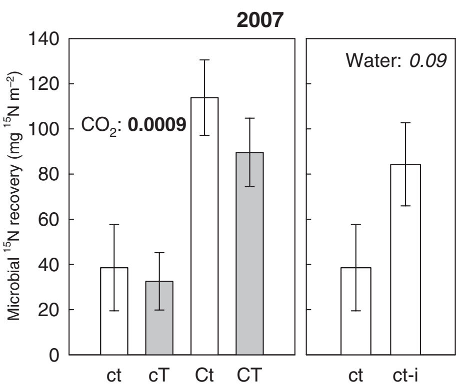

Fig. 5 ^{15}N recovery in microbial biomass at 0–15 cm soil depth averaged by CO_2, warming, and irrigation treatments. Treatments: ct, ambient CO_2 and ambient temperature; cT, ambient CO_2 and elevated temperature; Ct, elevated CO_2 and ambient temperature; CT, elevated CO_2 and elevated temperature; ct-i, ambient CO_2 and ambient temperature, but irrigated. Error bars indicate ± 1 SE. ANOVA *P*-values are reported when $P < 0.05$ (in bold) or $P < 0.1$ (in italics).

Our results are consistent with others that have shown reduced inorganic N in the soil because of increased plant and/or microbial N immobilization under elevated CO_2 (Díaz *et al.*, 1993; Hu *et al.*, 2001, 2005), which could ultimately limit the CO_2 fertilization effect on plant productivity (Luo *et al.*, 2004; Reich *et al.*, 2006). However, N mineralization and plant N uptake showed sustained increases after 5 yr of elevated CO_2 in a semiarid grassland in northern Colorado (Dijkstra *et al.*, 2008). Net N mineralization also increased after 5 yr of elevated CO_2 in a calcareous grassland in Switzerland (Ebersberger *et al.*, 2003). Despite a reduction in soil inorganic N availability as a result of increased microbial N immobilization, the plant N pool sizes in the present study were not affected after 3 yr of elevated CO_2, suggesting that net N mineralization was not affected by elevated CO_2. Root biomass significantly increased under elevated CO_2 in 2008 (Table 1), and it is possible that more roots proliferating into unexplored soil (including into deeper soil) under elevated CO_2 may have intensified plant N uptake, resulting in a similar total plant N pool as under ambient CO_2 (Finzi *et al.*, 2007; Zak *et al.*, 2007). We should note, however, that an increase in root biomass itself does not provide decisive evidence for increased soil exploration, as that would also require measurement of root length density, something we did not do. It remains to be seen how plant productivity and N cycling in this system will be affected by elevated CO_2 in the long term. Parton *et al.* (2007) predicted increased decomposition, net N mineralization and plant productivity under elevated CO_2 during most years of a 10-yr simulation period for the PHACE experiment.

Because microbial NO_3^- uptake also increased with irrigation, it is possible that the CO_2-induced increase in soil moisture caused the increased microbial NO_3^- uptake under elevated CO_2 in June 2007. An increase in microbial N immobilization under elevated CO_2 could also be attributable to increased labile C inputs (Barnard *et al.*, 2006; De Graaff *et al.*, 2006). However, labile soil C measured also in June 2007 was not consistently higher under elevated CO_2 (based on respiration measurements in laboratory incubations; Y. Carrillo *et al.*, unpublished), and there was no significant relationship between soil labile C and ^{15}N recovery in microbial biomass. Our results are consistent with results from a Mediterranean grassland where a CO_2-induced increase in soil moisture best explained the increase in microbial N immobilization (Hungate *et al.*, 1997). While an increase in soil moisture usually increases net N mineralization in semiarid grasslands (Burke *et al.*, 1997), our results support the notion that soil N availability may sometimes decrease with increased soil moisture as a result of increased N immobilization within the plant–soil system (McCulley *et al.*, 2009).

Warming effects

Experimental warming often increases soil inorganic N availability and net N mineralization in systems that are not water limited (Rustad *et al.*, 2001; Pendall *et al.*, 2004). In the semiarid grassland that we studied, soil water availability is a limiting factor for biological activity, and warming decreased soil water content. Nevertheless, warming significantly increased soil inorganic N (in midsummer in 2007 and 2008) and plant N pool size (in 2008), suggesting that it increased net N mineralization (Rustad *et al.*, 2001; Pendall *et al.*, 2004). During March–April 2008, soil inorganic N was not affected (PRS probes) or was only marginally affected (soil cores) by warming. Because plant activity is low this early in the season, it seems unlikely that soil inorganic N was influenced by plant N uptake. Plant and microbial ^{15}N recovery measured in June 2007 was not affected by warming, further suggesting that warming did not influence plant and microbial NO_3^- uptake in late spring (although we should note that the ^{15}N recovery study was performed only 2½ months after the warming treatment started). Further research is needed to determine whether the lack of a warming effect on soil inorganic N early in the growing season is persistent across years. Nevertheless, our results suggest that, despite increased plant N uptake, warming made the N cycle more open because of a greater increase in net N mineralization during much of the growing season. Increased net N mineralization and plant N uptake with warming were also predicted by Parton *et al.* (2007).

Parton *et al.* (2007) suggested that increased net N mineralization and plant N uptake with warming were more likely to be driven by direct warming effects on soil temperature than by indirect effects on soil moisture. Above we suggested that increased soil moisture may have increased microbial NO_3^- immobilization under elevated CO_2 and with irrigation. Thus, a decrease in soil moisture with warming could then reduce microbial NO_3^- immobilization and potentially increase net N mineralization. However, we observed no change in microbial NO_3^- immobilization with warming in 2007. While it is possible that our ^{15}N recovery measurements were obtained too recently after the warming treatment began to reveal significant reductions in microbial NO_3^- immobilization, it is also possible that the increase in soil temperature (on average by 2.45 and 1.81°C at 3 and 10 cm soil depth, respectively) stimulated microbial NO_3^- immobilization, thereby offsetting any soil moisture effects. The increase in soil temperature with warming may then have stimulated gross and net N mineralization, causing an increase in plant N uptake. Liu *et al.* (2009) suggested that the decrease in soil moisture (on average an absolute decrease of 2.8% v/v) was more important than the increase in soil temperature (average increase of 1.2°C) in decreasing microbial respiration with experimental warming in a semiarid grassland in Inner Mongolia, China. Verburg *et al.* (2009) also suggested that the lack of an effect of experimental warming (an average increase in soil temperature of 2.3°C) on inorganic soil N availability and plant N uptake in a tallgrass prairie was attributable to a reduction in soil moisture offsetting a potential increase in net N mineralization caused by a higher soil temperature. Our results support predictions by Parton *et al.* (2007) that the increase in soil temperature with warming had a greater effect on soil N mineralization and plant N pools than the reduction in soil moisture.

$CO_2 \times$ warming interactions

In support of our third hypothesis, there were no significant $CO_2 \times$ warming interactions for plant N pool sizes, plant and microbial NO_3^- uptake or soil inorganic N. By contrast, there was a significant $CO_2 \times$ warming interaction for soil inorganic N availability in a temperate grassland in Tasmania, Australia (Hovenden *et al.*, 2008). In that experiment, elevated CO_2 reduced soil inorganic N measured on ion exchange membranes (similar to our PRS probes) without warming, but not in combination with warming. Hovenden *et al.* (2008) could not ascribe this $CO_2 \times$ warming interaction to soil moisture differences, but suggested that the $CO_2 \times$ warming interaction for soil inorganic N availability was related to changes in C cycling. Because our measurements were made during the first 2 yr of the warming treatment, it is possible that a $CO_2 \times$ warming interaction for N cycling could occur after long-term changes in C cycling.

Conclusions

Based on our point-in-time and seasonally integrated N measurements, we conclude that elevated CO_2 and warming had contrasting effects on the N cycle in this semiarid grassland. Our results show that elevated CO_2 decreased the soil inorganic N pool, probably because of increased microbial immobilization, while warming increased the soil inorganic N pool and plant N uptake, probably because of increased gross and net N mineralization. Irrigation effects on the N cycle were often similar to the effects of elevated CO_2, suggesting that the elevated CO_2-induced increase in soil moisture played a critical role in the more closed N cycle under elevated CO_2. By contrast, direct effects of warming were probably more important than a warming-induced decrease in soil moisture in causing a more open N cycle with warming. It remains to be seen if a more closed N cycle under elevated CO_2 will reduce N loss, thereby supporting greater N mineralization as predicted by Parton *et al.* (2007). Similarly, a more open N cycle with warming could potentially have the opposite effect on N loss and long-term N mineralization. Nevertheless, effects of elevated CO_2 and warming on N cycling were additive, and our results indicate that both global climate change factors have important impacts on the N cycle in this semiarid grassland system, with potentially large consequences for plant productivity and C sequestration.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Soil temperature at 3 and 10 cm soil depth averaged by CO_2 and warming treatments.

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