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Does the accelerated soil N cycling sustain N demand of *Quercus mongolica* after decade-long elevated CO_2 treatment?

Jianfei Sun · Weiwei Dai · Bo Peng · Jun Liu · Tongxin He · Ping Jiang · Shijie Han · Edith Bai

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Abstract The stimulation of plant growth and biomass accumulation by elevated CO_2 may be limited by soil nitrogen (N) availability. However, our understanding of the response of soil N cycling to elevated CO_2 and when progressive N limitation occurs remains limited. Here, we used an open top chamber experiment to examine the effects of 10 years of elevated CO_2 on ecosystem carbon (C) and N dynamics in a *Quercus mongolica* (oak) dominated system in northeastern China. Elevated CO_2 increased oak biomass, C and N stocks and C/N by 26.4, 26.2, 16.5 and 8.6% respectively, which suggests increased plant N demand. Soil gross N mineralization, re-mineralization of microbial N and nitrification were accelerated likely due to increased photosynthesis (by 34.9%) and microbial biomass (by 24.2%) under elevated CO_2. Thus, the supply of soil available N can sustain the tree growth stimulated by elevated CO_2, and to date progressive N limitation has not happened. Nevertheless, both the annual increase of oak biomass, C and N stocks and C/N ratio and the seasonal variations of soil available N and microbial N concentrations, and net N transformation rates indicated that gradual N deficiency may be occurring and the CO_2 fertilization effect has weakened with increasing treatment duration.

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J. Sun · W. Dai · B. Peng · J. Liu · T. He · P. Jiang · S. Han · E. Bai (✉)
CAS Key Laboratory of Forest Ecology and Management, Institute of Applied Ecology, Chinese Academy of Sciences, No. 72 Wenhua Road, Shenyang 110016, China
e-mail: baie@iae.ac.cn

J. Sun · T. He
Key Laboratory of Beibu Gulf Environment Change and Resources Use (Guangxi Teachers Education University), Ministry of Education, No. 175 Mingxiu East Road, Nanning 530001, China

J. Sun · B. Peng · J. Liu · T. He
College of Resources and Environment, University of Chinese Academy of Sciences, Beijing, China

E. Bai
School of Geographical Sciences, Northeast Normal University, No. 5268 Renmin Road, Changchun 130024, China

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Introduction

Carbon dioxide (CO_2) concentration in the atmosphere has been rising since the industrial revolution and has reached about 400 ppm in 2013, 40% above preindustrial levels (IPCC 2013). Higher CO_2 typically increases plant photosynthesis, biomass and ecosystem net primary productivity (Ainsworth and Long 2005; Finzi et al. 2007), and may increase ecosystem carbon (C) storage (Luo et al. 2006), thereby slowing the rate of further increases in atmospheric CO_2 concentration. However, any CO_2 fertilization effect could be tempered by increased nitrogen (N) limitation with time (Luo et al. 2004; Reich et al. 2006). Increased ecosystem C storage may lead to sequestration of available N in longer-lived plant and soil pools and reduce available N in the soil, causing progressive N limitation (Luo et al. 2004). When and if progressive N limitation occurs is still under debate (Luo et al. 2004; Rütting et al. 2010; Leuzinger et al. 2011; Feng et al. 2015; Rütting and Andresen 2015; Liang et al. 2016). On one hand, increasing N deposition has enhanced N supply to terrestrial ecosystems, attenuating N limitation (Xia and Wan 2008). On the other hand, elevated CO_2 may stimulate soil organic matter (SOM) decomposition (Schneider et al. 2004; Finzi et al. 2007; Müller et al. 2009), accelerate soil N turnover (Williams et al. 2000; Finzi et al. 2007; McKinley et al. 2009), increase biological N fixation (Hu et al. 2006; Liang et al. 2016), or allow plants to mine deeper soil N by increasing fine root production (McKinley et al. 2009; Iversen et al. 2011). These changes under elevated CO_2 may attenuate N limitation to plants by increasing soil N availability. In this context, it is important to study the responses of soil N cycling to elevated CO_2 in order to better understand when progressive N limitation happens and better predict future ecosystem trajectories in the twenty-first century.

To date, there have been a variety of observed responses of the production (e.g. mineralization and nitrification) and consumption (e.g. immobilization) processes of soil available N (inorganic N) to elevated CO_2. This begs the question of how predictable those N cycle responses may be. For example, elevated CO_2 increased N mineralization and ammonium immobilization in a temperate heathland dominated by *Calluna vulgaris* and *Deschampsia flexuosa*, but also decreased gross nitrification (Björsne et al. 2014). In a Florida scrub oak ecosystem dominated by an N_2 fixing plant, *Galactia elliottii*, Hungate et al. (1999) also found NH_4^+ immobilization was stimulated and gross nitrification was reduced by elevated CO_2, but they did find reduced gross N mineralization. In contrast, in two different annual grasslands, gross N mineralization increased under elevated CO_2, while NH_4^+ immobilization did not change, leading to increased nitrification due to higher NH_4^+ availability (Hungate et al. 1997a). These studies indicated that the effect of elevated CO_2 on soil internal N cycling depends on ecosystem nutrient status (de Graaff et al. 2006; Rütting and Andresen 2015). It is likely that a better understanding of the responses of microbial biomass C and N, and isotopic characteristics may help elucidate soil N cycle responses to elevated CO_2, because microbes are the main driver for soil C and N cycles, and their biomass C and N and isotopic characteristics may indicate these changes (Booth et al. 2005; Dijkstra et al. 2008a; Björsne et al. 2014; Rütting and Andresen 2015). Moreover, the re-mineralization of microbial N as an important process for replenishing available N for plants and microbes should be considered, especially in systems with high microbial N immobilization (Zak et al. 1993; Hungate et al. 1999; Thornley and Cannell 2000; Rütting et al. 2010). However, information on how elevated CO_2 influences microbial N turnover is scarce (Zak et al. 1993; Rütting et al. 2010).

The conditions of N limitation of plant growth usually vary with time due to changing plant N demand throughout the growing season in temperate forest ecosystems (Chapin III et al. 2002). During the first half of the growing season, plants need more N to support rapid growth (Jaeger III et al. 1999), and elevated CO_2 may enhance plant and microbial N demands and intensify the competition between plants and microbes for available N (Williams et al. 2000; Barnard et al. 2004, 2006). In the late growing season, N may not be limiting due to reduced plant growth and N demand (Jaeger III et al. 1999; Williams et al. 2000; Zhou et al. 2010). Therefore, it is important to assess the temporal variations of plant and soil N cycles to

better understand when progressive N limitation occurs under elevated CO_2.

In this study, we investigated the effects of 10 years of elevated CO_2 on temporal dynamics of coupled C and N cycling processes and N availability in a *Quercus mongolica* dominated system in open top chambers. Our objective was to understand if progressive N limitation has diminished the CO_2 fertilization effect in this system. Because previous studies at this site suggested that belowground C inputs increased under elevated CO_2 (Li et al. 2010; Zheng et al. 2010; Zhou et al. 2010), we hypothesized that (1) elevated CO_2 would accelerate soil N cycling due to increased microbial biomass and activity caused by increased C inputs; and (2) accelerated soil N cycling may sustain plant N demand and progressive N limitation to date may not have occurred under 10 years of elevated CO_2 treatment.

Materials and methods

Site description

The experimental open top chamber (OTC) facility was established in 2004 in the Changbai Forest Ecosystem Research Station (42°24′N, 128°06′E) in Jilin Province, northeastern China. The study area is characterized by typical temperate climate, with a cold, long winter, and a warm, rainy summer. Mean annual precipitation is 745 mm, with rains mainly between June and September. Mean annual temperature is 3.6 °C, and the warmest month, July, averages 17.5 °C. *Quercus mongolica* (oak) is a widely distributed dominant tree species from approximately 700 to 1100 m asl (Li et al. 2010). The soil is dark brown soil developed from volcanic ash (Albic Luvisol), with well-drained, loamy sand texture. Basic soil characteristics were measured as part of a series of studies at this site in April 2014 (Table 1).

Table 1 Soil (0–10 cm) properties under ambient and elevated CO_2

Soil properties	Ambient CO_2	Elevated CO_2
SOC (g kg^{-1})	28.9 ± 1.4	29.1 ± 2.7
TN (g kg^{-1})	2.2 ± 0.1	2.1 ± 0.2
Soil C/N	13.4 ± 0.1	13.9 ± 0.1*
pH (H_2O)	6.7 ± 0.1	6.7 ± 0.1
Clay content (%)	10.4 ± 0.7	9.5 ± 0.4
Silt content (%)	13.6 ± 0.2	14.4 ± 0.3
Sand content (%)	76.0 ± 0.9	76.1 ± 0.6
Bulk density (g cm^{-3})	0.8 ± 0.2	1.0 ± 0.2

Data are presented as mean ± standard error (n = 3)

SOC soil organic carbon, *TN* total nitrogen

*Significant difference at $p < 0.05$

OTC description

The facility consists of six hexagonal OTC experimental plots, 4.4 m in diameter by 4 m high, and the area of each OTC is 12.6 m^2. As the oak is continually growing, the height was increased to 6 m in 2010. Three plots were treated with pure CO_2 to elevate the CO_2 concentration by 180 μmol mol^{-1} above ambient, while the other three chambers were maintained at ambient CO_2 concentration. Fans equipped in OTCs were used to increase air circulation. Infrared gas analyzers (A-SENSE-D, SenseAir, Delsbo, Sweden) placed in OTCs were used to monitor the CO_2 concentration. A computerized control system recorded 10-s averages of CO_2 concentration every 3 min, and periodically adjusted the flow of pure CO_2 into the OTCs to maintain the elevated CO_2 concentration.

Two years old oak seedlings were transplanted into these OTCs in the autumn of 2004. Twenty-two oak seedlings were planted in each OTC, and the seedlings were from the same nursery and had the same genotype (Li et al. 2010). CO_2 fumigation started in 2005 and had been carried out for 10 years until 2014 (turned off). Plants were exposed to elevated CO_2 during daytime from May to October in the growing season every year. Mean plant height and diameter at breast height significantly increased respectively under elevated CO_2 (4.53 ± 0.07 m, 2.45 ± 0.04 cm) compared with that under ambient CO_2 (4.15 ± 0.09 m, 2.20 ± 0.06 cm).

Oak biomass and C and N stocks measurement

The dry biomass of oak leaf, branch, stem and root were calculated according to the regression equation from Huo et al. (2011). The amount of leaf C or N of each OTC was calculated by multiplying the total leaf dry biomass of 22 oaks by the mean leaf C or N

concentration which was measured during the growing season (May–September) in 2014. Similarly, the amount of C or N of branch, stem and root was calculated by multiplying total dry biomass by the corresponding mean C or N concentration which was based on the concentration ratios of leaf to branch, stem or root in Lu et al. (2015). C or N stock of different oak components in each OTC was calculated by dividing the amount of C or N of corresponding components by the area of OTC respectively. Total C or N stock was the sum of leaf, branch, stem and root C or N stock respectively.

Leaf sampling and plant photosynthesis measurement

Plant photosynthetic rate was measured from June to September in 2014, three times in each month using Li-6400 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA) by the artificial light source (LED-6400-02B) at approximate light saturation point (1300 μmol m^{-2} s^{-1}). The fixed photosynthetically active radiation (PAR) was set to eliminate the effect of different PAR on photosynthesis. Undamaged growing leaves (six in each OTC) were selected and measured from 9:00 to 12:00 on sunny days. Measurements were consecutively repeated ten times for each leaf and the time interval was 10 s. Because the growing leaves are more sensitive to the change of soil available N than old leaves (Hikosaka et al. 1994), we chose to sample the growing leaves to explore the seasonal responses of plant to elevated CO_2. After the photosynthesis measurement, three growing leaves were sampled, dried, mixed and ground for C and N concentrations and isotopic compositions analysis.

Soil $NH_4^+$and NO_3^- concentrations and isotopic compositions

Soil samples were taken six times each month from June to October in 2014. Two soil subsamples (0–10 cm) were randomly taken in each OTC, and fully mixed and sieved (< 2 mm) for analysis of soil properties. Soil (20 g) was extracted with 100 ml 2 M KCl shaking for 60 min at 200 rpm at 25 °C. The extracts were filtered with ash-less filter papers (Qualitative Filter Paper, BH92410262) and 10 ml of the extracts were used to determine the concentrations of NH_4^+ and NO_3^- with a Continuous Flow Analyzer (Bran-Luebbe Inc., Germany). The ^{15}N natural abundance of NH_4^+ and NO_3^- were measured using the modified "ammonium diffusion" method according to Sebilo et al. (2004) with the details described by Sun et al. (2016), and were analyzed by an Elemental Analyzer (Thermo-Element Flash EA 1112, USA) coupled with an Isotope Ratio Mass Spectrometer (Thermo Fisher MAT 253, USA).

Microbial biomass C and N and isotopic compositions

The rest sieved soil samples were used to analyze microbial C, N, DOC and DON concentrations and isotopic compositions. Chloroform fumigation method was used for microbial C and N analysis (Brookes et al. 1985). The method to measure microbial C and N isotopic compositions was described in Dijkstra et al. (2006). Briefly, soil (20 g) was extracted with 100 ml 0.05 M K_2SO_4 and shaken for 60 min at 200 rpm at 25 °C, while the other 20 g soil was fumigated with chloroform followed by K_2SO_4 extraction. The extracted solution was dried at 60 °C and ground to fine powder for analysis of C and N concentrations and isotopic compositions.

The C and N concentrations and isotopic compositions of the soil, leaf, and K_2SO_4 extracts were analyzed at the Stable Isotope Faculty of University of California, Davis. About 5 mg leaf, 60 mg soil and 70 mg K_2SO_4 extracts were separately loaded into capsules and measured using an Elementar Vario EL Cube (Elementar Analysis system GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK), with an overall precision better than 0.2‰. Carbon or nitrogen isotope ratios are presented in δ notation:

$$\delta = [(R_{SAMPLE} - R_{STD})/R_{STD}] \times 10^3, \quad (1)$$

where R_{SAMPLE} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ of a sample and R_{STD} is the $^{13}C/^{12}C$ of Pee Dee Belemnite or $^{15}N/^{14}N$ of atmospheric N_2. Microbial C and N were calculated as:

$$\begin{aligned} M_{(C)} &= (C_f - C_e) \div 0.45 \text{ and } M_{(N)} \\ &= (N_f - N_e) \div 0.54, \end{aligned} \quad (2)$$

where $M_{(C)}$ and $M_{(N)}$ are the microbial C and N respectively; C and N are the dissolved organic C and

N in K_2SO_4 extracts respectively; subscript e and f represent before and after chloroform fumigation respectively. The extraction efficiency factors were 0.45 for microbial C (Vance et al. 1987) and 0.54 for microbial N (Brookes et al. 1985). Microbial N isotopic composition was calculated according to Dijkstra et al. (2008a):

$$\delta^{15}N_{Mic} = (\delta^{15}N_f \times N_f - \delta^{15}N_e \times N_e)/(N_f - N_e), \quad (3)$$

where $\delta^{15}N_{Mic}$ is the isotopic composition of microbial N; $\delta^{15}N_e$ and $\delta^{15}N_f$ are the isotopic compositions of N in the K_2SO_4 extracts before and after chloroform fumigation respectively. Microbial $\delta^{13}C$ was calculated based on the same principle.

Net N transformation rates

Net N mineralization, nitrification, and ammonification rates were measured in situ with the intact-core incubation method (Raison et al. 1987) from April to October in 2014. On the 17th of each month, three pairs of soil cores (0–10 cm) were randomly collected from each OTC using PVC soil corers (5 cm diameter by 12 cm deep). Three soil cores (one core from each pair) were immediately brought to the lab to measure NH_4^+, NO_3^-, and soil gravimetric water content. The other three PVC tubes were taken out and packed with absorbent gauze at the bottom of the tubes respectively, preventing the roots from penetration. Then, the PVC tubes were covered with lids to allow air circulation but prevent water exchange, and immediately placed back in the soil. After 30 days, the incubated samples were collected and lab analysis was repeated. Net N mineralization rate was determined from changes in the sum of NH_4^+ and NO_3^- concentrations; net nitrification rate was determined from changes in NO_3^- concentration; net ammonification rate was determined from changes in NH_4^+ concentration over 30 days.

Gross N transformation rates

Gross N transformation rates were determined by the FLUAZ ^{15}N tracing model (Mary et al. 1998). Five soil cores (0–10 cm) were collected in each OTC and mixed to one sample at the end of the growing season (17th October). The soil samples were sieved (< 2 mm), then air dried for the ^{15}N tracing incubation experiment.

Each soil sample was divided into 16 subsamples (32.5 g air dried soil per subsample) and each subsample was put into a 200 ml plastic bottle. Half of the 16 bottles were used for $^{15}NH_4NO_3$ treatment and the other half were used for $NH_4^{15}NO_3$ treatment. Deionized water (4.5 ml) was added to each bottle, and then sealed with parafilm which prevented water evaporation but still allowed airflow. The bottles were pre-incubated for 24 h at 20 °C prior to ^{15}N tracer addition. After the pre-incubation, 2.5 ml $^{15}NH_4NO_3$ or $NH_4^{15}NO_3$ solution (99.2 at.% ^{15}N, 32.4 mg l^{-1}) was added to each bottle. In order to reduce the effect of N addition on N cycling processes, the added N was minimized to 0.89 μg N g^{-1} soil. The soil was adjusted to 60% field capacity and incubated for 16 days at 20 °C. Soils were retrieved at 0 h (instantly extracted after ^{15}N application), 1, 2, 4, 6, 8, 13 and 16 d after ^{15}N application to determine NH_4^+ and NO_3^- concentrations and atomic percent excess (at.% excess). The residual soil after the KCl extraction was subsequently washed with 100 ml deionized water for 3 times, oven-dried at 60 °C and ground for soil organic ^{15}N analysis. The ^{15}N at.% and at.% excess are defined as:

$$\text{atom}\,\% = {}^{15}N/({}^{14}N + {}^{15}N) \times 100, \quad (4)$$

$$\text{atom}\,\%\ \text{excess} = {}^{15}N/({}^{14}N + {}^{15}N) \times 100 - 0.3663\%, \quad (5)$$

where 0.3663% is the at.% of the standard (atmospheric N_2).

Soil moisture, temperature, texture and pH

Standard oven-drying method was carried out to measure soil gravimetric water content. The temperatures of air and soil at 10 cm depth were recorded in situ at each sampling time using a digital thermometer. Soil texture was determined by the pipette sedimentation method (Gee and Bauder 1986). Soil pH was measured in a 1:5 (w/v) soil to water (CO_2-free) ratio using a pH detector.

Statistical analyses

The paired-samples *t* test was used to compare the differences of mean gross N transformation rates between treatments. Traditional statistical tests were not appropriate for the results of the FLUAZ model due to the large number of iterations (Yoccoz 1991). The statistical significance of gross N transformation rates under both treatments was indicated by comparing the standard deviations (68%) and 95% confidence intervals to distinguish three cases: (a) if the 95% confidence intervals did not overlap, the parameters were deemed significantly different; (b) if the standard deviations did not overlap, but the 95% confidence intervals overlapped, the parameters were not considered significantly different, but presented a clear tendency of difference; (c) if the standard deviations overlapped, the parameters were not considered different (Müller et al. 2009; Sun et al. 2016). Linear correlation was used to analyze the correlations between microbial C and N and isotopic compositions and plant and soil variables. Independent-samples *t* test was used to compare the differences of plant, soil and microbial properties between control and elevated CO_2 treatment for each sampling time separately. Repeated measures ANOVA was used to examine the effects of elevated CO_2 on plant photosynthetic rate, leaf C and N, soil NH_4^+, NO_3^-, DON, DOC, microbial C and N concentrations and isotopic compositions, and net N transformation rates using CO_2 fumigation treatment as the between subject factor and sampling time as the within subject factor. Data were transformed to normalize variance across treatments before analysis when necessary. Repeated measures ANOVA was carried out with R 3.2.2 software, and other analyses were carried out with SPSS 17.0 software (SPSS, Chicago, IL, USA).

Results

Elevated CO_2 for 10 years did not change soil organic C, total N, pH or soil texture, but significantly increased soil C/N by 3.4% ($p < 0.05$, Table 1). Air and soil (10 cm depth) temperatures and soil moisture were almost identical under the two treatments (Fig. S1).

Oak biomass and C and N stocks

Elevated CO_2 significantly increased the mean biomass of oak leaf, branch, stem, root and total biomass by 22.1, 43.1, 28.5, 23.6 and 26.4% respectively compared with the increase under ambient CO_2 ($p < 0.05$, Table 2). Elevated CO_2 significantly increased the mean oak leaf, branch, stem, root and total C stocks by 21.8, 43.0, 28.3, 23.3 and 26.2% respectively, and increased N stocks by 13.6, 25.0, 17.5, 15.6 and 16.1% respectively throughout the growing season in 2014 ($p < 0.05$, Table 3). The mean annual increases in oak biomass and C and N stocks after elevated CO_2 treatment for 3 years (7.6, 10.2 and 7.1% respectively) were higher than those after elevated CO_2 treatment for 10 years (2.6, 2.6 and 1.6% respectively) ($p < 0.05$, Fig. 1a). Moreover, the foliar litter C/N increased by 7.2% after elevated CO_2 treatment for 3 years, which was lower than that after treatment for 10 years (16.9%) ($p < 0.05$, Fig. 1b).

Plant photosynthetic rates, leaf C and N concentrations and isotopic compositions

Plant photosynthetic rates generally decreased with time during the growing season under both treatments (Fig. 2). Elevated CO_2 (7.2 ± 1.1 μmol CO_2 m^{-2} s^{-1}) increased mean plant photosynthetic rates by 34.9% compared with ambient CO_2 (5.3 ± 0.9 μmol CO_2 m^{-2} s^{-1}) over the growing season ($p = 0.05$).

Elevated CO_2 did not significantly change leaf C concentration (Fig. 3a). Leaf $\delta^{13}C$ was significantly lower under elevated CO_2 ($p = 0.01$), and decreased over the growing season from May to October under both treatments (Fig. 3c). Leaf N concentration gradually decreased from May to October under both treatments, and the mean leaf N concentration tended to decrease under elevated CO_2 throughout the growing season ($p = 0.06$, Fig. 3b). Especially from May to August, a significant decrease in leaf N concentration was found under elevated CO_2 ($p < 0.05$). Leaf $\delta^{15}N$ gradually increased from May to July and declined thereafter under both treatments, and elevated CO_2 significantly increased leaf $\delta^{15}N$ during the growing season ($p = 0.02$, Fig. 3d).

Table 2 Biomass of *Quercus mongolica* (mean values of the 22 oak trees in each OTC) under ambient and elevated CO_2 treatments

Biomass (g)	Allometric biomass equation	Ambient CO_2	Elevated CO_2
Leaf	$W = 0.02 \times D^{1.899}$	98.8 ± 4.0	120.6 ± 3.9*
Branch	$W = 0.004 \times D^{3.053}$	61.2 ± 3.9	87.6 ± 4.9*
Stem	$W = 0.147 \times D^{2.3}$	1064.3 ± 52.0	1368.1 ± 51.6*
Root	$W = 0.199 \times D^{1.998}$	1079.0 ± 46.1	1334.0 ± 44.1*
Total		2303.3 ± 105.9	2910.3 ± 102.7*

Data are presented as mean ± standard error (n = 3)

W dry biomass, *D* diameter at breast height

*Significant difference at $p < 0.05$

Table 3 The stocks of C and N of different components of *Quercus mongolica* under ambient and elevated CO_2 treatments

C and N stocks (g m^{-2})	Ambient CO_2	Elevated CO_2
Leaf C stock	78.8 ± 2.3	96.0 ± 3.1*
Branch C stock	46.3 ± 2.1	66.2 ± 3.8*
Stem C stock	824.5 ± 28.3	1057.5 ± 40.9*
Root C stock	736.9 ± 22.1	908.9 ± 30.8*
Total C stock	1686.5 ± 54.6	2128.6 ± 77.6*
Leaf N stock	4.4 ± 0.2	5.0± 0.2*
Branch N stock	0.8 ± 0.0	1.0 ± 0.1*
Stem N stock	4.0 ± 0.2	4.7 ± 0.2*
Root N stock	6.4 ± 0.2	7.4 ± 0.3*
Total N stock	15.5 ± 0.6	18.0 ± 0.8*

Data are presented as mean ± standard error (n = 3)

*Significant difference at $p < 0.05$

Soil NH_4^+, NO_3^-, DON and DOC concentrations and isotopic compositions

Mean soil NH_4^+-N concentration (5.6 ± 0.2 mg N kg^{-1} dry soil) was 4.7 times higher than mean soil NO_3^--N concentration (1.2 ± 0.1 mg N kg^{-1} dry soil) under both treatments throughout the growing season (Fig. 4a, c). Elevated CO_2 did not change mean soil NO_3^- or NH_4^+ concentration throughout the growing season. However, elevated CO_2 increased soil NO_3^- on June 26 and October 5, 10 and 15 ($p < 0.05$), and increased soil NH_4^+ in the early and late growing season ($p < 0.05$). Soil NH_4^+ concentration dropped from early to mid growing season, and remained constant thereafter. Neither $\delta^{15}N$ of soil NO_3^- nor $\delta^{15}N$ of NH_4^+ was different between treatments, and $\delta^{15}N$ of soil NO_3^- was significantly lower than that of NH_4^+ ($p < 0.05$, Fig. S2). The $\delta^{15}N$ of NH_4^+ was positively correlated with leaf $\delta^{15}N$ ($p = 0.04$), but no relationship was found between $\delta^{15}N$ of soil NO_3^- and leaf $\delta^{15}N$ (Fig. S3a and c).

On average, elevated CO_2 significantly increased soil DON (19.9 ± 0.7 mg N kg^{-1} dry soil) by 19.8% compared with ambient CO_2 (16.6 ± 0.5 mg N kg^{-1} dry soil) during the growing season ($p = 0.02$, Fig. 4b). Mean increase of soil DOC under elevated CO_2 (64.4 ± 2.0 mg C kg^{-1} dry soil) was 17.1% compared with that under ambient CO_2 (55.0 ± 1.4 mg C kg^{-1} dry soil) ($p = 0.01$, Fig. 4d), and soil DOC generally decreased with time during the growing season under both treatments. When data under both treatments were combined, a significant positive correlation between photosynthetic rate and soil DOC was found ($p < 0.01$, Fig. S4).

Soil microbial biomass C and N and isotopic compositions

During the growing season, soil microbial N initially decreased and then increased from August 28 to October 15 under both treatments (Fig. 5a). Elevated CO_2 did not change mean microbial N throughout the growing season. However, a significant increase was found on August 20 and September 5, 15 and 25 under elevated CO_2 ($p < 0.05$). On average, elevated CO_2 (210 ± 8 mg C kg^{-1} dry soil) significantly increased microbial C by 24.2% compared with ambient CO_2 (169 ± 8 mg C kg^{-1} dry soil) over the growing season ($p < 0.01$, Fig. 5b). When data under both treatments were combined, microbial C was positively correlated with soil NH_4^+, NO_3^-, DOC and DON, and microbial

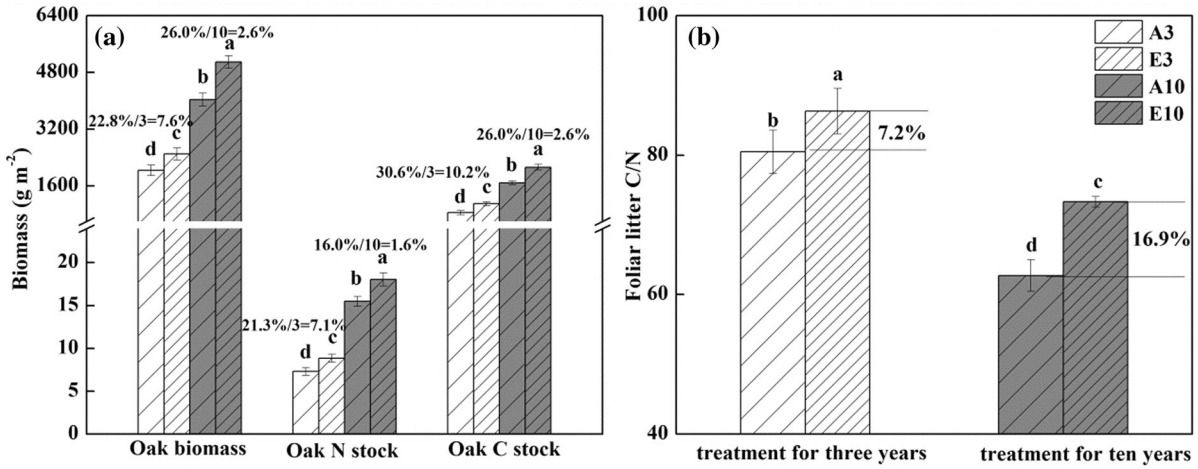

Fig. 1 The biomass and C and N stocks (**a**) and foliar litter C/N (**b**) of *Quercus mongolica* after treatment for 3 years (A3 and E3 for ambient and elevated CO_2 respectively) and ten years (A10 and E10 for ambient and elevated CO_2 respectively). Different letters indicate statistical significance at $p < 0.05$ among the four values according to one way ANOVA (Tukey's HSD test). Annual mean increases in biomass and C and N stocks were calculated in (**a**) and the absolute increase in litter C/N was presented in (**b**). Data of foliar litter C/N and diameter at breast height of *Quercus mongolica* (for biomass calculation) after 3 years of CO_2 treatment were from Zheng et al. (2010) and Zhou et al. (2010) respectively

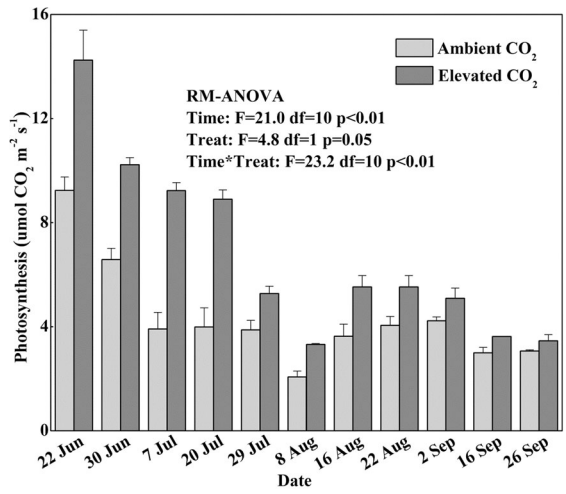

Fig. 2 Plant photosynthetic rates of *Quercus mongolica* under ambient and elevated CO_2 during the growing season of 2014. Values are mean ± standard error (n = 3). "Treat" represents ambient and elevated CO_2 treatments, "Time" represents sampling time in repeated measures ANOVA (RM-ANOVA), *F* represents F ratios and *df* represents degree of freedom (the same below)

N was positively correlated with soil NH_4^+ and DOC (Table S1).

Mean microbial $\delta^{15}N$ was significantly higher under elevated CO_2 (8.6 ± 0.3‰) than under ambient CO_2 (7.0 ± 0.4‰) during the growing season ($p = 0.02$, Fig. 5c). Microbial $\delta^{15}N$ was significantly higher than $\delta^{15}N$ of soil NH_4^+ and NO_3^- ($p < 0.05$, Fig. S2), and was negatively correlated with $\delta^{15}N$ of soil NH_4^+ ($p = 0.03$) (Fig. S3b). But no relationship between microbial $\delta^{15}N$ and $\delta^{15}N$ of soil NO_3^- was found (Fig. S3d). Microbial $\delta^{13}C$ was not significantly changed under elevated CO_2 throughout the growing season (Fig. 5d).

N transformation rates

Elevated CO_2 did not affect mean net N transformation rates throughout the growing season, while significant interaction effect between treatment and time was found (Fig. 6). Net N mineralization and ammonification rates significantly increased in April and May, and significantly decreased in June, July and August under elevated CO_2 ($p < 0.05$, Fig. 6a, b). Elevated CO_2 significantly decreased net nitrification in April and May, but had no effect from June to September (Fig. 6c).

The lab assay results showed that potential gross N mineralization and nitrification increased by 41.4 and 237.5% respectively under elevated CO_2 (Table 4). Elevated CO_2 significantly increased NH_4^+ and NO_3^- immobilization by 82.6 and 68.8% respectively ($p < 0.05$). NH_4^+ immobilization was much higher than NO_3^- immobilization under both treatments. The

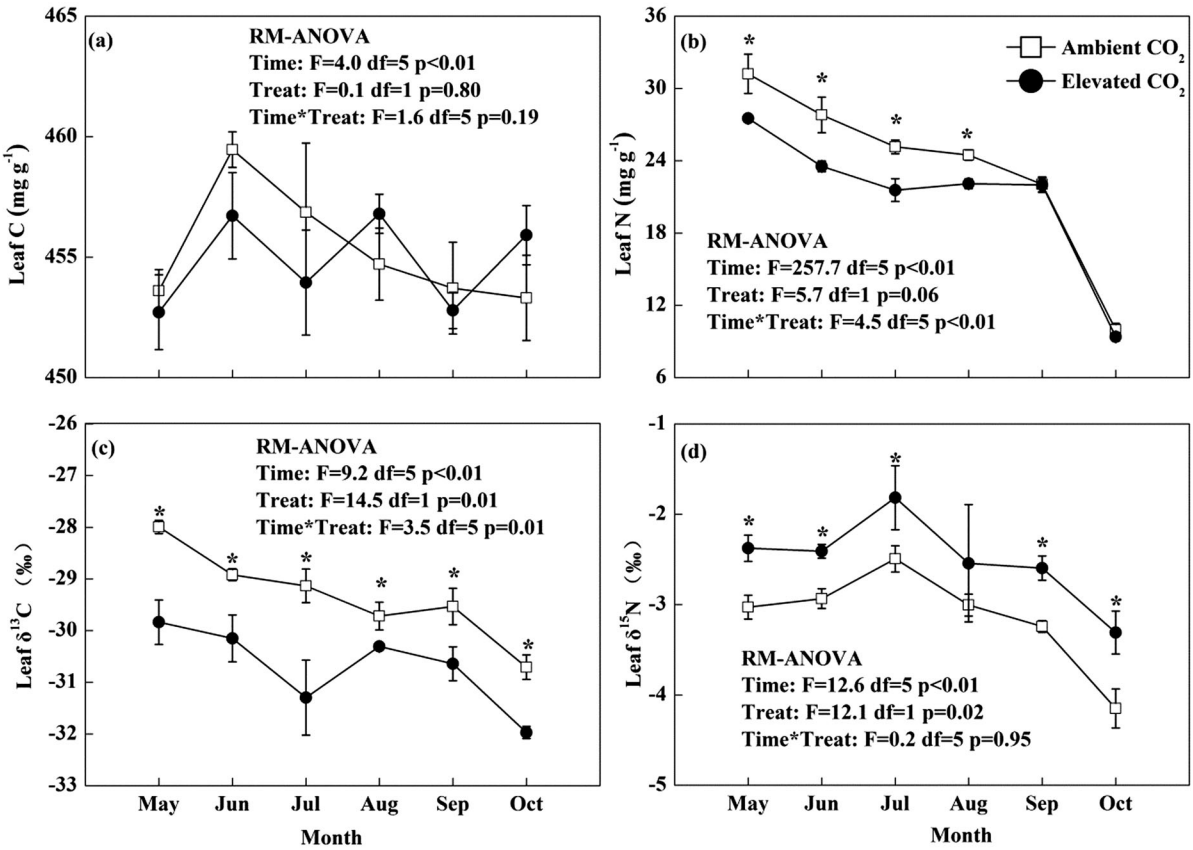

Fig. 3 Leaf C (**a**) and N (**b**) concentrations and $\delta^{13}C$ (**c**) and $\delta^{15}N$ (**d**) of *Quercus mongolica* under ambient and elevated CO_2 during the growing season of 2014. Values are mean ± standard error (n = 3). *$p < 0.05$ based on *t* test for each sampling time separately (the same below)

$I_{NH_4^+}/I_{NO_3^-}$ (the ratio of gross NH_4^+ immobilization to gross NO_3^- immobilization) was higher under elevated CO_2 (35.1) compared with that under ambient CO_2 (32.4) ($p < 0.05$). The re-mineralization rate was 149% higher under elevated CO_2 compared with that under ambient CO_2 ($p = 0.03$).

Discussion

Effects of elevated CO_2 on soil N cycling

Consistent with our hypothesis that elevated CO_2 would accelerate soil N cycling, and we found elevated CO_2 increased gross N mineralization by 41.1% and nitrification by 237.5%. We believe the increased gross N mineralization was mainly attributable to increased microbial biomass C under elevated CO_2 (Fig. 5b) because mineralization process is an aggregated process driven by most microbes (Schimel and Bennett 2004). The significant relationship between microbial C and products of N mineralization (DON and NH_4^+) (Table S1) also indicated that the increased microbial biomass stimulated N mineralization under elevated CO_2. Although previous studies demonstrated that increased soil moisture enhanced soil N mineralization under elevated CO_2 (Hungate et al. 1997a; Dijkstra et al. 2008b), this was not the case in our research because soil moisture was not different between the treatments (Fig. S1). Earlier studies in temperate forest ecosystems suggested that increased labile C inputs via rhizodeposition under elevated CO_2 enhanced microbial biomass and activity, which may cause a priming effect on SOM decomposition and N mineralization (Drake et al. 2011; Phillips et al. 2011; Zak et al. 2011). In our

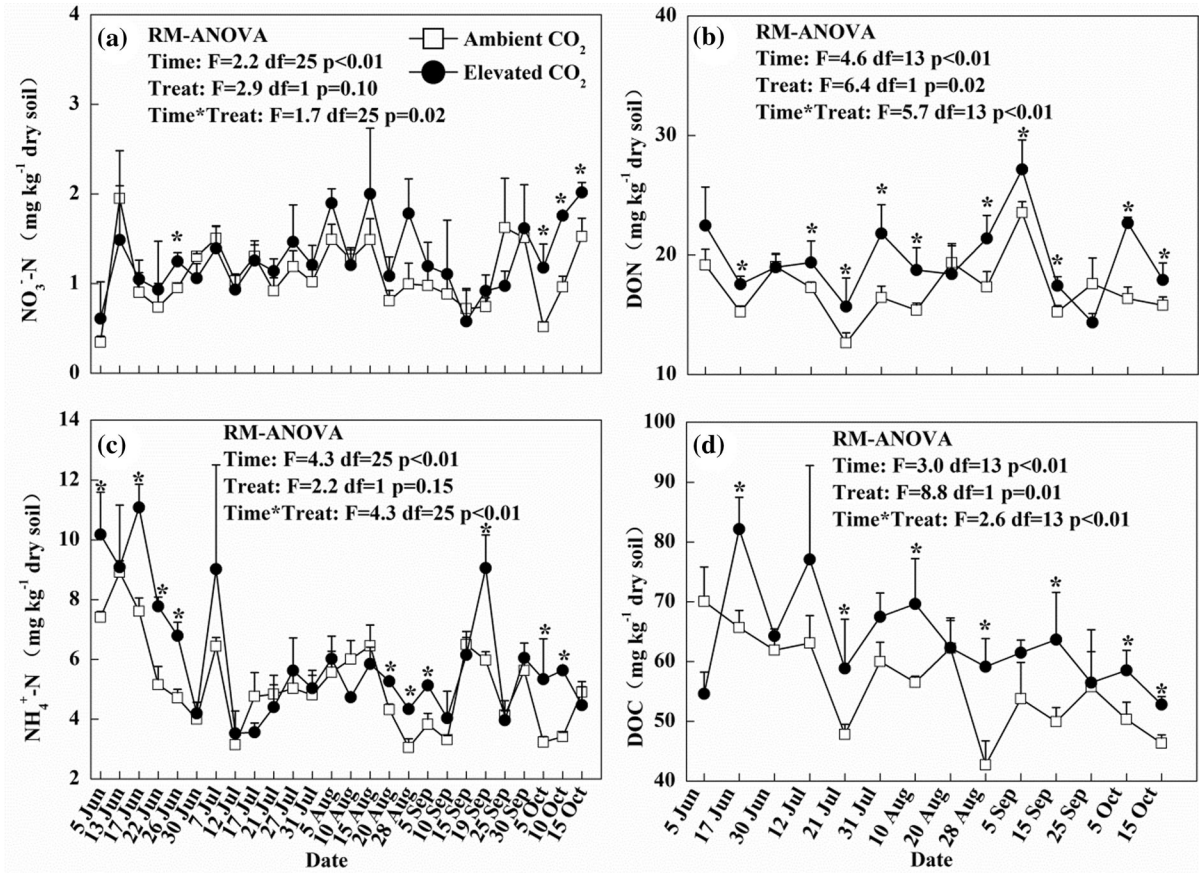

Fig. 4 Soil NO_3^- (**a**), DON (**b**), NH_4^+ (**c**) and DOC (**d**) under ambient and elevated CO_2 during the growing season of 2014. Values are mean ± standard error (n = 3)

study, the increased photosynthesis, root biomass and soil DOC, combined with the significantly positive correlation between photosynthetic rate and soil DOC implied potential higher labile C inputs into the soil under elevated CO_2 compared with under ambient CO_2. In addition, elevated CO_2 was found to increase fine root decomposition due to increased soil microbial biomass and enzyme activities in our study site (Li et al. 2010). Therefore, there also might be a priming effect on SOM decomposition and N mineralization induced by increased soil labile C inputs in our study (Li et al. 2010; Drake et al. 2011; Phillips et al. 2011). Although our result was contrary to other previous studies (Berntson and Bazzaz 1997; Hungate et al. 1999; Richter et al. 2003), which found decreased gross N mineralization under elevated CO_2, the underlying mechanism was consistent between our results and those studies. They found elevated CO_2 increased C partitioning to N_2-fixing microbes and therefore decreased C partitioning to other soil microbes, resulting in unchanged microbial biomass (Hungate et al. 1999; Richter et al. 2003). Elevated CO_2 decreased gross N mineralization in their studies (Berntson and Bazzaz 1997; Hungate et al. 1999; Richter et al. 2003), because of decreased litter quality (higher plant C/N) and the unchanged microbial activities. Therefore, the responses of belowground C inputs, microbial biomass and activity are central to the response of gross N mineralization under elevated CO_2.

N mineralization can induce ^{15}N enrichment in microbes and ^{15}N depletion in mineralization products (Collins et al. 2008), and microbial ^{15}N abundance is generally higher when N mineralization is higher (Dijkstra et al. 2006, 2008a). In the present study, higher microbial $\delta^{15}N$ compared with $\delta^{15}N$ of mineralization products (e.g. NH_4^+ and NO_3^-) indicated that N mineralization induced ^{15}N enrichment in

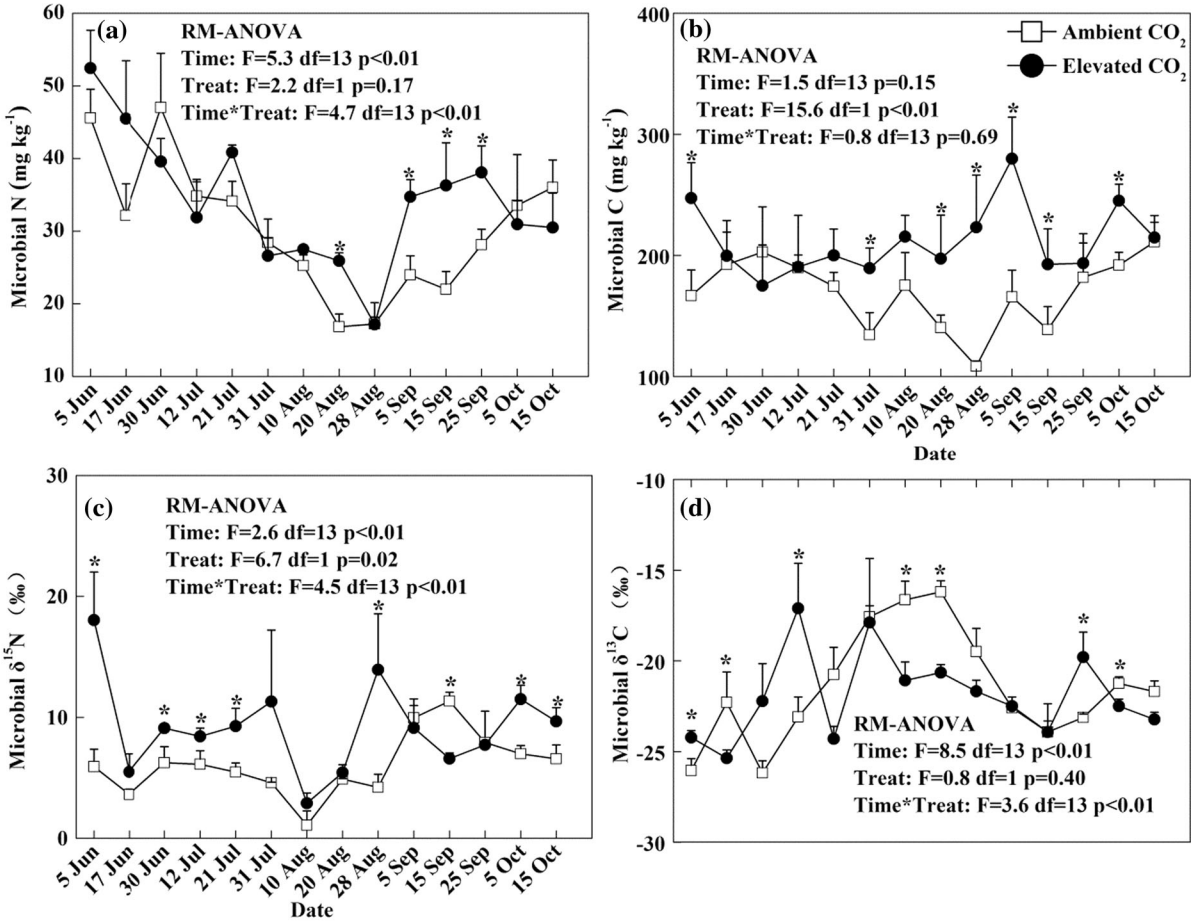

Fig. 5 Soil microbial N (**a**), C (**b**), $\delta^{15}N$ (**c**) and $\delta^{13}C$ (**d**) under ambient and elevated CO_2 during the growing season of 2014. Values are mean ± standard error (n = 3)

microbes. Thus, the higher microbial $\delta^{15}N$ under elevated CO_2 was consistent with the higher gross N mineralization under elevated CO_2 compared with under ambient CO_2. An indicator for the occurrence of progressive N limitation is the decrease in gross N mineralization (Luo et al. 2004). Our finding of increased potential soil gross N mineralization suggested that to date progressive N limitation has not occurred in our studied system.

We found elevated CO_2 increased gross nitrification, which was likely due to increased gross N mineralization and the higher NH_4^+ availability (Fig. 4c), because the change in nitrification is strongly dependent on N mineralization (Booth et al. 2005). Nitrification is a critical process for long-term ecosystem N availability (Schimel and Bennett 2004) and its increase is beneficial to those plant species which preferentially use NO_3^- as their N source. Our previous study found oak in the study area prefers NO_3^- to NH_4^+ (Liu et al. 2017). Therefore, the increased nitrification under elevated CO_2 could benefit its growth. Our result is consistent with Hungate et al. (1997a) which also indicated increased C supply and N mineralization under elevated CO_2 were beneficial to nitrifiers and nitrification process in two different annual grassland ecosystems. However, some studies found decreased gross nitrification under elevated CO_2 (Müller et al. 2009; Rütting et al. 2010), because of enhanced NH_4^+ immobilization and decreased NH_4^+ availability for nitrification (Rütting et al. 2010; Björsne et al. 2014). In our study, although NH_4^+ immobilization increased under elevated CO_2, NH_4^+ availability did not decrease, even increased

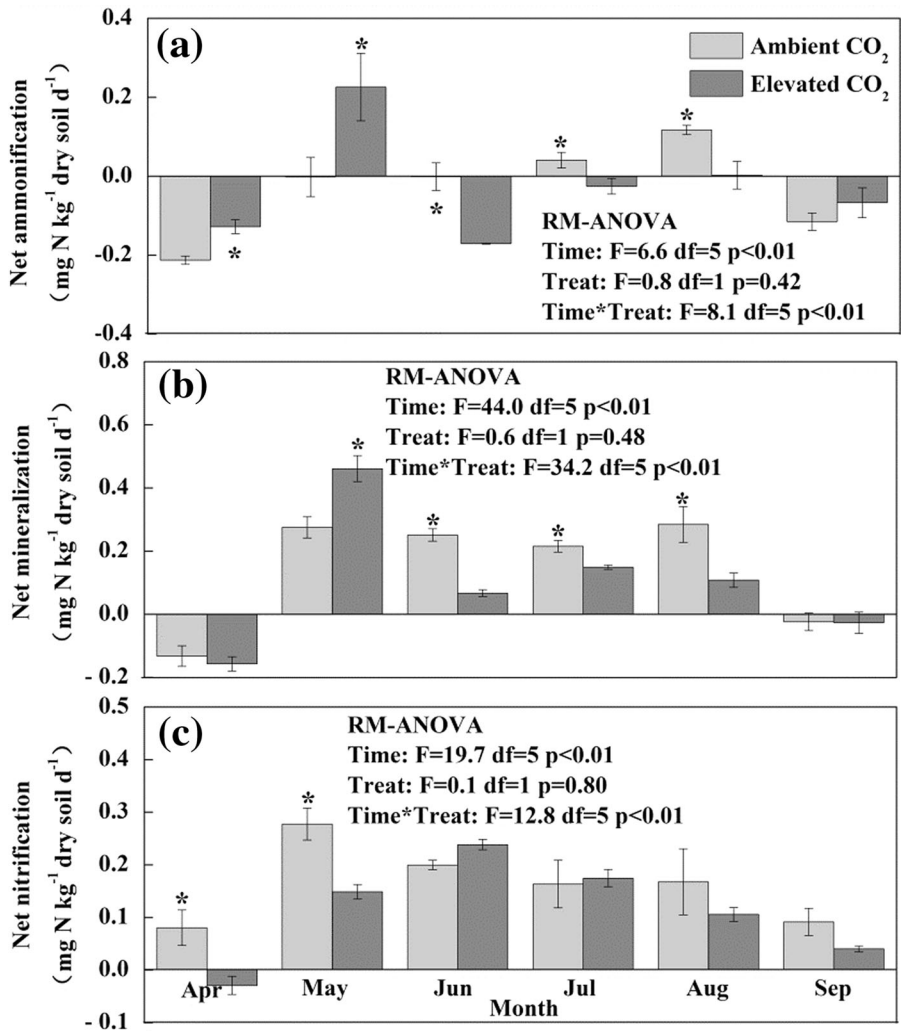

Fig. 6 Soil net N transformation rates under ambient and elevated CO_2 during the growing season of 2014. Values are mean ± standard error (n = 3)

Table 4 Mean gross N transformation rates modeled by FLUAZ for the seven incubation intervals

	Gross N transformation rates (mg N kg^{-1} dry soil day^{-1})		*p* value
	Ambient CO_2	Elevated CO_2	
N mineralization	3.12 ± 1.68	4.41 ± 1.28[b]	0.29
Nitrification	0.08 ± 0.03	0.27 ± 0.10[b]	0.17
NH_4^+ immobilization	5.18 ± 1.10	9.46 ± 1.45[a]	0.02
NO_3^- immobilization	0.16 ± 0.05	0.27 ± 0.06[a]	0.04
Re-mineralization	2.46 ± 0.84	6.12 ± 1.37[a]	0.03

Data are reported as mean ± standard error (n = 7)

[a]No overlap of 95% confidence intervals

[b]No overlap of standard deviations

sometimes during the growing season due to the enhanced gross N mineralization.

The observed increase in the gross NH_4^+ and NO_3^- immobilization was consistent with increased microbial biomass and gross N mineralization under elevated CO_2, which illustrated the higher microbial N demand under elevated CO_2. The higher microbial biomass C and N corresponded to the higher soil respiration under elevated CO_2 as reported in our previous study (Fig. 7, Sun et al. 2017). Many previous studies found higher gross N immobilization under elevated CO_2 due to increased microbial biomass (Hungate et al. 1997b, 1999; de Graaff et al. 2006; van Groenigen et al. 2006; Müller et al. 2009; Rütting et al. 2010; Björsne et al. 2014). However, the enhanced competition between plants and microbes for available N may induce unchanged, or even decreased microbial N immobilization under elevated CO_2 (Hungate et al. 1997a; Hu et al. 2006) because plants are found to out-compete microbes for the available N (Schimel and Bennett 2004; Hu et al. 2006). We found microbial N did not change during the early growing season (from June to mid August) under elevated CO_2 when plants grew fast, while it increased during the late growing season, when plants N demand decreased. Therefore, the impact of elevated CO_2 on microbial immobilization is not constant across different systems, or even is not constant in the same system at different times, and we should carefully consider the specific circumstances, especially the response of microbial N under elevated CO_2.

We found elevated CO_2 increased re-mineralization of microbial N by 149%, which even exceeded the rate of N supply derived from gross N mineralization. It is not the size of the readily available N pool but rather the speed of replenishment of the N pool, which determines the availability of N for plants and microbes in N limited ecosystems (Thornley and Cannell 2000). Although the higher N immobilization occurred under elevated CO_2, the faster microbial N turnover decreased the N limitation for plants and microbes. Microbial N re-mineralization was found to be higher when environmental conditions promote

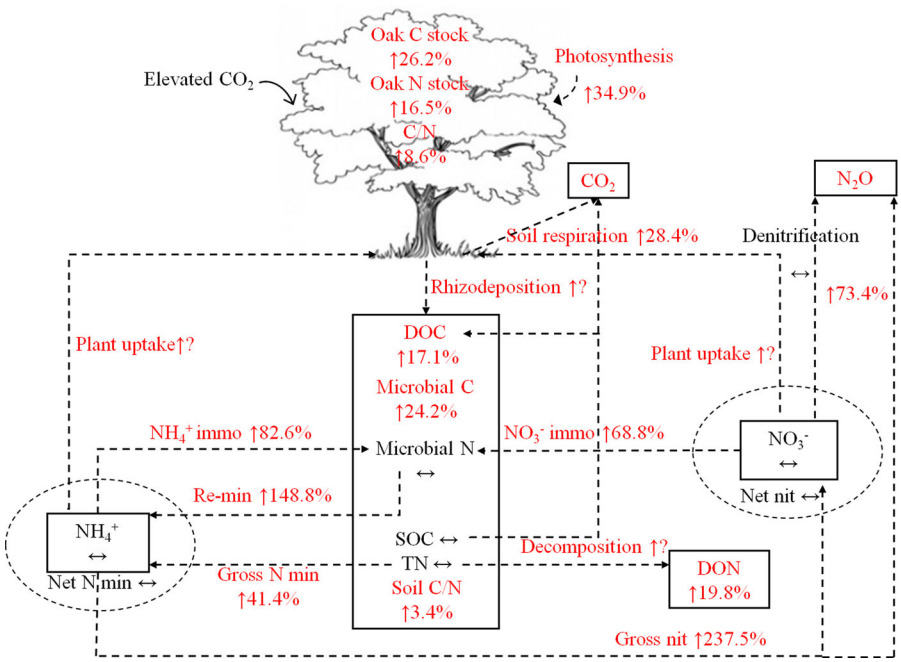

Fig. 7 Summary scheme showing the responses of ecosystem C and N pools and processes to consecutive 10 years of elevated CO_2 in our studied oak dominated system. Dashed line represents C or N cycling processes. *min* mineralization, *nit* nitrification, *immo* immobilization. ↑, ↓ and ↔ refer to increases, decreases and no change under elevated CO_2 respectively. Red color indicates significant difference between treatments, and black color indicates no significantly statistical discrepancy between treatments. "?" was marked because the processes were not evaluated. Data of soil respiration and N_2O flux were from Sun et al. (2017). (Color figure online)

high microbial growth rates (e.g. increased higher quality C availability) (Bengtson and Bengtsson 2005). We believe the higher soil DOC under elevated CO_2 suggested higher quality of soil C for microbes, which mainly contributed to the increased microbial N re-mineralization.

NH_4^+ was the dominating inorganic N form at our research site, which was 4.8 times higher than NO_3^- concentration on average under both treatments. The significant relationship between $\delta^{15}N$ of soil NH_4^+ and plant/microbial $\delta^{15}N$, and no relationship between $\delta^{15}N$ of soil NO_3^- and plant/microbial $\delta^{15}N$, further indicated the importance of NH_4^+ for ecosystem N demands. The increased plant N stock and $I_{NH_4^+}/I_{NO_3^-}$ under elevated CO_2 implied increased plant and microbial NH_4^+ demand. Thus, although elevated CO_2 increased gross N mineralization, the seasonal mean soil NH_4^+ did not change. The concentration of soil NH_4^+ was mainly controlled by the NH_4^+ production and consumption, and these processes are complex and changeable, which induced various responses of soil NH_4^+ to elevated CO_2 in previous studies, including no change (Arnone and Bohlen 1998; Niklaus et al. 1998; Johnson et al. 2001; Niklaus et al. 2001; Carrillo et al. 2012; Schleppi et al. 2012; Liang et al. 2016), decrease (Berntson and Bazzaz 1997; Barnard et al. 2004) or increase (Barnard et al. 2004; Carrillo et al. 2012; Liang et al. 2016). In our study, because both the production (gross N mineralization and re-mineralization) and the consumption (microbial immobilization and plant uptake) of soil NH_4^+ increased, the unchanged soil NH_4^+ concentration was reasonable. Similarly, the interaction of different NO_3^- production and consumption processes may cause the unchanged seasonal mean soil NO_3^- in our study. The seasonal variations of the effects of elevated CO_2 on the concentrations of soil NH_4^+ and NO_3^- also suggested that the effects were not constant and were determined by various N cycling processes.

Implications for our understanding of progressive N limitation

After 10 years of elevated CO_2 treatment, the biomass and C and N stocks of *Q. mongolica* were significantly higher than those under control treatment, which indicated that elevated CO_2 stimulated oak growth and enhanced plant C and N sequestration. It is hypothesized that after the initial increase of plant N sequestration, the stimulation of biomass would decline or disappear within a few years in strongly N limited systems due to decreased soil available N supply (Luo et al. 2004). However, we found soil inorganic N generally did not change, or even increased sometimes under elevated CO_2. Combined with increased plant photosynthesis, these results suggested that to date progressive N limitation has not happened in our studied system. This was because increased gross N mineralization and hastened microbial N turnover under elevated CO_2, providing additional available N for plants growth. However, this still did not prevent the increase in plant C/N under elevated CO_2, hinting the potential limitation of N for plant growth. The increase in gross N mineralization does not provide new N inputs into the system and would cause the decrease in organic N. Although the pool size of organic N is large, the mechanism of supplying the plant N demand still may not be sustainable over the long term.

Indeed, our results showed that the mean annual increase rate of oak biomass, C and N stocks was lower after 10 years of treatment compared with that after 3 years of treatment ($p < 0.05$). Moreover, the enhancement of litter C/N under elevated CO_2 increased after treatment for 10 years compared with that after treatment for 3 years. Collectively, these results indicated that soil N have constrained CO_2 fertilization effect with increasing duration of treatment. The lower leaf N concentration under elevated CO_2 compared with under ambient CO_2 also implied the tendency of N deficiency under elevated CO_2. Although some studies suggested that increased plant N use efficiency can decrease leaf N demand under elevated CO_2, leading to lower leaf N concentration (Long et al. 2004; Ainsworth and Long 2005). This may not be the case for our study because our study area is typically N limited, which induced a gradual decrease in leaf N concentration from May to August, suggesting insufficient soil N supply. In N limited ecosystems, the higher temperate forest productivity under elevated CO_2 was supported by increased plant N uptake rather than N use efficiency (Finzi et al. 2007). Indeed, elevated CO_2 increased soil NH_4^+ and NO_3^- during the early and late growing seasons, but had no effect during the mid growing season when plant N demand was highest, which suggested increased plant N uptake under elevated CO_2.

Additionally, the lower net N mineralization during the mid growing season indicated higher microbial N demand under elevated CO_2. Thus, the intensified competition between plants and microbes for available N under elevated CO_2 decreased plant N acquisition. This was in line with Feng et al. (2015) who found that lower plant N concentration under elevated CO_2 was due to deceased N acquisition, rather than decreased N demand. Together, these results suggested that although gross N mineralization and re-mineralization were accelerated under elevated CO_2, both increase in plant and microbial N demand during the period of fast plant growth caused insufficient soil N supply, which retarded the pace of enhancement of plant productivity.

Conclusions

In the *Quercus mongolica* dominated system, elevated CO_2 increased plant biomass, C and N stocks and C/N by 26.4, 26.2, 16.5 and 8.6% respectively, indicating increased ecosystem C and N sequestration, and plant N demand. Consistent with our hypothesis, elevated CO_2 increased photosynthesis and microbial biomass, which accelerated soil N cycling and supplied additional N for plant growth. Thus, progressive N limitation for plant growth has not happened in this oak dominated system after 10 years of elevated CO_2 treatment, but the annual and seasonal dynamics of ecosystem N status indicated that gradual N deficiency may be occurring.

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