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Ten years of elevated CO_2 affects soil greenhouse gas fluxes in an open top chamber experiment

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Abstract
Background The production and consumption of greenhouse gases (GHGs) in soils are largely regulated by biological processes. Increasing atmospheric CO_2 may alter these processes, thereby affecting GHG emissions and their feedbacks to climate.
Methods and aims Here, we used an open top chamber (OTC) experiment to examine the effects of elevated CO_2 for ten years on soil GHG fluxes in a *Quercus mongolica* dominated system in northeastern China.
Results Our results showed that elevated CO_2 increased soil CO_2 emissions, consistent with increased microbial biomass and the abundance of arbuscular mycorrhizal fungi and actinomycetes. Additionally, elevated CO_2 increased CH_4 uptake due to stimulated growth of methanotrophs. The seasonal mean soil N_2O flux was not changed by elevated CO_2, consistent with unchanged ammonia oxidizing bacteria, archaea and denitrifiers, which was probably due to large variations between the individual OTCs and with time. However, seasonal cumulative soil N_2O emissions increased by 64.7% under elevated CO_2. Our results also hinted that nitrification by ammonia oxidizing archaea was the major process of soil N_2O emissions.
Conclusions In our study elevated CO_2 increased soil GHG emissions and the cumulative global warming potential by 27.8%, causing an important positive feedback to climate change.

Keywords Global warming potential · Microbial community composition · Ammonia oxidizing archaea and methanotrophs · Methane uptake · Nitrous oxide emission · *Quercus mongolica*

Abbreviations

N	Nitrogen
C	Carbon
GHG	Greenhouse gas
CO_2	Carbon dioxide
N_2O	Nitrous oxide
CH_4	Methane
GWP	Global warming potential

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SOM	Soil organic matter
SOC	Soil organic carbon
TN	Total nitrogen
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
NH_4^+	Ammonium
NO_3^-	Nitrate
WFPS	Water filled pore space
AOA	Ammonium oxidizing archaea
AOB	Ammonium oxidizing bacteria
OTC	Open top chamber

Introduction

Atmospheric concentrations of the greenhouse gases (GHGs) carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) have reached 391 ppm, 1803 ppb, and 324 ppb in 2011, which exceeded the pre-industrial levels by about 40%, 150%, and 20%, respectively (IPCC 2013). CO_2 is by far the most abundant GHG in the atmosphere, while CH_4 and N_2O are powerful GHGs which are 25 and 298 times more potent than CO_2 over a 100 year lifespan (Forster et al. 2007).

Terrestrial ecosystems are large sources and sinks of GHGs. Increasing atmospheric CO_2 may alter the direction and strength of GHG fluxes in terrestrial ecosystems, which in turn could feedback to our climate. Elevated atmospheric CO_2 concentrations could increase plant photosynthesis (Ainsworth and Long 2005) and water use efficiency (Jackson et al. 1994), and indirectly alter soil properties (e.g. soil carbon (C) and nitrogen (N) availability, soil moisture and oxygen content), all of which may affect soil GHG fluxes. Most previous studies found that elevated CO_2 generally stimulated soil CO_2 emissions in different ecosystems, including grasslands (Luo et al. 1996; Zak et al. 2000; Reich et al. 2001), forests (Zak et al. 2000; Deng et al. 2010) and croplands (Smith et al. 2010). However, the effects of elevated CO_2 on soil-atmosphere exchange of CH_4 and N_2O varied widely among different ecosystems (Phillips et al. 2001; Dijkstra et al. 2010; van Groenigen et al. 2011; Dijkstra et al. 2013) and have not reached a consensus.

The source of soil CO_2 emissions is mainly from soil respiration, which includes autotrophic respiration (mainly respiration of plant roots) and heterotrophic microbial respiration. Three potential reasons of increased soil CO_2 emissions under elevated CO_2 have recently been explored. First, increased belowground C allocation under elevated CO_2 could stimulate plant root growth and increase autotrophic respiration (Zak et al. 2000; Deng et al. 2010; Zhou et al. 2010). Second, elevated CO_2 could increase root exudation and cause more labile C inputs into the rhizosphere (Zak et al. 1993), resulting in a priming effect on soil organic matter (SOM) decomposition and thereby an increase of heterotrophic respiration (Zak et al. 1993; Cheng 1999). Third, increased plant residues under elevated CO_2 could also stimulate heterotrophic respiration (Zak et al. 1996; Deng et al. 2010). Additionally, elevated CO_2 may increase plant water use efficiency (Jackson et al. 1994), which could induce higher soil moisture and benefit SOM decomposition (Hungate et al. 1997a; Dijkstra et al. 2008).

Soil net CH_4 flux is the balance between CH_4 production and uptake by soil microbes, and both processes are regulated by climatic and soil conditions. Elevated CO_2 may influence soil CH_4 flux by altering soil C, N availability and soil moisture (Dijkstra et al. 2010; Dijkstra et al. 2013). For soil CH_4 uptake which occurs in aerobic soils via oxidation of CH_4 by methanotrophs, previous studies found it could be stimulated by elevated CO_2 due to increased labile C (Dijkstra et al. 2010). However, this stimulation may be counterbalanced by increased soil moisture under elevated CO_2 which may limit CH_4 and/or O_2 diffusion (Ambus and Robertson 1999; Phillips et al. 2001). On the other hand, CH_4 can be produced in anaerobic soils by methanogens during SOM decomposition. Increased soil moisture and labile C under elevated CO_2 were beneficial to CH_4 production (Dijkstra et al. 2012). Therefore, the net soil CH_4 flux is the complex result of CH_4 uptake and production and its responses to elevated CO_2 still needs to be further explored.

Soil N_2O is mainly produced by microbial processes such as nitrification, denitrification, and nitrifier denitrification (Zhu et al. 2013). Altered soil C, N availability and soil moisture under elevated CO_2 could influence microbial activities and therefore soil N_2O emissions. Increased labile C inputs under elevated CO_2 were linked to increased denitrification rates, thereby increasing soil N_2O emissions, in particular when soil N availability was high (Ineson et al. 1998; Kammann et al. 2008; Dijkstra et al. 2012). Increased soil moisture or soil respiration under elevated CO_2 was also found to stimulate soil N_2O emissions due to their beneficial effects on denitrification (Hungate et al. 1997a; Robinson and Conroy 1998;

Kammann et al. 2008). However, increased N limitation under elevated CO_2 could often offset those stimulation effects, resulting in unchanged N_2O fluxes (Hungate et al. 1997b; Mosier et al. 2002; Dijkstra et al. 2013). Additionally, elevated CO_2 may decrease nitrification due to increased NH_4^+ limitation for autotrophic nitrifiers growth, reducing nitrification-derived N_2O flux (Müller et al. 2009; Rütting et al. 2010). Therefore, the effects of elevated CO_2 on soil N_2O emissions are regulated by many processes and factors, and are most likely system-specific.

The above introduction shows that microorganisms are key drivers of soil C and N cycling and play an important role in regulating terrestrial ecosystem GHG emissions (Singh et al. 2010). Thus, a robust prediction of future climate change requires mechanistic understanding of how elevated CO_2 affects soil microbial activities and community composition (Carney et al. 2007; He et al. 2010). Generally, elevated CO_2 increases soil C inputs and alters soil physico-chemical properties, which influences soil microbes and thereby the corresponding microbially regulated GHG processes. However, depending on the direction and magnitude of changes in soil C, N availability, soil moisture and oxygen content, and other soil properties under elevated CO_2 (He et al. 2010; Garcia-Palacios et al. 2015), different responses of soil microbes to elevated CO_2 were observed. For instance, elevated CO_2 was found to stimulate the growth of fungi (Carney et al. 2007; Cheng et al. 2012; Lipson et al. 2014) and actinomycetes (Billings and Ziegler 2005; Drigo et al. 2010) and alter the microbial community composition (de Graaff et al. 2006; He et al. 2010). Contrary, in a *Populus grandidentata* dominated ecosystem, Zak et al. (1996) found unchanged microbial community composition under elevated CO_2. In a temperate forest ecosystem, elevated CO_2 stimulated the growth of ammonium oxidizing archaea (AOA), but had no effect on ammonium oxidizing bacteria (AOB), probably due to N limitations (Long et al. 2012). However, He et al. (2010) found elevated CO_2 increased AOB, but decreased AOA in a grassland ecosystem. Recently, Bodelier and Steenbergh (2014) found that elevated CO_2 enhanced competition between methanotrophs and nitrifiers for NH_4^+ which altered soil methane uptake and N cycling. These potential changes of soil microbes under elevated CO_2 are important to understand the mechanisms underlying the responses of GHGs to the rising atmospheric CO_2.

The aims of this study was to investigate the effect of ten consecutive years of elevated atmospheric CO_2 concentrations on soil GHG fluxes from a *Quercus mongolica* (oak) dominated system and to explore factors affecting changes of soil GHG fluxes under elevated CO_2. We hypothesized that (1) elevated CO_2 increases soil CO_2 emissions due to increased belowground C inputs, which stimulates microbial activity; (2) Elevated CO_2 increases soil CH_4 uptake due to increased labile C inputs and well-aerated soil conditions, which are beneficial to methanotrophs; (3) Elevated CO_2 would not change soil N_2O fluxes, because our research site is typically N limited.

Materials and methods

Site description

The open top chamber (OTC) experiment was established in Changbai Forest Ecosystem Research Station in Jilin province, Northeastern China (42°24′N, 128°06′E). The study area is characterized by a typically temperate climate, with a mean annual temperature of 3.6 °C and mean annual precipitation of 745 mm (Wang et al. 2009; Hu et al. 2010). Six hexagonal OTC experimental plots (4.2 m in diameter and 4 m in height originally, and due to the growth of oak, the height was increased to 6 m in 2010) were utilized for CO_2 fumigation. The plots were arranged in a completely randomized design to lessen the effects of topographic variation and potential soil properties heterogeneity. Three chambers were dispensed with pure CO_2 to elevate the CO_2 concentration by 180 umol 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. More details about the OTC can be found in Li et al. (2010).

Two year 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 came from the same nursery and had the same genotype (Li et al. 2010). The CO_2 fumigation experiment started in April 2005, and was carried out for ten years until October 2014. Oaks were exposed to elevated CO_2

during daytime in the growing season, from May to October every year.

The soil at our experimental site is a dark brown soil developed from volcanic ash (Albic Luvisol), approximately neutral (pH = 6.7), with a well-drained loamy sand texture (clay, silt and sand content were about 10.4%, 13.6% and 76.0%, respectively in control plots). The above soil properties were unchanged under elevated CO_2 (Table S1).

Measurement of greenhouse gas fluxes

Soil CO_2, CH_4 and N_2O fluxes were measured using the static chamber method (Dijkstra et al. 2013). One squared stainless steel base (0.3 m × 0.3 m × 0.05 m) was placed in the center of each OTC in order to reduce any edge effects of the CO_2 fumigation system and inserted into soil 3 cm depth. The bases were installed into the soil approximately two months before the initial gas sampling to avoid disturbance on soil. A stainless steel chamber (0.3 m × 0.3 m × 0.3 m) was placed on the base every time gases were sampled, and the connection between the chamber and the base was sealed by water to prevent gas leakage. Gas samples (100 ml) were collected from each chamber every 20 min over a 1 h period, and stored in aluminum foil air bags (150 ml) for GHG measurements within 12 h using a gas chromatograph (HP 5890-II, Agilent, USA) equipped with FID and ECD detectors combined with Nickel converter. The carrier gas was high pure N_2, and the standard gas concentrations were 0.362, 2.03 and 455 ppm for N_2O, CH_4 and CO_2 respectively. The minimum detectable fluxes of CO_2, CH_4 and N_2O were 10, 0.4 and 0.1 ppm respectively.

Gas samples were taken six times (5th, 10th, 15th, 20th, 25th, 30th) each month from June to October, in 2014. Samples were taken between 9:00 and 10:00 as this time period was shown to represent daily average GHGs fluxes best (Xu and Qi 2001; Alves et al. 2012; Dijkstra et al. 2013). Furthermore this time slot was verified on two measurement occasions of the diurnal dynamics of GHG fluxes at our research site (Table S2).

Air and soil temperatures at 10 cm depth were recorded in situ at each sampling event using a digital thermometer. CO_2, CH_4 and N_2O fluxes were calculated as:

$$F = \rho \times V/A \times (\Delta c/\Delta t) \times 273/(273 + T) \times 0.6 \quad (1)$$

where F is the CO_2, CH_4 flux (mg or μg C m^{-2} h^{-1}) or N_2O flux (μg N m^{-2} h^{-1}); ρ is the density of C in CO_2 and CH_4 (0.54 mg C cm^{-3}) or the density of N in N_2O (1.25 mg N cm^{-3}) in the standard atmosphere environment; V is the volume of the static chamber (cm^3); A is the base area of the static chamber (cm^2); Δc/Δt is the slope of the linear correlation between CO_2 (ppm min^{-1}), CH_4 (ppb min^{-1}) or N_2O (ppb min^{-1}) concentration and time; T is air temperature (°C); 0.6 is conversion coefficient due to the different unit between Δc/Δt of CO_2, CH_4 or N_2O and F.

CO_2, CH_4 and N_2O fluxes measured on each sampling date were extrapolated to diurnal fluxes according to Eq. (2):

$$F_{diurnal} = F_9 \times 24 \quad (2)$$

where $F_{diurnal}$ is the diurnal GHG flux; F_9 is the GHG fluxes measured from 9:00 to 10:00 at each sampling time. The above calculated diurnal GHG fluxes were integrated into the growing season cumulative GHG emissions using Origin 8.5 software (Origin, OriginLab, USA). In details, using the time interval as X axis, and the diurnal flux as Y axis, we calculated the integral area as the cumulative GHG emissions during the time interval. The cumulative global warming potential (GWP) was calculated by summing the GWP of CO_2, CH_4 and N_2O (in g CO_2 eq. per m^2). The GWP of CH_4 was calculated by cumulative emissions of CH_4 multiplied by 25 and the GWP of N_2O was calculated by cumulative emissions of N_2O multiplied by 298 (Dijkstra et al. 2013).

Measurement of soil NH_4^+ and NO_3^- concentrations

Two soil subsamples (0–10 cm) were randomly taken close to the center of each OTC every time fluxes were measured. The two soil subsamples were fully mixed and sieved (< 2 mm) for analysis of soil NH_4^+, NO_3^-, dissolved organic C (DOC), dissolved organic N (DON), microbial C, N and community composition and the abundance of five specific functional bacteria genes (ammonia oxidizing bacteria-AOB *amoA*, ammonia oxidizing archaea-AOA *amoA*, methanotroph-*pmoA*, N_2 fixing bacteria-*nifH*, denitrifer-*nirS*). NH_4^+ and NO_3^- were extracted from 20 g soil with 100 ml of 2 M KCl, and filtered with ash-less filter papers (Qualitative Filter Paper, BH92410262) and measured with a Continuous Flow Analyzer (Bran-Luebbe Inc., Germany).

Measurement of soil microbial C, N and community composition

The chloroform fumigation method was used for microbial C and N analysis (Brookes et al. 1985). Unfumigated and fumigated soils (20 g) were extracted with 100 ml of 0.05 M K_2SO_4, and the extract solutions were dried in a ventilated oven at 60 °C, and ground to fine powder and analyzed for C and N concentrations using an Elementar Vario EL Cube (Elementar Analysis system GmbH, Hanau, Germany) instrument interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotope Faculty of University of California, Davis. Microbial C and N were calculated as:

$$M_{(C)} = (C_f - C_e) \div 0.45 \text{ and } M_{(N)} = (N_f - N_e) \div 0.54 \quad (3)$$

where $M_{(C)}$ and $M_{(N)}$ are microbial C and N respectively; e and f represent before and after chloroform fumigation respectively. DOC and total dissolved N concentrations were obtained from the C and N concentrations in the K_2SO_4 extract before chloroform fumigation. DON was calculated by subtracting dissolved inorganic nitrogen from the total dissolved N. The extraction efficiency factors were 0.45 for microbial C (Vance et al. 1987) and 0.54 for microbial N (Brookes et al. 1985).

PLFA analysis

Phospholipids were extracted and fractionated from lyophilized soil (4 g) according to Wang et al. (2013), and identified by gas chromatography (Agilent 7890, Agilent technologies, USA) equipped with FID detector and the soil microbial identification system (MIDI Inc., Newark, DE). Methyl nonadecanoate (19:0, Sigma-Aldrich, St. Louis, MO, USA) was used as the internal standard for quantifying the PLFAs. The abundance of specific PLFAs was calculated according to Eq. (4). Terminally branched saturated PLFAs (i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0), monounsaturated PLFAs (16:1 w7c, 16:1 w9c, 18:1 w5c, 18:1 w7c, cy 17:0 and cy 19:0), and the 14:0, 15:0, 16:0, 17:0, 18:0 were used as bacterial indicators (Zak et al. 1996; Moore-Kucera and Dick 2008; Wang et al. 2013); 16:1 w5c was used to represent arbuscular mycorrhizal fungi (AM fungi) (Olsson et al. 1995); 18:1 w9c and 18:2 w6c represent fungi (Zak et al. 1996). This interpretation requires some caution because 18:1 w9c can also exist in some bacteria (Frostegård et al. 2011). Nevertheless, 18:1 w9c is a relatively good indicator of fungi in forest soil (Frostegård et al. 2011); 10Me 16:0, 10Me 17:0 and 10Me 18:0 represent actinomycetes (Moore-Kucera and Dick 2008).

$$C_{PLFAs} = \left(B/A \times 40\text{ng ul}^{-1} \times 400\text{ ul}\right)/\left(4\text{g} \times M_{PLFAs}\right) \quad (4)$$

where C_{PLFAs} (nmol g^{-1} dry soil) is specific microbial biomass; B is the characteristic peak area of specific PLFA; A is the characteristic peak area of 19:0; 40 ng ul^{-1} is the concentration of 19:0; 400 ul is the volume of the sample; M_{PLFAs} (g mol^{-1}) is PLFA molecular weight.

Soil DNA extraction

Soil DNA was extracted from 0.25 g lyophilized soil sample with MOBIO PowerSoil® DNA Isolation Kit (Carlsbad, USA) according to the manufacturer's instructions. The extracted DNA which had high quality with the ratio of A260/A280 in the range of 1.78 to 1.85 was stored at −20 °C for quantitative real time PCR analysis.

Quantitative real time PCR

Quantitative PCR was conducted on a LightCycler 1.0 (Roche, Switzerland) and each measurement was performed in triplicates. Data analysis was carried out using the LightCycler software (version 5.23). The plasmids containing each target gene were extracted using Takara MiniBEST plasmid purification Kit (Takara), and quantified with a NANODROP 2000 UV-Vis Spectrophotometer (Thermo scientific, USA). Real time PCR was performed in 20 ul volumes which contained 10 ul SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara), 0.8 ul forward and reverse primers respectively (0.4 uM), 2 ul extracted DNA solution, and sterile water to fill up the volume to 20 μl. The target genes which were related to the processes producing or consuming soil N_2O and CH_4, including ammonia oxidizing bacteria (AOB *amoA*), ammonia oxidizing archaea (AOA *aomA*), denitrifier (*nirS*), N_2 fixing bacteria (*nifH*) and methanotroph (*pmoA*), and the lengths of target

genes, primer sets, nucleotide sequences of primers, annealing temperature and elongation time are described in Table 1. The thermal cycling conditions for the assays were: denaturation of DNA at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at the specific temperature (see Table 1) for 30 s and elongation at 72 °C for 30 s or 60 s (Table 1). The fluorescence signals were measured once per cycle at the end of the elongation step. In order to verify no nonspecific and primer dimer amplification, a melting curve was obtained by gradually increasing the temperature from 65 °C to 95 °C. A sample was only considered positive if it exhibited a log-linear amplification in the fluorescence curve (r^2 = 0.999) with an amplification efficiency at about 0.95 and a specific peak in the melting curve (between 85 and 92 °C).

Measurement of soil C and N concentrations, moisture, texture and pH

Soil organic C (SOC) and total N (TN) were measured using an Elementar Vario EL Cube (Elementar Analysis system GmbH, Hanau, Germany). Standard oven-drying method was carried out to measure soil gravimetric water content. Soil texture was determined by the pipette sedimentation method (Gee and Bauder 1986). Bulk density (BD) was determined by the core method (Burke et al. 1986). Soil pH was measured in a 1:5 (*w*/*v*) soil to water (CO_2-free) ratio using a pH detector (E-201-C, Leici, China). Water filled pore space (WFPS) was calculated based on Eq. (5):

$$WFPS = soil\ gravimetric\ water\ content \times \mathrm{BD/PS} \quad (5)$$

where PS is soil pore space and was calculated from Eq. (6) according to Parton et al. (2001).

$$\mathrm{PS} = (1-\mathrm{BD}/2.65) \times 100 \quad (6)$$

Q_{10} was calculated using Eq. (8) according to Epron et al. (2001).

$$\mathrm{R} = \mathrm{A}e^{BT} \quad (7)$$

$$Q_{10} = e^{10B} \quad (8)$$

where R represents soil respiration; T is soil temperature; A is the basic soil respiration rate at soil temperature 0 °C; B represents the temperature sensitivity of soil respiration.

Statistical analyses

Repeated measures ANOVA was performed to examine the effects of elevated CO_2 on soil properties, PLFAs, microbial C, N, functional bacteria genes and GHG fluxes during the growing season in 2014 using CO_2 fumigation treatments as the between subject factor and sampling time as the within subject factor. One way ANOVA was used to identify elevated CO_2 effects on cumulative GHG emissions and GWP. Principal components analysis (PCA) was used to analyze the effect of

Table 1 Measured soil genes and primer properties and the conditions of quantitative real time PCR assays

Target gene	Length of target gene (bp)	Primer	Nucleotide sequence	Annealing temperature (°C)	Elongation time (s)	Reference
amoA (AOA)	635	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	60	60	Francis et al. 2005
amoA (AOB)	491	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCC TTCTTC	60	30	Rotthauwe et al. 1997
nifH	360	PolF PolR	TGCGAYCCSAARGCBGACTC ATSGCCATCATYTCRCCGGA	55	30	Poly et al. 2001
nirS	410	cd3aF R3cd	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	57	30	Throbäck et al. 2004
pmoA	506	A189F Forest 675R	GGNGACTGGGACTTCTGG CCYACSACATCCTTACCGAA	63	60	Kolb et al. 2003

elevated CO_2 on microbial community composition. Data were transformed to normalized 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 using SPSS 17.0 software (SPSS, Chicago, IL, USA).

Results

Effects of elevated CO_2 on greenhouse gas fluxes

Elevated CO_2 significantly increased mean oak height and diameter at breast height (4.5 ± 0.1 m and 2.5 ± 0.1 cm, respectively) compared to that under ambient CO_2 (4.2 ± 0.1 m and 2.2 ± 0.1 cm, respectively), and increased soil C/N, DON and DOC ($p < 0.05$), but had no effect on SOC, TN, NH_4^+, NO_3^-, soil temperature (10 cm in depth), soil moisture and WFPS (Table 2).

The seasonal mean soil CO_2 flux under elevated CO_2 was 78.7 ± 6.0 mg C m^{-2} h^{-1}, and 61.7 ± 4.9 mg C m^{-2} h^{-1} under ambient CO_2, meaning a significant increase rate of 27.5% by elevated CO_2 (p = 0.04, Fig. 1a). During the growing season, seasonally cumulative CO_2 emissions increased by 27.7% under elevated CO_2 (250.4 ± 19.2 g C m^{-2}) compared to that under ambient CO_2 (196.0 ± 14.8 g C m^{-2}) ($p < 0.05$, Fig. 2c). When data for both treatments were combined, the soil CO_2 flux had significant positive correlations with soil temperature, WFPS, DOC, the photosynthetic rate ($p < 0.01$, Table S3, Fig. S3), and with the microbial C and the biomass of AM fungi and actinomycetes ($p < 0.05$, Fig. 3a. b and c). Q_{10} was higher under elevated CO_2 (2.83) than under ambient CO_2 (2.69) (Fig. S3a). Elevated CO_2 significantly increased soil CO_2 fluxes throughout the sampling days ($p < 0.01$, Fig. S2a, d) and diurnal cumulative soil CO_2 emissions ($p < 0.05$, Fig. S2 g) during both diurnal measurements.

CH_4 fluxes were always negative under both treatments throughout the growing season in 2014, and varied between −45.1 ± 8.3 and −7.4 ± 3.1 μg C m^{-2} h^{-1} under ambient CO_2 and between −77.3 ± 9.8 and −17.7 ± 2.6 μg C m^{-2} h^{-1} under elevated CO_2 (Fig. 1c), suggesting net soil uptake of CH_4. Elevated CO_2 significantly increased mean CH_4 uptake (−29.3 ± 2.2 μg C m^{-2} h^{-1}) by 32.4% compared to ambient CO_2 (−22.1 ± 1.4 μg C m^{-2} h^{-1}) (p = 0.02), and significantly increased seasonally

Table 2 Soil (0–10 cm) properties under ambient and elevated CO_2. p values of repeated measures ANOVA on the effects of elevated CO_2 and sampling time on these properties were shown in the last three rows

Soil properties		SOC (g kg^{-1})	TN (g kg^{-1})	Soil C/N	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	DON (mg kg^{-1})	DOC (mg kg^{-1})	Soil T (°C)	Soil M (kg H_2O kg^{-1})	WFPS (%)
Apr	A CO_2	28.2 ± 2.7[a]	2.0 ± 0.2	13.9 ± 0.1	10.8 ± 0.8	3.4 ± 0.5	0.1 ± 0.0	47.3 ± 2.4	ND[b]	29.1 ± 1.3	36.2 ± 6.1
	E CO_2	28.1 ± 2.6	2.0 ± 0.2	13.8 ± 0.2	11.3 ± 0.9	2.4 ± 0.5	1.1 ± 0.2	46.1 ± 6.0	ND	26.5 ± 1.9	40.9 ± 7.9
Jun	A CO_2	28.8 ± 4.4	2.2 ± 0.3	13.3 ± 0.1	8.9 ± 0.4	1.9 ± 0.5	19.0 ± 1.1	64.5 ± 2.9	14.4 ± 0.0	28.4 ± 1.2	35.2 ± 5.9
	E CO_2	27.7 ± 1.8	1.9 ± 0.2	14.0 ± 0.4	9.1 ± 2.1	1.5 ± 0.6	19.0 ± 1.1	86.9 ± 5.4	14.3 ± 0.1	31.8 ± 6.7	50.9 ± 16.2
Sep	A CO_2	28.8 ± 2.4	2.2 ± 0.2	13.2 ± 0.2	6.5 ± 0.4	0.7 ± 0.2	14.1 ± 0.6	47.6 ± 2.4	11.8 ± 0.0	13.1 ± 1.0	16.1 ± 2.9
	E CO_2	29.3 ± 6.0	2.1 ± 0.4	13.9 ± 0.2	6.2 ± 0.6	0.6 ± 0.4	17.4 ± 0.8	63.7 ± 7.9	12.5 ± 0.4	11.5 ± 2.9	18.6 ± 6.8
Oct	A CO_2	29.7 ± 1.1	2.2 ± 0.1	13.4 ± 0.1	4.9 ± 0.4	1.5 ± 0.2	14.2 ± 0.7	43.8 ± 1.4	4.5 ± 0.1	23.8 ± 1.1	29.0 ± 3.8
	E CO_2	31.4 ± 3.0	2.3 ± 0.2	13.6 ± 0.2	4.5 ± 0.6	1.9 ± 0.2	17.9 ± 1.4	52.8 ± 1.3	5.7 ± 0.7	20.1 ± 1.9	31.3 ± 6.9
Mean	A CO_2	28.9 ± 1.4	2.2 ± 0.1	13.4 ± 0.1	7.8 ± 1.3	1.9 ± 0.3	11.9 ± 1.9	50.8 ± 2.3	10.2 ± 1.1	23.6 ± 1.7	29.1 ± 2.9
	E CO_2	29.1 ± 2.7	2.1 ± 0.2	13.9 ± 0.1	7.7 ± 0.9	1.6 ± 0.3	13.8 ± 2.3	62.4 ± 5.3	10.8 ± 1.2	22.5 ± 2.8	35.4 ± 5.7
CO_2		0.48	0.63	0.03*	0.71	0.54	0.02*	0.03*	0.14	0.72	0.54
Time		0.22	0.22	0.11	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**
Time* CO_2		0.57	0.79	0.02*	0.69	0.19	0.10	0.02*	0.05*	0.18	0.18

A CO_2, ambient CO_2; E CO_2, elevated CO_2; SOC, soil organic carbon; TN, soil total nitrogen; DON, dissolved organic nitrogen; DOC, dissolved organic carbon; Soil T, soil temperature; Soil M, soil moisture; WFPS, water filled pore space. "CO_2" represents ambient and elevated CO_2 treatments, "Time" represents sampling time in repeated measures ANOVA (the same below)

[a] Data are presented as mean ± standard error (n = 3), and the values are expressed on a dry weight basis

[b] no data

* $p \leq 0.05$ and ** $p \leq 0.01$

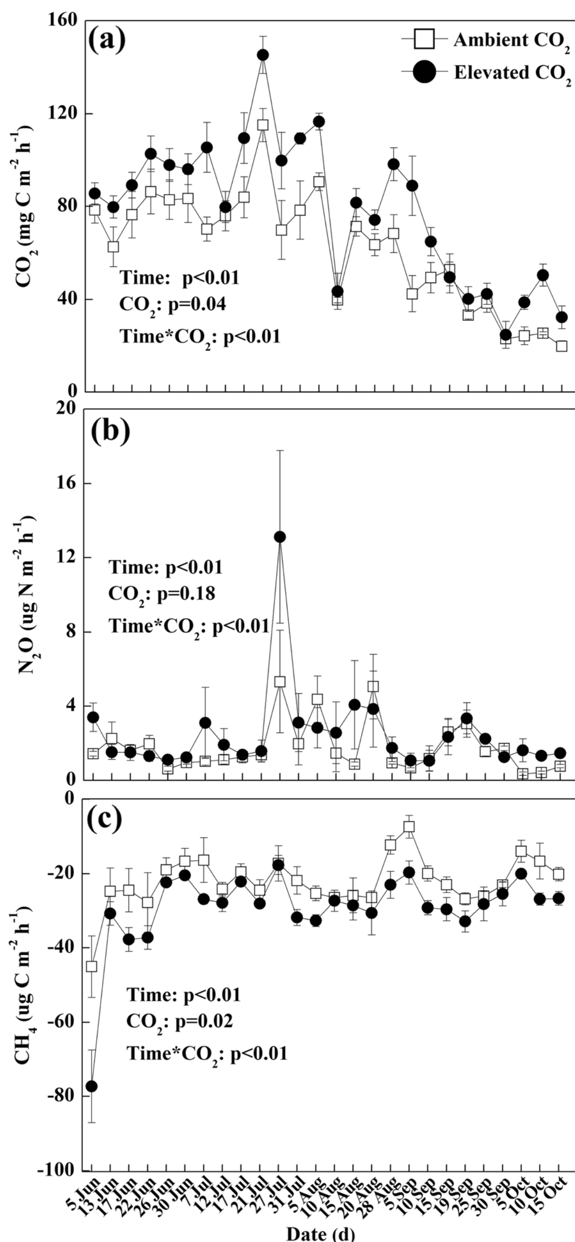

Fig. 1 Soil CO_2 (**a**), N_2O (**b**) and CH_4 (**c**) fluxes under ambient and elevated CO_2 during the growing season in 2014. Negative values indicate soil uptake and positive values indicate soil emission. Values are mean ± standard error (n = 3). "CO_2" represents ambient and elevated CO_2 treatments, "Time" represents sampling time in repeated measures ANOVA (the same below)

cumulative CH_4 uptake by 34.7% ($p < 0.05$, Fig. 2a). When data under both treatments were combined, soil CH_4 flux was significantly positively correlated with WFPS ($p = 0.01$), and negatively correlated with NH_4^+ ($p = 0.01$), DOC ($p = 0.03$) (Fig. S4) and the abundance of methanotroph *pmoA* ($p = 0.01$, Fig. 3d). Two diurnal changes also showed that elevated CO_2 significantly increased both daily mean CH_4 uptake ($p < 0.01$, Fig. S2b, e) and cumulative CH_4 uptake ($p < 0.05$, Fig. S2 h).

Elevated CO_2 had no effect on mean soil N_2O flux across the growing season (Fig. 1b). However, the seasonally cumulative N_2O was 64.7% higher under elevated CO_2 compared to that under ambient CO_2 ($p < 0.05$, Fig. 2b). When data were aggregated across both treatments, the soil N_2O flux was significantly negatively correlated with WFPS ($p = 0.05$) and microbial N ($p = 0.04$) (Fig. S5a, d), and positively correlated with soil NH_4^+ concentration ($p = 0.06$, Fig. S5c). The soil N_2O flux did not correlate with soil NO_3^- (Fig. S5b). Our results also showed that the soil N_2O flux was significantly positively correlated with the abundance of AOA *amoA* ($p = 0.05$), but not with the abundance of *nirS* (Fig. 3e, f). The two diurnal changes showed that elevated CO_2 significantly increased both daily mean N_2O fluxes ($p < 0.01$, Fig. S2c, f) and cumulative N_2O emissions ($p < 0.05$, Fig. S2i).

Elevated CO_2 significantly increased cumulative GWP by 27.8% ($p < 0.05$), which was mainly due to increased soil CO_2 emissions (Fig. 2d). The GWP caused by increased N_2O emissions was mostly offset by increased CH_4 uptake.

Effects of elevated CO_2 on soil microbial C, N and community composition

During the growing season microbial C and N concentrations were on average 16.7% and 26.3% higher under elevated CO_2 than that under ambient CO_2, respectively ($p < 0.05$, Fig. 4). Elevated CO_2 significantly increased the mean abundance of AM fungi, actinomycetes and bacteria by 11.5%, 16.7% and 11.0% respectively ($p < 0.05$), and decreased the ratio of bacteria to actinomycetes by 4.8% compared to ambient CO_2 throughout the growing season ($p < 0.01$, Table 3). The microbial community composition was not changed by elevated CO_2 in Apr. and Jun., whereas in Sep., elevated CO_2 significantly changed the microbial community composition on both PC1 and PC2 which accounted for 67% and 21% of the total variation respectively in the PCA analysis (Fig. S6).

The seasonal mean abundance of the methanotroph *pmoA* gene was $4.4 \pm 0.4 \times 10^7$ copies g^{-1} dry soil under elevated CO_2, which was significantly higher than under ambient CO_2 ($2.8 \pm 0.2 \times 10^7$ copies g^{-1} dry soil) ($p < 0.01$, Fig. 5a). The abundance of *nifH* was

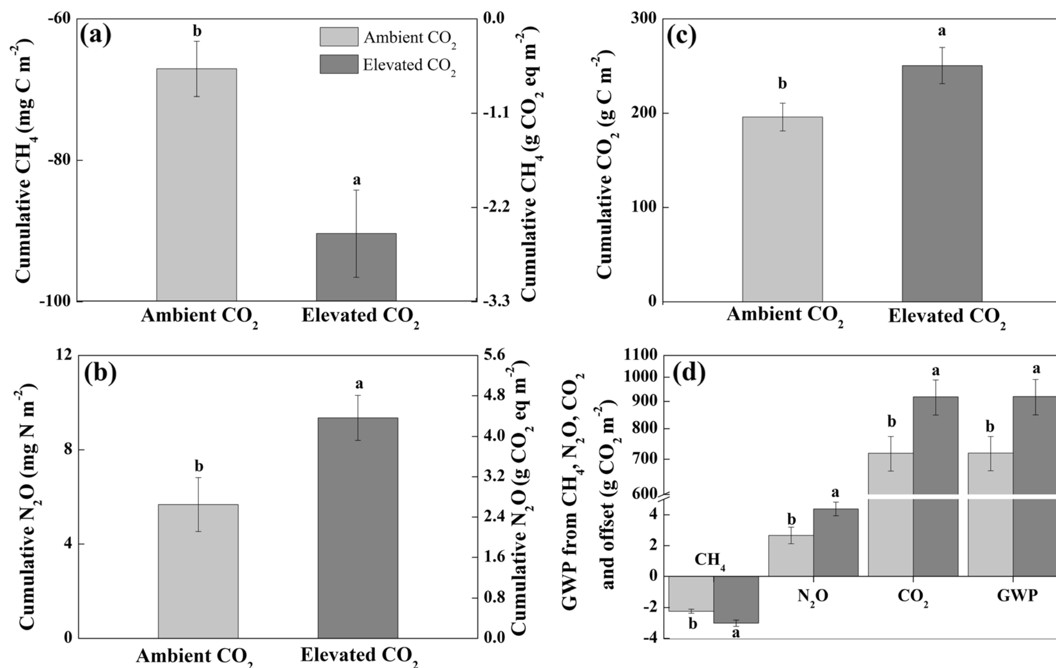

Fig. 2 Cumulative CH_4 (**a**), N_2O (**b**) [in mg C or N m^{-2} (left y-axis) and in g CO_2 eq. m^{-2} (right y-axis)], CO_2 emissions (**c**) and their global warming potential (GWP) (**d**) under ambient and elevated CO_2 during the growing season in 2014. Values are mean ± standard error (n = 3). Different letters above bars indicate significant differences between ambient and elevated CO_2 treatments at $p < 0.05$ based on one way ANOVA

$8.5 \pm 1.6 \times 10^7$ copies g^{-1} dry soil under elevated CO_2, which was not significantly different from that under ambient CO_2 ($5.7 \pm 1.0 \times 10^7$ copies g^{-1} dry soil) throughout the growing season. However, one way ANOVA showed elevated CO_2 significantly increased the abundance of the N_2 fixing *nifH* gene in Sep. ($p = 0.02$, Fig. 5b). The mean abundance of the denitrifier *nirS* gene was $1.9 \pm 0.3 \times 10^6$ copies g^{-1} dry soil under ambient CO_2, and $1.6 \pm 0.2 \times 10^6$ copies g^{-1} dry soil under elevated CO_2 during the growing season, which was not affected by elevated CO_2 (Fig. 5c). Elevated CO_2 did not significantly change the abundance of AOB *amoA* ($2.6 \pm 0.4 \times 10^6$ copies g^{-1} dry soil) compared to ambient CO_2 ($2.7 \pm 0.5 \times 10^6$ copies g^{-1} dry soil) (Fig. 5d). The abundance of AOA *amoA* under elevated CO_2 ($3.5 \pm 0.7 \times 10^7$ copies g^{-1} dry soil) was not significantly different compared to that under ambient CO_2 ($2.6 \pm 0.4 \times 10^7$ copies g^{-1} dry soil) based on repeated measures ANOVA, while in Sep. a significant increase in the abundance of AOA *amoA* was found under elevated CO_2 based on one way ANOVA ($p = 0.03$, Fig. 5e). The abundance of AOA *amoA* was one order magnitude higher than that of AOB *amoA* with the mean AOA/AOB *amoA* ratios at 10.2 and 15.9 under ambient and elevated CO_2 respectively (Fig. 5f). Results of the one way ANOVA showed that the ratio of AOA/AOB tended to increase under elevated CO_2 ($p = 0.06$, Fig. 5f).

Discussion

Effect of elevated CO_2 on soil CO_2 emissions

Elevated CO_2 significantly increased the mean soil CO_2 flux and its cumulative emissions during the growing season in 2014 ($p < 0.05$), which was consistent with our first hypothesis. Some previous studies found that higher soil moisture under elevated CO_2 stimulated SOM decomposition (Hungate et al. 1997a; Dijkstra et al. 2008), resulting in increased CO_2 fluxes. Our result also suggested a positive correlation between soil CO_2 flux and WFPS, indicating important effects of soil moisture on soil respiration. However, we did not find significant differences of soil moisture or WFPS between the two treatments, indicating that the increased soil respiration under elevated CO_2 was not caused by soil moisture. Elevated CO_2 significantly increased plant photosynthesis (Zhou et al. 2010), soil DOC and

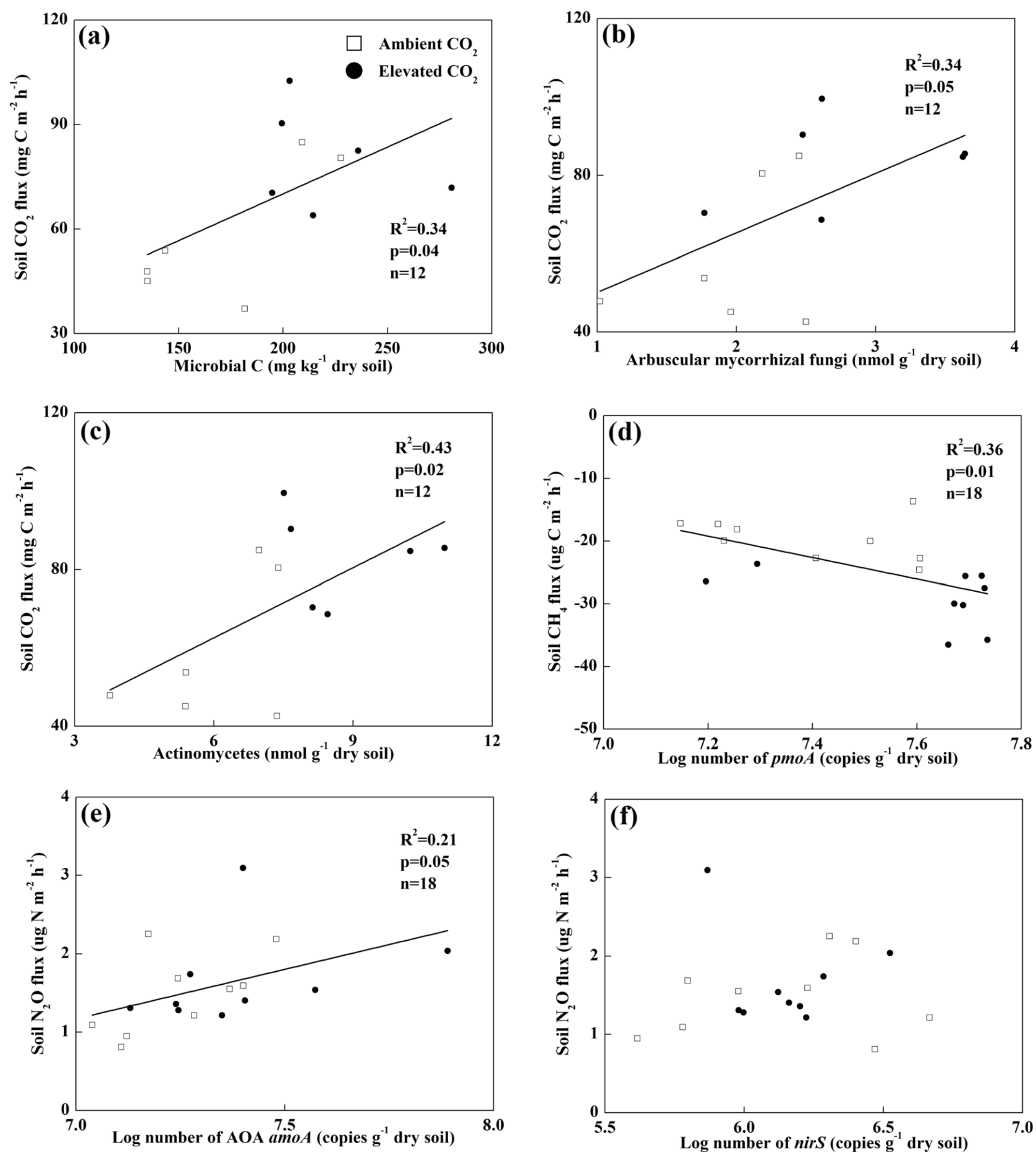

Fig. 3 Relationships between soil CO_2 flux and microbial biomass C (**a**) and the biomass of arbuscular mycorrhizal fungi (**b**) and actinomycetes (**c**), and relationship between soil CH_4 flux and the abundance of methanotrophs *pmoA* (**d**), and relationships between soil N_2O flux and the abundance of AOA *amoA* (**e**) and *nirS* (**f**). Each data point in the figures is the mean of the three replicates at each sampling time in Jun. and Sep. for microbial biomass C and PLFAs, and in Jun., Sep. and Oct. for functional genes under each treatment. Regression lines are only shown when significant ($p \leq 0.05$)

microbial biomass, and these factors were significantly positively correlated with soil CO_2 flux, which hinted that enhanced C inputs was the dominant reason for increased soil respiration under elevated CO_2. A previous study in the same site as our study found elevated CO_2 stimulated root growth and increased autotrophic respiration (Zhou et al. 2010), which further supported this conclusion. Microbes benefit from increased C inputs, especially AM fungi and actinomycetes (Treseder 2004; Billings and Ziegler 2005; Cheng et al. 2012). Indeed, our results showed that elevated CO_2 increased the biomass of AM fungi and actinomycetes, and a

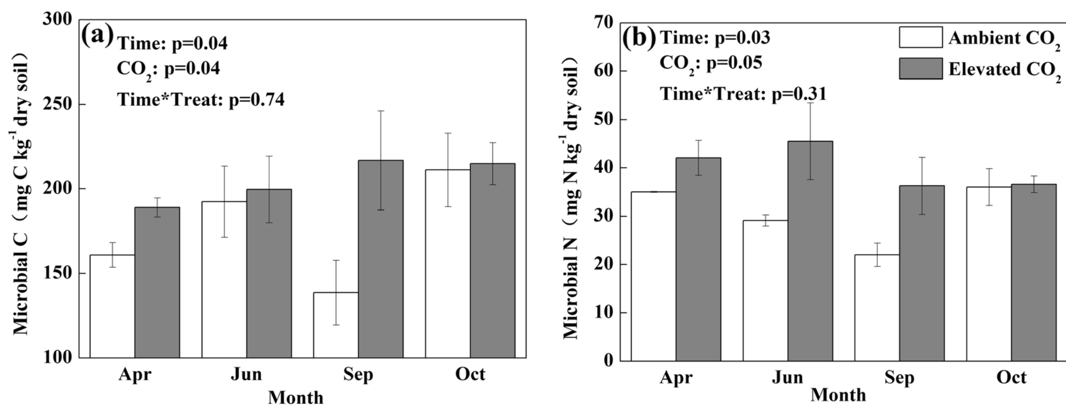

Fig. 4 Soil microbial C (**a**) and N (**b**) under ambient and elevated CO_2 during the growing season in 2014

significant positive correlation was found between soil CO_2 flux and the abundance of AM fungi and actinomycetes ($p < 0.05$). Because AM fungi and actinomycetes were found to decompose more recalcitrant SOM (Killham 1994; Cheng et al. 2012), their increases partly contributed to the higher soil CO_2 emissions under elevated CO_2.

Elevated CO_2 did not affect soil temperature, but did increase Q_{10}, which indicated that soil respiration got more sensitive to the change of soil temperature under elevated CO_2. This was possibly because more recalcitrant C was decomposed due to increased AM fungi and actinomycetes under elevated CO_2, and recalcitrant C generally has higher Q_{10} than labile C (Xu et al. 2014; Leitner et al. 2016). Therefore, our results suggest the interaction of soil warming and elevated CO_2 would accelerate the decomposition of SOM more than a single factor would.

Effect of elevated CO_2 on soil CH_4 uptake

WFPS was the dominating factor regulating the net CH_4 flux in our study (Table S3). Previous studies showed that higher WFPS may constrain and slow down the diffusion of CH_4 and O_2 from the atmosphere to the water-film covered microbes and suppress CH_4 uptake (Phillips et al. 2001; McLain et al. 2002). Moreover, the

Table 3 Phospholipid fatty acid (PLFA, nmol g^{-1} dry soil) and two PLFA ratios under ambient and elevated CO_2 at different sampling times during the growing season in 2014. *p* values of repeated measures ANOVA on the effects of elevated CO_2 and sampling time on these PLFAs were shown in the last three rows

PLFA (nmol g^{-1})		AM fungi	Fungi	Actinomycetes	Bacteria	B:F	B:A
Apr	ACO_2	2.8 ± 0.2 [a]	7.9 ± 0.6	7.0 ± 0.7	66.2 ± 1.6	7.4 ± 0.8	8.4 ± 0.3
	E CO_2	2.9 ± 0.1	8.2 ± 0.4	7.4 ± 0.6	66.5 ± 2.2	7.5 ± 0.1	8.2 ± 0.2
Jun	A CO_2	2.2 ± 0.2	7.3 ± 0.9	6.9 ± 0.4	58.2 ± 3.2	8.0 ± 1.0	8.6 ± 0.1
	E CO_2	2.9 ± 0.6	8.7 ± 1.9	8.5 ± 1.5	56.7 ± 6.7	8.1 ± 1.0	8.2 ± 0.3
Sep	A CO_2	2.6 ± 0.3	7.7 ± 0.9	7.5 ± 0.3	49.6 ± 5.9	8.5 ± 1.0	8.3 ± 0.2
	E CO_2	3.0 ± 0.5	7.9 ± 1.2	9.2 ± 1.6	69.9 ± 2.6	8.9 ± 0.2	7.7 ± 0.1
Mean	A CO_2	2.6 ± 0.1	7.7 ± 0.2	7.2 ± 0.2	58.0 ± 2.9	8.0 ± 0.3	8.4 ± 0.3
	E CO_2	2.9 ± 0.1	8.3 ± 0.3	8.4 ± 0.4	64.4 ± 2.9	8.2 ± 0.2	8.0 ± 0.3
	CO_2	0.04*	0.32	0.05*	0.05*	0.93	0.01**
	Time	0.98	0.89	0.03*	0.01**	0.03*	0.98
	Time* CO_2	0.29	0.42	0.32	0.27	0.59	0.17

A CO_2, ambient CO_2; E CO_2, elevated CO_2; *B* bacteria, *F* fungi, *A* actinomycetes

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

* $p \leq 0.05$ and ** $p \leq 0.01$

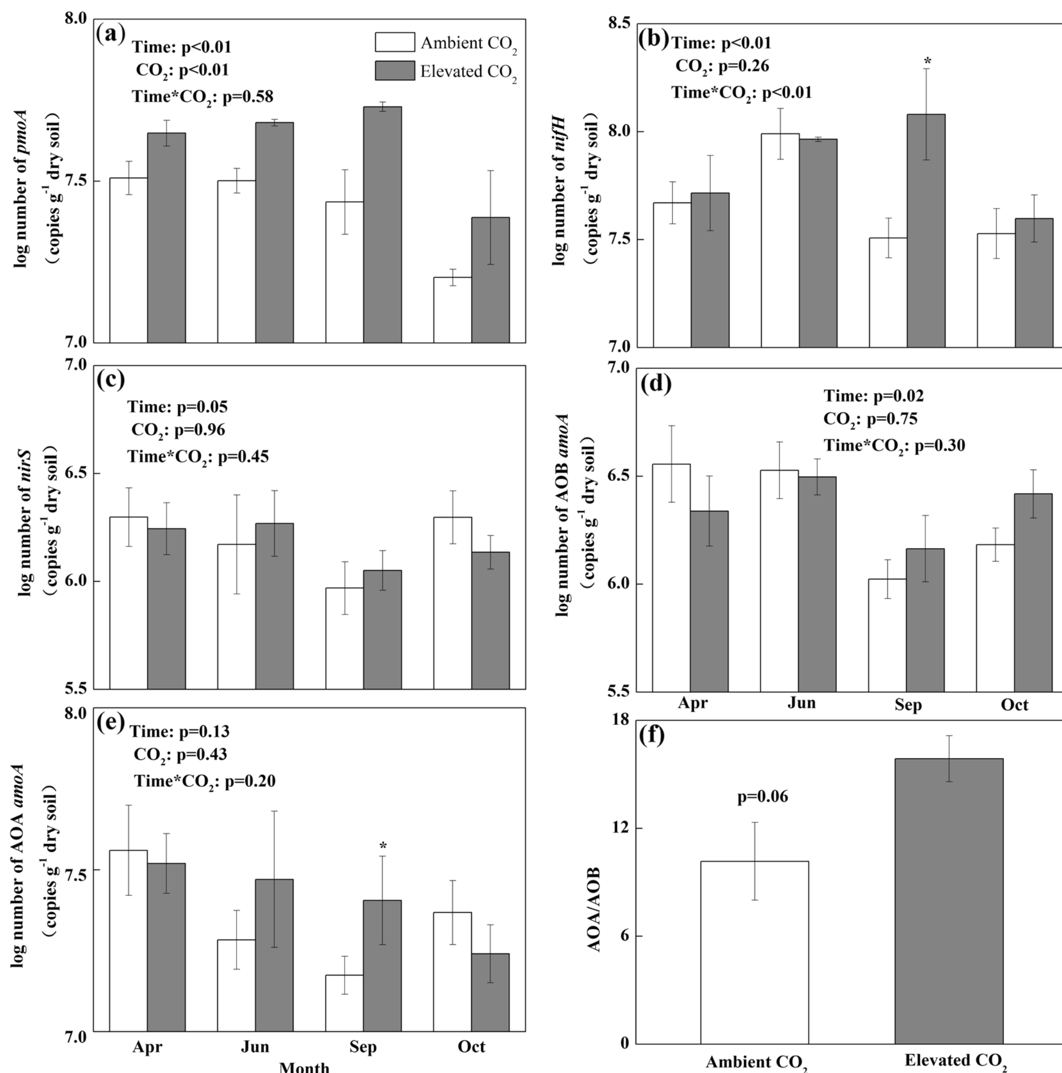

Fig. 5 Soil methanotroph gene (*pmoA*) (**a**), N_2 fixing bacteria gene (*nifH*) (**b**), denitrifier gene (*nirS*) (**c**), ammonia oxidizing bacteria gene (AOB *amoA*) (**d**), ammonia oxidizing archaea gene (AOA *aomA*) (**e**) and AOA/AOB (**f**) under ambient and elevated CO_2 during the growing season in 2014. Values are mean ± standard error (n = 3). *: significant difference at $p < 0.05$ based on one way ANOVA for each sampling time separately

anaerobic conditions induced by higher soil moisture may stimulate methanogens activity and increase CH_4 production (McLain and Ahmann 2008), counterbalancing the amount of CH_4 uptake. The relatively dry soil conditions at our site were beneficial for aerobic methanotrophs, but not for anaerobic methanogens, resulting in net CH_4 uptake.

Higher N availability was previously reported to alleviate N limitation of methanotrophs and stimulate CH_4 uptake (Bodelier et al. 2000; Dijkstra et al. 2010; Bodelier and Steenbergh 2014). However, when NH_4^+ concentrations are too high, it may inhibit methanotrophic activity due to competitive inhibition of the key enzyme (methane monooxygenase) (Bédard and Knowles 1989; Bodelier and Steenbergh 2014). Our study area is typically N limited, and thus, higher NH_4^+ concentration was found to stimulate CH_4 uptake (Fig. S4b).

The increased CH_4 uptake under elevated CO_2 was associated with the increase of the methanotroph *pmoA* gene, which was mainly due to increased soil DOC under elevated CO_2. Benstead et al. (1998) and Goldman et al. (1995) also found increased labile C substrates stimulated soil CH_4 uptake. In a semiarid grassland ecosystem, Dijkstra et al. (2010) speculated that increased labile C

inputs stimulated methanotrophs to consume CH_4 under elevated CO_2. Our results provided direct evidence of the increase of both methanotrophs and DOC, and therefore supported their viewpoints.

Effect of elevated CO_2 on soil N_2O emissions

The N_2O flux was low in our study due to N limitations. The elevated CO_2 therefore had no effect on the seasonal mean N_2O flux during the growing season in 2014. Previous studies in N limited ecosystems also found low N_2O fluxes and non-significant effects of elevated CO_2 (Ambus and Robertson 1999; Billings et al. 2002; Mosier et al. 2002). Some studies found that the greater plant and microbial N utilization under elevated CO_2 decreased N availability, resulting in unchanged soil N_2O fluxes (Hungate et al. 1997b; Mosier et al. 2002), or even reduced N_2O fluxes (Pleijel et al. 1998; Kettunen et al. 2007). The significant negative correlation between microbial N and N_2O flux in our research site suggested that the higher microbial N immobilization under elevated CO_2 may have reduced N_2O emissions, counterbalancing the potential stimulation effect of increased C inputs on N_2O emissions. However, we found seasonally cumulative N_2O emissions were higher under elevated CO_2 than that under ambient CO_2. We speculated that the large variations among individual OTC partly contributed to the lack of significant effects of elevated CO_2 on the seasonal mean N_2O flux. Moreover, the interaction effects between "time" and "elevated CO_2" based on repeated measures ANOVA were significant (Fig. 1b), which means the effects of elevated CO_2 were influenced by time. Thus, the significant difference in seasonal cumulative fluxes was probably due to elevated CO_2 effects on a few days, when soil temperature and/or soil NH_4^+ concentrations were larger under elevated CO_2 compared to ambient CO_2 (Fig. S7). These conditions may have benefited the nitrifying population, and thereby significantly increased soil N_2O fluxes. Therefore, the stimulation effect of elevated CO_2 on cumulative N_2O emissions should not be ignored.

Soil N_2O production is mainly controlled by nitrifiers and denitrifiers which regulate nitrification and denitrification processes respectively. The non-significant effect of elevated CO_2 on seasonal mean N_2O flux was associated with the unchanged AOB *amoA*, AOA *amoA* and *nirS*. In a temperate forest ecosystem, Long et al. (2012) found elevated CO_2 did not affect AOB due to increased N limitation, but stimulated AOA growth, indicating that elevated CO_2 and N limitation benefited AOA growth. At our site, the abundance of AOA *amoA* was one order of magnitude higher than that of AOB *amoA* and denitrifier *nirS*, and the AOA/AOB ratio tended to increase under elevated CO_2. Especially, in Sep., elevated CO_2 significantly stimulated AOA growth. Therefore in this study the increased seasonal cumulative N_2O emissions under elevated CO_2 was most likely caused by the AOA community and increased nitrification rates. The significantly positive correlation between soil N_2O flux and AOA *amoA* abundance further supported this conclusion. Furthermore, we found a positive correlation between NH_4^+ and N_2O flux, but found no relationship between NO_3^- and N_2O flux, which also indicated that most N_2O production at our site was derived from nitrification. The soil at our study site was of well-aerated loamy sand texture, which is considered unfavourable for denitrifiers. Soil WFPS ranged from 16.1% to 50.9%, and N_2O is thought to originate mostly from nitrification when WFPS is below 60% (Linn and Doran 1984). The significant negative correlation between WFPS and N_2O flux also provided evidence of nitrification dominating N_2O production in our study site (Fig. S5a).

Although on average the N_2 fixing bacteria gene *nifH* was not affected by elevated CO_2 during the study period, in Sep. a significant increase in N_2 fixing bacteria occurred under elevated CO_2. This was probably because N limitation was most severe in Sep. under elevated CO_2, inducing N_2 fixation. The stimulation of the growth of N_2 fixing bacteria under elevated CO_2 has been reported previously, which could relieve N limitations (Berthrong et al. 2014; Liang et al. 2016).

Conclusions

Elevated CO_2 increased soil CO_2 emissions and CH_4 uptake throughout the growing season in the *Quercus mongolica* dominated OTC system. Although the effect of elevated CO_2 on seasonal mean soil N_2O flux was not significant during the growing season, on some individual measurement dates N_2O emissions were increased by elevated CO_2. Changes in GHG fluxes were closely related to changes in soil microbial biomass and community composition, which drive the GHG production and consumption processes. The increased microbial biomass and the abundance of arbuscular mycorrhizal fungi and actinomycetes partly contributed to the

increased soil CO_2 emissions under elevated CO_2. Elevated CO_2 increased CH_4 uptake rates due to stimulated growth of methanotrophs. Our results also hinted that nitrification by AOA was the major process of soil N_2O emissions in our studied system. We found elevated CO_2 increased cumulative GWP from CH_4, N_2O and CO_2, pointing to positive feedbacks to global warming.

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