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Ten years of elevated atmospheric CO_2 doesn't alter soil nitrogen availability in a rice paddy

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ABSTRACT

Elevated atmospheric carbon dioxide (CO_2) concentration can affect soil nitrogen (N) cycling in natural and seminatural ecosystems, but the response of soil N cycling to elevated atmospheric CO_2 in intensively managed agricultural ecosystems characterized by large N fertilizer inputs remains poorly understood. Here, we investigated the effects of seven and 10 years of elevated CO_2 levels on soil gross N transformation rates using the ^{15}N dilution technique at the Rice Free Air CO_2 Enrichment (Rice-FACE) experiment in China. Our results show that under aerobic incubation conditions after the first seven years of CO_2 enrichment, gross rates of N mineralization, NH_4^+ immobilization, nitrification, and NO_3^- immobilization were not significantly different between N application rates or between CO_2 treatments. None of the four rates were affected by elevated CO_2 levels under both aerobic and water-logged incubation for a further three years of CO_2 enrichment. As a result, elevated CO_2 levels did not result in changes in available N for plants and soil microbes, and thus did not increase the potential risks of N losses through leaching and runoff. These results are probably associated with the lack of changes in soil organic C and N concentrations due to elevated CO_2. In contrast, elevated CO_2 levels significantly increased N_2O emissions for both incubation conditions. In general, our results suggest that 10 years of elevated atmospheric CO_2 concentration had negligible effects on soil N availability in a rice paddy field.

1. Introduction

Atmospheric CO_2 concentrations are predicted to double by the end of the 21st century as a result of human-induced changes to the global environment (IPCC, 2007). Elevated CO_2 levels generally enhance above- and below-ground plant productivity by increasing photosynthetic rates (Ainsworth and Long, 2005; De Graaff et al., 2006), water use efficiency (Tyree and Alexander, 1993), and root exudation and fine root turnover (Zak et al., 2000). In addition, the microbial community composition and activity changes in response to increased soil C availability with increasing CO_2 concentrations, because soil microorganisms are considered to C-limited (Paul and Clark, 1996). All of these factors could affect microbe-mediated N transformation processes and thus soil N supply capacity. Net N mineralization and nitrification rates are commonly used as indexes of plant available N (Magill and Aber, 2000). However, they only reflect the results of simultaneously-occurring gross N rates, and can not identify the mechanisms responsible for the observed changes to N cycling. Thus, quantifying gross N transformation rates can improve our understanding of the modification of soil N cycling induced by elevated CO_2 levels (Murphy et al., 2003; Booth et al., 2005).

Previous studies have investigated the effects of elevated CO_2 levels on soil gross N transformation rates; the response of gross N transformation rates to elevated CO_2 was found to be highly variable across studies, and no mechanistic explanation for the observed variation has been presented. Gross N mineralization rates have been reported to increase (Holmes et al., 2006; Rütting et al., 2010), decrease (Hungate et al., 1999; McKinley et al., 2009), or remain unchanged (Finzi and Schlesinger, 2003; West et al., 2006; Müller et al., 2009) in response to elevated CO_2 levels. Gross nitrification rates can also increase (Jin and Evans, 2007) or decrease (Hungate et al., 1997; Müller et al., 2009;

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Rütting et al., 2010; Björsne et al., 2014) in response to elevated CO_2. A recent meta-analysis found that neither gross N mineralization nor gross nitrification was altered by elevated CO_2 levels, but when conducting an ecosystem-specific analysis, gross N mineralization was only stimulated in N-limited ecosystems, and was unaffected in phosphorus (P)-limited ecosystems (Rütting and Andresen, 2015).

However, it should be noted that all studies on the response of soil gross N transformation rates to elevated CO_2 levels have been primarily conducted in natural and seminatural ecosystems, such as grasslands, forests, deserts, and heathland, without the input of N fertilizer (Barnard et al., 2005; De Graaff et al., 2006; Rütting and Andresen, 2015), and data from intensively managed agricultural soils are entirely lacking. Compared with natural and seminatural ecosystems, intensively managed agricultural ecosystems are characterized by high degrees of human disturbance and the large input of synthetic N fertilizer. Therefore, the effects of elevated CO_2 levels on gross N transformation rates in intensively managed agricultural soils could differ from those in natural and seminatural soils. In intensively managed agricultural ecosystems in China, excessive synthetic N fertilizer inputs have caused a series of environmental problems, such as eutrophication of surface waters (non-point source pollution), nitrate pollution of groundwater, acid rain and soil acidification, and greenhouse gas emissions (Guo et al., 2004; Ju et al., 2009). Thus, understanding the effects of elevated CO_2 levels on gross N transformation rates in intensively managed agricultural soils could predict the soil N supply capacity and provide the basis for reasonable fertilization schemes, and finally reduce the input of N fertilizer and N-associated pollution to the environment in response to climate change in the future.

Our previous studies have shown that elevated CO_2 levels can increase biomass and grain yield in rice in the China Rice-FACE experiment (Chen et al., 2015; Zhu et al., 2015). At the time of this study, China Rice-FACE had been under elevated CO_2 levels (ambient + 200 μmol mol^{-1}) for >10 years. In this study, we hypothesized that elevated CO_2 would increase soil organic C concentrations and thus enhance gross N mineralization and immobilization rates, eventually causing an increase in soil N supply capacity and grain yield. In addition, a decline in gross nitrification rates could be expected as a consequence of increased competition among heterotrophic microorganisms for NH_4^+-N. Therefore, our objective was to determine how 10 years of elevated CO_2 levels alters soil gross N transformation rates using the ^{15}N dilution technique under aerobic and water-logged conditions in a rice paddy field.

2. Materials and methods

2.1. Study site

The Rice-FACE experiment is situated in Zhongcun Village (119°42′0″E, 32°35′5″N), Yangzhou City, Jiangsu Province, in a typical Chinese rice-growing region with a subtropical monsoon climate (Zhu et al., 2012). The soil is classified as Shajiang-Aquic Cambiosol with a sandy loam texture. The soil bulk density and pH were 1.16 g cm^{-3} and 6.82, respectively. Other soil properties are given in Table 1. The CO_2 enrichment experiment was initiated in June 2004. The experiment consists of three identical 14 m diameter octagonal rings receiving elevated (E) atmospheric CO_2 concentration 200 μmol mol^{-1} above the ambient concentration in a free air CO_2 enrichment (FACE) setup and three comparison rings with ambient (A) atmospheric CO_2. Each ring is separated into two plots receiving, in addition to the ambient or elevated CO_2 levels, either a low rate of N application (LN, 125 kg N ha^{-1} yr^{-1}) or a normal rate of N application (NN, 225 kg N ha^{-1} yr^{-1}). In total, the experiment provides a full-factorial design with all four combinations for the rates of N application and CO_2 enrichment. Rings are separated by 90 m to avoid CO_2 contamination between plots. In the six plots, pure CO_2 gas was released 24 h day^{-1} from peripheral emission tubes set at 50 cm above the crop canopy. CO_2 release was controlled by a computer program with an algorithm based on wind speed and direction to keep the target CO_2 concentration within the FACE plot. Elevated CO_2 concentrations were achieved within 80% of the set point >90% of the time for each year. All other environmental conditions were consistent with cultural agronomic practices for this region. The detailed description of FACE operation for this location has been reported elsewhere (Okada et al., 2001; Liu et al., 2002).

Table 1
Soil properties (average ± standard deviation) in a rice paddy field (China-FACE site) at low N (LN) and normal N (NN) inputs under ambient CO_2 (A) and elevated CO_2 (E) concentrations.

Year	Treatment	Total C (g kg^{-1})	Total N (g kg^{-1})	C:N ratio
2011	LNA	16.7 ± 0.63a	1.64 ± 0.05a	10.2 ± 0.39a
	LNE	18.0 ± 1.75a	1.73 ± 0.17a	10.4 ± 0.05a
	NNA	17.6 ± 0.12a	1.65 ± 0.04a	10.7 ± 0.17a
	NNE	18.5 ± 1.48a	1.75 ± 0.14a	10.6 ± 0.65a
2014	NNA	18.6 ± 1.56a	2.10 ± 0.10a	8.90 ± 0.46a
	NNE	18.5 ± 1.91a	2.07 ± 0.15a	8.90 ± 0.36a

Values followed by the same letter within each column are not significantly different (P > 0.05) between ambient CO_2 (A) and elevated CO_2 (E) concentrations for each N input treatment and soil sampling year. LNA, LNE, NNA, and NNE refer to low N input under ambient CO_2 concentrations, low N input under elevated CO_2 concentrations, normal N input under ambient CO_2 concentrations, and normal N input under elevated CO_2 concentrations, respectively.

The main rice cultivars were Wuxingjing 14 (a japonica cultivar) during the 2004 to 2010 rice growing seasons, and Wuyunjing 21 (another japonica cultivar) during the 2011 to 2014 rice seasons. Seeds of each line were sown on May 18–20, and seedlings were transplanted on June 18–22 every year. The spacing of the hills was 16.7 cm and 25 cm (equivalent to 24 hills m^{-2}). During the 2004–2012 growing seasons, two levels of N were supplied as urea (N, 46.3%) and compound chemical fertilizer ($N:P_2O_5:K_2O$ = 15:15:15, %): low (LN, 125 kg N ha^{-1} yr^{-1}) and normal (NN, 225 kg N ha^{-1} yr^{-1}). For the two N levels, phosphorous (P) and potassium (K) were applied as compound chemical fertilizer at a rate of 70 kg P_2O_5 ha^{-1} yr^{-1} and 70 kg K_2O ha^{-1} yr^{-1}. However, during the 2013–2014 growing seasons, the low N levels with both ambient and elevated CO_2 treatments were eliminated, and only normal N levels with both ambient and elevated CO_2 treatments were retained. Specifically, the N level was 225 kg N ha^{-1} yr^{-1}, P was applied at 90 kg P_2O_5 ha^{-1} yr^{-1}, and K was applied at 90 kg K_2O ha^{-1} yr^{-1}. For all seasons, N was applied as a basal dressing (40% of the total) one day prior to transplanting, as a top dressing at early tillering (30% of the total), and at the panicle initiation (PI) stage (30% of the total). Both P and K were applied as a basal dressing one day before transplanting.

2.2. Experimental design and setup

In November 2011, soil cores (2.5 cm diameter × 20 cm deep) were sampled in each plot at four randomly selected sites, pooled into a single sample and sieved (2 mm mesh). The fresh soil was then stored at 4 °C for the incubation studies within one week. In November 2014, the same protocol was used to obtain soil samples and to prepare the soil for subsequent analyses. Gross N transformation rates were determined by the ^{15}N dilution technique with a paired labeling experiment (Kirkham and Bartholomew,

1954; Hart et al., 1994). Half of each pair was labeled with $^{15}NH_4NO_3$ at 10 atom%, and the other half was labeled with $NH_4^{15}NO_3$ in a similar manner.

Under aerobic incubation, 288 flasks of 250-mL volume (2 CO_2 treatments × 2 N levels × 3 plots × 2 ^{15}N labeling × 4 sampling time points × 3 replicates) were prepared in 2011. Similarly, under aerobic incubation in 2014, 144 flasks of 250-mL volume (2 CO_2 treatments × 3 plots × 2 ^{15}N labeling × 4 sampling points × 3 replicates) were prepared. Fresh soil (equivalent to 20 g dry weight) was placed inside each flask. The soil samples in the sealed flasks were then pre-incubated in the dark at 25 °C at 30% water-holding capacity (WHC) in the laboratory for 24 h. After pre-incubation, 2 mL of either ^{15}N-enriched $^{15}NH_4NO_3$ or $NH_4^{15}NO_3$ solution (10 atom%) was applied to each soil sample by pipetting the solutions uniformly over the soil surface, resulting in an equivalent addition of 20 mg of NH_4^+-N and 20 mg of NO_3^--N kg^{-1} to the soil. Subsequently, the final moisture content of each labeled sample was adjusted to 60% WHC by the addition of deionized water. Flasks were sealed with rubber stoppers and incubated at 25 °C in the dark for 72 h. During incubation, the flasks were opened for 30 min each day to refresh the atmosphere inside each flask. The moisture content of the incubated soil samples was maintained by adding water when necessary to compensate for the amount of water lost through evaporation. Soil extractions were performed 0.5, 24, 48, and 72 h after ^{15}N labeling using 100 mL 2 M KCl solution to determine the concentration and isotopic composition of NH_4^+ and NO_3^-.

Simultaneously, we collected gas samples in the headspace of the flasks under aerobic incubation in 2014 to determine the concentration of N_2O and CO_2. Gas samples (three replicates) were taken from the flasks at 6, 24, 48, and 72 h. Before each gas sampling event, the flasks were opened for 30 min to renew the atmosphere inside and immediately sealed for 6 h using a silicone sealant. Prior to sampling, the headspace gas was mixed by withdrawing and reinjecting the headspace gas five times using a 25 mL gas-tight syringe fitted with a stopcock. A 20 mL gas sample was collected from the headspace of each flask at the end of the 6 h incubation period, and the sample was then injected into a pre-evacuated vial (18.5 mL) to determine the concentration of N_2O and CO_2. Likewise, gas sample at the beginning of the seal was also collected to determine initial concentration. After removing the gas samples, the flasks were opened again for 30 min to achieve a pressure balance.

For the water-logged incubation in 2014, 72 flasks of 250-mL volume (2 CO_2 treatments × 3 plots × 2 ^{15}N labeling × 2 sampling points × 3 replicates) were prepared. Fresh soil (equivalent to 20 g dry weight) was placed inside each flask, and the soils in the sealed flasks were then pre-incubated in the dark at 25 °C in the laboratory for 24 h. After pre-incubation, 50 mL of either ^{15}N-enriched $^{15}NH_4NO_3$ or $NH_4^{15}NO_3$ solution (10 atom%) was added to each soil sample, resulting in an equivalent addition of 20 mg of NH_4^+-N and 20 mg of NO_3^--N kg^{-1} to the soil, respectively. The solution addition formed an approximately 1.5-cm surface water layer, which mimicked waterflooding conditions in the rice paddy ecosystem. After 2 and 48 h of ^{15}N addition, the soil was destructively sampled with 50 mL of 4 M KCl to determine the concentration and isotopic composition of NH_4^+ and NO_3^-. Gas samples (three replicates) were taken from the headspace of the flasks at 1, 24, and 48 h. Prior to each gas sampling event, the flasks were opened for 30 min to renew the atmosphere inside and then immediately sealed for 1 h using a silicone sealant. The subsequent gas sampling procedure was the same as for the flasks incubated under aerobic conditions in 2014.

2.3. Analyses

NH_4^+ and NO_3^- concentrations were determined with a continuous-flow analyzer (Skalar Analytical, Breda, the Netherlands). The isotopic compositions of NH_4^+, NO_3^-, and organic N were measured using an automated C/N analyzer isotope ratio mass spectrometer (Europa Scientific Integra, UK). NH_4^+ and NO_3^- were separated for ^{15}N measurements by distillation with magnesium oxide and Devarda's alloy (Lu, 2000). Specifically, a portion of the extract was steam-distilled with MgO to separate NH_4^+ on a steam distillation system. The sample in the flask was distilled again after the addition of Devarda's alloy to separate out the NO_3^-. Liberated NH_3 was trapped using boric acid solution. To prevent isotopic cross-contamination between samples, 25 mL of reagent-grade ethanol was added to the distillation flasks and steam-distilled for 3 min between each distillation. Trapped N was acidified and converted to $(NH_4)_2SO_4$ using 0.005 mol L^{-1} H_2SO_4 solution. The H_2SO_4 solution (containing NH_4^+) was then evaporated to dryness at 60 °C in an oven and analyzed for ^{15}N abundance.

The N_2O concentration was determined using a 2 mm ID stainless steel column that is 3 m long, packed with Porapak Q (80/100 mesh), and an Agilent 7890 gas chromatograph fitted with an electron captured detector set at 300 °C. The column temperature was maintained at 40 °C and the carrier gas was argon–methane (5%) at a flow rate of 40 mL min^{-1}. CO_2 concentrations, meanwhile, were determined with the same gas chromatograph equipped with a thermal conductivity detector using a column packed with Porapak Q (80/100 mesh). The temperatures of the column oven, injector, and detector were 40 °C, 100 °C, and 300 °C, respectively. The carrier gas (H_2) flowratewas 80 mL min^{-1}.

2.4. Calculation and statistical analysis

Gross rates of N mineralization, nitrification, NH_4^+ and NO_3^- consumption under aerobic conditions were calculated for time intervals $h_{0\text{-}24}$, $h_{24\text{-}48}$, and $h_{48\text{-}72}$ using the analytical equations of Kirkham and Bartholomew (1954) and Hart et al. (1994). All the rates were calculated under water-logged conditions for time intervals $h_{0\text{-}48}$ in the same manner. The potential gross NH_4^+ immobilization rate was calculated by subtracting the gross nitrification rate from the NH_4^+ consumption rate, on the assumption that NH_4^+ consumption through volatilization was zero. We assumed that NO_3^- consumption via denitrification was negligible, and therefore the gross NO_3^- immobilization rate was equivalent to the gross NO_3^- consumption rate under aerobic incubation (Burger and Jackson, 2003; Murphy et al., 2003). However, denitrification may play a predominant role in NO_3^- consumption under water-logged conditions. Re-mineralization was considered not to occur within seven days of incubation (Barraclough, 1995; Accoe et al., 2004). Gross NH_4^+ and NO_3^- immobilization may be overestimated due to stimulation by ^{15}N substrate addition; however, any such stimulation would be consistent among all treatments thus permitting comparisons between treatments.

The three replication rings for each treatment were considered as spatial replicates, and thus the effects of N input levels (low vs. normal), CO_2 enrichment (ambient vs. elevated CO_2), time intervals, and their interaction on gross N transformation rates under aerobic incubation after seven years of CO_2 enrichment in 2011 were analyzed by three-way ANOVAs. Two-way ANOVAs were used to analyze the effects of CO_2 enrichment, time intervals, and their interaction on gross N transformation rates under aerobic incubation after 10 years of CO_2 enrichment in 2014. Three-way ANOVAs were used to test the effects of CO_2 enrichment, the duration of enrichment, time intervals, and their interaction on gross N transformation rates under aerobic incubation conditions. In

addition, one-way ANOVA was used to compare the difference in gross N transformation rates calculated under water-logged incubation and CO_2 and N_2O emissions between ambient and elevated CO_2 treatments. All data were natural log-transformed when necessary to meet the assumptions of normality and homoscedasticity. All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). All results are reported as mean ± standard deviation for oven-dried soils.

3. Results

3.1. Changes in concentrations and ^{15}N enrichment for NH_4^+ and NO_3^-

In the aerobic incubation in 2011, soil NH_4^+-N concentrations gradually decreased over the course of the 72 h incubation, and NO_3^--N concentrations correspondingly increased, regardless of N application rates and CO_2 enrichment (Fig. 1a,b), indicating the occurrence of net nitrification. In the aerobic incubation in 2014, soil NH_4^+-N concentrations gradually decreased during the first 48 h of incubation, and then leveled off for the remainder of the incubation period in both CO_2 treatments (Fig. 2a). Accordingly, NO_3^--N concentrations increased during the first 48 h of incubation, and then remained almost constant (Fig. 2b). These results indicate that the production of NO_3^--N was restrained by NH_4^+-N availability during the remaining 24 h of incubation. The ^{15}N enrichments of the NH_4^+ pool in the $^{15}NH_4^+$ labeling treatment decreased quickly during the first 48 h of incubation, and thereafter decreased slowly or stayed more or less stable in the aerobic incubations in 2011 and 2014, regardless of N application rates and CO_2 enrichment (Figs. 1 and 2). The same phenomenon was also observed for the ^{15}N enrichments of the NO_3^- pool in the $^{15}NO_3^-$ labeling treatment (Figs. 1 and 2). A decline in the ^{15}N enrichments of the NH_4^+ and NO_3^- pools indicated low ^{15}N-NH_4^+ input from the mineralization of organic N that diluted the labeled $^{15}NH_4^+$ and low ^{15}N-NO_3^- input from nitrification of low-enrichment NH_4^+, respectively.

In the water-logged incubation in 2014, soil NH_4^+-N concentrations decreased during the 48 h of incubation, whereas NO_3^--N concentrations did not change over time (Fig. 3a,b). The ^{15}N enrichments of the NH_4^+ pool in the $^{15}NH_4^+$ labeling treatment and the ^{15}N enrichments of the NO_3^- pool in the $^{15}NO_3^-$ labeling treatment decreased during the 48 h of incubation (Fig. 3c,d).

3.2. Gross N transformation rates

In the aerobic incubation in 2011, gross rates of N mineralization, NH_4^+ immobilization, nitrification, and NO_3^- immobilization were not significantly different between the N application rates or between the CO_2 treatments (Fig. 4). In contrast, all gross N transformation rates varied significantly among the time intervals $h_{0\text{-}24}$, $h_{24\text{-}48}$, and $h_{48\text{-}72}$ ($P < 0.05$), being consistently higher in the first two time intervals than in the last time interval (Fig. 4). Such results suggest that the flush of gross N mineralization at the beginning of the incubation due to ^{15}N addition and the NH_4^+ substrate limitation of nitrification in the remaining 24 h of incubation may have occurred. There were no significant interactions among N application rates, CO_2 treatments, and time intervals for any of the four gross N transformation rates.

In the aerobic incubation in 2014, elevated CO_2 levels had no significant effects on gross rates of N mineralization, NH_4^+

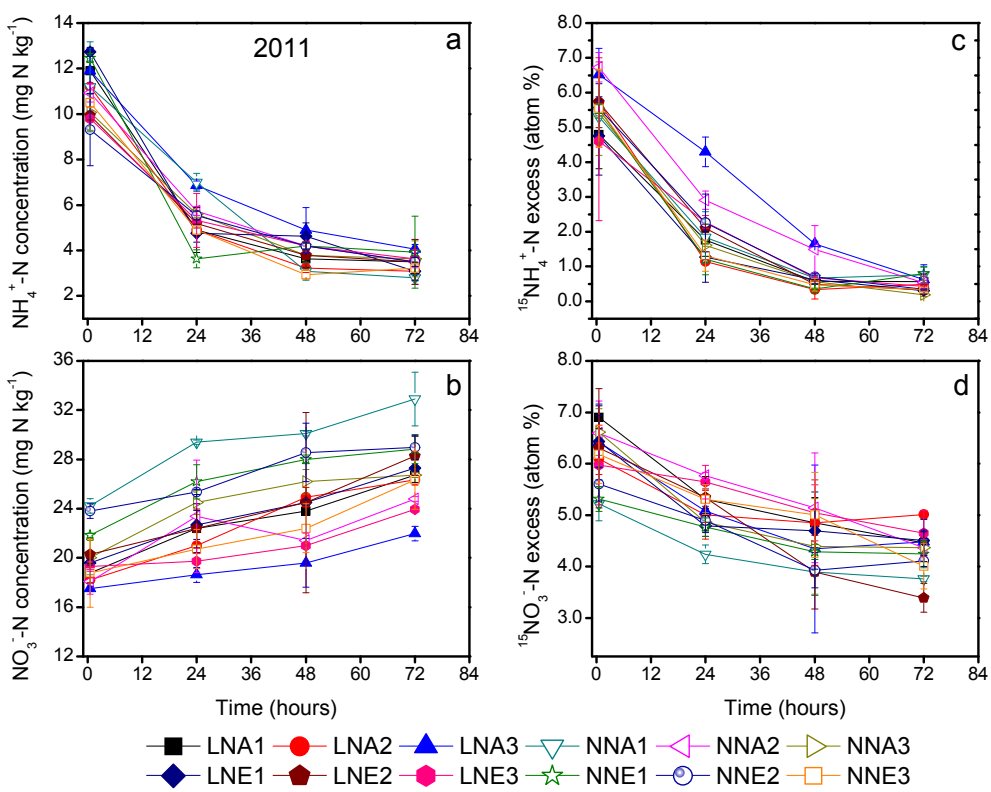

Fig. 1. Soil NH_4^+-N concentrations (a) and ^{15}N excess (c) in the $^{15}NH_4NO_3$ labeled treatments, and NO_3^--N concentrations (b) and ^{15}N excess (d) in the $NH_4^{15}NO_3$ labeled treatments during aerobic incubation under ambient CO_2 levels and after seven years of elevated atmospheric CO_2 concentrations (in 2011). Values are means with standard deviation (n = 3). LNA1, LNA2, and LNA3, NNA1, NNA2, and NNA3, LNE1, LNE2, and LNE3, and NNE1, NNE2, and NNE3 are three spatial replication rings of LNA, NNA, LNE, and NNE, respectively. LNA, NNA, LNE, and NNE indicate low N (125 kg N ha^{-1} yr^{-1}) input under ambient CO_2, normal N (225 kg N ha^{-1} yr^{-1}) input under ambient CO_2, low N (125 kg N ha^{-1} yr^{-1}) input under elevated CO_2 concentration, normal N (225 kg N ha^{-1} yr^{-1}) input under elevated CO_2.

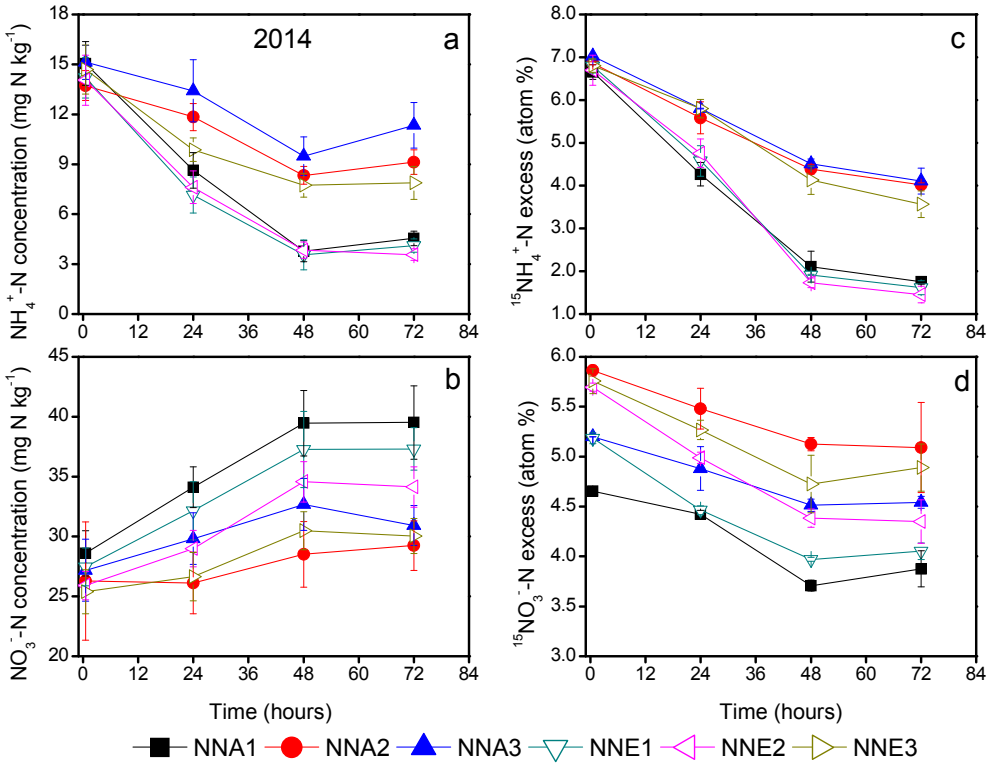

Fig. 2. Soil NH_4^+-N concentrations (a) and ^{15}N excess (c) in the $^{15}NH_4NO_3$ labeled treatments, and NO_3^--N concentrations (b) and ^{15}N excess (d) in the $NH_4^{15}NO_3$ labeled treatments during aerobic incubation under ambient CO_2 levels and after 10 years of elevated atmospheric CO_2 concentrations (in 2014). Values are means with standard deviation (n = 3). NNA1, NNA2, and NNA3, and NNE1, NNE2, and NNE3 are three spatial replication rings of NNA and NNE, respectively.

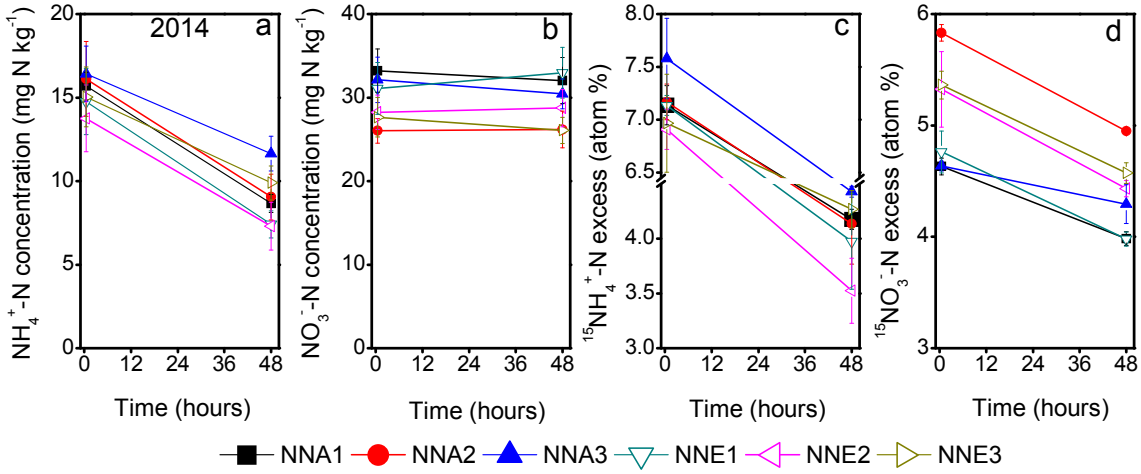

Fig. 3. Soil NH_4^+-N concentrations (a) and ^{15}N excess (c) in the $^{15}NH_4NO_3$ labeled treatments, and NO_3^--N concentrations (b) and ^{15}N excess (d) in the $NH_4^{15}NO_3$ labeled treatments during water-logged incubation under ambient CO_2 levels and after 10 years of elevated atmospheric CO_2 concentrations (in 2014). Values are means with standard deviation (n = 3). NNA1, NNA2, and NNA3, and NNE1, NNE2, and NNE3 are three spatial replication rings of NNA and NNE, respectively.

immobilization, nitrification, and NO_3^- immobilization (Fig. 4). However, time intervals significantly affected all gross N transformation rates except for gross NO_3^- immobilization rate ($P < 0.01$). There was no interaction between CO_2 treatments and time intervals. We further compared the CO_2 enrichment effects on gross N transformation rates between the duration of the CO_2 enrichment (7 vs.10 years). There were no interactions among N application rates, CO_2 treatments, and time intervals for each gross N transformation rate, except for the duration of the CO_2 enrichment × time interval interaction with respect to the gross N mineralization and NH_4^+ immobilization rates (Table 2). No CO_2 treatments-by-duration of the CO_2 enrichment interactions for all gross N transformation rates indicated that the CO_2 enrichment effect still had not taken place after a further three years of elevated CO_2 levels compared with the treatments that had received seven years of elevated CO_2. In the water-logged incubation in 2014, CO_2 enrichment had no significant effects on gross rates of N mineralization, NH_4^+ immobilization, nitrification, and NO_3^- consumption (Fig. 5).

Given that nitrification was almost limited by NH_4^+-N availability

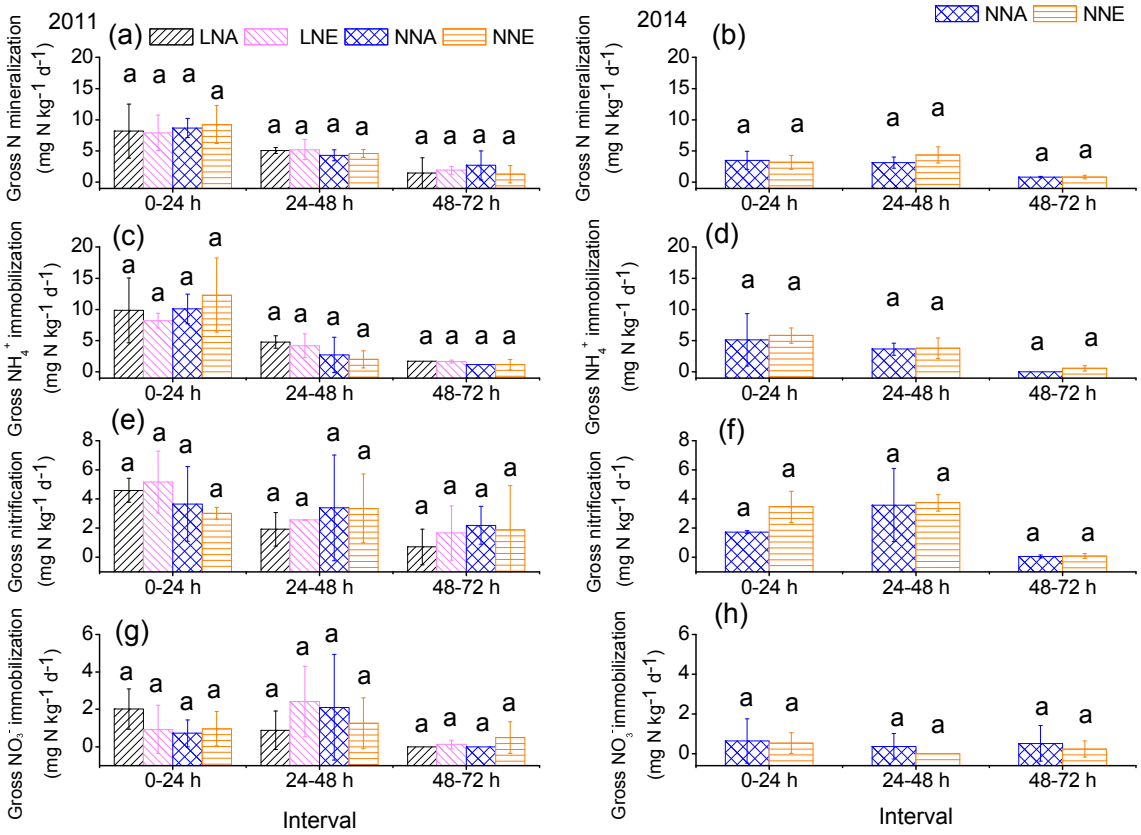

Fig. 4. Gross N mineralization (a, b), NH_4^+ immobilization (c, d), nitrification (e, f), and NO_3^- immobilization (g, h) for each time interval under aerobic incubation conditions. The left and right panels are gross N transformation rates in soil sampled in 2011 and 2014, respectively. Vertical bars are standard deviations of the mean (n = 3). Within each time interval, the same letter indicates no significant differences (P > 0.05) between ambient CO_2 (A) and elevated CO_2 (E) concentrations in the same N input treatment.

Table 2
Results of three-way ANOVA testing the effects of CO_2 enrichment (C), duration of the enrichment (D, 7 vs.10 years), time intervals (T), and their interactions on gross rates of N mineralization, NH_4^+ immobilization, nitrification, and NO_3^- immobilization during aerobic incubation.

Source of variation	C	D	T	C × D	C × T	D × T	C × D × T
	P values						
Gross N mineralization	0.915	**<0.001**	**<0.001**	0.626	0.485	**0.001**	0.623
Gross NH_4^+ immobilization	0.589	**0.048**	**<0.001**	0.993	0.255	**0.045**	0.366
Gross nitrification	0.801	0.132	**0.001**	0.350	0.871	0.146	0.185
Gross NO_3^- immobilization	0.444	0.058	0.262	0.995	0.551	0.103	0.665

in the remaining 24 h of incubation during the aerobic incubation (Figs. 1 and 2), the time-weighted average transformation rates were calculated over the first 48 h of the incubation period (Figs. 4 and 5). Gross N mineralization rates were almost equivalent to gross NH_4^+ immobilization rates, irrespective of N application rates, CO_2 treatments, duration of the CO_2 enrichment, and incubation conditions, indicating that NH_4^+ produced via mineralization was immediately consumed. Gross NO_3^- immobilization rates were generally lower than gross nitrification rates under aerobic conditions, irrespective of N application rates, CO_2 treatments, and duration of the CO_2 enrichment, resulting in net production of NO_3^- and an uncoupled microbial NO_3^- cycle. In contrast, gross nitrification rates were completely balanced by gross NO_3^- consumption rates (NO_3^- immobilization + denitrification) under water-logged conditions in both CO_2 treatments (Fig. 5b), leading to no net production of NO_3^- in the soil (Fig. 3b). Gross NH_4^+ immobilization rates were much greater than gross NO_3^- immobilization rates under aerobic conditions irrespective of N application rates, CO_2 treatments, and duration of the CO_2 enrichment, indicating a preferential utilization of NH_4^+ and a simultaneous utilization of NO_3^- by soil microbes. For the two main fates of NH_4^+, gross NH_4^+ immobilization rates generally exceeded gross nitrification rates, regardless of N application rates, CO_2 treatments, duration of the CO_2 enrichment and incubation conditions (Figs. 4 and 5).

3.3. Soil N_2O and CO_2 emissions

In 2014, we calculated soil N_2O and CO_2 emissions under both incubation conditions (Fig. 6). Elevated CO_2 levels significantly increased average N_2O emissions in both incubation conditions (Fig. 6a). Average CO_2 emissions were also enhanced by elevated CO_2 levels, but not significantly (Fig. 6b). Average N_2O and CO_2 emissions were significantly higher in the water-logged incubation than in the aerobic incubation regardless of the CO_2 treatment ($p < 0.001$).

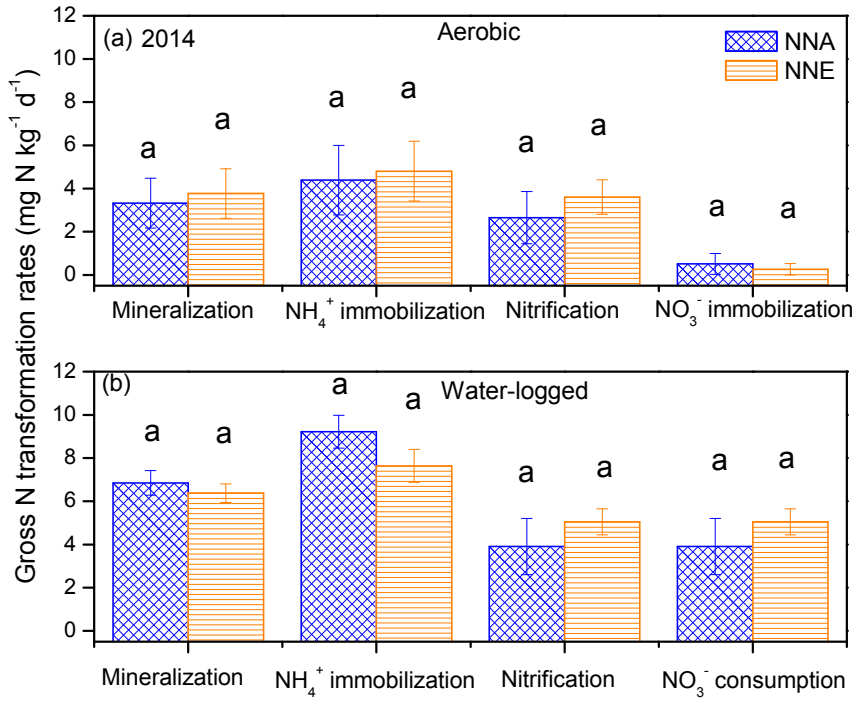

Fig. 5. Gross N mineralization, NH_4^+ immobilization, nitrification, and NO_3^- immobilization/consumption over a 48-h incubation period under ambient CO_2 levels and after 10 years of elevated atmospheric CO_2 concentrations (in 2014). The same letter indicates no significant differences ($P > 0.05$) between ambient CO_2 (A) and elevated CO_2 (E) concentrations. Vertical bars are standard deviations of the mean ($n = 3$).

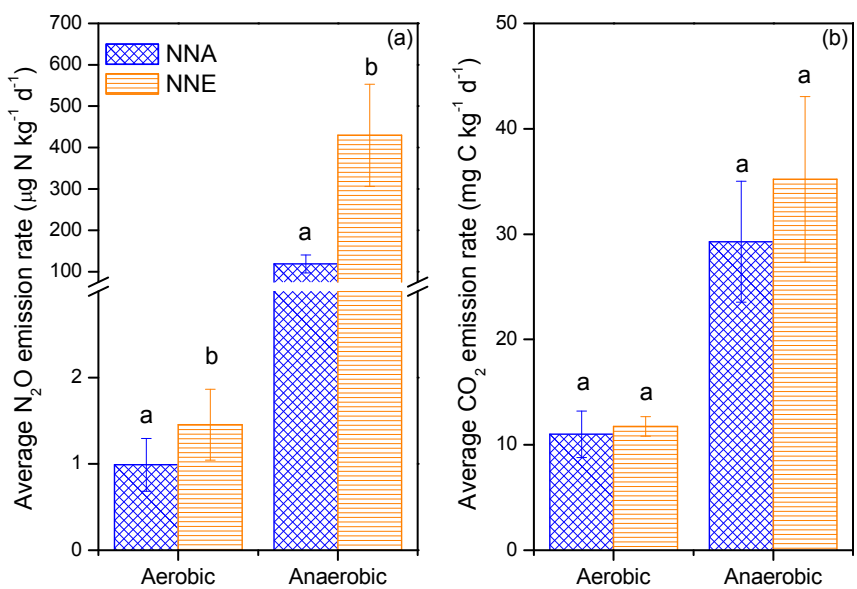

Fig. 6. Average N_2O (a) and CO_2 (b) emission rates under ambient CO_2 and after 10 years of elevated atmospheric CO_2 concentrations (2014). Average N_2O and CO_2 emission rates were calculated over the three (aerobic) and two (water-logged) days of incubation. Different letters indicate significant differences between ambient CO_2 (A) and elevated CO_2 (E) concentrations in the same incubation condition at $P < 0.05$. Vertical bars are standard deviations of the mean ($n = 6$).

4. Discussion

By combining our 2011 results after seven years of CO_2 enrichment and out 2014 results after 10 years of CO_2 enrichment, we found that 10 years of CO_2 enrichment had no effects on gross rates of N mineralization, NH_4^+ immobilization, nitrification, and NO_3^- consumption regardless of N application rate and incubation conditions in a rice paddy field, resulting in no changes in available N for plants and soil microbes. Until now, studies on the response of soil gross N transformation rates to elevated CO_2 levels were primarily conducted in natural ecosystems that are not supplemented with additional N, such as grasslands, forests, deserts, and heathland (Zak et al., 2003; Barnard et al., 2005; De Graaff et al., 2006; West et al., 2006; McKinley et al., 2009; Rütting et al., 2010; Rütting and Andresen, 2015), and data for intensively managed agricultural soils are entirely lacking. In addition, very few studies

have investigated the effects of elevated CO_2 levels on soil gross N transformation rates under water-logged conditions, because soil is often exposed to transiently flooded conditions. Therefore, the results of the rice paddy soil studied here could reinforce our understanding of the effects of elevated CO_2 concentrations on soil gross N transformation rates in intensively managed agricultural ecosystems. In addition our results will provide associated information to enable more complete assessments of the response of soil gross N transformation rates to elevated CO_2 levels in terrestrial ecosystems rather than in natural and seminatural ecosystems alone (Barnard et al., 2005; Rütting and Andresen, 2015).

4.1. Gross N mineralization and NH_4^+ immobilization rates in response to elevated CO_2 levels

Contrary to our hypothesis, gross N mineralization rates were not stimulated by elevated CO_2 levels under both aerobic and water-logged conditions, which is in agreement with previous studies on forest (Finzi and Schlesinger, 2003; Holmes et al., 2003; Zak et al., 2003; McKinley et al., 2009) and grassland soils (Hungate et al., 1997; West et al., 2006; Müller et al., 2009). Meta-analyses by De Graaff et al. (2006) and Rütting and Andresen (2015) further indicated that elevated CO_2 levels did not alter gross N mineralization rates when investigating results from free air carbon dioxide enrichment (FACE) and open top chamber (OTC) studies. It is well demonstrated that gross N mineralization is controlled by soil organic C and N concentrations (Booth et al., 2005). In this study, CO_2 enrichment did not affect soil organic C and N concentrations, irrespective of N application rates and the duration of the CO_2 enrichment (Table 1). This could result from the counteracting effects of increased total organic matter supply via plant production and root exudation versus decreased N concentrations in plant organic inputs (West et al., 2006), or of the counterbalancing of increased total organic matter supply against increased soil microbial C turnover (van Groenigen et al., 2014). Therefore, the lack of gross N mineralization in response to elevated CO_2 levels could be attributed to the absence of elevated CO_2 effects on soil organic C and N concentrations after seven and 10 years of continuous CO_2 enrichment.

Indeed, the stimulation of gross N mineralization by elevated CO_2 levels has been reported in several earlier studies (Holmes et al., 2006; Hu et al., 2006; Rütting et al., 2010). These authors suggested that gross N mineralization under elevated CO_2 levels might be site-specific or ecosystem N status-specific. Rütting and Andresen (2015) further conducted ecosystem specific analyses, and proposed that gross N mineralization is only stimulated in N-limited ecosystems, but is unaffected in phosphorus (P)-limited ecosystems. Rice paddy soil might not be constrained by N due to the large rate of N fertilizer applications (125–225 kg N ha^{-1} yr^{-1}), which is much higher than the amount required for optimal rice uptake (55.7–62.3 kg N ha^{-1} yr^{-1}) (Zhao et al., 2012), and thus gross N mineralization was insensitive to elevated concentrations of CO_2.

The response of gross N and NH_4^+ immobilization rates to elevated CO_2 levels was highly variable across studies, and the potential mechanisms are generally poorly understood. Elevated CO_2 levels have been found to stimulate gross N and NH_4^+ immobilization, which has been attributed to increased soil C availability via plant production and rhizodeposition, considering that soil microorganisms are generally C-limited (De Graaff et al., 2006; Holmes et al., 2006; Müller et al., 2009; Rütting et al., 2010; Björsne et al., 2014). In contrast, a review by Hu et al. (2006) found that elevated CO_2 levels did not affect gross N immobilization in nine out of 12 studies. Our results also showed that both gross N and NH_4^+ immobilization rates were not affected by elevated CO_2 levels irrespective of N application rates. Since gross N and NH_4^+ immobilization is mainly regulated by soil organic C concentration (Booth et al., 2005), the lack of elevated CO_2 effects on soil organic C concentration could be responsible for the lack of response of soil gross N and NH_4^+ immobilization rates to elevated CO_2 in the rice paddy soil studied. Holmes et al. (2003) also found that elevated CO_2 levels had no effect on soil organic C and N concentrations, and also on gross N mineralization and NH_4^+ immobilization rates in forest soils. Soil CO_2 emissions, as an index of soil microbial activity, were not significantly different between ambient CO_2 and elevated CO_2 treatments for both incubation conditions (Fig. 6b), which indirectly demonstrated that elevated CO_2 levels did not change soil microbial activity, and thus there was no additional heterotrophic N demand. A recent study confirmed that elevated CO_2 did not affect soil bacterial communities in a rice paddy soil (Ren et al., 2015). However, increased organic C concentration but no change in gross NH_4^+ immobilization by elevated CO_2 levels has been observed in a pine forest soil (Finzi and Schlesinger, 2003). These results may suggest that additional plant production and substrate inputs to soil by elevated CO_2 concentrations are not sufficient to overcome the effects of the native soil organic matter on microbial activity and soil N transformations (Finzi and Schlesinger, 2003; Holmes et al., 2003).

In summary, we did not detect a decrease in the potential plant-available N supply in elevated CO_2 soils as predicted by the progressive N limitation (PNL) hypothesis (Luo et al., 2004). PNL develops only if increased CO_2 levels cause long-lived plant biomass and soil organic matter to accumulate, sequestering substantial amounts of both C and N into long-term pools (Luo et al., 2004). However, such phenomenon did not occur in the rice paddy soil studied. Even if it did occur, the additional N supply from annual N fertilization was able to counteract N sequestration in plant biomass and soil organic matter, and thus prevent or alleviate PNL in an intensively managed agricultural ecosystem.

4.2. Gross nitrification and NO_3^- consumption in response to elevated CO_2 levels

In contrast to our hypothesis, gross nitrification rates were not sensitive to elevated CO_2 levels, irrespective of N application rates, incubation conditions, and the duration of the CO_2 enrichment. Numerous studies have reported that elevated CO_2 decreased gross nitrification rates (Hungate et al., 1997; Müller et al., 2009; Rütting et al., 2010; Björsne et al., 2014). A meta-analysis by Rütting and Andresen (2015) found that gross nitrification rates tended to decrease ($p < 0.09$), and varied with ecosystem type in response to elevated CO_2 levels. For example, gross nitrification rates decreased significantly in deserts, increased slightly in forest soils, and exhibited a numerical decrease in grassland FACE due to elevated CO_2 levels. NH_4^+ immobilization by microorganisms and oxidation of NH_4^+ to NO_3^- by nitrifiers are the two main fates of NH_4^+ in a plant-free environment. Therefore, a decline in gross nitrification rates by elevated CO_2 could be due to the stimulation of gross NH_4^+ immobilization, resulting in less available NH_4^+ for nitrifiers (Hungate et al., 1997). Our results showed that gross NH_4^+ immobilization rates were not affected by elevated CO_2 levels irrespective of N application rates, incubation conditions, and duration of the CO_2 enrichment. As a consequence, the balance between nitrification and NH_4^+ immobilization was not broken by elevated CO_2 (Holmes et al., 2003).

Generally, medium soil moisture content (usually 60–80% WFPS) is considered to be optimal for nitrification (Bateman and Baggs, 2005; Kiese et al., 2008). However, it is surprising to find similar rates of gross nitrification under aerobic and water-logged conditions. Under flooded conditions, the thicker the water layer

on the soil surface, the lower the oxygen content of the soil, and the lower the nitrification rate (Yoshida and Padre, 1974). It was likely that oxygen content under water-logged conditions (only 1.5-cm water layer) was sufficient for the occurrence of nitrirfication in the rice paddy soil studied. In addition, the surface water layer of paddy soil can be differentiated into oxidized and reduced layer. It has been demontrated that the number of nitrifiers in the oxidized layer was greater than that in the reduced layer (Chen et al., 1981; Li et al., 1983). As a result, sufficient oxygen and the existence of nitrifiers in the oxidized layer could be responsible for high rate of nitrification under water-logged conditions.

In non-planted systems, denitrification is considered to be negligible and NO_3^- consumption is often reported directly as NO_3^- immobilization under aerobic conditions, whereas denitrification may play a predominant role in NO_3^- consumption under water-logged conditions (Murphy et al., 2003). It is well documented that microorganisms prefer NH_4^+ over NO_3^- due to the higher energy costs associated with microbial assimilation of NO_3^- (Recous et al., 1992; Lindell and Post, 2001). The amount of available C is an important factor controlling microbial immobilization of NO_3^- (Shi and Norton, 2000; Bradley, 2001). In this study, elevated CO_2 did not change the soil organic C concentration, and thus could not stimulate the microbial demand for NH_4^+, let alone enhance NO_3^- immobilization. Under water-logged conditions, denitrification rates (NO_3^--N reduction rate) were not affected by elevated CO_2 levels. This was also due to the absence of changes in soil organic C concentration under conditions of elevated CO_2, as the denitrification rate was also shown to be limited by C availability (Devito et al., 2000; Pabich et al., 2001). Although very few studies have investigated the denitrification rates in response to elevated CO_2 levels under water-logged conditions, denitrifying enzyme activities (DEA) have been extensively examined and no consistent results were obtained (Billings et al., 2002; Barnard et al., 2004, 2005; Zhong et al., 2015).

Although our study showed that the duration of CO_2 enrichment did not affect the response of gross N transformation rates to elevated CO_2 levels, the duration of elevated CO_2 has been demonstrated to affect gross N transformation rates in other studies (Holmes et al., 2006; McKinley et al., 2009). For instance, in a woodland under elevated CO_2, the gross rates of N mineralization and N immobilization were reduced after six years, but were slightly increased when resampled after 11 years of CO_2 enrichment (McKinley et al., 2009). Therefore, caution is required in the interpretation of our results to predict the effects of elevated CO_2 on soil N availability and N losses in light of the fact that these phenomena can change over time with continued exposure to elevated concentrations of CO_2.

4.3. Soil N_2O emissions in response to elevated CO_2

The results of previous studies on the effects of elevated CO_2 levels on N_2O emissions are quite contradictory, with an increase (Baggs and Blum, 2004; Kammann et al., 2008) or no change (Billings et al., 2002; Rütting et al., 2010; Carter et al., 2011) in N_2O emissions in response to elevated CO_2. In a review of 20 experiments, Barnard et al. (2005) found that field N_2O fluxes were not altered by elevated CO_2 levels. In contrast, our results showed that elevated CO_2 significantly stimulated N_2O emissions for both incubation conditions. The magnitude of the N_2O emissions were assumed to depend on the rates of nitrification and denitrification ("size of the pipes") and the ratio of N_2O to the end products ("size of the holes in the pipes") in the 'hole-in-the-pipe' model (Firestone and Davidson, 1989). Since gross nitrification and denitrification rates were not affected by elevated CO_2 levels, the stimulation of N_2O emissions by elevated CO_2 could be attributed to an increased rate of N_2O emissions from nitrification and denitrification. A further investigation is required to identify N_2O production via nitrification and denitrification under conditions of elevated CO_2.

5. Conclusion

Throughout this study, we demonstrated that 10 years of CO_2 enrichment had no effects on gross rates of N mineralization, NH_4^+ immobilization, nitrification, and NO_3^- consumption regardless of N application rates and incubation conditions in a rice paddy field. This resulted in no changes in the available N for plants and soil microbes, and no enhanced risks of N losses through leaching and runoff. These results are probably tied to the fact that there were no changes in soil organic C and N concentrations due to elevated CO_2 levels. However, elevated CO_2 significantly stimulated N_2O emissions in both incubation conditions, but the possible mechanism behind this remains unclear. It should be noted that this study was conducted in the laboratory under controlled incubation conditions, in which cold storage, sieving, ^{15}N addition, water and temperature manipulation could change the true N transformation rates in situ, caution thus should be exercised when extrapolating these results to the field, and further in situ research needs to be taken into account to confirm our results.

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