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RESEARCH ARTICLE

Compositional variations of active autotrophic bacteria in paddy soils with elevated CO_2 and temperature

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ABSTRACT

Global warming is an increasingly serious ecological problem, we examined how the active autotrophic microbes in paddy soils respond to the elevated CO_2 and temperature. Here we employed stable isotope probing (SIP) to label the active bacteria using the soil samples from a fully factorial Simulated Climate Change (SCC) field experiment where soils were exposed to ambient CO_2 and temperature, elevated temperature, elevated CO_2, and both elevated CO_2 and temperature. Around 28.9% of active OTUs belonged to ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). *Nitrosospira* taxa was dominant in all soils and 80.4% of carbon-fixing bacteria under elevated temperature were classified as *Nitrosomonas nitrosa*. While no labeled NOBs were detected when temperature or CO_2 were elevated independently, diverse NOBs were detected in the ambient conditions. We found that elevated CO_2 and temperature had contrasting effects on microbial community composition, while relatively small changes were observed when CO_2 and temperature were elevated simultaneously. Summarily these results suggest that carbon-fixing bacteria can respond positively to elevated CO_2 concentrations, but when it's accompanied with increase in the temperature this positive response could be weakened. Multiple abiotic factors thus need to be considered when predicting how microbial communities will respond to multiple climatic factors.

1 Introduction

Climate model projections indicate that the atmospheric carbon dioxide (CO_2) concentration may double and the global average temperature will rise by another 1.1–6.4°C during the 21st century (Stocker et al., 2013). The properties and functions of terrestrial ecosystems would be profoundly altered under this unprecedented global climate change (Rosenzweig et al., 2007) and will lead to massive losses of habitat and organism diversity (Wetzel et al., 2012; Courchamp et al., 2014). Therefore, it is urgently necessary to relieve CO_2 concentration elevation by as many measures as possible. Terrestrial ecosystems are the dominant sink for

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global CO_2 emissions and are of significant interest because of their potential to alleviate atmospheric CO_2 levels (Lal, 2008). Compared to soils of upland crops, paddy soils tend to accumulate more soil organic carbon (SOC) (Sahrawat, 2004; Kögel-Knabner et al., 2010; Sun et al., 2015). This is mainly caused by the limited availability of oxygen under flooded conditions, which slows down decomposition of organic inputs, especially lignin and other phenolic compounds (Olk and Senesi, 2000; Gao et al., 2016).

Paddy soils cover a total area of approximately 165 million ha globally (Ge et al., 2015). 26% of cropland in China is used for rice cultivation, which is significant C sink, potentially sequestering up to 0.7 Pg C annually (Pan et al., 2004). Paddy ecosystems, especially in China, are thus critically important for CO_2 fixation. The role of plants in C fixation and belowground input is well known. Plant photosynthesis and C allocation belowground can buffer the increasing CO_2 on the global C budget (Piao et al., 2009; Bruggemann et al., 2011; Pausch and Kuzyakov, 2018). Microorganisms also play an important role for C cycle and CO_2 fixation (Long et al., 2015; Pratscher et al., 2011), but contributions made by active soil microorganisms in the field is less studied. Miltner et al. (2005) reported that the amount of fixed CO_2 in soil microorganisms reaches up to 3%–5% of the respiration emissions (Miltner et al., 2005). Yuan et al. (2012) showed that soil autotrophic microorganisms can convert CO_2 into soil organic matter (SOM) with an assimilation rate of about 0.0134 to 0.103 g cm^{-2} d^{-1}. Regrettably, there is a lack of understanding of the importance and distribution of soil microbial CO_2 fixation in paddy soils.

Nitrification, the biological oxidation of ammonia to nitrate via nitrite, is a quite important part of the global biochemical cycle (Galloway et al., 2008). As is well-known, autotrophic ammonia-oxidizing bacteria (AOB) perform the function of ammonia oxidation which is the first step of nitrification (Tourna et al., 2010). Meanwhile, Vuillemin et al. (2019) reported that nitrite-oxidizing bacteria (NOB) fix carbon via the rTCA cycle. This implies that AOB and NOB have coupling effect between carbon and nitrogen cycle. Paddy fields are a unique anthropogenic aquatic ecosystem. Irrigation management causes oxygen rapid depletion below the soil surface, where an oxygen gradient can be taken shape within a few millimeters of the soil surface (Liesack et al., 2000). The activity of AOB and NOB may be regulated by oxygen deficiency. Furthermore, irrigation often leads to the accumulation of electron donors and the successive decrease of electron acceptors in soil organic matter, for instance nitrate and sulfate (Kimura, 2000). These changes in soil physiochemical properties will also lead to changes in AOB or NOB communities (Ke et al., 2013).

A field study for Simulating Climate Change (SCC) experiment was conducted since 2011 with free air CO_2 enrichment and an elevated temperature in an annual rice–wheat rotation system in Changshu, Jiangsu Province, China and many researches has been published based on this experiment (Liu et al., 2014; Cai et al., 2016; Wang et al., 2016). Although a number of SCC experiments have been carried out for a broad range of questions (Del Galdo et al., 2006; Jin and Evans, 2007; Steven et al., 2014), the effects on soil microbial community, and particularly, the responses of active autotrophic microorganisms on elevated temperature and CO_2 are still unknown. Stable isotope probing (SIP) of nucleic acids, a culture-independent method for targeting the active microbes, allows specific identification of organisms assimilating labeled substances, most commonly carbon from a particular ^{13}C-labeled substrate (Radajewski et al., 2000; Dumont and Murrell, 2005). This method was available to identify the key autotrophic microbes which are the authentic drivers in the soil. We hypothesized that simultaneous existence of climate changes would result in more intense variation to bacterial community, since both CO_2 and temperature increases could be set up at the same time which gives rise to accelerated environmental change. This study aimed to answer the following main questions: (1) how do composition and diversity of active bacterial communities respond to climate change? (2) What are the primary ecological processes modifying the active bacterial communities in different climate scenario?

To test this, we collected soils from the SCC field experiment and used DNA-SIP technique to recognize the active bacterial species assimilating $^{13}CO_2$ from the atmosphere. We aimed to record possible shifts in the dominant active bacteria and identify the responsible ecological processes under elevated temperature and CO_2 in paddy soil.

2 Materials and methods

2.1 The field experiment design and soil sampling

A Simulated Climate Change (SCC) field experiment with an annual rice–wheat rotation has been running since 2011 in Changshu, Jiangsu Province, China (31°30′N, 120°33′E). The mean annual temperature and mean precipitation in the region are 16°C and 1100–1200 mm, respectively. A wheat (*Triticum aestivum* L.) – rice (*Oryza sativa* L.) crop rotation system has been maintained throughout the experiment. Seeds of rice and wheat are sown in November and June, respectively (Liu et al., 2014; Cai et al., 2016; Wang et al., 2016).

At the beginning of the experiment, the soil contained 16 g kg^{-1} organic matter, 1.9 g kg^{-1} total nitrogen (N), and 0.9 g kg^{-1} total phosphorus (P) and had a pH (H_2O) of 7.0. The experiment has four treatments consisting of 1) aCO_2-aTemp treatment (a control with ambient CO_2 and temperature), 2) eTemp treatment (elevated temperature by 2°C in canopy air), 3) eCO_2 treatment (elevated atmospheric CO_2 concentration of 500 μmol mol^{-1}), and 4) eCO_2-eTemp treatment (elevated CO_2 combined with elevated temperature). Each treatment was replicated in three plots (each plot = 50 m^2) and arranged using a randomized design and details regarding CO_2 concentration and temperature regulation during the field experiment have been described earlier (Liu et al., 2014).

Urea (46% N) and compound fertilizer containing N, P and K were used throughout the experiments and fertilization was applied four times during the rice and three times during the wheat crop seasons. Urea was applied two days before rice transplantation and wheat seeding at an average rate of 190 kg N ha^{-1} as a basal fertilizer. A compound fertilizer (375 kg ha^{-1} on average) was applied as topdressing fertilizer after heading. Thereafter, urea was applied two times during the rice and once during the wheat crop season as a topdressing fertilizer at an average concentration of 150 kg N ha^{-1}. Paddy rice was irrigated with a continuous flooding regime with two periods of drainage mid-season. Soil samples (the top 0–20 cm) were collected with a 2.5 cm diameter auger from three replicate plots within each treatment after the rice seeding in 2016. All samples were homogenized by passing through a 2.0 mm sieve after each soil sample was divided into two parts and approximately 200 g of fresh sample was stored at 4°C for the microbial DNA-SIP analysis and the second sample was air-dried for chemical analyses.

2.2 Soil chemical measurements

The soil moisture was determined by drying soils in an oven at 105°C for 12 h. The concentrations of soil NH_4^+ and NO_3^- were measured with a continuous-flow stream autoanalyser (Autoanalyzer 3, Bran Luebbe, Norderstedt, Germany) after shaking with 0.01 M $CaCl_2$ solution (1:10 of soil: solution by mass) for 30 min. Soil organic carbon (SOC) and total nitrogen (TN) were measured with a CN Analyzer (Vario Max CN, Elementar, Hanau, Germany). The electrical conductivity of the saturated paste extraction (EC) was measured using a conductivity meter (DDS-307A, REX, Shanghai), and pH was determined with air-dried soil (soil:water, 1:5) by pH meter (PHS-3C, REX, Shanghai)

2.3 Soil DNA-SIP microcosm experiment

DNA stable isotope probing (DNA-SIP) was used to determine the functionally active microbes in present in soil samples. To this end, 10 g of oven-dried and sieved fresh soil samples with a 40% soil maximum water holding capacity were transferred into 120-mL serum bottles which were sealed with rubber stoppers and aluminum caps for a 14-day pre-incubation period. Each independent soil sample for DNA-SIP microcosms was treated in two replicates with either $^{13}CO_2$ or $^{12}CO_2$ (100 Pa) injections. Five percent CO_2 (5% v/v $^{12}CO_2$ or $^{13}CO_2$) was added to the headspace, and $^{13}CO_2$ microcosms received 100 μg ^{13}C-urea-N g^{-1} once per week and the $^{12}CO_2$ microcosms received 100 μg ^{12}C-urea-N g^{-1}*d.w.s* once per week during the eight-week incubation period. Urea was added as a N source to simulate paddy soil environments in China which typically receive large amounts of N fertilizer. However, urea was only applied during the DNA-SIP experiment and not during the two-week long pre-incubation phase to reduce the dilution of $^{13}CO_2$ due to soil respiration. High soil urease activity rapidly hydrolyzed the urea into ammonium carbonate and ammonium carbonate easily released ammonia and CO_2 (Shi et al., 2007). In addition, the added ^{13}C-urea maintained a constant $^{13}CO_2$ release at a high concentration during the soil microcosm incubation. The ^{13}C-urea (99 atom % carbon) was purchased from the Shanghai Engineering Research Center of Stable Isotopes (Shanghai, China) and $^{13}CO_2$ (99 atom % carbon) was purchased from Cambridge Isotope Laboratories, Inc. The $^{12}CO_2$ was produced by acidifying calcium carbonate. The bottles were sealed tightly with black butyl stoppers and incubated at 25°C in the dark for 56 days. Every week, synthetic air (80% N_2, 20% O_2) was used to flush the bottles and replenish with 5% CO_2 ($^{12}CO_2$ or $^{13}CO_2$). Destructive sampling from each microcosm was performed at the end of the incubation period and the soil samples were transferred immediately to a –80°C freezer for subsequent molecular analysis.

2.4 DNA extraction, density gradient centrifugation and fractionation

A FastDNA Spin Kit for Soil (MP Biomedicals, Cleveland, OH, USA) was used to extract DNA from the soil samples of the field experiment and microcosm experiment. The concentration and quantity of extracted soil DNA were determined photometrically by a Nanodrop ND-1000 UV–vis Spectrophotometer (Nano Drop, ND2000, Thermo Scientific, 111 Wilmington, DE). Density gradient centrifugation and fractionation were used for soil samples from the DNA-SIP experiment and performed as reported previously with some modifications (Wang et al., 2015). Briefly, 3 μg of DNA was added to a 1.85 g mL^{-1} CsCl gradient buffer (0.1 M Tris-HCl, 0.1 M KCl, 1 mM EDTA, pH = 8.0) with an initial CsCl buoyant density of 1.725 g mL^{-1}, which was prepared by adjusting the refractive index to 1.4025 with an AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA). Density gradient centrifugation was performed in 5.1 mL Quick-Seal polyallomer ultracentrifugation tubes (Beckman Coulter, Palo Alto, CA, USA) in a VTi 90 vertical rotor (Beckman Coulter) and was subjected to centrifugation at 177 000 *g* for 44 h at 20°C. Different buoyant density DNA fractions were obtained by replacing the gradient medium with sterile water from the top of the ultracentrifuge tube using a syringe pump (Longer Pump. LSP01-1A, China) with controlled the flow rate at 0.38 mL min^{-1}. Finally, 14 DNA fractions were obtained with equal volumes of 380 μL, and the density of each collected fraction was determined from a small aliquot (50 μL) using AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA). The DNA fractions were purified and then dissolved in 30 μL of TE buffer as described previously (Freitag et al., 2006) before sequencing.

2.5 High-throughput sequencing

To verify the presence of ^{13}C-DNA in the density fractions before high-throughput sequencing we tested two marker genes that could be used to identify autotrophic CO_2-fixing bacteria: *cbbL* and *amoA* genes. The *cbbL* gene was chosen

because it encodes the key active-site residue in the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) (Kusian and Bowien, 1997; Tabita et al., 2007) affecting CO_2 fixation via the Calvin-Benson-Bassham (CBB) cycle (Ortiz et al., 2014). As a result, RuBisCO l-encoding *cbbL* genes could be used as a functional marker to differentiate the heavy and light fraction in SIP. However, due to unknown reasons we failed to amplify the *cbbL* genes in density fractions. As a result, we selected the bacterial *amoA* gene as the marker gene, which is indicative of autotrophic ammonia-oxidising bacteria, to distinguish the heavy and light fraction in DNA-SIP analysis. To quantify this, a quantitative real-time PCR was carried out in triplicate using an ABI 7500 real-time PCR system (Applied Biosystems, America) with SYBR Premix Ex *Taq* (TaKaRa) to determine gene abundances in fractionated DNA. The following targeted qPCR primers were used for the bacterial *amoA* genes: *amoA*-1F (5′-GGG GTT TCT ACT GGT GGT-3′) and *amoA*-2R (5′- CCC CTC KGS AAA GCC TTC TTC-3′). Each 25 μL qPCR reaction contained 12.5 μL of SYBR® Premix Ex Taq™, 1 μL of DNA, 0.5 μL of each primer (10 μM), 0.5 μL of ROX Reference Dye II (50 ×) and 10 μL of sterile water. All PCRs started with an initial enzyme activation step performed at 95°C for 5 min followed by 40 cycles of 95°C for 5 s, 60°C for 34 s, and a final temperature increase to 95°C for 15 s. Data were collected after each annealing step. Standard curves were constructed by a 10-fold serial dilution of the known copy numbers of plasmids harbouring the target gene. The efficiencies ranged from 102% to 103%, and the R^2 values ranged from 0.998 to 0.999.

Based on the qPCR results, ^{13}C-DNA was accurately distinguished into heavy fractions of the DNA-SIP samples. The DNA present in the heavy fractions of DNA-SIP samples and DNA collected from the field experiment samples were subsequently characterized using Illumina MiSeq System at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). To investigate the composition of the active bacterial communities, the V4–V5 region in the 16S rRNA gene was amplified using the primer pair 515F (5′- GTG CCA GCM GCC GCG GTA A-3′) and 907R (5′- CCG TCA ATT CMT TTR AGT TT-3′) (Krepski et al., 2012).

The raw high-throughput sequencing data was analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) toolkit (Caporaso et al., 2010) and the UPARSE pipeline (Edgar, 2013). The DNA sequences were filtered using quality files, and the remaining sequences were trimmed with barcodes and forward primers and sequences with low quality (quality score < 20, length < 300 bp) were excluded. And then the sequencing data were treated to remove chimeras from the data sets. After optimizing the sequences, the UPARSE pipeline was used to construct an OTU table. The 16S rRNA sequences were binned into OTUs using a 97% identity threshold, and the most abundant sequence from each OTU was selected as the representative sequence for that OTU. We used the SILVA database (https://www.arb-silva.de/, Version 119) to assign each representative sequence from 16S rRNA data to a taxon. All the OTUs belonging to archaea were removed because of the extremely low number of reads detected in all samples. The all selected active microbes were not autotrophic probably because of cross-feeding, which is nevertheless an important process that governs the growth and composition of microbial ecosystems in the exchange of essential metabolites. Some heterotrophic microbes inevitably incorporate ^{13}C into their DNA in this system and thus we referred to other studies to screen for the active autotrophs (Pratscher et al., 2011; Yuan et al., 2012; Long et al., 2015; Wang et al., 2015).

Ammonia-oxidizing bacteria (AOB) (Jia and Conrad, 2009) and nitrite-oxidizing bacteria (NOB) (Vuillemin et al., 2019) were identified as CO_2 fixing bacteria in some previous researches. All of the sequences were deposited in the NCBI Sequence Read Archive database (accession numbers SRP135916).

2.6 Statistical analysis

ANOVA was used to compare the means for soil physico-chemical and microbial diversity data with Duncan's least significant difference (p = 0.05) using IMB SPSS statistical software package version 20 (IBM Corporation, New York, USA). Principal component analysis (PCA) and PERMANOVA were used to analyze 16S rRNA communities in soils for all the treatments in R software with "vegan" package. To identify OTUs that were correlated with $^{13}CO_2$ assimilation, *DESeq2* R package was used to analyze differential OTU relative abundance based on the negative binomial distribution in the heavy fractions of the ^{13}C-treatments relative to the corresponding fractions from the $^{12}CO_2$-treated control (McMurdie and Holmes, 2014; Pepe-Ranney et al., 2016). Log2-fold change values higher than zero with adjusted p-values (FDR-adjusted p-value) lower than 5% were selected as ^{13}C-labeled OTUs.

To evaluate the degree of non-random phylogenetic structure of our communities, we calculated the nearest relative index (NRI) using 'ses.mpd' R function, NRI is equivalent to 1 times the output of 'ses.mdp' (Kembel et al., 2010), in which the mean phylogenetic distance (MPD) for each OTU was weighted by its abundance, and the observed MPD was compared with the null distribution of MPD generated by the 1000 randomizations of "phylogeny.pool" null model. The NRI (nearest relatedness index) is a standardized measure of the mean pairwise phylogenetic distance of taxa in a sample (Webb et al., 2002), and through which we could infer the importance of niche-based and neutral processes in driving community assembly (Kembel, 2009). In general, a mean NRI across all samples that is significantly greater than zero is correlated with phylogenetic clustering, equal to zero with random, and less than zero with over-dispersion (Kembel, 2009). The significant difference between NRI and null expectation of zero was tested using two-tailed t-test at the 95% confidence level.

The representative sequences with the longest lengths for the labeled OTUs were selected for a subsequent construc-

tion of phylogenetic tree, which was conducted with MEGA 6 through neighbor-joining trees using a Kimura 2-parameter distance model with 1000 bootstrap replicates. All analyses were conducted using IMB SPSS statistical software package version 20 (IBM Corporation, New York, USA) and R (2.15.3) and in all tests, p-values < 0.05 were considered as statistically significant differences.

3 Results

3.1 Responses of the soil chemical properties to the climate change

As Fig. 1 and Table S1 showed, compared to the aCO_2-aTemp soil, the pH increased for 0.18 units in the eTemp soil but decreased for 0.05 and 0.31 units in the eCO_2 and eCO_2-eTemp soil. Elevated temperature decreased both the total nitrogen (TN) and soil organic carbon (SOC) for about 7%, while TN and SOC increased about 13% and 33% in eTemp and eCO_2-eTemp soils, respectively. Elevated CO_2 can increase the storage of soil C pools and nitrogen pools. However, C:N ratio had a slight increase in treated soil compared to the control soil. As long as elevated temperature increased the electric conductivity (EC) of the soil for 31.9% and 11.2% in the eTemp and eCO_2-eTemp soils, while only elevated CO_2 decreased the soil EC by 11.9%.

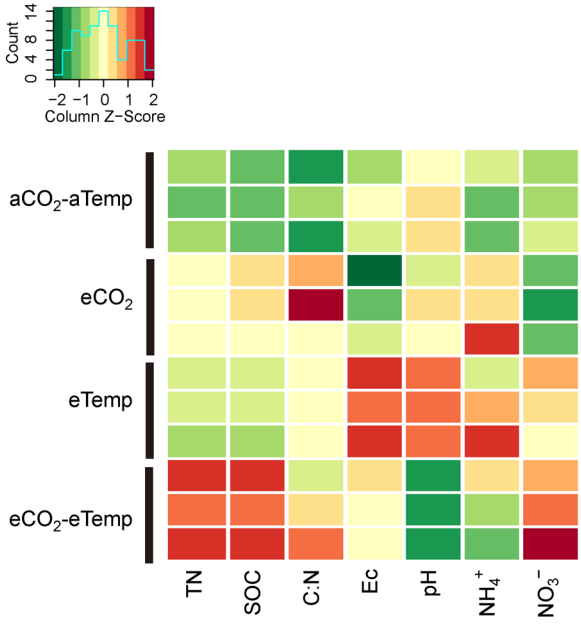

Fig. 1 Heatmap of the chemical properties of the four soils under climate change. SOC represents soil organic carbon, TN represents soil total nitrogen, DOC represents dissolved organic carbon, and NH_4^+ and NO_3^- represent ammonium nitrogen and nitrate nitrogen, respectively. Each treatment has three replications. aCO_2-aTemp: ambient CO_2 and temperature; eTemp: elevated temperature; eCO_2: elevated CO_2; eCO_2-eTemp: elevated CO_2 and elevated temperature.

3.2 Community composition of active bacteria assimilating CO_2

The marker gene, ammonia-oxidizing bacterial (AOB) gene, well discriminated the ^{13}C-DNA and ^{12}C-DNA in SIP into 15 density-gradient fractions (Fig. 2). In the ^{12}C-CO_2 microcosms, the relative abundance of the AOB gene was mainly distributed in the buoyant density 10–11th fractions (light fraction). DNA in ^{13}C-CO_2 microcosms distributed in the 5–6th fractions (heavy fraction). There was only obvious marker gene in the heavy fractions, indicating that active microorganisms took advantage the ^{13}C-CO_2 into the DNA and all ^{13}C-CO_2 treatments successfully labeled the DNA. Differently labeled OTU abundance analysis was conducted by *DESeq2*. The volcano plot indicated that 16, 2, 13 and 19 OTUs were labeled in aCO_2-aTemp, eTemp, eCO_2 and eCO_2-eTemp treatments, respectively (Fig. S1a). A Venn diagram showed (Fig. S1b) that there was only one OTU in the intersection of the all treatments, which allowed us to infer that this OTU may be widespread in active species in the paddy soils. The ^{13}C-labeled OTUs mainly belonged to *Nitrosomonadaceae, Bradyrhizobiaceae, Rhizobiaceae, Sphingomonadaceae,* and *Xanthomonadaceae* (Fig. 3). *Nitrosomonadaceae* was generally abundant in all soils, while the eTemp soil only contained *Nitrosomonadaceae* in the ^{13}C labeled fraction. The eCO_2 soil had the most categories of active microbes that included the typical families, mainly including *Microbacteriaceae, Nocardiaceae* and *Methylobacteriaceae.* However, the aCO_2-aTemp and eCO_2-eTemp soils had the most similar composition (Fig. 3). PCA (Fig. 4A) revealed clear temperature-only and CO_2-only effects on shift of active bacterial community composition, which somehow counteract toward an only smooth community variation if both factors increase simultaneously. The result of the two-way PERMANOVA analysis (Fig. 4B) more precisely revealed the aforementioned consequence, demonstrating that elevating temperatures or CO_2 individually dramatically affected community composition, but the interaction had little effect on the community composition.

3.3 Phylogenetic analysis of autotrophic bacteria

The nearest relative index (NRI) of the active bacterial community declined in the simulate climate change conditions, especially dramatically declined in the eCO_2 soil (NRI < 0) (Fig. 5). These results indicate that the phylogenetic structure of active bacterial communities in the control soil and in the eTemp soil were all the result of stochastic (= random) events, the eTemp trended to result in the clustered community more dispersed than control, and the elevated CO_2 made the soil active bacterial community over-dispersed.

According to the Fig. S3, 28.9% of active OTUs were founded to be two autotrophic groups: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). A total of seven OTUs were identified as AOB (Fig. S3) that were divided to three groups including *Nitrosospira* cluster 3, *Nitrosomonas* cluster 6b and *Nitrosomonas* cluster 8

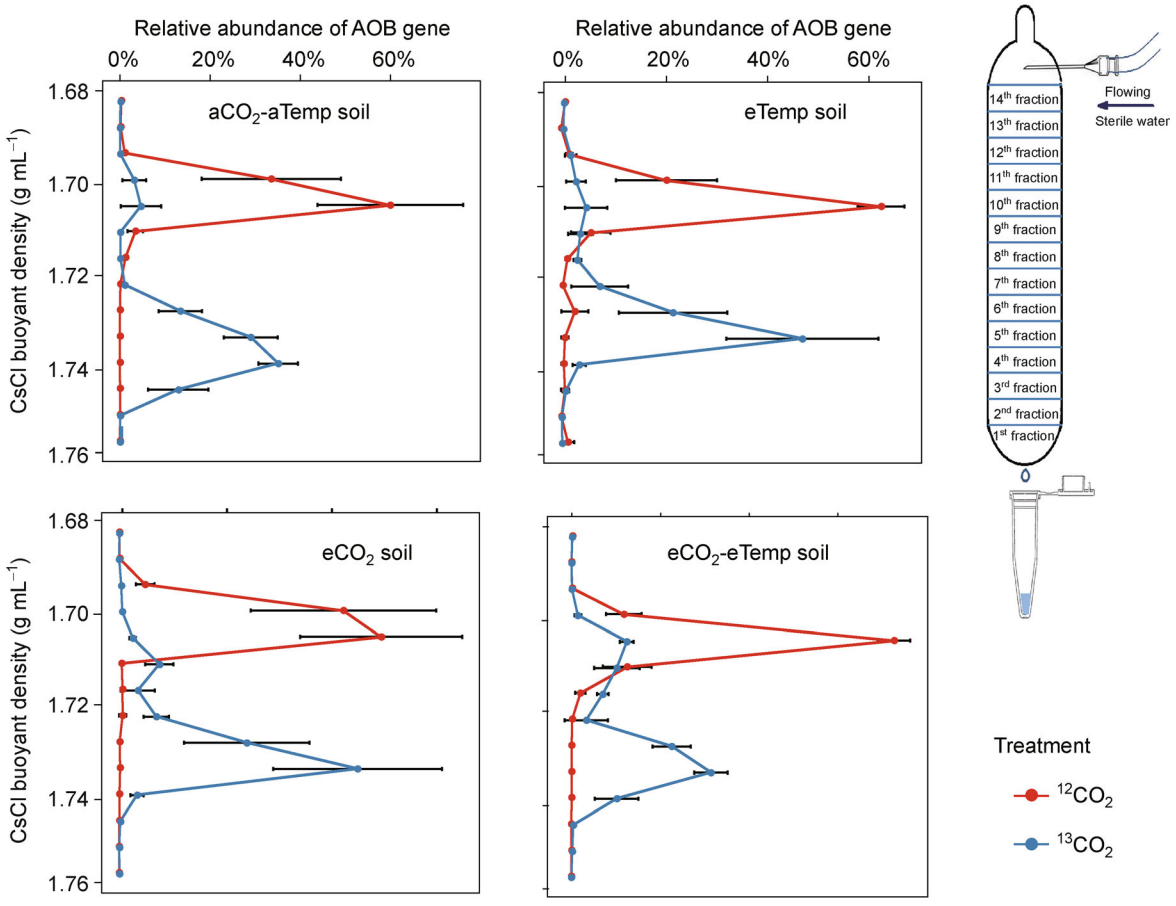

Fig. 2 The quantitative distribution of the AOB gene across the entire buoyant density gradient of the DNA fractions from soil microcosms incubated with $^{12}CO_2$ and $^{13}CO_2$ for 56 days. aCO_2-aTemp: ambient CO_2 and temperature; eTemp: elevated temperature; eCO_2: elevated CO_2; eCO_2-eTemp: elevated CO_2 and elevated temperature.

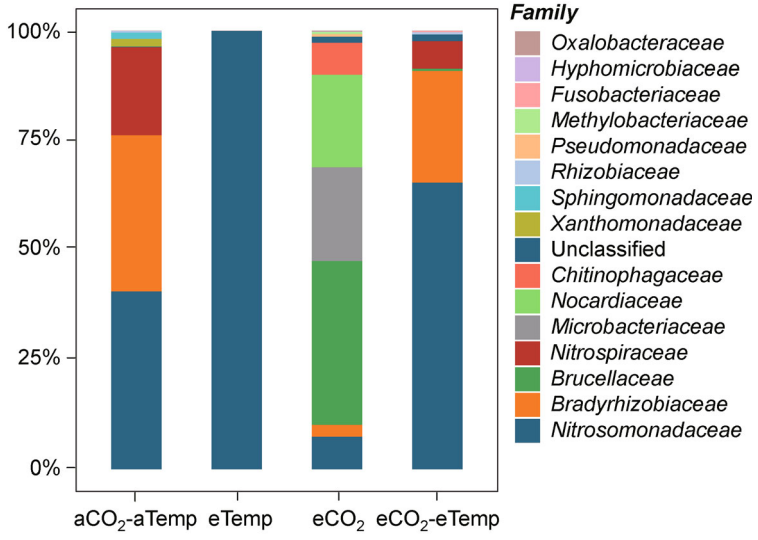

Fig. 3 Relative abundance of ^{13}C labeled OTUs in four soils after 56 days' incubation with $^{13}CO_2$. aCO_2-aTemp: ambient CO_2 and temperature; eTemp: elevated temperature; eCO_2: elevated CO_2; eCO_2-eTemp: elevated CO_2 and elevated temperature.

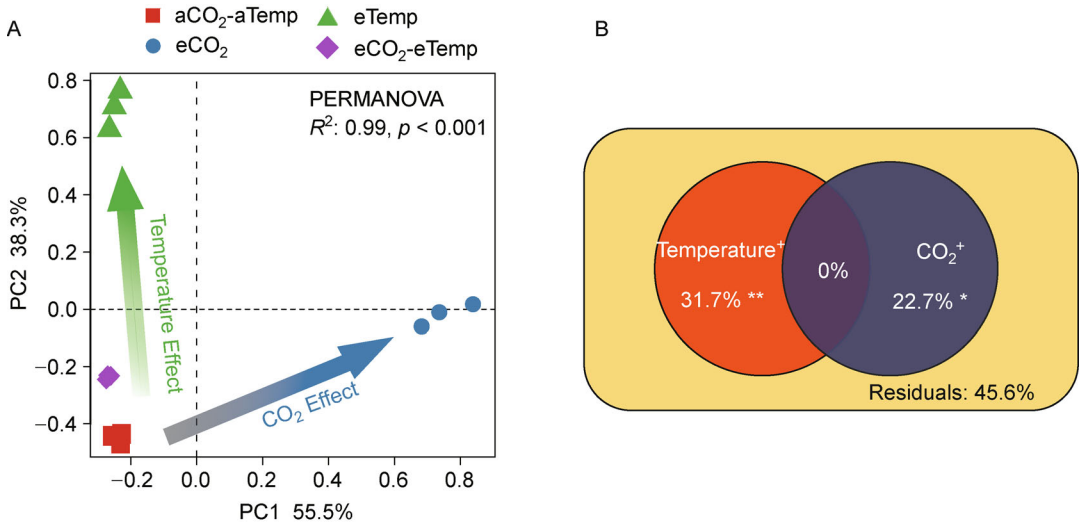

Fig. 4 (A) Principal component analysis (PCA) of ^{13}C labeled OTU matrices from: aCO_2-aTemp: ambient CO_2 and temperature; eTemp: elevated temperature; eCO_2: elevated CO_2; eCO_2-eTemp: elevated CO_2 and elevated temperature. (B) Two-way PERMANOVA was used to analyze the effects of elevated temperature and elevated CO_2 on community composition of active OTUs.

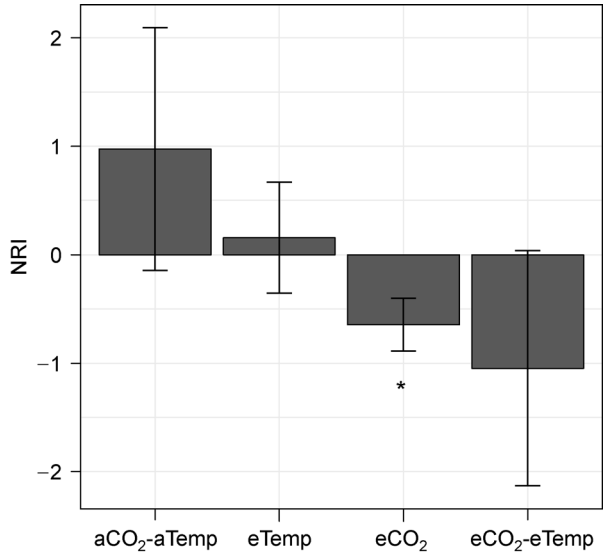

Fig. 5 The nearest relative index (NRI) of active bacterial community ($^{13}CO_2$ labeled) varied in aCO_2-aTemp: ambient CO_2 and temperature; eTemp: elevated temperature; eCO_2: elevated CO_2; eCO_2-eTemp: elevated CO_2 and elevated temperature. Bars represent means±SDs (n = 3). Asterisk indicates that the NRI is significantly different from zero after t-test (*p < 0.05)

(Fig. 6A). The eCO_2-eTemp soil had the most species of active AOB widely distributed in all three groups. However, the eCO_2 soil only contained one species of active AOB (OTU4). OTU4 was the most dominant active AOB in the aCO_2-aTemp, eCO_2 and eCO_2-eTemp treatments, at 95.6%, 100% and 82.5%, respectively (Fig. 6A). The primary AOB species in the eTemp treatment was OTU24 (80.4%), which belongs to the *Nitrosomonas* cluster 8. Thus, changes in climate reshape the active autotrophic microbial composition, leading to a low diversity of AOB in the eCO_2 soil and higher diversity in the eCO_2-eTemp soil.

Only the aCO_2-aTemp and eCO_2-eTemp soil had active autotrophic NOB species detected (Fig. 7A). Elevated CO_2 or temperature alone let to disappearance of the active autotrophic NOB. Under ambient conditions (aCO_2-aTemp), more NOB species were widespread, including OTU253 (2.0%), OTU131 (1.7%), OTU178 (5.4%), OTU308 (3.5%) and OTU12 (87.4%). The eCO_2-eTemp reduced the number of NOB species, which only contained OTU178 and OTU12. However, OTU12 occupied 97.7% of active NOB in the eCO_2-eTemp soil, which was the primary active autotrophic NOB in accordance with the aCO_2-aTemp soil. Thus, NOB was more sensitive to temperature and CO_2 increase than AOB.

4 Discussion

Elevated atmospheric CO_2 concentrations enhance plant photosynthesis and root growth, leading to an increased carbon input into the soil (Austin et al., 2009; Pritchard, 2011; Liu et al., 2014). Elevated CO_2 level increases SOC also in paddy soils (Chen et al., 2016), possibly through higher root rhizodeposition and organic matter release. Previous studies have reported that temperature increase stimulates net primary productivity (Trumbore, 1997) and soil respiration (Luo, 2007) in most terrestrial ecosystems. However, increase in temperature can also increase microbial activity (Blagodatskaya and Kuzyakov, 2013) resulting in the acceleration of SOC decomposition (Luo, 2007). Furthermore, elevated temperature likely decreases crop productivity owing to the enhanced respiration and decomposition of photosynthesized

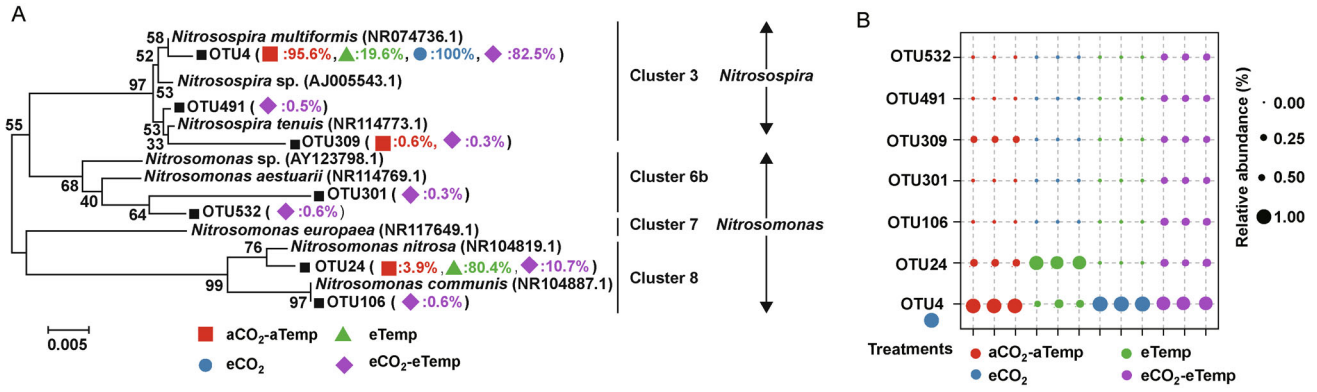

Fig. 6 (A) Neighbor-joining tree analysis of $^{13}CO_2$ labeled OTUs combined with relative abundance of AOB from four soils after 56 days' incubations with $^{13}CO_2$. Bootstrap values higher than 50% are indicated at branch nodes. The scale bar represents 2% nucleic acid sequence divergence. (B) Bubble plot represent abundances of labeled OTUs corresponding to the bubble sizes. The four colors represent the four treatments: aCO_2-aTemp: ambient CO_2 and temperature; eTemp: elevated temperature; eCO_2: elevated CO_2; eCO_2-eTemp: elevated CO_2 and elevated temperature.

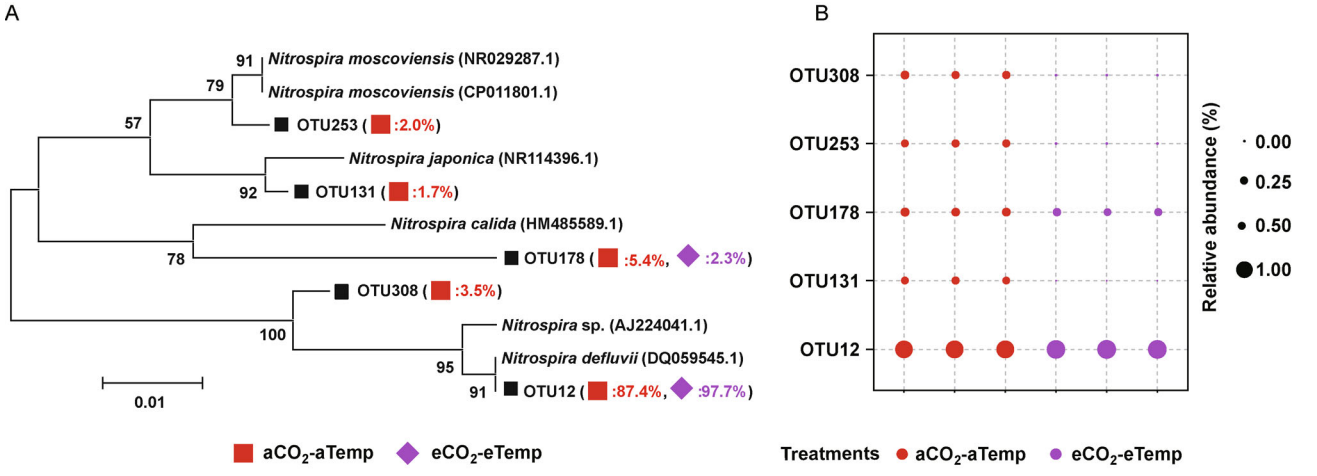

Fig. 7 (A) Neighbor-joining tree analysis of $^{13}CO_2$ labeled OTUs combined with relative abundance of NOB form different treated soils after 56 days' incubations with $^{13}CO_2$. Bootstrap values higher than 50% are indicated at branch nodes. The scale bar represents 2% nucleic acid sequence divergence. (B) Bubble plot represent different abundances of labeled OTUs with different bubble sizes. The different colors represent the different treatments: aCO_2-aTemp: ambient CO_2 and temperature; eCO_2-eTemp: elevated CO_2 and elevated temperature.

biomass, thus reducing carbon input into soil (Pan et al., 2004).

Previously it has been suggested that soil microbial diversity changes in response to pH, SOC, moisture and so on (Liu et al., 2014). In this study, the β-diversity of active bacteria varied under climate change scenarios (Fig. 4A) and the main factors affecting the community composition were EC, NO_3^- and NH_4^+ (Fig. S2). However, the impact of the combination of elevated temperature and CO_2 on β-diversity was far less than a single disturbance. This is in inconsistent with the two having additive effects on microbial communities in temperate agricultural soils (Pritchard, 2011). Some studies have reported that α-diversity and β-diversity shifts donnot occur at the same time and that β-diversity responds faster to environmental changes than α-diversity (Xiong et al., 2014; Zhu et al., 2016). Changes in active microbial diversity are likely to be different from those in general. To our interpretation, here the microbial functioning responded rapidly to the environment, and the compositional change of active microbes at taxonomic level could lag the variation in functioning.

Furthermore, we found that active microbial communities were phylogenetically random in the control, eTemp and eCO_2-eTemp treatments and over-dispersed in the eCO_2 treatment (Fig. 5), suggesting that the co-occurring active microorganisms were more distantly related in only high CO_2 concentration soil. The community in soil with only high CO_2 concentration is composed of less phylogenetically related species than expected by chance (overdispersal), and competition is likely to dominate because closely related or

identical species struggle for the same resources and therefore do not co-occur. Few studies showed that microbial communities were frequently clustered in natural undisturbed ecosystems (Saks et al., 2013; Horn et al., 2014; Liu et al., 2014). In this experiment, however, the soils under aCO_2-aTemp may also be random in ecological process. Nevertheless, the shifts of assemble patterns in eCO_2 treatment were likely to be related to increasing crop carbohydrates. Elevated CO_2 was often been found increasing rice primary biomass production, and therefore could lead to more carbon allocation into the soil for microbial utilization (Inubushi et al., 2003). High nutrient input results in a decrease in abundance of dominate species of *Glomus* within AMF (arbuscular mycorrhizal fungi) due to more opportunities for successful colonization of other genera of AMF (Liu et al., 2014). Thus, probably, in certain scenario, more resources may tend to increase competition between phylogenetically similar microorganisms resulting in the community change, for instance, under the scenario of elevated CO_2 in agroecosystem.

The active microbes that probably primarily determine the change of overall microbial community, account only a small proportion in the overall community. And the labeled microbial groups are not all autotrophic microbes probably because of cross-feeding. However, autotrophic microorganisms are the most important group of active microorganisms in our study, because we added ^{13}C-CO_2, and autotrophy is particularly important in the whole process. 28.9% of active OTUs were founded to be identified as ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Fig. S2).

Nitrosospira cluster 3 was the predominant AOB species in the aCO_2-aTemp, eCO_2 and eCO_2-eTemp soil. This result was not surprising: previous studies reported that *Nitrosospira* species are prevalent in terrestrial ecosystems, especially in agricultural soils (He et al., 2007; Shen et al., 2008; Jiang et al., 2014; Ouyang et al., 2016). However, the dominant species of AOB changed *Nitrosospira* clusters into *Nitrosomonas* clusters in the eTemp soil. Thus, elevated temperature alone had a much stronger effect on autotrophic AOB in paddy soil than elevated CO_2 alone. Microbial growth and vital functions are dependent on enzyme activity, which is temperature-sensitive (Razavi et al., 2017). Enzymes of microorganisms can directly respond to warming through physiological and genetic change (Blagodatskaya and Kuzyakov, 2013; Karhu et al., 2014). These variations may result in different microbial activity or loss of competitiveness, leading to the shift in microbial community diversity. In addition, soil warming could lead to functional gene expression, accelerate intracellular enzyme kinetics, and increase microbial cell turnover (Zhou et al., 2012; Nie et al., 2013; Hagerty et al., 2014). Due to microbial survival in dependent on enzyme functional activity (Burns 1982), the inactivity of certain intercellular enzymes under warming could be one of the factors leading to the change of AOB.

Soils containing *Nitrosomonas* phylotypes could be pre-adapted to relatively high temperatures and these phylotypes may have remained less active or dormant at lower temperatures (de la Torre et al., 2008; Hatzenpichler et al., 2008; Lennon and Jones, 2011). Nonetheless, elevated CO_2 affects the CO_2 concentrations within the soils only marginally (Drigo et al., 2008) and thus unlikely causes direct changes in the community composition. Numerous experiments have reported that under field conditions, elevated CO_2 concentration can intensify leaf photosynthesis in C3 species, such as wheat, rice, and many other food crops, resulting in promoted growth and increased yields (Horie et al., 2000; Kimball et al., 2002; Kim et al., 2003). In general, the amount of root exudates is positively correlated with the plant growth yield. Due to higher productivity, more carbon sources such as plant residues and root exudates, are allocated into the soils (Kuzyakov and Domanski, 2000). Thus, the above ground growth dynamics feed back to the soil microbial community composition via root exudates (Lange et al., 2015; Prober et al., 2015). Elevated CO_2 increases the input of plant residue first, consequently affecting the microbial communities (Singh et al., 2010). According to our results (Fig. 1), increasing the temperature and CO_2 at the same time can result in more C and N accumulation in soil. During simultaneous elevation of the CO_2 and temperature, more resources are available for the active microbes, which could lead to an increase in the number of species and change the composition of active microbes. To our interpretation, this is the reason why more OTUs of *Nitrosospira* and *Nitrosomonas* detected under the combination of elevated CO_2 and temperature. Moreover, the eCO_2-eTemp treatment altered the composition of autotrophic AOB to a certain extent, stimulated more autotrophic AOB to be active, particularly, the *Nitrosomonas* relative abundance increased from 3.8% to 10.7% (Fig. 6A). To sum up, simultaneous elevation of the CO_2 and temperature makes the community composition of active autotrophic bacteria much closer to the ambient condition treatment.

The results regarding to the active autotrophic NOB also revealed that the NOB composition in soil subjected to eCO_2-eTemp was similar to the composition in soil with aCO_2-aTemp, and the similarity is more pronounced than that found between the AOB communities in paddy soils. The change in composition of active autotrophic AOB and NOB could be to some extent attributed to the influence of plant root secreted oxygen. Lack of oxygen prevents nitrification in most soils (Geisseler et al., 2017), resulting in nitrifying microorganisms not getting enough resource and energy. As mentioned above, the higher inputs of plant root-secreted oxygen could be a key factor influencing the microbial communities indirectly under climate change, especially under elevated CO_2. This contrasts with the results in upland soils (increase of nitrification with warming) (Gao et al., 2016; Geisseler et al., 2017), because much less O_2 is available and O_2 is much faster depleted by overflooding in paddy soils. Another point is noteworthy: although active autotrophic NOB were not detected in the eTemp soil and eCO_2 soil, it did not lead to loss of nitrite oxidation function in soils. The capacity of DNA-

SIP depends on cell replication, thus excluding microbes that might be active but not replicating (Pritchard, 2011). RNA-SIP would be better for generating information regarding activity and it should be used instead of DNA-SIP to better explore active microorganism.

5 Conclusions

Increase of temperature, CO_2 or both shift the composition mainly of active microbes in paddy soils. AOB and NOB are major autotrophs in paddy soils. Increase of temperature for 2°C or increase of CO_2 concentration for 500 μmol mol^{-1} deactivate the NOB below the detection limit and decrease the OTU number of AOB by 33% and 66%, respectively. In comparison with the ambient conditions, increasing both CO_2 and temperature could recover activities of certain NOBs from the deactivated situations and stimulated more active OTUs of AOB (for instance, OTU301 and OTU532 belonging to Nitrosomonas cluster 6b) in the soil. Taken together, the individual effect of elevated temperature or CO_2 on the changes in active microbial community is greater than the additive effect.

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Electronic supplementary material

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