Changes of Soil Microbes Related with Carbon and Nitrogen Cycling after Long-Term CO_2 Enrichment in a Typical Chinese Maize Field

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Abstract: Elevated atmospheric CO_2 concentration (eCO_2) has been the most important driving factor and characteristic of climate change. To clarify the effects of eCO_2 on the soil microbes and on the concurrent status of soil carbon and nitrogen, an experiment was conducted in a typical summer maize field based on a 10-year mini FACE (Free Air Carbon Dioxide Enrichment) system in North China. Both rhizospheric and bulk soils were collected for measurement. The soil microbial carbon (MBC), nitrogen (MBN), and soil mineral N were measured at two stages. Characteristics of microbes were assayed for both rhizospheric soil and bulk soils at the key stage. We examined the plasmid copy numbers, diversities, and community structures of bacteria (in terms of 16s rRNA), fungi (in terms of ITS-internal transcribed spacer), ammonia oxidizing bacteria (AOB) and denitrifiers including nirK, nirS, and nosZ using the Miseq sequencing technique. Results showed that under eCO_2 conditions, both MBC and MBN in rhizospheric soil were increased significantly. The quantity of ITS was increased in the eCO_2 treatment compared with that in the ambient CO_2 (aCO_2) treatment, while the quantity of 16s rRNA in rhizospheric soil showed decrease in the rhizospheric soil in the eCO_2 treatment. ECO_2 changed the relative abundance of microbes in terms of compositional proportion of some orders or genera particularly in the rhizospheric soil-n particular, Chaetomium increased for ITS, Subgroups 4 and 6 increased for 16s rRNA, Nitrosospira decreased for AOB, and some genera showed increase for nirS, nirK, and nosZ. Nitrate N was the main inorganic nitrogen form at the tasseling stage and both quantities of AOB and denitrifiers, as well as the nosZ/(nirS+nirK) showed an increase under eCO_2 conditions particularly in the rhizospheric soil. The Nitrosospira decreased in abundance under eCO_2 conditions in the rhizospheric soil and some genera of denitrifiers also showed differences in abundance. ECO_2 did not change the diversities of microbes significantly. In general, results suggested that 10 years of eCO_2 did affect the active component of C and N pools (such as MBC and MBN) and both the quantities and relative abundance of microbes which are involved in carbon and nitrogen cycling, possibly due to the differences in both the quantities and component of substrate for relevant microbes in the rhizospheric soils.

Keywords: rhizosphere soil; elevated CO_2; microbial community; nitrifier; denitrifier; ammonia oxidizing bacteria

1. Introduction

The anthropogenic emission of greenhouse gases has caused a major increase in atmospheric CO_2 concentration. Atmospheric CO_2 concentration has risen to 407 $\mu mol \cdot mol^{-1}$ in 2017 from 280 $\mu mol \cdot mol^{-1}$ at the pre-industrial revolution period, due to the large amount of fossil fuel consumption by anthropogenic activities [1,2]. Enriched atmospheric CO_2 could enhance the production of above-ground and below-ground, as well as the labile carbon substrate input to the soil through the increased photosynthetic rate [3,4]. Plant derived carbon, including above-ground and below-ground residues and root exudation [5–7], usually provides an energy source for soil microbes. However, microbes could conversely affect the biogeochemistry of some elements [8] through the changes in the quantity and community structures in response to increased substrate availability under higher CO_2 conditions [9].

Some studies have suggested that increased input of photosynthetic carbon to the rhizospheric soil stimulated the decomposition of carbon in soils [10,11], which may accelerate the growth of microbes and thus increase losses of carbon in soils [9]. It has been found that the quantity and diversity of soil microbes would increase with elevation of CO_2 concentration [12,13]; and the same effect was observed in the bacterial abundance at rhizospheric soil [14,15]. Elevated atmospheric CO_2 (eCO_2) also showed an effect on the abundance of fungi, particularly by promoting the decomposition of resistant carbon substrate [9,16].

Due to the low mineral N content in the natural soil, synthetic nitrogen fertilizers are widely used in current agricultural production to obtain high yield output. However, available N in the chemical fertilizers is easily lost through ammonia volatilization, nitrate leaching, or transformation to nitrogen-containing gases via nitrification and denitrification processes. Ammonia-oxidizing bacteria (AOB) are a kind of dominant bacteria in the progress of nitrification in most alkaline soils [17,18]. Denitrification is an anaerobic process in which nitrate (NO_3^-) is reduced to nitrite (NO_2^-) and nitrogen gases (NO, N_2O and N_2) in sequence by denitrifiers [19] and could result in the loss of available N. The key enzymes in denitrification include nar, nor, nirS, nirK and nosZ [20]. Some denitrifiers have the multi-genes and are able to implement the entire four steps of denitrification, while other denitrifiers only have one or more of these genes and therefore can only perform one or more of these steps.

ECO_2 could increase both the content of soil dissolved carbon and dead fine root exfoliation [21,22]. This promotes the activity of microbes and strengthens the microbial demand for inorganic N [23], thus causing a temporary preservation of inorganic N in the form of microbial N and a decrease in the available N which may otherwise be lost to the environment [24]. An increase in atmospheric CO_2 concentrations affected root exudates and exfoliation of root cells, which influenced the abundance and communities of soil microbes. Horz [22] reported that soil AOB could respond significantly to changes in root exudates and plant residues induced to eCO_2. However, other studies have suggested that eCO_2 showed a decline in the abundance of AOB [25,26] and a significant increase in denitrifiers in the rhizospheric soil used for maize production [27]. The increase in soil microbial respiration would likely result in a change in soil oxygen concentration [28], which would decrease the loss of available soil N to the environment. Actually, in analyzing the nutrient cycling process, the tradeoff of different processes and their final balance is very important. Cleveland [29] found that more nitrogen was presented in microbes than in the soil, suggesting that the increase in atmospheric CO_2 concentrations may lead to an increase in nitrogen availability, or may alter the process of nitrogen turnover in response to the change of substrates in the soil. For example, root exudation could cause reductions of nitrate-N through the denitrification process, and the component of secretion had a notable effect on the forms of denitrification end products [30]. However, under eCO_2 conditions, data on the concurrent monitoring and analysis of the active C and N forms and the synchronized changes of functional microbes in regard to the soil C and N cycling are still limited.

Therefore, this study was conducted based on a mini FACE (free air carbon dioxide enrichment) system in a typical summer maize soil of North China, which has been running for 10 years. The purpose of this study was to examine the quantity, and diversity of the community structure of bacteria and

fungi in terms of 16s rRNA and internal transcribed spacer (ITS) respectively, regarding carbon cycling, as well as the microbes related to nitrogen cycling in the soil such as the functional genes of AOB, nirK, nirS, and nosZ in both the rhizospheric and bulk soils at the maize tasseling stage in response to the eCO_2. Soil MBC, soil MBN and mineral nitrogen content were also monitored. We hypothesized that eCO_2 would promote the C and N cycling in some extents and could be reflected in some soil index and microbes in some characters such as the abundance, diversity, or compositional structure.

2. Materials and Methods

2.1. Experimental Design

The mini FACE (Free Air Carbon Dioxide Enrichment) system was located in the northern suburban of Beijing, China (40°13′N, 116°14′E, 72 m above sea level) and was the only wheat-maize/soybean rotation FACE system in China. This region has a temperate and monsoonal type of climate with an annual average temperature of 10–12 °C and an annual average precipitation of 600 mm. The soil was aquic cinnamon soil in the Chinese Soil Taxonomy System, equivalent to the Cambisols of the semi-alfisol order in the FAO World Reference Base for Soil Resources System. The basic physical and chemical properties in October 2016 were as follows: organic carbon content: 11.8 g C kg^{-1}, total nitrogen: 0.7 g N kg^{-1}, Olsen-P: 11.8 mg P kg^{-1}, available potassium: 75.2 mg K kg^{-1}, and pH 8.6. The CO_2 enrichment experiment was started in October 2007. The fertilization rate was 180 kg N ha^{-1}, 165 kg P_2O_5 ha^{-1} and 90 kg K_2O ha^{-1} for each crop in the winter wheat-summer soybean/summer maize double cropping systems. The nitrogen fertilizers were divided into base fertilizer application (60%) and topdressing (40%). Phosphate and potassium were applied as base application.

This mini FACE system consists of 12 identical 4 m diameter octagonal rings, six rings of which were under aCO_2 condition (400 ± 15 μ mol·mol^{-1}), and the other six rings were under eCO_2 condition (550 ± 20 μ mol·mol^{-1}). The aCO_2 rings were 14 m away from eCO_2 rings to eliminate any cross-contamination of CO_2. The concentration of CO_2 was measured throughout the season by factory-calibrated sensors (Viasala, Vantaa, Finland) at the center of each octagonal plot about 10 cm above the canopy which adjusts the wind speed and direction in real time. The CO_2 concentration was stabilized at 550 ± 20 μ mol·mol^{-1} most of the time and CO_2 was released during the daytime only controlled by a software (Patent No. 2013SR007586) which adjusted the valves depending on the sunrise and sunset. The detailed description on this mini FACE system was fully described by Han [31].

2.2. Field Sampling

Summer maize ("Shandan 902" variety of Zea mays) was planted on 19 June, 2017 and the samples (rhizospheric and bulk soils) were taken at the 0–20 cm depth on 15 August, 2017, at the tasseling stage. The rhizospheric soil was collected by shaking sampling roots and the soil drill method was used for bulk soil sampling between the planting rows. Three random samples were collected for both rhizospheric and bulk soil from each plot and then put through a 2 mm sieve. A total of 12 composite samples were taken to the laboratory on ice and each sample was divided into two parts: one part was used to measure MBC, MBN, soil moisture and soil inorganic nitrogen; and the other one was stored at −80 °C for extraction of soil DNA.

2.3. Measurement of Soil Carbon and Nitrogen

The mineral nitrogen concentration (ammonium-N and nitrate-N) were measured by extracting the soil nitrogen with 1 M of KCl with a soil to water ratio of 1:10 and the filtrate of each sample was assayed using an AutoAnalyzer 3 (SEAL Analytical, Southampton, Hants, UK). The MBC and MBN were measured using the $CHCl_3$ fumigation direct extraction method. A 20 g subsample of fresh soil was fumigated for 24 h with C_2H_5OH-free $CHCl_3$ in a vacuum desiccator. A second 20 g subsample was simultaneously incubated in a desiccator without $CHCl_3$ as a control. Following fumigation, each

sample was extracted with 0.5 M K_2SO_4 with a soil to water ratio of 1:5. The filtrate of each sample was assayed using a total organic carbon analyzer (Elementar, Langenselbold, Germany).

2.4. DNA Extraction

Total soil DNA was extracted using AxyPrep Multisource Genomic DNA Kits (Axygen, Union City, CA, USA) according to the manufacturer's instructions. Then, the DNA purity was checked using a NanoPhotometer spectrophotometer (Implen, Munich, Germany). The quality of extracted DNA was checked using 1% agarose gel electrophoresis and spectrophotometry (optical density was set at 260 nm/280 nm ratio). All extracted DNA samples were stored at −20 °C for further assay.

2.5. PCR and Quantitative Real-Time PCR

The V3-V4 hypervariable regions of the 16s rRNA gene were subjected to a high-throughput sequencing by Beijing Allwegene Tech, Ltd., (Beijing, China) using the Illumina Miseq PE300 sequencing platform (Illumina, Inc., San Diego, CA, USA). Tenfold serial dilutions of the genomic DNA of 6 genes (16s rRNA, ITS, AOB, nirS, nirK, nosZ) from 10^{-1} to 10^{-6} were subjected to PCR and qPCR to generate a standard curve. The qPCR assay of standards, samples and no-template control was performed in triplicate on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA USA) with a 96-well format. The specific primer and conditions for PCR are described in the Supplementary Material (Table S1).

2.6. Bioinformatics Analysis

In this study, the compositional structure of microbial communities denotes the number of the Operational Taxonomic Unit (OTU) of a specific species or genus in all OTU numbers for a given functional gene (where groups with a composition of less than 1% were merged to one group together, referred to as "others"). Some researchers also called it the relative abundance of a specific species or genus. It can indicate the dominant species which plays a given role and can be used to interpret one of the aspects of a given functional gene.

2.7. Statistical Analysis

Data were analyzed to do the variance (ANOVA) using the statistical package SPSS 20.0 (IBM Corp, Armonk, NY, USA). Differences marked significant were checked using the least significant difference LSD method at $p < 0.05$.

3. Results

3.1. Effect of ECO_2 on the Concentration of Soil Inorganic N, MBC, and MBN

In this study, topdressing was applied on 2 August, 2017 in the summer maize growth period. Data showed that (Figure 1) the ammonium-N in the soils was lower than 1.0 mg kg^{-1} and the nitrate-N increased to around 90 mg kg^{-1} at the sampling date (13 days after topdressing). As the nitrate concentration in the soil was lower than 20 mg kg^{-1} at the seedling period (Figure S1 in Supplementary Material), we can deduce that the applied urea had been mostly nitrified to nitrate-N 13 days after topdressing in the soil. Both concentrations of ammonium-N and nitrate-N at the rhizospheric zone were lower than 25 mg kg^{-1}, obviously lower than at the bulk soils, possibly due to the uptake of roots (Figure 1a,b). For the ammonium-N, the concentrations at the bulk soil were 1.9 and 3.5 times that at the rhizospheric soils for eCO_2 and aCO_2 treatments respectively. The nitrate-N concentrations at the bulk soil were 4.8 and 4.2 times higher than that of the rhizospheric soils for the eCO_2 and aCO_2 treatments respectively.
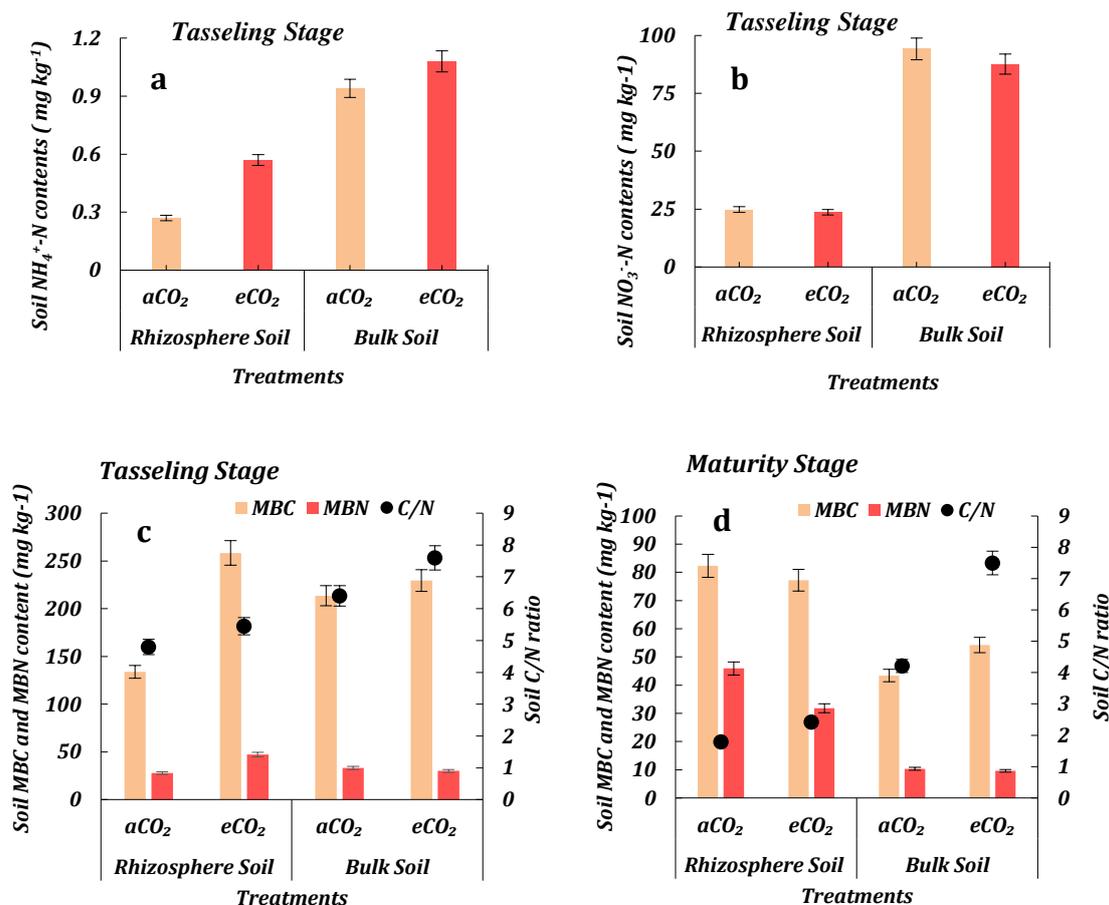
Figure 1. Effect of elevated CO_2 concentration on soil inorganic N contents at Tasseling stage (**a**: NTH_4^+ & **b**: NO_3^-) and soil MBC and MBN contents at Tasseling (**c**) stage and Maturity stage (**d**) in rhizospheric and bulk soil of maize (mg·kg^{-1}).

Tasseling stage is a transitional period when maize changes from vegetative growth to reproductive growth. At this stage, the biomass reaches its peak and the turnover and uptake of nutrients in the soil are active. Results showed that soil MBC was between 119.4 mg kg^{-1} and 248.7 mg kg^{-1} at the tasseling stage (as shown in Figure 1c,d) and decreased by 31%–59% at the maturity stage. Soil MBN also decreased somewhat at the mature stage compared with that at the tasseling stage. As MBC declined more rapidly after the tasseling stage, the C:N ratio decreased from 4.8–7.6 at the tasseling stage to 1.8–8.2 at the maturity stage. At the tasseling stage, MBC in the rhizospheric soil for eCO_2 treatment was significant higher, about 2.1-fold than that of aCO_2 treatment. MBC in all treatments decreased at the mature stage compared to that at the tasseling stage and there was no obvious difference between eCO_2 and aCO_2 treatments, no matter in the rhizospheric soil or in the bulk soil at the mature stage. For MBN in the rhizospheric soil, it was 1.7-fold in the eCO_2 treatment than that in the aCO_2 treatment at the tasseling stage. However, it was slightly lower in the eCO_2 treatment than in the aCO_2 treatment at the mature stage. For MBN at the bulk soils, it did not show significant difference between the eCO_2 and the aCO_2 treatments at the mature stage.

3.2. Responses of Microbes in Terms of Quantity under ECO_2 Conditions

There are a variety of microbes in the soil that play different or sometimes similar function in mediating the soil nutrient cycling. In this experiment, the plasmid quantities and communities of 16s rRNA and ITS, as well as amoA, nirS, nirK, and nosZ were measured to investigate their characteristics and the relationship with soil carbon and nitrogen. The copy number of 16s rRNA plasmids of different treatments ranged from 1.37×10^9 to 2.36×10^9 g^{-1} soil; while ranged from 0.16×10^7 to

2.01×10^7 g^{-1} for ITS, which was two to three orders lower in magnitude than that of 16s rRNA (Table 1). For the quantity of bacteria in terms of 16s rRNA plasmids, it showed that it was lower by 25% in the rhizospheric soil at the eCO$_2$ treatment compared to that at aCO$_2$ treatment. For the quantity of fungi in terms of the plasmid number of ITS, it was higher by 27.1% in the rhizosphere soil at the eCO$_2$ treatment than at the aCO$_2$ treatment. Moreover, there were significant difference in the bulk soil between the eCO$_2$ and aCO$_2$ treatments in the plasmid number of 16s rRNA and ITS. For the ratio of the plasmid numbers of 16s rRNA to ITS, it was significantly lower in the rhizospheric soil under eCO$_2$ (88.67 in eCO$_2$-R against 149.38 in aCO$_2$-R). However, the ratio was different in bulk soil, suggesting that the rhizosphere might be enriched by fungi-preferred substrate under the eCO$_2$ conditions.

Table 1. The copy number of 16s rDNA and ITS in rhizospheric and bulk soil of maize at tasseling stage under elevated CO$_2$ concentration. Data are the mean ± S.E., n = 3. Different letters indicate significants differences among CO$_2$ concentration, rhizophere and bulk soil at $p < 0.05$.

Treatment	16s rDNA (*10^9)	ITS (*10^7)	16s rDNA/ITS
aCO$_2$-R	2.36 ± 0.28 a	1.58 ± 0.05 b	149.38 b
eCO$_2$-R	1.77 ± 0.10 b	2.01 ± 0.16 a	88.67 c
aCO$_2$-B	1.37 ± 0.30 c	0.16 ± 0.02 d	547.90 a
eCO$_2$-B	1.95 ± 0.26 b	1.21 ± 0.09 c	137.35 b

For the quantity of AOB in terms of amoA gene plasmid copy numbers, there was no obvious difference between eCO$_2$ and aCO$_2$ treatments in the rhizospheric soil (Table 2). However, there was a significant difference in copy number of AOB in the bulk soil under different CO$_2$ concentration. For the quantities of denitrifiers in the soil, their quantities were shown in the descending order: nirK (10^9) > nosZ (10^8) > nirS (10^6) according to their magnitudes and they were all higher in the bulk soil than in the rhizospheric soil. Moreover, the quantities of three types of denitrifiers showed a similar pattern in that they were all higher in eCO$_2$ treatments than in aCO$_2$ treatments either in rhizospheric or in bulk soils. In addition, the ratio of nosZ to (nirS + nirK) was higher in the eCO$_2$ treatments than in the aCO$_2$ treatment, particularly in the bulk soil.

Table 2. The copy number of AOB, nirS, nirK and nosZ in rhizospheric and bulk soil of maize at tasseling stage under elevated CO$_2$ concentration. Data are the mean ± S.E., n = 3. Different letters indicate significants differences among CO$_2$ concentration, rhizophere and bulk soil at $p < 0.05$.

Treatment	AOB (10^7)	nirS (*10^6)	nirK (*10^9)	nosZ (*10^8)	nosZ/ (nirS + nirK)
aCO$_2$-R	6.38 ± 0.10 b	2.77 ± 0.17 d	4.64 ± 0.30 b	4.75 ± 0.48 c	0.16 b
eCO$_2$-R	6.56 ± 0.39 b	6.31 ± 0.37 b	7.63 ± 0.13 a	25.41 ± 5.53 b	0.59 a
aCO$_2$-B	6.58 ± 0.99 b	3.54 ± 0.39 c	5.29 ± 0.82 ab	8.56 ± 0.84 c	0.10 b
eCO$_2$-B	7.54 ± 0.23 a	9.29 ± 0.54 a	6.39 ± 0.55 ab	52.3 ± 2.51 a	0.33 ab

3.3. The Diversity of Microbes in Response to the ECO$_2$

The Shannon–Wiener index is a typical index to indicate the diversity of a series of microbes which have the similar function. A higher Shannon–Wiener index value implies the higher biodiversity of a group of microbes at a given classification level. In this study, the diversities of 16s rRNA, ITS, AOB and denitrifiers (i.e., nirS, nirK, and nosZ) did not show significant differences between eCO$_2$ and aCO$_2$ treatments in either the rhizospheric soil or the bulk soil (Table 3). Under the eCO$_2$ conditions, the diversity of microbes did not show obvious difference between the rhizospheric and the bulk soils for most microbes with the exception of nirS and nosZ under the aCO$_2$ conditions in the bulk soil.

Table 3. Effect of elevated CO_2 concentration on rhizospheric and bulk soil of maize on Shannon index at tasseling stage (Shannon–Wiener). Data are the mean ± S.E., n = 3. Different letters indicate significants differences among CO_2 concentration, rhizophere and bulk soil at $p < 0.05$.

Shannon	16s rDNA	ITS	AOB	nirS	nirK	nosZ
aCO₂-R	9.85 ± 0.04 a	5.96 ± 0.25 a	5.35 ± 0.38 a	7.09 ± 0.13 a	6.97 ± 0.27a	7.91 ± 0.29 a
eCO₂-R	9.82 ± 0.05 a	6.22 ± 0.08 a	5.31 ± 0.51 a	7.29 ± 0.02 a	6.54 ± 0.25a	8.08 ± 0.39 a
aCO₂-B	9.68 ± 0.08 b	5.93 ± 0.49 a	5.31 ± 0.61 a	6.37 ± 0.51 b	6.60 ± 0.13a	5.69 ± 1.43 b
eCO₂-B	9.76 ± 0.04ab	6.19 ± 0.13 a	4.96 ± 0.54 a	6.85 ± 0.11ab	6.87 ± 0.22a	6.02 ± 3.7ab

3.4. The Community Structure of Microbes in Response to ECO₂

3.4.1. 16s rRNA

At the order level in terms of 16s rRNA for bacteria, Subgroup 6 and Xanthomonadales were the two dominant orders (Figure 2). Subgroup 6 accounted for 5.5%–11.0% and Xanthomonadales accounted for 6.3%–7.9% in the compositional structure of 16s rRNA. Other major orders of 16s rRNA in this study included Sphingobacteriales, Subgroup 4, Rhodospirillales, Rhizobiales, and Sphingomonadales, and each of them accounted for around 5% in the compositional proportion. Overall, the proportion of Subgroup 6 was significantly higher in the rhizospheric soil than in the bulk soil. However, Sphingomonadales was higher in the bulk soil compared to that in the rhizospheric soil. Under eCO₂ conditions, both Subgroup 6 and Subgroup 4 were significantly higher in either the rhizospheric or bulk soils compared to that under the aCO₂ conditions (11.0% vs. 8.2% and 6.3% vs. 4.9%, respectively, in the rhizospheric soil and 6.5% vs. 5.5% and 4.6% vs. 3.9%, respectively, in the bulk soil). However, the compositional proportion of Sphingomonadales under the eCO₂ conditions was slightly lower than that under the aCO₂ conditions (3.5% vs. 4.7% in rhizospheric soil; 6.2% vs. 7.5% in bulk soil).
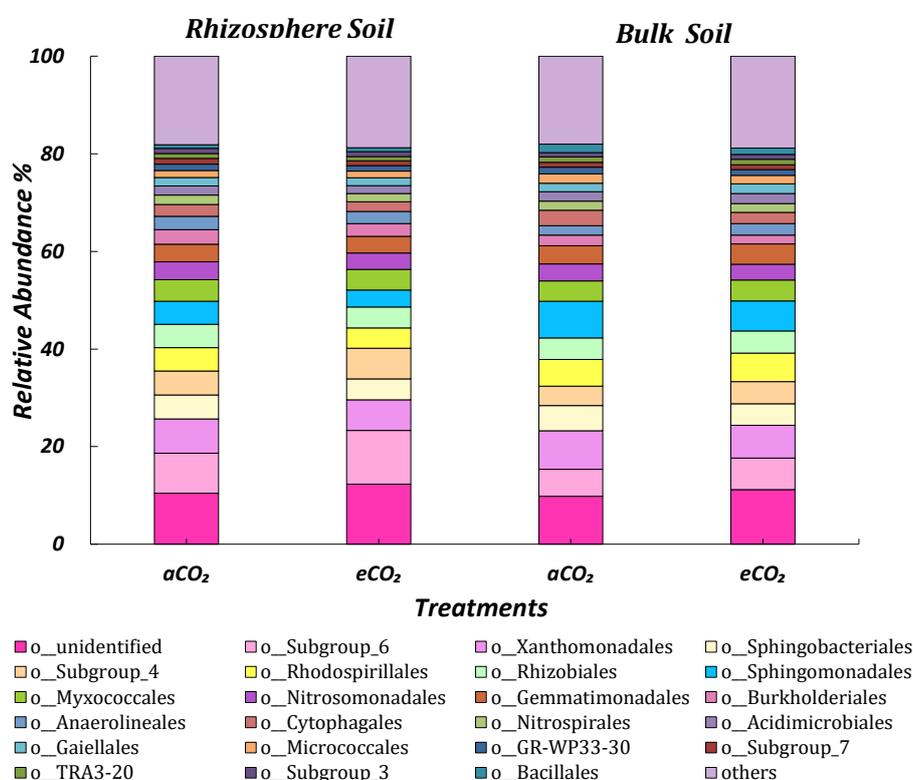
Figure 2. The compositional structure of 16s rDNA at the tasseling stage of maize growth period (at the order level).

3.4.2. ITS

In general, the dominant genera in the studied soil included Talaromyces, Chaetomium, Fusarium, Pseudallescheria, Mortierella, Humicola, and Monographella, and their compositional proportions were 1.4%–10.9%, 6.3%–8.3%, 5.9%–7.4%, 2.4%–5.5%, 5.0%–7.4%, 2.9%–4.7%, and 2.6%–4.5%, respectively (Figure 3). Of these, the proportion of Talaromyces was significantly higher in the rhizospheric soil than in the bulk soil; while the proportions of Chaetomium and Monographella were lower in rhizospheric soil than in the bulk soil. Under the eCO_2 conditions, the proportions of Humicola and Chaetomium were raised in both rhizospheric and bulk soil compared with that under aCO_2 conditions. The percentages of Humicola were 4.7% (for eCO_2) and 2.9% (for aCO_2) in the rhizospheric soil respectively; while they were 3.8% (for eCO_2) and 3.5% (aCO_2) in the bulk soil. For the Chaetomium, the compositional ratios were 8.2% (for eCO_2) and 6.3% (for aCO_2), in the rhizospheric soil respectively, and 8.3% (for eCO_2) vs. 7.6% (for aCO_2) for bulk soil respectively. However, the proportions of Talaromyces were lower under eCO_2 conditions than under aCO_2 conditions, in both rhizospheric (5.7% vs. 10.9%) and the bulk soils (1.4% vs. 1.7%), and these differences were more obvious in the rhizospheric soil.
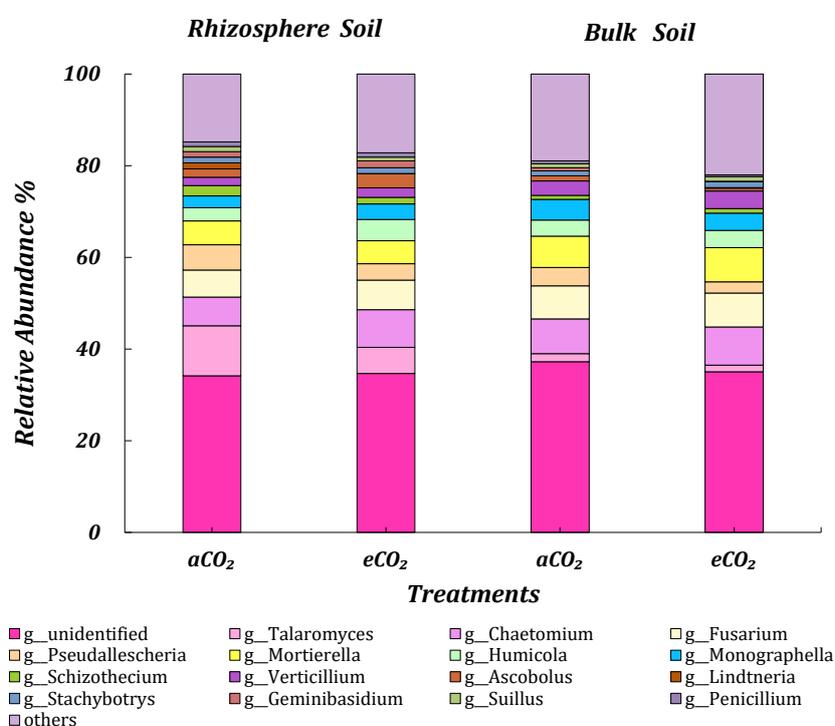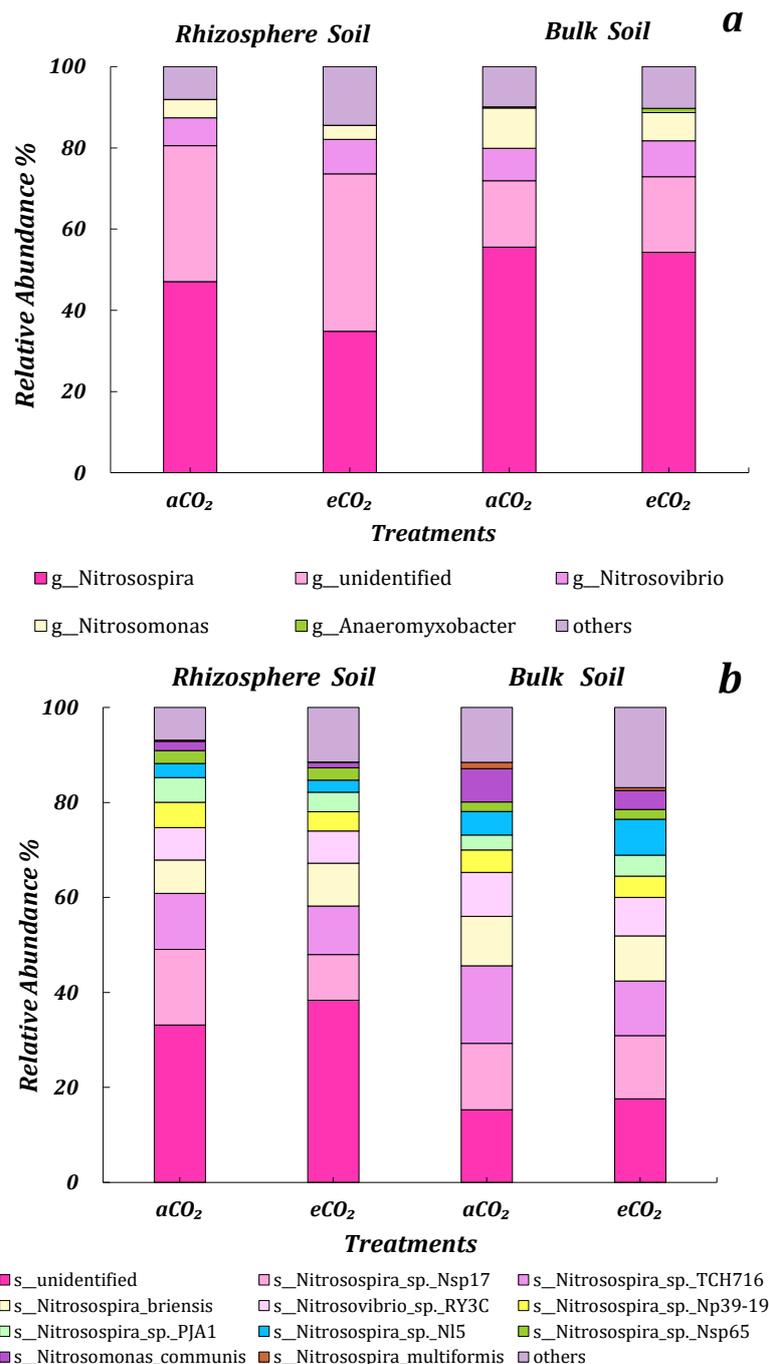

Figure 3. The compositional structure of ITS (internal transcribed spacer) at the tasseling stage of maize growth period (at the genus level).

3.4.3. AOB

In this study, we examined the community structure of AOB in rhizospheric soil and bulk soil under different atmospheric CO_2 concentrations. Figure 4a shows that, among the AOB that could be identified at the genus level, Nitrosospira was the dominant genus in the soil (34.8%–55.6%), higher (more than 50%) in rhizospheric soil than in bulk soil, followed by Nitrosovibro (6.8%–8.8%) and Nitrosomonas (4.5%–9.9%). Under the eCO_2 conditions, the compositional proportion of Nitrosomonas was lower than under the aCO_2 conditions in both the rhizospheric soils (3.5% vs. 4.5%) and the bulk soils (6.9% vs. 9.9%). The proportion of Nitrosovibro in the rhizospheric soil exhibited higher in the eCO_2 treatment than in the aCO_2 treatment (8.5% vs. 6.8%). However, for the Nitrosospira, its proportion was significantly lower in rhizosphere soil under the eCO_2 condition than under aCO_2 condition (34.8% vs. 47.1%), but not significant in the bulk soil. At the species level (Figure 4b), the

dominant species of Nitrosospira for the studied soil included Nitrosospira_sp. TCH716 (10.2%–16.3%), Nitrosospira_sp. Nsp17 (9.6%–16.0%), Nitrosospira_briensis (7.0%–10.4%), Nitrosospira_sp._N15 (3.0%–7.6%), and Nitrosospira_sp._Np39-19 (4.0%–5.4%), etc., in which Nitrosospira_sp. Nsp17 and Nitrosospira_sp._ TCH716 showed significant decrease in the compositional proportion under eCO_2 conditions in both rhizospheric and bulk soil. However, Nitrosospira_briensis showed significant increase in compositional proportion under the eCO_2 treatment than under the aCO_2 treatment in the rhizospheric soil. Additionally, the compositional percentage of unclassified genera were increased under the eCO_2 condition than under the aCO_2 condition (Figure 4b), particularly at the bulk soil (38.3% vs. 33.1%), implying that eCO_2 may promote the development of some unknown microbes.

Figure 4. The compositional structure of AOB at the tasseling stage of maize growth period at the genus level (**a**) and the species level (**b**).

3.4.4. nirS, nirK and nosZ

As shown in Figure 5a, the dominant genera which possess nirS gene included Rubrivivax (10.8%–12.8% in proportion), Rhodanobacter (3.1%–6.2% in proportion), Cupriavidus (4.6%–6.7% in proportion), Halomonas (4.7%–6.3% in proportion) and Herbaspirillum (4.1%–7.4% in proportion) etc. There were some genera which were higher in proportion in the rhizospheric soil than in the bulk soil, such as Rubrivivax, Rhodanobacter, Halomonas, Azoarcus and so on. However, some genera were lower in the proportion in the rhizospheric soil than in the bulk soil, for instance Thauera (7.1% vs. 12.7%). Under the eCO_2 condition, there was no significant difference in the compositional structure for nirS comparing with that under aCO_2 condition in the rhizospheric soil. However, in the bulk soil, the proportion of Thauera under the eCO_2 condition was higher in percentage than under the aCO_2 condition (12.7% vs. 7.1%).

As shown in Figure 5b, the dominant genera of microbes which possess nirK gene in the studied soil were Rhodopseudomonas (13.5%–21.1% in proportion), Bradyrhizobium (11.9%–18.4% in proportion), Rhizobium (11.2%–19.4% in proportion), Sinorhizobium (9.0%–12.2% in proportion) and Mesorhizobium (8.0%–14.9% in proportion) etc. Some genera were higher in proportion in the rhizospheric soil than in the bulk soil, which included Rhodopseudomonas (18.4%–21.1% vs. 13.5%–15.2%) and Bradyrhizobium (18.1%–18.4% vs. 11.9–14.4%); while, genera lower in proportion in the rhizospheric soil than in the bulk soil included Rhizobium (11.2%–14.3% vs. 14.3%–19.4%), Mesorhizobium (8.0%–11.8% vs. 14.5%–14.9%) and Sinorhizobium (9.0%–10.4% vs. 10.9%–12.2%). Under the eCO_2 condition, Mesorhizobium exhibited to be higher in the proportion in the rhizospheric soil than under the aCO_2 condition (11.8% vs. 8.0%). However, there was no significant difference in the proportion for Mesorhizobium in the bulk soil between eCO_2 and aCO_2 conditions. The Bradyrhizobium showed to be higher in proportion in the bulk soil under the eCO_2 condition than under the aCO_2 condition (14.4% vs. 11.9%).
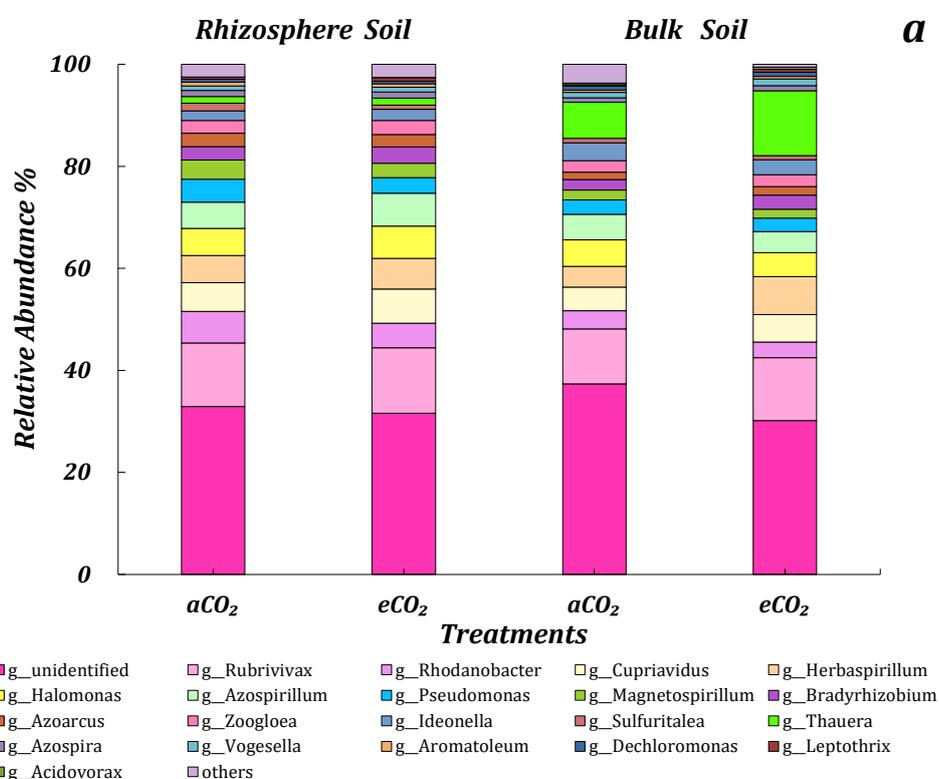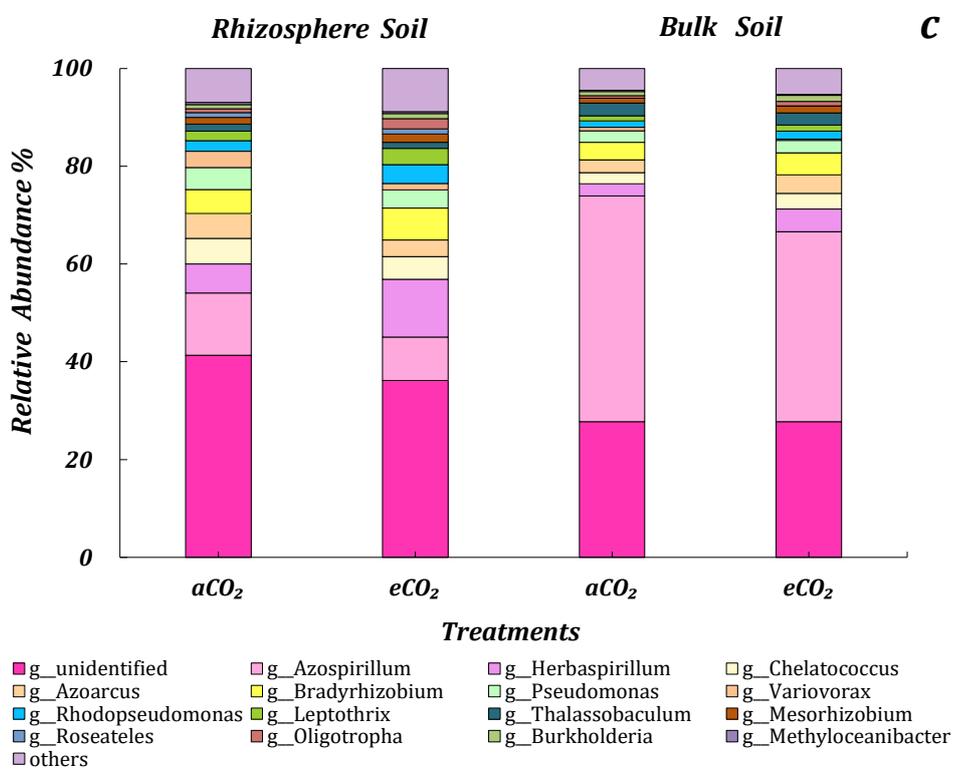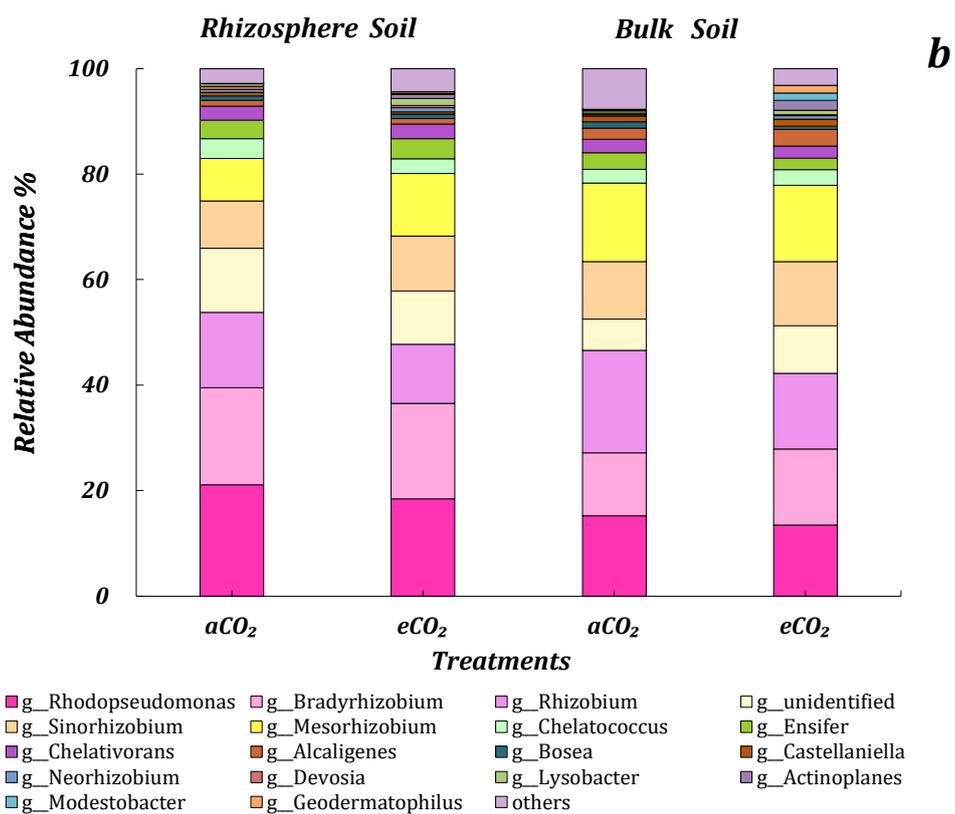

Figure 5. *Cont.*

Figure 5. The compositional structure of nirS (**a**), nirK (**b**) and nosZ (**c**) at the tasseling stage of maize growth period (at the genus level).

Results showed (Figure 5c) that Azospirillum was the main dominant species which possesses nosZ gene, particularly in the bulk soil, where it accounted for more than 1/3 in the compositional proportion. In addition, some other genera also had relatively high proportions in the studied soil, including Herbaspirillum, Chelatococcus, Azoarcus, Bradyrhizobium, and Pseudomonas. In the rhizospheric soil, some genera were significantly higher in the compositional proportion than in bulk soil, which included Herbaspirillum (6.0%–11.8% vs. 2.5%–4.6%) and Bradyrhizobium (4.9%–6.6% vs. 3.6–4.5%). However, some genera were significantly higher in proportion in the bulk soil than in the rhizospheric soil, such as the Azospirillum with the compositional proportion of 38.9%–46.2% in the bulk soil, while only 8.9%–12.8% in the rhizospheric soil. Under eCO_2 conditions, some genera were higher in their compositional proportion than under aCO_2 conditions. These genera mainly included Herbaspirillum (11.8% vs. 6.0% in the rhizospheric soil and 4.6% vs. 2.5% in the bulk soil) and Bradyrhizobium (6.6% vs. 4.9% in the rhizospheric soil and 4.5% vs. 3.6% in the bulk soil). However, some genera showed significantly lower in the compositional proportion under eCO_2 condition than under aCO_2 condition, for instance Azospirillum (8.9% vs. 12.8% in the rhizospheric soil and 38.9% vs. 46.2% in the bulk soil).

4. Discussion

4.1. Effects of Elevated CO_2 on Soil Carbon and Associated Microbes

Our results showed that soil MBC in the rhizospheric soil had significantly increased under the eCO_2 condition compared with that under the aCO_2 condition at the tasseling stage during summer maize growth period (Figure 1). This may be due to the increased root exudates and root exfoliations which came from photosynthetic products enhanced by eCO_2 [32,33]. In our study, the qPCR results showed that the quantity of bacteria in terms of 16s rRNA in the eCO_2 treatment was 75% of that in the aCO_2 treatment. However, the quantity of fungi was increased by 10% in the eCO_2 treatment than in the aCO_2 treatment (Table 1). These results illustrated that eCO_2 had an effect on the microbial population in the rhizospheric soil, which was mainly reflected in the increase of fungi population. This is in agreement with the research reported by Janus [34]. Fungi enable to decompose recalcitrant carbon substrate [35] and have lower oxygen demands than bacteria. Soil pH in the rhizospheric zone is usually lower due to the ammonium uptake by roots and nitrification in the dryland soils after fertilization [36,37]. Fungi were more suitable for growing in acidic conditions even when pH reached to pH 4.5 [38,39]. Therefore, we suggest that the increased MBC in rhizospheric soil at the tasseling stage was mainly attributable to the increase in fungi population. Paterson [40] compared the microbes in the rhizospheric and bulk soils with a ^{13}C labeling technique and found that eCO_2 promoted the turnover rate of soil carbon by increasing the decomposition rate of carbon derived from plants. He also observed that, under the eCO_2 condition, the new labeled 13C accounted for 80% of total carbon in PLFAs in fungi, while it was only 25% in bacteria. In a 13C-depleted FACE experiment [41], it was found that δ13C decreased by 3.1‰ under the eCO_2 condition, indicating that fungi participated more actively in utilizing the photosynthetic product in the rhizospheric soil. Therefore, we suggest that eCO_2 could increase the substrate for fungal activities, particularly at the mild acidic rhizospheric soil, and thus promote the soil carbon turnover.

As for the compositional structure of microbes regarding the bacteria, the percentage of Subgroup 6 and Subgroup 4 for 16s rRNA were significantly higher than under the eCO_2 conditions in both rhizospheric and bulk soils (Figure 2). It was reported that Subgroups 3, 4, 6 and 7 belonged to Acidobacteria groups that are hard to be isolated, hence there is only limited information available on these groups [42]. It was observed that pH could affect the abundance of Subgroups 3, 4, 6 and 7 [38,43]. Moreover, some orders showed slight decrease in the compositional proportion in the eCO_2 treatment (Figure 2), for instance Sphingomonadales, a kind of obligate aerobe that can produce catalase [44] and convert hydrogen peroxide into water and oxygen to eliminate the negative effects of peroxide to other microbes. Therefore, we suggest that oxygen content may be altered under the eCO_2 conditions and

this could lead to a decline in the relative abundance of aerobic microbes such as Sphingomonadales in the soil. Further study may be needed to consider this.

As for fungi communities, under eCO_2 conditions, the compositional proportions of Chaetomium and Humicola in both rhizospheric and bulk soils were higher than under the aCO_2 conditions (Figure 3). However, the proportion of Talaromyces decreased under the eCO_2 condition. According to Gautam [45], both Chaetomium and Humicola were thermophilic microbes that can synthesize cellulase, hemicellulase and amylase to decompose cellulose and organic materials. In this study, the compositional proportion of these two genera increased under the eCO_2 conditions, possibly due to the increase in dead root litter in the soil, which may promote the utilization of substrate by fungi. Soil humus could be formed and derived from the decomposed plant products of macromolecular organic compounds mediate by Humicola. Clare [46] found that increase in fungal populations under the eCO_2 conditions could stimulate the formation of soil aggregates, possibly due to the cementing function of mycelia produced by fungi. Talaromyces can synthesize chitinase and therefore decompose recalcitrant compounds [47]. In this study, the proportions of Talaromyces showed a decrease in the rhizospheric soil under the eCO_2 condition, possibly due to the increase in the labile and resistant carbon substrate instead of recalcitrant; this may lead to an increase in the quantity of some bacteria and fungi that preferentially decompose those plant-derived substrates; also, in turn, cause a decrease in the proportion of some genera that utilize recalcitrant carbon substrates (such as lignin).

We measured the content of soil organic carbon (SOC) in this field almost every year and it did not show significant change between eCO_2 and aCO_2 plots (data unpublished) for the bulk soil. Therefore, analyzing our results combined with the literature mentioned above, we suggest that eCO_2 increased soil MBC at the rhizospheric zone and thus may result in changed pH and oxygen conditions. As a consequence, the abundance of some fungi (such as Chaetomium, Humicola) increased, which prefer to decompose cellulose/semi-cellulose/starch. Moreover, some bacteria genera (such as Subgroups 4 and 6) may increase to some extent in response to changed substrate condition at the rhizospheric soil under eCO_2. Ten years of enrichment in atmospheric CO_2 did not change the SOC except for the MBC pool at the rhizospheric zone at the key growing period for maize. The comprehensive balance between carbon input in different pools and the decomposition of SOC still needs further detailed study.

4.2. Effects of Elevated CO_2 on Soil N and Relevant Nitrifiers and Detrifiers

In general, multiple N processes occur concurrently in the soil, including the biochemical processes such as mineralization, nitrification, denitrification and physical processes such as ammonia volatilization, nitrate leaching and ammonium fixation. ECO_2 could influence the N conversion due to changed soil environment such as the changed substrate in quantity and components for microbes [48,49].

In this study, the sampling period for measuring the quantities and communities of soil microbes was at the 13th day after topdressing, when most of applied ammonium-N had been nitrified to nitrate-N (Figure 1). The concentration of nitrate-N also showed slightly lower in eCO_2 treatments than that in the aCO_2 treatments. Data also indicated that the AOB quantity, in terms of copy number of amoA genes, did not show significant difference in the rhizospheric soil as that in the rhizospheric soil under the eCO_2 condition (it showed a little higher in the bulk soil under the eCO_2 condition). This was in agreement with the results reported by Lu [50] and Liu [51]. Nelson [52] found that eCO_2 had no effect on AOB; however, Lin [53] demonstrated that the quantity of AOB could decline under eCO_2 conditions in the winter wheat growth period for the rice-winter wheat cropping system. These differences may likely be due to differences in the soil N forms, plant types (dryland, paddy field), fertilization, crop growth period and other abiotic factors.

Nitrosospira showed to be a dominant genus (39.8%–55.6% in compositional proportion) in the nitrification process for the studied soil, consistent with the findings of Kowalchuk and Stephen [54]. However, the compositional percentage of Nitrosospira declined under the eCO_2 conditions in both the rhizospheric soil and the bulk soil (Figure 4a). It was reported that Nitrosospira was the main

contributor to the nitrification process in dryland soils when the ammonium-N content was high [55,56]. In this study, we collected soil samples at the 13th day after topdressing and found that most of the ammonium-N in soil had been converted to nitrate-N by nitrifiers at this period. Therefore, the significant reduction in the Nitrosospira in percentage in the rhizospheric soil under the eCO_2 condition was likely due to the change in substrate concentration. Data also showed that the proportion of Nitrosospira sp. 17 was one of the dominant species at this period (Figure 4b); however, its percentage showed decrease under eCO_2 at the sampling time. Since ammonium-N was the substrate for nitrification, the AOB community structure was closely related to the level of ammonium-N [57]. AOB community, particularly the Nitrosospira, was altered in the calcareous fluvo-aquic soil due to the different N forms at different periods [58,59]. Other studies also have shown that Nitrosomonas could mostly be found in the calcareous soil [58] and the manure-added soils [60]. Hence, we suggest that Nitrosomonas other than Nitrosospira may play a dominant role at this stage when nitrate N had become the main N form in the soil, possibly due to a change with the substrate change.

For the denitrifiers, our results showed that the quantities of three kinds of denitrifying functional genes (nirS, nirK, and nosZ) were significantly higher under eCO_2 conditions than under aCO_2 conditions and the ratio of nosZ to (nirS + nirK) also showed similar characteristics. In this study, after 13 days of N fertilizer application, nitrate-N exhibited to be the main inorganic N form in the soil (Figure 1). Data also indicated that some genera such as Mesorhizobium for nirK was increased in the proportion at the rhizospheric soils under the eCO_2 condition (Figure 5b). Jung [61] reported that the community of nirK gene was more sensitive to environmental changes than that of nirS gene, consistent with our results. For microbes bearing nosZ genes, the compositional ratio of some genera was increased significantly in the rhizospheric soil in the eCO_2 treatment than in the aCO_2 treatment, including Herbaspirillum (11.8% vs. 6.0%), Bradyrhizobium (6.6% vs. 4.9%) and Rhodopseudomonas (3.9% vs. 2.2%); while the Azospirillum showed a decrease in compositional proportion under eCO_2 conditions (Figure 5c). Many researchers reported that Herbaspirillum was the dominant genus with the nosZ gene in the rhizospheric soils of arable land for either rice or maize planting [30,62]. Herbaspirillum could convert N_2O to N_2 and it can also oxidize glucose, galactose and L-arabinose, other than hydrolyze starch [63]. We hypothesize that the reason that the relative abundance of Herbaspirillum was higher in rhizosphere soil was due to the lower oxygen resulted by both hetero- and autotrophic respiration in the rhizospheric zone. Studies reported that Azospirillum can reduce the release of N_2O in the denitrification, but the rate of N_2O reduction in the denitrification was much lower than that of Herbaspirillum [64].

Soil MBN is a kind of temporary N pool to resist the loss of soil N to the environment and could be converted into the available N when crops need it. In our results, the total inorganic N in the rhizospheric soil for eCO_2 treatment was lower to some extent due to crop uptake, while the content of soil MBN was increased at the same time. Our measurements showed that soil total N had no significant difference after ten years FACE (2007–2018, unpublished data). It may be because that there was an N-balance between input from fertilizers, and output from uptake, mineralization and loss under the current management practices. Cheng [8] indicated that ten years of eCO_2 did not change the availability of soil N in a rice paddy soil. The effect of eCO_2 was also reflected in the active component of carbon and nitrogen, particularly in the rhizospheric soil even after 10 years enrichment in atmospheric CO_2; this coincided with previous studies done by Liu [65] and Deng [66]. Studies indicated that net N mineralization rate and nitrification rate were positively correlated with soil MBC under both ambient and high CO_2 concentrations [67,68]. The nitrification rate was usually controlled by AOB for dryland soils, especially in N-rich alkaline soils [58,68]. Liu [65] also showed that AOB abundance was significantly increased in eCO_2 plots at the heading stage of rice and the same results were observed in a forest soil reported by Long [69]. As for the denitrifiers, it was found that the abundance of nirS-bearing denitrifiers was highly correlated with potential denitrification rates in the sediments of San Francisco Bay [70]. In our study, the copy number of nirS-bearing denitrifiers and other denitrifiers were all promoted by eCO_2, especially in the rhizospheric soil. Thus,

we suggest the increase of soil MBC in eCO$_2$ conditions came from influx of rhizodeposition such as root exudates and secretions, and in response to that, other processes including mineralization, nitrification, denitrification and biological preservation (related to MBN) may be promoted in the meantime. Besides, an increase in soil MBC could provide more energy for denitrifiers during the denitrification process. The promoted C and N cycling processes coincided with the increase in the quantities of ITS, denitrifiers and the compositional changes in some genera for 16s rRNA (reflected in Subgroups 4 and 6), ITS (reflected in Chaetomium and Humicola), AOB (reflected in the decrease of Nitrosospira), nirK (reflected in Mesorhizobrium), and nosZ (reflected in Herbaspirillum). Moreover, the integrated rate balance among multiple C and N cycling processes would be more important in analyzing the relationship between the nutrient status and microbes.

Actually, the effects of eCO$_2$ on soil nutrient cycling remain unclear at many aspects, such as increasing or decreasing the soil carbon sequestration, the influences on N N$_2$O emission contributions from different pathways and so on. Further studies are needed to address the comprehensive processes of carbon and nitrogen cycling and their final balance and associated microbes.

5. Conclusions

Throughout this study, we demonstrated that the eCO$_2$ significantly increased the soil MBC and MBN in the rhizospheric soil at the active tasseling stage during maize growth period. The change of microbes which were associated with carbon substrate was reflected in the significant increase in the quantity of fungi in terms of ITS and their change in the compositional proportion of some genera such as increase in Chaetomium for ITS, and increase in Subgroups 4 and 6 for 16s rRNA. ECO$_2$ also increased the quantity of AOB at the bulk soil slightly, as well as the denitrifiers significantly, in terms of the plasmid copy number of nirS, nirK, and nosZ genes, especially at the rhizospheric soil. The related changes in compositional proportion for nitrogen cycling microbes were reflected as the decrease in the ratio of nitrosospira for AOB, increase in the proportion of Mesorhizobium for nirK, and increase in the percentage of Herbaspirillum and Bradyrhizobium for nosZ. The changes in compositional proportion of the functional genes, which are associate with C and N cycling, were mostly the response of microbes on the form and quantity of soil C and N in the soils, particularly in the rhizospheric soil. More studies are needed for further detailed explanations between nutrient cycling and microbes.

References

1. Ruddiman, W.F. The Anthropocene. *Annu. Rev. Earth Planet. Sci.* **2013**, *41*, 45–68. [CrossRef]
2. Pieter Tans, R.K. NOAA/ESRL. 2017. Available online: www.esrl.noaa.gov/gmd/ccgg/trends/ (accessed on 9 July 2017).
3. Ainsworth, E.A.; Rogers, A. The response of photosynthesis and stomatal conductance to rising [CO$_2$]: Mechanisms and environmental interactions. *Plant Cell Environ.* **2007**, *30*, 258–270. [CrossRef] [PubMed]

4. Austin, E.E.; Castro, H.F.; Sides, K.E.; Schadt, C.W.; Classen, A.T. Assessment of 10 years of CO_2 fumigation on soil microbial communities and function in a sweetgum plantation. *Soil Boil. Biochem.* **2009**, *41*, 514–520. [CrossRef]

5. Zak, D.R.; Pregitzer, K.S.; Curtis, P.S.; Holmes, W.E. Atmospheric CO_2 and the composition and function of soil microbial communities. *Ecol. Appl.* **2000**, *10*, 47–59.

6. Li, Y.; Huang, G.; Shi, Y. Effect of atmospheric CO_2 enrichment on soil microbes and related factors. *Ying yong The J. of Appl. Ecol.* **2003**, *14*, 2321–2325. (In Chinese)

7. Phillips, R.P.; Finzi, A.C.; Bernhardt, E.S. Enhanced root exudation induces microbial feedbacks to N cycling in a pine forest under long-term CO_2 fumigation. *Ecol. Lett.* **2011**, *14*, 187–194. [CrossRef]

8. Cheng, Y.; Zhang, J.; Zhu, J.; Liu, G.; Zhu, C.; Wang, S. Ten years of elevated atmospheric CO_2 doesn't alter soil nitrogen availability in a rice paddy. *Soil Boil. Biochem.* **2016**, *98*, 99–108. [CrossRef]

9. Carney, K.M.; Hungate, B.A.; Drake, B.G.; Megonigal, J.P. Altered soil microbial community at elevated CO_2 leads to loss of soil carbon. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 4990–4995. [CrossRef]

10. Langley, J.A.; McKinley, D.C.; Wolf, A.A.; Hungate, B.A.; Drake, B.G.; Megonigal, J.P. Priming depletes soil carbon and releases nitrogen in a scrub-oak ecosystem exposed to elevated CO_2. *Soil Boil. Biochem.* **2009**, *41*, 54–60. [CrossRef]

11. Sayer, E.J.; Heard, M.S.; Grant, H.K.; Marthews, T.R.; Tanner, E.V. Soil carbon release enhanced by increased tropical forest litter fall. *Nature Clim. Change.* **2011**, *1*, 304–307. [CrossRef]

12. Drissner, D.; Blum, H.; Tscherko, D.; Kandeler, E. Nine years of enriched CO_2 changes the function and structural diversity of soil microorganisms in a grassland. *Eur. J. Soil Sci.* **2007**, *58*, 260–269. [CrossRef]

13. He, Z.; Xu, M.; Deng, Y.; Kang, S.; Kellogg, L.; Wu, L.; Van Nostrand, J.D.; Hobbie, S.E.; Reich, P.B.; Zhou, J. Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO_2. *Ecol. Lett.* **2010**, *13*, 564–575. [CrossRef] [PubMed]

14. Zak, D.R.; Pregitzer, K.S.; Curtis, P.S.; Teeri, J.A.; Fogel, R.; Randlett, D.L. Elevated atmospheric CO_2 and feedback between C and N cycles. *Plant and Soil.* **1993**, *151*, 105–117. [CrossRef]

15. Schortemeyer, M.; Hartwig, U.A.; Hendrey, G.R.; Sadowsky, M.J. Microbial community changes in the rhizospheres of white clover and perennial ryegrass exposed to Free Air Carbon dioxide Enrichment (FACE). *Soil Boil. Biochem.* **1996**, *28*, 1717–1724. [CrossRef]

16. Cheng, L.; Booker, F.L.; Tu, C.; Burkey, K.O.; Zhou, L.; Shew, H.D.; Rufty, T.W.; Hu, S. Arbuscular Mycorrhizal Fungi Increase Organic Carbon Decomposition Under Elevated CO_2. *Science* **2012**, *337*, 1084–1087. [CrossRef]

17. Verhamme, D.T.; Prosser, J.I.; Nicol, G.W. Ammonia concentration determines differential growth of ammonia-oxidizing archaea and bacteria in soil microcosms. *The ISME Journal.* **2011**, *6*, 1067e1071.

18. Xia, W.; Zhang, C.; Zeng, X.; Feng, Y.; Weng, J.; Lin, X.; Zhu, J.; Xiong, Z.; Xu, J.; Cai, Z.; et al. Autotrophic growth of nitrifying community in an agricultural soil. *ISME J.* **2011**, *5*, 1226–1236. [CrossRef]

19. Philippot, L.; Hallin, S.; Schloter, M. Ecology of Denitrifying Prokaryotes in Agricultural Soil. *Adv. Agron.* **2007**, *96*, 249–305.

20. Shapleigh, J.P. Denitrifying Prokaryotes. In *The Prokaryotes*; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2013; pp. 405–425.

21. Hungate, B.A.; Canadell, J.; Chapin, F.S. Plant Species Mediate Changes in Soil Microbial N in Response to Elevated CO_2. *Ecology* **1996**, *77*, 2505–2515. [CrossRef]

22. Horz, H.-P.; Barbrook, A.; Field, C.B.; Bohannan, B.J.M. Ammonia-oxidizing bacteria respond to multifactorial global change. *Proc. Nat. Acad. Sci. USA* **2004**, *101*, 15136–15141.

23. Barnard, R.; Barthes, L.; Le Roux, X.; Harmens, H.; Raschi, A.; Soussana, J.-F.; Winkler, B.; Leadley, P.W. Atmospheric CO_2 elevation has little effect on nitrifying and denitrifying enzyme activity in four European grasslands. *Glob. Chang. Boil.* **2004**, *10*, 488–497. [CrossRef]

24. Barnard, R.; Leadley, P.W.; Hungate, B.A. Global change, nitrification, and denitrification: A review. *Glob. Biogeochem. Cycles* **2005**, *19*, 1007. [CrossRef]

25. Hunger, S.; Schmidt, O.; Hilgarth, M.; Horn, M.A.; Kolb, S.; Conrad, R.; Drake, H.L. Competing Formate- and Carbon Dioxide-Utilizing Prokaryotes in an Anoxic Methane-Emitting Fen Soil▽†. *Appl. Environ. Microbiol.* **2011**, *77*, 3773–3785. [CrossRef] [PubMed]

26. Petersen, D.G.; Firestone, M.; Herman, D.J.; Turetsky, M.; Waldrop, M.; Blazewicz, S.J. Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. *Environ. Microbiol.* **2012**, *14*, 993–1008. [CrossRef] [PubMed]

27. Lee, S.-H.; Megonigal, P.J.; Kang, H. How do Elevated CO_2 and Nitrogen Addition Affect Functional Microbial Community Involved in Greenhouse Gas Flux in Salt Marsh System. *Microb. Ecol.* **2017**, *81*, 169–680. [CrossRef] [PubMed]

28. Huang, T.; Gao, B.; Hu, X.-K.; Lu, X.; Well, R.; Christie, P.; Bakken, L.R.; Ju, X.-T. Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous Fluvo-aquic soil. *Sci. Rep.* **2014**, *4*, 3950. [CrossRef] [PubMed]

29. Cleveland, C.C.; Diana, R.N.; Steven, K.S.; Alan, R.T. Increases in soil respiration following labile carbon additions linked to rapid shifts in soil microbial community composition. *Biogeochemistry* **2007**, *82*, 229–240. [CrossRef]

30. Henry, S.; Texier, S.; Hallet, S.; Bru, D.; Dambreville, C.; Chèneby, D.; Bizouard, F.; Germon, J.C.; Philippot, L. Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: Insight into the role of root exudates. *Environ. Microbiol.* **2008**, *10*, 3082–3092. [CrossRef]

31. Han, X.; Hao, X.; Lam, S.K.; Wang, H.; Li, Y.; Wheeler, T.; Ju, H.; Lin, E. Yield and nitrogen accumulation and partitioning in winter wheat under elevated CO_2: A 3-year free-air CO_2 enrichment experiment. *Agric. Ecosyst. Environ.* **2015**, *209*, 132–137. [CrossRef]

32. Drigo, B.; Kowalchuk, G.A.; Van Veen, J.A. Climate change goes underground: Effects of elevated atmospheric CO_2 on microbial community structure and activities in the rhizosphere. *Boil. Fertil. Soils* **2008**, *44*, 667–679. [CrossRef]

33. Lukac, M.; Lagomarsino, A.; Moscatelli, M.C.; De Angelis, P.; Cotrufo, M.F.; Godbold, D.L. Forest soil carbon cycle under elevated CO_2 - a case of increased throughput? *Forestry* **2009**, *82*, 75–86. [CrossRef]

34. Janus, L.R.; Angeloni, N.L.; McCormack, J.; Rier, S.T.; Tuchman, N.C.; Kelly, J.J. Elevated Atmospheric CO_2 Alters Soil Microbial Communities Associated with Trembling Aspen (Populus tremuloides) Roots. *Microb. Ecol.* **2005**, *50*, 102–109. [CrossRef] [PubMed]

35. Crawford, R.L. *Lignin biodegradation and transformation*; Wiley: New York, NY, USA, 1981; p. 137.

36. Phillip, B.; Babou, O.J.; Armand, R.K.; Lloyd, A.P.; David, A.L. Effects of long-term soil acidification due to nitrogen fertilizer inputs in Wisconsin. *Plant and Soil.* **1997**, *197*, 61–69.

37. Guo, L.; Wang, X.; Diao, T.; Ju, X.; Niu, X.; Zheng, L.; Zhang, X.; Han, X. N2O emission contributions by different pathways and associated microbial community dynamics in a typical calcareous vegetable soil. *Environ. Pollut.* **2018**, *242*, 2005–2013. [CrossRef] [PubMed]

38. Rousk, J.; Bååth, E.; Brookes, P.C.; Lauber, C.L.; Lozupone, C.; Caporaso, J.G.; Knight, R.; Fierer, N. Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J.* **2010**, *4*, 1340–1351. [CrossRef] [PubMed]

39. Rousk, J.; Brookes, P.C.; Bååth, E. Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization▽†. *Appl. Environ. Microbiol.* **2009**, *75*, 1589–1596. [CrossRef] [PubMed]

40. Paterson, E.; Thornton, B.; Midwood, A.J.; Osborne, S.M.; Sim, A.; Millard, P. Atmospheric CO_2 enrichment and nutrient additions to planted soil increase mineralisation of soil organic matter, but do not alter microbial utilisation of plant- and soil C-sources. *Soil Boil. Biochem.* **2008**, *40*, 2434–2440. [CrossRef]

41. Sharona, A.B.; Susane, E.Z. Altered patterns of soil carbon substrate usage and heterotrophic respiration in a pine forest with elevated CO_2 and N fertilization. *Glob. Change Biol.* **2008**, *14*, 1025–1036.

42. Kristin, K.; Bernd, W.; Vera, K.; Franziska, W.; Heiko, N.; Ingo, S.; Marion, S.; Rolf, D. Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests. *Sci. Report.* **2016**, *6*, 33696.

43. Jones, R.T.; Robeson, M.S.; Lauber, C.L.; Hamady, M.; Knight, R.; Fierer, N. A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME J.* **2009**, *3*, 442–453. [CrossRef]

44. Takeuchi, Y.; Yasukawa, H.; Yamaoka, Y.; Takahashi, N.; Tamura, C.; Morimoto, Y.; Fukushima, S.; Vasavada, R.C. Effects of Oleic Acid/Propylene Glycol on Rat Abdominal Stratum Corneum: Lipid Extraction and Appearance of Propylene Glycol in the Dermis Measured by Fourier Transform Infrared/Attenuated Total Reflectance(FT-IR/ATR) Spectroscopy. *Chem. Pharm. Bull.* **1993**, *41*, 1434–1437. [CrossRef]

45. Gautam, S.P.; Bundela, P.S.; Pandey, A.K.; Jamaluddin; Awasthi, M.K.; Sarsaiya, S. Diversity of Cellulolytic Microbes and the Biodegradation of Municipal Solid Waste by a Potential Strain. *Int. J. Microbiol.* **2012**, *2012*, 1–12. [CrossRef] [PubMed]

46. Clare, E.K. Soil carbon cycling responses to elevated CO$_2$ and nitrogen addition. University of Minnesota, 2017. Available online: https://conservancy.umn.edu/handle/11299/193426 (accessed on 6 November 2008).

47. Madi, L.; Katan, T.; Katan, J.; Henis, Y. Biological Control of Sclerotium rolfsii and Verticillium dahliae by Talaromyces flavus Is Mediated by Different Mechanisms. *Phytopathology* **1997**, *87*, 1054–1060. [CrossRef] [PubMed]

48. Norby, R.J.; Cotrufo, M.F.; Ineson, P.; Canadell, J.G.; Cortufo, M.F.; O'Neill, E.G. Elevated CO$_2$, litter chemistry, and decomposition: A synthesis. *Oecologia* **2001**, *127*, 153–165. [CrossRef]

49. Nowak, R.S.; Ellsworth, D.; Smith, S.D. Functional responses of plants to elevated atmospheric CO$_2$- do photosynthetic and productivity data from FACE experiments support early predictions? *New Phytol.* **2004**, *162*, 253–280. [CrossRef]

50. Lu, Z.W.; Wan, G.F.; Zhang, P.; Zhang, S.L.; Guo, Y.; Zhang, W.H. Effects of doubled CO$_2$ and enhanced UV-B radiation on rhizosphere ammonia-oxidizing bacteria and soil enzymes in soybean (Glycine max Merr.). *Soybean Sci.* **2012**, *31*, 69–72. (In Chinese)

51. Liu, Y.; Wang, G.L.; Li, L.Q.; Pan, G.X. Response of Soil Nitrifier and Denitrifier Community and Activity to Elevated Atmospheric CO$_2$ Concentration and Temperature. *Environ. Sci.* **2017**, *38*, 1245–1252. (In Chinese)

52. Nelson, D.M.; Cann, I.K.O.; Mackie, R.I. Response of Archaeal Communities in the Rhizosphere of Maize and Soybean to Elevated Atmospheric CO$_2$ Concentrations. *PLoS ONE* **2010**, *5*, e15897. [CrossRef]

53. Lin, X.G.; Hu, J.L.; Chu, H.Y. Response of soil ammonia-oxidizing bacteria to elevated atmospheric CO$_2$. *Rural Eco-Environ.* **2005**, *21*, 44–46.

54. Kowalchuk, G.A.; Stephen, J.R. Ammonia-Oxidizing Bacteria: A Model for Molecular Microbial Ecology. *Annu. Rev. Microbiol.* **2001**, *55*, 485–529. [CrossRef]

55. Kowalchuk, G.A.; Stienstra, A.W.; Heilig, G.H.; Stephen, J.R.; Woldendorp, J.W. Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. *Environ. Microbiol. 2*, 99–110. [CrossRef]

56. Kowalchuk, G.A.; Stienstra, A.W.; Heilig, G.H.; Stephen, J.R.; Woldendorp, J.W. Molecular analysis of ammonia-oxidizing bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). *FEMS Microbiol. Ecol. 31*, 207–215. [CrossRef] [PubMed]

57. Ai, C.; Liang, G.; Sun, J.; Wang, X.; He, P.; Zhou, W. Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil. *Soil Boil. Biochem.* **2013**, *57*, 30–42. [CrossRef]

58. Shen, J.-P.; Zhang, L.-M.; Zhu, Y.-G.; Zhang, J.-B.; He, J.-Z. Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. *Environ. Microbiol.* **2008**, *10*, 1601–1611. [CrossRef] [PubMed]

59. Wu, Y.; Lu, L.; Wang, B.; Lin, X.; Zhu, J.; Cai, Z.; Yan, X.; Jia, Z. Long-Term Field Fertilization Significantly Alters Community Structure of Ammonia-Oxidizing Bacteria rather than Archaea in a Paddy Soil. *Soil Sci. Soc. Am. J.* **2011**, *75*, 1431–1439. [CrossRef]

60. Fan, F.; Yang, Q.; Li, Z.; Wei, D.; Cui, X.; Liang, Y. Impacts of Organic and Inorganic Fertilizers on Nitrification in a Cold Climate Soil are Linked to the Bacterial Ammonia Oxidizer Community. *Microb. Ecol.* **2011**, *62*, 982–990. [CrossRef] [PubMed]

61. Jung, J.; Yeom, J.; Kim, J.; Han, J.; Lim, H.S.; Park, H.; Hyun, S.; Park, W. Change in gene abundance in the nitrogen biogeochemical cycle with temperature and nitrogen addition in Antarctic soils. *Res. Microbiol.* **2011**, *162*, 1018–1026. [CrossRef]

62. Dambreville, C.; Hallet, S.; Nguyen, C.; Morvan, T.; Germon, J.-C.; Philippot, L.; Hallet, S. Structure and activity of the denitrifying community in a maize-cropped field fertilized with composted pig manure or ammonium nitrate. *FEMS Microbiol. Ecol.* **2006**, *56*, 119–131. [CrossRef]

63. Baldani, J.I.; Baldani, V.L.D.; Seldin, L.; Döbereiner, J. Characterization of Herbaspirillum seropedicae gen. nov., sp. nov., a Root-Associated Nitrogen-Fixing Bacterium. *Int. J. Syst. Bacteriol.* **1986**, *36*, 86–93. [CrossRef]

64. Ishii, S.; Ohno, H.; Tsuboi, M.; Otsuka, S.; Senoo, K. Identification and isolation of active N2O reducers in rice paddy soil. *ISME J.* **2011**, *5*, 1936–1945. [CrossRef]

65. Liu, Y.; Zhou, H.M.; Wang, J.Q.; Liu, X.Y.; Cheng, K.; Lia, L.Q.; Zheng, J.W.; Zhang, X.H.; Zheng, J.F.; Pan, G.X. Short-term response of nitrifier communities and potential nitrification activity to elevated CO$_2$ and temperature interaction in a Chinese paddy field. *Appl. Soil Ecol.* **2015**, *96*, 88–98. [CrossRef]

66. Deng, Q.; Cheng, X.; Bowatte, S.; Newton, P.C.; Zhang, Q. Rhizospheric carbon-nitrogen interactions in a mixed-species pasture after 13 years of elevated CO_2. *Agric. Ecosyst. Environ.* **2016**, *235*, 134–141. [CrossRef]

67. Booth, M.S.; Stark, J.M.; Rastetter, E. Controls on nitrogen cycling in terrestrial ecosystems: A synthetic analysis of literature data. *Ecol. Monogr.* **2005**, *75*, 139–157. [CrossRef]

68. Sterngren, A.E.; Hallin, S.; Bengtson, P. Archaeal Ammonia Oxidizers Dominate in Numbers, but Bacteria Drive Gross Nitrification in N-amended Grassland Soil. *Front. Microbiol.* **2015**, *6*, 1620. [CrossRef] [PubMed]

69. Long, X.; Chen, C.; Xu, Z.; Oren, R.; He, J.-Z. Abundance and community structure of ammonia-oxidizing bacteria and archaea in a temperate forest ecosystem under ten-years elevated CO_2. *Soil Boil. Biochem.* **2012**, *46*, 163–171. [CrossRef]

70. Mosler, A.C.; Francls, C.A. Denitrifier abundance and activity across the san Francisco Bay estuary. *Environ. Microbiol.* **2010**, *2*, 667e676.