Effects of artificial simulated acidification on soil potential nitrification activity and ammonia oxidizing microbial community in greenhouse

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Background. A large amount of nitrate leaching produced by nitrification during vegetable production can lead to soil acidification in greenhouse system. It is of great significance to clarify the nitrification and its microbial mechanism in acidification soil. **Materials and Methods.** Simulated acidification experiment with artificially manipulated pH gradients (T1: pH 7.0, T2: pH 6.5, T3: pH 6.0, T4: pH 5.5, T5: pH 4.5) was conducted in a tomato potted experiment in the greenhouse. The abundance and community structure of ammonia oxidizers under different pH gradients was analyzed by q-PCR and high-throughput sequencing methods, respectively. **Results and discussions.** Soil acidification was accompanied by reduction of soil organic matter (SOM), total nitrogen (TN), NH_3 concentration and enzyme activities. The abundance of ammonia-oxidizing archaea (AOA) in soil was higher than that of ammonia oxidizing bacteria (AOB) in pH ranged from 6.93 to 5.33, and the opposite trend was observed when soil pH was 4.21. In acidification soils, the dominant strain in AOB was *Nitrosospira*, while the dominant AOA strain was *Nitrososphaera*. The abundance and community structure of ammonia oxidizers were mainly affected by soil pH, NH_4^+ content and microbial biomass. Soil nitrification activity (PNA) has a relationship with both AOA and AOB, in which the composition of AOA was the crucial factor affecting PNA. **Conclusions.** PNA was co-dominated by AOA and AOB in simulated acidification soils. Changes of soil pH, NH_4^+ and microbial biomass caused by acidification were the main factors for the differences of ammonia oxidizing microbial community in greenhouse soils. In addition, under acidic conditions (pH < 5), pH significantly inhibited nitrification and had a strong negative effect on production of tomatoes in greenhouse.

Effects of artificial simulated acidification on soil potential nitrification activity and ammonia oxidizing microbial community in greenhouse

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Abstract

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Materials and Methods. Simulated acidification experiment with artificially manipulated pH gradients (T1: pH 7.0, T2: pH 6.5, T3: pH 6.0, T4: pH 5.5, T5: pH 4.5) was conducted in a tomato potted experiment in the greenhouse. The abundance and community structure of ammonia oxidizers under different pH gradients was analyzed by q-PCR and high-throughput sequencing methods, respectively.

Results and discussions. Soil acidification was accompanied by reduction of soil organic matter (SOM), total nitrogen (TN), NH_3 concentration and enzyme activities. The abundance of ammonia-oxidizing archaea (AOA) in soil was higher than that of ammonia oxidizing bacteria (AOB) in pH ranged from 6.93 to 5.33, and the opposite trend was observed when soil pH was 4.21. In acidification soils, the dominant strain in AOB was *Nitrosospira*, while the dominant AOA strain was *Nitrososphaera*. The abundance and community structure of ammonia oxidizers were mainly affected by soil pH, NH_4^+ content and microbial biomass. Soil nitrification activity (PNA) has a relationship with both AOA and AOB, in which the abundance of AOA was the

crucial factor affecting PNA.

Conclusions. PNA was co-dominated by AOA and AOB in simulated acidification soils. Changes of soil pH, NH_4^+ and microbial biomass caused by acidification were the main factors for the differences of ammonia oxidizing microbial community in greenhouse soils. In addition, under acidic conditions (pH < 5), pH significantly inhibited nitrification and had a strong negative effect on production of tomatoes in greenhouse.

Introduction

High multiple cropping index and large amount of fertilizer input are common characteristics of tomato (*Lycopersicon esculentum Miller*) production systems in greenhouse, which caused a large accumulation and loss of soil nitrogen *(Zhu et al., 2005; Fan et al., 2014; Bai et al., 2020)*. With the increase of planting years and the large-scale application of chemical fertilizers, the nitrate leaching in the root zone is serious, and H^+ accumulate continuously in the process of nitrate production in soil, resulting in serious soil acidification *(Han et al., 2014; Ju et al., 2007; Min et al., 2011; Zhou et al., 2010)*. There is an important relationship between nitrate leaching due to nitrification and soil acidification *(Shi, Yao & Yan, 2009)*, however, the relationship between soil acidification and nitrification in greenhouse requires to be further clarified.

Nitrification performs a greatly vital role in the biogeochemical cycle of nitrogen *(Gruber & Galloway, 2008)*. The ammonia oxidation, driven by ammonia-oxidizing bacteria (AOB) and archaea (AOA), is the first and the rate-limiting step in the nitrification *(Kowalchuk & Stephen, 2001)*. The majority of prior research has shown that soil pH *(Nicol et al., 2008; Bru et al., 2011; Gubry-Rangin et al., 2011; Hu et al., 2015)* and substrate (ammonia/ammonium) *(Martens-Habbena et al., 2009; Schleper, 2010)* are the most important factors affecting the abundance and community compositions of both AOA and AOB. Furthermore, AOA was more competitive than AOB in a low-pH and low-ammonia environment owing to long-term niche differences *(Wang et al., 2015a)*, whereas AOB grows generally at high ammonia concentration *(Verhamme, Prosser*

& Nicol, 2011). There was increasing evidence shows that AOB may dominate the autotrophic ammonia oxidation process in alkaline soils *(Jia & Conrad, 2009; Xia et al., 2011)*, while AOA is the main ammonia oxidizers in acidic soils *(Zhang et al., 2012; Yao et al., 2013; Wang et al., 2015a; Song et al., 2016)*. However, there were different perspectives that AOB featured in nitrification in acidic soils *(Lin et al., 2021)*. In acid tea gardens, the abundance of AOB had a positive correlation with pH changes, but AOA showed no correlation *(Yao et al., 2011)*. This suggested that ammonia oxidizers have more complex ecological dispensing and metabolic diversity in acidic soils.

Current researches on the relationship of soil pH and nitrification were mainly basing on the different types or different amounts of fertilizer. The application of chemical and organic fertilizers changed the soil physicochemical properties, soil nitrification potential and the composition of AOA and AOB was also affected *(Chu et al., 2007; Schroder et al., 2011)*. The application of NPK fertilizers for 23 consecutive years resulted in soil acidification and enrichment of some AOA species, while the abundance of AOA , AOB and the potential nitrification activity increased significantly after the addition of organic fertilizers *(Xue et al., 2016)*. A single application of nitrogen fertilizer led to the soil pH decreased slightly, besides, the effect on ammonia oxidizers was different under different experimental conditions *(Wessén et al., 2010; Tao et al., 2017)*. In addition, there are some simulated acidification experiments by adding an acid solution. In forest and grassland soils, total and net nitrification rates were significantly stimulated by elevated pH, whereas soil nitrification rate was reduced due to the acidification *(Cheng et al., 2013)*. In forest and farmland soils, by adding different amounts of H_2SO_4 to generate pH gradients showed that the reduction in soil pH stimulates heterotrophic nitrification *(Zhang et al., 2020)*. However, few experiments have been investigated to explore the relationships between nitrification and acidification by simulating acidification in greenhouse system.

In order to elucidate the effect of soil acidification on nitrification and its microbial mechanism in the greenhouse system, a simulated acidification experiment was conducted in the greenhouse, where we added different amounts of H_2SO_4 to neutral field soils to achieve different pH gradients (pH7.0, pH6.5, pH6.0, pH5.5, pH4.5) in the tomato potted experiment. This study mainly investigated the changes in soil properties, potential nitrification activity (PNA), abundance and community structure of ammonia oxidizers under acidification conditions by using shaken slurry, q-PCR, and high-throughput sequencing methods. The purpose of this research was to correlate soil properties with ammonia oxidizers under simulated acidification conditions to evaluate the effects of soil acidification on ammonia oxidizing microbial community, then to further enhance our understanding on soil nitrification and its microbial mechanisms of acidified soils in greenhouse.

Materials & Methods

Experimental design and soil sampling

The experiment site was located in the solar greenhouse of the Horticulture College research basement of Shenyang Agriculture University (41°82 N, 123°56 E) in Liaoning Province, China. The area features a typical temperate continental climate with an annual average temperature and precipitation of 8.4 ℃ and 658 mm, respectively. We choose neutral field soil and added different concentrations of H_2SO_4 solution for simulated acidification experiment of potted tomato plants. The chemical properties of the original soil were, pH 7.21, SOM 47.38 $mg \cdot kg^{-1}$, TN 3.61 $g \cdot kg^{-1}$, AP 317.68 $mg \cdot kg^{-1}$, and AK 515.08 $mg \cdot kg^{-1}$.

The experiment set up 5 pH gradient treatments (T1: pH 7.0, T2: pH 6.5, T3: pH 6.0, T4: pH 5.5, T5: pH 4.5), meantime, the corresponding acid solution concentration was 0.10 $ml \cdot L^{-1}$, 0.13 $ml \cdot L^{-1}$, 0.20 $ml \cdot L^{-1}$, 0.53 $ml \cdot L^{-1}$, and 1 $ml \cdot L^{-1}$. The tomato potted experiment began in March 2021, and each treatment was repeated for 15 pots. Cultivation pot (30 cm diameter and 30 cm depth) was filled with 14 kg soil to plant one tomato plant, and a medium-fruit disease-resistant

variety “Meisheng” tomato was planted in the cultivation pot. Before the start of experiment, the soil was pre-poured with acid solution according to the pH gradient set in the experiment, and tomato planting was carried out after the soil of each treatment reached the preset pH gradient. During the whole growth period of the tomato, acid solution watering was performed once in the middle of two watering, and the amount of acid poured in each time was kept the same. Before planting, 190 g of chicken manure was applied to each pot as base fertilizer, and 15 g of NPK (15:15:15) compound fertilizer was applied to each pot after planting. Tomato plants were managed with single stem pruning, and plant height and stem diameter were measured 60 days after planting. When the tomato plant has four ears of fruits, pinch off the growth point.

At the end of June 2021, tomato fruits were harvested after they were fully ripe. We added the weights of four ears as the yield per tomato plant. After harvesting the fruit, the roots, stems and leaves of the plants were dried to constant weight in a drying oven at 80 ℃. The dry weight of stems and leaves was used as aboveground biomass, the dry weight of roots was used as underground biomass, and they were added together as total biomass. After tomato plants were removed, we collected 3 replicates rhizosphere soils (0-20cm) randomly for each treatment to analysis. Soil samples were collected in each pot from 5 positions using an auger and then mixed thoroughly to form one sample. The soil samples were sieved with a 2 mm sieve after removing visible plants residues and then divided into three parts. One part was naturally dried for determination of soil chemical properties, a second part was stored at 4 °C until the soil inorganic nitrogen (NH_4^+-N and NO_3^--N), microbial biomass, enzyme activity and potential nitrification activity were analyzed, and a third part was stored at -80 ºC for later molecular analysis.

Soil chemical analysis

Soil pH was measured with a pH meter (pHS-25, INESA) in a 1:2.5 soil: water slurry using air-dried soil and CO_2-free distilled water. Soil organic matter (SOM) was determined by oxidative digestion with $K_2Cr_2O_7$ and concentrated H_2SO_4 and then titrated with 0.5 M $FeSO_4$ under the

condition of o-phenanthroline indicator. Total soil nitrogen (TN) was digested with 5 ml concentrated H_2SO_4 and mixed catalyst at 350 ℃ and then analyzed by Automatic Kjeldahl nitrogen analyzer (BUCHI, Switzerland). Soil inorganic nitrogen were measured with a SAN++ continuous flow analyzer (Skalar, Netherlands) after extraction with 2 M KCl (soil: KCl = 1:10) for 1 h. The concentration of NH_3 was calculated according to the formula：$NH_3=NH_4^+*10^{(pH-pka)}$，pka=9.245, 25 ℃ *(Norman & Barrett, 2014)*.

Soil microbial carbon (MBC) and microbial nitrogen (MBN) were determined by the chloroform fumigation-extraction method. Briefly, 3 parts of 10 g fresh soil were weighed and placed in a glass dish, one part was fumigated with ethanol-free chloroform under 25°C dark conditions for 24 hours, while other part was placed under the same conditions without chloroform. There was also one part for the determination of moisture. Fumigated and unfumigated soil samples were then leached with 40 mL of 0.5 M K_2SO_4 at 220 rpm for 30 minutes. The solution was filtered through quantitative filter paper and analyzed by a TOC analyzer (Multi C/N 3100, Analytik Jena, Germany). Soil urease was measured by the phenol-sodium hypochlorite method, and soil protease was measured by the ninhydrin colorimetric method.

Potential nitrification activity (PNA)

Potential nitrification activity(PNA) was determined by the modified shaken slurry method *(Xue et al., 2009)*. Briefly, weighed 9 g fresh soil into a 100 mL glass bottle, added 60 ml of phosphate buffer containing 1.5 mmol·L^{-1} $(NH_4)_2SO_4$ to the bottle, then added air-permeable sealing film, and placed it in a constant temperature shaking box at 25 °C for culture. Extracted 2 ml of soil homogenate at 2 h, 4 h, 6 h, 8 h, and 10 h respectively, centrifuged to obtain the supernatant, and measured the nitrite nitrogen content in the solution by colorimeter at 540nm. The dynamic change trend of nitrite nitrogen content was used as the linearity of ammonia oxidation. By calculating the amount of nitrite produced per gram of fresh soil per unit time as the PNA.

Extraction of soil DNA and quantitative analysis of AOA and AOB

Soil DNA was extracted from 0.30g of fresh soil using the FastDNA SPIN kit for Soil (MP Biomedicals, USA). DNA concentration and quality were determined with NanoDrop2000 (Thermo Fisher, USA). Abundance of the *amoA* gene of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) were determined by an ABI7300 real-time PCR system (Applied biosystems, USA). The primers Arch-*amoA*F/Arch-*amoA*R and *amoA*-1 F/*amoA*-2R (**Table 1**) were used for quantifying the AOA and AOB *amoA* genes, respectively. The standard curve was made by referring to the method of *He et al. (2007)*. Each DNA was repeated 3 times, and the quantitative reaction system was 25ul.

High-throughput sequencing and data processing

Before high-throughput sequencing, we used the same primers and conditions as quantitative analysis of AOA and AOB for amplification, and then purified the product. The purified products were sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA). Before data analysis, the original data was spliced for quality control and optimized data, and OTU clustering was performed according to the sequence similarity of 97%. Complete data have been uploaded to the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA837458 and PRJNA 837646.

Statistical analysis

All statistical analysis was performed using the SPSS 18.0 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by a Duncan's least significant differences test was carried out to determine significant differences between the treatments, and $p < 0.05$ were considered statistically significant. Differences in microbial communities among all treatments were investigated using Principal component analysis (PCA). In addition, permutation multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis distance algorithm was used to represent significant differences in changes among all treatments. Pearson's

correlation coefficients were used to test the relationships among environmental factors (soil chemical properties and microbial activities), potential nitrification activity (PNA), and ammonia oxidizers communities. Redundancy analysis (RDA) was conducted to represent the correlation of sample distribution and environmental factors. To evaluate the relative effects of environmental factors on PNA and ammonia oxidizing microbial communities, a Random Forest analysis method using a decision tree approach to assess the importance of variables was used. Random Forest Analysis can predict how much other variables explain the target variable and the importance of other variables to the target variable.

Results

Soil chemical analysis

Watering with different concentrations of acid solutions produced significantly different pH gradient, soil pH values of each treatment were 6.93, 6.45, 5.93, 5.33 and 4.21 ***(Table 2)***. Changes in soil pH significantly affected other soil chemical properties ($p < 0.05$) ***(Table S1)***. Soil SOM and TN content showed a decreasing trend with the decrease of soil pH. In T4 (pH=5.33) and T5 (pH=4.21) treatments, soil SOM and TN content were the lowest and significantly different from other treatments ($p < 0.05$). The content of NH_4^+-N was ranged from 3.48 to 19.02 $mg \cdot kg^{-1}$, and it showed an increasing trend as the soil pH decreased. In T4 (pH=5.33) and T5 (pH=4.21) treatments, the content of NH_4^+-N were significantly higher than other treatments ($p < 0.05$). The NO_3^--N content of T5 (pH=4.21) treatment was the lowest compared with other treatments ($p < 0.05$), and the differences between other treatments were not significant. In addition, the highest NO_3^--N content was detected in T4 (pH=5.33) treatment, 25.95 $mg \cdot kg^{-1}$. The NH_3 concentration showed a decreasing trend with the decrease of soil pH, among them, the T1 (pH=6.93) treatment had the highest NH_3 concentration, 15.65 $mg \cdot m^{-3}$, which was higher than others ($p < 0.05$).

The effect of soil pH on microbial biomass and enzyme activities were shown in ***Fig.*1**. The MBN and MBC of T5 (pH=4.21) treatment were significantly lower than other treatments ($p < 0.05$). There was a very significant positive correlation between microbial biomass and NO_3^--N content ($p < 0.01$). In addition, soil MBN also had a very significant positive correlation with soil pH and a very significant negative correlation with NH_4^+-N ($p < 0.01$) ***(Table S1)***. Soil urease and protease of T4 (pH=5.33) and T5 (pH=4.21) treatments were significantly lower than T1 (pH=6.93), T2 (pH=6.45) and T3 (pH=5.93) treatments ($p < 0.05$). Correlation analysis of soil enzyme activities and soil chemical properties showed that soil urease and protease were positively correlated with soil pH, SOM, TN, and NH_3 concentration ($p < 0.01$), and negatively correlated with NH_4^+-N content ($p < 0.01$). Soil urease was also positively correlated with soil NO_3^--N content ($p < 0.05$) ***(Table S1)***.

Ammonia oxidation rate and potential nitrification activity (PNA)

The ammonia oxidation rate was expressed by the change of nitrite nitrogen content with the incubation time, and the ammonia oxidation showed a linear relationship ***(Fig.2)***. The slope magnitude of the linear relationship was ranked as T2 (pH=6.45) (k=2.249, R^2=0.9861) > T1 (pH=6.93) (k=1.909, R^2=0.9995) > T3 (pH=5.93) (k=1.476, R^2=0.9995) > T4 (pH=5.33) (k=0.3387, R^2=0.8897) > T5 (pH=4.21) (k=0.0752, R^2=0.6382). The variation range of PNA for different treatments was 0.26~2.32 NO_2^--N $ug \cdot g^{-1} \cdot h^{-1}$ ***(Fig.2F)***, the highest and lowest PNA were detected in T2 and T5 treatments, respectively, and the PNA of T2 treatment was significantly higher than other treatments ($p < 0.05$). The correlation between PNA and soil environmental factors was shown in ***Table S2***. PNA was extreme significant positive correlation with soil pH, SOM, TN, NH_3 concentration, urease, and protease ($p < 0.01$), and was extreme significant negative correlation with NH_4^+-N content ($p < 0.01$). Besides, PNA also has a significant positive correlation with MBN ($p < 0.05$).

amoA gene abundance of AOA and AOB

amoA gene copy numbers were used to indicate the abundance of ammonia oxidizing bacteria (AOB) and archaea (AOA). The AOA and AOB *amoA* gene copies were in the range 7.84×10^8 to 1.41×10^{10} and 4.53×10^8 to 1.15×10^9 copies g^{-1} d.w.s, respectively ***(Fig.3)***. The ratios of AOA to AOB were in the range 0.62 to 18.46 among all treatments. Except for T5 (pH=4.21) treatment, the abundance of AOA was higher than that of AOB in each treatments. The abundance of AOA decreased with the decrease of soil pH, the copy number of AOA *amoA* genes of T4 (pH=5.33) and T5 (pH=4.21) treatments were significantly lower than other treatments. In T1 (pH=6.93) treatment, the copy number of AOA *amoA* gene was 28.54 times that of T5 (pH=4.21) treatment. The highest and lowest AOB *amoA* gene copies appeared at T3 (pH=5.93) and T5 (pH=4.21), respectively, and the copy number of bacterial *amoA* genes in T5 (pH=4.21) treatment was significantly lower than the other treatments ($p < 0.05$).

Community diversity and composition of AOA and AOB

The sequencing results of AOA and AOB community were shown in ***Table 3***. After all the sequencing results were standardized, the AOA and AOB of each sample had 8222 and 8244 reads, respectively. The coverage value of AOA and AOB were both above 0.99, indicating that the sequencing depths of all samples were sufficient and the sequencing data was reasonable. ACE and Chao1 indices were used to represent the richness of species, Shannon index was used to characterize species diversity. For AOA community, the number of OTUs in T5 (pH=4.21) treatment was significantly lower than other treatments ($p < 0.05$). Likewise, its richness was also significantly lower than other treatments. The diversity of T4 (pH=5.33) treatment was significantly higher than other treatments ($p < 0.05$). For AOB, as the soil pH decreased, the number of OTUs showed a decreasing trend. The richness of T4 (pH=5.33) and T5 (pH=4.21) treatments were significantly lower than other treatments ($p < 0.05$). The diversity of T1 (pH=6.93) treatment was significantly higher than that of other treatments ($p < 0.05$), while the

diversity of T5 (pH=4.21) treatment was significantly lower than that of other treatments ($p < 0.05$).

All AOA OTUs were assigned into 4 phyla and 6 genera, *Thaumarchaeota* was the dominant group with a proportion of 10.69–83.60% in all treatments at the phylum level, and the proportion of *Thaumarchaeota* in T5 (pH=4.21) treatment was the smallest ***(Fig.4A)***. At genus level, among 6 species, only one species was named, namely *Nitrososphaera* ***(Fig.4B)***. The relative abundance of *Nitrososphaera* was the highest in T4 (pH=5.33) treatment, accounting for 46.87%, followed by T3 (pH=5.93) treatment, accounting for 15.52%, significantly higher than other treatments ($p < 0.05$) ***(Fig.4C)***. For the AOB community, the OTUs were assigned into 3 phyla and 6 genera. At the plylum level, *Proteobacteria* had the largest relative abundance, and the proportion of all treatment were accounting for 76.11%-95.10% ***(Fig.4D)***. At genus level, among 6 species, only one has been named, namely *Nitrosospira* ***(Fig.4E)***. Among them, this specie had the largest overall proportion in T5 (pH=4.21) treatment, accounting for 88.05%, significantly higher than other treatments ($p < 0.05$) ***(Fig.4F)***.

Based on the Bray-Curtis distance matrix, PCA was used to analyze the differences in AOA and AOB community composition among different treatments. PCA showed that the two axes explained 67.58% of the total variation of the AOA community (PERMANOVA: $R = 0.5378$, $P = 0.001$). And the AOA community composition of T4 (pH=5.33) and T5 (pH=4.21) treatments were clearly separated from other treatments on the first axis ***(Fig.5A)***. Similarly, PCA showed that the two axes explained 100% of the total variation of the AOB community composition (PERMANOVA: $R = 0.8874$, $P = 0.001$). And the AOB community composition of T3 (pH=5.93) and T4 (pH=5.33) treatments were clearly separated from other treatments on the first axis ***(Fig.5B)***.

Correlation between the abundance, diversity and composition of ammonia oxidizers with environmental factors

The correlations between the abundance and diversity of ammonia oxidizers and environmental factors were shown in ***Table S2***. The abundances of AOA and AOB were both significantly positively correlated with soil pH, SOM, TN, MBN, urease, and protease ($p < 0.05$), significantly negatively correlated with NH_4^+-N content ($p < 0.01$). In addition, AOA abundance was also significantly positively correlated with NH_3 concentration ($p < 0.01$). The diversity of AOB was significantly correlated with all measured environmental factors ($p < 0.05$), but the diversity of AOA was not. The relationships between AOA or AOB community compositions and environmental factors were tested using the RDA analysis ***(Fig.6)***, whose results showed that the X-axis and Y-axis can explain 64.82% and 67.79% of the total variation of AOA and AOB community composition, respectively. It was observed that soil pH, SOM, TN, NH_4^+-N, NH_3, urease and protease have longer projection lengths on the first ordinal axis of AOA and AOB, respectively, indicating that they had a significant impact on community composition ***(Table S4)***.

Relative importance of environmental factors on potential nitrification activity (PNA) and ammonia oxidizing microbial communities

In order to assess the relative impact of environmental factors on the ammonia-oxidizing microbial community, we used the Random Forest analysis to calculate the relative importance of each environmental factor to the differences in the ammonia-oxidizing microbial community. The model showed that NH_4^+-N content explained the most to the abundance and composition of AOA ***(Fig.7A and E)***. Among all environmental factors, soil pH has the greatest impact on AOB abundance and diversity ***(Fig.7B and D)***. MBN and MBC exerted a strong effect on the diversity of AOA ***(Fig.7C)*** and composition of AOB ***(Fig.7F)***, respectively. In addition, the analysis results showed that the soil parameter that had the greatest effect on PNA was the soil pH ***(Fig.7G)***; the abundance of AOA had the greatest effect on the PNA ***(Fig.7H)***. Besides, correlation analysis data showed that PNA was significantly associated with abundance, diversity and community composition of ammonia oxidizers ***(Table S3)***.

Effects of simulated acidification on tomato growth and yield

The effect of long-term acidification on the growth of potted tomato was shown in ***Table 4***. The plant height of T2 (pH=6.45) and T4 (pH=5.33) treatments were significantly higher than that of T1 (pH=6.93) treatment, and the differences of stem diameter among treatments were not significant. The total biomass of T4 (pH=5.33) treatment was the highest, and the total biomass of T5 (pH=4.21) was significantly lower than the other treatments ($p < 0.05$). The aboveground biomass of T4 treatment was 1.3 times that of T5 treatment, and the underground biomass of T4 treatment was 2.18 times that of T5 treatment. The yield analysis of all acidification treatments showed that when pH is T5 (pH=4.21), the yield was significantly lower than other treatments ($p < 0.05$) *(**Table 4**)*. The correlation of total biomass and yield with environmental factors was shown in ***Table S2***. The total biomass of tomato plant was significantly positively correlated with soil nitrate nitrogen content and microbial biomass ($p < 0.01$). In addition to being affected by pH, yield was also significantly positively correlated with soil NO_3^--N content, microbial biomass, and enzyme activity ($p < 0.01$). Ammonium nitrogen content was negatively correlated with tomato yield ($p < 0.05$).

Discussion

Effects of acidification on AOA and AOB abundance

Both AOA and AOB abundances decreased with the decreasing of soil pH and were significantly positively correlated with soil pH *(**Fig.3, Table S2***), which was similar to the results of previous studies *(Shen et al., 2008; Chen et al., 2013)*. In addition, the Random Forest analysis also indicated that the most important factor affecting AOB abundance was soil pH *(**Fig.7B**)*. When the soil pH was 6.93-5.33, the abundance of AOA was higher than that of AOB *(**Fig.3**)*, which was consistent with the previous report that AOA was dominant in acidic soils *(Leininger et al., 2006; Di et al., 2009; Ai et al., 2013)*. However, when pH=4.21, the abundance of AOB was higher than AOA *(**Fig.3**)*. The analysis may be due to the long-term acidification, which makes

some acid-resistant strains appear in AOB *(Li et al., 2018; Picone et al., 2021)*, the presence of acid-tolerant strains resulted in higher abundance of AOB than AOA.

Besides pH, ammonia concentration was considered to be the main factor driving ammonia oxidizing microbial community and abundance *(Prosser & Nicol, 2012; Yao et al., 2013)*. The availability of ammonia was considered to be an important factor in inducing the growth and niche of AOA and AOB *(Erguder et al., 2009; Martens-Habbena et al., 2009)*. The different responses of AOB and AOA to pH may be due to their different affinities for ammonia substrate *(Levičnik-Höfferle et al., 2012)*. In our study, with the decreasing of soil pH, NH_4^+ increased ***(Table 2)***, the AOA abundance decreased ***(Fig.3)***, and the AOA abundance was significantly negatively correlated with NH_4^+ content ***(Table S2)***. The relative importance analysis showed that NH_4^+-N content was the most important factor affecting the abundance of AOA ***(Fig. 7A)***. This may be due to the change in the balance between NH_3 and NH_4^+ as a result of the decreased of the soil pH, led to an increase in NH_4^+, which inhibited the growth of AOA and reduced the activity and abundance sensitivity of AOA *(Boer & Kowalchuk, 2001; Verhamme, Prosser & Nicol, 2011)*. The abundance of AOB decreased with decreasing pH, probably because NH_3 concentration decreased with decreasing of soil pH ***(Table 2)***, while AOB preferred a high ammonia environment *(Norman and Barrett, 2014)*. When the soil pH was 4.21, the abundance of AOB decreased less than that of AOA, possibly because there were some species in the AOB which had higher tolerant to soil NH_4^+ when compared with AOA *(Prosser & Nicol, 2012)*. Some studies have also shown that the increase of NH_4^+ will lead to the increase of AOB quantity and nitrification activity *(Okano et al., 2004; Dang et al., 2018)*.

Effects of acidification on the community structure of AOA and AOB

Soil pH has effects on diversity of ammonia-oxidizers *(Nicol et al., 2008)*. The results of this study showed that the Shannon index of AOA increased with decreasing of soil pH ***(Table 3)***, which was in contrast to the previous finding that AOA diversity increased with increasing of soil

pH *(Gubry-Rangin et al., 2011; Pester et al., 2012)*. This may be due to the increase of NH_4^+ with decreasing of soil pH ***(Table 2)***, AOA can grow in NH_4^+ rich conditions and exhibit a high degree of diversity *(Francis et al., 2005)*. The Shannon index of AOB in this study was significantly positively correlated with soil pH ***(Table S2)***, and the Random Forest analysis suggested that the most various of AOB diversity were primarily ascribed to the soil pH ***(Fig.7D)***, which was consistent with previous findings that pH was the main factor affecting AOB diversity *(Guo et al., 2017)*.

Soil pH is a key explanatory variable for the variation in community structure of AOA and AOB *(Yao et al., 2011; Zhou et al., 2014)*, our study also confirmed this. AOA and AOB community compositions had different responses to pH, which can be seen from the PCA results ***(Fig.5)***. AOA community composition of T4 and T5 treatment were separated from other treatments on the PCA1 axis ***(Fig.5A)***, and AOB community composition of T3 and T4 treatments were separated from other treatments on the PCA1 axis ***(Fig.5B)***. The Random Forest analysis showed that NH_4^+-N content and MBN were the most important factor affecting the composition of AOA and AOB communities, respectively. Soil acidification leads to changes in chemical properties and microbial biomass that affect the composition of ammonia-oxidizing microbial communities.

Under the simulated acidification conditions, *Nitrososphaera* was the main AOA among different treatments ***(Fig.4E)***. This was in contrast to previous studies that the dominant AOA cluster in acidic soils was *Nitrosotalea (Lehtovirta-Morley et al., 2011; Lu et al., 2012; Wang et al., 2015b)*. However, active AOAs were also reported to belong to the *Nitrosotalea* and *Nitrososphaera* clusters in 5 strongly acidic soils (pH < 4.5) *(Zhang et al. 2012)* . A recent study has provided strong evidence for the adaptive growth of *Nitrososphaera*-like AOA in acidic soil (pH 4.92) *(Wang et al., 2014)*. *Tourna et al., (2011)* showed that most cultivable *Nitrososphaera* are neutrophilic, and the nitrification activity of these AOA was significantly reduced or absent at

pH values below 5.5. Similarly, it was also observed that when the pH was 5.93, *Nitrososphaera* has the largest proportion, and when the pH was 4.21, its proportion is the smallest. *Nitrosospira* was the main AOB in different acidification treatments ***(Fig.4F)***, which was consistent with most studies *(Avrahami & Conrad, 2003; Chen et al., 2011)*. In acidic soils, *Nitrosospira* cluster 2, *Nitrosospira* Cluster 3 and Cluster 9 are often found to be the main active AOB *(Kowalchuk & Stephen, 2001; Wang et al., 2015a)*. In our study, *Nitrosospira* accounted for the highest proportion in T5(pH=4.21) treatment, which may be related to the previous report that *Nitrosospira* cluster 3a.2 had higher nitrogen (perhaps NH_4^+-N) demand *(Avrahami, Conrad & Braker, 2003)*.

The contribution of AOA and AOB to nitrification under the acidification soils

Our research showed that soil pH and PNA had a very significant positive correlation under long-term acidification conditions (r = 0.888, p =0.000) ***(Table S2)***, which was consistent with the results of previous studies about the effect of acidification on nitrification *(He et al., 2007)*. It was also observed that when the pH decreased from 6.93 to 6.45, the PNA increased by 1.18 times ***(Fig.2F)***. There was the same report showed that in acidic soils, the nitrification rate increased by 4.6 times when the soil pH decreased from 6.2 to 5.7 *(Zhu et al. 2011)*. When the soil pH was 6.93, the PNA was lower, and the analysis may be due to high NH_3 concentration, which inhibited the activity of ammonia oxidant. Afterwards, as the pH decreased, the PNA also decreased, and validated previous findings on acidification in acidic forests and grasslands reducing soil nitrification rate *(Cheng et al. 2013)*.

There has been existing controversies whether AOA or AOB dominates nitrification in acidic soil *(Boer & Kowalchuk, 2001; Li et al., 2018)*. Many studies claimed that AOA plays a more important role than AOB in autotrophic ammonia oxidation in strongly acidic soils *(Zhang et al., 2012)*. There were also studies show that the AOB community dominates the nitrification process *(Di et al., 2009)*, the abundance of AOB has a significant relationship with the

nitrification rate *(Xia et al., 2011)*, and AOB dominates ammonia oxidation in specific acidic soils *(Huang et al., 2018; Lin et al., 2021)*. In our study, PNA was significantly positively correlated with AOA and AOB abundance, AOB diversity and AOA community composition, significantly negatively correlated with AOA diversity ***(Table S2)***. Indicating that nitrification was driven by both AOA and AOB under long-term acidification condition in greenhouse systems ***(Fig. 8)***. Besides, the AOA abundance was the most important factors affecting PNA according to Random Forest analysis ***(Fig.7H)***.

Conclusions

Acidification led to changes in soil chemical properties, affected microbial activity as well as the abundance and community structures of AOA and AOB involved in ammonia oxidation. The PNA of acidification soil was co-dominated by AOA and AOB, and the abundance of AOA has the greatest effect on the PNA. The effect of acidification on soil nitrification and ammonia oxidizers was not only from the pH, but also from the substrate and microbial biomass changes caused by acidification. Under acid-neutral conditions (pH=6.93-5.33), plant growth would not be significantly affected. When the pH was less than 5, the nitrification activity was greatly weakened, which adversely affected the yield of tomatoes in greenhouse.

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Figure 1

Soil microbial activity of the different treatments.

(A) Soil microbial nitrogen. (B) Soil microbial carbon. (C) Soil urease. (D) Soil protease. Letters at the top of the bar chart represent significant differences at 0.05 probability level.

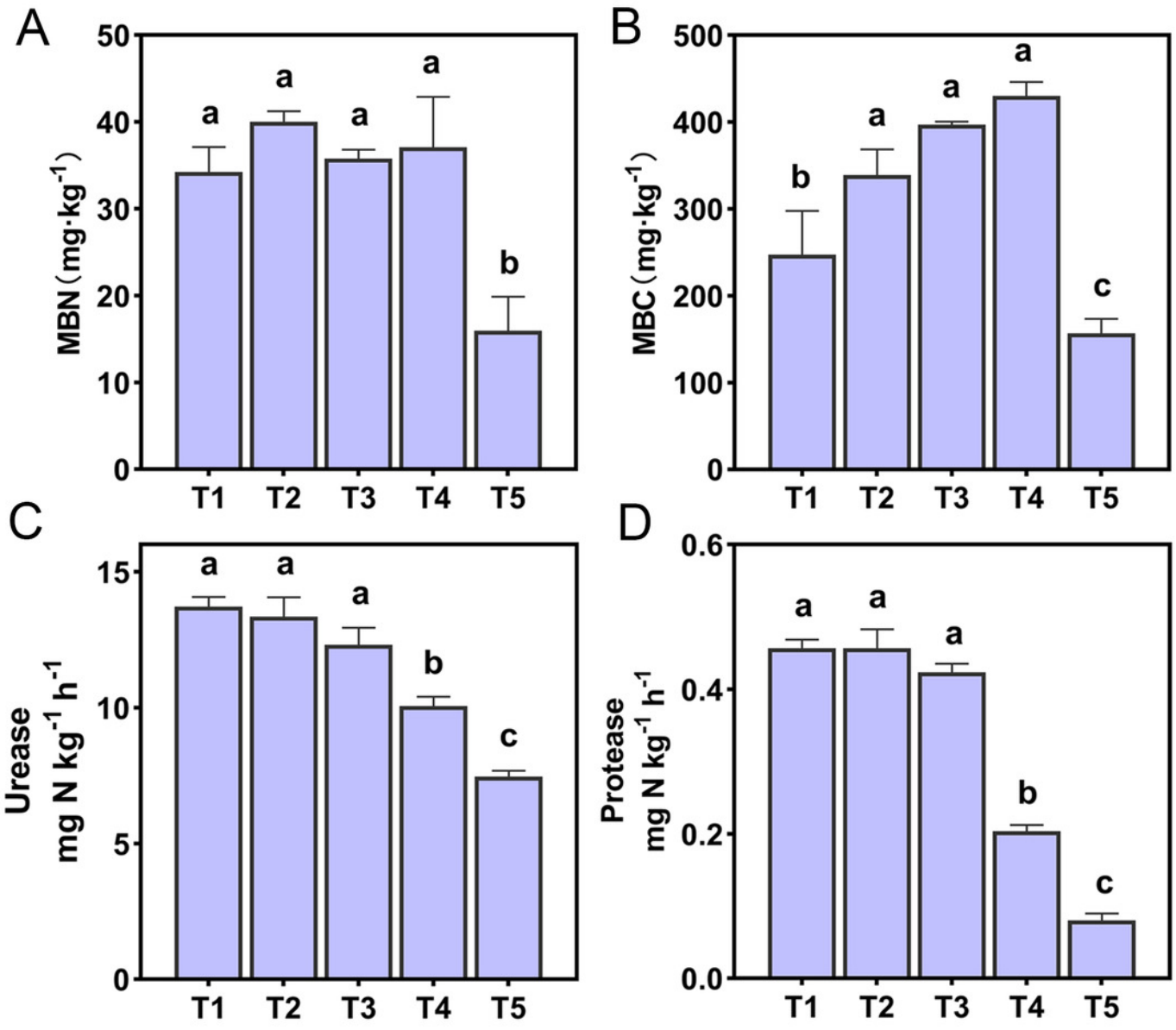

Figure 2

Ammonia oxidation linear relationship and potential nitrification activity (PNA) of different treatments.

R^2 indicates the linear relationship fit, the letters at the top of the bar chart represent significant differences at 0.05 probability level.

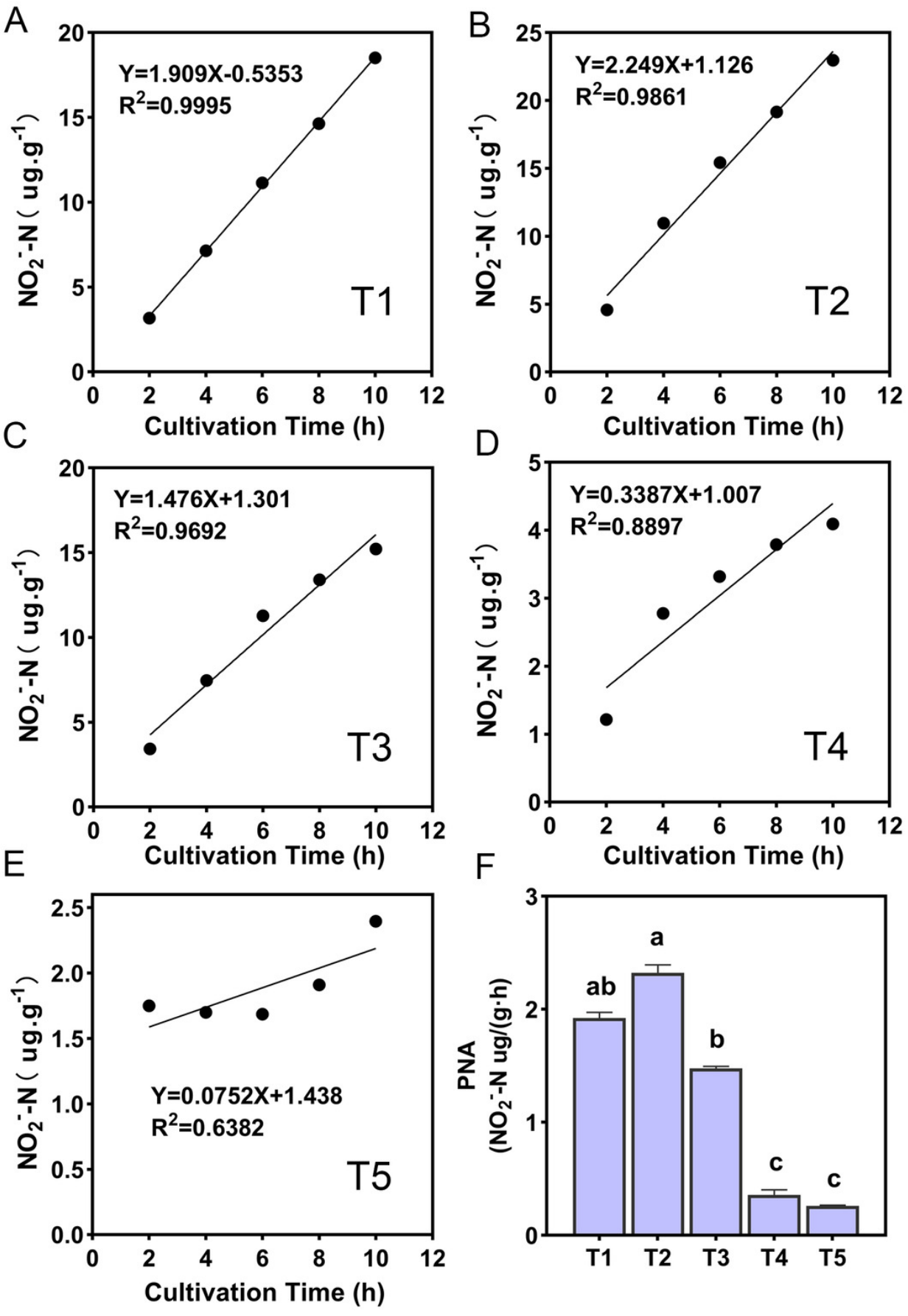
A
Y=1.909X-0.5353
R2=0.9995
T1
NO2--N (ug.g-1)
Cultivation Time (h)
B
Y=2.249X+1.126
R2=0.9861
T2
C
Y=1.476X+1.301
R2=0.9692
T3
D
Y=0.3387X+1.007
R2=0.8897
T4
E
Y=0.0752X+1.438
R2=0.6382
T5
F
PNA
(NO2--N ug/(g·h)
ab
a
b
c
c
T1
T2
T3
T4
T5

Figure 3

Abundance of AOA and AOB *amoA* gene copy numbers under different treatments.

amoA gene copy numbers were log-transformed, and the different letters indicate significant differences at 0.05 probability level (Capital letters indicate the significance of AOA, lowercase letters indicate the significance of AOB). The ratios of AOA to AOB were shown in the boxes above the chart.

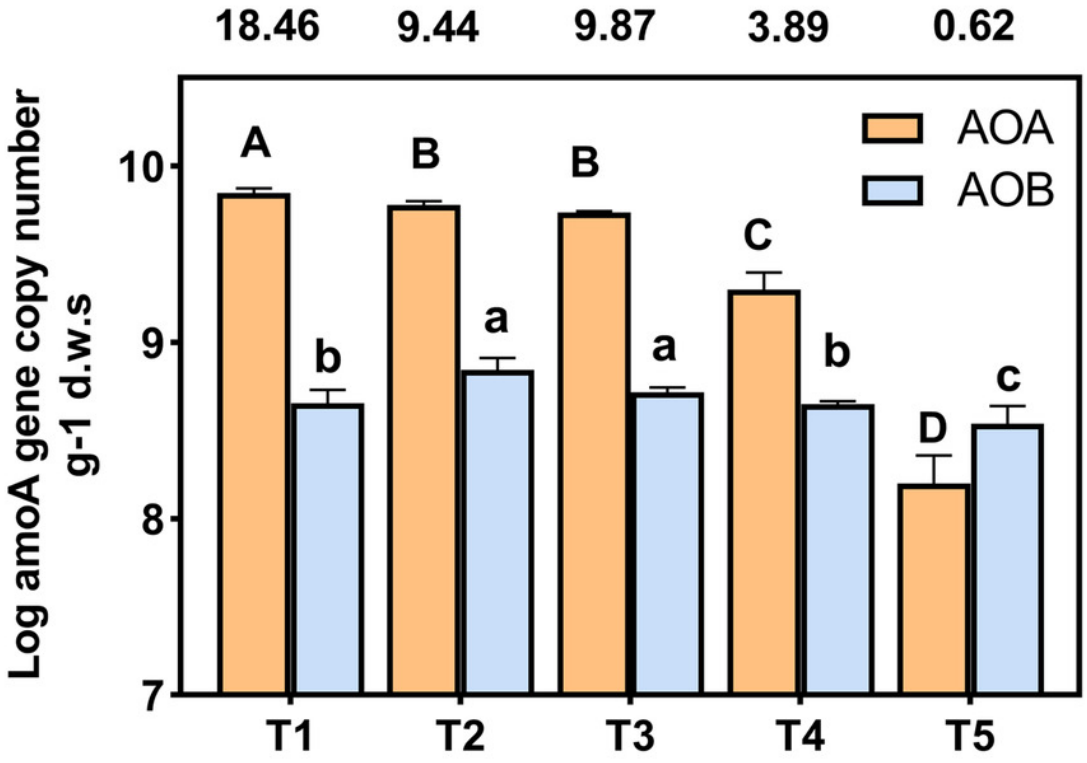

Figure 4

Relative abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB) at phylum and genus level.

(A) Relative abundance of AOA at the phylum level. (B) Relative abundance of AOA at the genus level. (C) Relative abundance of dominant AOA taxa (*Nitrososphare*). (D) Relative abundance of AOB at the phylum level. (E) Relative abundance of AOB at the genus level. (F) Relative abundance of dominant AOB taxa (*Nitrosospira*).

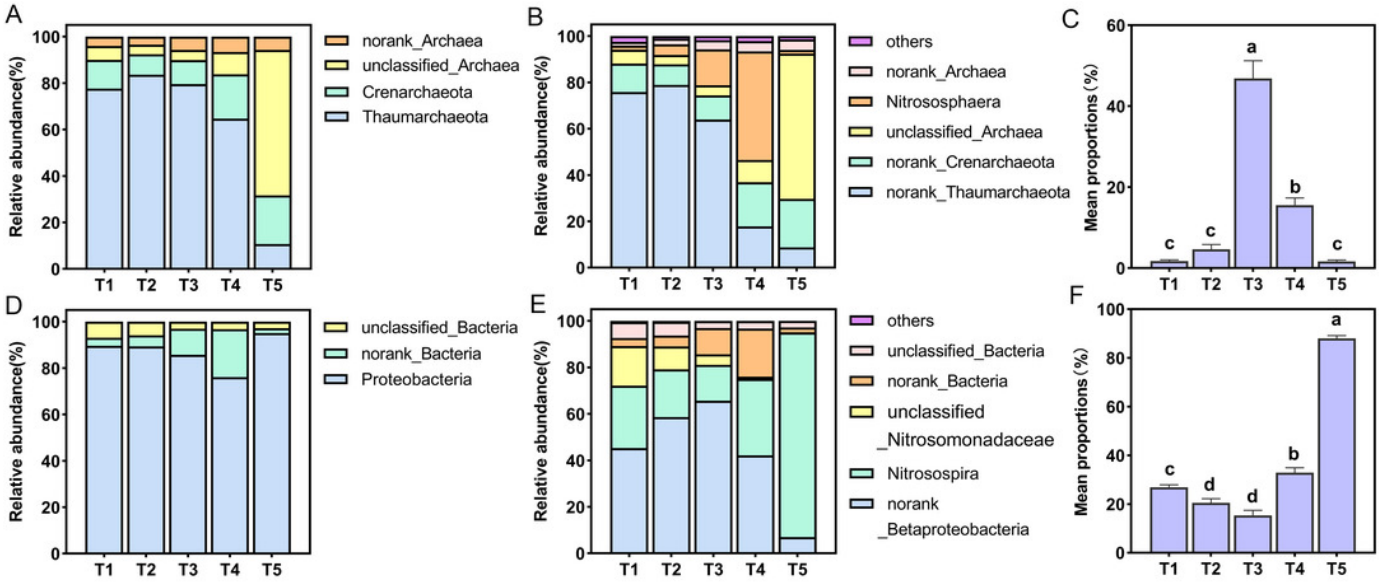

Figure 5

Principal Component analysis (PCA) of ammonia-oxidizing archaea (AOA) and bacteria (AOB) based on the Bray-Curtis distance matrix at the genus level.

(A) PCA of AOA. (B) PCA of AOB. The annotations on the top of the figure represent the significance of differences.

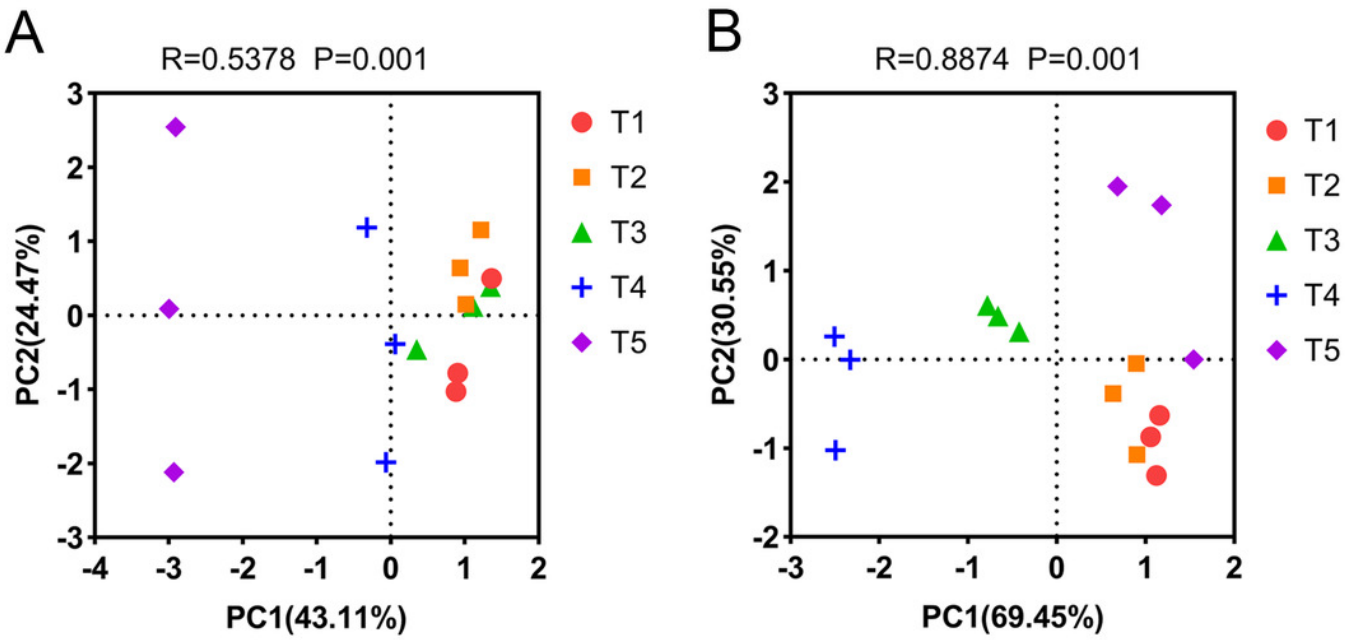

Figure 6

Distance-based redundancy analysis (db-RDA) of ammonia-oxidizing archaea (AOA) and bacteria (AOB).

(A) db-RDA of AOA. (B) db-RDA of AOB. In the graph, * indicates the p-value of correlation, * $0.01< p \leq 0.05$,** $0.001 < p \leq 0.01$,*** $p \leq 0.001$.

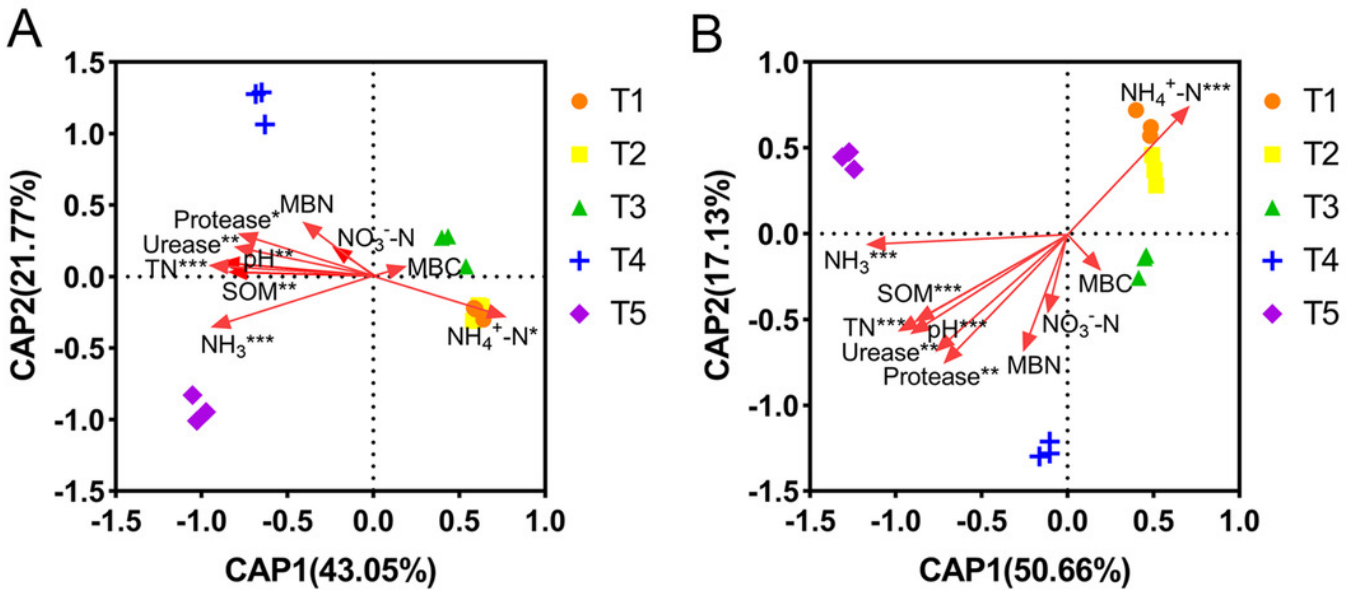

Figure 7

Relative variable importance of environmental factors for the ammonia oxidizing microbial community.

(A) Abundance of AOA. (B) Abundance of AOB. (C) Shannon index of AOA. (D) Shannon index of AOB. (E) PCA axis1 of AOA. (F) PCA axis1of AOB. (G and H) Potential nitrification activity (PNA).

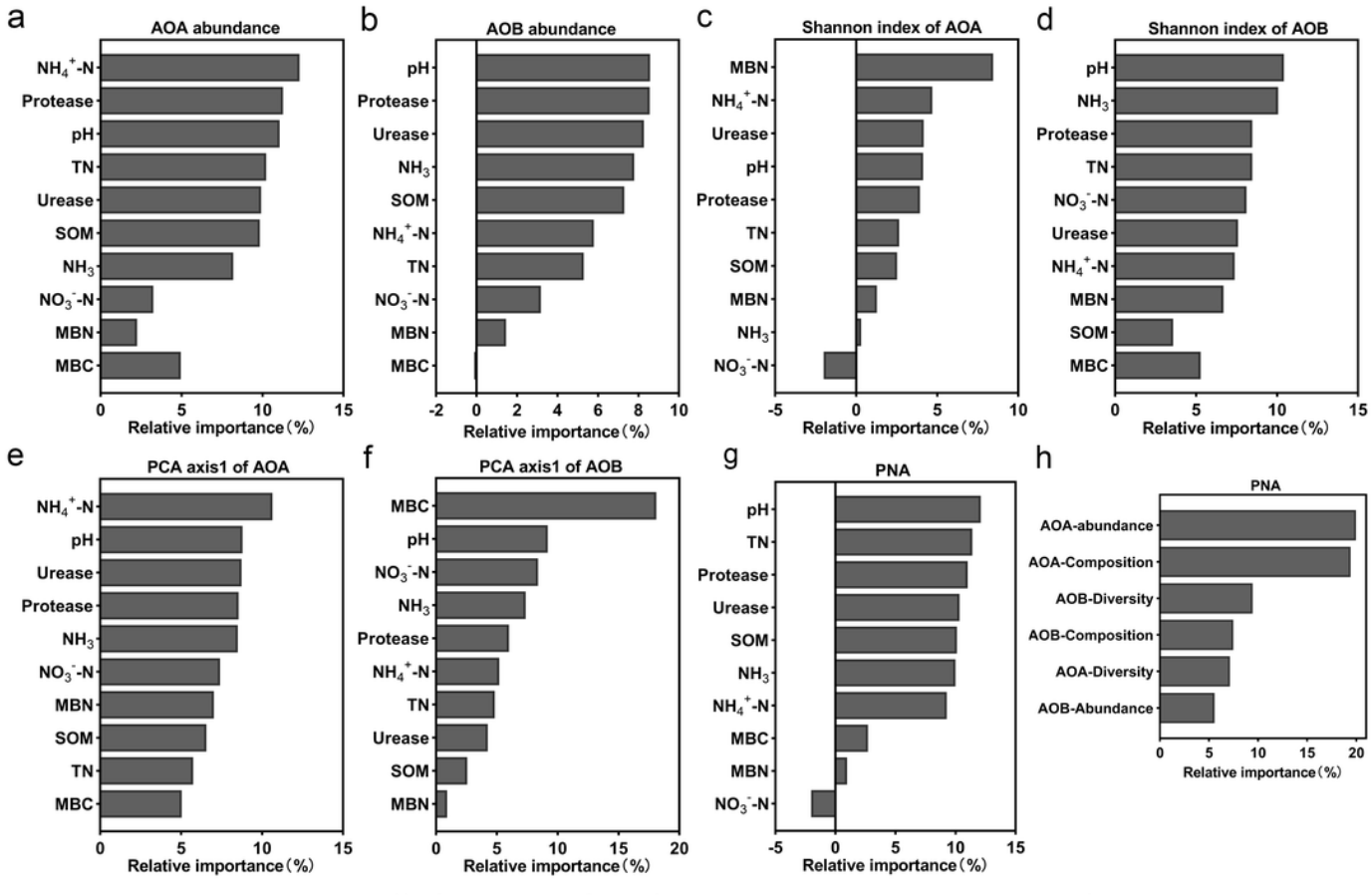

Figure 8

Summary of this study.

The arrows represent the correlation between the two indicators.

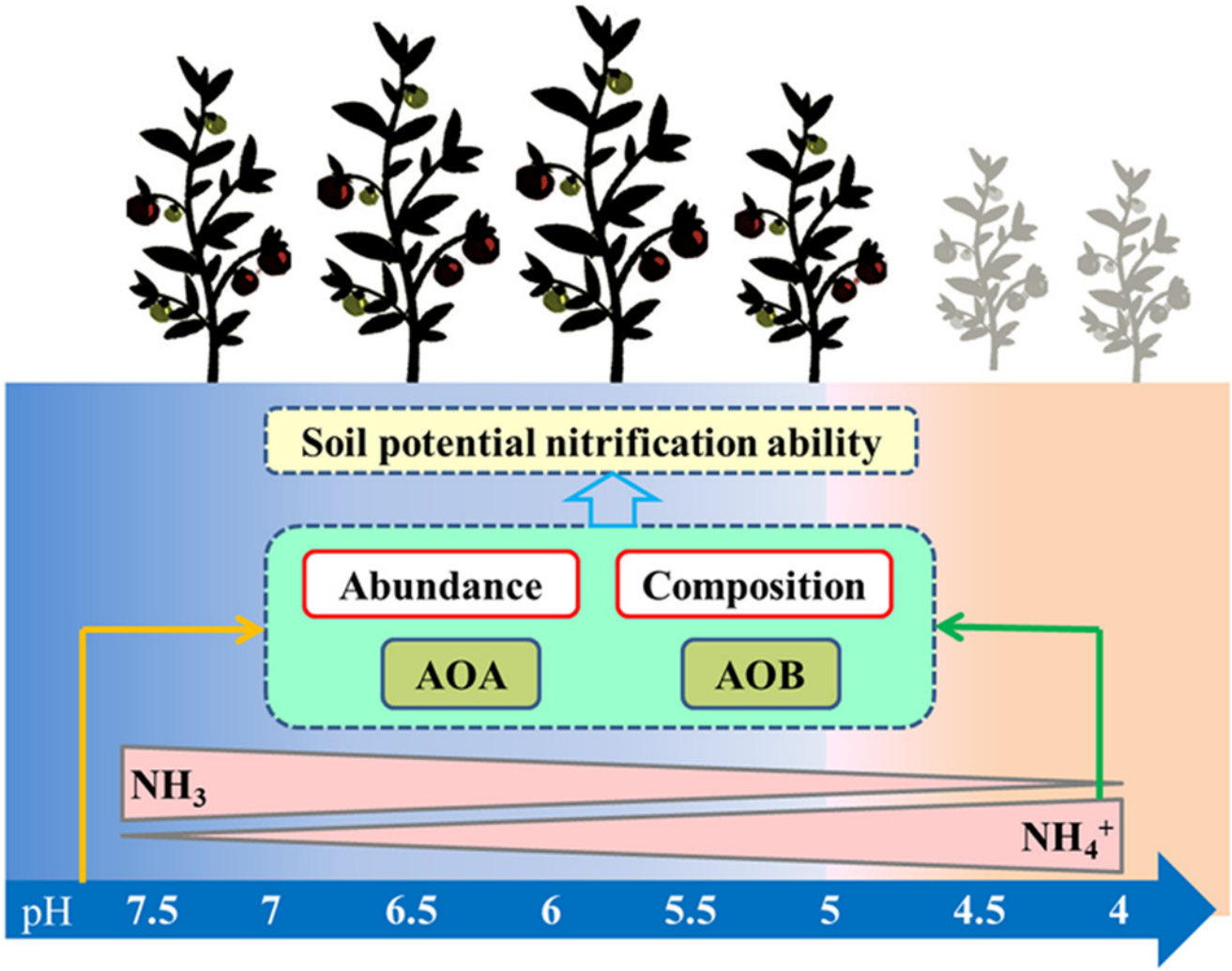

Table 1(on next page)

Primers for quantitative analysis of *amoA* gene.

1

Target group	Primer	Sequence (5–3)	real-time PCR conditions	Reference
AOA	Arch-amoA F Arch-amoA R	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	5 min at 95 °C, 35 cycles of 45 s at 95 °C, 30 s at 57°C for AOB, and 60 s at 53 °C for AOA, 1 min at 72 °C	(Francis et al. 2005)
AOB	amoA1 F amoA2 R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC		(Rotthauwe et al. 1997)

2

1

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Table 2(on next page)

Soil chemical properties of the different treatments.

Values are mean ± standard deviation (n = 3). Different letters indicate significant differences at 0.05 probability level.

1

Treatment	pH	SOM (g·kg^{-1})	TN (g·kg^{-1})	NH_4^+-N (mg·kg^{-1})	NO_3^--N (mg·kg^{-1})	NH_3 (mg·m^{-3})
T1	6.93±0.02a	45.35±0.57a	3.45±0.04a	3.48±0.10c	17.66±2.77a	15.65±1.74a
T2	6.45±0.03b	42.62±1.03a	3.35±0.04a	3.79±0.23c	21.46±4.14a	6.38±0.79b
T3	5.93±0.01c	41.90±1.91a	3.12±0.05b	4.41±0.42c	18.03±1.26a	2.09±0.19c
T4	5.33±0.02d	35.94±0.63b	2.95±0.05c	14.38±1.10b	25.95±3.93a	1.63±0.08c
T5	4.21±0.01e	34.24±0.79b	2.83±0.06c	19.02±1.14a	13.01±4.70b	0.16±0.02c

2

Table 3(on next page)

Illumina Miseq sequencing results and alpha diversity analysis.

Values are mean ± standard deviation (n = 3). Different letters indicate significant differences at 0.05 probability level.

1

Treatment		Sequencing results		Richness		Diversity	Coverage
		Read	OTUs	ACE	Chao1	Shannon	
AOA community	T1	8222	61a	69.20±0.63a	78.96±6.34a	1.50±0.12b	0.9987
	T2	8222	62a	72.19±3.46a	70.49±2.04a	1.31±0.06b	0.9985
	T3	8222	59a	67.28±3.27a	67.00±4.04a	1.69±0.05b	0.9988
	T4	8222	68a	73.71±1.48a	77.99±2.39a	2.32±0.02a	0.9989
	T5	8222	33b	35.98±11.11b	34.50±10.77b	1.70±0.36b	0.9996
AOB community	T1	8244	59a	63.98±1.25a	61.04±0.87a	2.63±0.01a	0.9993
	T2	8244	62a	66.67±1.47a	64.72±1.66a	2.38±0.07b	0.9992
	T3	8244	49b	61.69±5.48a	58.53±3.79a	2.04±0.08c	0.9990
	T4	8244	39c	41.93±2.63b	42.17±3.35b	2.20±0.03bc	0.9994
	T5	8244	29d	39.61±11.05b	37.15±10.23b	1.12±0.10d	0.9993

2

Table 4(on next page)

Tomato plant growth indexes of different treatments.

Values are mean ± standard deviation (n = 3). Different letters indicate significant differences at 0.05 probability level.

1

Treatment	Plant height (cm)	Stem diameter (cm)	Aboveground biomass (g/plant)	Underground biomass (g/plant)	Total biomass (g/plant)	Yield (kg/plant)
T1	158.33±0.88b	1.08±0.08a	189.52±4.15d	5.42±0.27b	194.60±4.06c	1.98±0.02a
T2	165.00±1.00a	1.11±0.01a	201.92±4.49c	8.60±0.28a	209.93±4.11b	1.97±0.04a
T3	162.67±0.33ab	1.20±0.02a	213.49±3.64b	7.46±0.61a	221.17±5.11b	2.12±0.03a
T4	166.33±4.91a	1.25±0.09a	242.10±2.95a	8.06±0.80a	250.10±2.10a	2.10±0.08a
T5	164.33±0.88ab	1.17±0.04a	173.34±2.01e	3.69±0.09c	177.05±2.10d	1.63±0.03b

2