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Soil pH and phosphorus drive the canonical nitrifiers and comammox *Nitrospira* communities in citrus orchards with different cultivation ages

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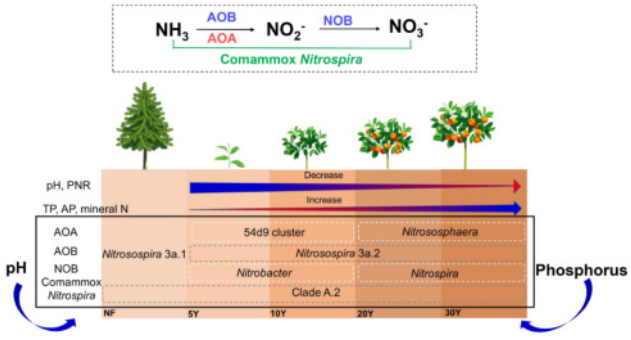
ABSTRACT

- Comammox *Nitrospira* clade A and B showed contrasting responses to citrus planting.
- 54d9-like AOA and *Nitrobacter*-NOB dominated in the 5Y and 10Y soils.
- *Nitrososphaera*-like AOA and *Nitrospira*-like NOB dominated in the 20Y and 30Y soils.
- Soil pH and P content were the major factors shaping nitrifying communities.



Ammonia oxidizing bacteria (AOB), archaea (AOA), nitrite oxidizing bacteria (NOB) and complete ammonia oxidizers (comammox *Nitrospira*) are major players in nitrification. However, the distribution and community composition of these nitrifiers in intensively managed orchard soils are still unclear. Here, we chose soil samples from citrus orchards that had been planted for 5 years (5Y), 10 years (10Y), 20 years (20Y) and 30 years (30Y), and adjacent woodland (NF), to study the response of nitrifiers to long-term citrus plantation using quantitative PCR and MiSeq sequencing. Our results revealed that the ammonia and nitrite oxidation potentials in the 5Y soil were the highest, and decreased with increasing plantation age. The AOB abundance was higher in 5Y and 10Y soils than that in 20Y and 30Y soils. The abundance of comammox *Nitrospira* clade A increased with increasing plantation age, but comammox *Nitrospira* clade B showed the opposite tendency. MiSeq sequencing results indicated 54d9-like AOA and *Nitrobacter*-NOB were the dominant populations in 5Y and 10Y soils whereas *Nitrososphaera*-like AOA and *Nitrospira*-like NOB dominated in 20Y and 30Y soils. The conversion of woodland to orchard resulted in a significant shift of AOB population from *Nitrosospira* cluster 3a.1 to cluster 3a.2. In addition, soil pH and phosphorus (P) content were the major factors shaping nitrifying communities. This work suggested citrus plantation altered the distribution of community composition of nitrifiers by affecting soil chemical and physical conditions, and comammox *Nitrospira* could potentially play an important role in nitrification in intensive managed orchard soils.

Keywords AOB, AOA, comammox *Nitrospira*, NOB, soil pH, phosphorus content

1 Introduction

Citrus, as one of the most valuable fruits in the world, is grown in more than 80 countries. China is the world's largest citrus producer with 29.6% of the global citrus production (FAO, 2021). For higher economic benefits, most of the orchards are intensively managed, which can result in high density of productive trees with yields significantly higher than in traditional orchards. However, the intensive tillage and scant plant cover promote land degradation and soil erosion (Cao et al., 2021; Garcia-Franco et al., 2021). In addition, the average application rate of nitrogen (N) fertilizer in citrus orchards in China is 500 kg ha^{-1}, which is significantly higher than that in advanced citrus producing countries such as Brazil (150−200 kg ha^{-1}) (Li et al., 2019b). The excessive application of N fertilizer not only decreases fertilizer efficiency but also causes severe environmental pollution such as nitrate (NO_3^-) leaching and runoff from soil and nitrous oxide (N_2O) emissions. NO_3^- is a water contaminant, and

N_2O is both a greenhouse gas and an ozone-depletion substance (Di et al., 2010). Previous study showed the conversion of woodland to orchard significantly increased soil nitrification rate (Zhang et al., 2015). Therefore, understanding the detail of soil nitrification in orchard soils has important practical significance in orchard management and environmental protection.

Traditionally, nitrification is considered as a two-step process with ammonia (NH_3) oxidized to nitrite (NO_2^-) followed by oxidation to NO_3^-. Ammonia-oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) are generally thought to drive the first and rate-limiting step of nitrification (Könneke et al., 2005). The distribution, activity and community composition of AOA and AOB in terrestrial ecosystems have been widely studied (Shen et al., 2008; Di et al., 2010; Tzanakakis et al., 2019). Numerous studies have shown that AOB dominate nitrification in N-rich soils while AOA play more important role in N-depleted and acidic soils (Di et al., 2009; Lu and Jia 2013; Sterngren et al., 2015). The second step is performed by nitrite-oxidizing bacteria (NOB) (Gruber and Galloway, 2008). *Nitrobacter* and *Nitrospira* are the two major nitrite-oxidizing performers in terrestrial ecosystems (Bartosch et al., 2002; Ke et al., 2013; Stempfhuber et al., 2016). *Nitrobacter*- and *Nitrospira*-like NOB are hypothesized as r-strategists and K-strategists, and prosper under high and low N environments, respectively (Attard et al., 2010).

However, the discovery of the complete ammonia oxidation (comammox) process, in which NH_3 can be oxidized to NO_3^- directly by a single organism (comammox *Nitrospira*) challenges our previous perceptions of the two-step nitrification and creates a need to re-evaluate the relative contribution of nitrifiers to nitrification (Daims et al., 2015; van Kessel et al., 2015; Hu and He, 2017). At present, comammox *Nitrospira* is found to be distributed widely in terrestrial ecosystems (e.g., agricultural, paddy and forest soils, and sediments) (Xia et al., 2018; Wang et al., 2019a; Xu et al., 2020; Liu et al., 2023), indicating comammox *Nitrospira* is ubiquitous along with canonical ammonia oxidizers in soil habitats. However, the issues of importance and ecological niche of comammox *Nitrospira* are still controversial. For example, the *amoA* gene copy number of comammox *Nitrospira* has been found to far exceed than that of canonical oxidizers and exhibited nitrification activity in some acidic soils (Hu and He 2017; Takahashi et al., 2020; Hu et al., 2021), indicating that comammox *Nitrospira* might functionally outcompete AOA under acidic environments. Some other studies found comammox *Nitrospira* could be enriched and play an active role in soils with fertilizer application (Orellana et al., 2018; Li et al., 2019a; Lin et al., 2020), which was contrary to that comammox *Nitrospira* preferred oligotrophic conditions as has been concluded from kinetic analysis (Kits et al., 2017; Sakoula et al., 2021).

Long-term continuous monoculture has been seen to induce soil acidification and has significant effect on other soil properties (Zhang et al., 2016). However, the response of canonical nitrifiers and comammox *Nitrospira* to long-term citrus plantation is unclear. We collected soil samples from citrus orchards of different plantation ages in the same region. The objectives of this study were to elucidate (i) soil property and nitrification activity variations between citrus orchards with different ages; (ii) the distribution and community composition of nitrifiers in citrus plantations of different ages; and (iii) the main factors to explain observed changes in nitrifying communities.

2 Materials and methods

2.1 Soil sampling

The studying sites were located at Zigui County, Three Gorges reservoir area (TGRA), Hubei Province, China, which is characterized by a subtropical monsoon climate. Mean annual rainfall and temperature at the study sites are 1 164 mm and 18°C. Four citrus orchards, 5, 10, 20 and 30 years old (hereafter referred to as 5Y, 10Y, 20Y and 30Y) and one adjacent natural forest (NF) were selected. The types of vegetation in citrus orchards and natural forest are Newhall navel orange and cypress, respectively. The altitude, longitude and latitude of sampling sites, and morphological characteristics of trees are shown in Fig. S1 and Table S1. All soils were classified as Inceptisol (USDA Soil Taxonomy) and developed from purple sandstone. All selected orchards had similar managements such as fertilization, irrigation and weeding. At each citrus orchard and adjacent forest, three trees were randomly selected. Five soil cores of each tree were randomly collected with a depth of 0–20 cm under the crowns of trees, and then mixed as one soil sample. Soil samples were packed into ice packs and transported to the laboratory. A proportion of each sample was air-dried for physiochemical analysis and another part was stored at −80°C for molecular analysis.

2.2 Soil chemical properties

Soil pH was determined with a 1:2.5 soil to water ratio using a pH meter (FE28, Shanghai, China). Soil total carbon (TC) and nitrogen (TN) were determined by an elemental analyzer (Vario Macro Cube, Germany). Soil total phosphorus (TP) was determined following H_2SO_4-$HClO_4$ digestion, and then measured by the molybdenum blue method (Olsen, 1954). Soil available P (AP) and potassium (AK) were determined by the molybdenum blue method and flame photometry (nova 300, Analytic Jena, Germany), respectively. Soil NH_4^+-N, NO_3^--N and NO_2^--N concentrations were extracted

with 1 M KCl and determined spectrophotometrically at 625 nm, 220 and 275 nm, and 540 nm, respectively.

2.3 Potential ammonia oxidation and nitrite oxidation

Soil potential ammonia oxidation (PAO) rate was determined according to Kurola et al. (2005). Briefly, 5 g fresh soil was added into 50 mL centrifuge tubes with 20 mL phosphate buffer solution (NaCl, 8.0 g L^{-1}; KCl, 0.2 g L^{-1}; Na_2HPO_4, 0.2 g L^{-1}; NaH_2PO_4, 0.2 g L^{-1}; pH 7.4) and 1 mM $(NH_4)_2SO_4$. To inhibit nitrite oxidation, 10 mM potassium chlorate was added into the mixed liquor. The suspension was incubated at 25°C with shaking at 170 r min^{-1} for 22 h. After incubation, NO_2^--N was extracted with 2M KCl and determined by a spectrophotometer at 540 nm. Soil potential nitrite oxidation (PNO) rate was determined using the method described by Attard et al. (2010). Briefly, 5 g fresh soil was incubated with 30 mL $NaNO_2$ for 30 h with shaking at 150 r min^{-1}. During incubation, 2 mL aliquots of the suspension were taken at 0, 9, 24 and 30 h and centrifuged at 5000 r min^{-1} for 2 min. Then, these suspensions were filtered through 0.2 μm filter and NO_2^--N concentration was measured.

2.4 Soil DNA extraction, qPCR analysis and Miseq sequencing analysis

Soil DNA from each sample was extracted using FastDNA spin kit for soil (MP Biomedicals, OH, USA) according to the manufacturer's protocol. The DNA quality and concentration were checked by gel electrophoresis and a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Quantitative PCR (qPCR) assays targeting the AOA, AOB, comammox *amoA* genes and NOB *nxrA* and *nxrB* genes were performed on an ABI 7500 real-time PCR system (Applied Biosystems, Santa Clara, CA). Primer sets and conditions used for qPCR are shown in Table S3. Each PCR reaction system (20 μL) contained 10 μL SYBR Premix Ex Taq (TaKaRa, Dalian, China), 500 nM of each primer, 1 μL of DNA template and milli-Q water to the final volume. Standard curves of each target gene were generated as previously described by Liu et al. (2019). The amplification efficiencies were 80%–101%, with R^2 values ranging between 0.991 and 0.999.

Miseq sequencing, of AOA, AOB and comammox *Nitrospira amoA* genes, was performed using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The primers, and conditions of AOA, AOB and comammox *Nitrospira* are shown in Table S3. The purified PCR products were used to generate sequencing libraries using TruSeq Nano DNA LT library Prep Kit (Illumina, USA). Finally, paired-end sequencing was performed on an Illumina MiSeq platform by the Personal Biotechnology Co., Ltd. (Shanghai, China). In addition, the primer pair of 515f and 907r (Stubner, 2002) was used to amplify V4–V5 regions of the 16S rRNA gene and sequenced on the Illumina MiSeq platform. Sequences were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) under studies PRJNA770137, PRJNA770379, PRJNA770466 and PRJNA770468 for AOA, AOB and comammox *Nitrospira amoA* genes and 16S rRNA gene, respectively.

The raw data of *amoA* and 16S rRNA gene sequencing were analysed using the QIIME software (Caporaso et al., 2010). Low-quality sequences with ambiguous nucleotides, improper primers, quality score < 20 and read length below 200 bp were discarded. A total of 581895, 215610, 366600 and 306765 high quality sequence reads of AOA, AOB, comammox *Nitrospira amoA* gene and 16S rRNA gene were obtained, respectively. Operational taxonomic units (OTUs) were clustered at 97% identity similarity using the UCLUST (Edgar, 2010). The representative sequence of each OTU was chosen from the most abundant sequence of that OTU. Then, each representative sequence was compared to the NCBI database using BLAST, and sequences that did not belong to AOA, AOB or comammox *Nitrospira* were excluded from the following analysis. In addition, 16S rRNA gene reads classified as NOB (*Nitrobacter*, *Nitrospira*, *Nitrotoga*, *Nitrolancetus*, *Nitrococcus* and *Nitrospina*) were screened out to analyze the NOB community (Zhang et al., 2019; Liu et al., 2021). To depict AOA, AOB and comammox *Nitrospira* community compositions clearly, OTUs with relative abundance higher than 0.1% were used for phylogenetic tree construction. Neighbor-joining phylogenetic trees were constructed by the Molecular Evolutionary Genetics Analysis (MEGA 6.0) using 1000 bootstrap replicates (Tamura et al., 2013).

2.5 Statistical analysis

Statistical analysis was performed using SPSS 17.0 (IBM, Armonk, NY, USA). Differences in soil properties, nitrification potential and functional gene abundances were conducted by a one-way analysis of variance (ANOVA), and $P < 0.05$ was considered to be significant. The relationships between functional gene abundances, PAO, PNO and soil properties were analyzed by Pearson correlation analysis in R software with the 'corrplot' package. The relative abundance of nitrifiers in each OTU was depicted by heatmap using the 'pheatmap' package of R software. BIO-ENV analysis was used to show the optimal group of soil properties affecting nitrifying communities. Canonical correspondence analysis (CCA) and redundancy analysis (RDA) were conducted to identify the dominate factors affecting nitrifying community composition. BIO-ENV, CCA, and RDA were conducted by R software in the 'vegan' package. The main predictors of nitrifying abundances and community compositions were

identified using the 'Random Forest' (RF) package in R (Bahram et al., 2018). Random Forest mean predictor importance (MPI) (i.e., percent increase in mean square error (MSE)) was used to characterize each predictor (Luo et al., 2019).

3 Results

3.1 Soil properties

Long-term citrus planting accelerated soil acidification with soil pH decreasing significantly from 8.48 in the 5Y soil to 4.29 in the 30Y soil ($P < 0.05$) (Table S2). The planting of citrus trees resulted in a decrease in TC content. The TC contents in 10Y, 20Y, and 30Y soils were significantly lower compared to those in NF and 5Y soils ($P < 0.05$). Soil TN, NH_4^+-N and NO_3^--N contents in 20Y and 30Y soils were significantly higher than those in 5Y and 10Y soils ($P < 0.05$). Soil TP and AP increased significantly with planting year increase ($P < 0.05$). After forest land reclamation, soil AK content increased significantly, but no regular change was observed among citrus orchard soils with different ages. However, there was no significant difference of NO_2^--N among soils except in the cases of NF and 30Y soils ($P > 0.05$).

3.2 Potential nitrification rate and nitrifying abundance

The highest PAO and PNO were both observed in the 5Y soil with values of 1.25 μg NO_2^--N h^{-1} g^{-1} *d.w.s* and 0.6 μg NO_2^--N h^{-1} g^{-1} *d.w.s*, respectively (Fig. 1a). The PAO and PNO in 10Y and 20Y soils were significantly lower than those in the 5Y soil but higher than those in the NF soil ($P < 0.05$). No significant difference of PAO was observed between NF and 30Y soil ($P > 0.05$), while PNO in the 30Y soil was significantly lower than that in the NF soil ($P > 0.05$) (Fig. 1a). PAO and PNO were positively correlated with pH ($r = 0.62$ and 0.66, $P < 0.01$), TC ($r = 0.82$ and 0.7, $P < 0.01$) and NO_2^--N ($r = 0.23$ and 0.23, $P < 0.01$), but negatively correlated with TN ($r = -0.3$ and -0.38, $P < 0.01$), TP ($r = -0.29$ and -0.29, $P < 0.01$), AP ($r = -0.47$ and -0.5, $P < 0.01$), NH_4^+-N ($r = -0.5$ and -0.57, $P < 0.01$) and NO_3^--N ($r = -0.29$ and -0.35, $P < 0.01$) (Fig. S2).

The abundance of AOA *amoA* gene ranged from 4.49×10^5 copies g^{-1} soil (NF) to 1.35×10^7 copies g^{-1} soil (30Y) and was significantly lower in NF and 20Y soils than that in the 5Y, 10Y and 30Y soils ($P < 0.05$) (Fig. 1b). The abundance of AOB *amoA* gene ranged from 6.02×10^6 copies g^{-1} soil (NF) to 1.32×10^8 copies g^{-1} soil (5Y) and decreased in the order 5Y > 10Y > 30Y > 20Y > NF (Fig. 1b). On the whole, the AOA/AOB ratio increased with increasing years of planting except in the case of the 20Y soil (Fig. 1b). The *Nitrospira*

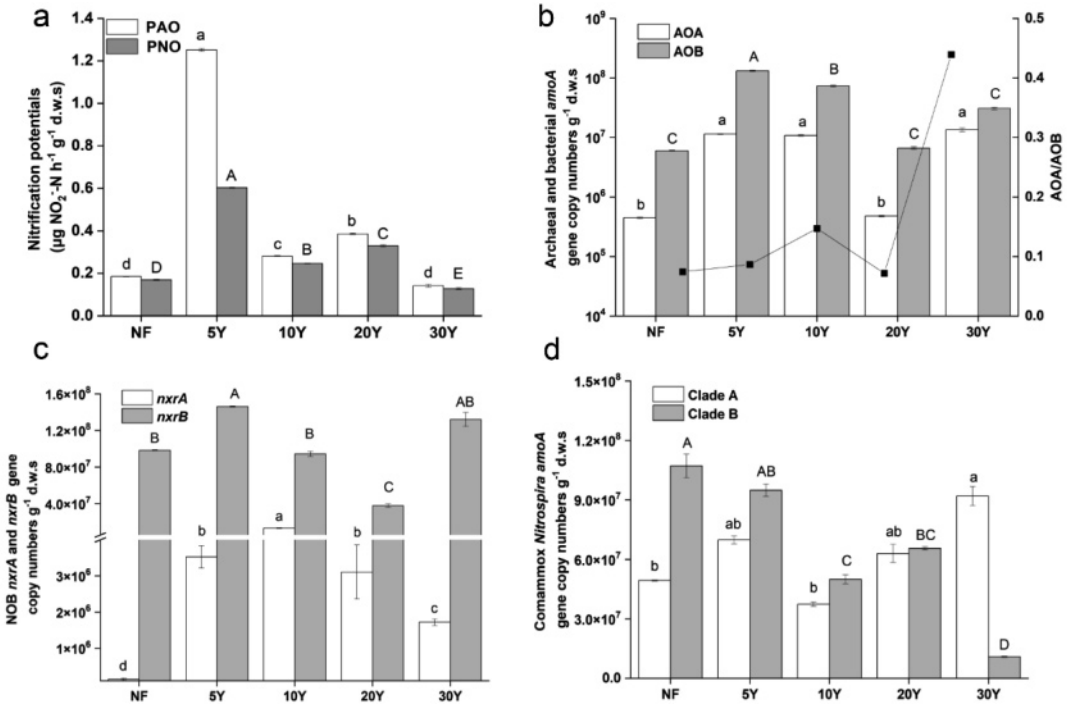


Fig. 1 The changes of potential nitrification rate (a); the abundances of AOA and AOB *amoA* genes (b); the abundances ofNOB *nxrA* and *nxrB* genes (c); the abundances of and comammox *Nitrospira* clade A and clade B *amoA* genes (d), in forest soil and orchard soils with different plantation ages. The error bars represent the standard errors of the mean of the triplicate microcosms. Different letters above the columns indicate significant differences ($P < 0.05$). NF represents natural forest soil. 5Y, 10Y, 20Y and 30Y represent soil samples collected from citrus orchards planting for 5 years, 10 years, 20 years and 30 years, respectively. PAO and PNO represent soil potential ammonia oxidation and potential nitrite oxidation, respectively.

nxrB gene abundance was much higher than that of the *Nitrobacter nxrA* gene in the five soils. The *Nitrobacter nxrA* gene copy numbers increased with planting year increase and reached the highest in the 10Y soil and then decreased after long-term planting (Fig. 1c). The *Nitrospira nxrB* gene abundance reached the highest value in the 5Y soil (1.46×10^8 copies g^{-1} soil) and was lowest in the 20Y soil (3.80×10^7 copies g^{-1} soil) (Fig. 1c). The abundance of comammox *Nitrospira* clade A and clade B varied from 3.74×10^7 copies g^{-1} soil (5Y) to 9.20×10^7 copies g^{-1} soil (30Y) and 1.09×10^7 copies g^{-1} soil (30Y) to 1.07×10^8 copies g^{-1} soil (NF), respectively (Fig. 1d). Overall, long-term citrus planting increased the abundance of comammox *Nitrospira* clade A but decreased the abundance of comammox *Nitrospira* clade B when compared with the NF soil (Fig. 1d). PAO and PNO showed strong positive correlations with AOB abundance (r = 0.82 and 0.84, P < 0.001) (Fig. S2). In addition, PAO and PNO were also positively correlated with NOB *nxrB* (r = 0.45 and 0.33, P < 0.01 and P < 0.05), comammox clade B *amoA* (r = 0.3 and 0.2, P < 0.01) gene abundances (Fig. S2). A significant positive relationship was observed between PAO and comammox *Nitrospira* clade A abundance (r = 0.03, P < 0.05). However, a negative relationship was found between PNO and comammox *Nitrospira* clade A abundance (r = −0.11, P < 0.05) (Fig. S2).

3.3 The composition of soil nitrifying communities

According to the phylogenetic analysis of AOA, the majority of AOA *amoA* gene sequences fell within soil group 1.1b, containing four distinct clusters: *Nitrososphaera* cluster, 54d9 cluster, *Nitrosocosmicus* cluster, and unclassified cluster (Fig. S3). The relative abundance of the 54d9 cluster increased in the order NF > 5Y > 10Y, accounting for 26.0%, 46.5% and 80.4% of total AOA sequences, respectively (Fig. 2a). The proportions of the *Nitrososphaera* cluster in old orchard soils (20Y and 30Y) were significantly higher than those in young orchard soils (5Y and 10Y) and NF soil (Fig. 2a). In addition, the relative abundance of *Nitrosocosmicus* cluster varied among different soils, accounting for 66.5%, 29.3%, 11.9%, 53.6% and 15.4% of AOA populations in NF, 5Y, 10Y, 20Y and 30Y soils, respectively (Fig. 2a).

As for AOB community composition, phylogenetic analysis showed the dominant AOB taxa exclusively belonged to the *Nitrosospira* cluster (Fig. S4). The relative abundance of *Nitrosospira* cluster 3a.1 in orchard soils was significantly lower than that in the NF soil (P < 0.05) (Fig. 2b). *Nitrosospira* cluster 3a.2 dominated bacterial *amoA* gene sequences in the orchard soils, accounting for up to 70.7%, 41.8%, 73.9% and 90.3% of AOB populations in the 5Y, 10Y, 20Y and 30Y soils, respectively. In addition, *Nitrosospira* cluster 0 occupied 24.2% of AOB *amoA* gene sequences in the NF soil, and *Nitrosospira* cluster 4 accounted for 9.9%, 14.0% and 9.2 of AOB populations in 10Y, 20Y and 30Y soils, respectively (Fig. 2b).

As shown in the NOB phylogenetic tree, *Nitrospira* and *Nitrobacter* were the two major genera across all soil samples (Fig. S5). In NF, 20Y, and 30Y soils, *Nitrospira* was the dominant genus, accounting for up to 81.5%, 66.6%,

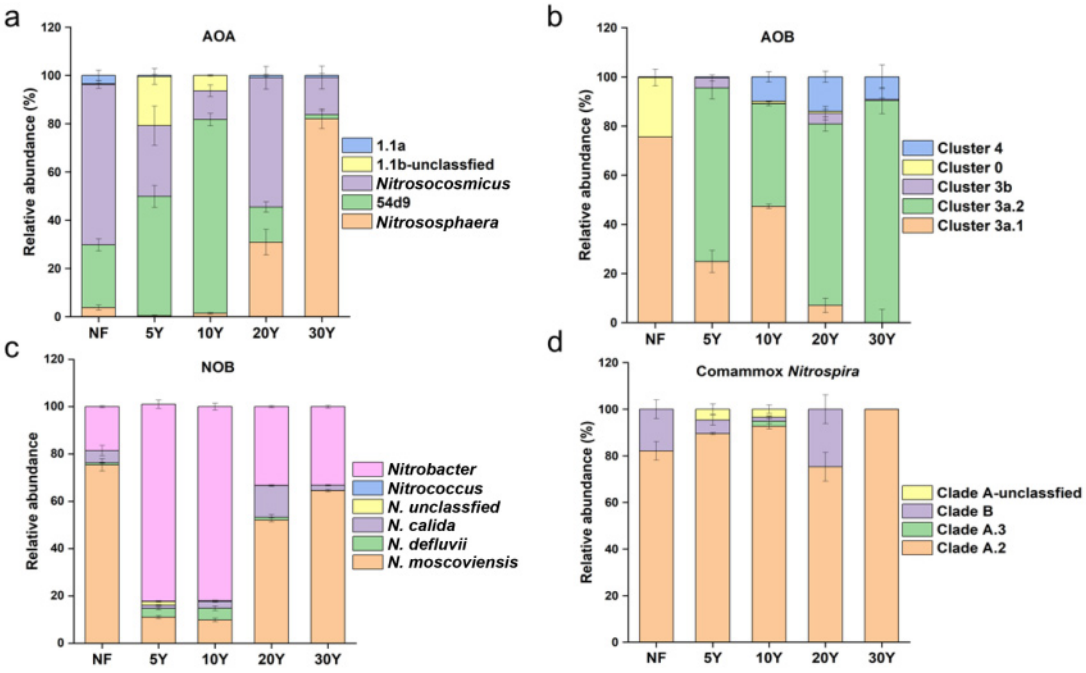


Fig. 2 Proportional changes of AOA (a), AOB (b), NOB (c) and comammox *Nitrospira* (d) phylotypes in response to long-term citrus plantation. The error bars in the columns represent the standard errors of the mean of triplicate samples.

and 66.8% of NOB populations, respectively (Fig. S5). The *Nitrospira moscoviensis* lineage was abundant in NF (75.4%), 20Y (52.1%) and 30Y (64.5%) soils (Fig. 3c and Fig. S5). However, *Nitrobacter* was predominant in 5Y and 10Y soils, accounting for up to 83.3% and 82.0% of all NOB 16S rRNA gene sequences, respectively (Fig. 2c and Fig. S5). Moreover, *Nitrospira defluvii*-like and *Nitrospira calida*-like NOB together accounted for 6.1%, 5.0%, 7.8%, 14.5% and 2.3% of NOB in NF, 5Y, 10Y, 20Y and 30Y soils, respectively. Small proportions of NOB in the 5Y (0.1%), 10Y (0.3%) and 20Y (0.1%) were grouped into the *Nitrococcus* cluster (Fig. 2c and Fig. S5).

Regarding comammox *Nitrospira*, comammox *Nitrospira* clade A dominated in all soil samples, accounting for up to 82.1%, 94.2, 98.4%, 75.3%, and 99.9% of the comammox *Nitrospira amoA* gene sequences in NF, 5Y, 10Y, 20Y and 30Y soils, respectively (Fig. 2d). In NF and 20Y soils, 17.9% and 24.7% of the comammox *Nitrospira* sequences belonged to clade B, respectively (Fig. 2d). The phylogenetic tree of comammox *Nitrospira* supported a designation of

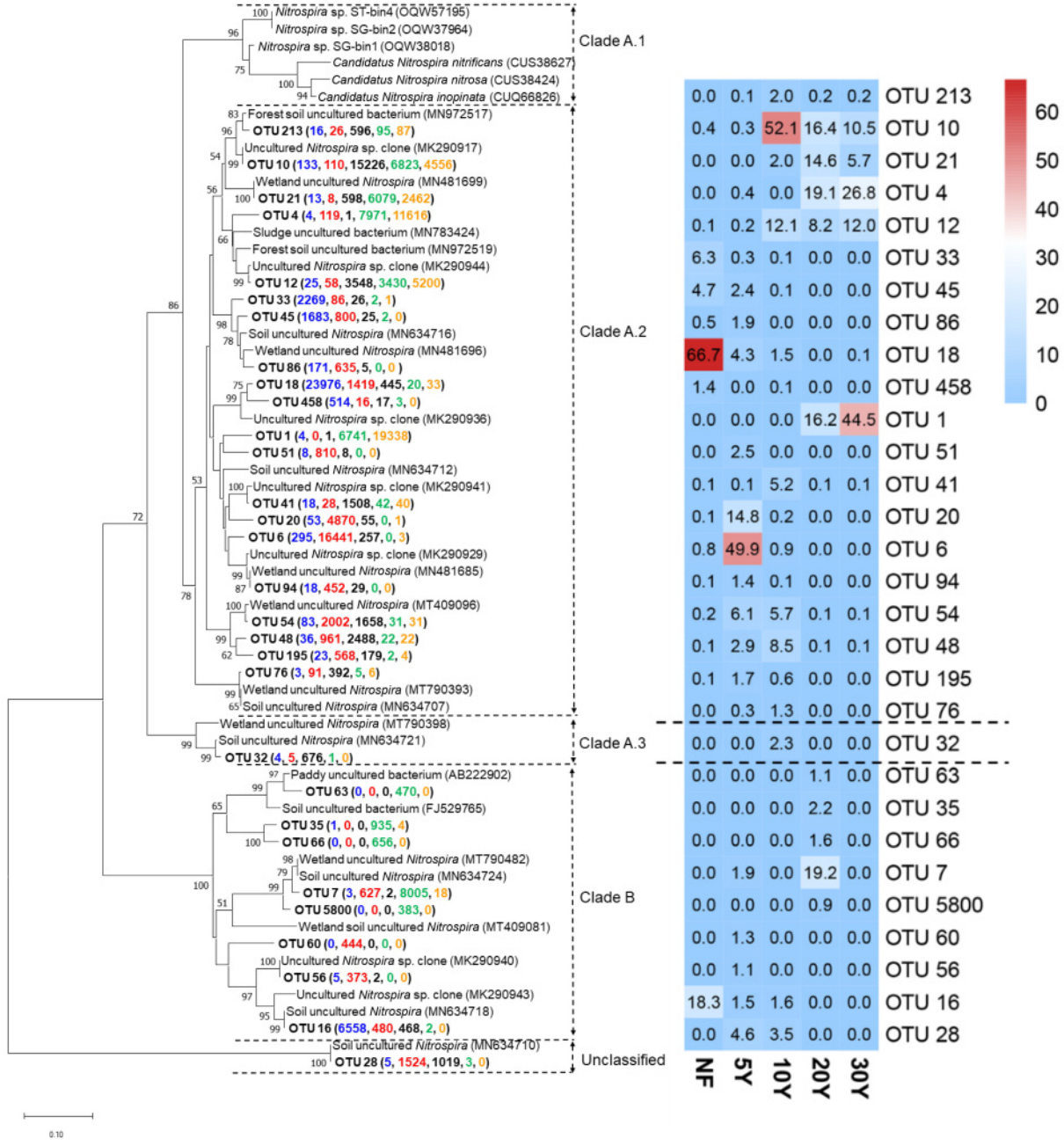


Fig. 3 Phylogenetic analysis of the comammox *Nitrospira amoA* gene in different soils, and heatmap displaying the proportion of each OTU to total OTUs in each soil. Numbers with blue, red, black, green and yellow colors represent sequencing numbers in NF, 5Y, 10Y, 20Y and 30Y soils, respectively. Bootstrap values higher than 50% are indicated at the branch nodes. The scale bars represent 10% nucleic acid sequence divergence.

three subclades within clade A, namely clade A.1, A.2 and A.3 (Fig. 3). Most OTUs belonged to clade A.2, and occupied 82.1%, 89.5%, 92.7%, 75.3% and 99.9% of the comammox *Nitrospira* sequences in NF, 5Y, 10Y, 20Y and 30Y soils, respectively (Figs. 2d and 3). Only one OTU was grouped into clade A.3 and accounted for 2.3% in the 10Y soil (Fig. 3).

3.4 The influence of soil properties on nitrifying population size and communities

The abundance of AOA *aomA* gene was significantly positively correlated with soil AK content (r = 0.5, P < 0.05) but negatively correlated with NO_2^--N content (r = −0.57, P < 0.05) (Fig. S2). The abundance of AOB *amoA* gene was positively correlated with soil pH (r = 0.55, P < 0.001) and TC (r = 0.49, P < 0.01), but negatively correlated with TN (r = −0.52, P < 0.001), TP (r = −0.15, P < 0.001), AP (r = −0.32, P < 0.001), NH_4^+-N (r = −0.4, P < 0.001) and NO_3^--N (r = −0.36, P < 0.001) (Fig. S2). The abundance of NOB *nxrA* gene showed a negative correlation with TN (r = −0.61, P < 0.05). However, the abundance of NOB *nxrB* gene was influenced by multiple factors, being positively correlated with pH (r = 0.3, P < 0.05) and TC (r = 0.36, P < 0.05), but negatively correlated with TN (r = −0.37, P < 0.01), TP (r = −0.17, P < 0.05), AP (r = −0.15, P < 0.05), NH_4^+-N (r = −0.08, P < 0.05) and NO_3^--N (r = −0.29, P < 0.05). Comammox *Nitrospira* clade A abundance was significantly positively correlated with TN (r = 0.49, P < 0.001), TP (r = 0.45, P < 0.001), AP (r = 0.49, P < 0.001), AK (r = 0.39, P < 0.01), NH_4^+-N (r = 0.59, P < 0.001) and NO_3^--N (r = 0.53, P < 0.001), but negatively with pH (r = −0.48, P < 0.001) and NO_2^--N (r = −0.42, P < 0.001). The relationships between comammox *Nitrospira* clade B abundance with soil properties were counter to those of comammox *Nitrospira* clade A abundance. Moreover, significant positive correlation was found between comammox *Nitrospira* clade B abundance and TC (r = 0.41, P < 0.001) (Fig. S3).

CCA and RDA were performed to illustrate the associations between soil properties and nitrifying populations (Fig. 4). The first two axes explained 53.5%, 55.4%, 76.7% and 67.5% of the total variation in the AOA, AOB, NOB and comammox *Nitrospira* community structures, respectively. Soil pH, AP and TP were found to be highly significantly

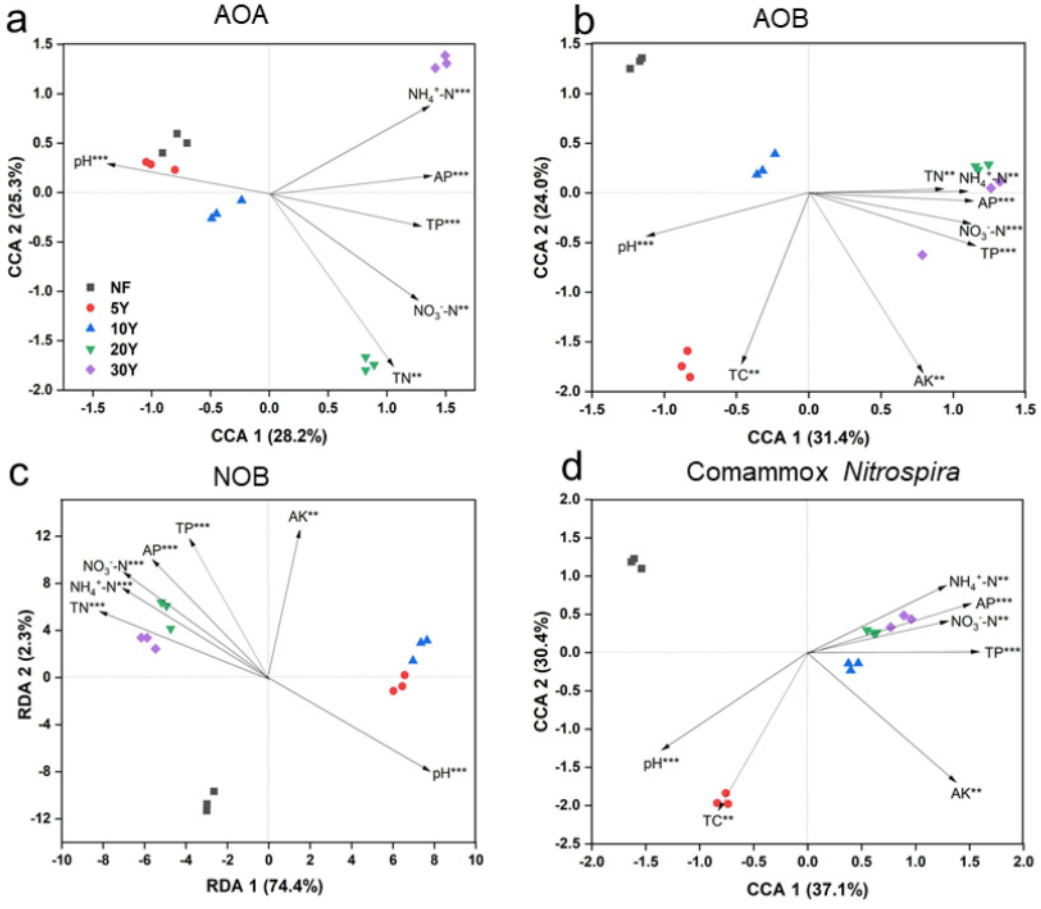


Fig. 4 Canonical correspondence analysis (CCA) and redundancy analysis (RDA) ordination plots quantifying the impacts of edaphic factors on AOA (a), AOB (b), NOB (c) and comammox *Nitrospira* (d) community structures. Single asterisk, double asterisk and three asterisks mark significance at P < 0.05, 0.01 and 0.001, respectively, based on 999 Monte Carlo permutations.

correlated with the AOA, AOB, NOB and comammox *Nitrospira* communities ($P < 0.001$) (Fig. 4). NH_4^+-N and NO_3^--N also had extremely significant effects on AOA and AOB communities, respectively ($P < 0.001$) (Fig. 4a, b). For the NOB community, soil pH, TP, AP, NO_3^--N, NH_4^+-N and TN were the most influential factors in driving NOB community composition (Fig. 4c). Furthermore, the BIO-ENV analysis showed the best correlations of pH, AP and NH_4^+-N with AOA population. AOB population was related to pH, TC, TP, AK and NO_3^--N. Soil pH, TC, TP, AP and AK affected comammox *Nitrospira* population, and NOB population was related to TP, pH and NO_3^--N (Table 1).

Random Forest modeling revealed that AK exhibited the highest Random Forest MPI (9.4%) in the prediction of AOA abundance, followed by TC (8.7%) and TN (7.0%) (Fig. 5a). TC (12.9%) and pH (12.8%) were the main predictors of AOB abundance, followed by TN (8.0%) (Fig. 5b). TN was the best predictor for the abundances of NOB *nxrA* and *nxrB* genes (Fig. 5c and d). TP (10.3%) and pH (10.0%) were the top two factors shaping the abundance of comammox *Nitrospira* clade A while TN (11.7%) and NH_4^+-N (10.6%) were the top two factors shaping the abundance of comammox *Nitrospira* clade B (Fig. 5e and f). For nitrifying communities, pH and TP were identified as the main predictors of the composition of AOA, NOB and comammox *Nitrospira* communities (Fig. 5g, i and j). In addition, TP (13.1%) exhibited the highest MPI in the prediction of AOB community, followed by AP (12.5%) and pH (11.6%) (Fig. 5h).

Table 1 BIO-ENV analysis based on the Spearman rank correlation coefficient (ρ), showing the association between nitrifying community composition and environmental variables.

Nitrifier	Combined variables	Size	Spearman's coefficient (ρ)
AOA	pH	1	0.8654
	pH+AP	2	0.8831
	pH+AP+NH_4^+-N	3	0.893
	pH+AP+NH_4^+-N+NO_3^--N	4	0.8667
	pH+TN+TP+AP+NH_4^+-N	5	0.8536
AOB	pH	1	0.818
	pH+TP	2	0.8763
	pH+AK+NO_3^--N	3	0.8972
	pH+AP+AK+NO_3^--N	4	0.9055
	pH+TC+TP+AK+NO_3^--N	5	0.9152
	pH+TC+TP+AK+NH_4^+-N+NO_3^--N	6	0.9094
Comammox *Nitrospira*	TP	1	0.8971
	pH+AK	2	0.8995
	pH+TP+AK	3	0.9052
	pH+TC+AP+AK	4	0.9266
	pH+TC+TP+AP+AK	5	0.9433
	pH+TC+TP+AP+AK+NO_2^--N	6	0.9269
NOB	TP	1	0.5218
	pH+NO_3^--N	2	0.626
	TP+pH+NO_3^--N	3	0.6331
	TP+pH+NO_2^--N+NO_3^--N	4	0.5888

4 Discussion

4.1 Response of soil properties and potential nitrifying activity to citrus planting

As shown in Table S2, long-term citrus planting caused severe soil acidification especially in 20Y and 30Y soils. Soil acidification has also been found in other terrestrial ecosystems such as agricultural soil (Schroder et al., 2011; Zeng et al., 2017; Tao et al., 2019), tea garden (Yang et al., 2018) and tobacco plantation soil (Zhang et al., 2016), because of excessive chemical fertilization application and intensive soil management practices (Guo et al., 2010; Bortoluzzi et al., 2012). Evidences have shown that the conversion of forest or grassland soils into agricultural soils usually caused a reduction in SOC stocks (Laganière et al., 2010; Shi et al., 2015); SOC loss rate was high in the first 5–7 years, slowed down after 15−20 years and gradually reached a new equilibrium over a longer period of time (Dick et al., 1998). TC contents in orchard soils observed in this study basically followed this pattern except in the case of the 5Y soil, which may have been because of the short-term effect of manure application. In addition, our results showed long-term citrus planting caused the accumulation of N and P contents due to the long-term massive fertilization application.

PAO and PNO decreased with planting age increase (Fig. 1a) and were significantly positively correlated with soil pH ($P < 0.001$) (Fig. S2). These results agreed with previous findings which showed that significantly higher soil nitrification occurs in alkaline soil than that in neutral and acidic soils (Jiang et al., 2015; Wang et al., 2019c). The low nitrification rate in acidic soils was due to the decrease in NH_3 availability with decreasing pH (De Boer and Kowalchuk, 2001; Norton and Stark, 2011). In addition, soil pH had an effect on the distribution and activity of nitrifiers and selected dominant microflora in their influence on nitrification rate (Avrahami et al., 2002; Nicol et al., 2008; Jiang et al., 2015). Interestingly, PAO and PNO were also positively correlated with TC (Fig. S2). Previous studies have indicated a positive correlation between heterotrophic nitrification rate and soil C content in forest and cropland soils (Zhang et al., 2011; Zhang et al., 2013). Therefore, heterotrophic nitrification may occur in the 5Y soil with the highest TC content, and further studies with stable isotope labeling and inhibitors need to quantify the contribution of heterotrophic nitrification to nitrification.

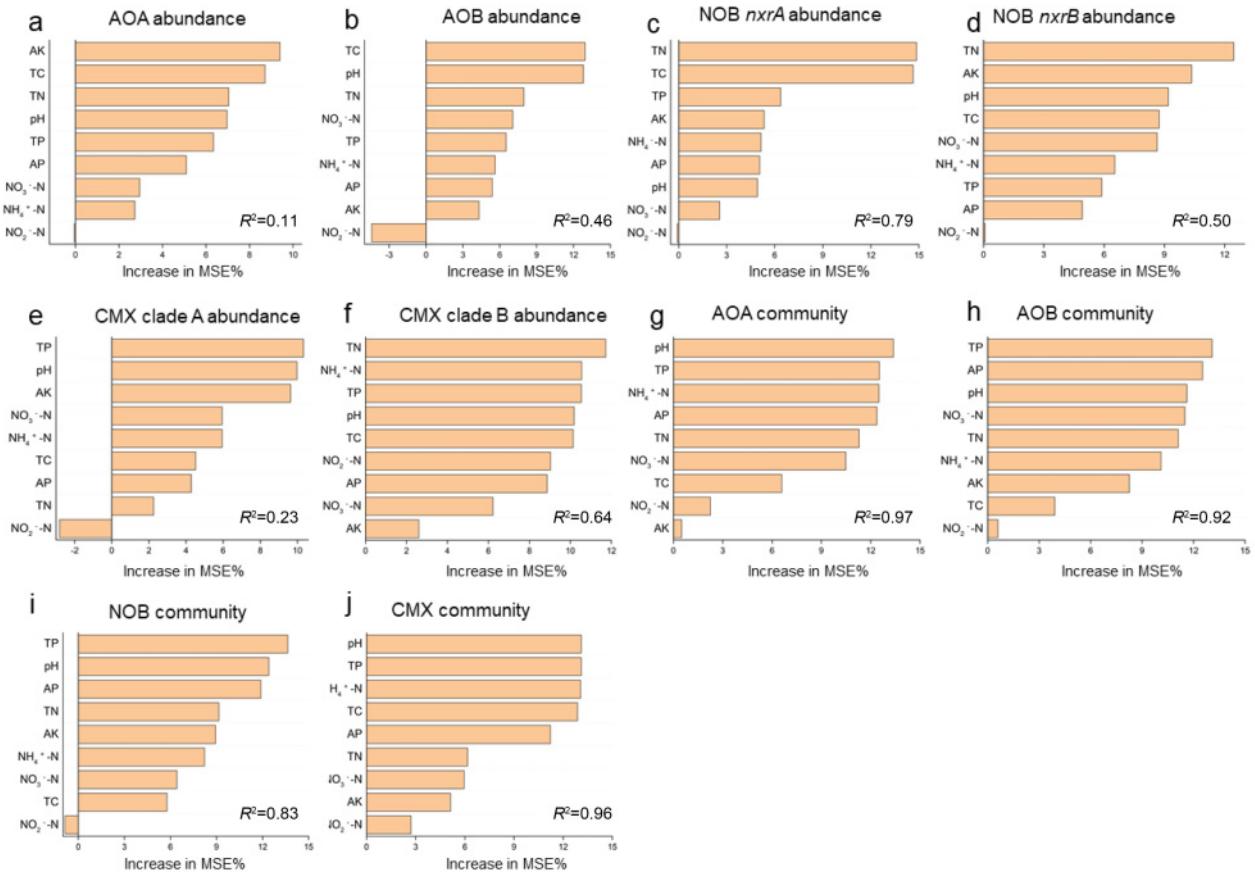


Fig. 5 Random Forest analysis to identify the main predictors of abundance and community composition of AOA, AOB, NOB and comammox *Nitrospira*. CMX represents comammox *Nitrospira*.

4.2 Response of canonical nitrifiers to citrus planting

The significant increases of AOA *amoA* gene abundance and higher AOA/AOB ratios in alkaline 5Y, neutral 10Y and acidic 30Y soils, compared with the NF soil (Fig. 1b), indicated AOA were not restricted to acidic environments. These results were inconsistent with previous studies which suggested AOA play more important role in acidic soils (Zhang et al., 2012; Lu and Jia, 2013). The significantly higher abundance of AOB in new orchard soils (5Y and 10Y) rather than old orchard soils (20Y and 30Y), compared with NF (Fig. 1b), indicated AOB were more abundant in neutral and alkaline soils. These results were further supported by Pearson correlation analysis, which showed the abundance of AOB rather than of AOA had significant relationship with soil pH (Fig. S2) as previously reported (Hu et al., 2014a; Scarlett et al., 2021). Meanwhile, the significant increase of the abundances of AOA and AOB in 5Y and 10Y soils (Fig. 1b) revealed AOA and AOB might conduct nitrification simultaneously. Some previous studies have also shown that both AOA and AOB were involved in nitrification in neutral and slightly alkaline soils (Wang et al., 2015; Nguyen et al., 2019), as they share the same energy source and substrate.

The phylogeny of the archaeal *amoA* gene showed distinct community compositions in orchard soils with different years. The 54d9-like and *Nitrososphaera*-like AOA were respectively dominant in young (5Y and 10Y) and old orchard soils (20Y and 30Y) (Fig. 2a). BIO-ENV results indicated pH, TP and NH_4^+-N were the best group to explain the variation of AOA populations (Table 1). RDA and RF analysis further indicated soil pH, N and P had significant effect on AOA community composition (Figs. 4a and 5g). The 54d9-like AOA was previously found to be dominant in neutral and alkaline soils (Bertagnolli et al., 2015; Wang et al., 2019b) and alkaline soil with high N application (Dong et al., 2019). Phylogenetic analysis found 30.9% and 82.1% of AOA reads in 20Y and 30Y, respectively, were phylogenetically most closely related *Nitrososphaera* JG1 (Fig. S3). A physiologic study showed the optimal pH for strain JG1 growth was 6.5 to 7.0, and it could not grow at pH below 6.0 (Kim et al., 2012). However, the high proportion of neutrophilic *Nitrososphaera* JG1 in acidic old orchard soils suggested a greater metabolic versatility than previously appreciated. Similarly, global analysis showed the distribution of AOA within the *Nitrososphaera* cluster even in strongly acidic soil, although the predominance of this cluster was observed in neutral and alkaline soils (Gubry-Rangin et al., 2011; Hu

et al., 2013). A study based on DNA-based stable-isotope probing found *Nitrososphaera*-like AOA played an active role in acidic soil nitrification as the estimated NH_3 content was high enough to meet the substrate demand of the strain JG1 (Wang et al., 2014). However, in an alkaline soil, high N application completely inhibited the growth of *Nitrososphaera*-like AOA (Dong et al., 2019). Therefore, the low NH_3 contents in 20Y (1.17 μM) and 30Y (0.35 μM) soils would be suitable for *Nitrososphaera* JG1 growth whereas the high NH_3 contents in 5Y (707 μM) and 10Y (102 μM) soils would inhibit the growth of this strain. In addition, P addition can accelerate N mineralization (Bauhus and Khanna, 1994), which can indirectly support enough substrate for strain JG1 even in an acidic environment. That would also explain why pH, N and P had significant effect on AOA community composition.

Most AOB *amoA* gene sequences were affiliated with the *Nitrosospira* cluster 3 (Fig. 2b and Fig. S4), which were frequently detected in previous studies (He et al., 2007; Zhang et al., 2019; Liu et al., 2021). The conversion of woodland to orchard soil decreased the relative abundance of *Nitrosospira* cluster 3a.1 especially in the 30Y soil while *Nitrosospira* cluster 3a.2 became dominant in orchard soils (Fig. 2b). Our results agreed with previous fertilizer studies which showed N fertilizer decreased the importance of *Nitrosospira* cluster 3a.1 but that *Nitrosospira* cluster 3a.2 became predominant (Zhong et al., 2016; Guo et al., 2017). In addition, *Nitrosospira* cluster 3a.1 were found to be predominant in neutral and alkaline soils (Hu et al., 2014b; Li et al., 2018). Thus, soil pH decline and N accumulation caused by long-term citrus planting would be the reason for the enrichment of *Nitrosospira* cluster 3a.2 in orchard soils. Besides soil pH and N content, P was also an important factor shaping AOB communities (Figs. 4b and 5h). A recent study found AP had negative correlation with AOB richness (Yang et al., 2020). In our study, the OTU numbers in 20Y (567 OTUs) and 30Y (108 OTUs) soils with high P concentration were significantly lower than those in 5Y (1 192 OTUs) and 10Y (1 363 OTUs) soils with low P concentrations (data not shown). The significant negative relationship between soil P content with AOB *amoA* gene abundance (Fig. S2) further indicated high P content in soil had negative effect on AOB. However, some other studies showed AOB preferred high P environments (Norman and Barrett, 2014; Sun et al., 2019). They believed P addition would increase N mineralization and increased availability of substrate for AOB (Norman and Barrett, 2014). The reason for the distinct results may be that AOB community was influenced by multi-factors, not one factor, and more research is needed to further study the relationship between soil P availability and AOB.

Nitrospira and *Nitrobacter* are the two major genera of NOB in terrestrial ecosystems (Attard et al., 2010; Ke et al., 2013). In our study, most sequences affiliated with these two groups, and *Nitrospira*- and *Nitrobacter*-like NOB respectively dominated in old (20Y and 30Y) and young orchard (5Y and 10Y) soils (Fig. 2c and Fig. S5). This could be explained by the actions of r-strategists for *Nitrobacter* and K-strategists for *Nitrospira* (Attard et al., 2010). For instance, *Nitrobacter*-like NOB are frequently observed in N-rich environments like fertilized soils and wastewater environments because of their low N substrate affinity (Wagner et al., 2002; Xia et al., 2011; Han et al., 2018). In young orchard soils, the high NH_3 concentrations and PAO may support sufficiently high substrate for *Nitrobacter*-like NOB. However, the low pH in old orchard soils may restrict the speed of nitrification as the limitation of NH_3 availability, and thus selects *Nitrospira*-like NOB.

4.3 Response of comammox *Nitrospira* to citrus planting

The comparable abundances of comammox *Nitrospira* clade A and clade B *amoA* genes, as well as those of AOA and AOB *amoA* genes in orchard soils (Fig. 1) indicate that comammox *Nitrospira* also plays an important role in nitrification. The highest abundance of comammox *Nitrospira* clade A in the 30Y soils (Fig. 1d) suggested that it prefers acidic environments, which is consistent with previous studies (Hu and He 2017; Takahashi et al., 2020; Hu et al., 2021) because of the overlapped NH_3 affinity between comammox *Nitrospira* and AOA isolates (Hu and He 2017; Kits et al., 2017). However, the comammox *Nitrospira* clade A *amoA* gene copy number was the second highest in the 5Y soil, in which the NH_3 content was the highest (Fig. 1d). Some recent studies have indicated that comammox *Nitrospira* clade A played an active role in agricultural and forest soils amended with N fertilizers (Li et al., 2019a, 2020). Genome analysis has found that comammox *Nitrospira* clade A, like most of β-AOB, possesses Rh-type ammonia transporters with a lower substrate affinity and higher uptake capacity (Weidinger et al., 2007; Palomo et al., 2018). These results indicate that comammox *Nitrospira* clade A are not strictly restricted to oligotrophic habitats. The abundance of comammox *Nitrospira* clade B was found to be higher in soils with high TC content (Fig. 1d), and there was a significant positive correlation between clade B abundance and TC (Fig. S2). A prior study showed pig manure significantly increased the relative abundance of clade B (Lin et al., 2020). Comammox *Nitrospira* clade B contain a 4-oxalocrotonate tautomerase, which is essential in the conversion pathway of various aromatic compounds (Harayama et al., 1989; Palomo et al., 2018). These results indicate comammox *Nitrospira* clade B has the potential to grow mixotrophically. In addition, comammox *Nitrospira* genomes harbor an alkaline phosphatase, which is highly expressed under P limiting environments (Palomo et al., 2018). Comammox

Nitrospira clade B rather than clade A abundance showed significant negative correlation with P (Fig. S2), but positive correlation with *phoD* gene abundance (Fig. S6), which suggested soil P content might be a key factor influencing the niche differentiation between comammox *Nitrospira* clade A and clade B.

MiSeq sequencing of comammox *Nitrospira amoA* gene revealed that clade A was the dominated cluster (Figs. 2d and 3). Clade A was further divided into clades A.1, A.2 and A.3, as in previous studies (Xia et al., 2018; Li et al., 2020). Clade A.1 contained the isolated and identified comammox species obtained from biofilm samples (Daims et al., 2015; van Kessel et al., 2015). In our study, most clade A sequences were affiliated with clade A.2, which was in line with previous studies in terrestrial ecosystems (Xu et al., 2020; Hu et al., 2021; Li et al., 2021; Liu et al., 2023). A previous study has suggested clade A.2 and A.3 play an important role in nitrification in soils with intensive agricultural practices (Li et al., 2020). However, clade A.3 only constituted a small proportion in orchard soils. Previous studies have indicated A.3 are favored in wet, hot and N-limited environments (Li et al., 2021), where water contents in the tested soils were between 6%–10% with the TN higher than 1g kg^{-1}. Thus, the dry orchard soils with intensive management might be unsuitable for clade A.3 but beneficial to clade A.2.

5 Conclusions

Our results showed that the conversion of woodland to orchard, and plantation ages, dramatically affected soil properties, nitrifying abundance and community. Long-term citrus planting increased soil acidification and caused nutrient accumulation. Soil potential nitrification activity decreased with increasing plantation age. The comparable abundance of comammox *Nitrospira amoA* genes and those of AOA and AOB *amoA* genes in orchard soils indicated that comammox *Nitrospira* were as important as canonical ammonia oxidizers for nitrification in intensively managed orchard soil. Significant shifts of nitrifying communities were observed in orchard soils with different cultivation ages. For instance, 54d9-like and *Nitrososphaera*-like AOA, and *Nitrobacter*- and *Nitrospira*-like NOB respectively dominated in young and old orchard soils. Soil pH and P content affected by citrus planting were the major factors shaping both canonical nitrifying and comammox *Nitrospira* communities. Our study is the first to clarify the abundances and communities of canonical nitrifiers and comammox *Nitrospira* in intensively managed orchard soils with different ages. Further studies should reveal the functional activity and relative contribution to nitrification of canonical nitrifiers and comammox *Nitrospira* in intensively managed orchard soils.

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Conflicts of interest

The authors do not have any personal, financial, or other conflicts of interest with other people or organizations that could inappropriately influence, or be perceived to influence, the submitted manuscript.

Author contributions

WFT designed the study; HYL wrote the manuscript; HYL, ZKT, WX and YYQ collected and analyzed the data; ZJN and HEL edited the manuscript. All authors contributed substantially to the discussion and the manuscript writing.

Electronic supplementary material

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