



OPEN

Ammonia oxidizing archaea and bacteria respond to different manure application rates during organic vegetable cultivation in Northwest China

Zhan Wang^{1,2}, Yinkun Li¹✉, Wengang Zheng¹, Yuru Ji¹, Minjie Duan³ & Li Ma⁴

Ammonia oxidization is a critical process in nitrogen cycling that involves ammonia oxidizing archaea (AOA) and bacteria (AOB). However, the effects of different manure amounts on ammonia-oxidizing microorganisms (AOMs) over the course of organic vegetables production remains unclear. We used the *amoA* gene to evaluate AOMs abundance and community structure in organic vegetable fields. Quantitative PCR revealed that AOB were more abundant than AOA. Among them, the *amoA* copy number of AOB treated with 900 kg N ha⁻¹ was 21.3 times that of AOA. The potential nitrification rate was significantly correlated with AOB abundance ($P < 0.0001$) but not with AOA, suggesting that AOB might contribute more to nitrification than AOA. AOB sequences were classified into *Nitrosomonas* and *Nitrosospira*, and AOA into *Nitrosopumilus* and *Nitrososphaera*. *Nitrosomonas* and *Nitrosopumilus* were predominant in treatments that received manure nitrogen at ≥ 900 kg ha⁻¹ (52.7–56.5%) and when manure was added (72.7–99.8%), respectively, whereas *Nitrosospira* and *Nitrososphaera* occupied more than a half percentage in those that received ≤ 600 kg ha⁻¹ (58.4–84.9%) and no manure (59.6%). A similar manure rate resulted in more identical AOMs' community structures than greater difference manure rate. The bacterial *amoA* gene abundances and ratios of AOB and AOA showed significantly positive correlations with soil electrical conductivity, total carbon and nitrogen, nitrate, phosphorus, potassium, and organic carbon, indicating that these were potential key factors influencing AOMs. This study explored the AOMs' variation in organic vegetable fields in Northwest China and provided a theoretical basis and reference for the subsequent formulation of proper manure management.

Organic cultivation is one of the leading agricultural patterns worldwide, including vital organic vegetable cultivation^{1,2}. The global organic agricultural cultivation area continuously increases and reached over 74.0 million hectares in 2020³. Since 2015, Ningxia has served as a major organic vegetable cultivation province with an inland area of 6670 ha for organic vegetable export^{4,5}. The organic vegetable cultivation area has been increasing in Ningxia due to unique climatic conditions such as extended daylight, considerable temperature differences, intense solar radiation, and favorable primary conditions, including advanced animal husbandry, rich manure resources, and no pollution^{6,7}. A large amount of organic fertilizer is usually applied during organic vegetable production to enrich the soil. However, nitrogen is often still deficient during crop growth because the nitrogen in organic fertilizer is mainly organic nitrogen, which needs to be decomposed into inorganic nitrogen before it can be absorbed and utilized by crops^{4,5}.

Manure produced by animal husbandry is the primary fertilizer nitrogen source for organic vegetable production. Several organic nitrogenous compounds in manure are easily transformed into ammonia through

¹Research Centre of Intelligent Equipment, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China. ²Guyuan Branch, Ningxia Academy of Agricultural and Forestry Sciences, Guyuan 756000, China. ³Beijing Key Laboratory of Ecological Function Assessment and Regulation Technology of Green Space, Beijing Urban Ecosystem Positioning Observation and Research Station, Beijing Institute of Landscape Architecture, Beijing 100102, China. ⁴Wuzhong National Agricultural Science and Technology Park Management Committee, Wuzhong 751100, Ningxia, China. ✉email: lykun1218@163.com

ammonification, which can then be oxidized to nitrate via nitrification⁸. Ammonia oxidation is the first and rate-limiting nitrification step because of its final product, nitrate⁹. The main reasons for excessive manure application during vegetable production are the lack of knowledge and control of the amount of nitrogen supplied by manure and changes in the critical microbial community structure^{4,7}. The ammonia oxidation process was long thought to be primarily restricted by ammonia-oxidizing bacteria (AOB)¹⁰. Known AOB are categorized into two monophyletic groups, *Gammaproteobacteria*, comprising members of the genus *Nitrosococcus*, and *Betaproteobacteria*, including the genera *Nitrosospira* and *Nitrosomonas*^{11,12}. However, this long-held view changed after the gene encoding ammonia monooxygenase (*amoA*) was discovered in ammonia-oxidizing archaea (AOA) within the phylum *Thaumarchaeota*¹³. *Thaumarchaeota* was shown to aerobically oxidize ammonia to nitrite, indicating that both AOA and AOB can be responsible for the conversion of ammonia¹⁴. Since then, the abundance, diversity, and community structures of ammonia-oxidizing microorganisms (AOMs) in distinct environments have been extensively researched, indicating they often coexist in various habitats^{12,15}.

The AOA and AOB community structures are essential for assessing nitrification. The AOA and AOB distribution reportedly depends on habitat type^{16,17}. AOA outnumber AOB in many ecosystems, such as drinking water bioreactors, river sediment, river water, acidic soils, terrestrial environments, and paddy soils^{18–20}. Additionally, AOB, rather than AOA, may dominate nitrogen-rich grassland soils, agricultural soils, lakes, and rivers^{21,22}. These differences may be due to the effect of environmental factors and the distinct habitat types.

Moreover, AOA's and AOB's abundance and diversity are reportedly affected by many abiotic factors, including pH, ammonium levels, electrical conductivity, and organic matter content^{20,23,24}. Generally, ammonia, in ammonia oxidation substrate, has been considered the primary factor in manipulating the distribution of AOMs²⁵. AOB growth was favored at high ammonium levels in field soils. Moreover, freshwater river studies reported that the AOMs' community distribution was related to organic matter and ammonia. pH is another essential factor influencing the AOM's distribution^{26,27}. AOB reportedly become more competitive in environments with higher ammonium concentrations, while AOA may dominate ammonia oxidation in ecosystems with low pH and ammonium concentrations. In low nitrogen and strong acidic environments, AOA was the main nitrification driver, while AOB primarily have functional activity in high nitrogen, neutral, and alkaline environments^{28,29}. The varied AOA and AOB communities' responses to different niches and physiochemical characteristics highlight the need for an increased focus on agricultural research to improve manure application for organic vegetable production systems.

Therefore, this study designed a field plot experiment in which soils were amended with different amounts of manure. The objectives of this study were to compare the relative contributions of AOA and AOB to the process of ammonia oxidation and the changes in AOM abundance and community structure in response to different amounts of manure and to determine which major soil physicochemical variables affect AOA and AOB segregation in the organic vegetable field.

Material and methods

Site description and soil sampling. This study was conducted at the Wuzhong National Agricultural Science and Technology experimental station, located in Ningxia province, China (106°6'26" E, 37°57'10" N). The station is situated in the semi-humid and semi-arid temperate continental monsoon climate with an average annual temperature of 5.3–9.9 °C and a yearly average of 105–163 frost-free days. According to the international classification system World Reference Base, the soil in the area is classified as sandy soil³⁰. The field capacity and soil bulk densities were 29.7 m³ m⁻³ and 1.65 g cm⁻³, respectively. The total nitrogen organic matter, available phosphorous, and potassium in the 0–20 cm soil layer were 0.20, 1.57, 0.013, and 0.13 g kg⁻¹, respectively.

The experiment was carried out from May to October 2016 in three growth cycles for Chinese Flowering Cabbage (*Brassica parachinensis* L. ssp. *Chinensis* var. *utilis*; Fig. S4). Following local production methods, the first, second, and third round crops were sowed on May 13, July 15, and September 10, respectively, and harvested on June 23, August 18, and October 22, respectively^{4,6,7}. The experiment employed a randomized complete block design with five manure dosage treatments, replicated thrice. Each treatment was applied to a plot with an area of 10 × 4 m with a buffer zone of 0.5 m surrounding each plot to explore the effect of manure dosage on AMOs^{7,31}. The treatment dosages were as follows: 300 kgN ha⁻¹ (M1), 600 kgN ha⁻¹ (M2), 900 kgN ha⁻¹ (M3), 1200 kgN ha⁻¹ (M4), and 0 kgN ha⁻¹ (M0, the control)^{4,31}. Cow dung was used as an only-nitrogen fertilizer source and was applied once a year before the first crop was planted according to the local customary production method⁶. The cow dung has a pH of 6.9 and contained 12.9 g kg⁻¹ total nitrogen, 10.1 g kg⁻¹ available potassium, 1.2 g kg⁻¹ available phosphorous, and 195.0 g kg⁻¹ organic matter.

Topsoil samples (0–20 cm) were collected from each plot in October 2016 after harvesting the Chinese Flowering Cabbage. A total of six soil cores (5 cm) were taken from each field, and samples of the same manure dosage area were mixed thoroughly to form one composite sample. After collection, all samples were divided into three parts; one was transported to the laboratory on dry ice and stored at –80 °C for molecular analysis, and the second was kept at 4 °C for 24 h until the potential nitrification determination was completed. The third was used to determine the soil's physicochemical properties.

Potential nitrification rate measurement. As previously described³², 300 μM of 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) was used in the current study since a 200 μM PTIO concentration may be insufficient in inhibiting AOA while a concentration of 400 μM might result in inhibiting AOB. A total of 4 μM 1-octyne was added to inhibit AOB³³. The PNR was measured as previously described^{34,35}. Briefly, each sample was divided into three subsamples. All subsamples (10.0 g fresh soil) were placed in 120-mL serum vials with phenolic caps and butyl stoppers containing 20 mL deionized water and 1 mM NH₄Cl. The samples were shaken at 24 °C (110 rpm) for 2 h in the dark to prepare soil slurries. This soil slurry was then exposed to

4 μM 1-octyne, 300 μM PTIO, or none. Then, 15 mg mL^{-1} KClO_3 was added to all samples to inhibit NO_2^- oxidation. Soil slurry (1 mL) samples were collected for NO_2^- analysis at 2, 12, 24, 48, and 72 h after adding 1-octyne, PTIO, and KClO_3 . The aliquots were centrifuged at $15,000 \times g$ for 5 min. The supernatant were frozen until NO_2^- measurement under unifying conditions. However, no significant NO_3^- accumulation was observed after KClO_3 was added to inhibit NO_2^- oxidation in this study, indicating that NO_2^- oxidation was satisfactorily inhibited.

The PNR was calculated from the rate of the linear accumulation in NO_2^- concentrations over time. The PNR of AOA and AOB was calculated by subtracting the NO_2^- accumulation in the PTIO or 1-octyne treatment from the values measured with only KClO_3 . The NO_3^- and NO_2^- concentrations were measured using a flow injection autoanalyzer (SKALAR, San++, Netherlands).

DNA extraction. Soil DNA was extracted using an EZNA DNA Soil kit (Omega Bio-Tek Inc., Norcross, GA, USA) per the manufacturer's protocols. The extracted DNA was visualized using 1% agarose gel during electrophoresis, diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0), and stored at -20°C before use.

Quantitative PCR. The *amoA* gene's abundance in AOA and AOB was quantified by real-time PCR using the Arch-*amoA*F/Arch-*amoA*R primers for the AOA *amoA* genes and *amoA*1F/*amoA*2R for the AOB *amoA* genes. All qPCR assays were performed in triplicate with an ABI PRISM R 7500 Sequence Detection System (Applied Biosystems, Waltham, MA, USA) using the SYBR Green I method. The 20 μL qPCR reaction system contained 10 μL FastStart Universal SYBR Green Master (ROX) (Roche, NJ, USA), 0.4 μL 10 μM each forward and reverse primer, 7.2 μL sterilized MilliQ water, and 2 μL standard or extracted soil DNA. Amplification was conducted in a LightCycler[®] 480 (Roche Applied Science, Penzberg, Germany) as follows: 95°C for 10 min, followed by 40 cycles of 15 s at 95°C , 45 s at 54°C and 60 s at 72°C for AOA, or 40 cycles of 15 s at 95°C and 2 min at 58°C for AOB. Standard melting curves were generated using 10-fold serial dilutions of a plasmid containing the AOA or AOB target gene inserts.

Barcoded pyrosequencing. Pyrosequencing of the AOA and AOB *amoA* gene was performed using the specific primer pairs Arch-*amoA*F/Arch-*amoA*R and *amoA*1F/*amoA*2R, respectively. Barcodes were ligated to the 5'-ends of the primers to distinguish each sample. Each sample was then PCR amplified in 25 μL reactions containing 10 ng DNA under the following conditions: 98°C for 5 min for initial denaturation, followed by 30 cycles of 98°C for 30 s, 50°C for 45 s, and 72°C for 60 s, and final extension at 72°C for 7 min. After purification with an Agarose Gel DNA purification kit (TaKaRa, Dalian, China), pyrosequencing was conducted using a 454 Life Science Genome Sequencer FLX Titanium instrument (Roche, NJ, USA) by Personal Biotechnology Co., Ltd., Beijing, China.

Statistical analysis. The raw sequences obtained by pyrosequencing that matched the barcodes and primers were retained. Data were processed using QIIME Pipeline Version 1.9.0 (<http://qiime.sourceforge.net/>)³⁶. Briefly, reads with a mean quality score of <20 or >200 bp and <500 bp in length were removed, and the 10 bp barcode was examined to assign sequences to each sample. The sequences were trimmed using the UCHIME algorithm to remove the barcodes and primers³⁷. After quality control, USEARCH was performed to cluster the high-quality sequences into OTUs with a 97% similarity cutoff³⁸. The richness indices (ACE estimator), diversity indices (Shannon index), and Good's coverage were also processed using QIIME Pipeline Version 1.9.0³⁶. Phylogenetic analysis was performed using MEGA6³⁹, while redundancy analysis was performed using CANOCO for Windows (version 4.5) to identify the correlations between soil parameters and microbial communities⁴⁰. Pearson's correlation analysis of the abundance, transcript abundance, PNR, and environmental factors was performed using the SPSS statistics software (version 19.0 for Windows). Principal coordinate analysis (PCoA) and analysis of similarities (ANOSIM) were used to measure community similarity based on the algorithm of the Bray-Curtis matrix, and 999 Monte Carlo permutations were used to assess the statistical significance of diversity metrics.

DNA sequence accession numbers. The raw data generated from pyrosequencing were submitted to the National Center for Biotechnology Information Sequence Read Archive database under accession numbers SRP425288 and SRP425614.

Results

AOA and AOB abundance and potential nitrification rates. In organic vegetable fields, the abundance of soil AOA and AOB *amoA* genes were $6.63 \pm 0.24 \times 10^5$ – $11.14 \pm 0.90 \times 10^5$ and $1.20 \pm 0.11 \times 10^6$ – $18.99 \pm 0.99 \times 10^6$ copies/g of dry soil, respectively (Table 1). The AOA *amoA* abundance initially decreased, then increased with increasing manure levels, whereas the *amoA* abundance of AOB increased continuously. In addition, the ratios of AOB and AOA *amoA* gene copy numbers varied from 1.08 to 20.67 among all treatments and decreased in the order of $\text{M4} > \text{M3} > \text{M2} > \text{M1} > \text{M0}$, indicating that the *amoA* copy numbers of AOB were consistently greater than those of AOA (Table 1).

Overall, total potential nitrification rates (PNR) increased as the manure amount increased. The addition of ampicillin to separate the AOA's PNR from the total PNR revealed that the AOB PNR was significantly greater than that of AOA for all samples ($P < 0.001$, Table 1). Pairwise regression analysis revealed that PNR was not correlated with the AOA abundance (Fig. 1a) but was highly correlated with the AOB abundance ($P < 0.0001$, Fig. 1b). These results suggest that AOB might contribute more to nitrification than AOA in organic vegetable fields.

Sample	EC (cm ⁻¹)	pH	TC (g kg ⁻¹)	TN (mg kg ⁻¹)	NH ₄ ⁺ (mg kg ⁻¹)	NO ₃ ⁻ (mg kg ⁻¹)	AP (mg kg ⁻¹)	AK (mg kg ⁻¹)	TOC (g kg ⁻¹)	AOA (copy number 10 ⁵ g ⁻¹ soil)	AOB (copy number 10 ⁶ g ⁻¹ soil)	AOB/AOA abundance	AOA PNR (mg NO ₂ ⁻ -N kg ⁻¹ soil h ⁻¹)	AOB PNR (mg NO ₂ ⁻ -N kg ⁻¹ soil h ⁻¹)	Total PNR (mg NO ₂ ⁻ -N kg ⁻¹ soil h ⁻¹)
M0	179.6	8.07	11.1	333.0	28.52	1.54	29.52	177.18	2.85	11.14	1.20	1.08	0.22	0.79	1.01
M1	187.8	8.16	12.3	433.0	28.75	3.39	60.89	194.58	4.27	7.35	4.58	6.23	0.52	1.56	2.08
M2	213.4	8.12	12.9	461.0	28.93	4.52	98.71	218.26	5.42	6.63	8.21	12.38	1.03	2.20	3.23
M3	227.3	8.21	13.6	530.0	28.78	6.80	144.21	235.51	7.35	7.85	16.70	21.28	1.50	2.71	4.21
M4	253.0	8.03	15.4	750.0	28.56	7.41	174.32	251.27	9.43	9.19	18.99	20.67	2.46	3.07	5.53

Table 1. Basic properties of the sandy loam soil under different fertilization treatments. *EC* electrical conductivity, *TC* total carbon, *TN* total nitrogen, *NH₄⁺* ammonium nitrogen, *NO₃⁻* nitrate nitrogen, *AP* available phosphorus, *AK* available potassium, *TOC* total organic carbon, *M0* without manure application, *M1* annual manure application 300 kgN ha⁻¹, *M2* annual manure application 600 kgN ha⁻¹, *M3* annual manure application 900 kgN ha⁻¹, *M4* annual manure application 1200 kgN ha⁻¹, *PNR* potential nitrification rates, *AOA* ammonia-oxidizing archaea, *AOB* ammonia-oxidizing bacteria.

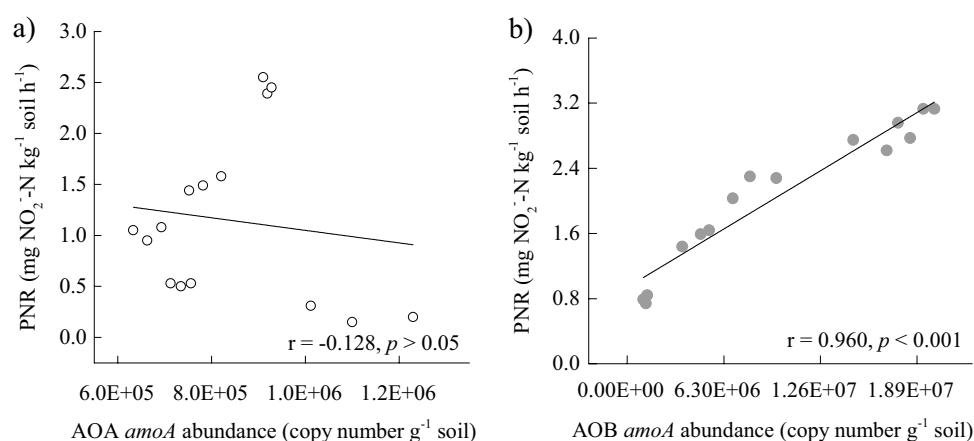


Figure 1. Relationship between AOA and AOB *amoA* gene abundance and potential nitrification rates (PNR) in organic vegetable field soils.

***amoA* gene diversity.** As previously described²³, the α - and β -diversity of AOA and AOB were measured using *amoA* gene clone libraries. A total of 27,111 high-quality archaeal *amoA* gene sequences were generated, divided into 31 operational taxonomic units (OTUs) using a 3% cutoff (Table 2). Regarding AOB, 84,863 high-quality bacterial *amoA* gene sequences were grouped into 66 OTUs. AOB displayed higher α -diversity (Ace and Shannon indexes) than AOA in all treatments. Individually, the AOA diversity decreased and then increased with increasing manure application, while AOB levels increased continuously. For each sample, AOA had the highest diversity at M1, whereas AOB had the highest variety at M4. The coverage exceeded 0.99 in all treat-

Samples	<i>amoA</i> sequence/no. of OTUs		Ace index		Shannon index		Coverage	
	AOA	AOB	AOA	AOB	AOA	AOB	AOA	AOB
M0	27111/24	44378/35	50.30	46.66	1.62	1.78	0.9995	0.9986
M1	15467/22	51618/38	46.14	48.21	1.61	1.81	0.9998	0.9992
M2	13412/17	54822/43	37.00	52.23	1.30	1.92	0.9998	0.9993
M3	16501/17	74171/48	36.72	56.26	1.30	2.05	0.9999	0.9993
M4	18834/14	84863/52	31.73	61.57	1.23	2.23	0.9998	0.9991

Table 2. Diversity of archaeal and bacterial *amoA* gene sequences. *M0* without manure application, *M1* annual manure application 300 kgN ha⁻¹, *M2* annual manure application 600 kgN ha⁻¹, *M3* annual manure application 900 kgN ha⁻¹, *M4* annual manure application 1200 kgN ha⁻¹, *AOA* ammonia-oxidizing archaea, *AOB* ammonia-oxidizing bacteria.

ments, indicating that the *amoA* gene sequences retrieved from the treatments could represent most AOA and AOB communities.

Principal coordinate analysis (PCoA) and analysis of similarities (ANOSIM) were used to evaluate the β -diversity of ammonia-oxidizers in organic vegetable fields (Fig. 2). The OTU-based PCoA results indicated that the AOA and AOB communities differed significantly between the fertilization and no fertilization groups ($P < 0.01$). Furthermore, the community structure of AOA and AOB became more similar when the same amount of manure was added and vice versa, indicating that the manure quantity significantly influenced the microbial community structure of organic vegetable fields ($P < 0.05$).

Phylogenetic analysis and AOA community structure. The AOA phylogenetic tree was constructed using known AOA isolated strains and *amoA* gene sequences of representative OTUs (Figs. 3 and S1). All sequences were grouped into two clusters, namely *Nitrosopumilus* and *Nitrososphaera*. The *Nitrosopumilus* cluster had the greatest abundance group, accounting for 40.4–99.8% of all archaea *amoA* gene sequences with nine OTUs in the organic vegetable field (Fig. S3a). *Nitrososphaera*, which had four OTUs and a relative abundance of 0.2–59.6%, was the second most abundant. Notably, the *Nitrosopumilus* cluster was continuously increasing with the increasing manure application rate, whereas the *Nitrososphaera* cluster was consistently decreasing.

Cluster analysis showed that the AOA community could be classified into two branches (Fig. S3a). The *Nitrosopumilus* cluster was the predominant AOA in manure samples M1, M2, M3, and M4, which accounted

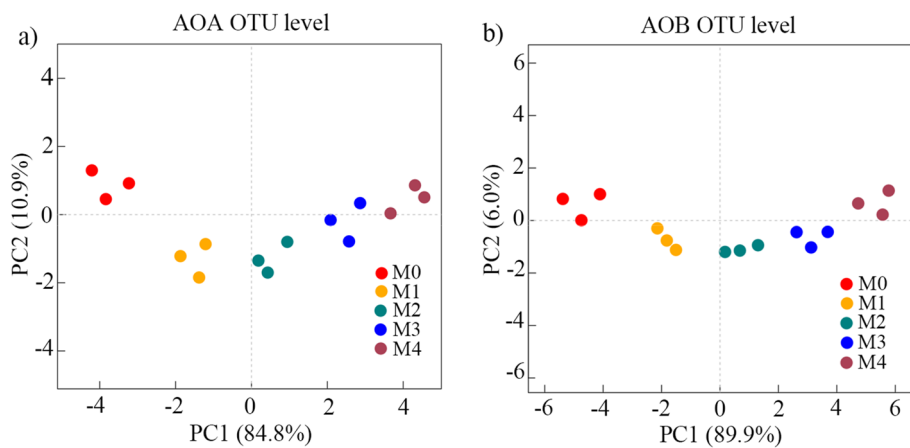


Figure 2. A PCoA plot at OTU level of (a) ammonia-oxidizing archaea (AOA); and (b) ammonia-oxidizing bacteria (AOB).

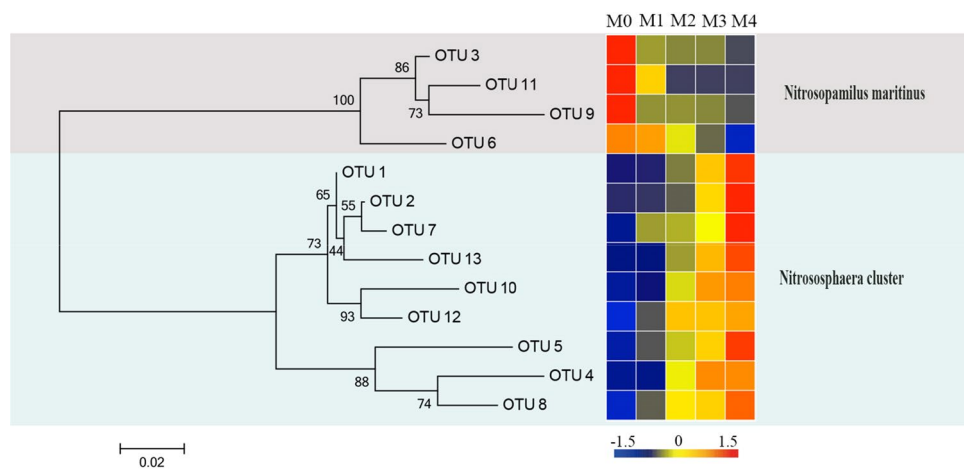


Figure 3. Neighbor-joining phylogenetic tree and community distributions of AOA *amoA* gene sequences from organic vegetable fields under different manure application rates. Bootstrap values $> 50\%$ based on 1000 replicates are shown next to each branch, and the scale bar represents a divergence of 0.02 nucleic acid sequences. Each OTU relative proportion value is color-coded in the corresponding heat map legends. M0: without manure application; M1: 300 kgN ha⁻¹ annual manure application; M2: 600 kgN ha⁻¹ annual manure application; M3: 900 kgN ha⁻¹ annual manure application; M4: 1200 kgN ha⁻¹ annual manure application.

for 72.7, 91.9, 94.9, and 99.8% of the total sequences, respectively. For no manure samples, the *Nitrososphaera* cluster occupied more than a half percentage in the M0 treatment. These results imply that AOA communities may be influenced by manure.

Phylogenetic analysis and AOB community structure. The AOB *amoA* gene phylogenetic tree showed that all retrieved sequences could be categorized into two major clusters, *Nitrosospira* and *Nitrosomonas* (Figs. 4 and S2). The *Nitrosospira* cluster, containing 16 OTUs and accounting for 43.4–84.9% of all bacterial *amoA* gene sequences, was the most abundant group in the organic vegetable field, followed by the *Nitrosomonas* cluster, which contained four OTUs and had a relative abundance of 15.1–56.6% (Fig. S3b).

Similarity analysis indicated that the AOB community could be clustered into two branches (Fig. S3b). The *Nitrosomonas* cluster accounted for more than half of all bacterial *amoA* gene sequences in the M3 (52.7%) and M4 (56.5%) treatments. Furthermore, the *Nitrosospira* cluster showed relatively higher ratios of 58.4, 67.7, and 84.9% in vegetable field soil samples of M0, M1, and M3, respectively, than the *Nitrosomonas* cluster. These results imply that the manure addition rate had an apparent effect on microbial communities.

Impact of soil parameters on AOA and AOB communities. Spearman's correlation analyses were conducted to illustrate the relationship between soil parameters and ammonia-oxidizing microbial abundance, α -diversity, and predominant taxa (Fig. 5). The bacterial *amoA* gene abundances in organic vegetable fields showed significant positive correlations with all soil parameters ($P < 0.05$) except for pH and ammonium. In contrast, except for ammonium, no significant correlations were detected between archaeal *amoA* gene abundances and soil parameters. Additionally, the ratios of AOA and AOB *amoA* gene abundances were positively correlated with electrical conductivity (EC), total carbon (TC), nitrate, available phosphorus (AP), available potassium (AK), and total organic carbon (TOC) ($P < 0.05$). The AOB α -diversity (OTU number, Ace, and Shannon indexes) had notable positive correlations with all soil properties. Interestingly, AOA α -diversity was significantly negatively correlated with soil parameters ($P < 0.05$).

The genera *Nitrososphaera* (AOA) and *Nitrosomonas* (AOB) were also notably and positively correlated with the EC, TC, TN, nitrate, AP, AK, and TOC concentrations ($P < 0.05$). In contrast, the *Nitrosopumilus* (AOA) and *Nitrosospira* (AOB) clusters showed no significant correlations with soil parameters, except ammonium.

Discussion

Fertilization considerably affects the ammonia-oxidizing microorganism (AOM) distribution in soil^{11,12,14}. However, little is known about AOM distribution patterns in organic vegetable fields. This study's quantitative PCR (qPCR) indicated that bacterial *amoA* gene copy numbers were always greater than archaea's under the same

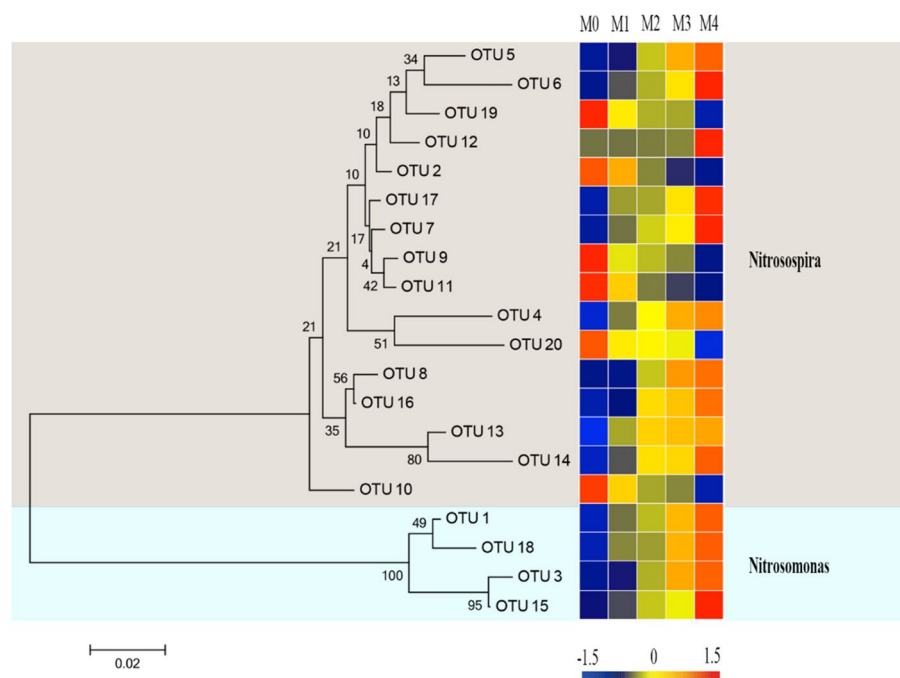


Figure 4. Neighbor-joining phylogenetic tree and community distributions of AOB *amoA* gene sequences from organic vegetable fields under different manure application rates. Bootstrap values $> 50\%$ based on 1000 replicates are shown next to each branch, and the scale bar represents a divergence of 0.02 nucleic acid sequences. Each OTU relative proportion value is color-coded in the corresponding heat map legends. M0: without manure application; M1: 300 kgN ha⁻¹ annual manure application; M2: 600 kgN ha⁻¹ annual manure application; M3: 900 kgN ha⁻¹ annual manure application; M4: 1200 kgN ha⁻¹ annual manure application.

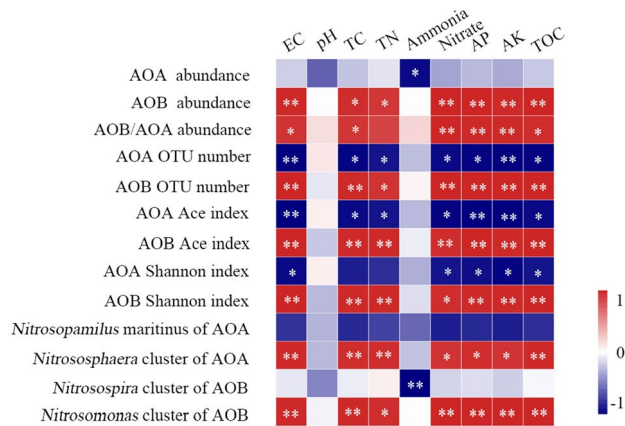


Figure 5. Heat maps visualizing the correlation between soil parameters, ammonia-oxidizing microbial abundance, α -diversity, and predominant taxa. Correlation coefficient values are color-coded in the corresponding heat map legends. ** $P < 0.01$ and * $P < 0.05$ indicate significant correlation effects at different significance levels. EC: electrical conductivity; TC: total carbon; TN: total nitrogen; AP: available phosphorus; AK: available potassium; TOC: total organic carbon.

manure dosage. These higher ammonia-oxidizing bacteria (AOB) abundances in organic vegetable fields revealed that AOB are more adapted to organic vegetable cultivation conditions and, therefore, might have more versatile metabolisms than ammonia-oxidizing archaea (AOA)^{18,41}. Different nitrogen concentrations and pH values reportedly cause differential growth of AOB and AOA, with AOB growing at high nitrogen concentrations in alkaline environments (pH7.0–8.5) and AOA being predominant at low nitrogen concentrations and acidic and neutral conditions (pH3.7–7.0)^{21,27,42}. Due to total nitrogen concentration ($> 333 \text{ mg kg}^{-1}$) and alkalinity of soils (pH > 8.03) were relatively high in this study (Table 1), it is reasonable that higher AOB abundance was detected in the organic vegetable field. With the increase of fertilization within a reasonable range, the crop developed excellently, the root system absorbed more particles and the soil pH increased⁴³. For example, the long-term winter wheat and summer corn fertilization experiment on red loam in Hunan Province indicated that after 18 years of continuously adding pig manure (nitrogen dosage 850 kg ha^{-1}), the soil pH increased from 5.70 to 6.40⁴⁴. In this study, the amount of 900 kg N ha^{-1} manure used was similar to that added to the Hunan red loam, and the pH increased from 8.07 to 8.21 (Table 1). However, if the manure dosage was too high (such as $1200 \text{ kg N ha}^{-1}$ in this study), it was not conducive to crop growth, resulting in soil acidification and pH drop⁴⁵.

Slight changes in pH can impact the AOMs' community structure^{11,12,20}. In this study, soil pH increased with manure dosage (Table 1). This increase may benefit AOB for abundance and diversity but is not conducive to AOA, which may be a fundamental reason for the decreased AOA and the rise of AOB. Furthermore, the AOA and AOB quantities in the organic field had a significantly positive correlation with total carbon (TC, $P < 0.05$, Fig. 5). Carbon concentration is also a key factor causing the AOMs abundance to vary. AOMs are reportedly autotrophic microorganisms capable of using carbon dioxide as the only carbon source^{46,47}. However, AOMs have been described as mixotrophic or heterotrophic^{48,49}. Thus, AOMs' response to carbon sources is very complex, and enhancing carbon can increase the abundance of some members but inhibit that of others⁴⁹. Moreover, two closely-related *Nitrosotalea* strains displayed differences in physiological features, as indicated by variations in growth rate in response to organic compounds⁵⁰. In the organic field soil in the present study, Spearman's statistical analyses showed that both TC and several AOA and AOB lineages were significantly positively correlated (Fig. 5), supporting this complex response.

The ammonia oxidation rate may or may not be directly related to the AOA and AOB *amoA* abundance. Furthermore, AOB *amoA* abundance is more positively correlated with potential nitrification rates (PNR) than AOA in neutral and alkaline soils⁵¹. However, AOA communities are more critical than AOB for ammonia oxidation in acidic soils⁵². A paddy soil survey soil revealed that AOA and AOB abundance was significantly positively correlated with PNR, with a stronger correlation for AOB ($r = 0.724$) than AOA ($r = 0.406$)⁴⁷. In the present study, the soil was alkaline (Table 1), and the abundance of AOB was positively correlated with PNR ($r = 0.960$, $P < 0.001$), whereas AOA showed no significant relationships (Fig. 1). This correlation is consistent with the results reported in previous studies, which showed AOB were more suitable for alkaline soils and high nitrogen concentrations than AOA^{39,53}. These findings indicate that AOB, rather than AOA, contributed considerably to ammonia oxidation in the organic vegetable field. However, the AOA abundance was lower than that of AOB, and the relationship between relative abundances of different lineages of AOA and AOB and PNR varied greatly (ANOSIM test, data not shown). Therefore, the DNA-SIP method or more detailed experiments (e.g., using different inhibitors) should be used in further studies to assess the contribution of several AOA and AOB lineages to ammonia oxidation in organic vegetable field soil.

All known archaeal genera related to ammonia oxidizers have been classified as members of *Nitrosopumilus*, *Nitrososphaera*, *Nitrosoarchaeum*, and unclassified as *Thaumarchaeota*^{34,54}. Phylogenetic analysis indicated that in the soils of organic vegetable fields, the *Nitrosopumilus* cluster was the main species (72.7–99.8% of all archaeal sequences) for manure-added treatments (M1, M2, M3, and M4). However, in treatments with no added manure,

the percentage of *Nitrosopumilus* cluster was much lower, although it was still high (up to 40.4%; Fig. S3a). These findings were consistent with those observed for other soil environments in the Chinese black zone, Greenland, Costa Rica, Namibia, and Austria^{55,56}. These findings suggest that *Nitrosopumilus* are the dominant species in organic field soils and most other soils.

Interestingly, the *Nitrosopumilus* cluster continuously increased with the manure rate, whereas the *Nitrososphaera* cluster continuously decreased. Principal coordinate analysis (Fig. 2a) and cluster analysis (Fig. S3a) showed treatments that received similar amounts of manure had more identical AOA community structures. In contrast, greater differences in the manure quantity were associated with more divergent AOA and AOB community structures. These findings imply that manure rate had an evident influence on AOA microbial communities, and *Nitrosopumilus* may be more adaptive to a higher manure quantity, whereas *Nitrososphaera* showed an opposite trend.

The AOA distribution is reportedly closely associated with pH^{47,52}. However, we found that the distribution of AOA lineages at the genus level was not significantly related to pH. The inconsistent relationships between soil pH and different AOA lineages may be associated with the various pH ranges tested in previous studies and this study⁴⁷. In this study, the pH value range was narrower (8.03–8.21, Table 1) than in previous research^{47,54}, which may lead to the impact of pH on AOA lineage distribution in the organic vegetable soils being masked by other environmental parameters^{16,54}. We found that other factors, such as the TC, total nitrogen (TN), electrical conductivity (EC), nitrate, available phosphorus (AP), available potassium (AK), and total organic carbon (TOC) concentrations, were associated with the distribution of specific AOA lineages in organic vegetable soils.

Among bacterial communities, *Nitrosospira* and *Nitrosomonas* were the dominant terrestrial genera⁵⁶. The relative abundance of the *Nitrosospira* genus is greater than that of the *Nitrosomonas* genus in many samples collected from paddy soils⁴⁷, sediment³⁷, and water³⁹. In contrast, some studies reported that *Nitrosomonas* might be dominant in the black soil zone of northeast China²³, as well as in aquatic environments and paddy soils^{58,59}. These findings suggest that AOB has different distribution characteristics in various settings.

In this study, *Nitrosospira* showed greater abundance in M0, M1, and M2 (58.4–84.9%) samples (organic nitrogen dosage of less than 600 kg ha⁻¹), whereas *Nitrosomonas* accounted for more than half of the percentage in the M3 and M4 treatments (organic nitrogen dosage of more than 900 kg ha⁻¹). Thus, the AOB community compositions in organic vegetable field soils differed distinctly under different manure application rates. *Nitrosomonas* was more adaptable to large manure amounts, whereas the opposite was true for *Nitrosospira*. However, in organic vegetable fields, the application of different amounts of manure had little effect on soil pH; therefore, the impact of pH on soil microorganisms can be overshadowed by other environmental factors such as large quantities of organic carbon, organic nitrogen, and inorganic nitrogen quantities present in manure. We also observed that the distribution of some AOB lineages at the genus level was positively or negatively related to the EC, TC, TN, nitrate, AP, AK, TOC, and ammonia levels (Fig. 5). Furthermore, AOB were reportedly not significantly correlated with pH but were significantly associated with different environmental factors²³. This study and previous studies' results indicate that multiple soil factors influence the distribution of the AOB *amoA* gene in organic vegetable fields.

Conclusions

The abundance, diversity, and community AOMs structure in organic vegetable field soil receiving different manure amounts were analyzed using the functional marker gene *amoA*. The AOB *amoA* genes showed higher abundance and diversity than the AOA *amoA* genes. There was also a positive relationship between bacterial *amoA* gene copy numbers and PNR, whereas the archaeal *amoA* gene copy numbers were not correlated with PNR. Separation of the AOMs' total PNR revealed that AOB's PNR was higher than AOA's in all samples. Therefore, AOB may dominate the soil nitrifying processes in organic vegetable fields. In addition, AOMs communities analysis in organic vegetable fields revealed they were altered by variations in physicochemical characteristics caused by different manure amounts. The AOA *Nitrosopumilus* and AOB *Nitrosomonas* clusters may be more adaptable to large manure quantities, whereas the opposite was true for the *Nitrososphaera* and *Nitrosospira* clusters. These results provide additional valuable information for future studies investigating nitrogen cycles in organic field systems.

Data availability

The data used to support the findings of this study are included within the article.

Received: 8 October 2022; Accepted: 12 May 2023

Published online: 18 May 2023

References

- Constantino, L. V. *et al.* Post-harvest characterization and sensory analysis of Roma tomato cultivars under organic cultivation: A strategy using consumers and chefs. *Int. J. Gastron. Food Sci.* **29**, 100564 (2022).
- You, P. S. & Hsieh, Y. C. A computational approach for crop production of organic vegetables. *Comput. Electron. Agric.* **134**, 33–42 (2017).
- Willer, H., Trávníček, J., Meier, C. & Schlatter, B. The world of organic agriculture statistics and emerging trends 2022. *FiBL* 31–37 (2022). <http://www.organic-world.net/yearbook/yearbook-2022.html>.
- Wang, Z. *et al.* Yield, nitrogen use efficiency and economic benefits of biochar additions to Chinese Flowering Cabbage in Northwest China. *Nutr. Cycl. Agroecosyst.* **113**, 337–348 (2019).
- Sha, X. L. *et al.* Effects of different organic fertilizer on pepper growth in new irrigation areas by pumping Yellow River. *Ningxia J. Agric. For. Sci. Tech.* **58**, 11–13 (2017).
- Wang, Z. *et al.* Effects of organic fertilizer combined with biochar on soil moisture and water use efficiency in vegetable field. *Chin. J. Agrometeorol.* **12**, 771–779 (2017).

7. Wang, Z., Li, Y. K., Wang, L. C., Guo, W. Z. & Xu, Z. G. Effects of biochar on yield, quality and water utilization of organic Flowering Chinese Cabbage. *Trans. Chin. Soc. Agric. Mach.* **49**, 273–280 (2018).
8. Inácio, C. D. T. *et al.* Organic, conventional and hydroponic vegetables: Can ¹⁵N natural abundance of farm N inputs differentiate mode of production. *Sci. Hortic.* **265**, 109219 (2020).
9. Li, M. Y., He, H., Mi, T. Z. & Zhen, Y. Spatiotemporal dynamics of ammonia-oxidizing archaea and bacteria contributing to nitrification in sediments from Bohai Sea and South Yellow Sea, China. *Sci. Total Environ.* **825**, 153972 (2022).
10. Beekman, F., Motte, H. & Beekman, T. Nitrification in agricultural soils: Impact, actors and mitigation. *Curr. Opin. Biotechnol.* **50**, 166–173 (2018).
11. Sun, Y. *et al.* Correlation between ammonia-oxidizing microorganisms and environmental factors during cattle manure composting. *Rev. Argent. Microbiol.* **51**, 371–380 (2019).
12. Zhong, W. H. *et al.* Nitrogen fertilization induced changes in ammonia oxidation are attributable mostly to bacteria rather than archaea in greenhouse based high N input vegetable soil. *Soil Biol. Biochem.* **93**, 150–159 (2016).
13. Stieglmeier, M. *et al.* *Nitrososphaera viennensis* gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum *Thaumarchaeota*. *Int. J. Syst. Evol. Microbiol.* **64**, 2738–2752 (2014).
14. Wang, X. Y., Wang, C., Bao, L. L. & Xie, S. G. Abundance agriculture and community structure of ammonia-oxidizing microorganisms in reservoir sediment and adjacent soils. *Appl. Microbiol. Biotechnol.* **98**, 1883–1892 (2014).
15. Liu, J. J. *et al.* Ammonia-oxidizing archaea show more distinct biogeographic distribution patterns than ammonia-oxidizing bacteria across the black soil zone of northeast China. *Front. Microbiol.* **9**, 1–13 (2018).
16. Ouyang, Y., Reeve, J. R. & Norton, J. M. Soil enzyme activities and abundance of microbial functional genes involved in nitrogen transformations in an organic farming system. *Biol. Fertil. Soils* **54**, 437–450 (2018).
17. Dong, X. C. *et al.* Chronic nitrogen fertilization modulates competitive interactions among microbial ammonia oxidizers in a loess soil. *Pedosphere* **29**, 24–33 (2019).
18. Leininger, S. *et al.* Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442**, 806–809 (2006).
19. Zhao, D. *et al.* Vertical distribution of ammonia-oxidizing archaea and bacteria in sediments of a eutrophic lake. *Curr. Microbiol.* **67**, 327–332 (2013).
20. Li, H., Weng, B. S., Huang, F. Y., Su, J. Q. & Yang, X. R. pH regulates ammonia-oxidizing bacteria and archaea in paddy soils in Southern China. *Appl. Microb. Biotechnol.* **99**, 6113–6123 (2015).
21. Zheng, Y. *et al.* Community dynamics and activity of ammonia-oxidizing prokaryotes in intertidal sediments of the Yangtze Estuary. *Appl. Environ. Microbiol.* **80**, 408–419 (2014).
22. Zhang, G. *et al.* Ammonia-oxidizing bacteria and archaea: response to simulated climate warming and nitrogen supplementation. *Soil Sci. Soc. Am. J.* **83**, 1683–1695 (2019).
23. Liu, H. *et al.* Ammonia oxidizers and nitrite-oxidizing bacteria respond differently to long-term manure application in four paddy soils of south of China. *Sci. Total Environ.* **633**, 641–648 (2018).
24. Qi, X. *et al.* Changes in alpine grassland type drive niche differentiation of nitrifying communities on the Qinghai-Tibetan Plateau. *Eur. J. Soil Biol.* **104**, 103316 (2021).
25. Sun, W. *et al.* Distribution and abundance of archaeal and bacterial ammonia oxidizers in the sediments of the Dongjiang River, a drinking water supply for Hong Kong. *Microbes Environ.* **28**, 457 (2013).
26. Liu, S. *et al.* Spatial distribution and factors shaping the niche segregation of ammonia-oxidizing microorganisms in the Qiantang River, China. *Appl. Environ. Microb.* **79**, 4065–4071 (2013).
27. Verhamme, D. T., Prosser, J. I. & Nicol, G. W. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *ISME J.* **5**, 1067–1071 (2011).
28. Ouyang, Y., Norton, J. M., Stark, J. M., Reeve, J. R. & Habteselassie, M. Y. Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. *Soil Biol. Biochem.* **96**, 4–15 (2016).
29. Ouyang, Y., Norton, J. M. & Stark, J. M. Ammonium availability and temperature control contributions of ammonia oxidizing bacteria and archaea to nitrification in an agricultural soil. *Soil Biol. Biochem.* **113**, 161–172 (2017).
30. Němeček, J. & Kozák, J. Approaches to the solution of a soil map of the Czech Republic at the scale 1:250 000 using SOTER methodology. *Plant Soil Environ.* **49**, 291–297 (2003).
31. Liu, R. L. *et al.* The study on rice organic fertilizer suitable application amount in Yellow River irrigation area of Ningxia. *Ningxia J. Agric. For. Sci. Technol.* **7**, 1–2 (2016).
32. Sauder, L. A. *et al.* Cultivation and characterization of *Candidatus Nitrosocosmicus exaquare*, an ammonia-oxidizing archaeon from a municipal wastewater treatment system. *ISME J.* **11**, 1142 (2017).
33. Taylor, A. E. *et al.* Use of aliphatic n-alkynes to discriminate soil nitrification activities of ammonia-oxidizing Thaumarchaea and bacteria. *Appl. Environ. Microbiol.* **79**, 6544–6551 (2013).
34. Bernhard, A. E. *et al.* Abundance of ammonia-oxidizing archaea and bacteria along an estuarine salinity gradient in relation to potential nitrification rates. *Appl. Environ. Microbiol.* **76**, 1285–1289 (2010).
35. Duan, P. P., Wu, Z., Zhang, Q. Q., Fan, C. H. & Xiong, Z. Q. Thermodynamic responses of ammonia-oxidizing archaea and bacteria explain N₂O production from greenhouse vegetable soils. *Soil Biol. Biochem.* **120**, 37–47 (2018).
36. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335–336 (2010).
37. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194–2200 (2011).
38. Edgar, R. C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **10**, 996–998 (2013).
39. Li, M. C. *et al.* Distinct distribution patterns of ammonia-oxidizing archaea and bacteria in sediment and water column of the yellow river estuary. *Sci. Rep.* **8**, 1–10 (2018).
40. Ter Braak, C. & Šmilauer, P. CANOCO Reference Manual and CanoDraw for Window User's Guide: Software for Canonical Community Ordination (Version 4.5) (Microcomputer Power, Ithaca, 2002).
41. Zhang, H., Cheng, F., Sun, S. Y. & Li, Z. K. Diversity distribution and characteristics of comammox in different ecosystems. *Environ. Res.* **214**, 113900 (2022).
42. Pearson, A. *et al.* Factors controlling the distribution of archaeal tetraethers in terrestrial hot springs. *Appl. Environ. Microbiol.* **74**, 3523–3532 (2008).
43. Wen, Y. C. *et al.* Crop yield and soil fertility response to commercial organic fertilizer substituting chemical fertilizer. *Sci. Agric. Sin.* **51**, 2136–2142 (2018).
44. Wang, B. R., Cai, Z. J. & Li, D. C. Effect of different long-term fertilization on the fertility of red upland soil. *J. Soil Water Conserv.* **24**, 85–88 (2010).
45. Zhang, X., Guo, J., Vogt, R. D., Mulder, J. & Zhang, X. Soil acidification as an additional driver to organic carbon accumulation in major Chinese croplands. *Geoderma* **366**, 114234 (2020).
46. Tournai, M. *et al.* *Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil. *Proc. Natl. Acad. Sci. U. S. A.* **20**, 8420–8425 (2011).
47. Hu, H. W. *et al.* The large-scale distribution of ammonia oxidizers in paddy soils is driven by pH, geographic distance, and climatic factors. *Front. Microbiol.* **6**, 1–14 (2015).
48. Walker, C. B. *et al.* *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine Crenarchaea. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 8818–8823 (2010).

49. Zhalnina, K., de Quadros, P. D., Camargo, F. A. & Triplett, E. W. Drivers of archaeal ammonia-oxidizing communities in soil. *Front. Microbiol.* **3**, 1–9 (2012).
50. Lehtovirta-Morley, L. E. *et al.* Characterisation of terrestrial acidophilic archaeal ammonia oxidisers and their inhibition and stimulation by organic compounds. *FEMS Microbiol. Ecol.* **89**, 542–552 (2014).
51. Bai, X. *et al.* Ammonia oxidizing bacteria dominate soil nitrification under different fertilization regimes in black soils of northeast China. *Eur. J. Soil Biol.* **111**, 103410 (2022).
52. Zhang, L. M., Hu, H. W., Shen, J. P. & He, J. Z. Ammoniaoxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. *ISME J.* **6**, 1032–1045 (2012).
53. Zhou, L. L., Wang, S. Y., Zou, Y. X., Xia, C. & Zhu, G. B. Species, abundance and function of Ammonia-oxidizing Archaea in inland waters across China. *Sci. Rep.* **5**, 1–13 (2015).
54. Jiang, H. *et al.* Latitudinal distribution of ammonia-oxidizing bacteria and archaea in the agricultural soils of eastern China. *Appl. Environ. Microbiol.* **80**, 5593–5602 (2014).
55. Pester, M. *et al.* amoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions. *Environ. Microbiol.* **14**, 525–539 (2012).
56. Zou, W. X., Lang, M., Zhang, L., Liu, B. & Chen, X. P. Ammonia-oxidizing bacteria rather than ammonia-oxidizing archaea dominate nitrification in a nitrogen-fertilized calcareous soil. *Sci. Total Environ.* **811**, 151402 (2022).
57. Chen, Y. *et al.* Diversity, abundance, and spatial distribution of ammonia-oxidizing β -proteobacteria in sediments from Changjiang estuary and its adjacent area in East China Sea. *Microb. Ecol.* **67**, 788–803 (2014).
58. Hu, A. *et al.* Community structures of ammonia-oxidizing archaea and bacteria in high altitude lakes on the Tibetan Plateau. *Freshw. Biol.* **55**, 2375–2390 (2010).
59. Hou, Q. *et al.* Responses of nitrification and bacterial community in three size aggregates of paddy soil to both of initial fertility and biochar addition. *Appl. Soil Ecol.* **166**, 104004 (2021).

Acknowledgements

This work was supported by S&T Program of Hebei (21327005D and 21326904D), the National Natural Science Foundation of China (41501312), Funds for Science and Technology Innovation Pilot of Ningxia Academy of Agricultural and Forestry Sciences (NNKZZCGZH-2021-06) and Natural Science Foundation of Ningxia (2022AAC05048).

Author contributions

Z.W. analyzed the data and wrote the paper, Y.L. designed the experiment and review the manuscript, Z.W. and L.M. performed the field experiment, M.D. and Y.J. prepared the tables and figures, and W.Z. contributed ideas.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-023-35134-3>.

Correspondence and requests for materials should be addressed to Y.L.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2023