Resuscitation of inactive ammonia-oxidizing archaea and complete nitrifiers by extracellular electrons from a heterotrophic bacterium, *Bacillus amyloliquefaciens*

Shanghua Wu
Research Center for Eco-Environmental Sciences Chinese Academy of Sciences
Yuzhu Dong
Research Centre for Eco-Environmental Sciences Chinese Academy of Sciences
Haonan Fan
Research Centre for Eco-Environmental Sciences Chinese Academy of Sciences
Ye Deng
Research Centre for Eco-Environmental Sciences Chinese Academy of Sciences
Xuliang Zhuang (xlzhuang@rcees.ac.cn)
Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences
https://orcid.org/0000-0001-5902-4267

Research

Keywords: ammonia-oxidizing bacteria and archaea, complete nitrifiers, Bacillus amyloliquefaciens, extracellular electron transfer, nitrous oxide, nitric oxide

Posted Date: September 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-76082/v1

Abstract

Background: Nitrification in the soil is dominated by ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB) and the newly discovered complete nitrifiers, which are members of complex communities. Complicated relations have been found in microbial communities, but the relations between heterotrophic bacteria and autotrophic ammonia oxidizers (AOM) are unclear, and experimental models are lacking. Here, we constructed a microcosm based on DNA stable isotope probing that was established between a heterotrophic bacterium, *Bacillus amyloliquefaciens* (BA), and the soil autotrophic AOM.

Results: The inoculation of BA changed the activities of three indigenous nitrifiers. The originally marginal AOA and complete nitrifiers were active in the nitrification process as well as the AOB, which led to mitigation of N_2O and an increase in NO. The network analysis showed that the inoculated BA was indirectly and positively linked to AOA through a *Microbacteriaceae* that contains genes encoding proteins responsible for transporting electrons. Further, the microbial fuel cell system indirectly confirmed the potential regulation of extracellular electron transfer (EET) between these two species.

Conclusions: With our findings, a useful model to investigate the relations between heterotrophic bacteria and AOM was constructed, and evidence of EET in complex regulations was provided.

Background

Nitrous oxide (N_2O) is the third most abundant and long-lived greenhouse gas in the atmosphere. Anthropogenic emissions are the most important sources of global N_2O production, comprising nearly 30–45% of total emissions, with approximately two-thirds of this derived from agriculture and soil[1].

Microbial transformations of nitrogenous compounds, especially heterotrophic denitrification and chemolithoautotrophic aerobic nitrification, are the two dominant contributors to N_2O emissions[2]. Classically, heterotrophic denitrification has been considered to be the major biological source and sink of N_2O in the terrestrial nitrogen cycle, but N_2O emissions from nitrification are thought to be of equal importance to those from denitrification in recent decades. N_2O results directly from the ammonia-oxidizing activity of traditional nitrifiers and indirectly from the activities of ammonia-oxidizing bacteria, ammonia-oxidizing archaea, and the newly discovered complete nitrifier (complete ammonia oxidation; comammox) *Nitrospira inopinat*a[1–9]. Nitrifier denitrification may account for up to 100% of N_2O emissions from ammonia in soils and is more significant than classical denitrification at low oxygen concentrations, especially fluctuating aerobic-anaerobic conditions[10, 11]. Given predicted increases in farmland and fertilizer application (EPA reference), agricultural soils are likely to contribute up to 59% of total N_2O emissions by 2030[12], increasing the need to explore approaches to manage and reduce N_2O emissions from agricultural systems[13, 14].

In our previous work, a strain of *Bacillus amyloliquefaciens* (BA) that significantly reduced N_2O emissions by 35%-50% after inoculation was isolated from rice rhizosphere, and the results suggested that the mitigation was mostly due to the inhibition of nitrification[12]. However, the detailed process of inhibition and relations of organisms behind the regulation of BA have not been studied, especially those between heterotrophic BA and three groups of autotrophic AOM.

AOA *amoA* genes are abundant and widespread in soils, frequently outnumbering AOB *amoA* genes[15], and comammox bacteria are also abundant in some soils[16, 17]. The relative abundance and dominance of these three types of ammonia-oxidizing microorganisms (AOM) are affected by abiotic factors, including pH, temperature and moisture content[18], and by several nitrification inhibitors. AOM may also be influenced by interactions with other microorganisms, e.g., through cross-feeding and quorum sensing[19, 20], and by direct or indirect electron transfer between species. The latter is rarely studied and can be investigated using microbial fuel cells (MFCs). *Geobacter sulfurreducens* and *Shewanella oneidensis* are the two most commonly used model strains in MFCs because of their good efficiency in transferring electrons; in MFCs, protons are the only thing allowed to pass through the membrane between the anode and cathode chambers and complete the circuit[21].

In this study, we constructed microcosms to investigate the influence of the heterotrophic bacterium BA on nitrification and three different types of nitrifiers. We also constructed MFCs to study the possible extracellular electron transfer between BA and AOA. Together, these results suggest a complex regulation strategy in which heterotrophic bacteria affect autotrophic organisms, and extracellular electron transfer may be a possible link between the two types of microbes.

Methods

Soil microcosms and stable isotope probing (SIP)

The influence of BA on nitrification was investigated in soil microcosms consisting of 10 g of sieved acidic soil (mesh size of 3.35 mm) from Southwest China used in our previous work[12]. Microcosms were adjusted to 60% maximum water-holding capacity in 120-ml serum bottles, sealed with rubber stoppers and aluminium caps and incubated at 30 °C in the dark for 4 weeks. The headspace was sampled and adjusted to 5% (vol/vol) CO_2 by injection through the rubber septum, and each bottle was flushed for 5 min weekly with synthetic air (20% O_2, 80% N_2) between sampling and injection. For every week, 100 μg urea-N g^{-1} dry weight soil was added by liquid to each bottle.

Three treatments were established, each in triplicate microcosms. In Treatment 12C-B, the headspace contained ^{12}C-CO_2, and active BA was inoculated. The inoculum was grown in liquid batch culture to stationary phase in M9 Minimal Medium (^{13}C-glucose was used in treatments of ^{13}C-CO_2 to avoid additional ^{12}C into the microcosms) at 30 °C and 180 rpm for 28 h. Cells were separated by centrifugation at 8000 rpm and washed three times with sterilized water to remove the remaining culture medium. The final cell suspension was adjusted to 3×10^8 CFU ml^{-1}, and 1 ml of this suspension was

added weekly by injection through the rubber septum into each microcosm for 3 consecutive weeks. Treatment 13C-B was identical to Treatment 12C-B, except that the headspace contained ^{13}C-CO_2. Treatment 13C-BS was identical to Treatment 13C-B but was inoculated with *B. amyloliquefaciens* sterilized by autoclaving as a control.

Soil chemical analysis

Soil pH was determined in water at a ratio of 1:2.5 (w/v) using a pH meter (Accumet Excel XL 60, Fisher Scientific, Singapore). The NO_3^- and NH_4^+ concentrations were determined after extraction in 2 M KCl (soil:solution ratio of 1:10 (w/v)) and measured using a continuous flow analyser (SAN++, Skalar, Breda, Holland).

Instantaneous CO_2, NO and N_2O measurements

N_2O and CO_2 concentrations in the headspace were measured using an Agilent gas chromatograph (Agilent Technologies Inc., Wilmington, DE) equipped with a micro-TCD and a flame ionization detector, respectively. NO concentration was measured using a NO-NO_2-NO_x chemiluminescent analyser (42i Thermo Environmental Instruments Inc., USA)[22].

DNA extraction and SIP gradient fractionation

DNA was extracted from 0.5 g of dry soil using the FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The extracted DNA was dissolved in 80 µL of sterile, deionized, nuclease-free water and divided into two parts: one part was stored at 4 °C prior to quantitative PCR and the other was stored at -20 °C for further use.

The quantity and purity of soil DNA were determined by a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). SIP fractionation was performed as described previously[15, 23]. For each sample, 3.5 µg of soil DNA was mixed with CsCl (buoyant density of 1.725 g ml^{-1}) before ultracentrifugation at 44,100 r.p.m. (~ 177,000 *gav*) in a Vti 65.2 rotor (Beckman Coulter, cat. no. 362754) at 20 °C for 44 h with vacuum and maximum acceleration and without braking (requiring an additional 2 h to stop). DNA fractionation was carried out by displacing the gradient medium with sterile water from the top of the ultracentrifuge tube using an NE-1000 single syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA) with a precisely controlled flow rate of 0.375 ml min^{-1}. Up to 15 DNA gradient fractions were generated with equal volumes of approximately 380 ml, and a 65-ml aliquot of each fraction was used for refractive index measurement using an AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA). The fractionated DNA was purified and dissolved in 30 µL of sterile, deionized, nuclease-free water[24].

Quantitative PCR

Functional marker genes (bacterial and archaeal *amoA*) and *amoA* of *Nitrospira* clade A were quantified using Premix Ex Taq (TaKaRa, Japan) and gene-specific primers. The primers used for *Nitrospira* clade A

were coma-244f/coma-659r. Details of plasmid standards, gene-specific qPCR primers, reaction mixtures and thermal programmes are described in previous studies[17, 25]. Each sample was quantified in duplicate using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad laboratories). The algorithm that was used to calculate the efficiency (E) and threshold cycle (CT) was based on the kinetics of each individual reaction.

BA abundance was determined using Q-PCR based on the method reported by Yong *et al.* [26]. Primers targeting the *pgsB* gene were used for Q-PCR: pgsB726-f (5’-TGGCGCCATGAGA-ATCCT-3’), pgsB791-r (5’-GCAAAGCCGTTTACGAAATGA-3’) and pgsB-probe (5’-FAM-CCGCTGCTCAGCACGAAGGAGC-TAMRA-3’). Thermal cycling consisted of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 34 s.

High-throughput sequencing

The heavy fractions (considered as the active species including ammonia oxidizers and their mostly closely linked species incorporating ^{13}C into their genomes during the DNA-SIP incubation) of Treatment 13C-B were sent for 16S rRNA sequencing of both bacteria and archaea. To construct a network to investigate the niches of AOA and AOB, we used universal primers for bacteria and archaea. The V3-V4 region of the bacterial 16S rRNA gene was amplified using the primers 341F (5'-CCTAYGGGRBGCASCAG – 3') and 806R (5'-GGACTACNNGGGTATCTAAT – 3')[27]. The V4 region of the archaeal 16S rRNA gene was amplified using the primers 524F10extF (5'-TGYCAGCCGCCGCGGTAA-3') and Arch958RmodR (5'-YCCGGCGTTGAVTCCAATT-3')[28]. Fragments from each soil sample were sequenced using MiSEq. Following gene amplification, 3 µl of the PCR product was used for agarose gel electrophoresis (1%) to confirm the results of amplification. PCRs of 4 replicates for each sample preparation were combined and quantified with PicoGreen. From each sample, 200 ng of the PCR product was collected and pooled with other samples for one sequencing run. The pooled mixture was purified with a QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD, USA) and re-quantified with PicoGreen. The purified mixture was then diluted and loaded as described in the MiSeq Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA).

Construction of a microbial fuel cell (MFC) system

A MFC system was constructed to assess possible extracellular electron transfer between heterotrophic BA and autotrophic AOM. Two-bottle MFC reactors (total volume of each bottle was 100 mL) were constructed as described by McAnulty in 2017[21]. The anode side was the soil used in this experiment, and the cathode side was subjected to 3 different treatments: BA, sterile BA as the negative control and *Shewanella oneidensis* MR-1 as the positive control. The MFC systems were incubated at 30 °C in the dark for 14 days.

Data analysis

After assigning each sequence to the appropriate sample according to its barcode and allowing up to two mismatches, a total of 30,000 reads from both ends were obtained as a partitioned run for each sample. All the OTUs belonging to ammonia-oxidizers were picked up from the bacterial and archaeal OTU table after classification. Network analysis was performed and visualized using Cytoscape (Version 3.72). All statistical analyses were carried out in the R platform (3.6.2, http://www.r-project.org). The Shapiro–Wilk test and Bartlett test were used to check whether the data conformed to normality and homoscedasticity, respectively. The post hoc Tukey HSD test was used to check if the relationship between two sets of data was statistically significant.

Results

Inhibition of nitrification by BA

The influence of BA on nitrification was assessed by measuring differences in NH_4^+, NO_3^-, CO_2, NO and N_2O concentrations during incubation of microcosms (Fig. 1). The increases in NO_3^-, NO and N_2O concentrations in Treatments 12C-B and 13C-B were significantly less than those in Treatment 13C-BS. The nitrate concentration in Treatments 13C-B and 12C-B was significantly ($P < 0.05$) lower than that in 13C-BS after incubation for 28 days. The nitric oxide concentration in 13C-B and 12C-B was significantly ($P < 0.05$) higher than that in 13C-BS. The N_2O concentration in 13C-B and 12C-B was significantly ($P < 0.05$) higher than that in 13C-BS. In contrast, the CO_2 concentration decreased after inoculation of microcosms with active BA. The CO_2 concentration in 13C-B and 12C-B was significantly ($P < 0.05$) higher than that in 13C-BS. These factors all lead to the potential inhibition of the nitrification process by BA. However, NH_4^+ in Treatments 13C-B and 12C-B was also less than that in Treatment 13C-BS. The ammonium concentration in Treatments 13C-B and 12C-B was significantly ($P < 0.01$) lower than that in Treatment 13C-BS.

DNA-SIP

To identify which ammonia oxidizers incorporated $^{13}CO_2$ during incubation, both archaeal and bacterial *amoA* genes were quantified in DNA from different fractions (Fig. 2a and b). The quantitative abundance of AOB was greatest around a buoyant density of ~ 1.71–1.72 g ml $^{-1}$ in Treatment 12C-B and ~ 1.73–1.75 g ml $^{-1}$ in Treatments 13C-B and 13C-BS. In contrast, the archaeal abundance was greatest at ~ 1.71–1.72 g ml $^{-1}$ in 12C-B and 13C-BS and ~ 1.73–1.75 g ml $^{-1}$ in 13C-B. This indicates that bacterial *amoA* was labelled in Treatment 13C-BS, while in Treatment 13C-B and 12C-B, both ammonia-oxidizing archaea and bacteria were labelled after incubation for 28 days.

Sequencing of 16S rRNA genes in the heavy fractions indicated that neither the AOA nor AOB were the most abundant species in the ^{13}C-labelled 16S rRNA-based communities. Within Thaumarchaeota, 93.6% of the ^{13}C-labelled AOA community fell within the Soil Crenarchaeotic Group and 1.5% within South African Gold Mine Gp 1. Within the bacteria, 80% of the AOB and NOB sequences fell within the *Nitrospira*

group, while few traditional AOB, e.g., *Nitrosococcus*, or NOB, e.g., *Nitrobacter*, were observed in labelled DNA (Fig. 2c and d).

Activity of comammox after stimulation by BA

Given the relatively high proportion of *Nitrospira* 16S rRNA sequences in ^{13}C-labelled DNA, specific *amo*A primers for comammox were quantified in DNA from heavy fractions. This analysis indicated that comammox, especially *Nitrospira* clade A, was active in our BA-induced systems (Fig. 3). The relative abundance of *Nitrospira* clade A was greatest around a buoyant density of ~ 1.71–1.72 g ml $^{-1}$ in Treatments 12C-B and 13C-BS and at ~ 1.73–1.75 g ml $^{-1}$ in Treatment 13C-B. Moreover, we amplified clade B at first but did not obtain any positive results.

Network of the microcosms

In order to elucidate the niche of BA after inoculation into the microcosms, we constructed a network of microorganisms, including archaea and bacteria (Fig. 4). The network showed that both nitrifiers and BA were not heavily connected to other organisms because of their small size in the whole community. However, BA and Thaumarchaeota were linked indirectly by *Microbacteriaceae*, and the edge indicated positive relations between both BA-*Microbacteriaceae* and *Microbacteriaceae*-Thaumarchaeota.

AOA in the MFC system

To further investigate whether AOA provides positive feedback to electron transfer from the outside, we constructed a MFC system, and the abundance of AOA was quantified by Q-PCR. The abundance of AOA in the presence of BA was significantly higher than that in Treatment SBA (Fig. 5), but it was not significantly higher between *Shewanella* and SBA. The abundance of *amoA* in Treatment C was 2.62×10^5 g^{-1} dry weight soil, which was much lower than that in the other three treatments with inoculation of both active and sterile microbes. The abundance of AOA in Treatment BA was 6.65×10^5 g^{-1} dry weight soil, which was significantly higher than that in the presence of *Shewanella* and Sterile BA (6.08×10^5 ($P < 0.05$) and 5.71×10^5 ($P < 0.05$), respectively).

Discussion

This study suggests that the three autotrophic AOM, AOA, AOB and comammox bacteria *Nitrospira* clade A, were active in ammonia oxidation. The system involved a heterotrophic bacterium (HB), BA, which suggested a special relation between the HB and autotrophic AOM in the soil to be confirmed in the future. This provides compelling evidence that the regulation of the extraneous HB on the nitrification process involved the enhancement of oxidation of NH_2OH to NO through AOA and *Nitrospira* clade A and the inhibition of the production of N_2O through AOB, leading to a significant increase in NO and mitigation of N_2O in the nitrification process. It also provides evidence that the potential regulating strategy is extracellular electron transfer between HB and AOM, especially AOA.

Previous studies have suggested a complex relation between heterotrophic bacteria and autotrophic AOM, including competition for NH_4^+ and cooperation where organic carbon is low[29–31]. There are

different ways microbes interact, e.g., cross-feeding, co-metabolism, cell-to-cell communication[20, 29, 32] and interspecies extracellular electron transfer (IEET)[33]. In our constructed microcosms, we found the co-activity of AOA, AOB and the newly discovered comammox in the ammonia-oxidizing process. After BA was inoculated into the microcosms, it successfully resuscitated the inactive ammonia-oxidizing archaea and complete nitrifier *Nitrospira* in the soil, which was previously dominated by ammonia-oxidizing bacteria[15, 34].

Nitrification is a two-step process:[14] ammonia (NH_3) is oxidized via hydroxylamine (NH_2OH) to nitrite (NO_2^-) and, subsequently, nitrite is oxidized to nitrate (NO_3^-). Within these two steps, two processes contribute to N_2O emissions. The first is aerobic N_2O formation from the abiotic reaction of the intermediates NH_2OH, NO and NO_2^-, termed chemodenitrification. NH_3 is first oxidized to NH_2OH by the enzyme ammonia monooxygenase (AMO), which belongs to the superfamily of copper-dependent membrane-bound monooxygenases[35]. Subsequently, NH_2OH is oxidized to NO by hydroxylamine dehydrogenase (HAO) under oxic and anoxic conditions[1, 4, 7]. NH_2OH released in this process may be oxidized to N_2O by oxidants, including Fe^{3+} and MnO_2, while NO_2^- and NO may be reduced by reductants such as Fe^{2+}, Cu^{2+} or humic substances[36]. The second is nitrifier-denitrification, an enzymatic process in which NO_2^- is reduced to N_2O via NO; reduction of NO to N_2O is catalysed by two classes of cytochrome *c* nitric oxide reductases (NORs). Chemodenitrification is associated with ammonia oxidation by ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) and comammox, while nitrifier denitrification has only been reported in AOB[1, 3, 4, 8, 37], and all genome-sequenced AOA and comammox lack NOR[1, 5, 38, 39]. The inoculation of BA and the change in AOM status *in situ* obviously changed the rate of nitrification. The consumption of CO_2 and production of N_2O and NO_3^- were lower in Treatment 13C-B compared to Treatment 13C-BS, indicating that BA inhibited the nitrification process[40–43]. However, NH_4^+ remained in the soil, and the higher emissions of NO in Treatment 13C-B after inoculation did not support the inhibition of nitrification. The concentrations of NH_4^+ in Treatment 13C-B were less than those in Treatment 13C-BS, and the concentration of NO was slightly higher in 13C-B, which led to an enhancement of the ammonia-oxidizing process. The analysis of ammonia-oxidizing microorganisms in this system helped us to resolve this problem. Our soil used in this work was agricultural soils collected from a typical acidic soil area in China, in which AOB play a dominant role, as previous works have suggested[34, 44]. The results of SIP in Treatment 13C-BS confirmed the dominance of AOB in our experimental soils. However, the results also suggested that both AOA and *Nitrospira* clade A become other active organisms that oxidize NH_3 to NH_2OH in Treatment 13C-B. This indicated that the initially inactive and feeble AOA in agricultural soils was resuscitated by the inoculation of BA and began to be active in function with AOB *in situ*.

The labelled AOA in Treatment 13C-B mainly belong to Soil Crenarchaeotic Group, which indicates their dominant function in the ammonia-oxidizing process. After that, the AOB in Treatment 13C-B were sequenced, and we found that only a few AOB were classified, including *Nitrosospira* and *Nitrosococcus*. We wondered if any other species participated in this process, especially the newly isolated comammox.

Interestingly, the results of Q-PCR of comammox showed that *Nitrospira* clade A was labelled in the heavy fractions. Taken together, after the inoculation of BA into the acidic soils, the whole ammonia-oxidizing process was redefined; instead of the sole activity of AOB as usual, both AOA and comammox were more active and made use of NH_3.

The results further revealed the abnormal increase in the production of NO in Treatment 13C-B. As we have mentioned before, NO is an important intermediate in the nitrification process both in biotic and abiotic pathways[1, 4, 8, 45]. However, in almost all the AOA and comammox genomes sequenced to date, no canonical nitric oxide reductases (NORs) have been detected, despite the wide presence of a nitrite reductase gene (*nirK*) in AOA. Although cytochrome P450 and other enzymes possibly involved in the production of N_2O and acting as NOR may be detected in the future, it can only take action in some AOA in the presence of excess nitrite[1, 8, 46]. After the inoculation of BA and the recovery of AOA and comammox in the soil, more NO was produced in the microcosms (Fig. 6a).

The results and relations between the inoculated BA and ammonia-oxidizing microorganisms indicate a complex competence and cooperation among these species. BA is a widely isolated heterotrophic bacterium that holds a good NH_4^+ affinity in oligotrophic environments, and the inoculation of BA consumes NH_4^+ in soil, which makes it more competitive for nitrification by AOM[47]. This in turn provides opportunities for AOA and comammox, whose affinity for ammonium is better than AOB[1, 38, 39, 47, 48], and makes the co-function of AOM in soils possible[49]. In addition, the inoculation of BA into the soils changed the original niche *in situ*, and new relations between BA and the ammonia-oxidizers were constructed. The network showed that BA and the AOA Thaumarchaeota are at the edge of the network, and they are indirectly and positively connected by a *Microbacteriaceae*.

Both BA and *Microbacteriaceae* contain genes encoding multiheme cytochrome *c*, which is good for the transport of electrons and facilitates ammonia oxidation, especially AOA[50–52]. To date, no *cytochrome c* has been found in AOA compared to its wide expression in AOB and comammox[53–55]. Previous studies have confirmed the influence of IEET on anaerobic methane oxidizing microbes[33], which is similar to ammonia-oxidizing microbes. Therefore, we constructed a MFC system to investigate the potential IEET between BA and AOA. Although the MFC systems were carried out for only 14 days, the abundance of AOA in Treatment BA was significantly higher than that in the control. This indicated that the electrons transferred from BA were as high as the typical *Shewanella* at the cathode side, and the electrons may play a positive role in the abundance of AOA in the soil. Of course, this is only preliminary evidence for the IEET between these two species, but the difference between the 3 treatments did provide evidence that AOA reply positively to the electron transferred from other organisms.

Altogether, this suggests that BA is regulated by all autotrophic AOM in acidic soils via the competence of NH_4^+ (competence resource) and the facilitation of electron transport (energy supplying), a hypothesis that warrants further experimental elucidation (Fig. 6b).

Conclusions

This is a novel discovery of the resuscitation of AOA and newly found complete nitrifiers under the originally dominant AOB in the ammonia-oxidizing process. The results were detected based on the complex interactions between microbes. In our work, after the inoculation of BA, the whole nitrification in soil was changed, the ammonia-oxidizing process was inhibited; in addition, the emission of N_2O was much lower, and the production of NO increased owing to the lack of NOR in AOA and comammox. We provide a possible explanation for the special relationship between BA and AOM; both the competence of ammonium with AOB and the facilitation of electron transport with AOA may have led to these results. However, far more research needs to be performed, not only to testify and confirm the relations and electron transfer between these microorganisms but also to determine if some other microbes are involved in this process. Additionally, the mechanisms by which BA regulates the ammonia-oxidizing process at metabolic levels should be elucidated.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Sequences data of 16S rRNA high-throughput sequencing are available in NCBI Sequence Read Archive repository database (www.ncbi.nlm.nih.gov/sra) under accession number SRR12587975 to SRR12588174.

Competing interests

The authors declare that they have no competing interests

Funding

This research was supported by the National Natural Science Foundation of China (Nos. 41907273, 31670507 and 91951108).

Authors' contributions

S.W. conducted the experiments with the help of Y.D. and F.H. S.W. and Y.D. analyzed the data with the help of Y.D.. S.W. wrote the manuscript. S.W. and X.Z. initiated the project and directed the research.

Acknowledgements

The authors would like to thank Professor James I. Prosser for his general help in revising the manuscript and provide strains for further study.

Author information

Affiliations

Key Laboratory of Environmental Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

Shanghua Wu, Yuzhu Dong, Haonan Fan, Ye Deng & Xuliang Zhuang

College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 100049, China

Shanghua Wu, Yuzhu Dong, Haonan Fan, Ye Deng & Xuliang Zhuang

References

1. Kits KD, Jung MY, Vierheilig J, Pjevac P, Sedlacek CJ, Liu S, et al. Low yield and abiotic origin of N_2O formed by the complete nitrifier *Nitrospira inopinata*. Nat Commun. 2019;10(1):1836; doi: 10.1038/s41467-019-09790-x.
2. Kuypers MMM, Marchant HK, Kartal B. The microbial nitrogen-cycling network. Nat Rev Microbiol. 2018;16(5):263-76; doi: 10.1038/nrmicro.2018.9.
3. Stieglmeier M, Mooshammer M, Kitzler B, Wanek W, Zechmeister-Boltenstern S, Richter A, et al. Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. ISME J. 2014;8(5):1135-46; doi: 10.1038/ismej.2013.220.
4. Caranto JD, Vilbert AC, Lancaster KM. *Nitrosomonas europaea* cytochrome P460 is a direct link between nitrification and nitrous oxide emission. Proc Natl Acad Sci U S A. 2016;113(51):14704-9; doi: 10.1073/pnas.1611051113.
5. Kozlowski JA, Stieglmeier M, Schleper C, Klotz MG, Stein LY. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. ISME J. 2016;10(8):1836-45; doi: 10.1038/ismej.2016.2.
6. White CJ, Lehnert N. Is there a pathway for N_2O production from hydroxylamine oxidoreductase in ammonia-oxidizing bacteria? Proc Natl Acad Sci U S A. 2016;113(51):14474-6; doi: 10.1073/pnas.1617953114.
7. Caranto JD, Lancaster KM. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. Proc Natl Acad Sci U S A. 2017;114(31):8217-22; doi: 10.1073/pnas.1704504114.

8. Hink L, Nicol GW, Prosser JI. Archaea produce lower yields of N2 O than bacteria during aerobic ammonia oxidation in soil. Environ Microbiol. 2017;19(12):4829-37; doi: 10.1111/1462-2920.13282.
9. Hink L, Gubry-Rangin C, Nicol GW, Prosser JI. The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. ISME J. 2018;12(4):1084-93; doi: 10.1038/s41396-017-0025-5.
10. Zhu X, Burger M, Doane TA, Horwath WR. Ammonia oxidation pathways and nitrifier denitrification are significant sources of N_2O and NO under low oxygen availability. Proc Natl Acad Sci U S A. 2013;110(16):6328-33; doi: 10.1073/pnas.1219993110.
11. Wrage-Monnig N, Horn MA, Well R, Muller C, Velthof G, Oenema O. The role of nitrifier denitrification in the production of nitrous oxide revisited. Soil Biology & Biochemistry. 2018;123:A3-A16; doi: 10.1016/j.soilbio.2018.03.020.
12. Wu S, Zhuang G, Bai Z, Cen Y, Xu S, Sun H, et al. Mitigation of nitrous oxide emissions from acidic soils by *Bacillus amyloliquefaciens*, a plant growth-promoting bacterium. Glob Chang Biol. 2018;24(6):2352-65; doi: 10.1111/gcb.14025.
13. Harter J, Krause HM, Schuettler S, Ruser R, Fromme M, Scholten T, et al. Linking N_2O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. ISME J. 2014;8(3):660-74; doi: 10.1038/ismej.2013.160.
14. Prosser JI, Hink L, Gubry-Rangin C, Nicol GW. Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. Glob Chang Biol. 2020;26(1):103-18; doi: 10.1111/gcb.14877.
15. Xia W, Zhang C, Zeng X, Feng Y, Weng J, Lin X, et al. Autotrophic growth of nitrifying community in an agricultural soil. ISME J. 2011;5(7):1226-36; doi: 10.1038/ismej.2011.5.
16. Xu S, Wang B, Li Y, Jiang D, Zhou Y, Ding A, et al. Ubiquity, diversity, and activity of comammox *Nitrospira* in agricultural soils. Sci Total Environ. 2020;706:135684; doi: 10.1016/j.scitotenv.2019.135684.
17. Li CY, Hu HW, Chen QL, Chen DL, He JZ. Comammox Nitrospira play an active role in nitrification of agricultural soils amended with nitrogen fertilizers. Soil Biology & Biochemistry. 2019;138:107609; doi: ARTN 10760910.1016/j.soilbio.2019.107609.
18. Zhao J, Bello MO, Meng YY, Prosser JI, Gubry-Rangin C. Selective inhibition of ammonia oxidising archaea by simvastatin stimulates growth of ammonia oxidising bacteria. Soil Biology & Biochemistry. 2020;141:107673; doi: ARTN 10767310.1016/j.soilbio.2019.107673.
19. Kassotaki E, Buttiglieri G, Ferrando-Climent L, Rodriguez-Roda I, Pijuan M. Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products. Water Res. 2016;94:111-9; doi: 10.1016/j.watres.2016.02.022.
20. Gao J, Ma A, Zhuang X, Zhuang G. An N-acyl homoserine lactone synthase in the ammonia-oxidizing bacterium *Nitrosospira multiformis*. Appl Environ Microbiol. 2014;80(3):951-8; doi: 10.1128/AEM.03361-13.

21. McAnulty MJ, Poosarla VG, Kim KY, Jasso-Chavez R, Logan BE, Wood TK. Electricity from methane by reversing methanogenesis. Nat Commun. 2017;8:15419; doi: 10.1038/ncomms15419.
22. Wang R, Willibald G, Feng Q, Zheng X, Liao T, Bruggemann N, et al. Measurement of N_2, N_2O, NO, and CO_2 emissions from soil with the gas-flow-soil-core technique. Environ Sci Technol. 2011;45(14):6066-72; doi: 10.1021/es1036578.
23. Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, et al. DNA stable-isotope probing. Nat Protoc. 2007;2(4):860-6; doi: 10.1038/nprot.2007.109.
24. Wang B, Qin W, Ren Y, Zhou X, Jung MY, Han P, et al. Expansion of Thaumarchaeota habitat range is correlated with horizontal transfer of ATPase operons. ISME J. 2019;13(12):3067-79; doi: 10.1038/s41396-019-0493-x.
25. Xu HJ, Wang XH, Li H, Yao HY, Su JQ, Zhu YG. Biochar impacts soil microbial community composition and nitrogen cycling in an acidic soil planted with rape. Environ Sci Technol. 2014;48(16):9391-9; doi: 10.1021/es5021058.
26. Yong X, Zhang R, Zhang N, Chen Y, Huang X, Zhao J, et al. Development of a specific real-time PCR assay targeting the poly-gamma-glutamic acid synthesis gene, *pgsB*, for the quantification of *Bacillus amyloliquefaciens* in solid-state fermentation. Bioresour Technol. 2013;129:477-84; doi: 10.1016/j.biortech.2012.11.092.
27. Behrendt L, Larkum AW, Trampe E, Norman A, Sorensen SJ, Kuhl M. Microbial diversity of biofilm communities in microniches associated with the didemnid ascidian *Lissoclinum patella*. ISME J. 2012;6(6):1222-37; doi: 10.1038/ismej.2011.181.
28. Ohene-Adjei S, Teather RM, Ivan M, Forster RJ. Postinoculation protozoan establishment and association patterns of methanogenic archaea in the ovine rumen. Appl Environ Microbiol. 2007;73(14):4609-18; doi: 10.1128/AEM.02687-06.
29. Sedlacek CJ, Nielsen S, Greis KD, Haffey WD, Revsbech NP, Ticak T, et al. Effects of Bacterial Community Members on the Proteome of the Ammonia-Oxidizing Bacterium Nitrosomonas sp. Strain Is79. Appl Environ Microbiol. 2016;82(15):4776-88; doi: 10.1128/AEM.01171-16.
30. Sun H, Narihiro T, Ma X, Zhang XX, Ren H, Ye L. Diverse aromatic-degrading bacteria present in a highly enriched autotrophic nitrifying sludge. Sci Total Environ. 2019;666:245-51; doi: 10.1016/j.scitotenv.2019.02.172.
31. Kindaichi T, Ito T, Okabe S. Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence *in situ* hybridization. Appl Environ Microbiol. 2004;70(3):1641-50; doi: 10.1128/aem.70.3.1641-1650.2004.
32. Mellbye BL, Spieck E, Bottomley PJ, Sayavedra-Soto LA. Acyl-homoserine lactone production in nitrifying bacteria of the genera *Nitrosospira*, *Nitrobacter*, and *Nitrospira* identified via a survey of putative quorum-sensing genes. Appl Environ Microbiol. 2017. doi: 10.1128/AEM.01540-17.
33. Moscoviz R, Quemener ED, Trably E, Bernet N, Hamelin J. Novel Outlook in Microbial Ecology: Nonmutualistic Interspecies Electron Transfer. Trends Microbiol. 2020;28(4):245-53; doi:

10.1016/j.tim.2020.01.008.

34. Trivedi C, Reich PB, Maestre FT, Hu HW, Singh BK, Delgado-Baquerizo M. Plant-driven niche differentiation of ammonia-oxidizing bacteria and archaea in global drylands. ISME J. 2019;13(11):2727-36; doi: 10.1038/s41396-019-0465-1.
35. Alves RJE, Minh BQ, Urich T, von Haeseler A, Schleper C. Unifying the global phylogeny and environmental distribution of ammonia-oxidising archaea based on *amoA* genes. Nat Commun. 2018;9(1):1517; doi: 10.1038/s41467-018-03861-1.
36. Heil J, Vereecken H, Bruggemann N. A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. European Journal of Soil Science. 2016;67(1):23-39; doi: 10.1111/ejss.12306.
37. Vajrala N, Martens-Habbena W, Sayavedra-Soto LA, Schauer A, Bottomley PJ, Stahl DA, et al. Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. Proc Natl Acad Sci U S A. 2013;110(3):1006-11; doi: 10.1073/pnas.1214272110.
38. Kits KD, Sedlacek CJ, Lebedeva EV, Han P, Bulaev A, Pjevac P, et al. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. Nature. 2017;549(7671):269-72; doi: 10.1038/nature23679.
39. Palomo A, Pedersen AG, Fowler SJ, Dechesne A, Sicheritz-Ponten T, Smets BF. Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox *Nitrospira*. ISME J. 2018;12(7):1779-93; doi: 10.1038/s41396-018-0083-3.
40. Di HJ, Cameron KC, Podolyan A, Robinson A. Effect of soil moisture status and a nitrification inhibitor, dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. Soil Biology & Biochemistry. 2014;73:59-68; doi: 10.1016/j.soilbio.2014.02.011.
41. Scheer C, Rowlings DW, Firrel M, Deuter P, Morris S, Grace PR. Impact of nitrification inhibitor (DMPP) on soil nitrous oxide emissions from an intensive broccoli production system in sub-tropical Australia. Soil Biology & Biochemistry. 2014;77:243-51; doi: 10.1016/j.soilbio.2014.07.006.
42. Lam SK, Suter H, Mosier AR, Chen D. Using nitrification inhibitors to mitigate agricultural N2 O emission: a double-edged sword? Glob Chang Biol. 2017;23(2):485-9; doi: 10.1111/gcb.13338.
43. Sun L, Lu Y, Yu F, Kronzucker HJ, Shi W. Biological nitrification inhibition by rice root exudates and its relationship with nitrogen-use efficiency. New Phytol. 2016;212(3):646-56; doi: 10.1111/nph.14057.
44. Jia Z, Conrad R. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environ Microbiol. 2009;11(7):1658-71; doi: 10.1111/j.1462-2920.2009.01891.x.
45. Martens-Habbena W, Qin W, Horak RE, Urakawa H, Schauer AJ, Moffett JW, et al. The production of nitric oxide by marine ammonia-oxidizing archaea and inhibition of archaeal ammonia oxidation by a nitric oxide scavenger. Environ Microbiol. 2015;17(7):2261-74; doi: 10.1111/1462-2920.12677.
46. Jung MY, Gwak JH, Rohe L, Giesemann A, Kim JG, Well R, et al. Indications for enzymatic denitrification to N_2O at low pH in an ammonia-oxidizing archaeon. ISME J. 2019;13(10):2633-8; doi: 10.1038/s41396-019-0460-6.

47. Martens-Habbena W, Berube PM, Urakawa H, de la Torre JR, Stahl DA. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature. 2009;461(7266):976-9; doi: 10.1038/nature08465.
48. Daims H, Lucker S, Wagner M. A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. Trends Microbiol. 2016;24(9):699-712; doi: 10.1016/j.tim.2016.05.004.
49. Straka LL, Meinhardt KA, Bollmann A, Stahl DA, Winkler MH. Affinity informs environmental cooperation between ammonia-oxidizing archaea (AOA) and anaerobic ammonia-oxidizing (Anammox) bacteria. ISME J. 2019;13(8):1997-2004; doi: 10.1038/s41396-019-0408-x.
50. Pankratova G, Hederstedt L, Gorton L. Extracellular electron transfer features of Gram-positive bacteria. Anal Chim Acta. 2019;1076:32-47; doi: 10.1016/j.aca.2019.05.007.
51. Schmid MC, Hooper AB, Klotz MG, Woebken D, Lam P, Kuypers MM, et al. Environmental detection of octahaem cytochrome *c* hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. Environ Microbiol. 2008;10(11):3140-9; doi: 10.1111/j.1462-2920.2008.01732.x.
52. Arp DJ, Sayavedra-Soto LA, Hommes NG. Molecular biology and biochemistry of ammonia oxidation by Nitrosomonas europaea. Arch Microbiol. 2002;178(4):250-5; doi: 10.1007/s00203-002-0452-0.
53. van Kessel MA, Speth DR, Albertsen M, Nielsen PH, Op den Camp HJ, Kartal B, et al. Complete nitrification by a single microorganism. Nature. 2015;528(7583):555-9; doi: 10.1038/nature16459.
54. Stahl DA, de la Torre JR. Physiology and diversity of ammonia-oxidizing archaea. Annu Rev Microbiol. 2012;66:83-101; doi: 10.1146/annurev-micro-092611-150128.
55. Bertini I, Cavallaro G, Rosato A. Cytochrome *c*: occurrence and functions. Chem Rev. 2006;106(1):90-115; doi: 10.1021/cr050241v.

Figures

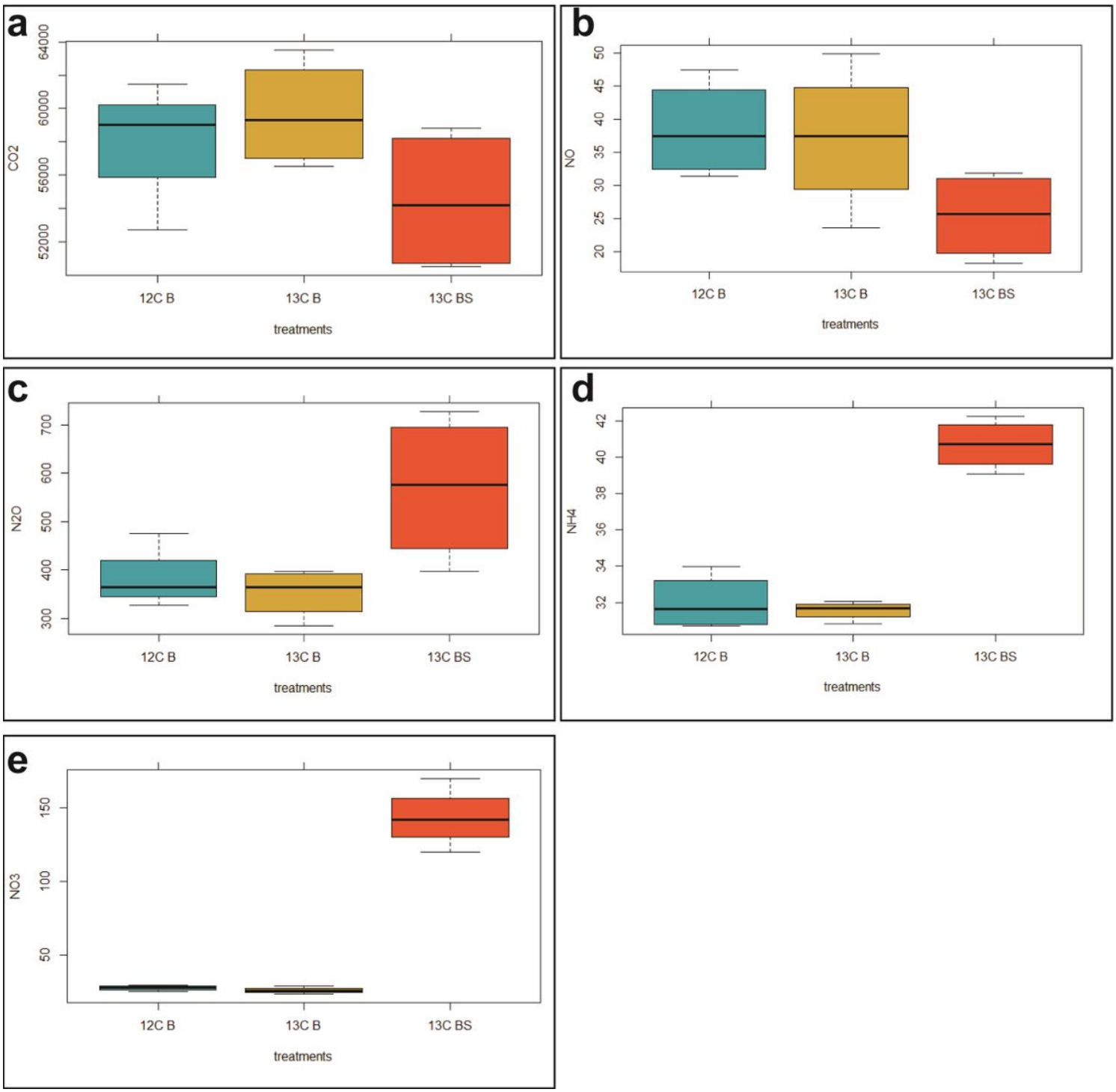

Figure 1

Concentrations of CO2 (a), NO (b), N2O (c), NH4+ (d) and NO3- (e) in surface agricultural soil incubated within Treatments 12C-B, 13C-B and 13C-BS after incubation for 28 days. The error bars of soil nitrogen contents indicate standard errors of triplicate microcosm incubations.

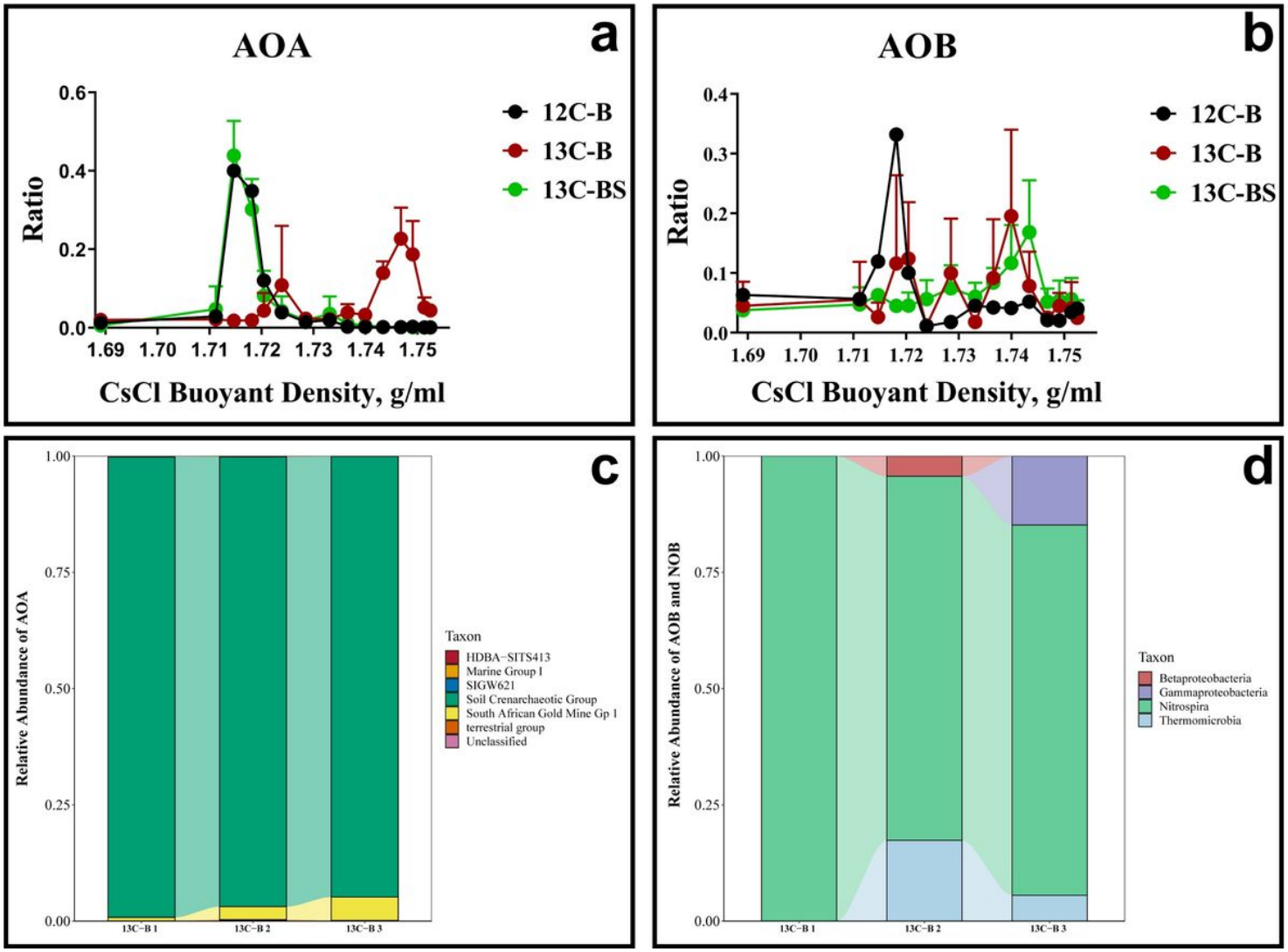

Figure 2

Abundance and community of AOA and AOB labelled in the heavy layers. (a and b) Quantitative distribution of the abundance of amoA genes in AOA (a) and AOB (b) across the buoyant density gradient of the fractionated DNA from soil incubated with either 12CO2 or 13CO2 after incubation for 28 days. The error bars indicate standard errors of triplicate microcosm incubations. (c and d) Communities of AOA (c), AOB and NOB (d) in the heavy layer DNA from the soil incubated with 13CO2 after incubation for 28 days. Treatments 13C-B 1 to 13C-B 3 were triplicates of microcosms.

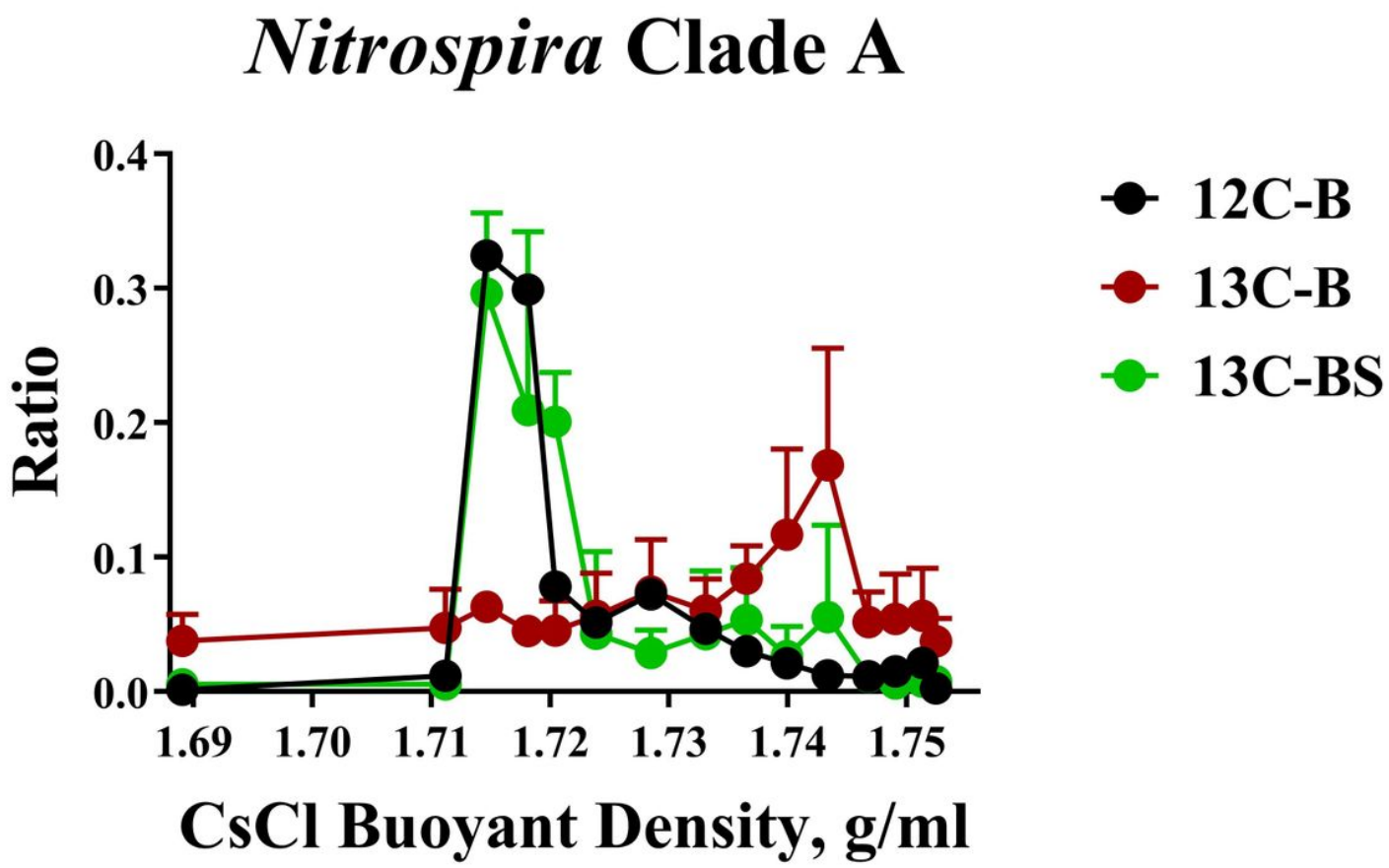

Figure 3

Quantitative distribution of the amoA genes of complete nitrifying Nitrospira across the entire buoyant density gradient of the fractionated DNA from the soil incubated with either 12CO2 or 13CO2 after incubation for 28 days. The error bars indicate standard errors of triplicate microcosm incubations (Fig. 3).

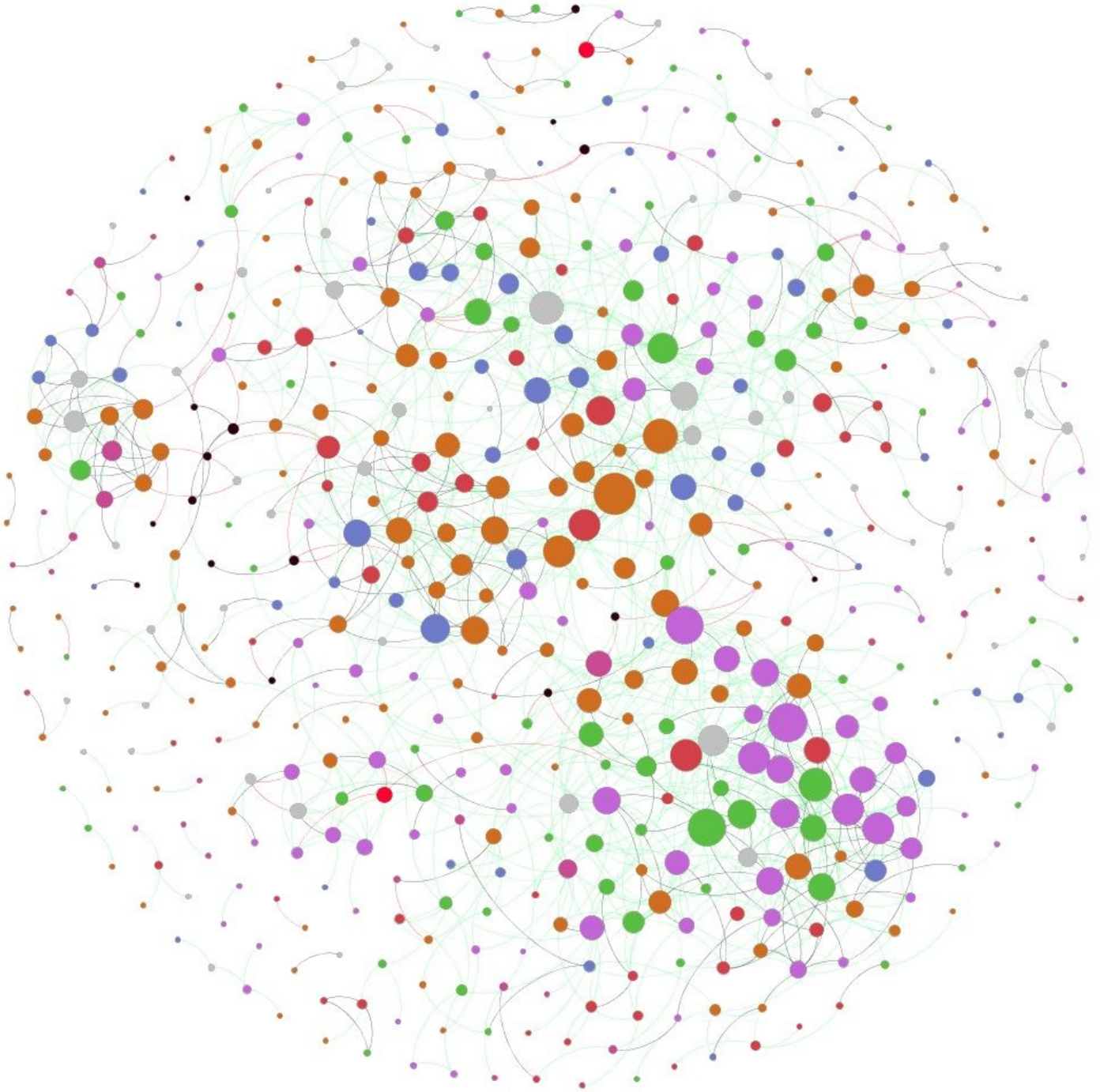

Figure 4

Network of bacteria and archaea in the heavy layer DNA from the soil incubated with 13CO2 and BA after incubation for 28 days. The red nodes belong to Bacillus, and the black nodes belong to AOA.

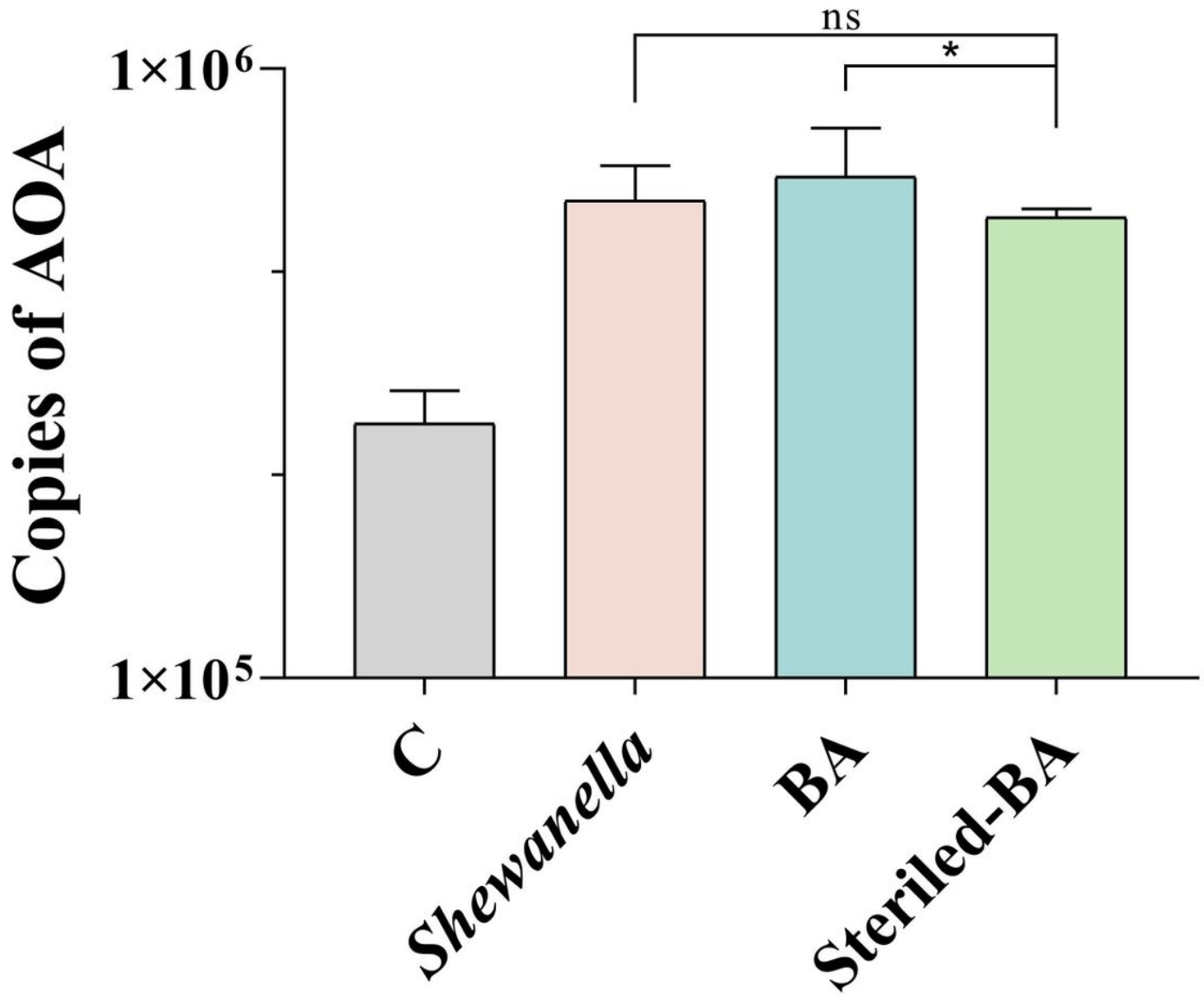

Figure 5

Abundances of AOA in the MFC systems. The error bars indicate standard errors of triplicate Q-PCRs.

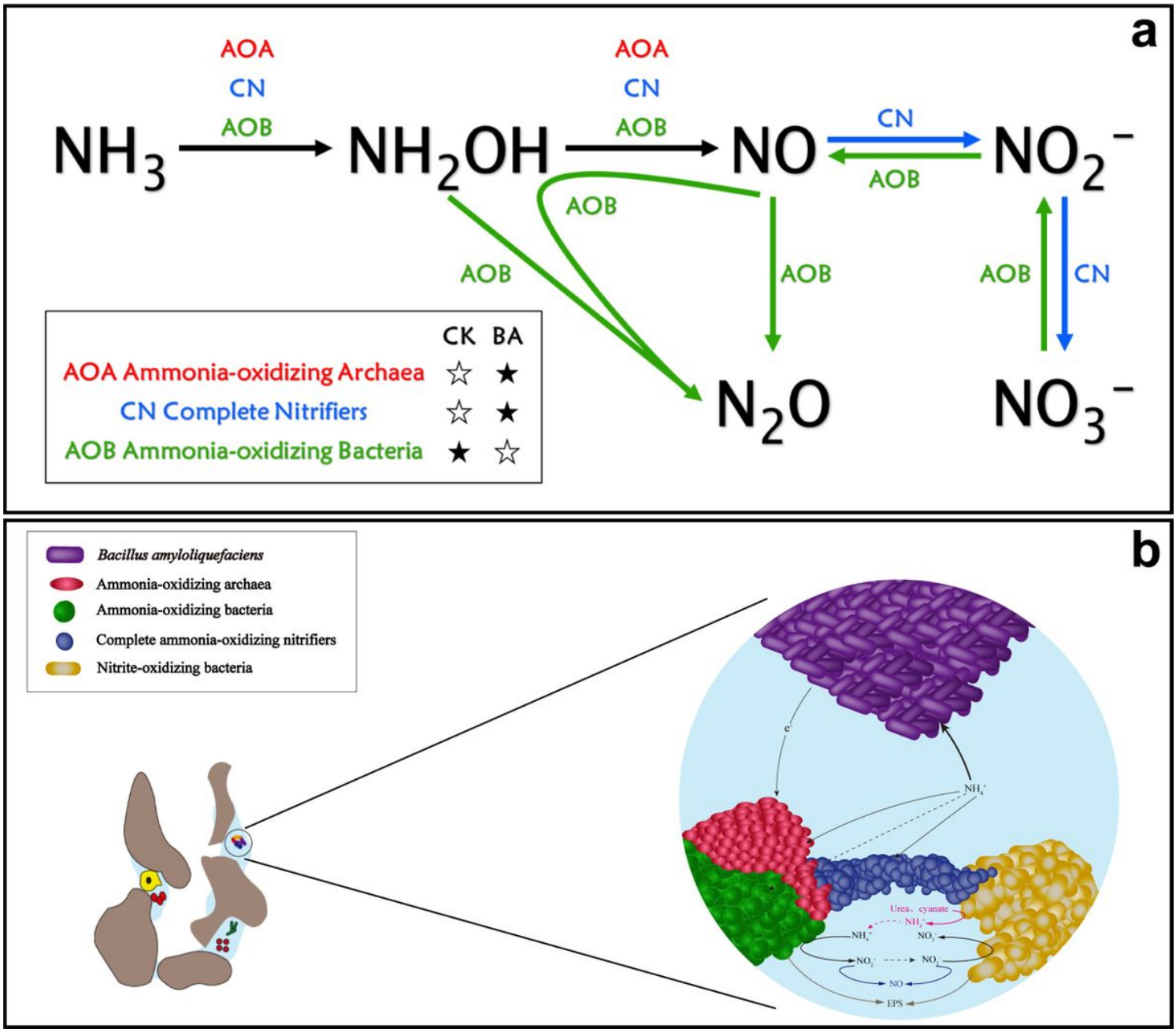

Figure 6

Potential relations between BA and AOM and regulations in the nitrification process. (a) Changes in the nitrification process after BA was inoculated. The solid stars indicate the dominant species in the nitrification process with BA or without BA. (b) The potential relations between BA and the three ammonia oxidizers[48].