Substrate competition contributes to the niche differentiation between ammonia-oxidizing bacteria and archaea in ammonium-rich alkaline soils

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Research

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Abstract

Background The niche differentiation between ammonia-oxidizing archaea (AOA) and bacteria (AOB) has been believed to primarily stem from their disparities in substrate affinity and tolerance threshold for ammonia, such that the latter group dominates in ammonium-rich neutral-alkaline soils. However, recent surveys indicate that direct competition between them might be of importance in driving this phenomenon. As such, diverse AOA lineages might grow in ammonium-rich alkaline arable soils if AOB growth was suppressed. To test this hypothesis, a microcosm study using three alkaline arable soils differing substantially in biogeochemical properties was established in which high ammonium concentration (200 μg N g -1 dry soil) was maintained by routinely replenishing urea, and activities of AOB were selectively inhibited by 1-octyne or 3, 4-dimethylpyrazole phosphate (DMPP). The dynamics of AOA and AOB population were monitored throughout the incubation.

Results Our results suggested that amendment with either 1-octyne or DMPP retarded the growth of AOB to varying extents, but AOA proliferation accelerated in both treatments; this fitted well with the magnitude to which AOB growth was inhibited. Unexpectedly, nearly identical AOA OTUs were enriched by high-ammonium amendment across the soils tested, and they were taxonomically related to Nitrososphaera sp. JG1, N. viennensis EN76, and N. garnensis . These phylotypes have been previously demonstrated to be sensitive to high ammonia concentrations adopted here. Besides, the changed OTUs in the same lineage of AOA showed a similar response pattern to a given treatment across soils.

Conclusions Our results demonstrate that some AOA phylotypes occupy a wider niche than previously believed, and it is the competitiveness for ammonia rather than disparities in substrate affinity and tolerance threshold for ammonia that drives niche differentiation between these AOA and AOB in ammonium-rich alkaline soils. These results further expand our understanding of microbial nitrogen cycling in the terrestrial environment.

Background

The relatively recent discovery of ammonia-oxidizing archaea (AOA), comprising diverse Nitrososphaeria within the phylum Thaumarchaeota, has fundamentally changed our understanding of the biogeochemistry of nitrogen cycling in aquatic and terrestrial environments [1, 2], since ammonia oxidization, the key component and rate-limiting step of nitrogen cycling, had been thought to be primarily conducted by certain lineages of Beta- and Gamma-proteobacteria for more than a century [3]. In many ecosystems, AOA are numerically more abundant than AOB; this leads to the speculation that AOA assume a functionally-significant role in nitrification and, consequently, regulation of the ecosystem N-cycling [1, 2].

In soils, AOB typically prevail in ammonium-rich conditions while AOA dominate environments with low NH_4^+ concentrations [4–6]. These niche-associated differences, linking to AOA and AOB numerical and functional dominance, have been frequently invoked to explain the variation in nitrification activity

among soil ecosystems [4–6], and demonstrated to have a profound mechanistic basis, as cultivated AOA and AOB show substantial disparities in physiological traits [4, 7, 8]. Importantly, most cultivated AOA have a higher affinity for ammonia/ammonium (i.e. K_m value) than AOB [4–6, 8], and this has been proposed as a key mechanism by which AOA dominate nitrification activity in oligotrophic environments including acidic soils where the availability of substrate, ammonia, is exponentially decreased due to the ionization [5, 9]. On the other hand, their higher sensitivity to ammonia/ammonium has been believed to restrict growth under high ammonium/ammonia concentrations [4, 5, 10], such that AOB dominate nitrification in ammonium-rich neutral-alkaline soils [10–12].

While these physiological disparities between AOA and AOB provide compelling explanations for their varying activities in different environments, these prevailing paradigms have recently been questioned. Emerging physiological analyses failed to support that there are meaningful differences in ammonia affinity between several typical AOA and AOB strains [13, 14]. And, isolates of the Nitrosocosmicus lineage that grow in ammonia concentration as high as 1.49 mM, similar to that of copiotrophic AOB, have been documented [15–17]. Therefore, it is likely that some, at least a few, AOA phylotypes potentially adapt to growth in ammonia-rich neutral-alkaline soils. This notion is supported by studies reporting that multiple phylotypes of Nitrosopumilales and Nitrososphaerales grew moderately in soils that were continually amended with a high amount of ammonium [10, 18], and further strengthened by recent researches reporting that AOA growth was accelerated under ammonium-rich conditions when the growth of AOB was selectively repressed by inhibitors, 1-octyne or DMPP [19, 20]. These studies point out that niche differentiation between AOA and AOB might stem not only from their differences in ammonia oxidization dynamics but also from direct inter-group competition for the substrate [19]. As such, it is reasonable to hypothesize that release from competition with AOB may support the growth of AOA in ammonium-rich alkaline soils; and if present, this phenomenon may not be soil specific or restricted to a narrow range of AOA lineages.

Therefore, the main objective of this study was to explore the possibility that diverse AOA lineages could thrive in high ammonium alkaline arable soils ($7.7 < pH < 8.4$). To achieve this, a microcosm experiment using three contrasting soils was established, in which a high concentration of NH_4^+ (i.e. 200 μg g^{-1} N dry weight soil) was maintained by routinely amending with urea and the growth of AOB was selectively inhibited by 1-octyne [21]. In comparison, DMPP was further selected to retard the growth of AOB based on earlier findings [20, 22]. Taking advantage of multiple molecular ecological tools, we focused on testing the hypothesis that diverse AOA lineages, in particular those of Nitrosocosmicus lineage, would prevail in ammonium-rich alkaline arable soils if the growth of AOB was suppressed.

Results

The changes in ammonium, nitrate, and pH

The dynamics of soil ammonium and nitrate concentration, as well as pH, were monitored over the incubation. Around 30–50% of added urea was hydrolyzed to ammonium within the 2 hours stabilization

period in urea-amended treatments (Fig. 1), and complete hydrolysis was achieved within 12 hours for all three soils tested (data not shown). The NH_4^+ concentrations of no amendment control (CK) approached the analytical detection limit throughout the whole incubation period for all soils, and a detectable buildup of NO_3^- concentration in this treatment was only observed in soil from Shihezi (one-way ANOVA; $P < 0.001$; Fig. 1E). The NH_4^+concentrations in the C_2H_2 treatments exhibited minor temporal variation, but no significant accumulation of NO_3^- was observed at the end of incubation across all soils (Tukey HSD test; $P > 0.05$). In contrast, NH_4^+ in the urea-only treatment (Urea) was rapidly nitrified into NO_3^- and replenished urea completely converted within 72 h after day 12, leading to a decline of 0.6, 1.0 and 0.5 units in pH for the Luoyang, Shihezi, and Tianshui soils, respectively, relative to their value of CK at the end of incubation (Fig. S1). These findings indicated the occurrence of strong autotrophic nitrification activity for all soils tested. Amendment with DMPP strongly retarded oxidation of NH_4^+ to NO_3^-(Fig. 1). Its effect was higher in soils from Shihezi (Fig. 1B) and Tianshui (Fig. 1C), resulting in relatively consistent nitrification rates and stable NH_4^+ concentrations during incubation, such that a decline of smaller magnitude in pH was observed in this treatment of Luoyang (0.31, $P < 0.001$) and Shihezi soils (0.22, $P < 0.001$), but a minor increase (0.07 relative to CK) was observed in the Tianshui soil at day 27 ($P < 0.02$) (Fig. S1). In comparison, 1-Octyne treatment was less effective at inhibiting nitrification, particularly in the Luoyang soil (Fig. 1D). In these treatments with 1-octyne, the pH dropped by 0.51, 0.95 and 0.27 units for Luoyang, Shihezi, and Tianshui soils, respectively (Fig. S1).

AOA and AOB amoA gene abundances

Across all soils tested, AOA growth was inhibited in the C_2H_2-treated microcosms (i.e. C_2H_2 treatment), but strongly promoted in both DMPP and 1-Octyne treatments (Fig. 2A-C). The highest AOA growth rates were generally found in the DMPP-treated soils; the exception was the Tianshui soil in which similar AOA growth was observed in DMPP and 1-Octyne treatments after day 15 (Tukey post hoc test, $P > 0.05$), while the growth of AOA was moderately stimulated in the Urea treatment in Luoyang and Tianshui soils (one-way ANOVA, $P < 0.001$).

For all soils, the Urea treatment led to the fastest increase in AOB amoA gene numbers, but the addition of C_2H_2 and DMPP with urea completely suppressed AOB growth in soils (Fig. 2D-F). In comparison, the inhibitory effect of 1-Ocytne treatment was moderate, especially in the Shihezi soil (Fig. 2E). The varying degrees of AOA and AOB growth led to different ratios of AOA to AOB among treatments, showing a trend of increase following the order of Urea, 1-Octyne, and DMPP across soils tested at the end of incubation, and this index was significantly higher in DMPP treated soils than in the rest treatments (Fig. S2).

Temporal changes in community structures of AOA and AOB

The temporal shift in AOA and AOB community structures was explored by terminal restriction fragment length polymorphism (T-RFLP) in combination with principal component analysis (PCA) ordination (Fig.

S3, S4). The major two principal axes accounted for > 80% of the overall separation among samples for all soils (Fig. S4). Permutational analysis of variance (PERMANOVA) tests revealed significant effects of sampling time and treatment on both guilds across soils (Table S1). The dominant AOA T-RFs differed among soils (Fig. S3A-C), while community structures of 1-Octyne and DMPP treatments in each soil increasingly converged by day 27, separating from other treatments (Fig. S4A-C). This was due to the gradual enrichment of nearly identical T-RFs over the incubation across soils (Fig. S3A-C). The major T-RFs contributing to the shift in overall AOB community structure were similar, but their response patterns to treatments varied among the soils (Fig. S3D-F). In PCA plots, the CK, C_2H_2, and DMPP treatments clustered together and were distinct from the 1-Octyne and Urea, and the latter two treatments were dissimilar to one another across all soils (Fig. S4D-F).

Phylogenetic composition of AOA and AOB

The phylogenetic composition of AOA and AOB ammonia oxidizers were analyzed by high-throughput sequencing of amoA genes from samples collected on day 27. Rarefaction curves indicated that the sequencing depth was sufficient to capture the vast majority of the total diversity present (Fig. S5). After equalizing effort, 407 295 AOA reads clustered into 113 OTUs at 3% distance sequence dissimilarity, with 90 OTUs being found for Luoyang, 91 OTUs for Shihezi, and 76 OTUs for Tianshui, respectively; while 192 150 AOB reads clustered into 78 OTUs (3% sequence dissimilarity), and 56 OUTs were found for Luoyang, 62 for Shihezi, and 48 for Tianshui (Table S2).

All amplified AOA OTUs were classified as Nitrososphaerales, and dominant lineages differed among soils (Fig. 3). Specifically, AOA assemblage in the Luoyang soil primarily comprised Nitrososphaerales-δ-1.1.2 (54% of relative abundance), α-3.2.1(20%), γ-1.1 (10%), and γ-2.1.1 (13%) lineages. A similar composition was found for Tianshui soil except for a considerable portion (6%) coming from Nitrososphaerales-β-1.1 therein. In the Shihezi soil, Nitrososphaerales-β-1.1 was dominant (34%); the α-3.2.3 and α-3.2.4 lineages which were rare in Luoyang and Tianshui soils, contributed another 27% to the reads. Notably, OTUs similar to Nitrosocosmicus were very rare across all soils.

For AOB taxa, only two OTUs clustered within the Nitrosomonas family (Shihezi soil specific), the remaining being Nitrosospira (Fig. S6). The dominant lineages of AOB were similar among soils and primarily composed of taxa from cluster 3. Cluster 3a.1 accounted for > 60% of reads across soils, followed by cluster 3a.2 in Luoyang (25% relative abundance) and Tianshui soils (22%), and cluster 3b (18%) in soil from Shihezi.

Treatment effects on AOA and AOB amoA genotypes

The effects of treatment on compositional changes in AOA and AOB amoA OTUs were analyzed by non-metric multidimensional scaling (NMDS) (Fig. 4) and further visualized with heatmaps (Fig. 5, S7). A similar resolution for β-diversity analysis of treatment effect was observed between the NMDS ordination and the PCA analysis of T-RFLP data (Table S1; Fig. 4), and heatmaps revealed strong differences in compositions of amoA OTUs among the soils tested.

For AOA, the relative abundances of 28, 37, and 18 OTUs significantly differed among treatments for the Luoyang, Shihezi, and Tianshui soils, respectively (Kruskal-Wallis rank test, $P < 0.05$; Fig. S8A), but none of them were in the ε-2.2, γ-2.2.3, or Nitrosocosmicus lineages (Fig. 3, 4). Among these, seven OTUs were shared by all soils, each of which showed a similar response pattern to given treatment (Fig. 5; S8A). Moreover, the heatmap shows that the major OTUs enriched by 1-Octyne treatment (i.e. OTUs 13, 21, 20, 22, 26, 75 and 77) were also promoted by both Urea and DMPP treatments in all soils (Fig. 5). These OUTs phylogenetically belonged to either Nitrososphaerale α-3.2.1 or α-3.2.4 lineages, which are related to Nitrososphaera viennensis EN76 and Nitrososphaera sp. JG1, as well as Nitrosophaera gargensis strains with high bootstrap confidence (Fig. 3). However, their close relatives in α-3.2.3 lineage declined in both Urea and C_2H_2 treatments compared with other treatments, implying diversification of physiology within phylogenetically associated lineages. In contrast, changed OTUs in β-1, δ-1, γ-1.1, and γ-2.1 lineages were generally reduced by 1-Octyne and DMPP treatments; interestingly, those of γ lineages mostly showed a trend of increase in C_2H_2 treatment (Fig. 5).

For AOB, the relative abundances of 17 OTUs for Luoyang, 18 OTUs for Shihezi, and 19 OTUs for Tianshui were significantly changed by treatments (Kruskal-Wallis rank test, $P < 0.05$; Fig. S8B). All of them were assigned to Nitrosospira clusters 2 or 3 (Fig. 4; S7). Seven of these treatment-responsive OTUs were shared across all soils, with six associated with Nitrosospira cluster 3a.1 and one to cluster 3a.2 (Fig. S6B; S7). The relative abundances of OTUs in cluster 3b were generally reduced by either Urea or 1-Octyne treatment, and OTUs in cluster 3a.2 tended to decline only in the Urea treatment (Fig. S7). In contrast, OTUs enriched by the Urea treatment (in all tested soils) mainly came from Nitrosospira cluster 3a.1, 2a, and 2b, and the majority of them were inhibited by both C_2H_2 and DMPP treatments. Meanwhile, diverse shift patterns were observed in cluster 3a. 1. For instance, OTUs 26, 47 and 69, all of which were related to Nitrosospira multiformis, showed a trend of increase in 1-Octyne treatment across the soils, while OTUs 54 and 80 were inhibited by 1-octyne (Fig. S7).

Discussion

Underlying ammonia oxidization dynamics in cultivated strains of AOA and AOB, alongside biogeochemical data from field and microcosm studies, has established an understanding that AOA are favored under oligotrophic conditions whereas AOB thrive in moderate acid-alkaline soils with elevated concentrations of NH_4^+ [4–6]. Extended from this, it has been proposed that the relative differences in abundance and functional role of AOB vs. AOA in various ecosystems are primarily driven by their disparities in metabolic traits including substrate affinity and tolerance threshold for ammonia, as well as preference for a supply rate of ammonia [4–6, 19, 23]. Here, by continually supplying urea and selectively inhibiting AOB, we demonstrate that taxonomically constrained AOA, which had been believed to be sensitive to high ammonia concentration, prevail in copiotrophic alkaline soil conditions. These findings strongly support the notion that it is direct competition for the substrate that plays a key role in driving niche differentiation between these AOA phylotypes and AOB in ammonium-rich alkaline soils.

Furthermore, our results revealed the coherence of ecophysiology within the same phylogenetic lineage of Nitrososphaerales.

Ammonia rather than ammonium serves as the direct substrate for autotrophic ammonia oxidizers; differences in substrate affinity and tolerance threshold for ammonia have been proposed as overarching mechanisms driving niche differentiation between AOA and AOB [4–6]. Indeed, the majority of cultivated AOA own a much higher substrate affinity for ammonia than their bacterial counterpart, which has been postulated to provide them with advantages under oligotrophic conditions [5, 6]. This appeared to be evident in the untreated Shihezi soil where no AOB growth was observed but AOA populations were sustained by scavenging ammonia released during organic matter mineralization [24]. However, it would be of little relevance in treatments such as 1-Octyne, as the soil NH_4^+ was maintained at a high concentration in the first 15 days, during which AOA and AOB both grew rapidly and simultaneously. In fact, the NH_4^+-N concentrations fluctuated between 20 and 200 μg g^{-1} soil during this period, in particular for Shihezi and Tianshui soils in which the lowest NH_4^+-N concentrations were found to be as high as 117 μg g^{-1}soil. According to the ionization equilibrium $NH_4^+ \rightleftharpoons NH_3 + H^+$ (pKa = 9.25; T = 25 °C), the lowest NH_3 concentrations were estimated to range from 88 to 528 μM. Such concentrations not only far exceed K_m values of both AOA and AOB, but are higher than the inhibitory concentration for most cultivated AOA [4–6]. Importantly, AOA grew in both 1-Octyne and DMPP treatments, and their growth coincided with the inhibition of AOB. This echoed the recent findings that alleviation of competition between AOA and AOB by either 1-octyne or DMPP accelerated the growth of AOA under copiotrohic conditions [19, 20]. Therefore, our results provide strong evidence that low AOA growth in ammonium-rich alkaline arable soils extends from their poor competitiveness with AOB rather than disparities in physiological characteristics such as substrate affinity and tolerance threshold for ammonia, as well as preference for a supply rate of ammonia.

The growth of AOA in neutral-alkaline arable soils amended with varying amounts of NH_4^+ has been sparsely documented. Multiple AOA lineages including Nitrosopumilales-η [22], Nitrosopumilales-γ [10], Nitrososphaerales-α-3.2.1 [12, 22], and Nitrosocosmicus lineage [25, 26] have been found to proliferate in these soil ecosystems, especially for the last one which could grow under ammonia concentrations preferred by copiotrophic AOB [15–17]. However, in all soils tested here, OTUs of Nitrosocosmicus lineage were in low abundance and not affected by any treatments, and phylotypes responding to treatments were restricted to Nitrososphaerales. Moreover, when released from competition with AOB, AOA OTUs stimulated by the addition of high urea concentrations were taxonomically constrained, with all belonging to Nitrososphaerales-α-3.2.1 and α-3.2.4 lineages. Phylogenetically, OTUs of Nitrososphaerales-α-3.2.4 lineage were closely related to N. gargensis, while those of Nitrososphaerales-α-3.2.1 were associated with N. viennensis EN76, Nitrososphaera sp. JG1, and N. evergladensis SR1. These strains grew optimally under pHs ranging from 6.5 to 7.5 [27–29], and phylotypes closely related to them have been classified as being alkalinophilic [30, 31]. Besides, when directly assessed in environmental studies including those using stable isotopic probing analysis, phylotypes within these lineages grew to a moderate extent in

microcosms that were regularly spiked with urea at a concentration of 100 μg NH_4^+-N g^{-1} soil [12, 22, 25, 26]. Thus, it appears that AOA affiliating to Nitrososphaerales-α-3.2.1 and α-3.2.4 lineages adapt to growth in ammonium-rich alkaline soils. Notwithstanding, their rapid growth under such high ammonium concentrations adopted here (i.e. 200 μg NH_4^+-N g^{-1} soil) was still unexpected, as the reported inhibitory concentrations of ammonia for these taxa are much lower than the theoretical values in 1-Octyne, especially for DMPP treatments (the highest value of 356 μM ammonia for EN76 vs. lowest concentration of 2 375 μM in DMPP treatments) [27, 28]. Thus, it is likely that these phylotypes occupy a much broader niche than previously described, and they are highly susceptible to the competition from their bacterial counterpart.

On the other hand, the promotion of growth of Nitrososphaerales-α-3.2.1 and α-3.2.4 lineages in 1-Octyne treatment might be associated with mixotrophic activities, since their representative strains are equipped with genes encoding transporters for organics [29, 32, 33], and their growth could be enhanced by the addition of low-molecular-weight organic compound [27, 34]. To exclude this possibility, DMPP, a nitrification inhibitor with distinctive heterocyclic chemical structure, was additionally adopted to inhibit the growth of AOB based on earlier findings [20, 22]. Our results suggested that the amendment of soils with 1-octyne or DMPP led to the enrichment of the same AOA phylotypes both within and across each soil tested, thus proving that the acceleration of AOA growth in 1-Octyne and DMPP treatments was indeed attributed to their chemolithotrophy. However, unexpectedly, 1-octyne was far less effective at reducing the growth of AOB than previously reported [19, 21]. Some AOB OTUs increased in the 1-Octyne treatment, particularly those related to Nitrosospira multiformis whose growth had been previously demonstrated to be abolished by a lower concentration of 1-octyne [21]. It is less likely that this stems from restricted diffusion or leakage of 1-octyne in the experimental systems, given that low concentrations of C_2H_2 completely abolished the growth of both AOA and AOB. Regardless of mechanisms, the lower inhibition efficiency of 1-octyne did not compromise our conclusion. Rather, the inhibition gradient created by Urea, 1-Octyne and DMPP treatments provided a unique opportunity to disentangle the effect of interaction between AOA and AOB. Hereby, we were able to show that the growth of AOA was closely coupled with the magnitude to which the growth of AOB was inhibited, and DMPP is a more effective and specific inhibitor against AOB than 1-octyne in the soils examined (see supplemental discussion).

Besides, the experimental design adopted here allowed us to explore the potential ecological coherence of major phylogenetic lineages. Indeed, we found that, in addition to those enriched by the high-ammonium amendment, the majority of changed OTUs within the same lineage showed a similar response pattern to a given treatment across soils. For instance, our results confirmed that the neutral-alkalinophilic Nitrososphaerales-δ lineage dominated the AOA assemblage in all soils tested [30], but most of OTUs therein were insensitive to amendment with urea or inhibitors across soils. Currently, no cultured representative has been identified in this lineage, therefore its ecological function in the soil is still unknown [18]. In contrast, changed OTUs in β-1, γ-1.1, and γ-2.1 lineages were generally reduced by 1-Octyne and DMPP treatments. In particular, those of γ-1.1 and γ-2.1 lineages were exceptionally enriched

in C_2H_2 treatment of all soils tested, implying a mixotrophic lifestyle of these lineages. Similarly, one earlier report documented that the addition of 10 Pa C_2H_2 failed to inhibit the growth of AOA in an alkaline arable soil [35]. Intriguingly, despite being closely related to α-3.2.4, OTUs of Nitrososphaeralesα-3.2.3 lineage were inhibited by Urea but not by DMPP and 1-Octyne treatment, this concurred with the finding that phylogenetically closely related AOA could show contrasting metabolic traits [36]. Overall, our results confirm that AOA are functionally heterogeneous [36], but the coherence of response to treatments indicates similar ecophysiology between phylotypes in the same AOA lineages [30].

Finally, our results reaffirmed that AOB of Nitrosospira cluster 3a dominated the nitrification activity in the ammonium-rich alkaline soils (see supplemental discussion)[10–12, 35, 37], whereas the growth of AOA in Urea and 1-Octyne treatments implies that they assume a considerable role in contributing to overall nitrification in these soils as well. Although our experimental design did not allow for an absolute partitioning of their relative contribution, the nearly linear buildup of NO_3^- and occurrence of only AOA growth in DMPP treatment across the soils enabled estimates to be calculated. Assuming that each AOA strain contains two amoA gene copies [32] and all AOA growth is due to autotrophic nitrification activity, the specific AOA nitrification rates estimated ranged from 1.06 to 2.48 fmol $cell^{-1}$ d^{-1}. This was very similar to that of Nitrososphaera sp. JG1 (1.4 fmol $cell^{-1}$ d^{-1}, [28]), and Nitrosarchaeum koreense MY1 (2.5 fmol $cell^{-1}$ d^{-1}, [38]), but lower than that of EN76 strain (62.6 fmol $copy^{-1}d^{-1}$, [39]); and Candidatus Nitrosocosmicus franklandus C13(13.92 fmol $cell^{-1}d^{-1}$, [16]). Consequently, it is estimated that AOA accounted for 2%, 9% and 10% nitrification activities in Urea treatments of the Shihezi, Tianshui, and Luoyang soils, respectively. These matched the estimates reported in ref. [12] (1.51%-23.4%) and were marginally lower than measurements in ref. [37] (11.55–16.77%). However, the partial inhibition of AOB by 1-octyne further increased AOA contribution ranging from 17–30%. Collectively, these results point to a fundamental role for AOA in nitrification within ammonium-rich alkaline arable soils.

Conclusions

Our results revealed that release from the competition by bacterial ammonia oxidizers allowed AOA to thrive under ammonium-rich alkaline arable soils. Phylotypes that were particularly supported by high ammonium were from a narrow taxonomical group closely related to Nitrososphaera sp. JG1, N. viennensis EN76, and N. garnensis; these isolated strains have been previously demonstrated to be sensitive to high ammonia concentration adopted here. Moreover, 1-Octyne and DMPP treatment of urea-enriched soils supported identical AOA lineages, highlighting that growth acceleration of these AOA under high ammonia conditions was attributed to chemolithotrophy. Together, these findings strongly support our hypothesis that it is direct competition for the substrate that drives the niche differentiation between these AOA phylotypes and AOB in ammonium-rich alkaline soils, thereby providing an alternative explanation for this phenomenon. Overall, this research strongly demonstrates that AOA plays an important role in nitrification in ammonium-rich alkaline arable soils, further expanding our understanding of microbial nitrification and N cycling.

Material And Methods

Soil sampling and determination of soil properties

Three alkaline arable soils (i.e. fluvo-aquic soil, grey desert soil, and a loessial soil), all previously characterized as being AOB-dominated with regard to nitrification activity [12, 40, 41] were used in this study. The fluvo-aquic soil was collected from Henan province, the grey desert soil from Xinjiang Uyghur autonomous region, and the loessial soil from the Zhongliang long-term fertilization experiment station, Gansu province (see Table 1 for georeferencing). These sites, spanning a wide geographic range, differed greatly in meteorological and physio-chemical properties, as well as management practices (Table 1). Bulk soil (0–20 cm) was collected at each site, sieved through a 2 mm mesh, and stored at 4 °C. The detailed sampling design and determination of basic soil properties were presented in supplemental materials.

Table 1
The meteorological parameters of sampling sites and basic physicochemical properties of soils

Item	Luoyang	Shihezi	Tianshui
Location	34°27'N, 113°01'E	44°23'N, 85°41'E	34°36'N, 105°38'E
Soil type	Fluvo-aquic soil	Grey desert soil	Loessial soil
Cultivated crops	Wheat/maize	Grape	Wheat/Canola
MAP (mm)	620	225	491
MAT (^{o}C)	15	7.8	11
Annual range of temperature (^{o}C)	-3^{o}C – 32^{o}C	-17^{o}C- 32^{o}C	-6^{o}C- 30^{o}C
pH (H_2O)	7.80	8.35	7.66
TN (g kg^{-1})	1.12	0.96	1.03
OM (g kg^{-1})	19.03	13.27	14.78
TP (g kg^{-1})	0.827	0.861	0.898
Available K (mg kg^{-1})	199.85	98.48	140.03
NH_4^+-N (μg g^{-1})	0.37	0.61	1.40
NO_3^--N (μg g^{-1})	35.73	19.40	73.40
Olsen P (mg kg^{-1})	34.58	34.42	15.13
WHC (%)	61.14	36.09	52.19

Microcosm experiment

The 27-day microcosm incubation experiment with five treatments for each of the three soil types was established. These treatments included no fertilizer control ('CK'), the addition of urea ('Urea'), the addition of urea with 10 Pa C_2H_2 ('C_2H_2'), the addition of urea with 1-octyne (C_{aq}=4 μM; '1-Octyne'), and addition of urea with DMPP (0.15% of added urea N; 'DMPP'). The C_2H_2 treatment was used to inhibit all autotrophic nitrification activity, while 1-octyne and DMPP are considered to have specificity to inhibition of AOB growth [20, 21]. All soils were pre-incubated at 25 °C for 15 days at 30% water holding capacity (WHC). Microcosms were established by weighing pre-incubated soil (15 g dry wt. equivalent) into 125 ml serum bottles sealed with butyl stoppers and aluminum caps. In each microcosm, the soil moisture was adjusted to 50% WHC with deionized water, urea, C_2H_2, 1-octyne, or DMPP according to treatment design. Three replicates were established for each treatment. For treatments other than CK, urea was initially

added at 200 μg N g^{-1} dry soil and maintained by regularly replenishing throughout incubation at an interval of 6 days. The amount of urea replenished was based on measured NH_4^+ concentration for each microcosm; the CK treatment was supplemented with an equivalent volume of water only. At the end of the incubation, the soil moisture of all treatments reached 60% WHC. All microcosms were incubated at 25 °C. Following microcosm setup, 2 h was allowed for solutions to adequately diffuse in soils and the first batch of samples was collected; these are designated as 'day 0'. Soil samples were destructively collected on days 3, 9, 15, 21, and 27 for the determination of NH_4^+ and NO_3^- contents, and pH, and molecular ecological analysis. All samples were stored at -80^{o}C until use. All microcosms were regularly ventilated for 30 min every 3 days; C_2H_2 and 1-octyne were refreshed in the headspace of their respective treatment flasks.

T-RFLP analysis and Quantitative PCR (q-PCR)

DNA was extracted from 500 mg of soil using Fast DNA SPIN Kit for soil with a FastPrep-24 machine (Qbiogene, Canada). T-RFLP analysis of bacterial and archaeal amoA genes was determined over the CK sample on day 0, and all treatment samples at days 15 and 27. The PCR amplification conditions were presented in Table S1 and raw data analysis procedures have been reported previously [41]. Bacterial and archaeal amoA genes were quantified in DNA samples from days 0, 3, 9, 15, 21, and 27. The qPCR reactions were carried out in triplicate per experimental sample on a LightCycler® 480II (Roche Diagnostics, Switzerland) using SYBR green chemistry. Primer pairs and thermal cycling conditions for amplification were supplied in Table S3. Each 15 μl PCR mixture contained 0.3 μM each primer, 7.5 μl of TSINGKEMasterqPCR mix (Qingke, China), and 2 μl of 20-fold diluted DNA. Three replicates of no-template negative control were included and gave negligible values.

Amplicon sequencing of AOA and AOB

At the end of the incubation (day 27), high throughput sequencing of AOA and AOB genes was conducted using the Illumina MiSeq sequencing platform (PE300 chemistry). For AOB, barcoded amoA1F/amoA2R primers were used. For archaeal amoA genes, barcoded Arch-amoAF/Arch-amoAR primer pairs were used, and PCR conditions for both amoA genes were presented in Table S3. Raw data were processed using the Majorbio Cloud Platform () with default parameters. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity. Representative OTUs of AOA and AOB were further validated by BLAST against the NCBI nucleotide database. Classification of representative AOA OTUs was made to the database curated by ref. [18]. Representative AOB OTUs were classified based on previously defined lineages within reference phylogenies.

Statistical analysis

The phylogenetic analysis of AOA and AOB was conducted in MEGA 6.0 [42] and visualized using iTOL (http://itol.embl.de) [43]. Statistical analysis was conducted in R. 3.6.1 [44]. One-way ANOVA was used to assess the temporal changes in pH, NH_4^+, and NO_3^- concentrations, as well as the abundances of AOA and AOB among soil types. Variation in treatment means was assessed using Tukey's post hoc test.

Differences in relative abundances of OTUs among treatments were assessed with the Kruskal-Wallis rank test at α = 0.05. Variation in the presence of amoA OTUs (AOA and AOB) in soils was conducted in the 'vegan' package[45]. PCA was used to explore the temporal shift in the occurrence of AOA and AOB genes across treatments and soils. PERMANOVA was used to test the effect size and significance of treatments, and these were visually interpreted using NMDS.

Abbreviations

AOA, ammonia-oxidizing archaea.

AOB, ammonia-oxidizing bacteria.

DMPP, 3, 4-dimethylpyrazole phosphate.

NMDS, non-metric multidimensional scaling.

PCA, principal component analysis.

PERMANOVA, permutation multivariate analysis of variation.

T-RFLP, terminal restriction fragment length polymorphism.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All raw *amoA*gene sequence data is available at NCBI under the accession number PRJNA579399.

Competing interests

The authors declare that they have no competing interests.

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Authors'contributions

CY and YL conceived and designed this study. CY, XP, SW, and YL wrote the manuscript with the contribution from TL and YP. CY, XP, and GY analyzed the data. CY, XP, HC, and MY conducted the experimental analysis. SE and ZC provided the experimental materials.YL supervised all aspects of experimentation, data analysis, and manuscript preparation. All authors read and approved the final manuscript.

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Figures

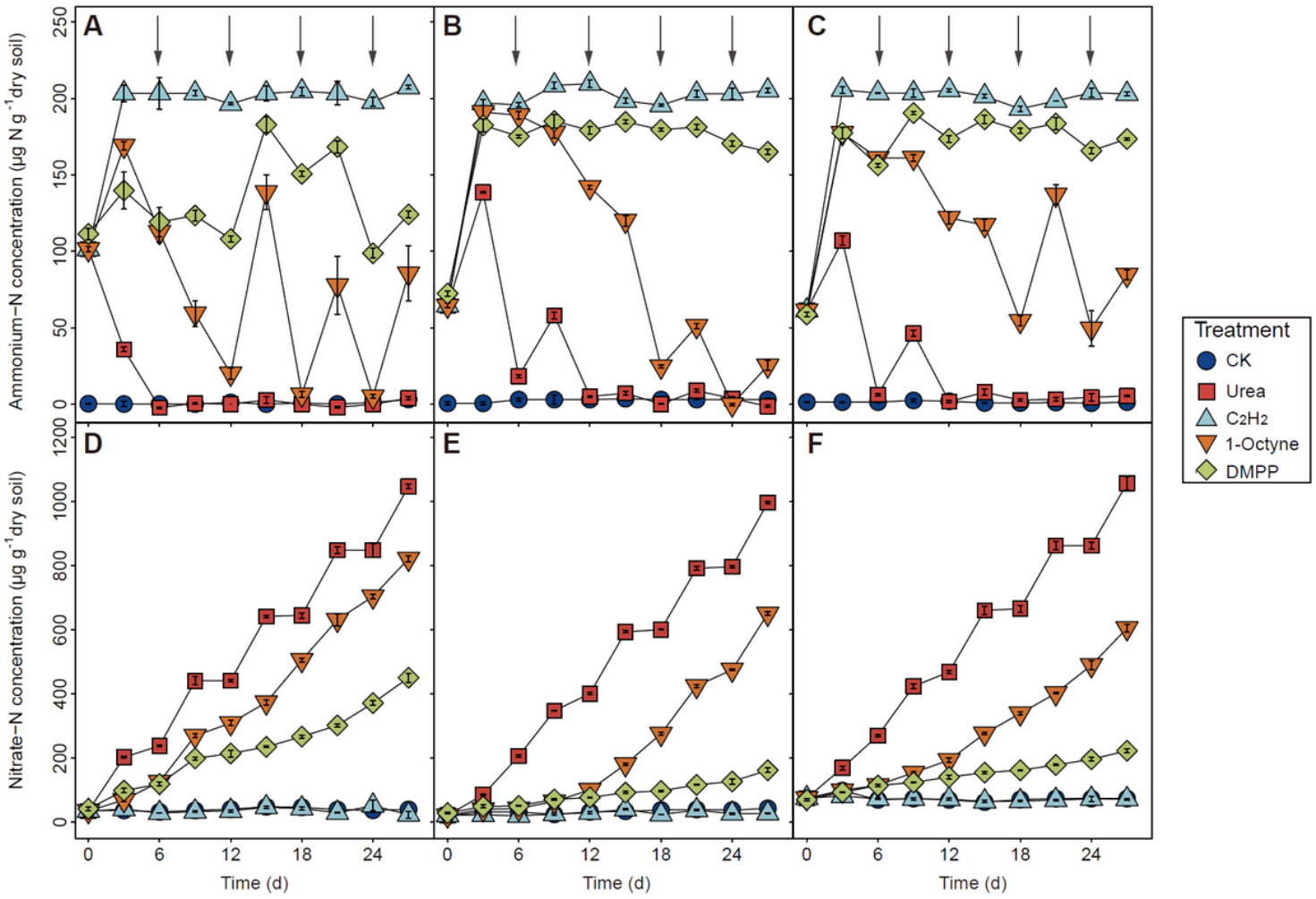

Figure 1

The dynamics of ammonium (A, B, and C) and nitrate (D, E, and F) in Luoyang (A, D), Shihezi (B, E) and Tianshui (C, F). Means ± 1SE (n = 3) were shown. The arrows indicated the time when urea was supplemented in all treatments except CK to reach the targeted ammonium concentration (i.e. 200 μg N g-1 dry soil)

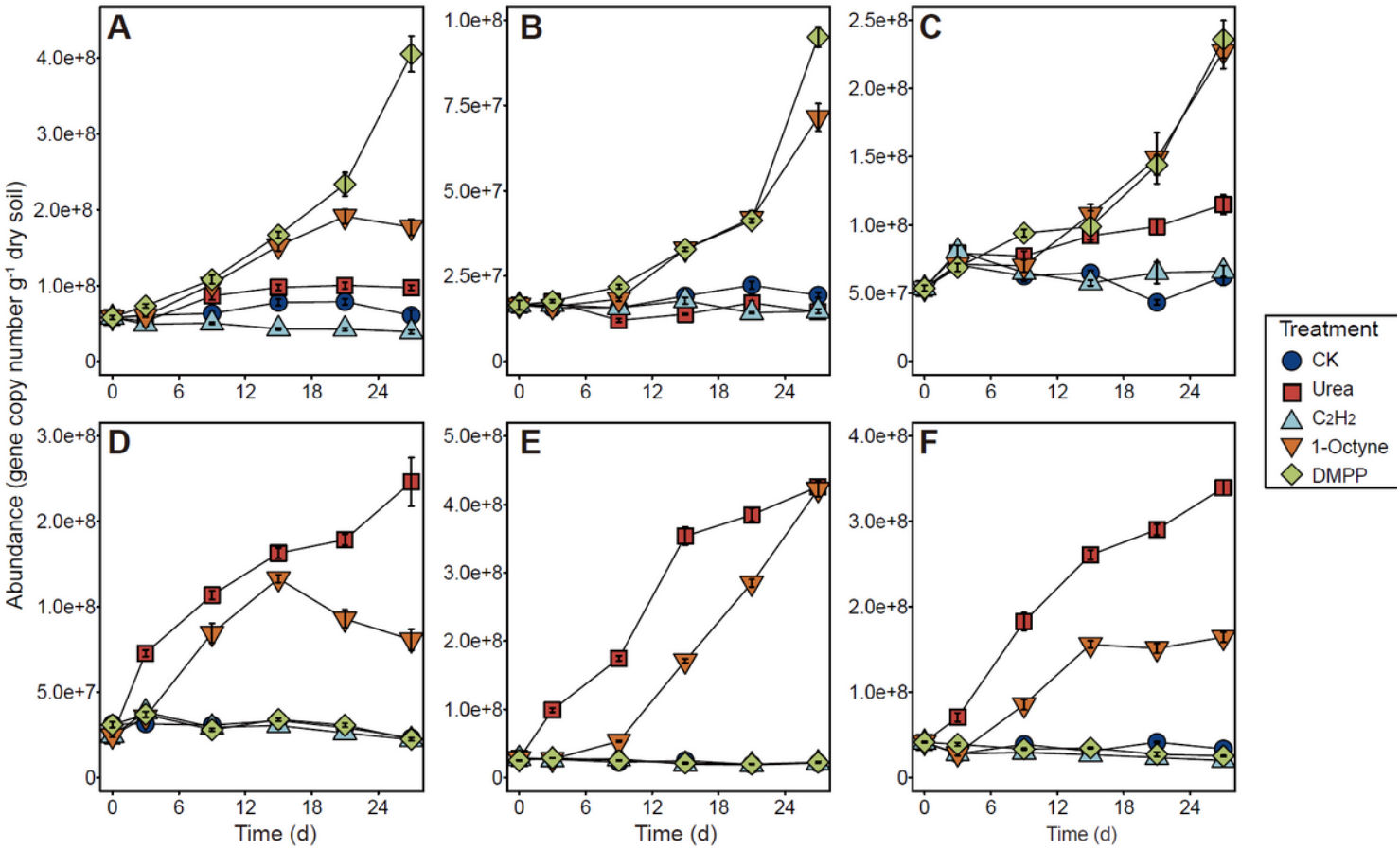

Figure 2

The changes in abundances of AOA (A, B, and C) and AOB (D, E, and F) in Luoyang (A, D), Shihezi (B, E) and Tianshui (C, F) soil during incubation. Means ± 1SE (n = 3) were shown.

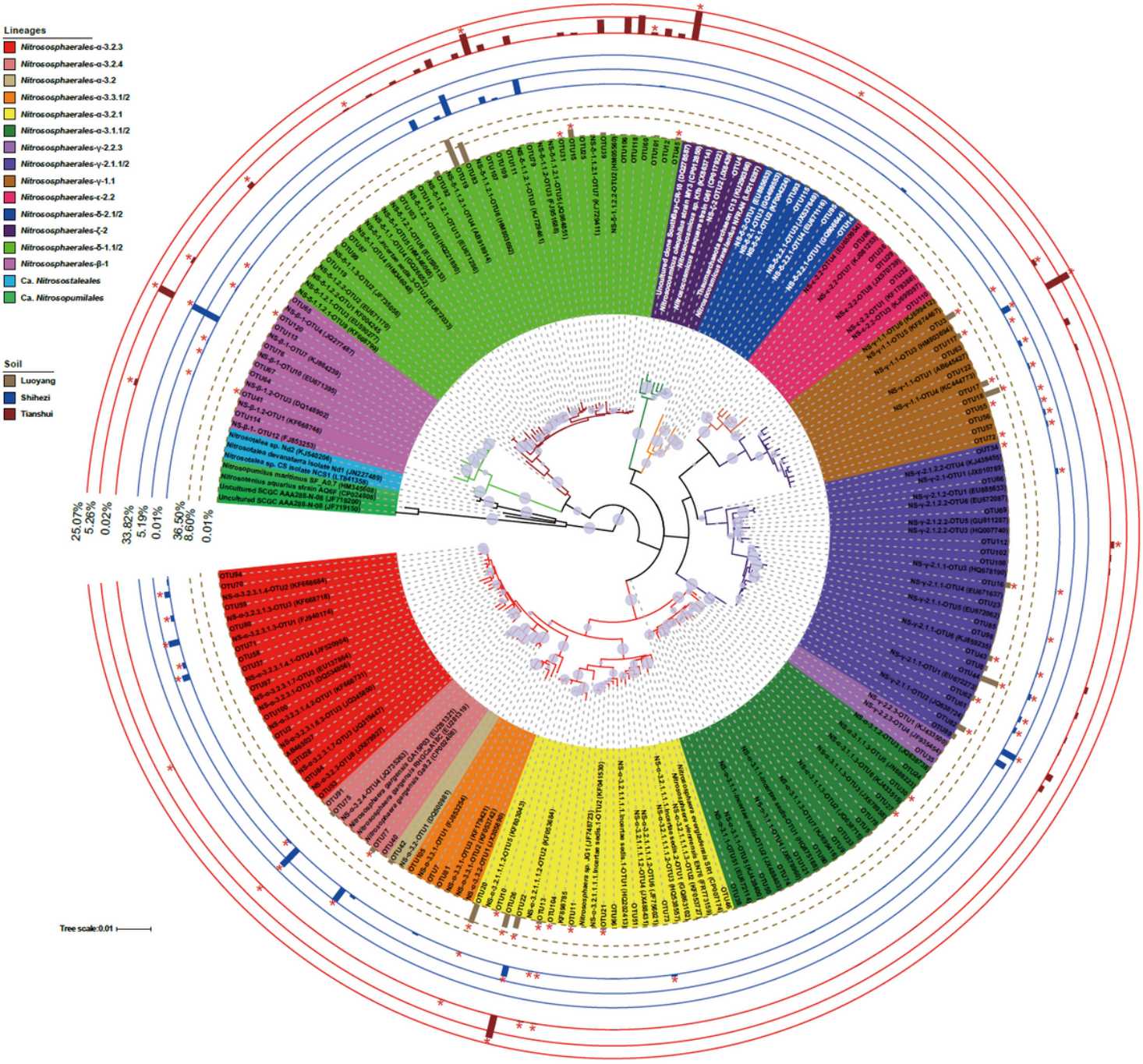

Figure 3

Phylogeny of AOA amoA gene sequences. The maximum likelihood tree was constructed with Gamma distributed with Invariant sites method (G+I) (1000 bootstrap iterations). The bootstrap values > 50% were shown. The relative abundance of each OUT across treatments was presented as bars for each tested soil. The sub-order lineages are indicated by colors of branches; lower-level lineages are indicated by different colors of outer circles. OTUs that were significantly different among treatments within soil were labeled with the red asterisk.

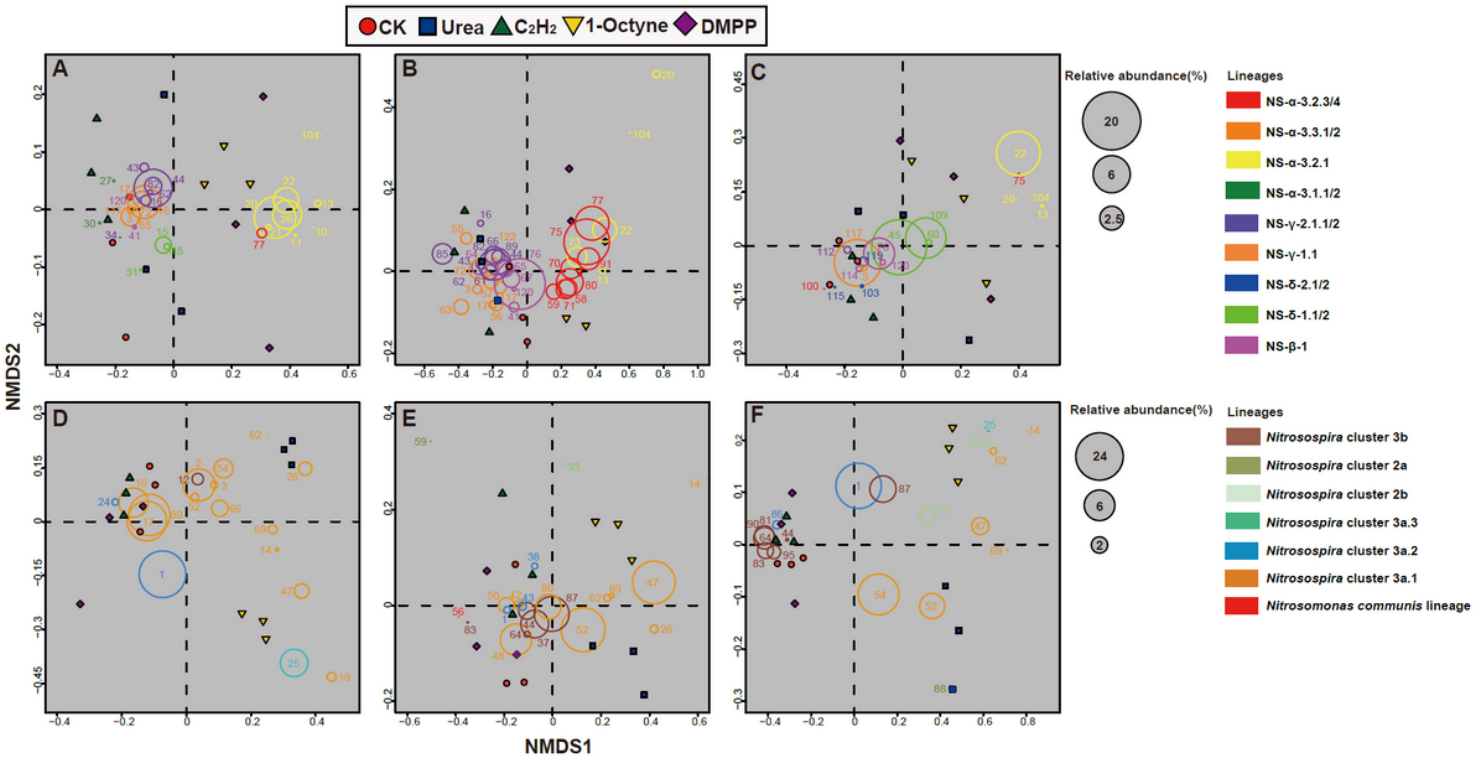

Figure 4

NMDS analysis of the community composition of AOA (A-C) and AOB (D-F) for Luoyang (A, D), Shihezi (B, D), and Tianshui (C, F). All OTUs whose relative abundances were significantly changed by treatments were shown as circles. Their relative abundances were log-scaled with the diameter of circles, and phylogenetic affiliations were mapped to different colors. NS, Nitrososphaerales.

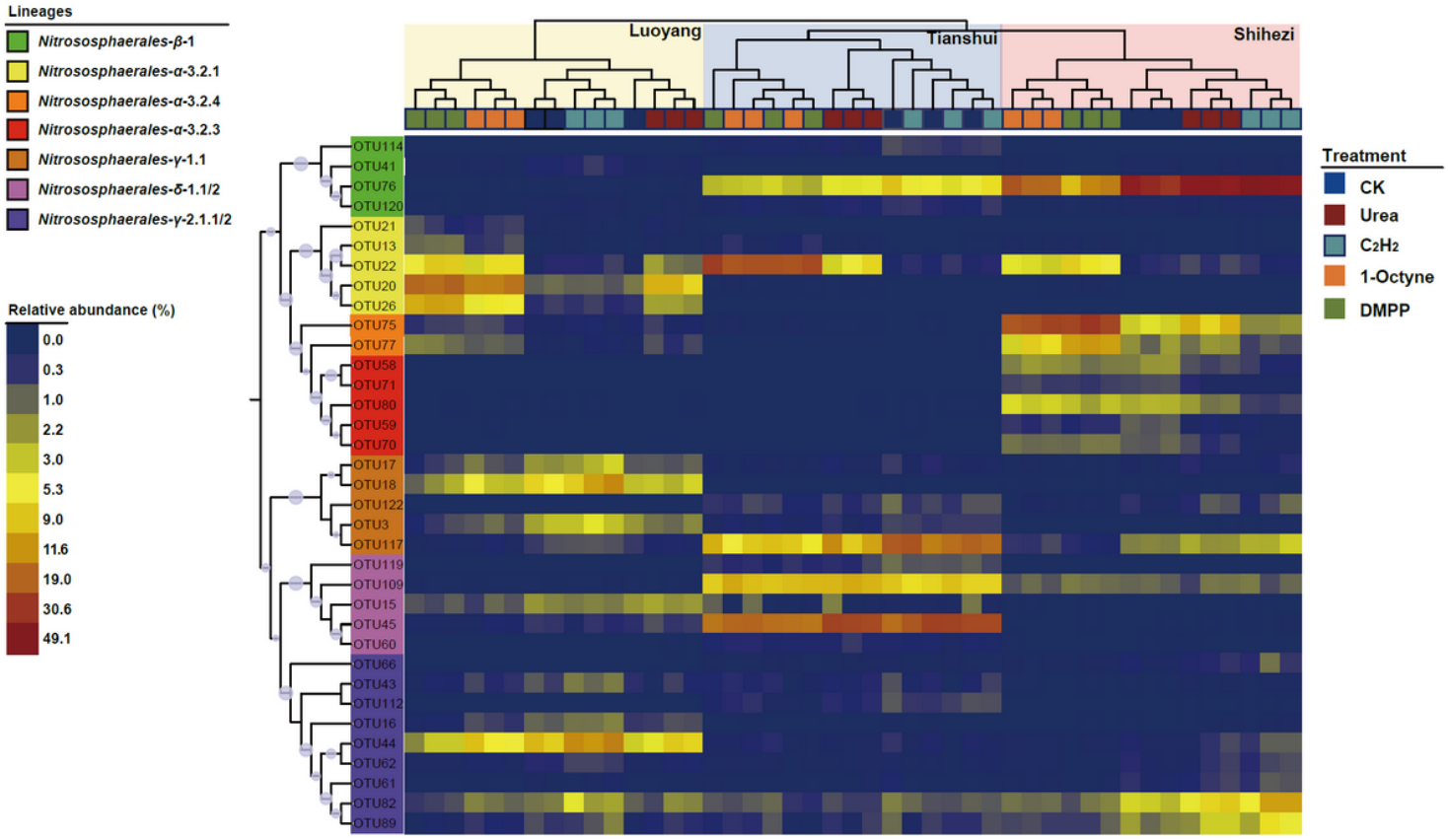

Figure 5

The composition of AOA OUTs that were significantly different among treatments within soils. The unrooted phylogenetic tree was shown on the left of the heatmap. The cluster analysis result of samples based on the Bray-Curtis dissimilarity matrix was shown above the heatmap. For clarity, only OTUs with relative abundance higher than 0.1% across all samples were shown.

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