



## Review

# Towards a more labor-saving way in microbial ammonium oxidation: A review on complete ammonia oxidization (comammox)



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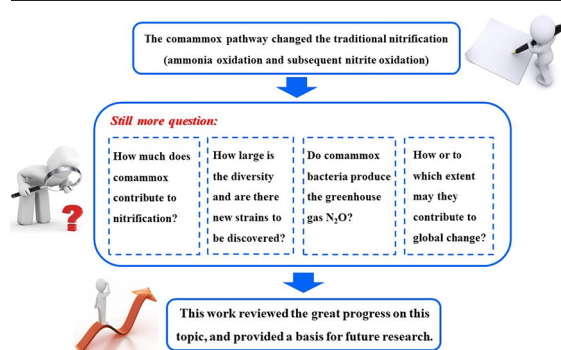
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## HIGHLIGHTS

- Comammox plays an important role in the global terrestrial nitrification process.
- Comammox is high heterogeneity and biodiversity with highest in wetlands.
- Comammox harbor N<sub>2</sub>O-production genes, but maybe not a main N<sub>2</sub>O source.
- Comammox plays a potential role in N management and global climate change.
- This article provided a critical review on comammox research in the past five years.

## GRAPHICAL ABSTRACT



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## ABSTRACT

In the Anthropocene, nitrogen pollution is becoming an increasing challenge for both mankind and the Earth system. Microbial nitrogen cycling begins with aerobic nitrification, which is also the key rate – limiting step. For over a century, it has been accepted that nitrification occurs sequentially involving ammonia oxidation, which produces nitrite followed by nitrite oxidation, generating nitrate. This perception was changed by the discovery of comammox *Nitrospira* bacteria and their metabolic pathway. In addition, this also provided us with new knowledge concerning the complex nitrogen cycle network. In the comammox process, ammonia can be completely oxidized to nitrate in one cell via the subsequent activity of the enzyme complexes, ammonia monooxygenase, hydroxylamine dehydrogenase, and nitrite oxidoreductase. Over the past five years, research on comammox made great progress. However, there still exist a lot of questions, including how much does comammox contribute to nitrification? How large is the diversity and are there new strains to be discovered? Do comammox bacteria produce the greenhouse gas N<sub>2</sub>O, and how or to which extent may they contribute to global climate change? The above four aspects are of great significance on the farmland nitrogen management, aquatic environment restoration, and mitigation of global climate change. As large number of comammox bacteria and pathways have been detected in various terrestrial and aquatic ecosystems, indicating that the comammox process may exert an important role in the global nitrogen cycle.

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## 1. Introduction

Nitrogen (N) is an essential nutrient for all organisms, and its distribution and transformation, driven by microorganisms, may restrict primary production across a large range of terrestrial and aquatic environments (Mills et al., 2004; Gruber and Galloway, 2008; Canfield et al., 2010). A century of rapid population growth and human development along with ammonia synthesis via the Haber-Bosch process has significantly altered the global N-cycle. The N cycle has been investigated for over 100 years, and with the discovery of new functional microorganisms involved in N cycling, our understanding of the processes and underlying mechanisms has steadily enhanced (Jetten et al., 1997; Zhu et al., 2008).

Nitrogen conversion mainly includes oxidation and reduction processes. The aerobic nitrification process includes two pathways in which either ammonia oxidizing archaea (AOA) or ammonia-oxidizing bacteria (AOB) oxidize ammonia (NH<sub>4</sub><sup>+</sup>) to nitrite (NO<sub>2</sub><sup>-</sup>). Then, NO<sub>2</sub><sup>-</sup> is oxidized to nitrate (NO<sub>3</sub><sup>-</sup>) by nitrite-oxidizing bacteria (NOB) (Könneke et al., 2005; Leininger et al., 2006; Jia and Conrad, 2010; Wang et al., 2011; Zhou et al., 2015). Based on kinetic considerations, Costa et al. (2006) hypothesized that organisms being capable of performing both steps of the nitrification process may exist in nature, i.e. complete oxidation of NH<sub>4</sub><sup>+</sup> to NO<sub>3</sub><sup>-</sup> by a single organism. The authors called this complete ammonia oxidation, i.e. comammox (Costa et al., 2006). Recently, following their identification, such microorganisms have been enriched, and metagenomic and physiological experiments demonstrated that they belong to the genus *Nitrospira* and have the ability to directly convert NH<sub>4</sub><sup>+</sup> into NO<sub>3</sub><sup>-</sup> (Wang et al., 2017a, 2017b; Daims et al., 2015). In addition, the discovery of comammox bacteria and their metabolic pathway has stimulated the study of their ecological significance in different ecosystems.

## 2. The widespread occurrence of comammox

The discovery of comammox challenges the traditional understanding that NH<sub>4</sub><sup>+</sup> oxidation to NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> can only be accomplished sequentially. Metagenomic analyses of the enriched comammox *Nitrospira* bacteria provided diagnostic *amoA* (and 16S rRNA) biomarkers and methods (Wang et al., 2017a, 2017b), contributing to demonstrating the wide distribution of the comammox in soils (Wang et al., 2017a, 2017b), rivers (Liu et al., 2020), lakes (Palomo et al., 2018a, 2018b), tidal flats (Wang et al., 2017a, 2017b), estuaries (Liu et al., 2020), and some artificial ecosystems, containing sewage treatment plants (Daims et al., 2015; Van Kessel et al., 2015; Roots et al., 2019; How et al., 2020), drinking water systems (Pinto et al., 2015; Wang et al., 2017a, 2017b) and freshwater aquaculture systems (Tatari et al., 2017). To the best of our knowledge, there is currently no marine comammox, which however may change with time. However, in the transient stage between land and ocean, in estuaries (Zhao et al., 2021), tidal flats (Sun et al., 2021a, 2021b), saltmarshes (Wang et al.,

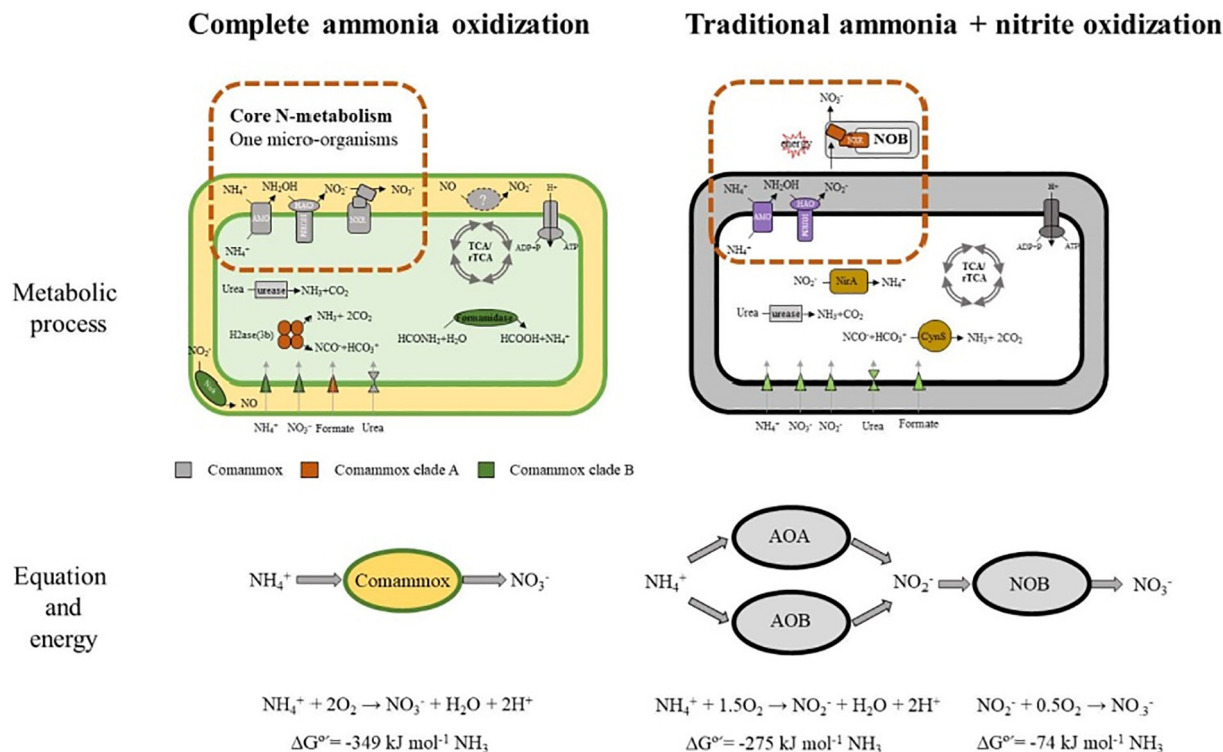
2021a, 2021b, 2021c, 2021d) and mangrove (Liu et al., 2019) ecosystems, comammox has been reported. Nevertheless, biogeographic distribution of comammox as well as its overall contribution to NH<sub>4</sub><sup>+</sup> oxidation and global N cycling across various ecosystems remains unclear.

Most recently, Shi et al. (2020) investigated the biogeography of 38 samples taken from five different habitat types including paddy fields, wheat fields, rivers, lakes and grasslands on national scale in China for the presence and composition of comammox communities. Samples were analyzed using qPCR and high-throughput sequencing approaches. Comammox bacteria were detected in all samples (*n* = 38), with *Candidatus Nitrospira nitrificans* (Ca. *N. nitrificans*) dominating the community, followed by Ca. *N. nitrosa*. Generally, in paddy fields, ammonia (NH<sub>4</sub><sup>+</sup>) was the key factor influencing comammox bacteria while in the wheat fields, rivers and grasslands of the Qinghai-Tibet Plateau, altitude was the most influential factor regulating presence and activity of comammox bacteria. In lakes, comammox bacteria presented temporal heterogeneity, occurring more abundantly in winter than in summer, especially in the riparian zone. In addition, Xia et al. (2018) also verified the widespread occurrence, high abundance and diversity of comammox in soil, sediment, sludge, and water. The results of a two-step PCR using highly degenerate primers (HYDEN) and real-time quantitative PCR (qPCR) supported the relatively high abundance of comammox in nearly half of all samples tested, which could sometimes even outnumber canonical ammonia-oxidizing bacteria (AOB). These results suggested that comammox bacteria are widespread in diverse habitats, which exhibit niche partitioning, and are affected by various environmental factors depending on habitat.

## 3. Comammox classification

The complete ammonia oxidation pathway consists of ammonium monooxygenase (fAMO), hydroxylamine dehydrogenase (HAO) and nitrite oxidoreductase (NXR) (Koch et al., 2019) (Fig. 1). Since the *amoA* genes of comammox and AOB have different gene sequences and thus form significantly separated branches in phylogenetic analysis, the *amoA* gene proves an ideal phylogenetic biomarker for identifying and quantifying comammox in complex microbial communities (Wang et al., 2017a, 2017b; Daims et al., 2015; Pinto et al., 2015).

The known comammox bacteria belong to *Nitrospira* Lineage II (Van Kessel et al., 2015). Based on the phylogenetic analysis of comammox bacterial *amoA* genes, comammox *Nitrospira* have been divided into clades A and B. Clade A can be subdivided into three clusters, respectively, clade A.1, clade A.2, and clade A.3 (Xia et al., 2018; Xu et al., 2020b) (Fig. 2). Till the present, four comammox bacterial species have been reported, namely, *Candidatus Nitrospira nitrosa*, Ca. *Nitrospira nitrificans*, Ca. *Nitrospira inopinata* and Ca. *Nitrospira kreftii* (Daims et al., 2015; Van Kessel et al., 2015; Kits et al., 2017; Sakoula et al., 2021). All these species



**Fig. 1.** Microbial metabolism and pathway of complete ammonia oxidation vs. canonical ammonia/nitrite oxidation. AMO, ammonia monooxygenase; HAO, hydroxylamine dehydrogenase; HURM, hydroxylamine-ubiquinone reaction module; NXR, nitrite oxidoreductase; NHase, nitrile hydratase; NirK, Cu-dependent nitrite reductase; FDH, formate dehydrogenase; H<sub>2</sub>ase, hydrogenase; CynS, cyanate hydratase; NirA, ferredoxin-nitrite reductase. The question mark indicates that the exact pathway of nitrite formation from NO is uncertain.

belong to comammox clade A.1 (Xia et al., 2018). Recently, Wang et al. (2017a, 2017b) extracted another three species (metagenome-assembled genomes [MAGS] *Nitrospira* sp. sg-bin1, sg-bin2 and sg-bin4) from the metagenome data of tap water. Although these three candidate species have yet to be named, they are generally considered to belong to clade A2.

At present, the reports on comammox mainly concentrate on ecosystems such as sewage treatment plants, drinking water plants, dryland farmlands, paddy fields, forests, grasslands, sediments, groundwater and wetlands. Totally 1330 comammox bacterial sequences were extracted from these systems and adopted for performing a pedigree analysis using known sequence divergence. It could be found that these comammox clones or macro gene sequences did not display spatio-temporal heterogeneity and temperature heterogeneity, which could indicate low diversity of comammox bacteria. Based on the different ecosystem sequences, we found that comammox *Nitrospira* clade A is more widely distributed than clade B. In clade A, clade A.1 is more widely distributed than clade A.2 and more widely distributed in comparison with clade A.3. Diversity and abundance were lower in clade B than in clade A.

The biodiversity of comammox *Nitrospira* in wetland systems is the highest based on the biodiversity index or OTU number, while that of rivers and lakes remains the lowest. In artificial ecosystems, the diversity of comammox was the highest in wastewater treatment plants and the lowest in drylands. Clades A and B are distributed in different ecosystems. Besides, so far, these distributions are rather inconsistent. Comammox *Nitrospira* clades A.1 and A.2 are dominant in both natural and artificial environments. However, clade B was not detected in pasture systems with only clade B being detected in forest systems.

#### 4. Niche distribution of comammox species

Through comparative genomes analysis, it is obvious that the genomic differences of comammox *Nitrospira* are mainly due to the niche adaptability of each species (Camejo et al., 2017). The comammox genome has a

complete set of genes encoding ammonia and nitrite oxidation. This usually leads to comammox *Nitrospira* that has a high affinity for ammonia than a two-step process with 2 different groups of nitrifiers involved. Compared with the *amoA* genes of AOA and AOB, the *amoA* genes of comammox bacteria showed higher diversity, indicating that the comammox process may prevail in competition with other ammonia oxidizers, especially in oligotrophic environments (Costa et al., 2006; Koch et al., 2019). In addition, an increase in comammox abundance was also detected in fertilized soils (Orellana et al., 2018), suggesting that comammox may also play an important role under non-oligotrophic conditions.

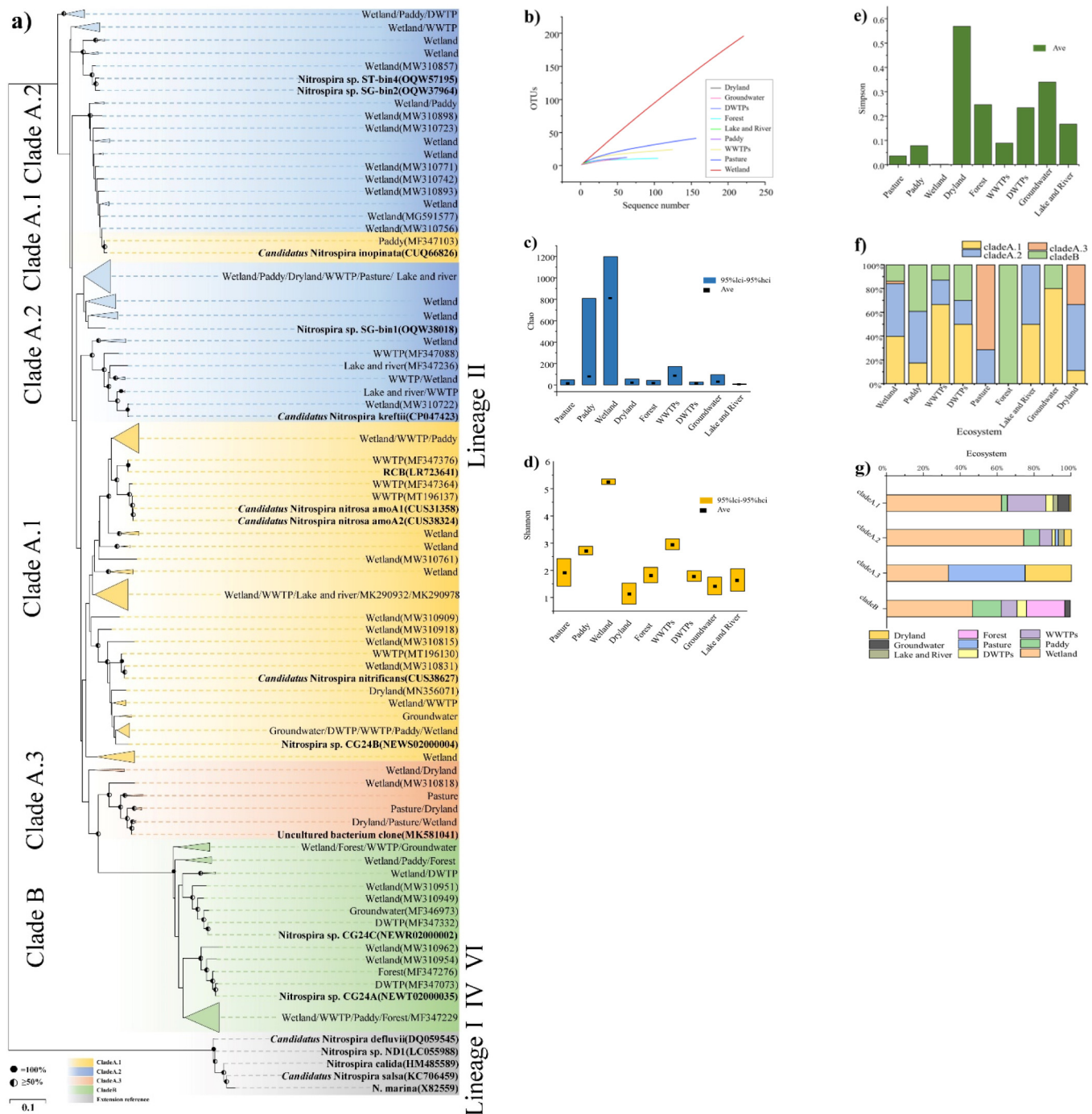
Increases in substrate availability will substantially influence the comammox bacterial population structure. Current research reveals that the application of nitrogen fertilizer significantly increases the abundance of comammox clade A (Liu et al., 2019; Li et al., 2019). Moreover, clade A.1 is the dominant cluster in natural (fresh water and groundwater (Wang et al., 2017a, 2017b) and artificial aquatic ecosystems (drinking water (Wang et al., 2017a, 2017b), activated sludge (Wang et al., 2021a, 2021b, 2021c, 2021d) and rapid sand filters (Fowler et al., 2018)), while clade A.2 is more common in soil and terrestrial sediment (Xu et al., 2020b; Li et al., 2020). Till the present, the only successfully cultured pure comammox strain is *Candidatus Nitrospira inopinata*, belonging to clade A.1 (Kits et al., 2017).

Clade B dominates in soils, and it contributed more to nitrification in soils not amended with  $\text{NH}_4^+$  (Wang et al., 2019). This may be due to the presence of an ammonium transporter in clade B and may indicate that clade B has a higher affinity for  $\text{NH}_4^+$  than clade A (which possesses Rhesus-type transporters) (Koch et al., 2019). As a result, soil with low ammonia content may be more conducive to the ammonia oxidation reaction conducted by comammox clade B.

#### 5. Genomics of comammox bacteria

Genomic studies of comammox *Nitrospira* underline its oligotrophic live style. Characterized by the presence of the *amoCAB* gene cluster and the





**Fig. 2.** Phylogenetic and diversity analysis of comammox in different ecosystems. WWTPs and DWTPs are the abbreviations of Wastewater Treatment Plants and Drinking Water Treatment Plants respectively. a) Phylogenetic analysis of the comammox *amoA* gene among different ecosystems constructed by neighbor joining methods. All sequences were collected from the NCBI database. Bootstrap values: black circles (100%), half black circles ( $\geq 50\%$ ). The scale bar represents 10% nucleic acid sequence divergence; b) Rarefaction curves of the number of OTUs in each ecosystem; c) Chao value of microbial community in each ecosystem; d) Shannon value of microbial community in each ecosystem; e) Simpson diversity indices among different ecosystems with OTU similarity thresholds of 97%; f) Proportion of each clade in different ecosystems; g) Proportions of OTU-derived ecosystem types within the four clades. All the clone sequences of comammox *amoA* gene from various ecosystems were downloaded from National Center for Biotechnology Information Search database (NCBI) (Supplementary Table S1 and S2).

*ntrAB* genes, the crucial metabolic feature of the comammox *Nitrospira* genome is complete nitrification (Palomo et al., 2018a, 2018b). The genomic comparisons have revealed the presence of highly conserved respiratory complexes I-V, along with the (reverse) tricarboxylic acid cycle for carbon fixation, like in the sublineage II *Nitrospira* NOB (Poghosyan et al., 2019). The ammonium metabolism is further supported by genes for ammonium

and urea transporters. Kits et al. (2017). proposed favorable kinetics as a key driver for the wide distribution of comammox. Comammox bacteria have a high affinity for ammonia, a low maximum rate of ammonia oxidation, and a high growth yield compared to canonical nitrifiers. *Nitrospira inopinata*, which has the highest substrate affinity of all ammonia oxidizers analyzed. Moreover, the species also exhibits high metabolic diversity.

Genomic data from long-term comammox enrichment indicated that comammox bacteria encoded genes that had higher nitrite tolerance (Zhao et al., 2021). Consequently, comammox genomes are acclimatized for oxidizing the ammonium in adverse conditions such as oxygen-deficient and nutrient-limited environments. Moreover, the presence of formate and hydrogen oxidation enzymes in comammox clades A and B may help them survive under starvation (Leung et al., 2021).

Nevertheless, different from the canonical ammonia-oxidizing microbes, the comammox genome is devoid of nitric oxide reductase genes. In addition, genes for cyanate hydratase and assimilatory octaheme nitrite reductase, and genes responsible for reverse electron transport from nitrite appear to be absent in the comammox genome. Therefore, comammox bacteria are likely not capable of using external nitrite as an energy and nitrogen source. Palomo et al. (2018a, 2018b) also discovered that comammox bacteria lacked the gene *nirA*, which encodes nitrite reductase, and that it is impossible for comammox bacteria to grow on or metabolize nitrite. On other comammox bacteria, *Ca. Nitrospira nitrosa*, Camejo et al. (2017) also found a similar binning in the reactor when nitrite was applied as the sole electron donor.

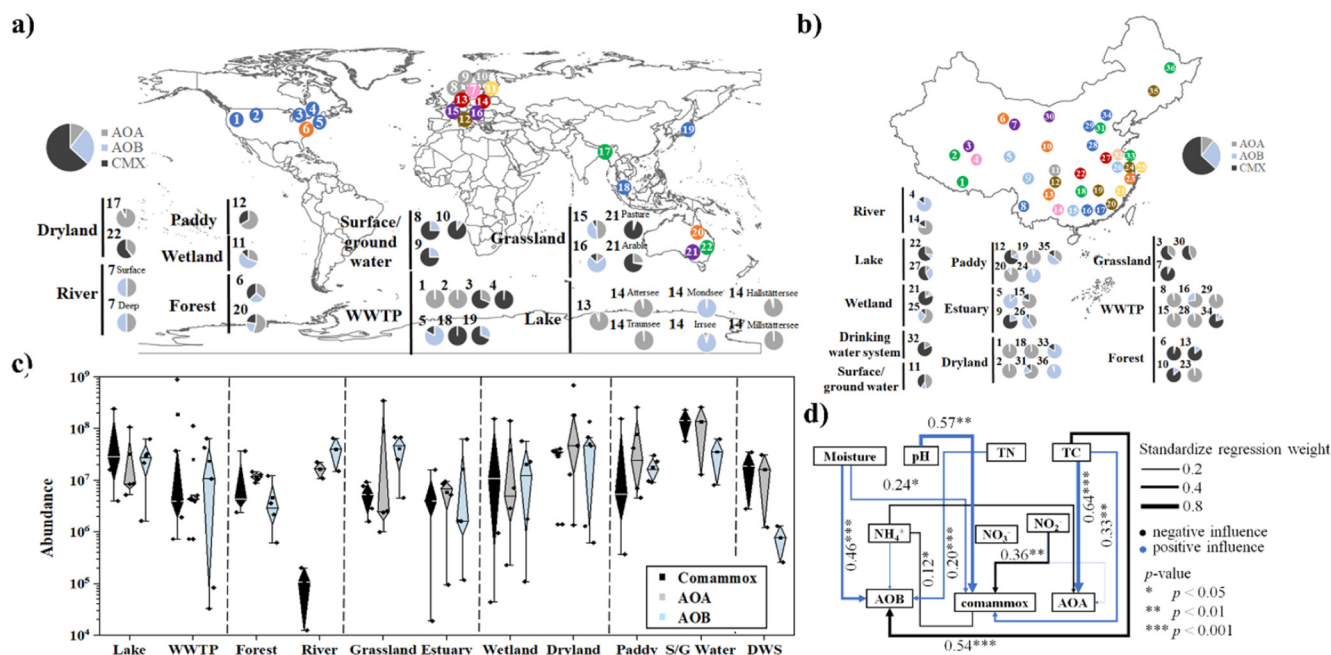
Comparative genomic analysis of the typical comammox clade A and clade B revealed the specificity metabolic substrate response (Supplementary Table S3). In terms of metabolic substrates, comammox clade A was found to have more metabolic diversity than clade B. In terms of comammox clade A, it not only utilized ammonia but also urea (*ureABCD*, *urtABCDE*), cyanate (*cyns*) and hydrogen (*3b<sup>[NiFe]</sup>* Hydrogenase gene) as substrates for chemolithoautotrophic complete nitrification (Van Kessel et al., 2015; Palomo et al., 2018a, 2018b; Camejo et al., 2017). Apart from that, it can metabolize sulfonamides and some trace pollutants through the unique AMO structure (Daims et al., 2015; Han et al., 2019; Zhou et al., 2019). For comammox clade B, it has formate dehydrogenase (*fdh*) gene, proving the potential process of formic acid oxidation (Palomo et al., 2018a, 2018b).

Assembled comammox *Nitrospira* MAGs had high pairwise average nucleotide identity (ANI) with genes of *Candidatus Nitrospira nitificans* and *Candidatus Nitrospira inopinata*, which may play nonnegligible impact on wastewater treatment systems (Yang et al., 2020; Wang et al., 2017a, 2017b). Meanwhile, for natural ecosystems, *Candidatus Nitrospira inopinata* – like comammox bacteria also plays a major role in both planktonic and benthic environment in Yangtze River (Liu et al., 2020). Phylogenetic analysis of 16S rRNA and *amo* gene revealed that the majority retrieved comammox *Nitrospira* belonged to clade A despite parts of the similarity below 95%. MAGs analysis also suggested that more novel comammox *Nitrospira* species need to be studied.

## 6. Ecological niche differentiation among comammox, AOA and AOB

Unravelling the ecological niche differentiation between comammox *Nitrospira*, AOA and AOB plays an essential role in understanding global N cycling and dynamics. Although genomic and physiological studies have suggested that comammox *Nitrospira* may outcompete AOA and AOB under oligotrophic conditions, there still lacks empirical evidence in terrestrial ecosystems. The biogeographic distribution and contribution to  $\text{NH}_4^+$  oxidation and global N cycling in natural ecosystems remain unclear.

At present, reports on comammox abundance mainly concentrate on ecosystems such as sewage treatment plants, drinking water plants, dryland farmlands, paddy fields, forests, grasslands, sediments, groundwater and wetlands. We extracted the comammox abundance reported in these systems from totally 95 publications and compared it with those of related traditional nitrifying microorganisms. Abundance of comammox was found to be highly heterogeneous at many sampling points in diverse systems, and to vary greatly between different ecosystems. Comammox has the highest abundance in groundwater systems (70.4%) and the lowest abundance in rivers (7.6%). Comammox abundance exhibits no spatial covariation with latitude, indicating quite a temperature adaptability of comammox



**Fig. 3.** The composition and abundance of microorganisms (AOA, AOB, Comammox = CMX) involved in ammonia oxidation processes in different ecosystems and the influence of biogeochemical factors on the three types of microorganisms. a) Locations of 22 different ecosystems in the world (excluding China) and the proportion of microorganisms in ammonia-oxidizing processes; Different color location codes represent different types of ecosystems and their location distribution characteristics on the world map, and the same as China map. WWTP = wastewater treatment plants; b) Locations of 36 different ecosystems in China and the corresponding proportions of the three ammonia-oxidizers; c) The abundance of three types of ammonia oxidizers in each ecosystem, DWS = drinking water systems, S/G Water = surface /ground water; d) Structural equation model analysis of the influence of biogeochemical factors on the three types of microorganisms, TN = total nitrogen, TC = total carbon. All the data in papers published before October 2021 were obtained by searching ISI Web of Science database and Chinese CNKI platform with query term “comammox”. Studies including abundance of comammox, AOA and AOB measured by qPCR or metagenome were selected (Supplementary Tables S5 and S6).

bacteria. Biogeochemical parameters, rather than biogeographic factors, are considered as the main determinants of comammox abundance. Structural equation model analysis showed that pH was the most important factor influencing comammox abundance on a global scale. In estuaries, besides ammonia, we also find that salinity is another important factor (negatively) affecting the abundance of comammox bacteria ( $R = -0.840^{**}$ ,  $P = 0.000$ ) (Supplementary Fig. S1 and Table S4).

In a comparison of different ecosystems, it could be found that the abundance of comammox in more than 50% ( $n = 111$ ) of samples exceeded the abundances of AOA or AOB. Highest abundance was observed in forest systems (81.8%) and the lowest in drylands (12.5%). According to correlation analysis, in groundwater systems, the abundance of comammox was significantly higher than that of AOA and AOB. In dryland and paddy field systems, the abundance of traditional ammonia oxidizing microorganisms is higher than comammox. In addition, correlation analysis also showed that in forest, estuary and river systems, pH is the most important influencing factor, while in dryland, paddy and drinking water systems,  $\text{NH}_4^+$  concentration is the most influential parameter.

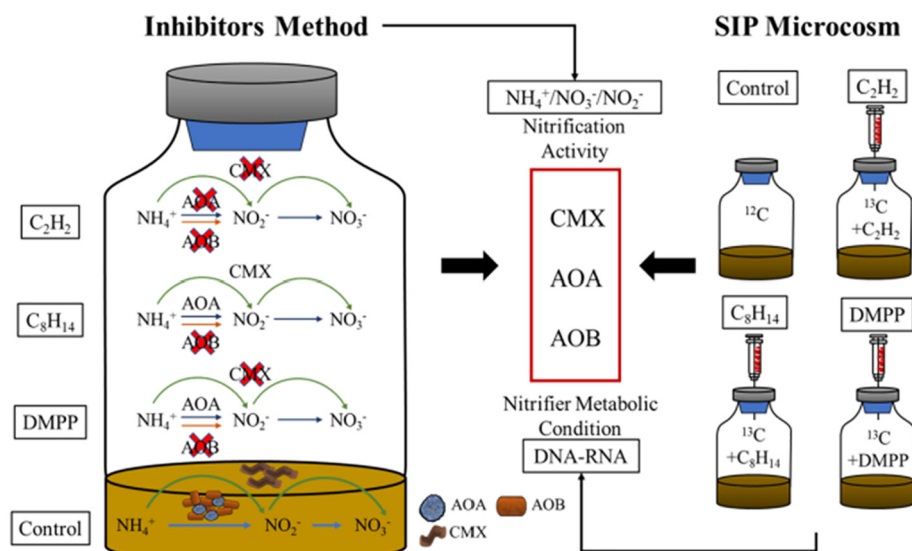
The relative abundance of comammox bacteria in identical ecosystems was significantly different in a variety of regions. We found the proportion of comammox bacteria to total ammonia-oxidizing microorganisms in Chinese artificial systems (including paddy fields, drylands, sewage treatment plants, drinking water systems, surface/ground water-fed rapid sand filters) was around 17.9%, which was lower than the proportion found in natural ecosystems (48.8%). Based on the structural equation model in Fig. 3, the concentrations of ammonia and nitrite are positively correlated with the abundance of AOA and AOB bacteria respectively, showing negative correlation with the abundance of comammox bacteria. The average concentration of ammonia and nitrite in artificial ecosystems is approximately  $11.35 \text{ mg kg}^{-1}$  and  $0.17 \text{ mg kg}^{-1}$ , respectively, and in natural ecosystems, concentration is approximately  $9.58 \text{ mg kg}^{-1}$  and  $0.12 \text{ mg kg}^{-1}$ , respectively. Hence, the proportion of comammox bacteria in artificial ecosystems is lower, which is consistent with previous results, indicating that comammox is more suitable for oligotrophic environments with low nitrogen (Wang et al., 2019). By contrast, the proportion of comammox bacteria within total ammonia-oxidizing microorganism in artificial

systems globally (excluding China) is 53.4%, which is higher than the proportion found in natural ecosystems (16.9%). Specifically, the average proportion of comammox bacteria in sewage treatment plants, surface/ground water-fed rapid sand filters and grassland ecosystems was 81.3%, 79.9% and 46.8%, respectively. In forest ecosystems, the proportion of comammox bacteria to total ammonia-oxidizing microorganisms across the world ranged from 3.7% to 85.1%. With positive correlation to soil pH, and negative correlation to the  $\text{NH}_4^+$ , these ratios were significantly related to physicochemical properties. In addition, we found a similar distribution trend in grassland ecosystems.

## 7. Comammox activity measurement

In order to investigate the contribution of comammox to nitrification in different niches, comammox bacterial activity needs to be measured. Because the substrates and products of comammox reaction remain the same as those of the traditional ammonia oxidation reaction, it is challenging to determine them by adopting the established  $^{15}\text{N}$  isotope labeling method. Currently, the methods most suitable are using inhibitors methods or DNA stable-isotope probing (DNA-SIP) method.

Wang et al. (2020a, 2020b, 2021a, 2021b, 2021c, 2021d) developed the double inhibitor method to distinguish the comammox, archaeal nitrification and bacterial nitrification processes as well as to evaluate the contribution of comammox to ammonia oxidation in dryland farmland soil and wetland sediments (Wang et al., 2020a, 2020b; Wang et al., 2021a, 2021b, 2021c, 2021d). The method employed two inhibitors consisting of potassium chlorate ( $\text{KClO}_3$ ) and Octyne ( $\text{C}_8\text{H}_{14}$ ) (Fig. 4).  $\text{KClO}_3$  can inhibit the  $\text{NO}_2^-$  oxidation and the  $\text{NO}_3^-$  reduction processes (Xu et al., 2011).  $\text{C}_8\text{H}_{14}$  can selectively distinguish bacteria and archaea ammonia monooxygenase (AMO) in soil, for instance, it inhibits AOB without affecting AOA, and the inhibition of AOB is irreversible (Taylor et al., 2013). Although this method is beneficial, it still has some limitations as the abilities of  $\text{C}_8\text{H}_{14}$  to inhibit the *amoA* gene of comammox and  $\text{KClO}_3$  to inhibit the  $\text{NO}_3^-$  reductase of comammox have not yet been well investigated. Recently, Sakoula et al. (2021) developed an activity-based protein profiling protocol specifically and efficiently staining AOB and comammox, which



**Fig. 4.** Sketch plot showing currently used methods in measuring comammox activity. Different inhibitors are used to inhibit the activities of different ammonia oxidizers (comammox = CMX, AOA, and AOB). The potential rates of these processes were calculated using the linear regression of substrate ( $\text{NH}_4^+ - \text{N}$ ,  $\text{NO}_2^- - \text{N}$ ,  $\text{NO}_3^- - \text{N}$ ) concentration over time in different treatments (left panel). The  $^{12}\text{CO}_2$  and  $^{13}\text{CO}_2$  substrates were replenished in the inhibitor – treatments to measure the metabolic activities of different ammonia oxidizers by DNA/RNA SIP methods (right panel). Both the activity and contribution to nitrification from comammox, AOA, and AOB could be measured.



could directly convey information on the functional potential of them (Fig. 5).

In addition to the double inhibitor method, DNA-SIP technology has also been adopted for evaluating the activity of comammox and its contribution to ammonia oxidation. Li et al. (2019) demonstrated the important contribution of comammox in farmland soil nitrification by combining acetylene ( $C_2H_2$ ) and  $C_8H_{14}$  inhibitors with DNA-SIP technology.  $C_2H_2$  can effectively and simultaneously inhibit the *amoA* gene expression in AOA and AOB (Offre et al., 2009), while  $C_8H_{14}$  can selectively inhibit the bacterial *amoA* gene expression (Taylor et al., 2013). It was first determined whether the two inhibitors would make an effect on comammox through different treatments. Then, DNA-SIP technology was used to combine different C markers ( $^{12}CO_2$  and  $^{13}CO_2$ ) with different inhibitor treatments to determine the metabolic activities of comammox, AOA and AOB. This method proved that  $C_2H_2$  generates a good inhibitory effect on comammox clade A, indicating that the comammox process in soil can be controlled by adding inhibitors.

Zheng et al. (2019) measured the transcriptional activity of comammox using the RNA transcription method, finding that comammox bacteria provide a high contribution for nitrification in sewage treatment plants. Based on an RNA perspective, the abundance of mRNA corresponding to the

*amoA* gene in comammox, AOA and AOB was measured to evaluate the activity of the corresponding ammonia oxidizing microorganisms. The proposed method is relatively simple, and would contribute comprehensively to measuring the activity and contribution of comammox when combined with other inhibitor methods.

Gülay et al. (2019) simultaneously adopted DNA-SIP, RNA-SIP technologies and the inhibitor method for exploring the activity of comammox in a groundwater biofilter, and highlighted the dominant role of comammox in the nitrification process of this system. The application of the two SIP technologies can simultaneously detect comammox bacteria with active or slow growth rates. Besides, the inhibitors allylthiourea (allylthiourea, ATU) and  $KClO_3$  were applied to inhibit ammonia oxidizing microorganisms and nitrite oxidizing microorganisms, respectively (Tatari et al., 2017; Ginestet et al., 1998). This method considers the changes of comammox activity at both DNA and RNA levels. However, there still exist some limitations. Studies have shown that ATU only exerts a good inhibitory effect on AOB, while the effect on AOA is not obvious (Ginestet et al., 1998; Taylor et al., 2010), and thus it is impossible to accurately distinguish comammox, AOA and AOB. At present, other inhibitors such as  $C_2H_2$  and  $C_8H_{14}$  can be employed to enhance selectivity in inhibiting the three ammonia oxidizing microorganisms.

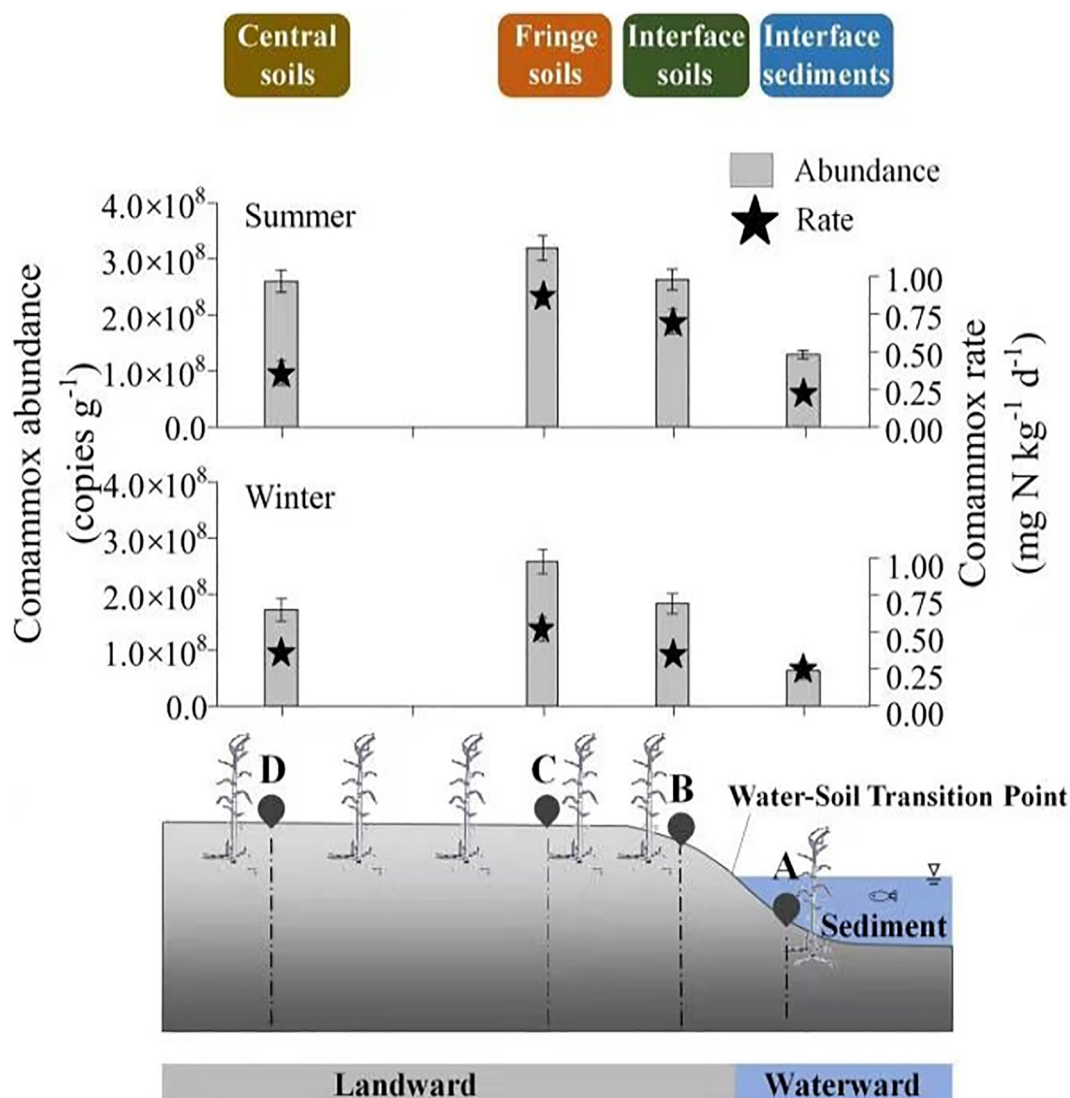


Fig. 5. Comammox hotspot occur at the fringe soils in riparian systems. The letters "A-D" represent riparian interface sediments (site A), riparian interface soils (site B), riparian fringe soils (site C), and riparian central soils (site D).

Currently, the reports on the activity and contribution of comammox are extremely limited. This may be due to fact that the inhibitor method is newly developed and has not been extremely applied. Another important consideration is that the ultra-high speed centrifuge required for SIP experiments is not ubiquitously available, limiting the application of this method. However, based on the existing reports, it is apparent that comammox plays a very important role in ammonia oxidation in terrestrial soil and aquatic ecosystems, with its contribution being even higher than AOA and AOB in some specific regions, so-called comammox “hot-spots”.

## 8. The comammox hot-spot

During the comammox process, nitrogen is directly oxidized from the lowest valence state ( $\text{NH}_4^+$ ) to the highest valence state ( $\text{NO}_3^-$ ). Hence, a large redox gradient would promote the occurrence of comammox. An oxic–anoxic interface, characterized by distinct redox gradients, represents the ideal habitat for the comammox process. In terrestrial ecosystems, the most typical anoxic aerobic interface is the riparian system.

Natural riparian zones are interfaces between terrestrial and aquatic ecosystems, and are also among the most diverse, dynamic, and complex habitats in terrestrial systems (Naiman and Decamps, 1997; McClain et al., 2003). These interfaces show unique characteristics defined by spatial and temporal scales and by the strength of interactions between adjacent ecosystems (Naiman and Decamps, 1997). As a result, riparian zones possess specific physicochemical and biotic properties as well as energy and material flows (Gregory et al., 1991). Due to water level fluctuations, riparian zones are frequently subjected to variable anoxic/aerobic conditions forming strong redox driving forces (Verhoeven et al., 2006; Zhu et al., 2013b). Recently, Wang et al. (2021a, 2021b, 2021c, 2021d) found that fringe soils in the riparian zone comprise a comammox hotspot, where the abundance ( $2.58 \times 10^8$  copies  $\text{g}^{-1}$ ) and rate ( $0.86 \text{ mg N kg}^{-1} \text{ d}^{-1}$ ) of comammox were not only higher than at other sampling sites but also higher than those of other ammonia oxidation processes. Thus, riparian hotspots yielded the highest comammox bacterial abundance and activity reported in nature.

## 9. Comammox, $\text{N}_2\text{O}$ production and global climate change

Recent studies indicated that global  $\text{N}_2\text{O}$  emissions are primarily driven by anthropogenic sources and have increased by 30% in the Anthropocene (Tian et al., 2016). It appears that up to 87% of this increase may be resulted from agricultural fertilizer emission. The traditional view is that  $\text{N}_2\text{O}$  is mainly produced in heterotrophic denitrification processes due to an insufficient carbon source, leading to incomplete denitrification and the cumulative release of  $\text{N}_2\text{O}$  (Beaulieu et al., 2011; Woodward et al., 2009; Vilain et al., 2014). Increasing evidence demonstrates that ammonia oxidation pathways, including nitrifier nitrification, nitrifier denitrification, and nitrification-coupled denitrification, could also produce a large amount of  $\text{N}_2\text{O}$  (Zhu et al., 2013a; Kool et al., 2010; Kool et al., 2011; Wrage et al., 2018; Shi et al., 2017). Among them, the main functional genes and enzymes involved primarily contain the hydroxylamine oxidoreductase (*hao*) and nitrate reductase (*nirK*) genes.

Current process–based  $\text{N}_2\text{O}$  models simply apply the  $\text{N}_2\text{O}/\text{N}_2$  ratio to represent the  $\text{N}_2\text{O}$  production rate during the denitrification process (Maavara et al., 2019; Fu et al., 2018; Marzadri et al., 2014). However, model parameters associate much better with microbially mediated  $\text{N}_2\text{O}$  production than nitrogen inputs. The possibility that the nitrification process contributes significantly to  $\text{N}_2\text{O}$  production is hardly considered, although there is increasing evidence that ammonia oxidation may contribute much more to  $\text{N}_2\text{O}$  production than denitrification, particularly at low  $\text{O}_2$  condition.

Numerous studies have proved that AOB and AOA are major producers of  $\text{N}_2\text{O}$  (Kozłowski et al., 2016; Liu et al., 2017; Wrage-Mönnig et al., 2018), while there is no clear report on how much the production of  $\text{N}_2\text{O}$  by comammox. Nevertheless, the comammox bacteria do harbor the enzymes

and functional genes related to  $\text{N}_2\text{O}$  production. Recently, Kits et al. (2019) reported that comammox *N. inopinata* may produce  $\text{N}_2\text{O}$  from abiotic hydroxylamine  $\text{NH}_2\text{OH}$  conversion that is comparable to AOA, but much low as for AOB, implying that comammox is not a main  $\text{N}_2\text{O}$  source.

Comammox, which may be considered as an “green process”, has been extensively detected in natural and engineered systems (Wang et al., 2021a, 2021b, 2021c, 2021d; Wang et al., 2020a, 2020b; Wang et al., 2017b; Annavajhala et al., 2018), sometimes at considerable abundance (Wang et al., 2021a, 2021b, 2021c, 2021d; Van Kessel et al., 2015; Daims et al., 2015; Palomo et al., 2018a, 2018b). The competition of the three ammonia oxidizers may influence the  $\text{N}_2\text{O}$  emission. For example, adoption of conditions that favor the growth of comammox over AOB in engineered systems and in soils may lower nitrification-dependent  $\text{N}_2\text{O}$  emissions, aiming to impact the global change.

## 10. Comammox and anammox

Comammox is closely associated with another nitrogen cycle process, namely, anaerobic ammonium oxidation (anammox) (Van Kessel et al., 2015; Mulder et al., 1995). In fact, Van Kessel et al. (2015) originally discovered comammox bacteria while enriching for anammox bacteria from an aquaculture filter. Under low oxygen, and low ammonium conditions, as confirmed recently, comammox and anammox bacteria may rather cooperate than compete for the substrates (Gottshall et al., 2021; Shao and Wu, 2021; Wu et al., 2019). A bench-scale bioreactor with continuous stirring was operated for more than 1000 days with limited oxygen supply to achieve efficient nitrogen removal ( $70.1 \pm 2.7\%$ ) at a low ammonium loading of  $35.2 \text{ mg-N L}^{-1} \text{ d}^{-1}$ . Under the above-mentioned conditions, comammox species dominated the ammonia-oxidizing community, with an abundance as high as  $89.2 \pm 7.9\%$  of total prokaryotic *amoA* copies (Shao and Wu, 2021). Microscale (within hydrogel beads) studies found that comammox-anammox consortia can form a good niche separation and symbiosis relationship (Gottshall et al., 2021). As a result, comammox microbes occupy the aerobic outer zone while anammox bacteria inhabit the oxygen-deficient layer. Comammox-anammox co-cultures present signs of cooperation, resulting in nearly complete  $\text{NH}_4^+$  removal of  $\text{NH}_4^+$ . Beside low-strength ammonium-containing wastewater, anammox and comammox can also co-occur in high-strength substrates. In a sludge digester liquor with a concentration of  $2100\text{--}2200 \text{ mg L}^{-1}$ ,  $\text{NH}_4^+$  was removed with the efficiency of 98.82%, due to partial-nitrification (55.11%), anammox (18.28%), and comammox (25.43%) (Wu et al., 2019). The obtained results identify comammox-associated partial nitrification–anammox as a potential biotechnological application for treating low- and high-strength ammonium-containing wastewater.

In addition, the coexistence of comammox and anammox was also detected in natural ecosystems. Previously, we proved that anammox occurs extensively in inland waters (Zhu et al., 2015; Wang et al., 2020a, 2020b), where low oxygen and low ammonia-nitrogen concentration are not the limiting factors of comammox. Thus, we may infer that comammox and anammox are likely to coexist extensively in natural water bodies. Recently, the coexistence of comammox and anammox was detected in riparian zones due to that their strong heterogeneity exhibits spatial differentiation, leading to establishment of microbial hot-spots. Anammox develops hot-spot zones in anoxic sediments (Zhu et al., 2013b), while comammox develops a hot-spot in aerobic fringe soils (Wang et al., 2021a, 2021b, 2021c, 2021d). Thus, natural ecosystems exhibit characteristics in ammonium oxidation compatible to results obtained by hydrogel beads experiments.

For technical applications, in particular wastewater treatment, a utilization of comammox complementary to anammox may prove to be highly beneficial. The biggest obstacle to the application of the anammox process in the field of sewage treatment refers to the production of nitrite (Zhu et al., 2008; Zhu et al., 2013b; Zhu et al., 2011a; Zhu et al., 2011b; van Dongen et al., 2001). Although various nitrite accumulation methods, e.g. low dissolved oxygen (DO) control, have been developed, routine application of the anammox process was still extremely limited in industrial



wastewater with high-strength ammonium loading (van Dongen et al., 2001; Peng and Zhu, 2006; Ma et al., 2020; Xu et al., 2020a; Lu et al., 2021a; Lu et al., 2021b). A potential utilization of the comammox process avoids these limitations, as it is independent of with a high tolerance towards nitrite.

## 11. Future directions

There are still several methodological problems to be solved in the basic scientific research of comammox. Regarding lipidomic, it has not been determined whether comammox bacteria contain any unique lipids and whether related analysis methods need to be developed. In terms of molecular biology, specific functional genes and enzymes of comammox bacteria and related analysis methods remain unclear. Although inhibitor, SIP and their combined methods have been developed for activity determination, each method still presents some limitations.

Sometimes, we even obtain completely opposite results. Recently, Wang et al. (2021a, 2021b, 2021c, 2021d) reported that the activity of comammox may be more inhibited by the long-term nitrogen fertilization than canonical ammonia oxidizers in agricultural soils. In addition, it is also carried out in the farmland soil with long-term fertilization. Results indicate that fertilization can stimulate nitrification, and comammox had higher abundance of *amoA* functional gene than AOB and AOA in paddy soils (Liu et al., 2019). Even though comammox bacteria clade A has an oligotrophic lifestyle, little is known about clade B. However, an increasing number of reports prove that the abundance of comammox increases when substrate concentration increases. This information emphasizes the potential for comammox applications although there remains a long way to go.

Based on the above discussions, our knowledge of terrestrial and aquatic comammox bacteria, and the magnitude and controls of the process, has evolved in recent years. However, there still exist a lot of questions. In fact, much of our understanding is derived from studies in waste water treatment systems. The *Nitrospira* comammox in wastewater treatment systems appears to be similar to those found in terrestrial and aquatic environments. Nevertheless, the overall stoichiometry of the comammox process in the various environments has not been identified.

Sewage treatment engineers around the world would consider another problem that how to apply the comammox process. While the ubiquitous presence of comammox in engineered systems provides the foundation of developing a novel biological nitrogen removal process, factors contributing to the comammox dynamics in engineered systems have not been well resolved. When compared with the traditional nitrification process, the comammox process would certainly cost less aeration. In addition, the coupled comammox and anammox processes are also expected to produce a nitrogen removal process that requires less energy and carbon input. However, at present, key design parameters of the comammox process remain unknown. Thus, a full-scale implementation of the comammox process will need more extensive research.

Comammox bacteria was a competitive advantage nitrifier over AOB and AOA in oligotrophic habitats (Kits et al., 2017). The kinetic characterization revealed that comammox processes the high ammonia uptake capacity in low ammonia availability environment. As a result, comammox bacteria maybe play an important role in ammonia-restricted ecological area. Moreover, based on our own research, it could be found that high activity of comammox also occurred in riparian where water level fluctuates frequently. Therefore, it is possible to strengthen application research in the environment which ammonia-limited riparian areas in wetlands (Wang et al., 2021a, 2021b, 2021c, 2021d).

## 12. Conclusion

To conclude, this review systematically summarizes the comammox distribution, mechanism and  $N_2O$  production in the global nitrogen cycle, provides new perspective of comammox “hot-spot”, proposes the possibility

and effectiveness that comammox and anammox coexistence in practical application as well as triggers the thinking of enhancing comammox process decreasing nitrification-dependent  $N_2O$  emission to alleviate global warming. At the same time, it also solves the following four problems that have plagued us for a long time.

- 1) Although comammox process has been discovered for a short time, it exerts an important role in the global terrestrial nitrification process.
- 2) The diversity of comammox *Nitrospira* varies significantly in different ecosystems, and fewer new comammox *Nitrospira* strains have been founded.
- 3) The comammox bacteria do harbor the enzymes and functional genes associated with  $N_2O$  production.
- 4) However, the contribution of  $N_2O$  generated by comammox to global climate change still needs to be further explored.

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## CRediT authorship contribution statement

The project was conceived and led by G.B.Z. X. W., S.W., L.Y., G.A., J.Y., L.J., D.Y., Z.G., H.Z., L.Z. contributed to the data collection and analysis. G.B.Z. wrote the manuscript and M.J., L.S., and Y.G.Z. substantially contributed by commenting upon and revising it. All authors discussed and interpreted the results and contributed to the manuscript. Correspondence and requests for materials should be addressed to G.B.Z. ([gbzhu@rcees.ac.cn](mailto:gbzhu@rcees.ac.cn)).

## Declaration of competing interest

The authors declare that they have no conflict of interest.

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