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# Occurrence and Roles of Comammox Bacteria in Water and Wastewater Treatment Systems: A Critical Review



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## ABSTRACT

Nitrogen removal is a critical process in water treatment plants (WTPs) and wastewater treatment plants (WWTPs). The recent discovery of a novel bacterial process, complete ammonia oxidation (comammox, CMX), has refuted a century-long perception of the two-step conversion of  $\text{NH}_3$  to  $\text{NO}_3^-$ . Compared with canonical nitrifiers, CMX bacteria offer undeniable advantages, such as a high growth yield propensity and adaptability to nutrient- and growth-limiting conditions, which collectively draw attention to validate the aptness of CMX bacteria to wastewater treatment. As there has been no comprehensive review on the relevance of CMX bacteria for sustainable water and wastewater treatment, this review is intended to discuss the roles and applications of CMX in the removal of nitrogen and pollutants from water and wastewater. We took into account insights into the metabolic versatilities of CMX bacteria at the clade and subclade levels. We focused on the distribution of CMX bacteria in engineered systems, niche differentiation, co-occurrence and interactions with canonical nitrifiers for a better understanding of CMX bacteria in terms of their ecophysiology. Conceptualized details on the reactor adaptability and stress response of CMX bacteria are provided. The potential of CMX bacteria to degrade micropollutants either directly or co-metabolically was evaluated, and these insights would be an indispensable advantage in opening the doors for wider applications of CMX bacteria in WWTPs. Finally, we summarized future directions of research that are imperative in improving the understanding of CMX biology.

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## 1. Introduction

Efficient biological nitrogen removal has attracted much attention in recent years [1–3]. The wondrous discovery of bacteria performing complete oxidation of ammonia (so-called comammox, CMX) to nitrate ( $\text{NO}_3^-$ ) via nitrite ( $\text{NO}_2^-$ ) has challenged the understanding of nitrogen removal. CMX bacteria have been widely reported in both natural [4–7] and engineered systems [8–11], implying their ubiquity as well as their contributions in various nitrifying habitats. CMX bacteria, having a high growth yield propensity [12], are well fitted to water treatment plants (WTPs) and wastewater treatment plants (WWTPs), particularly plants that have low oxygen accessibility and low cell washout rates (such as biofilms). Thus, revalidation of nitrifying environments

has attracted much attention, which likely provides meaningful insights into the understanding of CMX bacteria, particularly clade A *Nitrospira* sp. [13–17]. While producing a high-quality nitrate solution (to be used as hydroponic fertilizer) from organic nitrogen using a dual reactor system, Sato et al. [18] found a high abundance of CMX *Nitrospira*-related operational taxonomic units (OTUs) in the nitrification reactor, where the expression of CMX-ammonia monooxygenase (AMO) was found to be 500 times higher than that in the canonical reactor. In a long-term study, four antibiotics (i.e., ampicillin, kanamycin, lincomycin, and trimethoprim) could successfully enrich phylogenetically distinct CMX *Nitrospira* in the activated sludge system [19]. Likewise, in very recent investigations, CMX bacteria were found to offer undisputable advantages in WWTPs, such as biotransformation of antibiotic [20,21] and heavy metal tolerance [22], avoiding the generation of  $\text{N}_2\text{O}$  in the intermittently aerated lab-scale sequencing batch reactor [23], survivability at low temperature [24], dominance of tertiary rotating biological contactors (RBCs) biofilm samples [10], occurrence of

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CMX *Nitrospira* in urine-fed membrane bioreactors [25], and so forth, which strongly implies that CMX bacteria are fully functional in WWTP facilities.

The existence of CMX bacteria was hypothesized in 2006 [26]. The occurrence of CMX bacteria was confirmed in 2015 [7,27], although full-fledged operations of WTPs or WWTPs with CMX bacteria have not been reported. CMX bacteria have been studied from different angles, such as niche preference and differentiation [28–30], co-occurrence [31,32], cooperative and competitive interactions [11,33,34], metabolic versatility [35], biotransformation of pollutants [36], enzymology [37–39], biochemistry and physiology [40,41], and molecular detection at the clade level [42]. In addition, the response of CMX bacteria to seasonal changes and external perturbations has also been largely explored [21,43–47]. These attempts shed light on the potential vision that it is not far from now when CMX-based processes are applied in WTPs or WWTPs.

CMX bacteria have considerable disparity in many aspects, despite their uniqueness in their reaction mechanisms [38] and enzymes [27] responsible for  $\text{NH}_3$  oxidation to  $\text{NO}_3^-$ . For instance, not all types of CMX *Nitrospira* are cultivatable *in vitro*, which has been disclosed by emphasizing *Nitrospira* sp. at the subclade-level [48]. Additionally, there is a significant difference in the responsiveness of clade A and clade B CMX to external perturbations [49]. Thus, CMX *Nitrospira* have unique niche requirements [13,16,30], and knowledge of these requirements helps us not only understand how CMX bacteria withstand competition from canonical nitrifiers (e.g., ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB)) but also reveal their coexistence with canonical nitrifiers [8,32,50,51]. Importantly, the niche of CMX bacteria may raise doubt as to whether all clades and subclades of CMX bacteria have a similar type of coresident bacteria because such multispecies assemblages are the key drivers of community functions. Therefore, there should be close linkages between CMX bacterial functionalities and niche determinants. On the other hand, physiological, biochemical, and molecular studies have revealed that CMX bacteria have wide metabolic potential, such as the enhanced removal of micropollutants from wastewater by metabolic [36,52,53] and co-metabolic routes [54–58]. We believe that the above insights will aid in the deep understanding of CMX bacteria in bringing closure to engineered settings for sustainable water or wastewater treatment in nitrifying environments.

To date, the occurrence of CMX in WTPs or WWTPs has been reported widely [8,10,15,17,59–67]. Note that previous reviews have advanced the understanding of CMX in the following aspects: genomic insights [8]; nitrous oxide ( $\text{N}_2\text{O}$ ) emissions [64]; and enzymology and metabolic models [65]. Nonetheless, several key aspects of CMX bacteria are not yet fully understood, such as their distribution in natural and engineered systems, niche differentiation, co-occurrence, metabolic versatility, interactions of CMX bacteria with other bacteria, reactor adaptability, stress response, and co-metabolic biotransformation. Thus, the present paper aimed to critically review these factors, and these insights would be useful in bringing CMX bacteria much closer to WTPs and WWTPs.

## 2. Ubiquity of CMX bacteria

### 2.1. Occurrence in natural and engineered systems

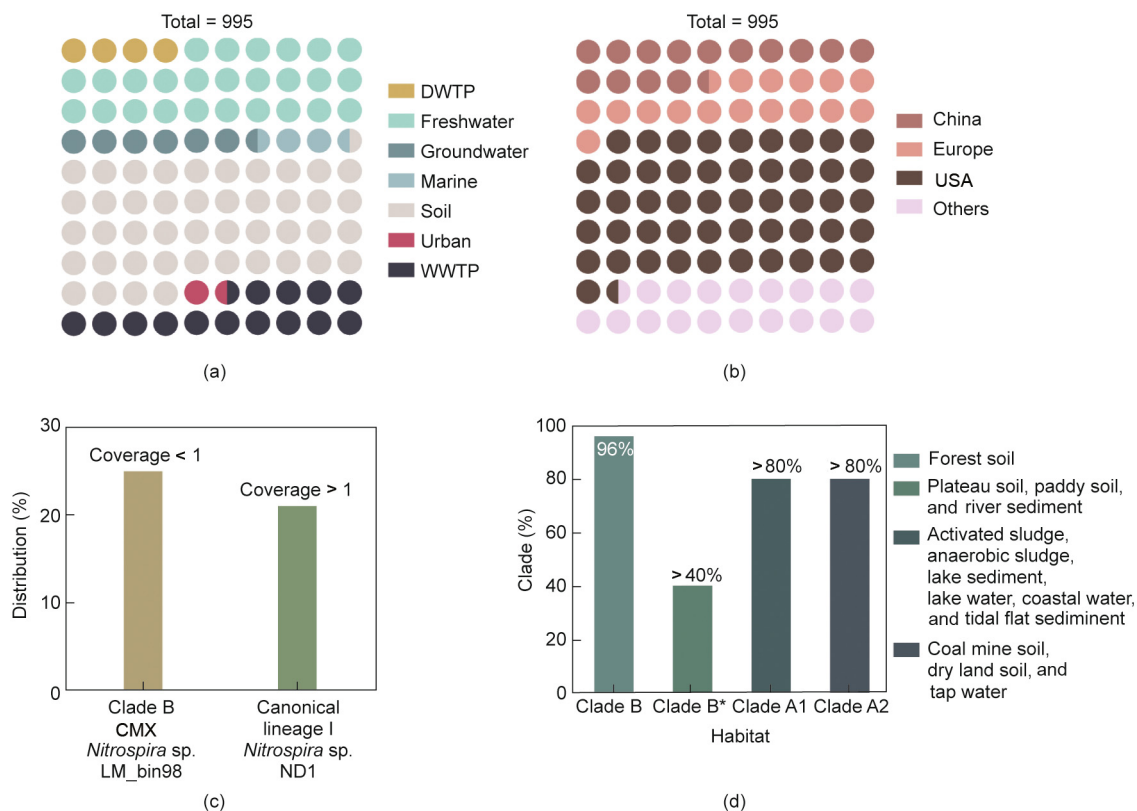
The distribution and abundance of CMX bacteria in different natural and engineered systems are summarized to reveal the ecology of microbial nitrification in the nitrogen cycle (Fig. 1 [48,68]). Based on the gene-encoding subunit A of the ammonia monooxygenase (*amoA*) phylogeny, there are two novel CMX *Nitrospira* rep-

resenting two divergent sister clades, namely, clade A and clade B [7]. Along these lines, CMX bacteria of clade A and clade B have been detected in various ecosystems [68], implying that they are environmentally widespread. Recently, cultivatable and uncultivable *Nitrospira* were found to be present in subclades A1 and A2, respectively [48]. CMX *Nitrospira* has been detected in different soil and freshwater ecosystems [69], WWTPs and WTPs [31], sand filters of groundwater [9], aquaculture ponds [70], and nitrifying biofilms [64], suggesting that these niches commonly serve as nitrification platforms. Although there have been limited studies, CMX *Nitrospira* seems to be more abundant in WTPs [9] than in WWTPs [60] and soils [71]. Additionally, within the same microenvironment, the abundance of clades A and B is normally different. For instance, clade B is more abundant than clade A in forest soils. In comparison, the abundance of clade B was 14 times lower than that of clade A in paddy soil [49]. It is noteworthy that CMX bacteria in clade B are more sensitive than those in clade A to the external environment. For example,  $\text{NH}_4^+$  amendment did not show a significant impact on the abundance of clade A, while clade B abundance significantly ( $P < 0.05$ ) increased with the same amendment [49]. Similarly, the presence of residual chloramine ( $(1.74 \pm 0.21) \text{ mg}\cdot\text{L}^{-1}$ ) in drinking water distribution systems favored a shift toward *Nitrospira*-like OTUs [72]. It is worth noting that there are several primers for the identification of clades of CMX bacteria with different matching percentages (Fig. 2 [48]). For example, primers A378f show high matching (80%–90% with the *amoA* gene) with CMX clade A (subclade A1 and subclade A2) but low matching (< 20%) with clade B and AOB (Fig. 2), which clearly suggests that there are massive differences in the genetic potential between clade A and clade B.

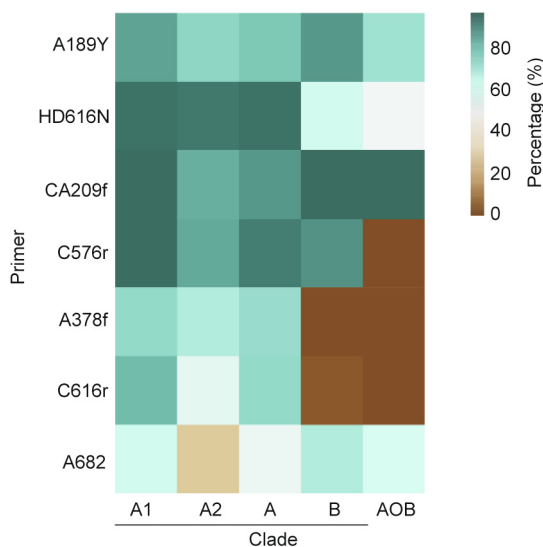
In full-scale WWTPs, CMX bacteria have been found to be an important  $\text{NH}_3$ -oxidizing group [7]. A primer pair specifically designed to target the CMX *amoA* gene revealed that the *Nitrospira nitrosa* cluster accounted for 94.34% of bacteria in WWTPs [60], which was about 183-fold (as determined by quantitative polymerase chain reaction (qPCR)) more abundant than the AOB *amoA* gene. The dominance of CMX *Nitrospira* over AOB was also found in a nitrification reactor fed real municipal wastewater, where 94% of the  $\text{NH}_3$ -oxidizing community at 400 d was the former group [73]. Similarly, CMX *Nitrospira* showed dominance over AOB *Nitrospira* in the organic carbon-rich nitrification-denitrification system [74]. These insights clearly suggest that CMX bacteria have solid competitive advantages over other nitrifying communities in WWTPs and particularly in WTPs, which is promising for the engineering application of mainstream CMX (Table 1 [8,12,14,41,43,63,70,75–77]). Notably, although CMX bacteria have a full suite of enzymes for both  $\text{NH}_3$  and  $\text{NO}_2^-$  oxidation, proper markers should be selected to characterize the presence and abundance of such enzymes. For instance, nitrite oxidoreductase (Nxr) of CMX *Nitrospira* is very similar to that of strictly  $\text{NO}_2^-$ -oxidizing *Nitrospira* [37], which is not a suitable marker of CMX *Nitrospira*.

### 2.2. Niche differentiation

In each ecological niche, CMX *Nitrospira* shows a complex response to the distribution of resources. It has been indicated that dissolved oxygen (DO) fluctuations in water may not affect the CMX community [28], likely because CMX *Nitrospira* contains genes that allow these bacteria to grow even at low levels of oxygen, as determined in a comparative genomic study [29]. However, the genome of CMX *Nitrospira* does permit assimilatory nitrate reduction by using an external nitrite nitrogen source, which is contrary to canonical *Nitrospira*. Other potential niche determinants in CMX *Nitrospira* include levels of nitrite (activated sludge) [13], elevated N deposition (forest soils) [30], and pH-dependent  $\text{NH}_3$  availability (agricultural/forest soils) [16,30]. Nonetheless,



**Fig. 1.** Ubiquitous distribution of CMX bacteria. Distribution of *Nitrospira* species (a total of 68) across 995 metagenomes across (a) seven broadly defined habitats and (b) the globe, and (c) CMX *Nitrospira* was the most widely distributed (low abundance with a coverage < 1) than canonical *Nitrospira* (high abundance with a coverage of > 1) [68]. (d) Clade-level distribution of CMX bacteria in different habitats analyzed by partial nested PCR with CMX-specific primer sets [48]. DWTP: drinking water treatment plant.



**Fig. 2.** Primers and their matching (%) with the *amoA* gene sequences of different CMX clades and AOB [48]. Sequences are extracted from metagenomic or metatranscriptomic data sets in public databases as described in the reference.

subclades of CMX *Nitrospira* can achieve rapid niche divergence from other canonical nitrifiers by horizontal transfer of genes encoding  $\text{NH}_3$  oxidation [29] and hydroxylamine oxidoreductase [68]. These studies have indicated that the genomic traits of CMX *Nitrospira* are impacted by biotic factors [32]. Niche differentiation is an important determinant shaping microbial community structure [78]. However, there is a scarcity of information on the niche

**Table 1**  
Comparison between CMX bacteria and canonical nitrifiers.

Character	CMX bacteria	Canonical nitrifiers
$K_m$	449.2 $\mu\text{mol}\cdot\text{L}^{-1}$ $\text{NO}_2^-$ [12]	6–27 $\mu\text{mol}\cdot\text{L}^{-1}$ $\text{NO}_2^-$ [12]
Energy yield during $\text{NH}_3$ oxidation	$\Delta G^0 = 384.9$ $\text{kJ}\cdot\text{mol}^{-1}$ [8]	$\Delta G^0 = 274.7$ $\text{kJ}\cdot\text{mol}^{-1}$ [8]
$\text{CO}_2$ fixation pathway	Calvin–Benson–Bassham (CBB) cycle [8]	Reductive tricarboxylic acid cycle (TCA) [8]
Oxygen consumption stoichiometry ( $Y_0$ )	Larger ( $Y_0 = 2$ )	Smaller ( $Y_0 = 1.5$ )
Withstand Cu deficiency	High [63]	Not withstand [75]
Urease transporters	High affinity [63]	–
Oxidative stress-evading mechanism	High [63]	Low
$\text{N}_2\text{O}$ production	Low [63]	High
$\text{H}_2$ and formate oxidation by 3b bidirectional [NiFe] hydrogenase	Yes [76]	–
High-affinity sulfate permease (SulP)/Solute Carrier 26 (SLC26)-type transporters for broad range substrates ( $\text{SO}_4^{2-}$ , $\text{HCO}_3^-$ , and $\text{Cl}^-$ )	Yes [76]	–
Correlation with dissolved oxygen (DO)	Negative ( $r = -0.77$ ) [43]	Positive ( $r = 0.14-0.48$ ) [70]
Half-saturation constant on $\text{NH}_3$	0.026 $\mu\text{g}\cdot\text{NH}_3\cdot\text{N}\cdot\text{L}^{-1}$ (oligotrophic lifestyle) [41]	0.077 $\text{mg}\cdot\text{NH}_3\cdot\text{N}\cdot\text{L}^{-1}$ [77]
$\text{O}_2$ requirement during enrichment	Low (0.2 $\mu\text{g}\cdot\text{L}^{-1}$ ) [14]	High (0.06–0.80 $\text{mg}\cdot\text{L}^{-1}$ ) [63]

$K_m$  is described as the concentration of the substrate to which the velocity of the reaction is half maximal.

specialization, preference and differentiation of CMX *Nitrospira* with respect to their habitats, such as trophic states and oxygen levels [28,40]. Niche separation between CMX *Nitrospira* and canonical nitrifiers should be further studied by focusing on the metabolic versatility and comparative genomics of the former community. Moreover, the niche divergence and separation of CMX *Nitrospira* have been explored in natural settings (aquatic [28] and soil [16,30,40] settings) and in engineered settings [73,79]. However, additional reactor-based studies are largely needed for a deeper understanding of niche differentiation of CMX *Nitrospira* in various facilities, including habitat heterogeneity, environmental conditions, and biointeractions.

### 2.3. Functionalities at the gene level

CMX bacteria are known to coexist with different canonical bacteria, such as NOB [32], AOA [8,50], AOB [8,51], and anammox bacteria [34], which clearly implies the considerable microbial interactions of CMX *Nitrospira* with other canonical communities. There is a significant positive relationship between the *amoA*-Niño gene of CMX bacteria and selective genes of canonical nitrifiers and other bacteria (Fig. 3(a) [43]). The presence of a large number of nitrogen-cycling genes in *Nitrospira inopinata* (CMX) could help *Nitrospira moscoviensis* (NOB) utilize different metabolic functions in a freshwater mussel habitat [32]. This is because *Nitrospira inopinata* has more N-cycling genes than *Nitrospira moscoviensis*, and the subsequent metabolic sharing between the two strains is the key factor for the survival of two bacteria in the mussel habitat. Furthermore, CMX *Nitrospira* is known to possess a complete set of genes (*urtABCDE*) for a high affinity of the urea transporter system, whereas *Nitrospira moscoviensis* lacks a full array of these genes (having only *UrtA*) [32]. In addition, CMX *Nitrospira* has a tendency to oxidize  $\text{NH}_3$  more than  $\text{NO}_2^-$

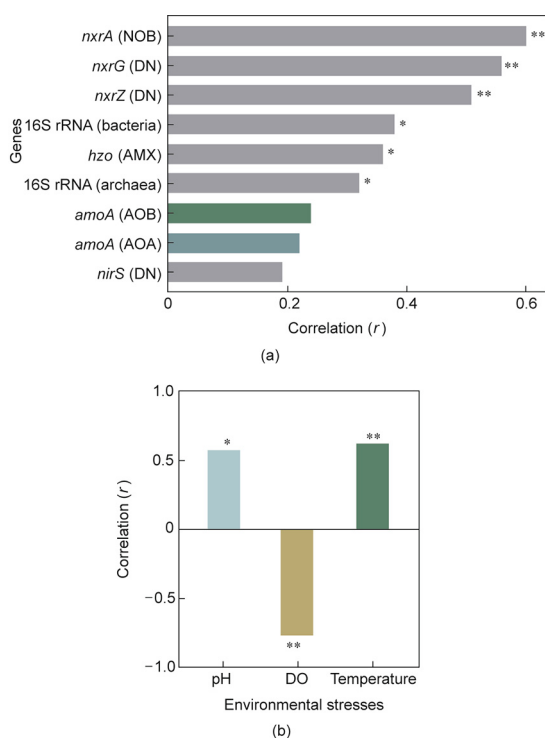
under very low oxygen concentrations, which favors the co-occurrence of CMX *Nitrospira* and anammox bacteria [27]. In addition, the concentrations of nitrate and total nitrogen show a positive correlation with CMX clade A1 and clade A2, respectively [51]. We can expect that these clades may have different responsive behaviors to co-resident canonical nitrifiers. Therefore, the co-occurrence of CMX *Nitrospira* should be evaluated at the subclade level to unravel the uncertainty in the cooperation and competence of CMX *Nitrospira* with others in nitrogen conversion systems during water and wastewater treatment [80]. It would aid in process design and operation in both WWTPs and WTPs.

In summary, CMX *Nitrospira* can be readily detected in natural waters, drinking water systems and oligotrophic wastewater. This is very similar to the AOA. This is likely due to the low growth rates of canonical nitrifiers in oligotrophic conditions. The high activity of CMX bacteria at low oxygen levels implies that it is possible to develop a low-cost nitrogen removal process. We should keep in mind that the boundary conditions of CMX bacteria and canonical nitrifiers in engineered systems should be determined with well-designed experiments.

## 3. Physiology and biochemical potentials of CMX bacteria

### 3.1. Metabolic versatility

Some strains of CMX *Nitrospira* can respire on  $\text{O}_2$  or  $\text{NO}_3^-$  as terminal electron acceptors and oxidize different substrates, such as  $\text{NH}_3$ ,  $\text{H}_2$ , formate, and  $\text{NO}_2^-$  [35]; thus, their versatility in metabolism allows these bacteria to colonize a broad range of habitats. They can oxidize  $\text{NH}_3$  by ammonia monooxygenase (AMO) and can fix  $\text{CO}_2$  by using the reductive citric acid cycle. Due to the high affinity for  $\text{NH}_3$ , as confirmed by whole-cell (*Nitrospira inopinata*) kinetics [38], the population of CMX *Nitrospira inopinata* is usually higher than that of many other  $\text{NH}_3$ -oxidizing microbes under oligotrophic conditions. The high  $\text{NH}_3$  affinity indicated by the half-saturation constant ( $K_{m(\text{app})}$ ) of a pure cultured CMX bacterium (e.g., *Nitrospira inopinata*) is  $63 \text{ nmol}\cdot\text{L}^{-1}$ , which is 4- to 2500-fold below the values reported for AOB [41]. Even a much lower  $K_m$  value (i.e.,  $49 \text{ nmol}\cdot\text{L}^{-1}$ ) for  $\text{NH}_3$  was observed for clade A CMX bacteria [41]. Nonetheless, CMX bacteria show a lower affinity toward  $\text{NO}_2^-$  than other NOB [41]. Similar to  $\text{NH}_3$ , cyanate can also be utilized by CMX *Nitrospira* during complete nitrification [74]. Furthermore, CMX *Nitrospira* is likely to adapt to oligotrophic conditions [35], which assists CMX bacteria in outcompeting AOA in oligotrophic environments [40]. It is also worth noting that if there is any overlap in  $K_{m(\text{app})}$  values, competition exists between CMX *Nitrospira inopinata* and other canonical nitrifiers (e.g., AOA) in oligotrophic and low- $\text{NH}_3$  available habitats, although there is a higher abundance of the *amoA* gene in CMX *Nitrospira* than in AOA or AOB *amoA*. Surprisingly, changes in soil pH ranging from 4.0 to 9.0 had a mild impact on the abundance of *amoA* gene copy numbers of both clades A and B of CMX bacteria, although the abundance of clade A was higher than that of clade B at each measured pH value [40]. Nevertheless, pH, DO, and temperature have a significant influence on CMX bacteria, as revealed by *amoA*-Niño gene abundance (Fig. 3(b)). CMX *Nitrospira* is able to thrive successfully in WWTPs with low and fluctuating urea concentrations [40], attributed to the presence of genes encoding urease proteins, high-affinity urea transporters (*urtABCDE*), urea carboxylase-related transporters (*uctT*), and outer-membrane porins (*fmdC*), as revealed by metagenomic analysis [7,27]. Recently, it was found that the release of  $\text{N}_2\text{O}$  by CMX microbes during nitrification is comparatively lower than that by AOB [6] because of the lack of NO reductases in the genome of *Nitrospira inopinata*. In fact, nitrifying biofilms of canonical nitrifiers have higher  $\text{N}_2\text{O}$  emissions



**Fig. 3.** Pearson relationship of *amoA*-Niño [43]. (a) Co-existence of CMX bacteria with other bacteria including canonical nitrifiers. Correlation between *amoA*-Niño and other gene appellation. (b) Correlation between *amoA*-Niño gene and environmental factors. AMX: anammox; DN: denitrifying bacteria; DO: dissolved oxygen; significance Two-tailed \*  $\leq 0.05$  and \*\*  $\leq 0.01$ .

[64]. Therefore, less emission of N<sub>2</sub>O by CMX helps to decrease the cost associated with improved plant design and operation to mitigate N<sub>2</sub>O emissions during wastewater treatment. In addition, the metabolic potentials of CMX *Nitrospira* due to the presence of [NiFe]-hydrogenases (group 3b) can allow these bacteria to exhibit distinct roles [12], such as ① production of H<sub>2</sub> by reoxidizing NAD(P)H and concurrent maintenance of cellular redox balance during fermentation, ② oxidation of H<sub>2</sub> to provide electrons for CO<sub>2</sub> reduction, and ③ reduction of elemental sulfur or polysulfide to H<sub>2</sub>S. Furthermore, it is not clear how CMX bacteria switch between NH<sub>3</sub> and NO<sub>2</sub><sup>-</sup> oxidation modes. When *Nitrospira* phylogenotypes were fed unlabeled NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> columns (composed of rapid gravity sand filter material), CMX *Nitrospira* did not show a preference for only oxidizing external NO<sub>2</sub><sup>-</sup> [81], which was in line with *Nitrospira inopinata* [7,27]. It is worth noting that ClO<sub>3</sub><sup>-</sup> and NO-chelating compounds (e.g., 2-phenyl-4,4,5,5-tetramethylimidazole-3-oxide-1-oxyl (PTIO)) exert inhibitory effects on NH<sub>3</sub> oxidation by CMX *Nitrospira* [81] and *Nitrospira inopinata* [6], respectively. In fact, reduction of ClO<sub>3</sub><sup>-</sup> generates toxic ClO<sub>2</sub><sup>-</sup>, which is toxic to CMX bacterial metabolism (including NH<sub>3</sub> oxidation). The metabolic potentials of different clades of CMX bacteria were compared with AOB and AOA, and their relevance to WWTPs is presented in Table 2 [8,82–91]. It is also worth noting that there is possible lateral gene transfer in CMX *Nitrospira*, by which these bacteria obtain certain additional metabolic functions [10,14,92].

### 3.2. Enzymology

Eight-electron oxidation reactions are mediated by CMX bacteria during the complete oxidation of NH<sub>3</sub> to NO<sub>3</sub><sup>-</sup> [38]. CMX-

mediated nitrification is potentially dependent on the expression of genes, such as *amo*, *hao*, and *nxr* [27]. A study based on metagenome-assembled genomes confirmed the potential of CMX in hydrolyzing urea to NH<sub>3</sub> by using the *ure* genes in the genome of CMX *Nitrospira* with RBCs [10]. These genes have also been reported in urea-enriched cultures of clade A CMX *Nitrospira* [29]. In contrast, clade A CMX *Nitrospira* cannot utilize formate as an alternative electron donor since *fdh* genes are absent in these bacteria, whereas clade B CMX *Nitrospira* are known to possess *fdh* genes [10]. In addition, some CMX *Nitrospira* can potentially use dihydrogen/protons as alternative electron donors/acceptors, which is attributed to genes such as *hyb* and *hyd* encoding group 3b [Ni–Fe] sulfur-reducing hydrogenase [11]. By lateral gene transfer, CMX *Nitrospira* acquire not only cyanase activity [10] but also two toxin–antitoxin genes adjacent to cyanase genes [14]. Nonetheless, horizontal gene transfer (also called vertical inheritance) helps in the recruitment of diverse genes from non-ammonia oxidizers to facilitate the overall ammonia oxidation pathway [92]. In fact, CMX bacteria inherently have cyanase activity [17]; therefore, these bacteria utilize diverse organic nitrogen compounds in addition to free ammonia as the substrate. It is noteworthy that the other metabolic versatility of CMX *Nitrospira* bring these bacteria much closer to WWTPs. Han et al. [36] found that CMX *Nitrospira inopinata* was able to biotransform a wide range of micropollutants, where carbendazim was exclusively biotransformed by CMX *Nitrospira inopinata*, where there was a supply of ammonia rather than nitrite as the energy source. These results strongly imply that the AMO of CMX *Nitrospira inopinata* has a much higher affinity toward ammonia than that of AOA- or AOB-*Nitrospira*. Additionally, the enzymatic system of CMX *Nitrospira*

**Table 2**  
Metabolic potential of CMX bacteria and their canonical nitrifiers.

Metabolism	Bacteria [8]			Relevance of metabolism to wastewater treatment		
	Clade A CMX	Clade B CMX	Canonical <i>Nitrospira</i>	AOB	AOA	
Ammonia oxidation	+++	+++	+++	–	+++	Anthropogenic discharge of nitrogen to freshwater (in China) is 14.5 ± 3.1 megatons of nitrogen per year which is 2.7 times higher than the estimated safe nitrogen discharge threshold ((5.2 ± 0.7) Mt of nitrogen per year) [82]
Nitrite oxidation	+++	+++	+++	–	–	
Nitrite reductase	+++	+++	+++	+	+	
Assimilatory nitrite reduction	–	–	+++	+++	+++	
NO reductase	–	–	–	+	–	
H <sub>2</sub> oxidation	+++	–	+	+	–	
Formate oxidation	–	+++	+++	–	–	H <sub>2</sub> can be an alternative energy source for aerobic respiration in the absence of nitrifying reactions. It suggests the ecological flexibility of N-cycling bacteria, which can fix CO <sub>2</sub> with H <sub>2</sub> as a sole electron source [83]
Cytochrome <i>bd</i> -like oxidase	+++	+++	+++	–	–	Formate oxidation propensity confirms the electrochemical active nature of bacteria [84], and such bacteria are effective in the removal of organic pollutants (e.g., 17β-estradiol) in aerobic aquatic environments [85]
Cytochrome <i>aa</i> <sub>3</sub> oxidase	–	–	–	+++	+++	
Cytochrome <i>bd</i> quinol oxidase	+	+	+++	–	–	
Urea degradation	+++	+++	+	+	+	Expression of oxidases could be considered as one of the stress-evading mechanisms. For instance, stress caused by DO perturbation led microbial community changes and higher activities of peroxidase and cytochromes, and there was a high percent removal (57%–92%) of organic micropollutants during wastewater treatment (WWT) [86]
Reductive TCA cycle	+++	+++	+++	–	–	Performance of many biological WWT processes are inhibited by urea [87], and urea removal is crucial for a successful treatment process
CBB cycle	–	–	–	+++	–	
Thaumarchaeal 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle	–	–	–	–	+++	Reductive TCA is an effective energy-yielding and carbon fixation pathway compared with CBB [8]. Type of C-fixation pathway is important in the C sequestration and suppression of CO <sub>2</sub> emission by bacteria; importantly, selection of the C-fixation pathway is highly influenced by NH <sub>4</sub> <sup>+</sup> -N and NO <sub>2</sub> <sup>-</sup> -N concentrations [88]
Polyhydroxy-alkaotes (PHA) synthesis	–	–	–	–	+	The most energy-efficient CO <sub>2</sub> fixation pathway in the presence of oxygen and nutrient-limited environments [89]
Glycogen synthesis	+++	+++	+++	+++	–	PHA and glycogen are important storages during biological phosphorous removal from the wastewater [90]. Alternative synthesis and oxidation of intracellular storages have significant impact on N <sub>2</sub> O emissions during biological nutrient removal [91]

+++ means function is encoded in all genomes; + means function is encoded in some to most genomes; – means function is not encoded.

*inopinata* enables these strains to emit lower levels of  $\text{NO}_y$  (nitrous acid (HONO) + nitric oxide, and  $\text{NO}$  + nitrogen dioxide ( $\text{NO}_2$ )) [93], which is a critical emission in global warming and environmental chemistry. In short, the presence of these functional enzymes can endow CMX bacteria with various metabolic potentials.

### 3.3. Interactions of CMX bacteria with other bacteria

Interactions between CMX bacteria and other bacteria (e.g., canonical nitrifiers) would greatly impact the design and optimization of WWTP facilities. *Nitrospira inopinata* showed significant positive correlations with NOB rather than AOA and AOB [43], implying that CMX-mediated  $\text{NO}_2^-$  oxidation is more active than  $\text{NH}_3$  oxidation. Similar results of CMX bacteria and NOB interactions have also been found in recirculating aquaculture systems [31]. Although these positive correlations are often present in other combinations (*Nitrospira inopinata*-like CMX and denitrifying microbes [32]), we are far from fully understanding the multiple correlations among CMX bacteria and canonical nitrifiers due to complex environmental factors. In particular, in natural circumstances, it is difficult to tackle biological factors in engineered systems. Cooperation among partial nitrifiers, CMX and anammox bacteria achieved the removal of 98.82% of  $\text{NH}_4^+\text{-N}$  in a sequencing batch reactor [34], suggesting that CMX bacteria can play cooperative roles in concurrent high nitrogen removal. In particular, under low oxygen conditions, CMX bacteria oxidize  $\text{NH}_3$  to  $\text{NO}_2^-$ , which favors interactions between CMX and anammox [11]. Cooperation between AOB and CMX *Nitrospira* has been found to ensure efficient Cu-stimulated nitrification in groundwater-fed rapid sand filters [45]. To understand the community behavior of CMX bacteria, research toward quorum sensing (QS) in CMX has already been undertaken, for example, CMX bacteria contain genes responsible for the synthesis of acyl homoserine lactones (AHLs) and extracellular polymeric substances (EPS) [77]. In fact, QS has been identified in pure [94] and mixed cultures [95] of canonical nitrifiers, as well as in autotrophic nitrification/denitrification biofilms [96]. The biofilm formation potential of CMX bacteria is well regulated by the genes present in ABC transporters (ko02010) and two components (ko02020) [97], corroborating that CMX can prefer to grow in biofilms. In fact, the CMX process has already been identified in biofilm reactors [98], but the molecular details of their QS in these settings are not yet known [99].

Overall, metabolic versatility is the unique feature of CMX *Nitrospira* because of the expression of various functional genes in CMX cells, implying that CMX *Nitrospira* can grow both in sewage and industrial wastewater. In addition, the low generation of  $\text{N}_2\text{O}$  by CMX *Nitrospira* over canonical nitrifiers suggests that the application of CMX would aid in developing sustainable nitrogen processes in the near future.

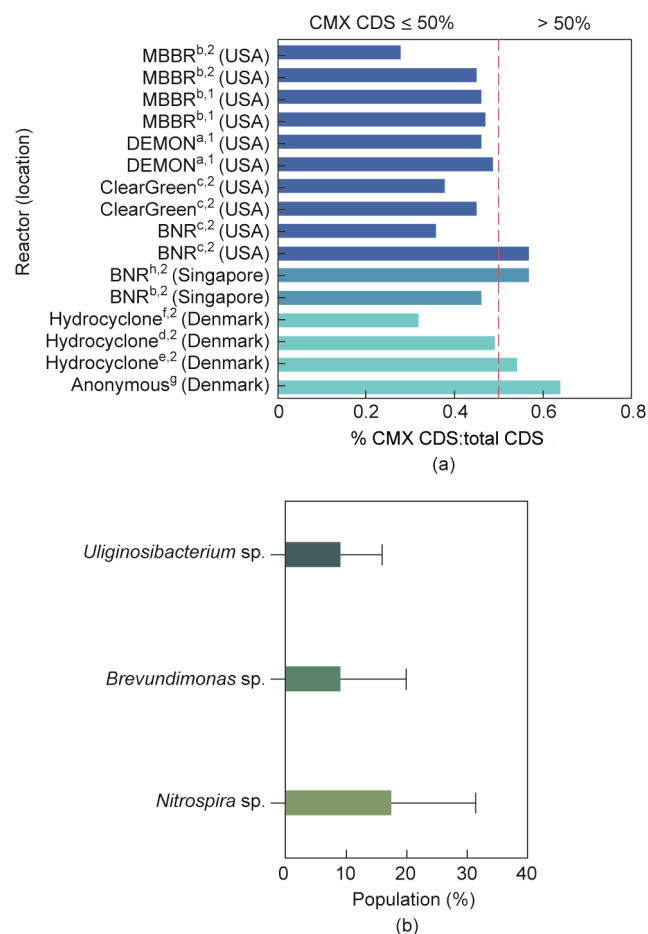
## 4. Behavior of CMX bacteria in engineered systems

### 4.1. Reactor adaptability

CMX *Nitrospira* possesses distinct physiological characteristics, such as a lower  $K_m$  value for  $\text{NH}_3$ , a higher  $\mu_{\text{max}}$ , a higher mg protein per mole  $\text{NH}_3$ , and a higher  $\text{O}_2$  consumption rate in terms of mole  $\text{O}_2:\text{NH}_3$  than other canonical nitrifiers [8]. These features enable CMX *Nitrospira* to have great resilience in WTPs and WWTPs, where fluctuations of water/wastewater compositions and ambient conditions often occur. A combination of molecular approaches (i.e., qPCR, 16S rRNA gene sequencing and metagenomics) confirmed the dominance of CMX *Nitrospira* over AOA and AOB in the tertiary rotating biological contractors of a municipal WWTP [10], indicating that CMX bacteria are ubiquitous in

tertiary WWTP systems. Additionally, the presence of CMX bacteria in the bioreactor could provide a way to understand the impacts of various environmental factors on the distinct spatial and temporal distributions of CMX [59]. Nevertheless, there is a significant contribution of CMX to nitrification [100]. The molecular analysis of samples collected from different engineered systems revealed that the ratio (%) of CMX-coding DNA sequences (CDS) and total CDS were between about 0.3% and > 0.6% (Fig. 4(a) [15]). The dominance of CMX *Nitrospira* sp. was also found in a dechlorinated reactor (Fig. 4(b) [72]), where the sequences comprised 7.2% to 27.4%, and these bacteria were the major candidates responsible for nitrification in the dechlorinated reactors. These studies clearly indicate the adaptability of CMX bacteria to reactor systems.

It is important to note that  $\text{NH}_3$  concentrations alone are not a suitable determinant for the abundance of CMX *Nitrospira* in engineered systems. Despite high ammonium concentrations, low [101] and high abundances [61] of CMX bacteria have also been reported in sludge samples. It is difficult to assess the abundance of CMX bacteria in reactors unless the studies examined substrate ranges, oxygen requirements, growth rates and yields, and biofilm formation propensities. Factors affecting the presence and dominance of clade A and clade B are not yet well unknown [10]. Roots et al. [73] found higher ammonium removal rates (about  $59 \text{ mg}\cdot\text{N}\cdot(\text{L}\cdot\text{d})^{-1}$ ) by CMX *Nitrospira* at a DO of  $0.2\text{--}1.0 \text{ mg}\cdot\text{L}^{-1}$  in a mainstream nitrification reactor fed real municipal wastewater



**Fig. 4.** Reactor adaptability of CMX bacteria. (a) Ratio of CMX coding DNA sequences (% CMX CDS) and total CDS in full-scale WWTPs surveyed in different countries [15]. Type of feed (<sup>1</sup>SS, <sup>2</sup>MS) and biomass (<sup>a</sup>granule, <sup>b</sup>biofilm, <sup>c</sup>mixed liquor, <sup>d</sup>overflow, <sup>e</sup>underflow, <sup>f</sup>ALT (mixed liquor), <sup>g</sup>inoculum, and <sup>h</sup>Activated sludge). (b) Dominance (mean with standard deviation) of *Nitrospira* sp. in a dechlorinated reactor, showed in a Scatter plot with bars [72]. MBBR: moving bed bioreactor; BNR: biological nutrient removal.

(operated for > 400 d) compared with a nitrifying conventional activated sludge reactor with  $\text{DO} = 3\text{--}5 \text{ mg}\cdot\text{L}^{-1}$ . This clearly suggests that CMX-driven low-DO nitrification is an alternative to conventional high-DO-driven nitrification. Furthermore, in a recent investigation, it was found that CMX bacteria were well adapted in a reactor fed either  $\text{NH}_3$  or  $\text{NO}_2^-$  as a sole nitrogen source [102]. However, the nitrification rates of CMX bacteria were nearly 2-fold higher when dosed with  $\text{NH}_3$  than when dosed with  $\text{NO}_2^-$ . This finding can largely strengthen the application of CMX-based processes. Nonetheless, the ratio of either CMX bacteria to canonical nitrifiers, or clade A to clade B, was significantly affected by nitrogen–phosphorous–potassium (N–P–K) fertilization (Fig. 5 [103]). A recent study also revealed that, in comparison with other ammonia-oxidizing microbes, CMX bacteria were more severely inhibited by free ammonia in a partial-anammox bioreactor [104].

Furthermore, long solid retention times (SRT > 10 d) and attached growth mode are favorable conditions for the growth of clade A CMX bacteria [11], and SRT was found to be the key determinant for the prevalence of CMX bacteria. Certainly, a long SRT has a significant impact on the microbial community in the reactor system, for example, biofilm thickness [105], microbial biodiversity [106], biomass concentration [107], and microbial volatile acid production [108]. Moreover, it was found that *Nitrospira* suppressed  $\text{NO}_2^-$  accumulation and inhibited  $\text{N}_2\text{O}$  generation under a long SRT [109]. It is worth noting that enrichment of CMX *Nitrospira* is feasible in nitrifying granules, and these enriched cultures showed satisfactory efficiency in nitrogen removal [110]. Interestingly, mucopolysaccharides from invertebrates can affect CMX bacteria in a natural setting (e.g., coastal sediments), that is, they can stimulate  $\text{NO}_3^-$ -oxidizing functional groups [111]. Recently, it was also found that CMX *Nitrospira* exhibited efficient nitrogen removal in engineered systems at low DO and  $\text{NH}_4^+$  and high SRT [73]. These insights provide potential clues for the optimization of bioreactor configuration and operating parameters. Furthermore, quantitative PCR and PCR cloning experiments revealed that CMX bacteria can better adapt to biofilms than to suspended

sludge in WWTPs [62]. Furthermore, according to phylogenetic analysis of *amoA* genes, it was found that uncultured CMX *Nitrospira* are predominant in biofilms [62]. As current WWTPs are mainly based on biofilm and activated sludge processes, these findings are of great importance for the operation and design of WWTPs. This may be a potential reason why CMX are ubiquitous in WTPs or drinking water distribution systems where bacteria can normally be present in the form of biofilms.

#### 4.2. Stress response

CMX *Nitrospira* can firmly withstand physically and chemically induced stresses. For instance, *Nitrospira* was constantly detected in activated sludge when subjected to  $\text{NH}_3$  starvation stress, where the DNA and complementary DNA (cDNA) of *Nitrospira* were not affected by fluctuations in temperature throughout the starvation experiments [44]. Some gene-encoding enzymes (e.g., superoxide dismutase, catalase, and several peroxidases) can protect bacteria against reactive oxygen species (ROS) [17]. CMX bacteria can adapt well to a common disinfectant, for example, peracetic acid (PAA), in aquaculture systems. PAA (application rate  $1.1 \text{ mg}\cdot\text{L}^{-1}$  twice per day) showed adverse effects on the gene copy numbers of CMX bacteria after exposure for eight weeks, but these effects gradually decreased at week 13 [112]. This strongly indicates the potential adaptability of CMX bacteria to long-term chemically induced stress. Such an adaptive response of CMX bacteria was also found when exposed to long-term fertilization [46]. There was an alleviation of tetracycline (TC) stress on the nitrifying population in the presence of a low Cu(II) content. The abundance of functional genes related to nitrification and denitrification was adversely affected at  $20 \text{ mg}\cdot\text{L}^{-1}$  TC, but these effects were diminished in the presence of a mixed stress of TC ( $20 \text{ mg}\cdot\text{L}^{-1}$ ) and Cu(II) ( $10 \text{ mg}\cdot\text{L}^{-1}$ ) [113] due to the decreased TC inhibition on enzyme activity at low Cu(II) content.

Importantly, the adaptability of CMX bacteria has been observed to switch between lifestyles in fluctuating environments. For example, CMX bacteria trigger the biosynthesis and degradation of intracellular compounds, such as polyphosphate, glycogen and polyhydroxyalkanoates, as a part of their survival under unfavorable conditions [17]. However, the stress response of other canonical nitrifiers is different from that observed in CMX bacteria. For example, a model AOA, that is, *Nitrosopumilus maritimus* SCM1, was adversely affected by  $\text{NH}_3$  starvation and Cu stress, leading to the downregulation of genes responsible for energy generation and biosynthesis [114]. These previous findings clearly imply that CMX bacteria have better stress adaptability than canonical nitrifiers.

Nonetheless, CMX *Nitrospira* seems to be a potential candidate in withstanding reactor conditions because of the adaptability of these bacteria. Resistance to physical and chemical stress is a key mechanism of the adaptability of CMX *Nitrospira*. It should be noted that the occurrence of some chemicals, such as ciprofloxacin, may significantly inhibit CMX *Nitrospira* and nitrogen removal [47]. Furthermore, in comparison with those in activated sludge, CMX *Nitrospira* in biofilm aggregates are expected to have higher operational flexibility, lower space requirements, and better resilience to environmental fluctuations [115], which has great implications for the real application of CMX-centered processes.

## 5. Pollutant removal in WTPs and WWTPs

### 5.1. Biodegradation of micropollutants

Interestingly, some micropollutants can be exclusively degraded by CMX bacteria, which provides promising application of CMX in WTPs and WWTPs. The fate of micropollutants in the

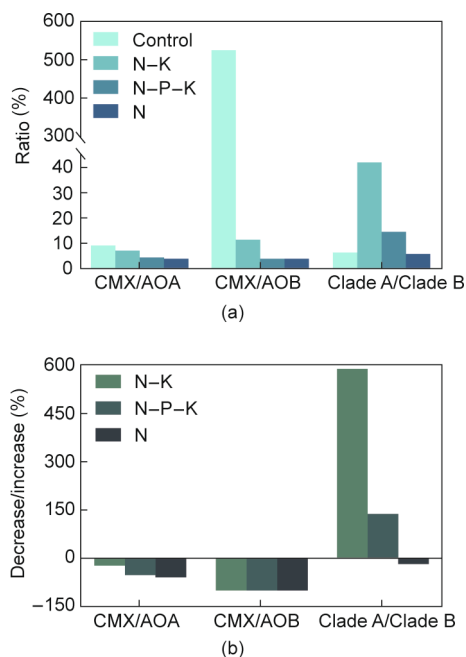


Fig. 5. Influence of nutrient amendments on the CMX bacteria and canonical nitrifiers [103]. (a) Ratios of *amoA* gene copy numbers under different long-term fertilization regimes. (b) Per cent decline or increment in the ratios of *amoA* gene copy number over untreated control.

nitrifying environment was investigated recently [36], and it was found that *Nitrospira inopinata* possesses a high affinity for the  $\text{NH}_3$ -degrading AMO system and degrades asulam, fenhexamid, mianserin, fantidine, and carbendazim in the presence of  $\text{NH}_3$ . The presence of active genes responsible for the utilization of urea, amines and cyanates strongly suggests that CMX bacteria can potentially degrade diverse organic nitrogen compounds except for free  $\text{NH}_3$  [52]. It is noteworthy that increasing the dosage (0–30  $\text{mg}\cdot\text{L}^{-1}$ ) of powdered activated carbon (PAC) to a nitrifying MBBR resulted in increased removal (up to 80%) of four different pharmaceuticals, where *Nitrospira* spp. were the dominant nitrifying species (8%–20%) in both the control and PAC-dosed reactors at all sampling times (79–164 d) [53]. However, the roles and mechanisms of CMX bacteria in the turnover of micropollutants are far from completely understood and deserve further study in the near future.

In WTPs or WWTPs, nitrifying bacteria normally play an important role in the degradation of different micropollutants, such as ibuprofen and naproxen [116], mianserin and ranitidine [117], sulfamethoxazole, erythromycin, roxithromycin and fluoxetine [118], and trichloroethane [119]. AMO could have a significant role in the hydroxylation of pharmaceuticals [118]. Enzyme-based studies also confirmed that AMOs can potentially oxidize a wide range of aliphatic and aromatic hydrocarbons [120] and micropollutants [121]. Importantly, other enzymatic systems in AMOs, such as hydroxylamine oxidoreductase (which catalyzes hydroxylamine to nitrite) and Nxr (which converts nitrite to nitrate), mediate oxidative biotransformation reactions and can play important roles in micropollutant removal [121]. The tolerance of pure-species cultures of ammonia oxidizers to heavy metals (e.g., Cu, Ni, Pb, and Cd) at varying concentrations (100–1000  $\mu\text{g}\cdot\text{L}^{-1}$ ) [122] implies that nitrifying populations seem to be potential candidates in the bioremediation of heavy metal-contaminated sites. To date, there is limited evidence for the successful applications of CMX bacteria either for the removal or biotransformation of micropollutants in engineered systems [20,36]. However, future studies are greatly warranted. On the one hand, more practical validation is needed to confirm the potential of CMX bacteria to degrade and/or transform micropollutants in different chemical groups. On the other hand, the ecotoxicity of micropollutants to CMX bacteria or the interaction between micropollutants and CMX bacteria should be investigated.

### 5.2. Co-metabolic biotransformation

CMX bacteria have a propensity to exhibit co-metabolic biotransformation of micropollutants through deamination during simultaneous  $\text{NH}_3$  oxidation, such as the transformation of pharmaceuticals [54,55], artificial sweeteners (e.g., acesulfame, aspartame, cyclamate, saccharin, and sucralose) [56], sulfamethoxazole [57], and 2-chlorophenol [58]. In an aerobic granular reactor, the removal of selected micropollutants (e.g., bisphenol A, naproxen, irgarol, terbuthryn, and iohexol) was particularly enhanced during simultaneous nitrification due to co-oxidation by AMOs [123]. Similarly, there was partial degradation of acidic pharmaceuticals (e.g., gemfibrozil, diclofenac, bezafibrate, and ketoprofen) and  $17\alpha$ -ethinylestradiol by nitrification, but the degradation rates of natural estrones (e.g.,  $17\beta$ -estradiol and estrone) were improved when nitrification was inhibited [124]. These previous attempts clearly imply that nitrifying enzymes (particularly AMOs) seem to be nonspecific [125] and could co-degrade several kinds of hydrocarbons during nitrifying reactions. It also indicates that the nitrifying community can be broadly applied to remove various micropollutants in WTPs or WWTPs. Certain micropollutants show either recalcitrance or inhibitory effects on the nitrifying process. For example, nitrifying activated sludge was found to be ineffective

against diclofenac, diazepam, carbamazepine and trimethoprim [118]. Nonetheless, some emerging contaminants are toxic to bacteria, for example, azoles are known to inhibit nitrification [126]. The toxicity of azoles is attributed to the modification of active sites of nitrifying enzymes with substituent groups. Although the specific roles of CMX, AOA, AOB, and NOB in the co-metabolic biotransformation of micropollutants have not been well revealed, the co-metabolic capability of such functional bacteria will provide more alternatives for water or wastewater treatment.

It is widely acknowledged that micropollutant removal and co-metabolic biotransformation can be considered effective features during the treatment of water and wastewater; fortunately, CMX *Nitrospira* has potential in these activities. Notably, an environmentally and economically sustainable WTP and WWTP have yet to emerge [127] because there is convincing evidence for the occurrence of micropollutants in treated water or wastewater [128–130]. On the other hand, co-metabolic biotransformation of organic micropollutants is ubiquitous in treatment facilities, such as activated sludge systems [131] and canonical nitrification systems [132,133]. Overall, CMX *Nitrospira* can aid in eliminating micropollutants via co-metabolic biotransformation.

## 6. Knowledge gaps

The shaping of CMX for sustainable water or wastewater treatment depends on many variables and requires comprehensive studies. These include:

(1) Niche preferences under different trophic levels and operational conditions of reactors should be clarified in future studies. Although niche preferences of CMX have been partially revealed [13,16,30], knowledge regarding resource utilization, niche specialization and niche overlaps of different clades of CMX bacteria is still limited. In particular, niche overlap other than competitive exclusion tends to occur when two species possess similar competitive abilities in source utilization [134].

(2) Switches between the modes of  $\text{NH}_3$  and  $\text{NO}_2$  oxidation in CMX bacteria remain unknown. Note that environmental factors are the key drivers of such switches [135]. It should be kept in mind that CMX bacteria harbor higher nitrification rates in a reactor dosed with urea than  $\text{NO}_2^-$  as a sole nitrogen source [102]. However, it is difficult to clarify the factors determining nitrogen preference [10].

(3) Reactor-centered studies focusing on the determination of boundary conditions should be extensively conducted, which can help to realize early large-scale application of CMX bacteria. Interactions of CMX bacteria with other bacterial groups are still limited in both natural and engineered systems. Therefore, future exploration of bacterial interactions between CMX and other bacteria will definitely facilitate the evaluation of reactor adaptability of CMX bacteria.

(4) Deep insights into the stress response of CMX bacteria remain to be explored. Stress-related genetic elements in CMX bacteria can help in understanding the mechanisms of bacterial persistence in nonhost environments [136], which deserve further investigation. It should be noted that water or wastewater has unique and distinct microbial and chemical compositions, which will also impose stresses on CMX.

(5) The role of CMX in the turnover of micropollutants is not completely known. This requires in-depth analysis of many issues, such as the abundance of CMX in engineered ecosystems, potential of CMX bacteria to degrade micropollutants, and factors influencing micropollutant removal by CMX bacteria. Addressing these issues could help us to design an efficient process to remove micropollutants using CMX bacteria in the future.



(6) Currently, we know that CMX *Nitrospira* is ahead compared with canonical nitrifiers by exhibiting higher DO affinity and thriving under low DO concentrations, but it is doubtful whether CMX *Nitrospira* is truly an effective energy saver while removing nitrogen. It is also worth focusing on manipulations of enzyme activities to reduce the energy demand of CMX *Nitrospira*.

## 7. Conclusions

CMX bacteria are ubiquitously distributed in WTPs and WWTPs. Importantly, CMX bacteria have several competitive advantages over coresident canonical nitrifiers, for example, the ability to grow at low DO levels, rapid niche divergence with horizontal gene transfer, urea utilization with high affinity, and efficient nitrogen removal at low DO and high SRT. Furthermore, CMX bacteria can endure long-term stress induced by diverse chemicals and can also co-metabolize certain pollutants (e.g., pharmaceuticals and personal care products (PPCPs)) during simultaneous  $\text{NH}_3$  oxidation. Although CMX bacteria coexist with various canonical nitrifiers, each clade of CMX bacteria has its own coresident bacterial community. It can be concluded that the use of CMX bacteria in WTPs or WWTPs for nitrogen and micropollutant removal is practically possible. However, future studies are largely needed for better design and operation of CMX bacteria-based reactors.

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## Compliance with ethical guidelines

Naga Raju Maddela, Zhihao Gan, Yabing Meng, Fuqiang Fan, and Fangang Meng declare that they have no conflicts of interest or financial conflicts to disclose.

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