

# Overlooked nitrogen-cycling microorganisms in biological wastewater treatment

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## HIGHLIGHTS

- AOA and comammox bacteria can be more abundant and active than AOB/NOB at WWTPs.
- Coupled DNRA/anammox and NO<sub>x</sub>-DAMO/anammox/comammox processes are demonstrated.
- Substrate level, SRT and stressors determine the niches of overlooked microbes.
- Applications of overlooked microbes in enhancing nitrogen removal are promising.

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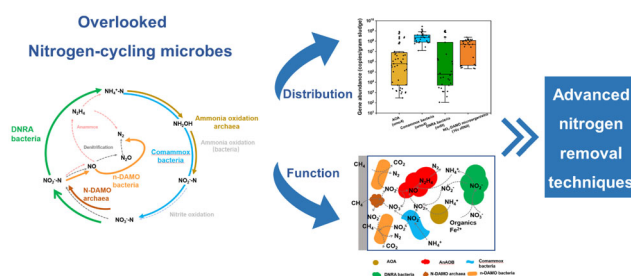
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Engineering application

## GRAPHIC ABSTRACT



## ABSTRACT

Nitrogen-cycling microorganisms play key roles at the intersection of microbiology and wastewater engineering. In addition to the well-studied ammonia oxidizing bacteria, nitrite oxidizing bacteria, heterotrophic denitrifiers, and anammox bacteria, there are some other N-cycling microorganisms that are less abundant but functionally important in wastewater nitrogen removal. These microbes include, but not limited to ammonia oxidizing archaea (AOA), complete ammonia oxidation (comammox) bacteria, dissimilatory nitrate reduction to ammonia (DNRA) bacteria, and nitrate/nitrite-dependent anaerobic methane oxidizing (NO<sub>x</sub>-DAMO) microorganisms. In the past decade, the development of high-throughput molecular technologies has enabled the detection, quantification, and characterization of these minor populations. The aim of this review is therefore to synthesize the current knowledge on the distribution, ecological niche, and kinetic properties of these “overlooked” N-cycling microbes at wastewater treatment plants. Their potential applications in novel wastewater nitrogen removal processes are also discussed. A comprehensive understanding of these overlooked N-cycling microbes from microbiology, ecology, and engineering perspectives will facilitate the design and operation of more efficient and sustainable biological nitrogen removal processes.

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## 1 Introduction

Reactive nitrogen is an indispensable nutrient for life, but excess emissions of reactive N species will cause air, soil and water pollution (Stein and Klotz, 2016). Nitrification-denitrification based nitrogen removal processes have been widely applied at wastewater treatment plants (WWTPs), where ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and heterotrophic denitrifiers are dominant microbes that have been extensively studied (Wiesmann, 1994). In recent years, considerable research efforts have been devoted to the autotrophic nitrogen removal by anaerobic ammonium oxidation (anammox) bacteria (Kartal et al., 2010). As a result, relevant review articles summarizing the abundances and activities of these key functional microorganisms have been published (Kartal et al., 2010; Lu et al., 2014; Soliman and Eldyasti, 2018). However, there are diverse groups of previously “overlooked” N-cycling microorganisms that may also play important roles in wastewater nitrogen removal, despite of their relatively low abundances in the community. These microorganisms include, but not limited to ammonia oxidizing archaea (AOA), the recently discovered complete ammonia oxidizing (comammox) bacteria, dissimilatory nitrate reduction to ammonia (DNRA) bacteria, and nitrate/nitrite-dependent anaerobic methane oxidizing ( $\text{NO}_x$ -DAMO) archaea and bacteria (Fig. 1) (Tiedje, 1988; Park et al., 2006; Ettwig et al., 2010; Haroon et al., 2013; Daims et al., 2015; Van Kessel et al., 2015).

With the rapid development of high-throughput molecular biology approaches, e.g., metagenomics and transcriptomics methods, the distribution, phylogeny, and metabolic properties of the previously overlooked N-cycling microorganisms are increasingly studied in a more

comprehensive manner. For example, AOA and comammox bacteria are the new members of autotrophic nitrifiers discovered by genomic sequencing rather than traditional culture-based method. Their functions in natural and engineered environments are also increasingly studied based on omics approaches (Palomo et al., 2018). It is previously believed that DNRA bacteria made limited contributions to nitrogen removal at WWTPs. However, recent studies based on metagenomics revealed their adverse roles in maintaining anammox process stability (Keren et al., 2020).  $\text{NO}_x$ -DAMO microorganisms are widespread in natural habitats but less detectable at WWTPs (Van Kessel et al., 2018; Ren et al., 2020). However, they could be the key microbes facilitating energy-neutral biological nitrogen treatment using methane as the electron donor. Although great efforts have been made in recent years to characterize these previously overlooked N-cycling microorganisms, critical knowledge gaps remain in their *in situ* activities and metabolic interactions with other microorganisms during biological nitrogen removal.

In this review, we synthesized the current knowledge on the distribution, ecological niche, and kinetic properties of overlooked N-cycling microorganisms, including AOA, comammox bacteria, DNRA bacteria and  $\text{NO}_x$ -DAMO microorganisms in wastewater treatment. Their potential applications in novel wastewater nitrogen removal processes, such as partial nitrification/denitrification, partial nitrification/anammox, partial denitrification/anammox,  $\text{NO}_x$ -DAMO/anammox, and  $\text{NO}_x$ -DAMO/comammox systems are also discussed. More insights into these previously overlooked microorganisms will contribute to a better understanding of their roles in wastewater N cycle, interactions with conventional N-cycling microorganisms

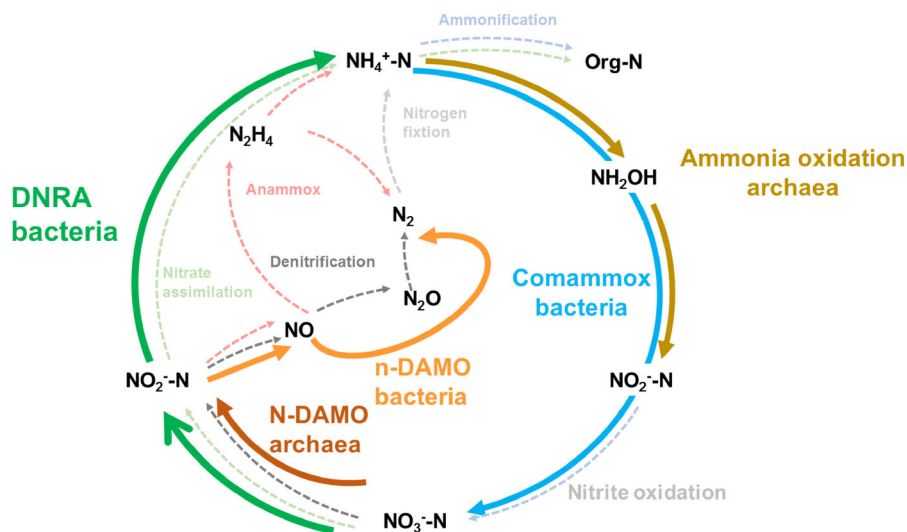


Fig. 1 Overlooked microorganisms in the wastewater microbial nitrogen cycle.

in community assembly and functioning, and facilitate the development of sustainable wastewater nitrogen removal techniques in the future.

## 2 Ammonia oxidizing microorganisms other than canonical AOB and NOB

### 2.1 Ammonia oxidizing archaea (AOA)

AOA belonging to *Thaumarchaeota* are prevalent in diverse environments, e.g., marine, hot spring, coral, and soils (Hatzenpichler, 2012). They possess the unique 16S rRNA and *amoA* genes that distinguish them from AOB (Könneke et al., 2005), which can be further divided into group I.1a (*Nitrosopumilus*), I.1a associated (*Nitrosotalea*), I.1b (*Nitrososphaera*) and ThAOA (*Nitrosocaldus*) (Table 1). AOA are much smaller, and have higher ammonia and oxygen affinity ( $K_{m,NH_3} = 0.003 - 4.4 \mu\text{mol/L}$ ,  $K_{m,O_2} = 2.01 - 10.38 \mu\text{mol/L}$ ) and lower ammonia utilization rate than AOB (Table 2) (Martens-Habben et al., 2009; Kits et al., 2017). They can grow in acidic/alkaline, hot/cold, and eutrophic/oligotrophic environments (Hatzenpichler, 2012). The monolayer cell membrane of AOA contains glycerol dialkyl glycerol tetraethers, forming stable ether bonds that are beneficial to their adaptations to extreme environments (Schouten et al., 2013).

The presence of AOA at WWTPs has been widely reported (Sauder et al., 2012; Pan et al., 2018). Their relative abundances varied from  $2.99 \times 10^2$  to  $8.65 \times 10^8$  *amoA* gene copies/g sludge, accounting for 0.07%–96.04% of the total ammonia oxidizers (Fig. 2) (Park et al., 2006; Limpiyakorn et al., 2011; Gao et al., 2014; Yin et al., 2018). DNA-SIP was always used to clarify the contribution of AOA and AOB, which demonstrated that AOA

were significantly dominant in winter despite numeral relationship (Pan et al., 2018). These AOA populations are mostly associated with group I.1a and group I.1b (Park et al., 2006). Factors determining the partitioning of AOA and AOB in wastewater nitrification reactors include substrate concentrations, solids retention time (SRT), temperature, and organics/toxicants. The following conditions favor the existence of AOA: **1) Nutrition levels (e.g., broad ammonia, diverse organics):** The abundances of AOA were found negatively correlated with ammonium concentration in the effluent of domestic WWTPs (Li et al., 2018). When treating landfill leachate with a high ammonia concentration ( $118 \pm 15 \text{ mgN/L}$ ), AOA could outcompete AOB and comammox bacteria at relatively low DO ( $\leq 1.5 \text{ mg/L}$ ) (Yang et al., 2020). Otherwise, AOA were abundant in some other industrial WWTPs (e.g., petroleum refinery), since they could utilize organics such as malate and succinate as alternative electron donors (Sauder et al., 2017). Another study demonstrated the effectiveness of adding N-acyl-L-homoserine lactones in upregulating *amoA* genes and the nitrification activity of AOA (Gao et al., 2019). **2) Operation parameters (e.g., low dissolved oxygen (DO), long SRT):** AOA could co-exist with anaerobic microorganisms under hypoxic conditions ( $\text{DO} < 0.1 \text{ mg/L}$ ), e.g., heterotrophic denitrifiers and anammox bacteria (AnAOB) (Straka et al., 2019). Longer SRT is usually required to sustain more AOA due to their relatively low growth rate ( $\mu_{\text{max}} = 0.010 - 0.050 \text{ /h}$ ). This explains why AOA exceed AOB as the dominant nitrifiers more frequently in biofilm systems than activated sludge (up to 1–2 orders higher than AOB) (Chen et al., 2017). **3) Environmental factors (e.g., high salinity, low temperature, sensitive to toxicants):** AOA can also outcompete AOB in treating wastewater with 0.25%–3.5% salinity (1.6–81.1 times more abundant) (Wu et al., 2020). In winter, *Candidatus Nitrosocosmicus exaquare* are the

**Table 1** Basic characteristics of the four overlooked nitrogen-cycling microorganisms

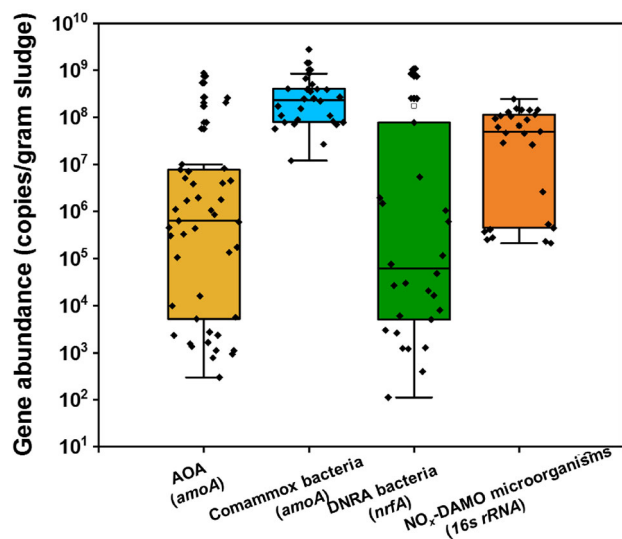
Microbes	Reaction equation	Key characteristics	Representative species at WWTPs	Reference
AOA	$\text{NH}_4^+ + 1.5\text{O}_2 = \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+$ ( $\Delta G^\circ = -274.7 \text{ kJ/mol}$ )	Aerobic, <i>Thaumarchaeota</i>	<i>Nitrosopumilus maritimus</i> <i>Candidatus Nitrosocosmicus exaquare</i> <i>Candidatus Nitrosotenuis cloacae</i>	Martens-Habben et al., 2009
Comammox bacteria	$\text{NH}_4^+ + 2\text{O}_2 = \text{NO}_3^- + \text{H}_2\text{O} + 2\text{H}^+$ ( $\Delta G^\circ = -348.9 \text{ kJ/mol}$ )	Aerobic, <i>Nitrospira</i> lineage II Mixotrophic (e.g., formate, urea)	<i>Nitrospira inopinata</i> <i>Candidatus Nitrospira nitrosa/nitrificans/kreftii</i>	Daims et al., 2015; Van Kessel et al., 2015
DNRA bacteria	$\text{C}_6\text{H}_{12}\text{O}_6 + 3 \text{NO}_3^- + 6\text{H}^+ = 6 \text{CO}_2 + 3\text{NH}_4^+ + \text{H}_2\text{O}$ ( $\Delta G^\circ = -623 \text{ kJ/mol NO}_3^- \text{-N}$ )	Respiratory/Fermentative Electron donors: organics, $\text{H}_2$ , $\text{HS}^-$ Enzymes: Nar/Nir, or Nap/Nrf	<i>Wolinella succinogenes</i> <i>Serratia marcescens</i> <i>Salmonella typhimurium</i> <i>Escherichia coli</i>	Tiedje, 1988; Holmes et al., 2019; Pandey et al., 2020
N-DAMO archaea	$4\text{NO}_3^- + \text{CH}_4 = 4\text{NO}_2^- + \text{CO}_2 + 2\text{H}_2\text{O}$ ( $\Delta G^\circ = -503 \text{ kJ/mol CH}_4$ )	Reverse methanogenesis, ANME-2D lineage Enzymes: NarGH, MCR	<i>Candidatus Methanoperedens nitroreducens</i>	Haroon et al., 2013
n-DAMO bacteria	$8\text{NO}_2^- + 3\text{CH}_4 + 8\text{H}^+ = 4\text{N}_2 + 3\text{CO}_2 + 10\text{H}_2\text{O}$ ( $\Delta G^\circ = -928 \text{ kJ/mol CH}_4$ )	Inter-aerobic pathway, NC10 phylum Enzymes: NirSJFD/GH/L, pMMO	<i>Candidatus Methylomirabilis oxyfera/sinica/limnetica/lanthanidiphila</i>	Ettwig et al., 2010; He et al., 2016; Graf et al., 2018

**Table 2** Kinetic parameters of the conventional and overlooked nitrogen-cycling microorganisms

Microbes	Electron donor/acceptor	Half saturation constant of electron donors $K_{m,d}$ ( $\mu\text{mol/L}$ )	Half saturation constant of electron acceptors $K_{m,a}$ ( $\mu\text{mol/L}$ )	Maximum specific growth rate $\mu_{\text{max}}$ (/h)	Cell yield (mg Protein /mol $\text{NH}_3$ or $\text{NO}_2^-$ )	Reference
Ammonia oxidizers	$\text{NH}_4^+$ -N/ $\text{O}_2$ (bacteria)	1.9–200	6.9–17.4	0.007–0.088	250	Laanbroek et al., 1994; Jiang and Bakken, 1999; Lawson and Lucker, 2018
	$\text{NH}_4^+$ -N/ $\text{O}_2$ (archaea)	0.003–4.4	2.01–10.38	0.010–0.050	298.4–304.3	Martens-Habbenha et al., 2009; Qin et al., 2014; Kits et al., 2017; Lawson and Lucker, 2018
Nitrite oxidizers	$\text{NO}_2^-$ -N/ $\text{O}_2$ ( <i>Nitrobacter</i> )	49–1380	5.31–165.63	0.0115–0.125	83–108	Laanbroek et al., 1994; Blackburne et al., 2007; Nowka et al., 2015; Lawson and Lucker, 2018
	$\text{NO}_2^-$ -N/ $\text{O}_2$ ( <i>Nitrospira</i> )	9–27	2.19–5.94	0.027–0.083	120–213	
Comammox bacteria	$\text{NH}_4^+$ -N/ $\text{O}_2$	0.049, 0.040	NA	0.0061	394.7	Kits et al., 2017; Sakoula et al., 2020
	$\text{NO}_2^-$ -N/ $\text{O}_2$	449.2, 12.5	NA	NA	NA	
DNRA bacteria	Organic/ ( $\text{NO}_3^-$ , $\text{NO}_2^-$ )	NA	NA	0.11–0.5	NA	Simon, 2002
	Inorganics/ ( $\text{NO}_3^-$ , $\text{NO}_2^-$ )	NA	NA	0.091	NA	
N-DAMO archaea	$\text{CH}_4/\text{NO}_3^-$	500	150 $\pm$ 28.6	NA	73–85*	
n-DAMO bacteria	$\text{CH}_4/\text{NO}_2^-$	2.6–5.9, 91.8 $\pm$ 5	4.28–7	0.0015–0.0583	NA	He et al., 2013; Winkler et al., 2015; Guerrero-Cruz et al., 2019; Lu et al., 2019
Anammox bacteria	$\text{NH}_4^+$ -N/ $\text{NO}_2^-$ -N	2.54–673.90	0.22–361.35	0.0022–0.014	30–70 <sup>#</sup>	Ali et al., 2015; Zhang and Okabe, 2020

Note: NA: not available, \* unit: mmol C/mol  $\text{CH}_4$ , <sup>#</sup> unit: mmol C/mol  $\text{NH}_4^+$ -N.

typical AOA population at WWTPs, which could be 52.95 times more abundant than AOB (Pan et al., 2018; Fan et



**Fig. 2** Abundances of AOA, comammox bacteria, DNRA bacteria and  $\text{NO}_x$ -DAMO microorganisms in wastewater treatment. Data are collected from published results, representing samples from municipal WWTPs, industrial WWTPs, recirculating aquaculture systems, and drinking water treatment systems.

al., 2019). AOA encode copper-based ammonia oxidation and respiration pathways, making them more copper-dependent than AOB and sensitive to the existence of metal-complexation organics in most industrial wastewater (Stahl and De La Torre, 2012; Gwak et al., 2020). For example, AOA could be strongly inhibited by 0.5 mmol/L cysteine (62.86%–98.90%), tryptone (70.75%–100%), and histidine (94.33%–96.51%) (Gwak et al., 2020). Otherwise, AOA are generally more sensitive to some toxicants than AOB, e.g., the NO-scavenger PTIO and short-chain alkynes (Beckman et al., 2018; Wright et al., 2020). Nevertheless, AOA could be more tolerant to antibiotics (e.g., streptomycin, kanamycin, ampicillin, and carbenicillin) than AOB, and the presence of spiramycin selected AOA as the dominant ammonia oxidizers (Zhang et al., 2015).

## 2.2 Complete ammonia oxidizing (comammox) bacteria

Comammox bacteria oxidize ammonia to nitrate via nitrite in a single microorganism. Thus far, all discovered comammox bacteria belong to *Nitrospira* sublineage II, and their 16S rRNA gene sequences are highly similar to those of canonical NOB-*Nitrospira* (Daims et al., 2015). Their *amoA* genes are different from those in AOB, but more identical to the particulate methane monooxygenase

gene *pmoA* (Palomo et al., 2018). Based on the *amoA* gene sequences, comammox bacteria are further divided into clade A and B (Daims et al., 2015; Pjevac et al., 2017), which differ in substrate transport, energy transfer, and adaptability to changing environment (Palomo et al., 2018). The representative clade A populations include *Nitrospira inopinata*, the only isolated comammox bacterium so far, and enriched cultures of *Candidatus Nitrospira nitrosa*, *Candidatus Nitrospira nitrificans*, and *Candidatus Nitrospira kreffii*, while clade B populations still lack representative cultures (Daims et al., 2015; Van Kessel et al., 2015; Sakoula et al., 2020).

Comammox bacteria are ubiquitous at WWTPs, accounting for 0.02%–3% of the total bacteria community in nitrifying activated sludge, and 1.8%–19.4% of the biofilm communities in rotating biological contactors (Fig. 2) (Annavaiah et al., 2018; Cotto et al., 2020; Spasov et al., 2020). However, unlike AOA, comammox bacteria were not detected in reactors receiving high concentrations of ammonia, e.g., side-stream partial nitrification/anammox systems (influent ammonium > 400 mgN/L) (Cotto et al., 2020). Compared to nitrification processes governed by AOB and NOB, comammox-dominated nitrification possesses advantage in maintaining performance efficiency and stability at extremely low ammonia and DO concentrations, having less N<sub>2</sub>O emissions, and might better cooperate with anaerobic bacteria (e.g., AnAOB) in facilitating total nitrogen removal. Some study revealed that currently genome-sequenced comammox species lack NO reductases, thus merely produced N<sub>2</sub>O abiotically (0.07% ± 0.006%), which was 1–2 orders lower than AOB (0.1%–8%) (Kits et al., 2019; Prosser et al., 2020).

Environmental factors shaping the ecological niches of comammox bacteria in wastewater nitrogen removal systems include: 1) Nutrition levels (e.g., low ammonia, simple organics): comammox bacteria are prevalent in natural and engineered systems with relatively low ammonium concentrations (Pjevac et al., 2017; Fowler et al., 2018). The ammonia affinity of comammox bacteria ( $K_{m,NH_3} = 0.049\text{--}0.040\ \mu\text{mol/L}$ ) is 1–3 magnitudes lower than AOB (Table 2) (Kits et al., 2017; Sakoula et al., 2020). As a result, drinking water treatment plants and recirculating aquaculture systems provide optimal conditions for comammox bacteria, where their relative abundances can be 2.1–60.7 times higher than AOA and AOB (Bartelme et al., 2017; Wang et al., 2017; Fowler et al., 2018). Comammox bacteria clade A usually dominate in these systems (mostly *Candidatus Nitrospira nitrosa*), whereas clade B are more frequently detected in rapid sand filters (up to 75% of the total nitrifying community) (Camejo et al., 2017; Palomo et al., 2018; Roots et al., 2019). In addition, mixotrophic growth of comammox bacteria on simple inorganic and organic substances (e.g., H<sub>2</sub>, formate, and urea) other than ammonia and nitrite further improves their survival and persistence in broad environments

(Palomo et al., 2018; Han et al., 2019). 2) Operation parameters (e.g., high SRT and low DO): Surveys on full-scale WWTPs revealed that longer SRT (activated sludge > 12.5 d, or biofilm) and lower DO (0.2–1.0 mg/L) favored comammox bacteria over canonical nitrifiers (Roots et al., 2019; Cotto et al., 2020). This can be explained by their relatively low growth rate ( $\mu_{max} = 0.0061\ \text{h}^{-1}$ ) (Table 2) and high oxygen affinity (Camejo et al., 2017; Kits et al., 2017; Roots et al., 2019). Molecularly, the latter is attributable to the bd-type terminal oxidase and the oxygen-sensitive carbon fixation pathway in comammox bacteria. 3) Environmental factors (e.g., metal deficient): Compared with AOB and NOB, comammox bacteria can better survive in copper and iron deficient conditions, because they possess enzymes to maintain copper homeostasis (CopABCD) and the high iron affinity of cytochrome c biogenesis system I (Palomo et al., 2018).

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### 3 Overlooked nitrate/nitrite reducing microbes other than heterotrophic denitrifiers

#### 3.1 Dissimilatory nitrate reduction to ammonia (DNRA) bacteria

DNRA bacteria reduce nitrate to ammonia via nitrite in a single microorganism, with organics or inorganics as electron donors. They can be heterotrophic, autotrophic, anaerobic or aerobic (Tiedje, 1988; Pandey et al., 2020). Heterotrophic DNRA bacteria break down large molecule organics and are represented by *Aerobacter aerogenes*, *Citrobacter freundii*, *Enterobacter spp.*, and *Denitrovibrio spp.* Autotrophic DNRA bacteria are less abundant, using hydrogen and sulfide as electron donors, such as *Desulfovibrio gigas* and *Veillonella alcalescens* (Holmes et al., 2019). Based on the means of ATP synthesis, DNRA bacteria are further classified into fermentative and respiratory, where the former derives energy directly from substrate-level phosphorylation and the energy for the latter is from oxidative phosphorylation (Pandey et al., 2020). *Escherichia coli* are representative fermentative DNRA bacteria possessing periplasmic nitrate reductase (Nap), respiratory nitrite reductase (Nrf), respiratory nitrate reductase (Nar), and cytoplasmic nitrite reductase (Nir) systems to support growth under low and high nitrate conditions (Pandey et al., 2020). *Wolinella succinogenes* is a typical strain of respiratory DNRA, utilizing non-fermentative organics or inorganics (e.g., formate or H<sub>2</sub>) as electron donors, and can grow with low nitrate as they possess the high affinity Nap/Nrf system (Simon, 2002). *nrfA* gene serves as the molecular biomarker for DNRA bacteria and its abundances positively correlated with DNRA activity in situ (Pandey et al., 2020).

DNRA bacteria account for 0.2%–4% of total communities at full-scale municipal WWTPs across the world

(Fig. 2). DNRA process is not trivial but can contribute significantly to nitrogen metabolism (9.5–15.7 nmol N/g/L) in activated sludge, granular and biofilm reactors (Chen et al., 2020; Wang et al., 2020c). They were found more abundant in systems operated under anaerobic, high C/N and high sulfide conditions, e.g., anaerobic digesters, biofloc aquaculture ponds, sulfate removing reactors (Tiedje, 1988; Akunna et al., 1993). With the upgrade of WWTPs, especially the nutrient removal processes, the ratios of DNRA/denitrifying bacteria detected in the receiving water bodies increased from 1.097 to 1.928 (Wang et al., 2020b). DNRA bacteria are important functional groups in simultaneous nitrification and denitrification processes, accounting for 37.15%–46.01% of total OTUs related to nitrogen removal (Xiang et al., 2020).

For a long time, scientists have been interested in the coexistence and competition between heterotrophic denitrifiers and DNRA bacteria. Factors influencing the relative abundances of the two populations at wastewater bioreactors mainly include: **1) Nutrition levels (e.g., electron acceptor availability, type of electron donor):** From the perspective of electron acceptor, DNRA bacteria transfer more electrons per mole of nitrate reduced, and have higher affinity for nitrate ( $\mu_{\max}/K_s, \text{NO}_3^-$ : 26.2  $\mu\text{mol/L/h}$  versus 8.6  $\mu\text{mol/L/h}$ ) but lower affinity for acetate (5.2  $\mu\text{mol/L/h}$  versus 8.6 Van Denmol/L/h) than denitrifiers (Van Den Berg et al., 2016). Hence, DNRA bacteria have competitive advantages over heterotrophic denitrifiers in nitrate limiting but electron donor abundant conditions (typically acetate/nitrate ratio higher than 1.5). The genome of *Shewanella loihica* strain PV-4 encodes the complete set of conventional denitrification and DNRA pathways, and relatively high C/N ratios upregulated their DNRA functional genes (e.g., *nrfA*) (Yoon et al., 2015). In another study, higher lactate/ $\text{NO}_3^-$  ratio (2.97) also promoted the co-existence of fermentative and respiratory DNRA, and enhanced their activities (Van Den Berg et al., 2017). Meta-analysis also suggested that DNRA rate was positively correlated with C/ $\text{NO}_3^-$  (Van Den Berg et al., 2016; Pandey et al., 2020). Additionally, nitrate other than nitrite serving as the electron acceptor favors DNRA process over denitrification (Kraft et al., 2014). Type of electron donor also was another important factor. Compared with heterotrophic denitrifiers, fermentative DNRA bacteria prefer to use less oxidative and labile organic carbon sources (Tiedje, 1988; Van Den Berg et al., 2017). L-sorbose or D-cellobiose enriched denitrifiers (e.g., *Klebsiella*), while D-glucose, D-fructose and citrate enriched DNRA bacteria (e.g., *Escherichia*, *Sulfurospirillum*) (Carlson et al., 2020). In a microbial community with both DNRA and denitrifying bacteria, DNRA process dominated when glucose and glycerol served as the carbon sources (nitrate removal rate: 2.7–10.1 mgN/g MLVSS/h), while denitrification mainly occurred when acetic acid and lactic acid were supplied (nitrate removal rate: 23.8–27.8

mgN/g MLVSS/h) (Akunna et al., 1993). Both autotrophic denitrifiers and respiratory DNRA bacteria can use inorganic compounds as electron donors (e.g.,  $\text{H}_2$ ,  $\text{S}^{2-}$ ,  $\text{Fe}^{2+}$ ), but FeS usually enriched denitrifiers, and  $\text{H}_2\text{S}$  favored the growth of respiratory DNRA bacteria (Yin et al., 2015; Pandey et al., 2020). **2) Environmental factors (e.g., high temperature and pH):** Higher diversity and activity of DNRA bacteria were observed in WWTPs operated at higher temperature despite variations in treatment configurations (Wang et al., 2020c). In *Shewanella loihica*, higher pH can regulate DNRA process, as the NrfA protein has pH optima at 8.0, while denitrification was observed in acid conditions (Kim et al., 2017).

### 3.2 Nitrate/nitrite-dependent anaerobic methane oxidizing ( $\text{NO}_x$ -DAMO) microbes

$\text{NO}_x$ -DAMO process is driven by a microbial consortium containing DAMO archaea and bacteria that can reduce nitrate to dinitrogen with methane as the electron donor (Ettwig et al., 2010; Haroon et al., 2013). N-DAMO archaea are members of the ANME-2D lineage capable of reducing nitrate to nitrite, with *Candidatus Methanoperedens nitroreducens* as the representative population (Haroon et al., 2013). Nitrate reduction is catalyzed by a pseudo-periplasm Nar-like protein complex, obtaining electrons from reverse methanogenesis (Arshad et al., 2015). Subsequently, nitrite is reduced to dinitrogen gas by n-DAMO bacteria belonging to NC10 phylum (e.g., *Candidatus Methyloirabilis oxyfera*, *Candidatus Methyloirabilis sinica*, and *Candidatus Methyloirabilis limnetica*) (Ettwig et al., 2010; He et al., 2016; Graf et al., 2018). Methylcoenzyme M reductase gene *mcrA* and particulate methane oxygenase gene *pmoA* are the biomarkers for DAMO archaea and bacteria, respectively (Luesken et al., 2011; Haroon et al., 2013).

$\text{NO}_x$ -DAMO microorganisms are widely present in full-scale anaerobic reactors (Luesken et al., 2011), and have been enriched in the laboratory (Hu et al., 2014; Kampman et al., 2014). N-DAMO archaea are less abundant than n-DAMO bacteria, due to the lack of lanthanide in wastewater, a special element required by methanol dehydrogenase in N-DAMO archaea (Ren et al., 2020). Overall, n-DAMO bacteria are present at  $10^6$ – $10^8$  16S rRNA copies/g sludge at WWTPs (Fig. 2), with an averaged activity of 1.61–22.31  $\mu\text{mol CH}_4/\text{g}$  sludge/d due to the shortage of nitrite and relatively short SRT of mainstream treatment processes (Hu and Ma, 2016; Meng et al., 2021). However, on account of the fact that methane can be produced *in situ* at WWTPs, this process still holds promise in future sustainable N removal techniques.  $\text{NO}_x$ -DAMO bioreactors have been established and studied more intensively at laboratory-scale. By combining anammox and  $\text{NO}_x$ -DAMO processes in a laboratory-scale membrane biofilm reactor (MBfR), enhanced total nitrogen (TN) removal rate

of 0.28 kg N/m<sup>3</sup>/d was achieved (Xie et al., 2018). Laboratory-scale membrane granular sludge reactor (MGSR) with NO<sub>x</sub>-DAMO/anammox consortium demonstrated high nitrogen removal capacity too (16.53 kg N/m<sup>3</sup>/d) (Fan et al., 2020).

The ecological niches of NO<sub>x</sub>-DAMO microorganisms in wastewater nitrogen removal reactors are influenced by: **1) Nutrition levels (e.g., substrate level, trace element):** The activity of n-DAMO bacteria increased with elevated methane concentration, whereas they can not tolerate high nitrite ( $K_{I,NO_2} = 4.1 \pm 0.5$  mmol/L) (He et al., 2013). Methanol and sodium acetate as additional electron donors can stimulate the activity of n-DAMO bacteria by up to 1.9 times (Ma et al., 2017). The nitrate affinities of N-DAMO archaea are similar to those of heterotrophic denitrifiers, but the nitrite affinities of n-DAMO bacteria are three orders of magnitude lower than those of denitrifiers (Table 2) (Guerrero-Cruz et al., 2019; Lu et al., 2019). In addition, trace amount of iron (20 μmol/L) and copper (10 μmol/L) promoted the growth of n-DAMO bacteria (He et al., 2015a). **2) Operation and environmental conditions (e.g., SRT, temperature and oxygen):** With relatively lower growth rate than heterotrophic denitrifiers, NO<sub>x</sub>-DAMO microorganisms can be readily washed out and a longer SRT or attached growth is generally required (Fan et al., 2020). Although n-DAMO bacteria can produce O<sub>2</sub> internally, the exposure to 2% oxygen reduced their nitrite reduction activity by 45%, indicating their sensitivity to oxygen (Luesken et al., 2012). In addition, mesophilic (35°C), neutral, and non-saline conditions are in favor of n-DAMO bacteria (He et al., 2015b).

## 4 Engineering applications of overlooked N-cycling microbes

Although overlooked N-cycling microorganisms make up rarely more than 1% of the microbial communities, they play important roles in sustaining community stability of wastewater nitrogen removal processes, helping them to cope with adverse conditions (e.g., cold, high salinity, copper deficient), and/or reducing energy consumption and greenhouse gas emissions.

### 4.1 AOA: ammonia oxidation under adverse conditions

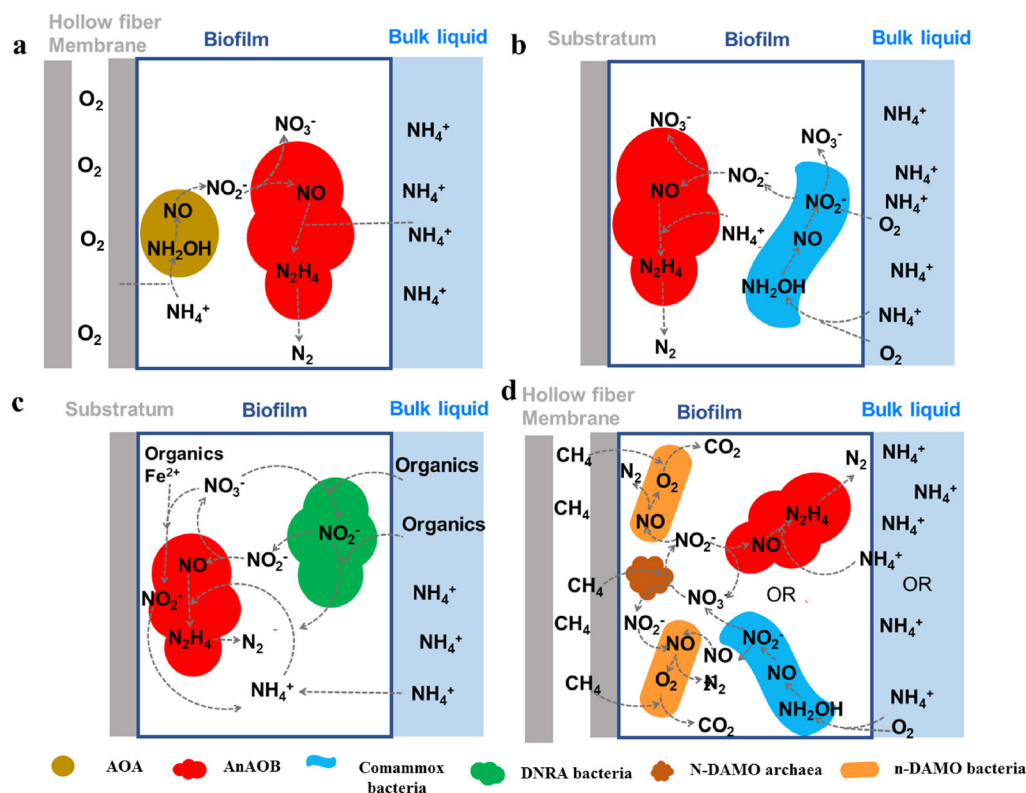
Due to their relatively high ammonia/oxygen affinity (Table 2) and tolerance to low temperature, high salinity, acidity and alkalinity, AOA are more promising in treating low strength ammonia wastewater under harsh conditions. For example, when treating saline wastewater, AOA can contribute to 94.9% and 48.2% of the overall ammonia oxidation at 10°C and 35°C, respectively (Lin et al., 2020). The much higher ammonia and oxygen affinities of AOA than AOB promoted their closer metabolic interactions

with AnAOB under nutrient- and oxygen-limited conditions, where the r-strategists NOB-*Nitrobacter* can be suppressed too. When treating low strength nitrogen sewage, AOA successfully cooperated with AnAOB in removing 90% of ammonium in a single-stage SBR (Pan et al., 2016). Reactors with efficient sludge retention (e.g., biofilm reactors, MbfR, sequencing batch biofilm reactors) were always used to sustain biomass of AOA (Straka et al., 2019; Wang et al., 2020a). For example, MBfR operated in a counter-diffusion mode, rather than diffusion better facilitate the AOA/AnAOB cooperation, as demonstrated in (Fig. 3(a)) (Straka et al., 2019). Theoretically, the TN removal by AOA-AnAOB consortia in MBfR can be up to 91.5%, and the coculture is more stable than AOB-anammox under fluctuated DO and HRT (Liu et al., 2016).

### 4.2 Comammox bacteria: nitrification at low N loading and low DO

Although raw domestic wastewater contains 20–50 mg TKN/L, the influent to biological nitrogen removal (BNR) systems could be as low as 9.86–14 mgN/L (Wang and Chen, 2016; Wang et al., 2018). It is highly possible that comammox bacteria can replace conventional AOB, AOA, and NOB as the dominant nitrifying bacteria under low DO conditions. Traditional nitrification process requires extensive aeration (DO > 2 mg/L), but comammox dominated nitrification reactor could be operated at DO < 1 mg/L and in the biofilm mode (Roots et al., 2019; Cotto et al., 2020). Simultaneous nitrification-denitrification (DO < 0.75 mg/L) can also occur in these systems, saving energy needed for aeration and internal recirculation (Holman and Wareham, 2005; Insel, 2007).

The unbalanced ammonia oxidation and nitrite oxidation activities of comammox bacteria lead to transient accumulation of nitrite, an important intermediate involved in diverse pathways including denitrification, DNRA, and anammox (Daims et al., 2015; Gottshall et al., 2020). With elevated ammonia addition from 0.01 to 1 mmol/L, transient nitrite accumulation up to 32.67% of converted ammonia was observed in comammox bacteria (Daims et al., 2015). Relatively high ammonium, limited oxygen supply, and addition of inhibitors (e.g., NH<sub>2</sub>OH) further enhance the nitritation activity (Kits et al., 2019; Wang et al., 2020d). Practically, comammox bacteria performing nitritation can be coupled with denitrifiers (i.e., nitritation-denitrification, or nitrite shunt) or AnAOB (partial nitritation-anammox, PN/A) to remove total nitrogen. In the attached growth mode, comammox bacteria on biofilm or granule surface oxidize ammonia to nitrite under hypoxic conditions, which is secreted and metabolized by AnAOB due to the relative higher nitrite affinity (Fig. 3(b), Table 2). A synthetic community comprising of AnAOB (*Candidatus* Brocadia anammoxidans) and comammox bacteria (*Nitrospira inopinata*) showed excellent nitrogen removal without nitrite accumulation (Gottshall et al., 2020).



**Fig. 3** Potential applications of AOA (a), comammox bacteria (b), DNRA bacteria (c) and  $\text{NO}_x$ -DAMO microorganisms (d) in novel wastewater nitrogen removal processes.

However, it should be cautious that comammox bacteria may replace AnAOB as the sole N-cycling microorganism in the integrated system, performing complete nitrification under low nitrogen concentrations and resulting in process failure (Roots et al., 2019). Additionally, comammox bacteria can also survive under metal-insufficient conditions (e.g. copper and iron), which future expands their ecological niches at WWTPs (Palomo et al., 2018; Koch et al., 2019).

#### 4.3 DNRA bacteria: coupling with anammox bacteria for enhanced N removal

One of the disadvantages of anammox is its inherent accumulation of nitrate (87% of influent nitrogen). DNRA coupled with anammox enhance total nitrogen removal by minimizing nitrate accumulation (Castro-Barros et al., 2017). DNRA bacteria were found coexisted with AnAOB in nearly all anammox systems, providing  $\text{NO}_2^-/\text{NH}_4^+$  for AnAOB (Fig. 3(c)) (Park et al., 2017). Typically, fermentative DNRA bacteria (e.g. *Chloroflexi*) utilize large molecule organics excreted by AnAOB in the form of extracellular polymeric substances (EPS) (Lawson et al., 2017). AnAOB also perform DNRA using small organic acids (e.g. formate, acetate, propionate) and inorganics (e.g. ferrous iron and hydrogen) (Castro-Barros et al., 2017). Thermodynamically, partial DNRA-AnAOB (1984 kJ/

acetate) could surpass heterotrophic denitrifiers (796.8 kJ/acetate) in carbon source limiting conditions. In the presence of high ammonium and sufficient inorganic carbon, the coupled partial DNRA-anammox process is in fact accomplished mainly by AnAOB rather than the mixed fermentative DNRA bacteria and AnAOB, representing a more sustainable autotrophic nitrogen removal technique (Castro-Barros et al., 2017). Autotrophic DNRA, e.g., Fe(II)-dependent DNRA coupled with anammox can achieve a total nitrogen removal rate of  $0.23 \pm 0.01 \text{ kg N/m}^3/\text{d}$  by controlling the EDTA-2Na/Fe(II) ratio and pH (Li et al., 2020). Notably, although heterotrophic/autotrophic DNRA bacteria can provide nitrite for AnAOB growth, they also compete with AnAOB for some metabolic necessities, such as amino acids and vitamins (e.g. VB12) (Keren et al., 2020). A recently study proposed another potential application of DNRA bacteria, i.e., nitrate recovery by bioelectrochemical ammonification mediated by the electroactive DNRA bacteria *Geobacter sp.* (Wan et al., 2020).

#### 4.4 $\text{NO}_x$ -DAMO microorganisms: sustainable nitrogen removal with methane

Applications of  $\text{NO}_x$ -DAMO microorganisms in wastewater nitrogen removal are under rapid development due to their advantages in energy conservation, greenhouse gas



mitigation, and sludge reduction (Van Kessel et al., 2018). Nitrate produced by AnAOB could be reduced by N-DAMO archaea, recirculating nitrite for AnAOB and n-DAMO bacteria (Fig. 3(d)). MBfR represents an optimal configuration facilitating the co-existence of NO<sub>x</sub>-DAMO microorganisms and AnAOB, resulting in a nitrogen removal rate of up to 190 mgN/L/d (Shi et al., 2013). In the recommended A-B process for achieving carbon neutral at WWTPs, the coupled NO<sub>x</sub>-DAMO and anammox process is promising in stage B because methane generated in stage A can be directly used without external addition of organic carbon sources. NO<sub>x</sub>-DAMO process enables a reduction in greenhouse gas emissions by at least one fifth compared with traditional treatment (Ma et al., 2017).

The coupled NO<sub>x</sub>-DAMO and comammox process was recently proposed as another sustainable nitrogen removal technology (Ren et al., 2020). Comammox bacteria supply electron acceptors (nitrate and nitrite) to DAMO archaea/bacteria under low N-loading and low DO conditions. Notably, the overdosed methane can be oxidized by ammonia monooxygenase in comammox bacteria, mitigating the accidental release of the potent greenhouse gas. However, the coupled process has not been demonstrated experimentally.

## 5 Conclusions and perspectives

This review summarized the distribution, ecological niche, kinetic properties and potential applications of the overlooked AOA, comammox bacteria, DNRA bacteria, and NO<sub>x</sub>-DAMO microorganisms in biological wastewater treatment processes. AOA and comammox bacteria are K-strategy nitrifiers growing slower than conventional AOB/NOB, but are better adapted to oligotrophic and adverse conditions. DNRA and NO<sub>x</sub>-DAMO microorganisms could surpass heterotrophic denitrifiers as the key nitrate reducers depending on substrate levels and operating parameters of bioreactors. Coupling them with AnAOB has been proven effective in enhancing total nitrogen removal, reducing costs and greenhouse gas emissions.

The following areas should be given priority in future studies on these overlooked microorganisms: 1) distribution pattern, *in situ* activities, and adaption strategies of overlooked microorganisms in traditional and novel nitrogen removal process should be evaluated more comprehensively. Efficient and accurate methods that can distinguish the contribution of specific microorganisms from similar function microbes (e.g., AOA/AOB/comammox bacteria, denitrifiers/DNRA bacteria) should be developed; 2) complex interactions (e.g., substrate cooperation/competition, toxicant detoxification) with other microorganisms in BNR systems, and the appropriate models describing these interactions could be constructed; 3) potentials and limitations of these microorganisms in

more sustainable nitrogen removal techniques (e.g., coupled AnAOB, comammox, and/or NO<sub>x</sub>-DAMO) could be assessed from engineering application points of view.

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