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Review Paper

Progress and Prospects of the Research of Complete Ammonia Oxidizers in Biological Wastewater Treatment

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Abstract. Comammox collapses the textbook two-step nitrification into a one-cell reaction: the same bug converts NH_3 straight to NO_3^-, upending the dogma that ammonia and nitrite oxidation must be split between different microbes. These single-step nitrifiers reset the playbook for microbial nitrogen turnover, global N-cycling and engineered denitrogenation. Here we chart their discovery trail, then condense what is known about their physiology, ecology and environmental footprints.We also map where comammox cells hide in treatment plants and how they can sway nitrogen conversions. Their edge under low O_2 and modest NH_3 hints at threats to partial nitritation and anammox stability. Closing sections flag open questions for future plant-focused work.

Keywords: *Complete ammonia oxidation bacteria; ammonia-oxidizing microorganisms; nitrifying bacteria; biological nitrogen removal; partial nitrification; wastewater treatment*

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Nitrification sits at the heart of Earth's nitrogen loop and remains the cornerstone tactic for scrubbing nitrogen out of sewage. Winogradsky [1] proposed in 1890 that nitrification is completed in two steps by two types of microorganisms: ammonia-oxidizing bacteria (AOB) use oxygen to oxidize ammonia nitrogen to nitrite, and then nitrite-oxidizing bacteria (NOB) use oxygen to oxidize nitrite to nitrate. For an extended period, nitrification was thought to be carried out by two distinct groups of bacteria under aerobic conditions. However, important progress has been made in research on the nitrogen cycle in recent years, deepening the understanding of microbial nitrogen metabolism. The 1995 unveiling of anammox microbes shattered the dogma that NH_4^+ demands oxygen to be oxidized, proving the reaction can proceed just as well under anoxia [2]. The 2005 isolation of ammonia-oxidizing archaea (AOA) overturned the long-held view that ammonia-oxidizing bacteria (AOB) alone handled NH_3 oxidation, proving archaea also catalyze the first step of nitrification [3]. Furthermore, the traditional view that nitrification is a two-step reaction mediated by distinct microorganisms has been challenged in recent years. In 2015, complete ammonia oxidation (comammox) microorganisms were identified and validated [4]. Since their unveiling, comammox bacteria have moved into the spotlight as researchers map where they thrive and how they perform, while probing their traits and head-to-head contests with other ammonia oxidizers.

1 Discovery and Confirmation of Comammox Microorganisms

Complete ammonia oxidation microorganisms possess the ability to directly oxidize ammonia nitrogen to nitrate independently, changing the understanding that "ammonia oxidation and nitrite oxidation are two completely independent processes" and redefining a key process in the Earth's nitrogen cycle (see Fig. 1).

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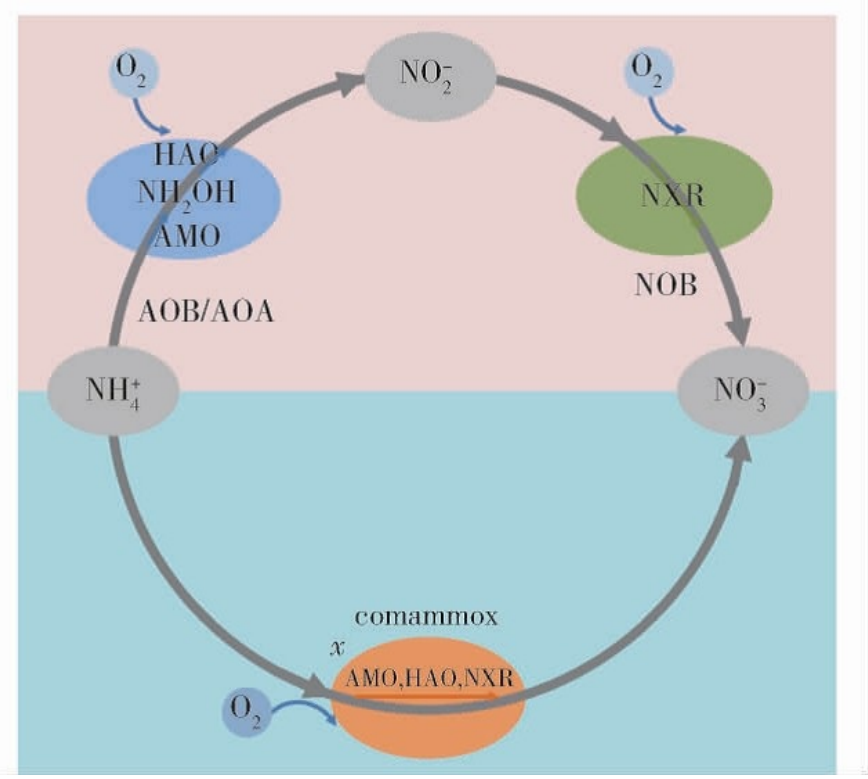

Figure 1 Schematic diagram of two-step and one-step ammonia oxidation process

In 2015, two research teams from Austria and the Netherlands, using a combination of stoichiometric analysis and metagenomics techniques, confirmed the existence of comammox bacteria, respectively [5].

Daims et al. [5] collected biofilm from the well walls of an oil exploration well in the North Caucasus region and cultured it at a constant temperature of 46°C. The enrichment culture could oxidize ammonia nitrogen to nitrate, suggesting the presence of both AOM and NOB in the culture. However, fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) assays failed to detect the ammonia monooxygenase gene (amoA) of ammonia‐oxidizing bacteria (AOB) or the 16S rRNA gene of ammonia‐oxidizing archaea (AOA) in the enrichment culture; only nitrite‐oxidizing bacteria (NOB) belonging to the Nitrospira genus were identified. Therefore, it was speculated that this species of Nitrospiramight have both ammonia oxidation and nitrite oxidation capabilities. Consequently, metagenomic sequencing of the enrichment culture was performed. Nitrospira dominated the enrichment, accounting for 68–80 % of the community. Gene screens showed the same Nitrospira cells carried both nitrite-oxidizing nxr and the classic ammonia-oxidation toolkit—amoA plus hao—revealing an all-in-one nitrifier. Because the enriched Nitrospira encoded the full set of $NH_3 \rightarrow NO_2^-$ and $NO_2^- \rightarrow NO_3^-$ genes and performed the entire conversion in one step, it was christened “Candidatus Nitrospira inopinata”—the first known comammox bug.

In the same year, van Kessel et al. [6] also reported the existence of comammox microorganisms. van Kessel et al. took sludge from an aquaculture wastewater trickling filter treatment system for culture. The medium contained low concentrations of ammonia nitrogen, nitrite, and nitrate, and the dissolved oxygen was always below 0.1 mg/L. After 12 months of enrichment, the culture showed anammox activity, simultaneously removing ammonia nitrogen and nitrite. However, FISH showed that the main microorganisms in the sludge were anammox bacteria of the Brocadiagenus, and 15% were NOB of the Nitrospiragenus. The high abundance of Nitrospiraunder low oxygen concentration was uncommon, so further analysis was conducted using molecular biology techniques. The results showed the system contained two different Nitrospiraspecies. In the high-quality draft genomes of the two Nitrospiraspecies, besides containing the nxr gene essential for nitrite oxidation, both also contained the amoA and hao genes that catalyze the ammonia oxidation process. Therefore, these Nitrospiraspecies were also identified as microorganisms with complete ammonia oxidation potential, named

CandidatusNitrospira nitrosa and CandidatusNitrospira nitrificans, respectively [6]. Fig. 2 shows the phylogeny of currently known complete ammonia oxidation microorganisms.

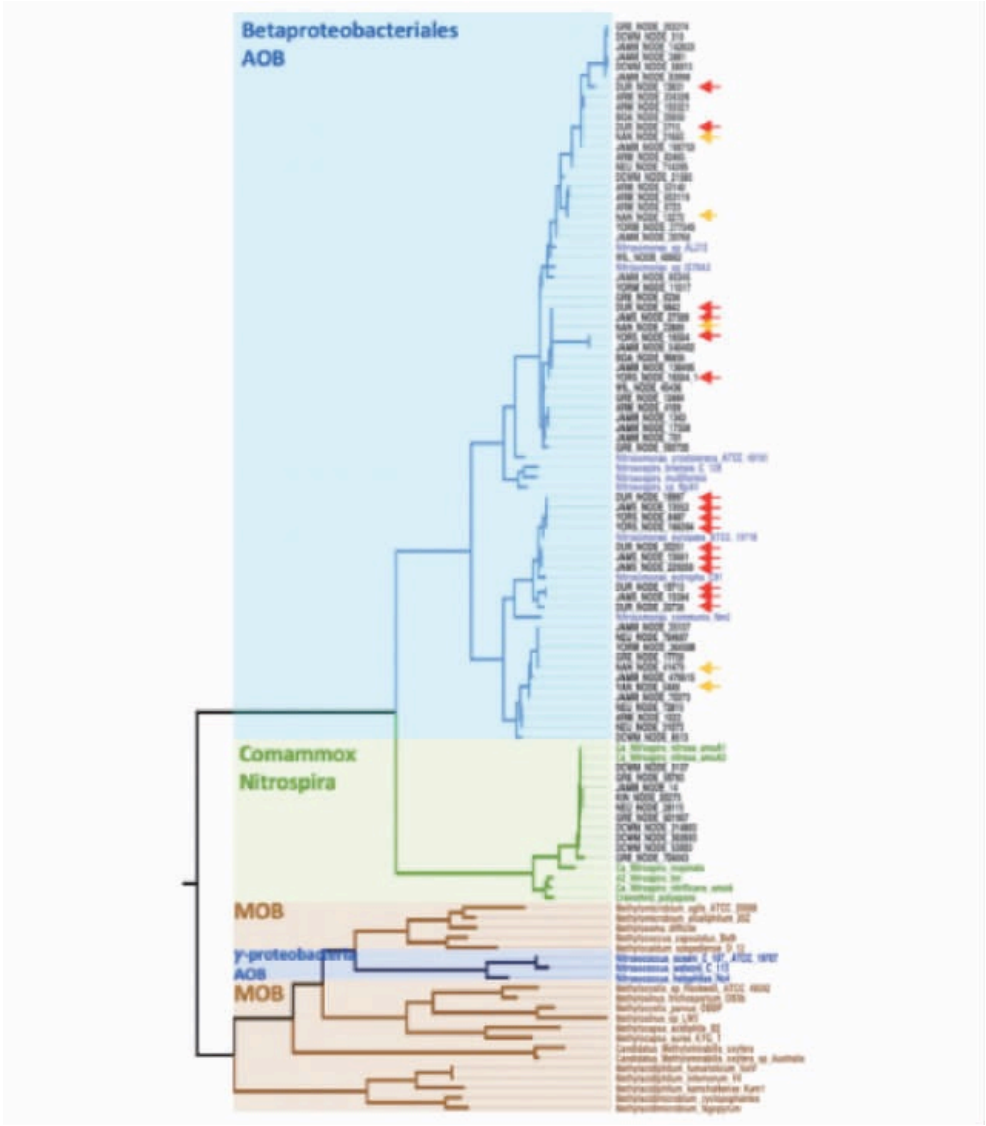

Figure 2 Comammox phylogenetic map

2 Physiological and Metabolic Characteristics of Comammox Bacteria

2.1 Phylogenetic Clade of Comammox Bacteria

Comammox rewrote the nitrogen-cycle script, turning the long-held two-step nitrification concept into a one-cell affair and opening a fresh research frontier. Since the discovery and confirmation of comammox, researchers have conducted extensive studies on the physiological, biochemical characteristics, and habitat distribution of comammox microorganisms, achieving phased progress. To date, all discovered complete ammonia oxidation microorganisms belong to Nitrospira lineage II (Nitrospira spp. Lineage II) [7]. Their genomes all contain the ammonia monooxygenase gene amoA, the hydroxylamine oxidoreductase gene hao, and the nitrite oxidoreductase gene nxr. Phylogenetic splits of comammox amoA place the organisms in two sister lineages—clade A and clade B [5].

The metabolic process of comammox microorganisms still needs in-depth research and analysis. Like AOB,

comammox cells can leak N_2O when the obligatory intermediate hydroxylamine (NH_2OH) is shunted off-pathway [8]. The genomes of comammox microorganisms also contain the nitrite reductase gene (nir), indicating that they possess the ability to reduce NO_2^- to NO, a trait shared with non-comammox Nitrospira [8]. However, akin to typical Nitrospira groups [9], the currently reported genomes of comammox clades A and B lack known nitric oxide reductase genes (nor) or proteins associated with NO metabolism [10], suggesting they may not be capable of performing nitric oxide reduction reactions.

2.2 Comammox Bacteria Are More Suitable for Low Ammonia Nitrogen and Low Oxygen Environments

Kinetic and genomic surveys show comammox bugs are engineered for life where NH_4^+ is scarce, letting them outcompete other ammonia oxidizers in oligotrophic niches [7]. Daims et al. measured an apparent K_m of 0.84 μmol/L NH_4^+ for *Ca.* N. inopinata [5], a value that rivals the 0.48 μmol/L reported for AOA [10]. The K_m values of both AOA and comammox bacteria are considerably lower than those of typical ammonia-oxidizing bacteria (AOB) (492 μmol/L NH_4^+) [17-23]. This suggests that, like AOA, comammox organisms are capable of growing and dominating in environments with low ammonia-nitrogen concentrations. AOB are in a competitive advantage compared to comammox bacteria in ammonia-rich environments [11], but comammox bacteria have a strong affinity for ammonia nitrogen [12], and comammox bacteria can encode various Amt-type transporter proteins with strong ammonia affinity [13], promoting their survival in environments with fluctuating ammonia nitrogen concentrations.

The carbon fixation pathways and electron transport characteristics of comammox microorganisms enable them to adapt to low-oxygen environments. There are notable differences in carbon fixation pathways among different ammonium-oxidizing microorganisms (AOM). AOB use the oxygen-tolerant Calvin cycle to fix CO2, AOA use the 3-hydroxypropionate/4-hydroxybutyrate cycle to fix CO2 [3], anammox bacteria use the reductive acetyl-CoA pathway [11], while comammox bacteria fix CO2 through the reductive tricarboxylic acid (rTCA) cycle which requires less oxygen [8]. Fig. 3 shows a schematic diagram of the carbon dioxide fixation pathways utilized by different AOM [14]. Comammox cells also pack a bd-type terminal oxidase with stronger O_2 affinity than the respiratory cytochromes of other ammonia oxidizers [12], handing them an edge when oxygen is thin.

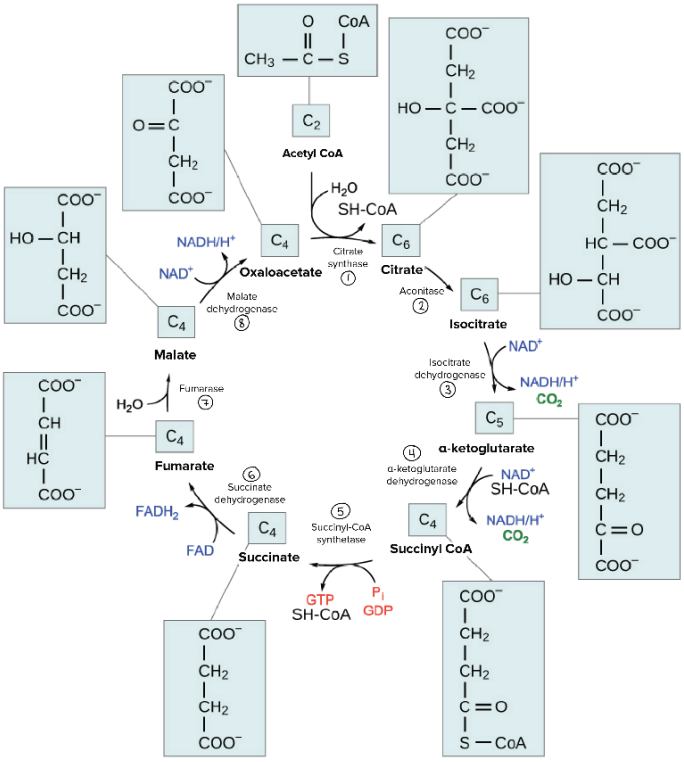

Figure 3 Schematic diagram of different carbon dioxide fixation pathways

2.3 Low Nitrite Affinity of Comammox Bacteria

Although comammox bacteria have the ability to oxidize nitrite, their affinity for nitrite is likely to be relatively low. Kits et al. [15] conducted a comparative analysis of the core genome of Nitrospira and found that although comammox bacteria are highly similar to NOB in all nitrifying genomes, the nitrite affinity of comammox bacteria is about 50 times lower than that of traditional NOB (about 500 μmol/L). However, the nitrite produced during the oxidation of ammonia by comammox bacteria can accumulate intracellularly, thereby overcoming the defect of poor nitrite affinity.

Comammox bacteria have complex metabolic pathways and can utilize various substances for metabolism, thus adapting to changes and fluctuations in environmental substrates. Studies have shown that in addition to using ammonia nitrogen as a nitrogen source,

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Comammox bacteria have complex metabolic pathways and can utilize various substances for metabolism, thus adapting to changes and fluctuations in environmental substrates. Studies have shown that in addition to using ammonia nitrogen as a nitrogen source, comammox bacteria can also use urea as an ammonia source to drive nitrification [6], and comammox bacteria also have the ability to oxidize formate and hydrogen [16]. In addition, the comammox microbial genome contains alkaline phosphatase genes [17] that are not found in AOA and AOB genomes. These genes can be highly expressed under conditions of insufficient phosphorus in the environment, giving comammox bacteria a competitive advantage in phosphorus-deficient environmental conditions.

3 Distribution and Impact of Comammox in the Natural Environment

3.1 Distribution of Comammox Bacteria in the Natural Environment

After the confirmation of the existence of comammox microorganisms, researchers compared the amoA gene data of comammox microorganisms on public data platforms and found that the amoA genes of comammox microorganisms are distributed in soils (paddy fields, plains, forests, etc.), freshwater environments (wetlands, riverbeds, lake sediments, aquifers) [18]. Recently, researchers have conducted extensive investigations on the habitat distribution of comammox bacteria in natural and engineered environments, confirming relevant patterns in new research progress [19]. Table 1 is an incomplete summary of the currently reported distribution of comammox microorganisms. Existing research results on the distribution of comammox bacteria further verify the characteristic that comammox bacteria can survive in low ammonia nitrogen concentration and micro-oxygen environments. It is worth pointing out that although the genes of comammox microorganisms share many similarities with those of AOA, no direct evidence has been found for the existence of comammox microorganisms in marine environments.

3.2 Impact of Comammox on the Earth's Nitrogen Cycle

Known comammox bugs are obligate autotrophs that share soil habitats with both AOB and AOA. Since they all utilize ammonia as a nitrogen source and CO_2 as their sole carbon source, it is likely that they compete with each other for these substrates. The metabolic versatility of comammox bacteria makes them competitive in various environments, but early studies on nitrification did not consider the influence of comammox, so the inferred activity of nitrifying bacteria in the environment may have a large error compared with the actual situation. Fitzgerald et al. [20] research showed that comammox bacteria may account for a high proportion in Nitrospiraand make an important contribution to ammonia oxidation. Therefore, in-depth study of the interaction between comammox bacteria and typical nitrifying bacteria is conducive to further understanding

and evaluating the contribution of comammox bacteria to the nitrogen cycle.

4 Impact of Comammox on Biological Wastewater Nitrogen Removal

4.1 Comammox Bacteria May Be Widely Present in Wastewater Treatment Systems

Nitrification is a key step and rate-limiting step in biological wastewater nitrogen removal. Currently, the nitrification reaction in the wastewater nitrogen removal process is still considered a two-step reaction, and research on the existence and impact of comammox bacteria is rarely involved. However, research shows that comammox bacteria may be widely present in biological wastewater nitrogen removal systems and play an important role. Chao et al. [21] collected activated sludge and biofilm samples from the aerobic reactor of a wastewater treatment plant and conducted metagenomic and 16S rRNA gene high-throughput sequencing, finding two genes highly similar to the amoA gene of Ca. N. inopinata. Paul et al. treated actual biological wastewater using an SBR reactor and found that after long-term operation under low oxygen, comammox bacteria dominated the nitrifying flora. Camejo et al. [10] detected high concentrations of comammox Nitrospirain a bioreactor system inoculated with activated sludge and operated under low DO concentration. Recent kinetic research by Kits et al. [15] found that the affinity of comammox for ammonia exceeds that of ordinary ammonia-oxidizing microorganisms, leading to its adaptation to highly oligotrophic environments for growth.

4.2 Competition between Comammox and AOB and Its Impact on Partial Nitrification Processes

Partial nitrification processes are based on the theoretical foundation of the two-step nitrification reaction. By enhancing AOB enrichment and NOB washing out, ammonia nitrogen is oxidized to nitrite without further oxidation to nitrate. Nitrogen removal processes based on partial nitrification are more economical and efficient. Currently, there is extensive research on the theory and practice of partial nitrification. When partial-nitration reactors are seeded, operators keep DO low to exploit the higher O_2 affinity of AOB over NOB, flushing nitrite oxidizers out of the system. As shown in Fig. 4, as dissolved oxygen decreases, NOB are gradually washed out, and the nitrite accumulation rate in the system increases.

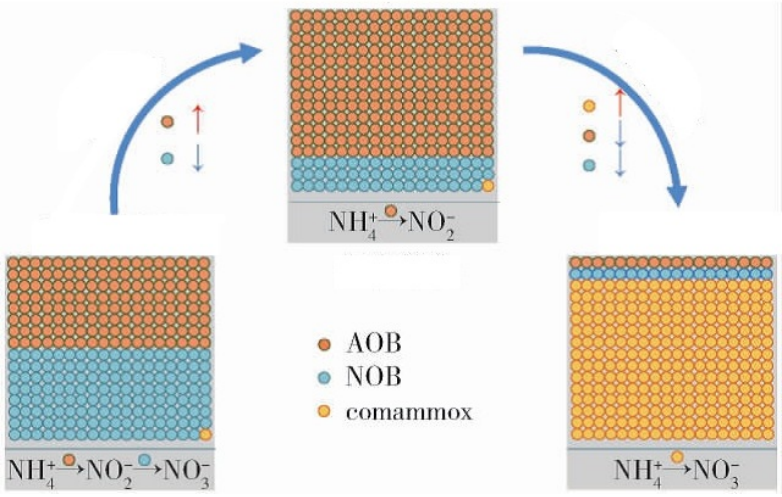

Figure 4 Possible impact of comammox on short-cut nitrification

Partial nitrification in municipal wastewater treatment may also experience unexplained failure under low dissolved oxygen conditions, which could be associated with the enrichment of comammox bacteria under low-oxygen environments. As ammonia nitrogen levels in the denitrification system continue to decline, comammox bacteria gain a competitive advantage over ammonia-oxidizing bacteria (AOB) under conditions of low ammonia nitrogen and low dissolved oxygen, leading to their gradual enrichment. Comammox bacteria oxidize ammonia nitrogen to nitrate in one step, leading to the failure of nitrite accumulation in the system. The potential impact of comammox on the partial nitrification process is shown in Fig. 4. Intermittent high dissolved

oxygen in municipal wastewater denitrification systems is also a feasible method to achieve partial nitrification, which may also be related to the enrichment of comammox microorganisms. When both AOB and comammox microorganisms are present in the system, comammox microorganisms have stronger oxygen affinity under low dissolved oxygen, and ammonia nitrogen will be oxidized to nitrate; whereas under high dissolved oxygen conditions, the relative activity of AOB is stronger, ammonia nitrogen is mainly oxidized to nitrite and accumulates, which instead favors the achievement of partial nitrification.

Comammox functional microorganisms may be K-strategist ammonia-oxidizing bacteria. When ammonia nitrogen is sufficient, the maximum specific growth rate is lower than that of AOB; when ammonia nitrogen is insufficient, due to its smaller K_m, it can achieve a higher specific growth rate than AOB, thus easily enriching under low ammonia nitrogen conditions. Accordingly, in high ammonia nitrogen wastewater treatment systems, comammox often does not dominate, and its impact on partial nitrification is weak. However, for municipal wastewater treatment with low ammonia nitrogen, the enrichment of comammox microorganisms and their impact on partial nitrification cannot be ignored. Therefore, in-depth research on comammox bacteria will enrich and improve the current partial nitrification theory and promote the stability of partial nitrification in practical applications.

4.3 Competition Between Comammox and Anammox Bacteria and Its Impact on Anaerobic Ammonium Oxidation (Anammox) Nitrogen Removal Processes

Anammox nitrogen removal processes are currently a hotspot in biological nitrogen removal research. Anammox microbes fuse NH_4^+ and NO_2^- into N_2 gas, slashing both aeration bills and the need for extra carbon in nitrogen-removal trains. Anammox-based nitrogen removal is typically implemented via the partial nitrification/anammox (PN/A) process. Fig. 5(a) shows the PN/A scheme: AOB and anammox cells share granule or biofilm real estate to strip nitrogen. Fig. 5(b) sketches how comammox bugs could crash this two-party system. In biofilms or granular sludge, the concentrations of ammonia nitrogen and DO gradually decrease along the biofilm depth. Due to their suitability for growth under low ammonia nitrogen and low dissolved oxygen conditions, comammox bacteria can gradually enrich and compete with AOB for substrates. Some ammonia nitrogen is directly oxidized to nitrate, thereby reducing the total nitrogen removal rate of the system.

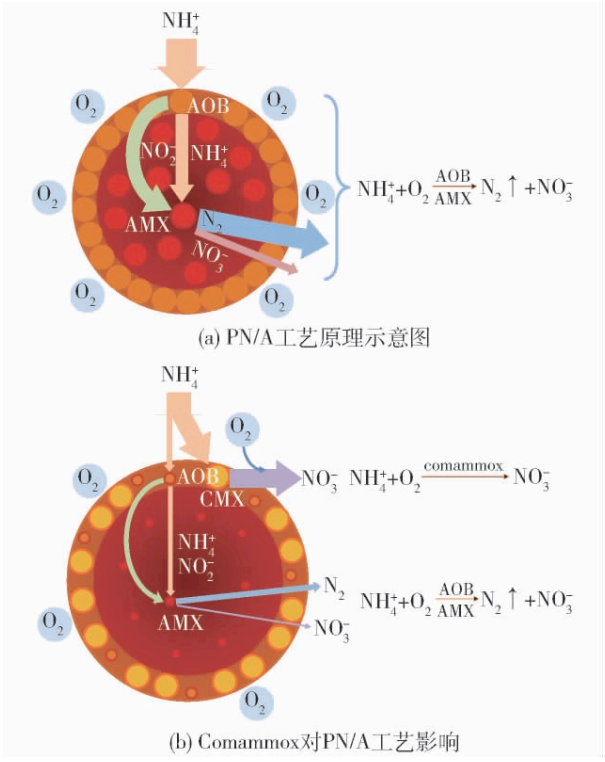

Figure 5 Schematic diagram of PN/A and possible enrichment of comammox and its effect on PN/A

It is currently generally believed that excessive proliferation of NOB and competition with anammox bacteria for the substrate nitrite is the main factor causing elevated nitrate in the effluent of PN/A processes. However, given the characteristics of comammox bacteria being suitable for low oxygen and low ammonia nitrogen environments, for PN/A processes, especially when treating low ammonia nitrogen municipal wastewater, there is a possibility of comammox bacteria aggregation leading to increased effluent nitrate. van Kessel reported finding a relatively high abundance of comammox in anammox enrichment cultures, and successful enrichment of comammox bacteria was achieved by culturing with anammox enrichment culture as seed sludge under low ammonia nitrogen and low oxygen conditions. The above reports indicate that excessive proliferation of comammox bacteria in anammox systems may lead to reduced total nitrogen removal efficiency [6]. It shows a comparison of the amount of carbon dioxide fixed and the energy required per single fixation process for different carbon fixation pathways. From the perspective of carbon fixation pathways, anammox bacteria require consuming 0.5 molecules of ATP per fixed molecule of CO_2 , while comammox bacteria require consuming 0.75 molecules of ATP per fixed molecule of CO_2 [6], and their cell yields are similar. Costa et al. [11] studied the trade-off mechanism between growth rate and growth yield, and the results showed that microorganisms with short metabolic pathways have a higher growth rate per unit time, while microorganisms with long metabolic pathways have more significant growth yield in long-term cultures. This is especially evident when microorganisms can grow slowly on biofilms. Both comammox microorganisms and anammox bacteria are suitable for growth in biofilm environments [18]. It is speculated that in the biofilm of the PN/A process, comammox bacteria and anammox bacteria are in a competitive relationship in terms of cell proliferation. However, under the low ammonia nitrogen concentration and low dissolved oxygen concentration environment of municipal wastewater treatment, comammox microorganisms have a more advantageous substrate competitiveness. Therefore, it is speculated that the proliferation of comammox bacteria is one of the potential reasons for the increase in effluent nitrate in the PN/A nitrogen removal process for municipal wastewater.

Currently, when elevated effluent nitrate is reported in PN/A processes, it is often accompanied by the enrichment of Nitrospira. The influent of anammox reactors contains trace oxygen, and besides anammox bacteria, a relatively high abundance of Nitrospirais usually detected in granular sludge [22]. For granular sludge PN/A processes, Li et al. [23] found that selective discharge of floc sludge would increase the abundance of Nitrospirain granular sludge, and these may be related to the enrichment of comammox bacteria, which requires further in-depth study. At Singapore's Changi plant, autotrophic nitrogen removal faltered under low DO—a slump linked to comammox thriving at modest oxygen and poaching NH_4^+ from anammox partners [24]. Therefore, in-depth research on the distribution and mechanism of comammox bacteria in anammox nitrogen removal processes is conducive to developing operational strategies that can simultaneously control the growth of comammox bacteria and NOB, and improve the stability of anammox, especially for municipal wastewater anammox processes.

4.4 Application Potential of Comammox in Enhanced Low Ammonia Nitrogen Removal Processes

Because comammox cells push NH_4^+ all the way to NO_3^-, they can serve as a one-step polish for micro-polluted raw water headed to drinking-water works. When source water contains ammonia‑nitrogen, its removal is required in an economical and efficient manner to enhance the performance of subsequent treatment processes. Biochemical treatment is an economical and efficient ammonia nitrogen removal process, but the nitrite accumulation caused by incomplete ammonia nitrogen oxidation poses potential health risks to aquatic organisms and humans. Enriching comammox bacteria in pretreatment reactors for slightly polluted water can achieve complete oxidation of ammonia nitrogen under low dissolved oxygen conditions, reducing aeration energy consumption while avoiding nitrite accumulation. Pinto et al. used metagenomics methods to sample from the active biological filter of a drinking water treatment plant in Ann Arbor (MI, USA), assembled the genome of Nitrospirabacteria capable of completely oxidizing ammonia nitrogen to nitrate, and confirmed it as comammox bacteria. For the treatment of slightly polluted source water, further research is still needed on reactor configurations and operating conditions suitable for the enrichment of comammox bacteria.

5 Advances in Enhanced Biological Nitrogen Removal Technology under Low-Temperature Conditions

Ammonia nitrogen is one of the important pollution factors in urban water bodies and also a key pollutant causing water eutrophication. Ammonia nitrogen pollution in urban water bodies mainly originates from the discharge of domestic sewage and industrial wastewater, the decomposition of nitrogen-containing organic matter (such as food waste and animal/plant residues), and the excessive application of fertilizers and feed in agriculture and livestock farming [1]. The "Second National Pollution Source Census Bulletin" [2] shows that the discharge of ammonia nitrogen pollutants into water bodies accounts for 4.6%, 22.4%, and 2.3% from industrial sources, agricultural sources, and domestic sources, respectively. Excessive discharge of ammonia nitrogen pollutants in wastewater can cause eutrophication, even blackening and odorization, of lakes, rivers, and other water bodies, leading to massive deaths of aquatic organisms. Therefore, ensuring and improving the stable and efficient nitrogen removal efficiency in wastewater is one of the key technical measures for safeguarding urban water quality. Currently, nitrogen removal from wastewater is primarily achieved through biological methods, i.e., using nitrogen-removing microorganisms to convert ammonia nitrogen compounds in water into gaseous nitrogen (e.g., N_2). Under low-temperature stress, the metabolic enzyme activity and cell proliferation rate of functional nitrogen-removing microbial communities are significantly attenuated [3-4], key pathways for nitrogen transformation are blocked, leading to a systematic decline in the treatment loading capacity of biological nitrogen removal units, and ultimately causing a stepwise decrease in nitrogen removal efficiency of wastewater treatment processes. Therefore, this paper systematically reviews the challenges associated with biological ammonia-nitrogen removal from wastewater under low-temperature conditions, enhanced biological nitrogen removal technologies for low-temperature applications, as well as the cold-response characteristics and potential cold-tolerance mechanisms of nitrogen-removing microbial communities. The aim is to provide a theoretical foundation and technical support for the biological removal of ammonia-nitrogen from contaminated water bodies in low-temperature environments.

The metabolic activity of microorganisms is highly dependent on environmental temperature, with the optimal growth temperature range for typical nitrogen-removing bacteria being 25-30°C. However, the temperature of natural water bodies in winter often drops below 20°C. When the water temperature falls below 15°C, microorganisms face low-temperature stress, and their physiological activity and biochemical functions are significantly inhibited, thereby affecting the treatment performance of biological nitrogen removal systems.

At the macroscopic system level, low temperature impairs the settling performance of activated sludge and reduces the nitrogen removal efficiency of biological wastewater treatment processes. Studies indicate that low temperature can trigger abnormal secretion of microbial extracellular polymeric substances and promote the proliferation of filamentous bacteria, leading to sludge bulking [5-6], thereby directly deteriorating the settling properties of activated sludge. Ou Jiali et al. [7] found that in an anaerobic granular sludge system at 10°C, the abundance of the filamentous bacterium Sphaerotilus natanswas the highest. The massive proliferation of filamentous bacteria caused sludge bulking, reducing the system's ammonia nitrogen removal rate by about 21.5% compared to conditions at 18°C.

At the whole-cell level, low temperature can significantly inhibit the energy metabolism process and cell membrane fluidity of microbial communities. Chen et al. [8] found that when the ambient temperature dropped from 25°C to 15°C, ATP content decreased by about 33%, revealing the regulatory effect of temperature gradient changes on the homeostasis of microbial energy carriers. Furthermore, low temperature leads to decreased fluidity of microbial cell membranes, hindering transmembrane substance transport and energy metabolism processes [9]. The gel state of the plasma membrane inhibits the formation of proton gradients, thereby further slowing down the growth rate and reproductive capacity of microorganisms.

Low temperatures in winter significantly affect the metabolic activity of nitrogen-removing microorganisms and the nitrogen removal efficiency of water treatment systems. Currently, technologies for enhancing biological nitrogen removal under low-temperature conditions mainly include operational parameter regulation (such as increasing dissolved oxygen concentration, insulation, extending hydraulic retention time, external carbon source addition, increasing sludge concentration or reflux ratio, and reducing hydraulic loading) [15-18], filler modification [19], bacterial quorum sensing regulation [20], and inoculation of cold-tolerant strains [21].

5.1 Low-Temperature Response Mechanisms of Nitrogen-Removing Microorganisms

Low-temperature environments frequently exist in some parts of the world (such as Northeast China and Northern Europe). Microorganisms in these cold environments have developed cold-adaptation mechanisms [20]. The process of microbial adaptation to low-temperature stress is shown in Fig. 6. Cold-tolerant microorganisms refer to those that can adapt to low-temperature environments, survive for long periods under low-temperature conditions, and maintain normal metabolic functions [21]. Morita [22] provided a clear definition for cold-tolerant microorganisms, classifying them as psychrophiles and psychrotrophs. Psychrophiles are microbes that can multiply at sub-zero Celsius, peak below 15°C and top out near 20°C. Psychrotrophs, in contrast, are microorganisms that can grow in environments ranging from 0°C to 5°C, but their optimal growth temperature is above 15°C, and they can proliferate at temperatures exceeding 20°C. In low-temperature water treatment systems, the response and cold-tolerance mechanisms of microorganisms to low-temperature environments are mainly related to cell membrane fluidity [23], cold-adapted enzymes [24], cold shock proteins and cold acclimation proteins [25], DNA transcription and translation, and cryoprotective substances.

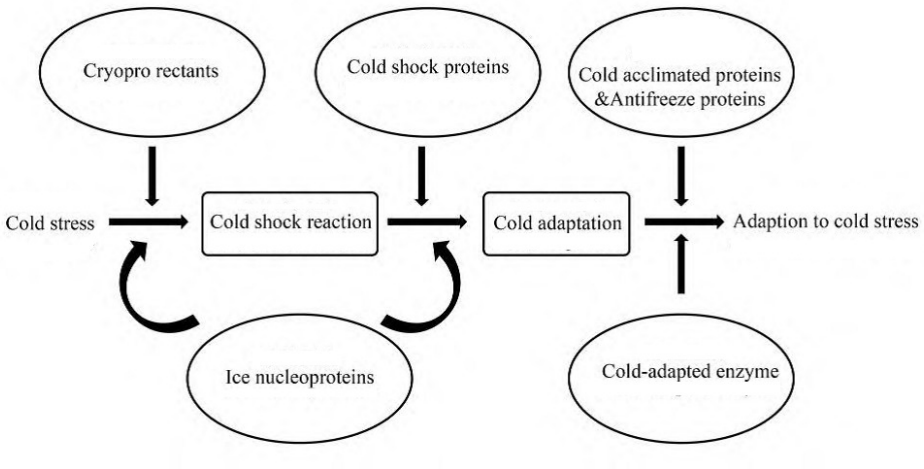

Figure 6 Potential mechanisms of microorganism's adaption to cold stress [27]

The cell membrane is the primary barrier for information, substance, and energy exchange with the surrounding environment, playing a crucial role in maintaining a stable intracellular metabolic environment and ensuring normal physiological and biochemical functions. Low temperature usually causes fatty acids on the membrane to pack more tightly, leading to a phase transition of the phospholipid bilayer from a liquid crystalline state to a gel state, thereby restricting bacterial growth and potentially causing death [26]. Cold-tolerant microorganisms maintain high fluidity of the membrane structure under low-temperature conditions by dynamically regulating the phase transition behavior of the cell membrane lipid bilayer. Relying on phosphorylation modification cascades mediated by membrane protein kinases/phosphatases, they reversibly regulate the conformation of transmembrane transport channels, promote the active transport of polar molecules and ions, forming a rapid physiological adaptation mechanism to low temperature [28]. Furthermore, cold-tolerant microorganisms can effectively regulate membrane structure and fluidity by adjusting the lipid composition within the cell membrane, thereby maintaining normal cellular physiological functions [29]. The regulatory mechanisms mainly include increasing the proportion of unsaturated fatty acids in membrane lipids, remodeling fatty acid structures, and synergistic effects of branched-chain fatty acids.

The normal operation of microbial metabolic activities is primarily maintained through a series of physiological metabolic reactions catalyzed by enzymes [30]. In the temperature response mechanism of enzyme-catalyzed reactions, protein conformational stability shows significant environmental dependence: high-temperature environments easily cause irreversible denaturation of enzyme proteins, while low-temperature conditions, by inducing phase transitions in the cell membrane lipid layer (liquid crystalline state → gel state), significantly reduce the efficiency of transmembrane substance transport [31]. Cold-adapted enzymes, as core regulatory factors of microbial low-temperature metabolism [32], have evolved unique molecular structural characteristics: they form a dynamic hydration layer by increasing the density of surface polar residues, exhibit anomalous thermal stability while maintaining low-temperature catalytic activity, and utilize reversible deformation of

flexible domains to achieve conformational regulation of substrate-binding sites, thereby ensuring the continuity of enzymatic reactions in cold environments. Cold-adapted enzymes enhance their structural stability at low temperatures and prevent enzyme activity loss through methods such as increasing hydrophobic interactions and forming disulfide bonds [33]. Additionally, cold-adapted enzymes can increase their activity at low temperatures by altering the content of amino acid composition (such as arginine and proline) and increasing the flexible regions of the protein. Therefore, under low-temperature conditions, cold-tolerant microorganisms can catalyze metabolic reactions by adjusting the molecular conformation of cold-adapted enzymes to resist external cold stress environments.

A decrease in environmental temperature induces microorganisms to produce a large amount of Cold Shock Proteins (CSPs) and Cold Acclimation Proteins (CAPs) [34]. Under sudden low-temperature stress, both mesophilic communities and cold-tolerant functional bacteria rapidly activate the stress-induced expression of cold shock proteins. In contrast, the cold acclimation protein synthesis pathway, unique to cold-tolerant bacteria, is specifically induced only under sustained low-temperature stress, forming a differentiated temperature response regulatory network. Studies have shown that cold shock proteins are primarily involved in regulating transcription and translation processes, such as maintaining transcriptional stability and translatability, inducing the synthesis of cold-adapted enzymes, compatible solutes, etc., to enhance cell tolerance to low temperatures. Additionally, cold shock proteins can assist in protecting protein function, preventing protein misfolding or aggregation, and maintaining their normal physiological and biochemical functions. Cold acclimation proteins, on the other hand, are mainly involved in various physiological processes such as cell metabolism, energy production, and substance transport, stabilize cell membrane and cell wall structures, prevent damage to cells caused by low temperature, Cryoprotectants produced by microorganisms are a key line of defense against low-temperature stress. These protectants can increase the concentration of intracellular solutes through covalent binding to resist low-temperature stress [35]. For example, in cryoprotection mechanisms, glycolipid antifreeze proteins can orderly guide water molecule arrangement near the phase transition critical temperature by regulating ice crystal nucleation kinetics, inhibiting the disordered crystallization of supercooled water, thereby effectively maintaining the integrity of the cell membrane lipid bilayer and avoiding irreversible damage to microbial subcellular structures caused by mechanical stress from ice crystals. Liu et al. [36] discovered a cell cryoprotectant analogous to DMSO, which stably binds to ice crystal surfaces and forms more stable hydrogen bonds with ice, significantly reducing osmotic pressure damage to cells. Extracellular Polymeric Substances (EPS) secreted by microbial communities are also an effective cryoprotective substance. Studies have found that EPS content increases as temperature decreases [37]. Microorganisms form multiple protective mechanisms by upregulating the synthesis level of EPS: the polysaccharide-protein complex matrix regulates solution crystallization kinetics through a hydrogen bond network, inhibiting disordered ice nucleus growth and lowering the phase transition critical temperature. Simultaneously, through the hydration of polar groups, it maintains the water and ion homeostasis of the extracellular microenvironment, enhances cell surface adhesion to drive the spatial structured assembly of biofilms. Furthermore, the three-dimensional network of EPS creates a molecular embedding effect for extracellular enzymes, effectively mitigating the perturbation of low temperature on enzyme protein conformation and ensuring the catalytic functional integrity of hydrolytic enzyme systems [30]. Additionally, in low-temperature adaptation mechanisms, microorganisms regulate the thermodynamic properties of intracellular solutions by selectively accumulating osmoregulatory substances such as glycine and betaine, reduce water activity, and disrupt the heterogeneous nucleation process of ice crystals, thereby maintaining the supercooled state stability of the cytoplasmic matrix and avoiding membrane system damage induced by phase transitions [31].

5.2 Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea

AOB and AOA are the main agents that kick-start nitrification by turning NH_3 into nitrite. Given that AOA exhibit higher ammonia affinity than AOB [32‑33], AOA are likely better adapted to low‑temperature environments.

The bulk of AOB are mesophiles whose optimum window sits at 15–40 °C. Their population make-up and reaction rate shift with salinity, actual temperature, organic-C load and inorganic-N makeup. Under short-term temperature changes, AOA exhibit greater stability than AOB, and AOA also have a broader temperature adaptation range (0.2-74°C). Studies have shown that within the ammonia-oxidizing functional microbial communities of wastewater treatment systems in winter, AOA demonstrate more significant niche

competitiveness than AOB due to their low-temperature metabolic advantages, becoming the dominant functional microbial group for ammonia nitrogen transformation during the cold period. For example, He et al. [37] found that in sediments near Rushan Bay on the Shandong Peninsula, ammonia-oxidizing microorganisms were predominantly AOB in summer (water temperature 21-25°C) and AOA in winter (water temperature 3-4°C), which also indicates that AOA have stronger adaptability than AOB in low-temperature environments. Furthermore, research by Yin et al. [38] also proved that the adaptability of AOA in low-temperature environments is superior to that of AOB, which is likely closely related to their unique cell membrane glycerol ether structure. This type of structure can stabilize the activity of ammonia monooxygenase, enabling it to efficiently catalyze the ammonia oxidation reaction even under low-temperature conditions.

5.3 Complete Ammonia Oxidation Bacteria

Based on kinetic principles, Costa et al. [39] hypothesized in 2006 the existence of a single microorganism capable of performing both ammonia oxidation and nitrite oxidation simultaneously, termed complete ammonia oxidation (comammox) bacteria (Fig. 7). In 2015, researchers successfully enriched and cultured pure Comammox strains and revealed that these bacteria belong to the genus Nitrospira[40].

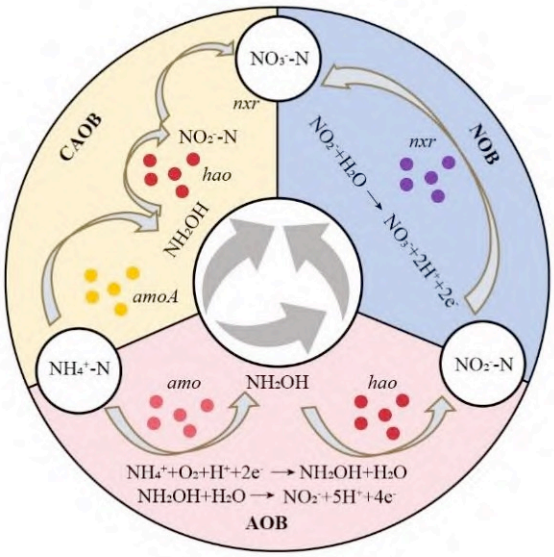

Figure 7 Schematic Diagram of Metabolic Pathways in Partial Nitrification Bacteria and Comammox Bacteria

Both kinetic characteristics and genomic analysis of Comammox bacteria indicate that this genus is more likely to survive in oligotrophic environments [41]. Relative to canonical two-step AOB, comammox cells grow slowly yet display heightened affinities for both ammonia and oxygen. These characteristics are likely important reasons why Comammox bacteria are more adaptable to low-temperature environments compared to other ammonia-oxidizing microorganisms. Zhou et al. [42] tracked how temperature shifts shape the comammox assemblage colonizing a GAC filter at a full-scale drinking-water works. They found that the abundance of Comammox bacteria accounted for 79%-91% in December (winter), significantly higher than their abundance in August (approximately 15%). Tang et al. [43] investigated the ammonia-oxidizing microbial community in Shanghai's groundwater. The results showed that Comammox bacteria were more dominant compared to other ammonia-oxidizing microorganisms and could grow dominantly at relatively low temperatures (<20°C). Zhang et al. [44] studied the abundance, community structure, and activity of ammonia-oxidizing microorganisms in five high-altitude rivers on the Qinghai-Tibet Plateau. They found that Comammox bacteria were the ammonia-oxidizing microorganisms in 23% of the samples, indicating that Comammox bacteria are more adaptable to low-temperature or low-oxygen environments. Li et al. [45] designed and operated a hydrogel reactor containing a coupled community of Comammox and Anammox bacteria, achieving highly efficient ammonia nitrogen removal (over 98%) at a low temperature of 10°C. Tang et al. [46], in their study of nitrification processes in inland and

coastal wetlands of Northern China during winter, found that Comammox bacteria had higher abundance than both AOA and AOB. Furthermore, after repeated freeze-thaw cycles of the samples, Comammox bacteria recovered their activity faster than AOA and AOB, indicating that Comammox bacteria have stronger freeze-thaw resistance. Hou et al. [47] discovered that Comammox bacteria were the dominant nitrifying microorganisms in sediments of near-shore lakes in Antarctica, and further confirmed the autotrophic nitrification activity of Comammox Strains possessing Heterotrophic Nitrification-Aerobic Denitrification (HN-AD) capability can perform heterotrophic nitrification using organic matter as an energy source and carry out denitrification under aerobic conditions to achieve nitrogen removal. Fig. 8 outlines the HN-AD route: NH_4^+-N is first pushed to NH_2OH, then HAO flips it to NO_2^--N. Thereafter, NIR, NOR and NOS sequentially trim NO_2^--N or NO_3^--N to NO, N_2O and finally N_2. Compared to traditional nitrogen-removing microorganisms, HN-AD bacteria can better adapt to environments with low temperature, high salinity, and oligotrophic conditions [42]. At 15 °C, *Pseudomonas putida* Y-12 removed NH_4^+-N, NO_3^--N and NO_2^--N at 2.14, 1.57 and 1.60 mg L^{-1} h^{-1}, respectively. The results indicate that this HN-AD bacterium has good low-temperature nitrogen removal performance at 15°C [43]. Zhao et al. [15] isolated a novel HN-AD strain TAC-1 from pig farm wastewater. Under low-temperature conditions of 5°C, its removal rates for ammonia nitrogen (400 mg/L) and nitrate nitrogen (400 mg/L) were as high as approximately 94.6% and 93.3%, respectively. Dong et al. [29] isolated and screened an efficient novel cold-tolerant HN-AD bacterium, PseudomonasNR-5, from river sediments in a cold region. At a low temperature of 10°C, its ammonia nitrogen removal rate reached over 97.3%, demonstrating good low-temperature nitrogen removal performance.

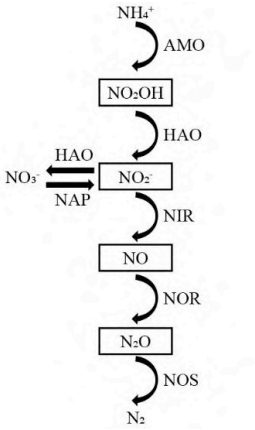

Figure 8 Schematic diagram of the HN-AD denitrification pathway

6 Conclusion

The decrease in microbial nitrogen removal efficiency under low-temperature environments is a key issue limiting the stable operation of wastewater treatment plants in winter. This paper systematically reviewed the main challenges faced by biological ammonia nitrogen removal under low-temperature conditions, enhanced technologies and measures for low-temperature biological nitrogen removal, as well as the low-temperature response characteristics and potential cold-tolerance mechanisms of nitrogen-removing microorganisms. The main conclusions are as follows: (1) Low temperature (<15°C) can lead to problems such as reduced cell membrane fluidity of microbial communities, decreased growth and reproduction rates, lowered ATP levels, declined enzyme activity, reduced sludge settling performance, and imbalanced microbial community structure, thereby significantly reducing the nitrogen removal efficiency of water treatment plants. (2) Under low-temperature conditions, the nitrogen removal performance of biological nitrogen removal processes can be improved through operational parameter optimization (such as regulating dissolved oxygen, extending hydraulic retention time, adding carbon sources, increasing sludge concentration or reflux ratio, and reducing hydraulic loading, etc.), filler modification, bacterial quorum sensing regulation, and the addition of cold-tolerant microorganisms. (3) Cold-tolerant microorganisms can adapt to low-temperature environments through strategies such as regulating cell membrane fluidity, maintaining cold-adapted enzyme activity, secreting cold shock proteins, and producing cryoprotective substances (e.g., EPS). (4) In the cold, AOA, comammox and HN-

AD organisms seize the advantage—maintaining activity while conventional AOB falter—thanks to tighter substrate binding and enzymes tuned for low-temperature catalysis. This edge likely stems from their unique ecological slots and cold-friendly biochemistry that let them keep metabolizing when others shut down.

Future research on low-temperature nitrogen removal should focus on the following directions: (1) Developing low-cost immobilization technologies for microbial agents, researching anti-washout strategies for cold-tolerant microorganisms, and improving the long-term performance of microbial communities; (2) Designing and preparing novel functional fillers, such as combining nanomaterials or designing bionic structures to enhance the low-temperature adsorption performance and biocatalytic efficiency of fillers; (3) In-depth analysis of the potential cold-tolerance mechanisms of nitrogen-removing microbial communities, such as the cold-tolerant growth mechanism of Comammox bacteria and their synergistic nitrogen removal mechanisms with other bacteria; (4) Systematically revealing the cold-tolerance regulation pathways of nitrogen-removing microorganisms by integrating multi-omics technologies (such as metagenomics, transcriptomics, and proteomics) to provide a molecular basis for developing cold-tolerant engineered nitrogen-removing bacteria and directed domestication of microbial

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