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Review

Advances in the Efficient Enrichment of Anammox Bacteria

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Abstract: Anaerobic ammonia oxidation (anammox) process is known as a low-energy and environmentally friendly process for treating nitrogen-rich wastewater. Anammox bacteria are the key microorganisms to achieve this biological process. However, the efficient enrichment of anammox bacteria has been a bottleneck for its practical application because of their slow growth and high sensitivity, and no pure culture has been found. Therefore, the development of efficient anammox bacterial enrichment techniques is of great theoretical and application value. Solving the problem of anammox bacterial activity and improving the process denitrification performance is one of the current research hotspots. In this paper, three aspects of anammox bacteria are described in terms of their physiological properties, environmental influencing factors, and short-term starvation tolerance; a systematic review of the latest research progress in accelerating the activity of anammox bacteria using enrichment strategies for process regulation, the construction of granulation models, suspended sludge biomass management, and strain preservation. Finally, the future frontier development of anammox bacteria was discussed and foreseen.

Keywords: anammox bacteria; short-term hunger tolerance; bacterial enrichment; bacterial dormancy

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1. Introduction

In recent decades, global energy depletion and environmental pollution have become increasingly serious problems. Green and sustainable solar energy is used as a clean energy source to solve the problem of energy depletion [1]. Heterogeneous photocatalysis is considered a strategy to deal with environmental pollution and alleviate the energy crisis [2]. Global water resources are under pressure due to increasing population and diminishing conventional water resources caused by global warming [3]. Water is an important resource for maintaining ecological balance and human survival, but water quality is currently seriously polluted and damaged globally. The substandard discharge of domestic sewage, industrial sewage, and agricultural sewage causes the excess of ammonia and nitrogen in water resources and serious eutrophication of water bodies, which endangers aquatic organisms and human health. In the traditional nitrification and denitrification process, ammonia nitrogen is produced via the ammonification reaction, nitrification reaction, and denitrification reaction to produce N_2, which finally achieves the purpose of denitrification, but this process requires an additional carbon source if the carbon source is insufficient, thus greatly increasing the treatment cost. As the effluent quality requirements continue to improve, the traditional process is difficult to meet the discharge standards. In recent years, more and more scholars have conducted research focusing on anammox, discovering new denitrification phenomena and seeking higher and economical denitrification pathways.

The anammox process has a broad application prospect due to its advantages of efficient nitrogen removal and low energy consumption, but the enrichment rate is still the bottleneck problem of the anammox process in the field of biological nitrogen removal due

to the slow reproduction rate of anammox bacteria. Anammox bacteria were discovered in the Netherlands in the 1820s. Under anaerobic conditions, ammonia is used as the electron donor and nitrite as the electron acceptor, and ammonia nitrogen is oxidized to nitrogen gas, which has the advantages of low energy consumption, low residual sludge, and no additional required carbon source. Processes derived based on anammox have also become a hot research topic, such as single-stage short-course nitrification–anammox (SNAP) [4], partial nitrification–anammox (PDA) [5], SHARON-ANAMMOX [6], restricted autotrophic nitrification–denitrification (OLAND) [7], total autotrophic denitrification (CANON) [8], and short-course nitrification/anammox/denitrification-coupled denitrification phosphorus removal (SNAD-DPR) [9], which are important in the management of produced wastewater, waste leachate, and environmental pollution. The PDA process has attracted the attention of many scholars with its advantages of low energy consumption, low cost, and high efficiency of nitrogen removal, and has become one of the mainstream practical engineering applications.

Although anammox bacteria play an important role in the denitrification process, anammox bacteria are slow growing and sensitive. The contribution of anammox bacterial activity to total carbon production ranged from 2.39 to 82.61%, indicating that anammox has an important role in controlling the ecosystem's nitrogen cycle [10]. Not only are the anammox bacteria themselves highly sensitive, but the wastewater composition is becoming increasingly complex, often accompanied by metal ions, antibiotics, and toxic substances that inhibit the activity of anammox bacteria, resulting in poor bacterial tolerance and reduced denitrification performance. This paper reviews the physiological characteristics of anammox bacteria, strategies to accelerate the activity of anammox bacteria, and the dormant resurrection of this bacterium, and provides an outlook on the research areas regarding anammox-related technologies.

2. Physiological Properties of Anammox Bacteria

2.1. Types of Anammox Bacteria

Anammox bacteria are unicellular organisms with different morphologies, both spherical and ovoid, ranging from 0.8 to 1.1 μm in diameter [11]. This bacterium is a Gram-negative bacterium with no pods and three intracellular parts: the anaerobic ammonia oxidizer, the ribosomal mass, and the outer chamber cytoplasm. Van Teeseling et al. observed under a microscope (TEM) that the cell surface (the outermost layer of the cell) was covered with a six-sided protein structure [12]. The anaerobic ammonia oxidizer is a unique structure of the bacterium, accounting for 50–80% of the cell volume, and is the site of the anammox reaction.

Anammox bacteria are a kind of floating mycobacteria, a clade of very early origin in the phylum floating mycobacteria. Anammox bacteria were discovered only after a long time, and the early Berger's manual had only five genera of anammox bacteria. Until today, researchers have identified a total of seven genera of anammox bacteria via deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) extraction, fluorescence in situ hybridization techniques, and other detection methods (Table 1), namely *Candidatus brocadia (Ca. B)*, *Candidatus kuenenia (Ca. k)*, *Candidatus scalindua (Ca. s)*, *Candidatus anammoxoglobus (Ca. a)*, *Candidatus jettenia (Ca. j)*, *Candidatus brasilis*, and *Candidatus anammoximicrobium*, and anammox bacteria obtained in pure culture have not been identified [13]. There are also large differences between different genera of anammox bacteria, and the reactors change depending on the operation or water quality to obtain a better enrichment of anammox bacteria. The anammox bacteria enrichment cultures were bright red. *Ca. k. stuttgartiensi*, *Ca. b. fulgida*, and *Ca. a. propionicus* among anammox bacteria can metabolize organotrophic type [14]. Anammox bacteria are widely distributed, not only in the ocean, but also in rivers, lakes, and other natural ecologies with thermal springs, hydrothermal mouths, and other extreme ecologies in rice paddies and artificial wetlands, and in artificial habitats such as wastewater treatment plants, where they are present in large numbers. According

to this study, the abundance of anammox bacteria in rice ecosystems was higher than in mangroves, followed by degraded and undisturbed mangroves [15].

Table 1. Population and discovery source of anaerobic anammox bacteria.

Bacillus spp.	Strain	Discover Time	Discover Country	Source	References
Candidatus Brocadia	*B. anammoxidans*	1999	The Netherlands	Wastewater	[16]
	B. fulgida	2008	The Netherlands	Wastewater	[17]
	B. sinica	2010	China	Bioreactors	[18]
	B. brasiliensis	2011	Brazil	Wastewater	[19]
	B. caroliniensis	2011	USA	Livestock manure sludge	[20]
	B. sapporoensis	2017	Japan	Bioreactors	[21]
Candidatus Jettenia	*J. asiatica*	2008	China	Bioreactors	[22]
	J. caeni	2012	Japan	Wastewater	[23]
	J. ecosi	2018	Russia	Bioreactors	[24]
	J. moscovienalis	2015	Moscow	Bioreactors	[25]
Candidatus Anammoxoglobus	*A. propionicus*	2007	The Netherlands	Bioreactors	[26]
	A. sulfate	2008	China	Biological turntable	[27]
Candidatus Scalindua	*S. brodae*	2003	Britain	Wastewater	[28]
	S. wagneri	2003	Britain	Wastewater	[28]
	S. sorokinii	2003	Britain	Seawater	[29]
	S. arabica	2008	Arabian	Seawater	[30]
	S. sinooilfield	2010	China	Oil reservoirs	[31]
	S. marina	2011	Sweden	Submarine sediments	[32]
	S. richardsii	2012	Black Sea	Black Sea sub-box area	[33]
	S. profunda	2013	Sweden	Submarine sediments	[34]
	S. rubra	2017	Red Sea	Seawater	[35]
	S. japonica	2017	Japan	Bay sediments	[36]
	S. zhenghei	2010	South China Sea	Seawater	[37]
Candidatus Kuenenia	*K. stuttgartiensis*	2000	Germany	Bioreactors Biofilm	[38]
Candidatus Brasilis	*B. concordiensis*	2011	Brazil	Bioreactors	[39]
Candidatus Anammoximicrobium	*A. moscowii*	2012	Moscow	River sediments	[40]

2.2. Environmental Factors Affecting the Growth and Adaptation of Anammox Bacteria

Temperature: In wastewater treatment, the temperature is the key parameter affecting the stable operation of anammox compared to other limiting factors [41]. Low temperature greatly slows down anammox bacterial activity, and the suitable growth temperature for anammox bacteria under strictly anaerobic conditions is 30–40 °C, and the survivable temperature is −2.5–100 °C, but most are mesophilic [42]. Different dominant strains of anammox bacteria have different tolerance to temperature. It was reported that the activity of anammox bacteria was measured at temperatures <15 °C, and a shift in the dominant system from Brocadia Ca. to Kuenenia Ca. was observed [43]. In practice, the best-tolerated temperature for anammox bacteria is in the range of 20 °C to 45 °C [44]. The cessation of anammox bacterial activity in high temperatures at 40 °C is mainly due to cell lysis and the release of cytochrome C from cells, which is an essential component of anammox enzymes [45]. The temperature instability in the anammox process resulted in the low activity of this bacterium, which formed the biggest obstacle to the anammox process. In recent years, there has been an increasing interest in studying the performance

of anammox bacterial activity at low temperatures to make the process usable at high latitudes. At high latitudes, the reduced growth rate of anammox bacteria is due to reduced cytoplasmic density, lower enzyme activity, and low mass transfer rate [46]. Yu et al. used 13C-DNA stable isotope probes combined with Illumina MiSeq high-throughput sequencing technology to analyze purple rice soils and found that anammox bacteria grow best at 25 °C [47]. The slow growth of anammox bacteria is the biggest obstacle to the development of this process. Compared to other microorganisms, anammox bacteria have an extremely long growth and reproduction time, but the advantage of anammox bacteria is that the bacteria undergo dormancy and resurgence under favorable or unfavorable conditions. All anammox sludge in all the storage systems recovered activity after 10 days under the condition of a 60-day storage period, and the best recovery of activity was 98.33% for the 4 °C storage system [48].

Pondus Hydrogenii (pH): pH changes have a great impact on the activity of anammox bacteria, so maintaining long-term pH stability in the culture solution is an important aspect of efficient denitrification in the anammox process. According to previous studies, pH values in the culture broth in the range of 6.5 to 8.3 are anaerobically conducive to anammox bacterial activity and growth [49]. The enzymatic reactions and cellular processes of anammox bacteria are inhibited when the pH is low and indirectly affect the activity of anammox via free nitrous acid (FNA) accumulation, Blum et al. showed that the optimal pH for the growth of anammox bacteria is between 8.0 and 8.3 [50]. Jetten et al. showed that anammox activity and growth were optimal in the pH range of 6.7 to 8.3, with the highest activity at pH 8.0 [51]. Waki et al. showed that anammox bacterial physiology can be guaranteed in the PH range of 6.6 to 8.3, and the best anammox performance was achieved in the PH range of 7.5 to 8.0 [52]. The anammox process can still operate normally for a long time in municipal wastewater with pH values below 6.2 [53]. The high sensitivity of anammox bacteria to the surrounding environment in anammox technology is one of the main bottlenecks at present. PH is a key parameter affecting the activity of anammox bacteria and must be maintained within the adaptation range of this bacterium. So far, effective methods to improve the resistance of anammox granular sludge in extreme pH conditions have been less studied. Zhang et al. showed that the addition of denitrification sludge EPS enhanced (DS-EPSCN) significantly increased the stability and activity of anammox granular sludge under extreme acid conditions [54]. It was shown that changes in hydraulic retention time (HRT) directly affect the pH of the anammox bioreactor, and the advancement of the denitrification process is reflected in an increase in effluent pH when the HRT and N loading rate (NLR) is kept constant [55].

Dissolved oxygen (DO): Anammox bacteria are sensitive to oxygen, and controlling DO is a key parameter that affects the activity of anammox bacteria in a long-term stable manner. Anammox bacteria under strictly anaerobic conditions are capable of stable growth and higher metabolic activity [56]. Yan et al. showed that moderate amounts of nano zero-valent iron (NZVI) (short-term dosing) can rapidly restore anammox activity when anammox bacteria are exposed to oxygen by chance, which is beneficial for the stable operation of the anammox process [57]. Granular anammox biomass can be a successful alternative to anammox biofilms when high concentrations of DO are required to promote partial nitrification reactions. The application of oxygen microelectrode profiles in sequencing batch reactor (SBR) anammox systems emphasizes the effect of low oxygen levels on the size and type of anammox biomass [58]. In one study, the anammox of granular biomass at DO concentrations of 1 and 8 mg/L produced similar nitrogen removal rates of 600 mg N/(L·d), even though oxygen could completely penetrate bacterial cells in the granular biomass at the latter DO concentration [58]. In contrast, in another study, DO concentration was maintained at 8 mg/L with no anammox growth or activity on the anammox biofilm [59]. The existence of a symbiotic relationship between anammox bacteria and nitrifying bacteria is reflected in the gradual increase in DO in the anammox reactor. Liu et al. showed a symbiotic relationship between anammox bacteria and nitrosomonas, an oxygen-dependent nitrifying bacterium that causes the partial oxidation of ammonia

to nitrite [59]. However, high DO levels can cause the dominant bacteria in the reactor to nitrify bacteria and interfere with the anammox reaction by completely oxidizing the nitrite to nitrate. Therefore, controlling DO levels is crucial to the success of anammox reactions.

Metabolic pathways: Although different genera of anammox bacteria have different phylogenetic distances, their cellular structures, and metabolic pathways are similar. In the metabolic activity of anammox bacteria, four main metabolic biological enzymes play an important role. The four key biological enzymes include nitrite reductase (Nir), hydrazine synthetase (HZS), hydrazine dehydrogenase (HDH), and nitrite oxidoreductase (Nxr) [60].

2.3. Short-Term Starvation Tolerance of Bacteria

Microorganisms are central to the wastewater treatment function, and anammox bacteria are very sensitive to changes in the external environment. The anammox process is considered to be susceptible to various environmental factors, and one of the most frequent problems is the starvation of anammox bacteria. Short- or long-term shelving of anammox wastewater treatment units due to seasonal closures, annual maintenance, and holidays in the industry has led to the starvation of anammox sludge [61]. In addition, the exposure of anammox sludge to starvation conditions is unavoidable for anammox sludge during long-distance transportation and preservation in wastewater treatment plants, thus triggering anammox bacterial starvation [62]. Faced with a starved environment, anammox bacteria will initiate programmed cell death to maintain some bacterial activity [63]. This is a genetically determined process of cellular self-destruction that results in the death of a large number of bacteria in starved anaerobic ammonia-oxidized sludge and a decrease in the number of viable bacteria [64]. Biomass in wastewater treatment systems is often exposed to starvation conditions imposed via fluctuations in domestic and industrial wastewater flow and composition. Under these conditions, which may last for days or weeks, biomass is converted to endogenous metabolism, consuming its intracellular macromolecules [65]. This consequently leads to a decrease in biomass and a decrease in bacterial activity, which ultimately affects the performance of the wastewater treatment system [66]. The decrease in the activity of anammox bacteria during short-term starvation is mainly attributed to active decay and is not related to cell death decay [67]. There have been no reports on the endogenous metabolic processes of anaerobic oxidizing bacteria, especially on the intracellular energy sources required by anaerobic oxidizing bacteria to maintain their cells in a starved state. However, in a study measuring changes in extracellular and intracellular macromolecules in the endogenous metabolism of starving anaerobic bacteria, it was hypothesized that extracellular polymeric substances (EPS) and HDH proteins are the sources of energy for cellular maintenance of starving anaerobic bacteria under this condition and that cellular macromolecules among them can also act as electron donors [68].

For the inevitable short-term starvation of anammox bacteria, finding a good way to counteract the negative effects of starvation would be the best option for anammox process development. Previous studies have shown the effect of anammox systems under starvation shock conditions. Lihong Ye et al. investigated the effect of a repeated short-term starvation (1–5 d) mode on the performance and microbial community of an anammox-activated sludge system, and the anammox activity recovered when the nitrite concentration was reduced from 60 mgNL^{-1} to 40 mgNL^{-1} [67]. Anammox bacteria are tolerant and adaptive in the face of short-term starvation and show better capacity after several repeated starvations and recoveries [68]. Phanwilai et al. investigated the effect of acetate on the recovery performance of starved anammox bacteria [69]. Dongdong Xu et al. developed a famine anaerobic ammonia oxidative denitrification (FANIR) system and showed that the FANIR system responded to starvation stress via two phases: a functional decline phase (0–54 d) and a functional stability phase (62–116 d) [70]. It has been demonstrated that anammox bacteria starved under anaerobic conditions have lower decay rates than anammox bacteria starved under aerobic conditions [67]. Anammox bacterial activity is influenced by the starvation effect. Anammox pellets starved for 2 months at 4 °C have a better recovery than those starved at −40 °C, and controlling temperature can be

used as a way to mitigate the starvation effect [71]. Intermittent starvation causes more damage to anammox bacterial mixes than chronic starvation, and higher concentrations of nitroso-nitrogen cause more damage [72].

3. Strategies for Accelerating the Enrichment of Anammox Bacterial Activity

3.1. Process Regulation

3.1.1. Addition of Carbon/Charcoal-Containing Material

Anammox bacteria, as autotrophic microorganisms, do not require an additional carbon source. However, some researchers have recently found that the addition of moderate amounts of inorganic carbon (IC) has a catalytic effect on the denitrification performance of anammox reactors [73]. Xu et al. showed that biochar with different surface functional groups as additives had different degrees of influence on the anammox process, with overall denitrification performance in the order CS300 > CS550 > CS800 [74]. JIN et al. added inorganic carbon to an anammox-EGSB reactor containing granular activated carbon, and the inorganic carbon concentration ranged from 55 to 150 mg/L, and the denitrification rate of an anammox-EGSB reactor reached more than 90%. However, the high concentration of inorganic carbon would generate calcium carbonate on the anammox sludge and hinder mass transfer [75]. Some studies concluded that the performance of anammox reactors was significantly improved when the IC/N ratio reached 1.20 [73]. However, some researchers suggested that the anammox process is favored when the IC/N ratio is in the range of 0.19 to 0.42 [76]. The addition of appropriate amounts of inorganic carbon can be used as a PH buffer to keep the anammox bacteria in a PH environment adapted to growth. Therefore, IC promotes the anammox process.

Biochar is an inexpensive redox-activated carbon material that has been shown to enhance the nitrogen conversion process via microorganisms. Adding biochar not only improves soil health but also increases microbial enzyme activity. The energy substance adenosine triphosphate (ATP) is synthesized via enzymes in anammox bacteria in the anaerobic ammonia oxidizer, thus increasing the activity of anammox bacteria [77]. Xu showed that the addition of redox-active biochar can increase specific anammox activity (SAA) and promote the proliferation of anammox bacteria [78]. In anammox bacterial matrices, EPS has many important, such as structural stabilization, the promotion of aggregation, the maintenance of the physical structure of the particles, water retention, and the protection of the cellular barrier [79]. The large surface area of graphene oxide (GO) facilitates the attachment and growth of anammox bacteria and promotes the secretion of EPS. It was shown that the addition of 100 mg/L of GO to an anammox reactor increased the biological denitrification activity by 10.26% [77]. YIN et al. found that 100 mg/L GO increased the denitrification rate of the anammox reactor by about 17.6%. In addition, the addition of 200 mg/L reduced graphene oxide (RGO) increased the hydrazine dehydrogenase activity by 1.75 times, and the addition of different concentrations of RGO increased the key enzyme activity by 0.7 to 2.75 times [80]. The addition of graphene oxide can create more growth sites for anammox bacteria and enhance the activity of anammox bacteria, thus allowing the anammox bacteria to increase in number. Chen et al. showed that biochar from coconut and apricot adsorbed 9.8 and 8.1 mgN/g of nitrogen, respectively, forming more available adsorption and redox sites, increased EPS content, improved electron transfer capacity of biochar, and the enrichment of anammox microorganisms such as *C. Brocadia* and *C. Kuenenia*, ultimately improving anammox activity and denitrification efficiency [81]. Biochar-mediated anaerobic ammonia oxidation granular sludge changes color with increasing nitrogen load, and different biochar combine with sludge to form a new type of anaerobic ammonia oxidation granular sludge [82].

Activated carbon is a kind of carbon material with a great specific surface area and strong adsorption and decolorization ability. Granular activated carbons (GAC) were reported to facilitate the colonization of anammox biofilms because of their high specific surface area and hydrophobicity, thus facilitating anammox reactor start up and improving denitrification performance [83]. Liang et al. showed that the addition of GAC promoted

the re-granulation of decomposed anammox-hydroxyapatite sludge under low phosphorus conditions, ensuring an effective nitrogen removal rate of 88% at a nitrogen removal rate (NRR) of 8.5 kg $N/m^3/day$ [84].

3.1.2. Addition of Hydrazine N_2H_4

N_2H_4 plays an important role in the biological denitrification cycle by inhibiting ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) activity. Hydrazine N_2H_4 is an intermediate product of the anammox reaction, and the addition of suitable hydrazine N_2H_4 can promote the improvement of nitrogen removal efficiency of the reactor and reduce the effect of nitrite on anammox bacteria, as well as accelerate the start-up time of the anammox reactor and enhance the activity of anammox bacteria, etc. [63]. The external addition of N_2H_4 enhances the anaerobic reaction process (Figure 1). Yao et al. found that the addition of trace amounts of N_2H_4 to the CANNO process enhanced the performance of the system's anammox reactor, inhibited NO_2^- oxidation, and reduced NO_3^- production, thus improving denitrification efficiency [85]. Similarly, Xiao et al. found via long-term experiments in SBR that the addition of small amounts of N2H4 inhibited the growth of AOB and NOB, but promoted the activity of anammox bacteria [86]. The long-term addition of N_2H_4 to the anammox SBR reactor influent, which was inhibited by NO_2^-, rapidly restored and enhanced the system denitrification performance, increasing the system total nitrogen removal rate to (0.833 ± 0.027) $kg/(m^{-3}d)$ and reducing the by-product NO_2^- production by about 21% [87]. Some researchers have suggested that N_2H_4 has a facilitative effect on anammox bacterial activity, and its mechanism of action is the oxidation of hydrazine added externally to release electrons in the form of external energy sources during the anammox reaction to produce the energy substance ATP, which generates additional ATP to maintain the cells and increase the glycogen content, a material that can be used as a carbon source when the bacteria are starved [63].

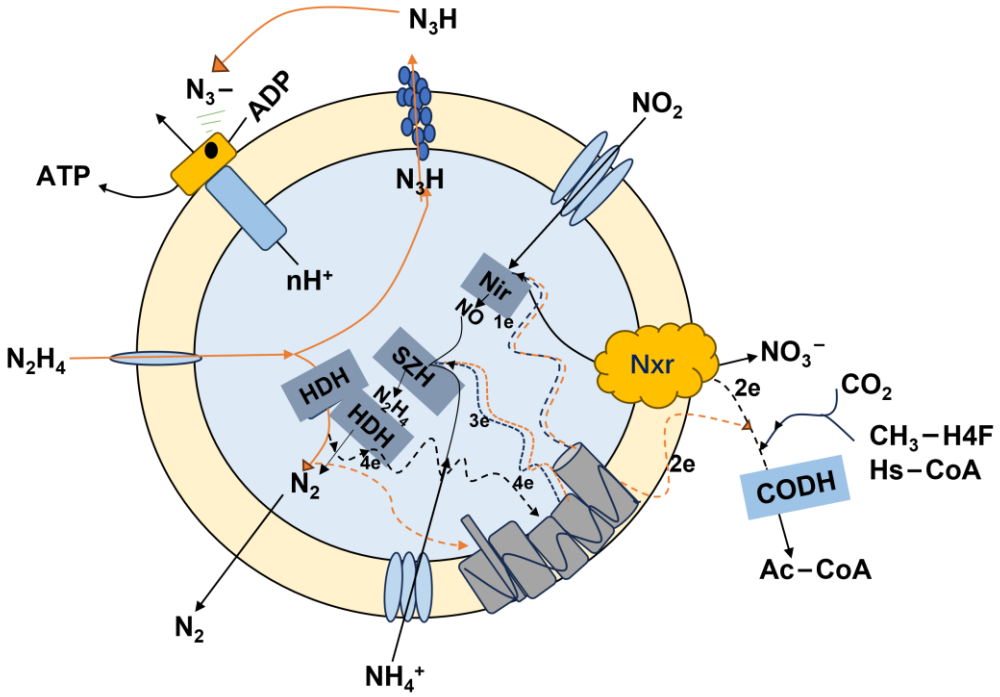

Figure 1. The mechanism of hydrazine addition strengthens the anammox process [86]. Note: See abbreviation list for abbreviations in the figure. Black line: conversion pathway of the substrate. Red line: conversion pathway.

3.1.3. Add Tourmaline

Tourmaline has the characteristics of electrode and permanent spontaneous polarization effect, which can make the water molecule association decrease and the water molecule clusters smaller, while the small molecule clusters are easily absorbed and used by cells so that the metabolic capacity of microorganisms can be improved [88]. The strong electric field on tourmaline decomposes to water and generates hydroxyl radicals to regulate the activity of proteins and lipids on cell membranes, thus regulating cell membrane fluidity, improving cell membrane permeability [89], improving the cellular uptake of nutrients, and increasing the activity of anammox bacteria. Dehydrogenase activity reflects the number and activity of microorganisms. The higher the rate of an enzyme-catalyzed reaction under certain conditions, the stronger the microbial metabolic capacity and the enhanced dehydrogenase activity under specific conditions; conversely, the lower the rate of enzyme catalysis, the lower the microbial metabolic capacity and the lower dehydrogenase activity [90]. The synthesis of the metallo-promoting factor heme C emitted from the tourmaline surface was used to increase the dehydrogenase activity, ultimately increasing the metabolic rate of anammox bacteria. Some researchers showed that the anammox bacteria solution was inoculated and incubated with different amounts of tourmaline added to the test and blank groups in the experiment, and it was found that the anammox bacteria dehydrogenase activity increased from 0.4 mgTF/(L·h) to 65.85% with tourmaline dosing from 0.68 mgTF/(L·h) after tourmaline dosing 0 to 5 g/L [91]. The results of Tan et al. showed that using SBR reactor experiments, the addition of tourmaline and the absence of tourmaline were labeled as SBR1 and SBR2, respectively, and the heme C of anammox sludge was consistently increased in both groups after the reactor system was stabilized, and the dehydrogenase activity was higher in SBR1 compared to SBR2 [92].

3.1.4. New Reactor Granules Circulating EGSB ($EGSB_{GC}$)

Up-flow anaerobic sludge blanket (UASB), expanded granular sludge bed reactor (EGSB), SBR, etc., are common reactors for anammox bacteria enrichment (Table 2). They have the advantages of some reactors that are more mature and provide better shear, enhancing the contact between the substrate and granular sludge in the water, which, in turn, improves the production and metabolism of functional bacteria. However, there are certain disadvantages, and the blockage and decay of floating anammox particles in conventional EGSB reactors severely limit their operation [93]. To improve the blockage problem, the researchers developed a new reactor, the $EGSB_{GC}$ reactor, using the EGSB reactor as a prototype. A new structure of a three-phase separator was used on the $EGSB_{GC}$ to stir the granular sludge in the water, thus improving the mass transfer between the granular sludge and the substrate. The reactor operated for 166 d. After 166 d of operation, the efficiency of nitrogen removal from the reactor was 1.28 times higher than that of the conventional EGSB [94]. YIN et al. used a three-phase electrode to enrich anammox bacteria on the electrode plate to enhance its activity, and the electrode had a voltage of 0.08 V. The denitrification efficiency was increased by 25.35%, and in addition, the activity of hydrazine dehydrogenase was increased by 57.8% [95]. The relative abundance of anammox bacteria in $EGSB_{GC}$ three-phase separator granules (28.5%) was higher than in conventional EGSB (16.1%), and in $EGSB_{GC}$, floating particles were adequately collected and efficiently separated in the three-phase separator, resulting in a faster circulation and a higher retention efficiency of anammox particles than in conventional EGSB [94]. Therefore, the development of new reactors to enrich anammox bacteria for more efficient denitrification will be one of the hot research topics.

Table 2. Advantages and disadvantages of commonly used anammox bacteria reactors.

Reactor Type	Advantages	Disadvantages	References
SBR	Highly efficient mud and water separation with good biological cut-off capacity, full mixing of the substrate, high impact resistance, no backflow, stable operation, and simple operation.	Low operating load and high automatic control requirements, not suitable for coupling with other processes, still some sludge loss	[96]
Membrane Bioreactor (MBR)	Good separation of mud and water, membrane structure can effectively stop anammox loss, high strain activity, short doubling time, good effluent quality, simple process, and easy operation.	High resistance, high reactor price, and easy clogging of membranes	[97]
EGSB	Enrichment of anammox has certain advantages, high sludge retention capacity, good mass transfer conditions, and less clogging	High operating conditions and control requirements, frequent and violent particle sludge collisions, easy loss, and high energy consumption	[98]
UASB	High sludge concentration, high treatment load, high impact resistance, high bioretention, short HRT and low energy consumption, easy separation of gas–liquid–solid phases, high confinement, stable operation, good denitrification, accelerated granular sludge formation	Poor mass transfer, uneven mixing within the sludge, easy formation of dead zones, short flows, and trench flows, affect the start-up effect	[99]
Anaerobic Baffled Reactor (ABR)	Good bioretention capacity, easy solid–liquid separation, easy formation of granular sludge, advantages of cultivating anammox.	Difficult to achieve uniform water distribution, prone to gullying and dead spots	[100]
Up-flow Blanket Filter (UBF)	Good adaptation to changes in water quality and quantity, long sludge age, the high flow rate in the tank, and good mass transfer conditions.	Granular sludge is unstable and easily lost	[101]
Sequencing Biofilm Batch Reactor (SBBR)	Good bioretention capacity, high impact resistance, stable operation, and simple operation.	Anammox enrichment is influenced by the type and nature of the filler, and the control system is complex	[102]
Rotating Biological Contactor (RBC)	High biomass per unit of sludge and low sludge loss.	High rotational energy consumption	[103]

3.1.5. Addition of Biological Carriers

Usually, carriers provide good growth substrates for anammox bacteria, while artificial immobilization (including encapsulation and particle immobilization) shows superior results over natural aggregation [104]. In some cases, microorganisms require a complex series of interactions with substrates to complete their metabolic activities. Therefore, for the aggregation of bacteria, artificial immobilization has become a highly preferred method. Researchers are actively exploring methods to artificially immobilize anammox bacteria to achieve a rapid initiation of anammox reactions and enhance bacterial activity. Among them, biosolids as novel carrier materials have received more and more attention from scholars. According to the literature, scholars are trying to convert a variety of carrier materials into carriers for the anammox of biomass with the aim of immobilization. Materials such as nonwoven fabrics [105], polyethylene sponges [106], bamboo charcoal [107], synthetic polymer gels [108], magnetic carbon microspheres [109], polyurethane foams [110], and zeolites [111] are among the materials that can be used to make polymeric materials. These carriers are used to physically or chemically immobilize microorganisms in a porous medium of a certain shape and size. Polymer gel carriers are one of the most promising

carriers in the field of biomass immobilization due to their excellent biological affinity, huge specific surface area, and wide pore channels [112]. Currently, PDA technology is considered one of the most promising technologies at present. Partial denitrification can make full use of organic matter to achieve simultaneous nitrogen and carbon removal, and partial denitrification provides a more stable supply of nitrite than partial nitrification and also reduces the negative effects of anammox bacteria [113]. Therefore, denitrification, partial denitrification, and partial denitrification using aerobic microorganisms have become a hot research topic. The construction of biofilms using carriers is a widely used technological tool for the retention of biomass [114]. Recently, a researcher successfully operated and treated municipal wastewater in an SBR-UASB system based on partial denitrification and anammox. By adding biological carriers, the system achieved an efficient enrichment of common activated sludge anammox bacteria with a TN removal rate of 90.5% [115]. Studies have shown that the integration of anammox with endogenous denitrification can yield excellent results of nitrogen removal rates of more than 90% [116].

3.2. Building Granulation Models

Anammox bacteria grow slowly and are highly sensitive to their surroundings. Therefore, the application and development of the anammox process are limited by the growth characteristics of anammox bacteria. Some researchers have investigated the discovery of pelletizing models to improve the efficiency of anammox reactors by increasing the retention of biomass via pelletizing. The natural aggregation of particles can be achieved via cell-to-cell contact by flow mixing in the reactor [117]. Pelletization is one of the most acceptable methods to improve biomass retention by improving the accommodation capacity, shock resistance, and settling [118]. Granular sludge has a wide range of uses as microbial aggregates formed during microbial self-fixation, and its main component is an extracellular polymer (EPS) generated via bacteria and microorganisms [119]. EPS in anammox bacteria have a large number of hydrophobic groups and a looser protein secondary structure because anammox bacteria have a higher protein/polysaccharide (PN/PS) compared to other activated sludge; therefore, anammox bacteria have a higher aggregation capacity [120]. The investigation by Jinhong Yang et al. showed that the PS content within EPS did not change much as the particle size of granular sludge increased, and PN/PS increased with PN; then, there was a positive correlation between the proportion of PN in EPS and particle size, which played a major role in the formation of sludge particles [121]. During the formation of anammox granular sludge, the granular sludge floats under the effect of high nitrogen-loading rates, causing the biomass to be washed away. The floating behavior is mainly influenced by the particle diameter (2.5~4.5 mm) and substrate concentration (NO_2^-N, 50~250 mg/L) in the transition zone (with some particles floating), and the optimal particle diameter to avoid floating and better settling performance is around 2.5 mm, and the sensitivity of particle size to substrate concentration is high [121]. Zhang Yachao et al. improved the activity and growth rate of anammox granular sludge by adding high serine lactone (AHL)-signaling molecules to effectively solve its floating problem in a high-loading reactor in the presence of floating phenomenon during granular sludge enrichment culture [122].

Some researchers have proposed a method to encapsulate live cells in a gel matrix via polymer cross-linking. The current gel-embedding technology is advantageous for the enrichment of anammox sludge. However, it has also been shown that encapsulation immobilization techniques lead to the degradation of performance under long-term reactor operation. Bae et al. showed that a large inactive dead space was formed in the dense gel that initially encapsulated the high concentration of inoculum, limiting the substrate transfer [123]. Synthetic polymer polyvinyl alcohol (PVA) has been used as a conventional encapsulation matrix to form PVA/sodium alginate (SA) gel beads [124]. PVA/SA is widely used as a cell immobilization carrier in wastewater treatment because of its low price, good durability, and high mechanical strength [125]. Wang et al. showed that the newly prepared PVA/SA anammox gel beads could induce microbial apoptosis

and, at higher PVA/SA ratios, could reduce the adverse effects of cell immobilization on microorganisms; cell immobilization had an enhanced effect on reactor denitrification efficiency, and the highest NRR and best pore characteristics of PVA/SA (12%/2%) gel beads with the best PVA: SA ratio gel beads [126]. Polyvinyl alcohol/chitosan (PVA/CS) and polyvinyl alcohol/chitosan/iron (PVA/CS/Fe) gel beads were recently synthesized by researchers and successfully applied for the immobilization of anaerobic species, including Mathanospirillum, Methanosaeta, and Methanobacterium [127]. The improved anammox reactor performance and high activity can be attributed to the tight aggregation and efficient substrate transport in porous gel beads [128]. The anammox bacteria proliferation found a suitable ecological niche with the addition of porous gel beads, and the roughness of the gel beads facilitated the entanglement of cells and EPS, thus forming tightly packed bacterial aggregates. Wang et al. showed that the immobilization of biomass by PVA/CS and PVA/CS/Fe-assisted particle-based anammox promoted SAA, and Fe in gel beads improved the aggregation of EPS in cells [129]. According to the literature, Fe^{2+} and Fe^{3+} ions induce flexible chelate bonds between PVA and CS in the form of knots, improving the mechanical strength of PVA/CS/Fe and thus preventing water shear erosion and cell detachment [130]. The denitrification performance in anammox reactors with and without gel beads at progressively lower temperatures has recently been shown that low temperatures have significantly fewer adverse effects on anammox bacterial activity inhibition and biomass washout and maintain higher nitrate reductase and nitrite reductase levels at lower temperatures. Gel beads alter the granulation of non-immobilized biomass, resulting in higher anammox bacterial activity [131].

Recently, a novel anammox granulation model regulated by *Epistylis* spp. has been proposed to investigate the potential mechanism of high enrichment of anammox bacteria in pellets [109]. Many reports have found protozoa in particles with bacteria and their bacterial extracellular polymeric material. The protozoa implicated in these study reports include ciliates, amoebae, and flagellates. Due to their high sedimentation properties, protozoa have a key role in stimulating granulation and biomass enrichment and retention. *Epistylis* spp., a common rootless ciliate in wastewater treatment bioreactors, has abundant stems and plays an important role in water purification and bioindicators [110]. Under the *Epistylis* spp.-regulated novel pelleting model, there are four main stages: nutrient uptake and proliferation of *Epistylis* spp. and formation of *Epistylis* spp. populations and agglomerates (stage 1); the particles in the flattened aggregates fix the leading branches and maintain the overall structural toughness (stage 2); the biomass layer becomes thick enough and smooth enough to form mature particles after hydraulic shear, aeration scouring, abrasion, and floating when the granular sludge settling performance is further improved (stage 3); excessive growth of *Epistylis* spp. is dislodged from the granules and a dynamic balance between the disintegration and formation of granules is formed, maintaining the stability of particle size and settling capacity of the granular sludge system (stage 4). The stems of *Epistylis* spp., in turn, provide attachment points for bacterial colonization and also enhance the rigidity of the overall structure. Therefore, the pelleting of anammox bacteria was facilitated by constructing an abundance suitable for *Epistylis* spp. The regulation of *Epistylis* spp. provides attachment sites for bacterial colonization and maintains the stability of the particle structure. During the pelletizing process, the expanded intermediate biomass layer, in turn, provides more space for non-walking, free-swimming animals. For *Epistylis* spp. to construct a novel granulation model and achieve an efficient enrichment of anammox bacteria, the investigators made the speculation that *Epistylis* spp. produces results that favor the predation pressure of anammox bacteria.

3.3. Biomass Management Strategies for Suspended Sludge

In the field of wastewater treatment, partial denitrification coupled with an anammox process (PDA) is a potential alternative for nitrogen removal in a cost-effective manner [132]. In some studies, this process is effective in treating high concentrations of ammonia nitrogen wastewater. Partial denitrification (PD) is a promising process for providing the required

NO_2^- for anammox under mainstream conditions [133]. The system takes advantage of the microbial reaction to nitrate reduction in an anaerobic environment. In the PDA process, NO_3^- is reduced to NO_2^- by denitrifying bacteria (DB), followed by anammox bacteria that act as electron acceptors for NH_4^+ oxidation to achieve the reaction. This process can treat both high and low concentrations of ammonia nitrogen. The PDA process has shown impressive nitrogen removal efficiency in actual municipal wastewater treatment, with nitrogen removal efficiencies of 93.0–94.6% [134]. The application of PDA in municipal wastewater treatment plants is limited by the biomass of anammox bacteria, resulting in a much lower contribution than expected. The imbalance between anammox bacteria and denitrifying bacteria biomass led to substrate competition and imbalance in ecological niches and, ultimately, a reduction in anammox bacteria activity. Some researchers have recently integrated biological carriers into suspended sludge to create, intending to create an ecological niche advantage for anammox bacteria. In this study, an anaerobic–anoxic–aerobic combined biological contact oxidation (A_2O-BCO) bioreactor (Figure 2), in which the A_2O unit was inoculated with pure suspended sludge and the BCO unit was inoculated with an aerobic carrier biofilm with a filling rate of up to 60%, was established and started. During the process of suspended sludge biomass management, Brocadia was enriched in the anoxic biofilm, and its concentration increased from 0.70% to 5.99%. In addition, the gradual reduction in suspended sludge can also effectively alleviate the competition between DB and anammox bacteria, thus achieving a balance between DB and anammox bacteria biomass and improving the ecological niche of anammox bacteria [135]. According to Cao et al., PD is capable of achieving a high nitrate–nitrite conversion ratio (NTR) at 25 °C; therefore, more and more people are showing interest in the coupling process of PD with anammox [136]. A recent study reported that coupling studies of S(S^{2-})AD and anammox reactions above 30 °C are feasible [137]. As an important environmental factor, sulfur plays a crucial role in the nitrogen cycle. Sulfur-driven autotrophic denitrification (SAD) is an alternative denitrification process that allows the use of sulfur compounds such as S(0), S^{2-}, and $S_2O_3^{2-}$. These sulfur compounds can be directly or indirectly involved in the utilization of nitrite nitrogen in microbial growth and reproduction. S(0) is an affordable, efficient, and convenient SAD electron donor that catalyzes the conversion of nitrate to nitrite and N_2 during sulfate production. In addition, sulfur promotes the ability of microorganisms to reduce ammonia nitrogen, and nitrite. S(0) has been reported by researchers to promote the accumulation of nitrite [138]. According to Zhang et al., the number and activity of anammox bacteria were significantly increased at a sulfate concentration of 400 mg/L [139]. Wang et al. integrated the partial S(0)-driven autotrophic denitrification (PSAD) process with anammox, resulting in the natural aggregation of anammox bacteria within 12 days during PSAD initiation and high total nitrogen removal efficiency (TNRE) during PSAD-anammox coupling [140].

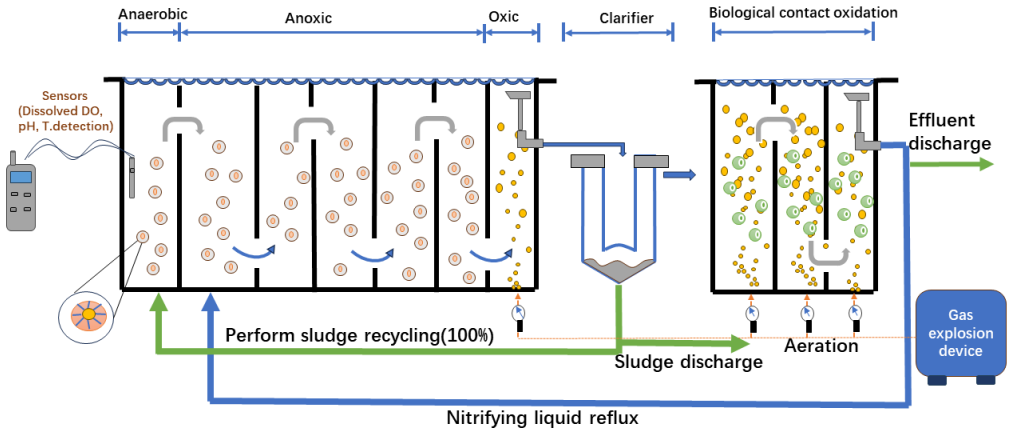

Figure 2. Schematic diagram of the A_2O-BCO bioreactor [135].

3.4. Strain Preservation

3.4.1. Bacterial Dormancy

Anammox technology breaks the inherent understanding of the nitrogen cycle, but the high sensitivity to the growth environment and the long reactor start-up cycle have prevented it from being widely used. Some researchers have shown that dormant bacteria stop growing and reproducing, exhibit lower metabolic activity, but survive for relatively long periods [141]. Once the bacterial growth environment becomes favorable, the dormant bacteria will be revived [142]. Therefore, the denitrification performance of anammox sludge, which has been left in the reactor for a long time to return to its original form in a dormant state, is of great interest.

Liu Xiruo et al. found via experiments that most of the otherwise clearly contoured cylindrical particles, as well as spherical particles of anammox bacteria in a dormant state, became angular and gritty on the surface, and this gritty substance was presumed by the researchers to be a substance produced via the self-protection of the bacterium in the dormant state [143]. Under suitable storage conditions, both ammonia-oxidizing bacteria and denitrifying bacteria in the sludge are in a strictly anaerobic and autotrophic state and die out, and anammox bacteria will become the dominant species. Xing et al. investigated the effect of prolonged starvation up to 50 d on anammox granular sludge and its reactivation process at two temperatures, 4 °C and 20 °C, and found that prolonged starvation at 4 °C was more suitable for maintaining the granules [144]. VIANCELLI et al. found that the abundance of *Ca. j* decreased by 35% and *Ca. B* decreased by 3%, while the abundance of Thermomonas (Th) increased by 58% and became the dominant species after 90 d of storage at −80 °C using Glycerin as a protectant; and the abundance of *Ca. a* increased by 95% and *Ca. B* decreased by 40% after 90 d of storage using skim milk as a protectant. B decreased by 40%, while the abundance of *Ca. a* increased by 95% and became the dominant species [145]. Anammox bacteria also change color differently under different preservation methods [86].

3.4.2. Activation Recovery

During the recovery of anammox bacterial activity, they awaken from their dormant state, intensify their metabolic activity, and begin to compete with other species for substrate substrates. In the suitable growth environment of anammox bacteria, the communication between bacteria becomes progressively more active, and the cells initiate the macro synthesis of oxidative nitrogen oxidoreductase, hydrazine synthase, and hydrazine dehydrogenase studies, etc., generating hydroxylamine and hydrazine in large quantities to accelerate the anammox reaction in brick-red anammox sludge [146].

Heterotrophic and aerobic bacteria find it difficult to survive under strictly anaerobic and autotrophic conditions, so anammox bacteria re-emerge as the dominant species. Li et al. showed that long-term low-temperature preservation of anammox sludge resulted in high EPS levels in the late stages of starvation but decreased dramatically in the early stages of recovery, and the researchers used glucose as an organic carbon source in the recovery of activity of anammox granular sludge, which was preserved for a long period of time, and EPS content and PN/PS were able to recover more quickly, thus recovering the anammox process [147]. The assimilation of cells is enhanced, PN and PS are produced, EPS content is also increased, the viscosity between granular sludge is increased, small particle size granular sludge is cross-linked with each other to produce large granular sludge, sludge settling properties are increased, and anammox bacteria have started to enrich [148]. Liu Xiruo et al. found via long-term experiments that the input of Fe_3O_4 and nano-CuO during the activity recovery of anammox bacteria could increase the NH_4^+-N removal rate by 16.13% and 15.36%, and the optimal dosage was 4 mg/L and 3 mg/L, respectively, and the dominant anammox bacteria genus *Ca. k* was further enriched, but more than 11 mg/L of nano-Fe_3O_4 and more than 8 mg/L of nano-CuO had an inhibitory effect on the recovery of activity of anammox bacteria [143]. Gao Yan et al. used an up-flow packed reactor, and the dominant species in the sludge before startup were *Anaerolineaceae*

(*An*), and *Ca. k*, during startup, the substrate content gradually increased, *Ca. b* competition increased, *Ca. k* genus continued to decline after 200 d in the beginning, and the abundance of species in the reactor was not high, An and Ca. *B* became the dominant species, and the relative abundance of the two genera accounted for 77.2% of all genera, where the relative abundance of *Ca. B* increased from 0.1% to 34.4% [149].

4. Summary and Outlook

The anaerobic ammonia oxidation process is a cost-effective biological treatment technology for wastewater. As the functional bacteria of the anaerobic ammonia oxidation process, anaerobic ammonia oxidation bacteria are an important microbial group mediating the nitrogen cycle and are an important microbial resource. The PDA process, a derivative of the anammox process, has achieved high denitrification efficiencies of 93.0–94.6% in municipal wastewater and is considered a future practical engineering application of the mainstream anammox process. The anammox process has been gradually advanced to engineering applications, but the problem of anammox bacterial activity needs further improvement and solution. Efficient enrichment of anammox bacteria was achieved using a novel granulation model involving *Epistylis* spp. The A_2O-BCO process effectively increased the enrichment of anammox bacteria on anoxic biofilms and contributed positively to the suspended sludge biomass management strategy. There are still many unknown blind spots for anammox bacteria, which need to be further explored in the future.

Although this paper provides an overview of the current methods for enriching anammox bacteria, there are still deficiencies and problems that need to continue to be improved and addressed in the future, so possible solutions include the following (Table 3).

Table 3. Possible solutions.

Serial Number	Possible Solutions
1	The current literature shows that no pure cultures of anammox bacteria have been found. Therefore, it is urgent to find a method that allows anammox bacteria to be isolated and cultured and remain active, contributing to the denitrification performance of the process.
2	A total of 4 mg/L of Fe_3O_4 is the most suitable input amount for the recovery of anammox bacterial activity, and NZVI has the advantages of a large specific surface area and strong reducing ability. Two substances, Fe_3O_4 and NZVI, were combined to explore the effect on the activity of anammox bacteria.
3	Seven genera of anammox microorganisms are known, and the exploration of other bacterial taxa with anammox functions will continue.

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Abbreviations

Abbreviation	Full form
Anammox	anaerobic ammonia oxidation
SNAP	single-stage short-course nitrification-anammox
PDA	partial nitrification-anammox
OLAND	restricted autotrophic nitrification-denitrification
CANON	total autotrophic denitrification
SNAD-DPR	short-course nitrification/anammox/denitrification coupled-denitrification phosphorus removal
OUT	operational taxonomic units
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
pH	pondus hydrogenii
FNA	free nitrous acid
DS-EPSCN	denitrification sludge EPS enhanced
HRT	hydraulic retention time
NLR	N loading rate
DO	dissolved oxygen
NZVI	nano zero-valent iron
Nir	nitrite reductase
HZS	hydrazine synthetase
HDH	hydrazine dehydrogenase
Nxr	nitrite oxidoreductase
EPS	extracellular polymeric substances
ATP	adenosine triphosphate
RGO	reduced graphene oxide
GAC	granular activated carbons
NRR	a nitrogen removal rate
AOB	ammonia-oxidizing bacteria
NOB	nitrite-oxidizing bacteria
$EGSB_{GC}$	granules circulating EGSB
UASB	up-flow anaerobic sludge blanket
EGSB	expanded granular sludge bed reactor
MBR	membrane bioreactor
ABR	anaerobic baffled reactor
UBF	up-flow blanket filter
SBBR	sequencing biofilm batch reactor
RBC	rotating biological contactor
PN/PS	protein/polysaccharide
AHLs	adding high serine lactones
PVA	polyvinyl alcohol
SA	sodium alginate
PVA/CS/Fe	polyvinyl alcohol/chitosan/iron
DB	denitrifying bacteria
A_2O-BCO	anaerobic-anoxic-aerobic combined biological contact oxidation
NTR	nitrate-nitrite conversion ratio
SAD	sulfur-driven autotrophic denitrification
PSAD	partial S (0)-driven autotrophic denitrification
TNRE	total nitrogen removal efficiency
CODH	CO dehydrogenase/acetyl coenzyme A (Ac-CoA) synthase
CH3-H4	methyltet-rahydrofolate
FDH	formic acid dehydrogenase
Ac-CoA	dehydrogenase/acetyl coenzyme A

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