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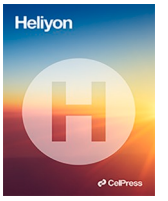

Review article

Nitrate formation in anammox process: Mechanisms and operating conditions

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is an energy-efficient technology for wastewater nitrogen removal. However, the byproduct nitrate has hindered development and application of anammox process. Meanwhile, the knowledge of nitrate formation during anammox process is insufficient, which prohibits high nitrogen removal. This review firstly summaries and discusses valuable findings on nitrate formation, including molecular mechanism of nitrate production, microbial pathway of nitrate reduction and its net formation. Specially, influences of operating conditions on mechanisms and patterns of nitrate formation are analyzed. Then, based on nitrate formation mechanism, current strategies of nitrate removal from anammox process are reevaluated. Finally, the key knowledge gaps and further process development are presented. Overall, this review sheds light on the understanding of nitrate formation of anammox process, which would further facilitate and optimize the process design and operation for high performance nitrogen removal.

1. Introduction

Anaerobic ammonium oxidation (Anammox) bacteria can oxidize anoxically ammonium utilizing nitrite as the electron acceptor with dinitrogen gas as the main product and nitrate as a byproduct [1]. Anammox process is regarded as a cost-effective and environment-friendly biological nitrogen removal process [2]. Until 2017, at least 200 full-scale installations based on anammox process have been implemented worldwide for treating these wastewaters [2,3]. More recently, implementation of anammox process is proposed as a powerful technology to achieve energy neutral or energy positive treatment of municipal wastewater. However, the byproduct nitrate has hindered its development and application. This is because that the maximum nitrogen removal efficiency of anammox process could reach 89 %, and high residual nitrate brings about low effluent quality [4,5]. In order to address nitrate

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dilemma, coupling anammox process with biological nitrate removal process is being extensively developed [6–9]. The strategies for nitrate removal in the coupling processes could be categorized into nitrate conversion to nitrogen and nitrate conversion to anammox substrates. In the former, nitrate generated by anammox bacteria (AnAOB) could be reduced to nitrogen by denitrification, such as simultaneous partial Nitrification, ANAMMOX and denitrification (SNAD) process. In the latter, nitrate could be converted to ammonium by dissimilatory nitrate reduction to ammonium (DNRA) and nitrite by partial DNRA or partial denitrification (PD) [7,10]. AnAOB or DNRA bacteria are responsible for partial DNRA, while denitrifiers are responsible for PD. Compared with SNAD process, the development of the coupling DNRA with anammox process and partial denitrification/anammox (PN/A) process is receiving great significance due to low carbon source demand [4].

Meanwhile, the understanding of nitrate formation in anammox process is no more than the widely acknowledged fact that the growth of anammox bacteria (AnAOB) is associated with nitrate production [11]. The qualitative relationship between them is not always effective due to variable nitrate production. Based on anammox mechanism, AnAOB seem to be the solely participant of nitrate formation. However, until now pure AnAOB cultures are still not available with slowly growing and anaerobic physiological characteristics [12]. The complex microbial communities are formed during the enrichment of AnAOB, and side population coexisting with AnAOB is capable of metabolizing nitrate [13]. It is evident that nitrate formation is a synergic effect of numerous functional microbial guilds in anammox process. Clarifying the complex relationships among them is of importance to understand anammox performance and optimize anammox process for minimizing nitrate formation.

Even though almost all aspects of anammox process have been reviewed well, there is lack of reviewing nitrate formation, which refers to nitrate production by Anammox and nitrate reduction by denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in pure anammox process. Nitrate formation in anammox-based processes is outside the scope of the current review. To the best of our knowledge, this review is the first to target the current knowledge of nitrate formation in anammox process. The review aims to summary and analyze nitrate formation mechanism (molecular mechanism of nitrate production, microbial pathway of nitrate reduction and net nitrate formation), with a special focus on influence of operating conditions (nitrogen load rate, substrate ratio, temperature and pH) on nitrate formation mechanism. Major perspectives and future trends about nitrate formation and removal will be put forward.

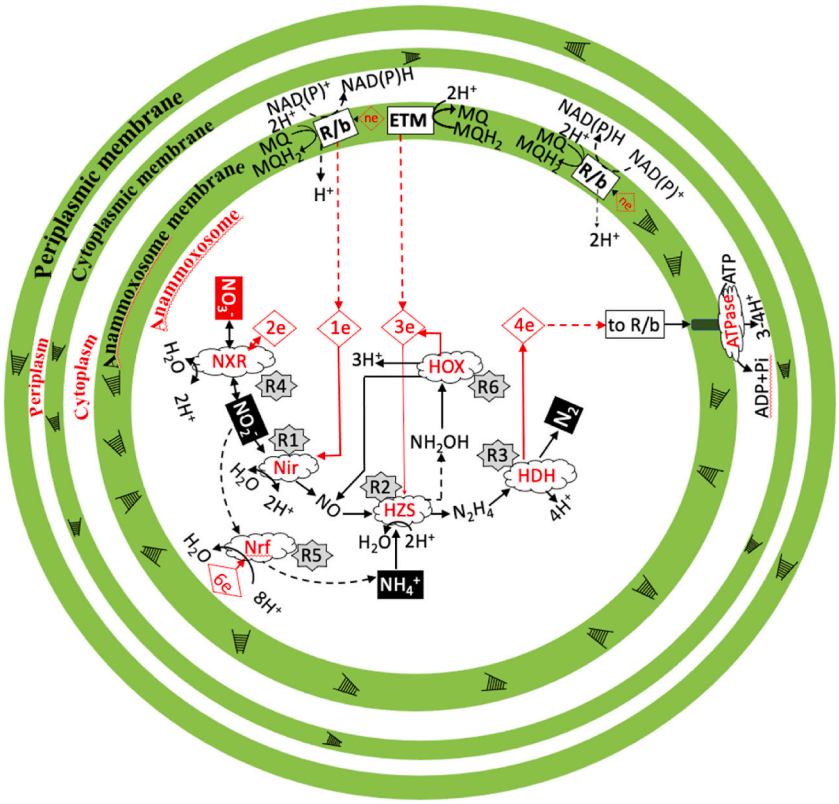

Fig. 1. Ammonium, nitrite and nitrogen are highlighted black and nitrate is highlighted red. Reactions are numbered (octagon with black numbers) and are catalyzed by the following enzymes (cloud shape): Nir, nitrite reductase; HZS, hydrazine synthase; HDH, hydrazine dehydrogenase; HOX, hydroxylamine oxidase; NXR, nitrite oxidoreductase; Nrf, nitrite reductase forming ammonium; ATPase, ATP synthase. Chemicals and electron flows are indicated by black and red arrows, respectively. Reactions and processes indicated by broken black and red lines are not completely established yet. Membrane ladderane is indicated by ladder shape.

2. The mechanism of nitrate formation

2.1. The molecular mechanism of nitrate production by AnAOB

Currently, the mechanism of anammox reaction has been proposed that the conversion of ammonium and nitrite into nitrogen proceeds in three consecutive redox reactions (R1-R3, Fig. 1), associated with two highly toxic intermediates of nitric oxide (NO) and hydrazine [14,15]. In R1, the reduction of nitrite to NO is catalyzed by nitrite reductase (Nir) at an expense of one electron. In R2, the combination reaction of ammonium and NO by hydrazine synthase (HZS) consuming three electrons results in the formation of hydrazine. At last, the oxidation of hydrazine into N_2 catalyzed by hydrazine dehydrogenase (HDH) in R3 releases four electrons, which drive the previous R1 and R2. Meanwhile, during the above-mentioned energy metabolism, cell carbon fixation of AnAOB using CO_2 as sole carbon source must be supplied with additional electrons. The available electron source is hydrazine oxidation process under normal conditions. However, the electron deficit would occur and suppress the efficiency of energy metabolism of AnAOB. In order to balance the budget of cyclic electron flow, the occurrence of nitrite oxidation to nitrate during anammox reaction (R4), catalyzed by nitrite oxidoreductase (NXR), could replenish the electrons that are withdrawn from the oxidation of hydrazine for cell carbon fixation. Briefly, nitrite disproportionation to NO and nitrate catalyzed by Nir and NXR guarantees the anabolism of AnAOB. In other words, high-rate anammox performance and quick growth of AnAOB would be consistent with more efficient and robust electron flow.

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (E_0' = +0.38V) \tag{R1}$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (E_0' = +0.06V) \tag{R2}$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (E_0' = -0.75V) \tag{R3}$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \ (E_0' = +0.43V) \tag{R4}$$

As known, denitrifying bacteria always release significant amounts of undesirable intermediates of NO and nitrous oxide (N_2O), especially during metabolic changes with varied operational conditions (pH, COD/N, nitrite, dissolved oxygen, etc.) [16,17]. Consistent with denitrifying bacteria, NO and N_2O release of AnAOB have been confirmed in spite of a lower loss [18–20]. In addition, anammoxosome as power station of AnAOB is analogous to mitochondria of eukaryotic cell. The endosymbiotic origin of mitochondria has been widely acknowledged [21]. Similarly, anammoxosome may be originated from endosymbiosis [22]. Mitochondrial proton and electron leak could bring about as high as 10 % energy loss [23]. Normally, ladderane phospholipids in anammoxosome membrane are tightly packed, which could provide protection against intermediates (proton, NO and hydrazine) leakage [24,25]. However, once the compositions and structure of densely packed anammoxosome membrane are altered or damaged [26,27], the barrier function would be ineffective. Thus, there is easily an overlooked fact that leakage of intermediates is underestimated [28]. The occurrence of the leakage would do harmful to slowly growing AnAOB. Maybe additional electrons also need to be replenished at the expense of nitrite oxidation to nitrate, which balance the budget of cyclic electron flow. Further research is required for confirming this hypothesis.

2.2. The microbial pathways of nitrate reduction

Nitrate reduction pathways in anammox sludge include denitrification and dissimilatory nitrate reduction to ammonium (DNRA). It is well known that extracellular polymeric substances (EPS) derived from AnAOB play a central role in the high aggregation ability of anammox sludge [29]. Moreover, EPS content of anammox sludge (111–410 mg/g VSS) is higher than anaerobic and nitrifying granules (60–74 mg/g VSS) [30]. In light of the source of SMP from EPS, high EPS offer more sufficient endogenous organics [30], indicating heterotrophs is important to anammox sludge. Among them, denitrifiers consist of a large share of anammox communities [31]. The common denitrifying genera of *Denitratisoma*, *Thauera*, *Dechloromonas*, *Bradyrhizobium*, *Rhodoplanes* and *Pseudomonas* could be usually detected in anammox sludge [13]. Additional coexisting *Chloroflexi* in anammox sludge is able to reduce nitrate with organic compounds, which are released from lysed anammox cell [32]. Anammox granular sludge could instantly exhibit significant denitrifying activity after a sudden exposition to acetate [33]. It should be emphasized that the used granular sludge was never fed with any organics before the acetate exposition in the research. These results indicate that nitrate reduction via denitrification process is an important nitrate reduction pathway in anammox sludge.

Differently from denitrification, researches on DNRA pathway of anammox sludge are at the initial stage. When ammonium is absent, AnAOB are capable of generating the substrate by DNRA with nitrite as intermediate using exogenous or endogenous organics (Fig. 1, R5) [11,34–36]. And even in the absence of ammonium and nitrite, AnAOB could feed itself by reducing part of nitrate to nitrite and one part to ammonium [37]. AnAOB are always kept in biofilms or cell aggregates, such as flocs and granules. Inside large aggregations, substrates famine inevitably occurs, and AnAOB have a great potential for implementing DNRA pathway. *Ca. Anammoxoglobus propionicus*, *Ca. Kuenenia stuttgartiensis*, *Ca. Brocadia fuldiga*, *Ca. Brocadia* sinica and *Ca. Jettenia caeni* have already been found to be capable of implementing DNRA pathway [10,34,37–39]. *Ca. Kuenenia stuttgartiensis* and *Ca. Brocadia sinica* could perform DNRA pathway using intracellular glycogen as electron donor [36,40], while the rest of AnAOB have been confirmed that the DNRA pathway could be driven by exogenous organics [41]. Whether their DNRA pathway could be driven by intracellular glycogen remains to be explored. Besides, DNRA bacteria are also the sink of nitrate while using endogenous organics and its relative abundance is

analogous to denitrifiers. DNRA bacteria could not only supply additional substrate supply for AnAOB, but also enhance stabilization of anammox sludge [32,42]. DNRA by AnAOB is only activated under substrate famine, while DNRA bacteria always take actively part in nitrate reduction for enhancing the growth of AnAOB [41]. Compared with DNRA by AnAOB, special researches on DNRA bacteria in anammox sludge are scarce. Future researches might shed more light on the difference of DNRA by AnAOB and DNRA bacteria in operational conditions and metabolic interactions.

2.3. Ecological niches of functional microorganisms

As described in section 2.2, anammox, denitrification and DNRA are involved in nitrate formation in anammox process, corresponding to functional microorganisms of AnAOB, denitrifiers and DNRA bacteria, respectively. AnAOB as the solely contributor of nitrate production is most critical to nitrate formation. To date, five known candidate genera of AnAOB with more than 20 species have been named and are classified to the order *Planctomycetales* [43]. Common anammox species possess different ecological niches due to substrate affinity and cell growth rate [12]. As a result, community compositions of AnAOB would be markedly varied with fluctuation of substrate supply. Besides, AnAOB could be always adaptive and enriched under different operating stressors (oxygen tolerance, salinity, aggregation ability, organic matter, temperature, etc.). For example, in the temperature tolerance, *Ca. Kuenenia* appear to well adapt to low temperature than *Ca. Brocadia* [44].

In spite of significantly different operating conditions in different researches, denitrifiers communities in anammox process are to a certain extent similar [13]. Generally, the type of carbon source is the decisive factor of community structure of denitrifiers [45]. A plausible explanation for the similarity of denitrifying communities may be a minor difference in compositions of endogenous organics. Based on different electron donors, DNRA consists of respiratory DNRA and fermentative DNRA. The former could be driven by non-fermentable organics (acetate and formate) and inorganics (sulfide and ferrous iron). The latter could be driven by organic macromolecules (glucose and glycerol) [46]. DNRA bacteria in anammox sludge are dominated by fermentative *Anaerolineaceae* and *Ignavibacteria*, which are abundant core taxa in the absence of exogenous organics [30]. Thus, in anammox process, the thriving reason of fermentative DNRA bacteria is that SMP serving as electron donor mainly consist of macromolecule organic compound, such as polysaccharides, protein and vitamin B_{12} [47].

Interestingly, dynamic competition and coexistence of denitrifiers and DNRA bacteria have been established. A balance between the population of two bacteria is controlled by several key factors, including C/N ratio, nitrate availability, types of electron donor. Fermentative DNRA bacteria have competitive advantages over denitrifiers in high C/N, insufficient nitrate, high nitrite/nitrate and sufficient fermentable organics [48]. In a recent research on impact of organics on anammox sludge communities, when acetate was replaced with glucose, DNRA bacteria became dominated over denitrifiers [30]. Generally, in order to quicken AnAOB enrichment, influent substrate is free from organics in anammox process. Thus, within anammox sludge, fermentative DNRA bacteria could be outcompeted by dentrifiers under oligotrophic-like conditions. On the other hand, denitrifiers could be favored by low molecule fermentation products serving as electron donor, which stem from DNRA bacteria utilizing SMP. Finally, a win-win cooperation among AnAOB, denitrifiers and DNRA bacteria is established with the cross-feeding of nitrate and endogenous organics (Fig. 2). However, there is an unexpected situation that DNRA bacteria could outcompete AnAOB on unknown operational mechanism, bringing about dysfunction of anammox process [49]. It is possible that versatile DNRA bacteria play double-edged sword effects on anammox process performance [49]. Thus, the importance of the mutual relationship to highly efficient performance and long-term stabilization of anammox process needs to be further evaluated.

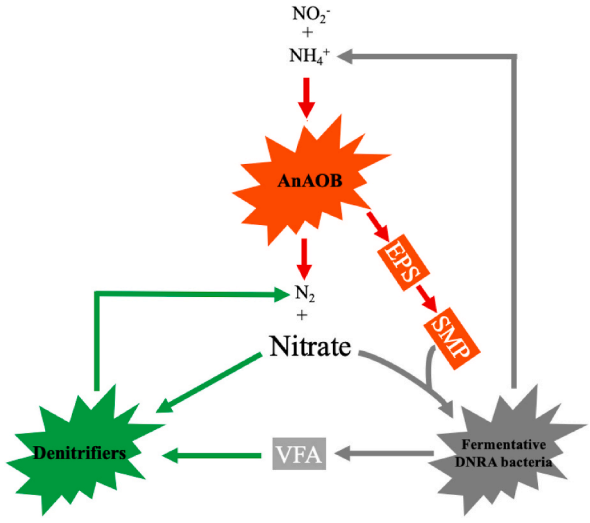

Fig. 2. A win-win cooperation for nitrate formation among AnAOB, denitrifiers and DNRA bacteria. AnAOB, anammox bacteria; DNRA, dissimilatory nitrate reduction to ammonium; EPS, extracellular polymeric substances; SMP, soluble microbial products; VFA, Volatile fatty acids.

2.4. Net nitrate formation

Net nitrate formation is critical in process design and optimization for nitrate removal. Based on the reported stoichiometry of anammox reaction (Table 1), the significant differences in net nitrate formation could be found. In the most cited anammox stoichiometry proposed by Strous et al. (1998), the amount of nitrate formation reached 11 % of the sum of removed ammonium and nitrite. In another batch experiment, a higher nitrate was produced with NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) of 15 % [50]. Due to a strong theoretical relationship between nitrate formation and anammox biomass production, the higher nitrate production is without oxygen leakage, the faster anammox biomass grow. Thus, it is reasonable that cell yield (0.17 g VSS/g NH_4^+-N) in the latter study is higher than that (0.11 g VSS/g NH_4^+-N) in the former. However, in a recent stoichiometry of anammox reaction proposed by Lotti et al. (2014), the value of NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) was reduced to 7.5 %. In the experiment, anammox cell suspension with high purity of 98 % was used in studying kinetics and stoichiometry of AnAOB. Anammox aggregates always hinder substrate diffusion, while the high purity free-cells suspension could not only guarantee high substrate availability, but also make sure that AnAOB almost are at the similar physiological states. Additionally, an appropriate nitrogen load rate (NLR) with the limiting substrate of nitrite protects AnAOB from nitrite inhibitory effect. Thus, compared with the previous researches, the stoichiometry of anammox reaction and its nitrate formation are considerably more accurate and representative. Consistent with the results from Lotti et al. (2004), NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) ratio of 7.5 % was also reported in anammox attached film expanded bed (AAEEB) reactor with NLR of 5.0 g-N/L/d [51]. The low ratio should ascribe to excellent settling property of granular sludge with long solid retention time (SRT), which has been reported in several similar researches. NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) of 2.9 % was achieved in an MBR reactor with an SRT of 500 days. Similarly, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) of 8 % was also observed in an up-flow membrane-aerated biofilm reactor with no wasted sludge discharge except a neglected small amount of sludge samples [52]. A plausible explanation for low nitrate formation may be that denitrifying or DNRA activity are enhanced by the release of endogenous carbon under long SRT [53,54]. Further investigation is required to identify what mechanism low nitrate formation is and what factors modulate nitrate formation under long SRT. Meanwhile, When NLR was improved from 5 to 50 g-N/L/d, the ratio went up to 10.5 % in Zhang et al. (2018) research, suggesting that minimizing nitrate formation could be performed by optimizing influent NLR.

Even though it is really a sight for sore eyes that anammox reaction in overwhelming majority of anammox researches is in line with the widely-cited anammox stoichiometry, abnormal nitrate overproduction has been recently reported in pure anammox process. In a biofilm reactor, as much as 60 % of influent nitrogen load was converted to nitrate, indicating that a shift of anammox metabolism towards nitrate has occurred [55]. Meanwhile, when AnAOB have entered a prolonged logarithmic growth phase, nitrate was overproduced [55]. However, the relative quantification data of AnAOB growth during the period was not given. Moreover, AnAOB possess two different types of NXR complex, periplasm-bound and membrane-bound NXR, respectively [56]. The periplasm-bound NXR is responsible for replenishing lost electrons during carbon fixation of AnAOB growth. Maybe the membrane-bound NXR plays a crucial role in nitrate overproduction. In another research, long-term performance of anammox process was evaluated under low ratio of food to microorganism (F/M) and moderately low temperature [57]. NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) ratio gradually and slowly increased during the first 973 days, and its peak value was 22.1 %, which was attributed to the growth of NOBs detected by fluorescence in situ hybridization (FISH). The results of NOB analysis by FISH only show the presence of NOBs, and do not represent high activity of nitrite oxidation. More importantly, *Nitrobacter* and *Nitrospira* are not distinguished by FISH analysis. Under low NLR, the enrichment of *Nitrospira* have been reported in various anammox-based biological nitrogen removal systems, and the enriched *Nitrospira* functioned as complete ammonia-oxidizing *Nitrospira* species (Comammox) [58,59]. In the recent publications, AnAOB could be fed with nitrite produced by comammox and a functional cooperation between comammox and anammox has been established [58,60,61]. Thus, the presence of NOB in anammox system is not equivalent to high nitrate formation. Further comprehensive analysis of high nitrate formation in these researches needs to be conducted. Besides, F/M applied in anammox SBR is always lower than actual nitrogen removal capacity of AnAOB, which maintains AnAOB in the status of long-term starvation. As a result, it may bring about a diversion of anammox metabolism towards nitrate formation as proposed in Kowalski et al. research. In the latest research on nitrate production of anammox process with low NLR of 0.8 g-N/L/d, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) ratio of 38.2 % was calculated based on the average influent and effluent concentration of ammonium, nitrite and nitrate [62]. TN removal efficiency of 57.9 ± 8.0 % was much lower than the theoretical TN efficiency of 89 % due to high nitrate overproduction. Metagenomic analysis found that high levels of reads predicted to be NXR genes always could be detected. It is notable that nitrate accumulation is not merely due to known NOB, but other NXR-containing bacteria coexisting in anammox sludge are of importance to nitrate production [62]. Further research on actual contributors of nitrate production in complex anammox communities is needed. For example, the importance of NXR-containing bacteria is evaluated according to fluorescence in situ hybridization and microautoradiography (MAR-FISH), which could be conducted using a reported procedure with slight modification [63]. First, Anammox sludge could be incubated with ammonium and ^{15}N-nitrite for ^{15}N fixation. Second, FISH is conducted using different specific oligonucleotide probes targeting nitrite-metabolism bacteria. Based on the above-described experimental results, insufficient substrate supply seems to be associated with nitrate overproduction.

3. Influence of operating conditions on nitrate formation mechanism

3.1. Influence of NLR and substrate ratio on nitrate formation mechanism

Consistent with species-specific responses to temperature, interspecific competitions among AnAOB compete for limiting substrates. For example, the genus *Ca. Brocadia* would possibly be an r-strategist (higher growth rate but lower substrate affinity, while the

Table 1
Summary of anammox metabolism stoichiometry. SBR - sequence batch reactor; MBR - membrane bioreactor; UASB - up-flow anaerobic sludge blanket; AAFEB – anammox attached film expanded bed reactor.

Reactionstoichiometry	$NO_2^- - N_r/NH_4^+ - N_r$	$NO_3^- - N_p/(NO_2^- - N_r + NH_4^+ - N_r)$(%)	Theoreticaltotalnitrogenremoval(%)	Cellyield(gVSS/gNH_4^+ − N)	Anammoxbacteriatype	Anammoxbacteriapurity(%)	Nitrogenloadingrate(g − N/L/d)	Reactortype	Biomasstype	References
$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O$	1.32	11.2	87.9	0.11	N.A.	N. A.	1.5	SBR	Flocculent sludge	[107]
$1NH_4^+ + 1.278NO_2^- + 0.105HCO_3^- + 0.101H^+ \rightarrow 0.944N_2 + 0.353NO_3^- + 0.105CH_2O_{0.5}N_{0.15} + 1.759H_2O$	1.278	15.5	82.9	0.17	*Candidatus Brocadia caroliniensis*	N. A.	0.35*	MBR*	Flocculent sludge	[50]
$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} + 2.002H_2O$	1.146	7.5	91.9	0.11	*Candidatus Brocadia* spp.	98	1.0	MBR	Free cell	[108]
$1NH_4^+ + 1.133NO_2^- + 0.092HCO_3^- + 0.038H^+ \rightarrow 0.980N_2 + 0.161NO_3^- + 0.092CH_{2.26}O_{1.07}N_{0.14} + 1.961H_2O$	1.133	7.5	91.9	0.09	N.A.	N. A.	5.0	AAFEB	Granular sludge	[109]
$1NH_4^+ + 1.300NO_2^- + 0.121HCO_3^- + 0.367H^+ \rightarrow 1.020N_2 + 0.242NO_3^- + 0.121CH_{1.74}O_{0.81}N_{0.15} + 2.139H_2O$	1.300	10.5	88.7	0.12	N.A.	N. A.	50.0	AAFEB	Granular sludge	[109]

NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r): the mole mass of produced nitrate to the sum of the mole mass of removed ammonium and nitrite based on reaction stoichiometry.
* indicates that the data is operational parameters of the seed sludge in parental MBR, but the stoichiometry experiment was conducted in flask serum bottles.

genus *Ca. Kuenenia* could be a K-strategist (lower growth rate but higher substrate affinity) [50]. Thus, *Ca. Brocadia* could proliferate at high NLR, while *Ca. Kuenenia* outcompete *Ca. Brocadia* at low NLR. Recently, an existence of similar competition between *Ca. Brocadia* and *Ca. Jettenia* has been reported [64]. species-specific responses to NLR suggest that the discrepancy in physiology of different AnAOB could lead to different behaviors of nitrate production. With NLR increase, anammox activity and EPS production are increased [65], which would release more SMP and favor fermentative DNRA bacteria. Based on mete-analysis of anammox communities, *Anaerolineaceae* positively correlated with NLR and NRE in the absence of influent organics [30]. It is probable that DNRA bacteria would outcompete denitrifiers in higher NLR. This is a guarantee of eliminating nitrate overaccumulation. Meanwhile, the activity and EPS production of AnAOB are decreased due to lower NLR, and even poor settleability and disintegration of anammox sludge happen, suggesting the breakdown of cooperation among the functional microorganisms. Worse still, NXR could be overexpressed in low NLR, leading to low nitrogen removal efficiency due to nitrate accumulation [62,66]. However, what microorganisms carrying NXR is responsible for nitrate overproduction still need to be investigated.

Additionally, nitrate formation is also affected due to improper substrate ratio. At a proper NO_2^--N/NH_4^+-N of 1.25 with 80 mg-N L^{-1} nitrite, DNRA bacteria utilizing SMP produced by AnAOB could convert nitrate to nitrite for maximizing nitrogen removal [67]. At higher influent NO_2^--N/NH_4^+-N than the stoichiometric ratio (1.32), nitrite inhibition on anammox activity possibly emerges. In the scenario, a difference in nitrate production could be attributed to different capability of nitrite inhibition tolerance in different AnAOB [68]. Furthermore, once nitrite is overdosed, the ratio of nitrite to nitrate increases. Fermentative DNRA bacteria could benefit temporally from the increasing nitrite/nitrate. In turn, more fermentation products from fermentative DNRA bacteria could favor reduction of overdosed nitrite by denitrifiers. A strategy to alleviate low level of nitrite inhibition is proposed based on side population of anammox sludge, but need to be further verified. However, if DNRA bacteria is enhanced, there is a risk of deteriorating anammox process. Because when nitrite is overloaded, DNRA bacteria could outcompete AnAOB [49,67]. At influent NO_2^--N/NH_4^+-N of about 1.2, relative high concentration of ammonium (350 mg-N L^{-1}) is unfavorable for fermentative DNRA bacteria to reduce nitrite to ammonium [10], which limits SMP fermentation. Furthermore, the activity of denitrification would be negatively affected due to lack of carbon source. As a result, nitrate formation is possibly higher than the stoichiometric value.

3.2. Influence of temperature and pH on nitrate formation mechanism

AnAOB, denitrifiers and DNRA bacteria are sensitive to temperature variation. Their abundance and activity are positively correlated with temperature, when temperature ranges from 10 to 40 °C [69–72]. Based on the consistent temperature dependence, nitrate formation would be manipulated by AnAOB. This is because denitrifiers and DNRA bacteria survive on metabolic products of AnAOB (nitrate and endogenous organic). The optimum temperature range for most of AnAOB in wastewater treatment plants is between 30 and 37 °C, where common *Ca. Brocadia sinica, Ca. Kuenenia stuttgartienis* and *Ca. Jettenia caeni* could be well enriched. Furthermore, the optimal range temperature for *Ca. Kuenenia stuttgartienis* and *Ca. Brocadia sinica*, is 25–37 °C and 25–45 °C, respectively. Diverse AnAOB in the same temperature range and their different optimal temperature suggest that the existence of physiological distinctions between AnAOB, bringing about the difference in nitrate production.

When anammox process is operated in a lower temperature (10–20 °C), a series of metabolites including polysaccharides, vitamins and cofactors are reduced for preserving energy [73]. Furthermore, the cost of AnAOB adaption to low temperature is the reduction of EPS, disintegration of anammox sludge and decreasing growth rate [74–76]. And even some AnAOB are able to shift high metabolic demand to low energy investment (pentose phosphate pathway) for survival at low temperature [74]. Metabolism shifts and biosynthesis limitation are likely to affect nitrate formation. Meanwhile, a synergic relationship among AnAOB, denitrifers and DNAR bacteria has been uncoupled due to low production EPS of AnAOB. A simple conclusion could be drawn that low nitrate formation would occur due to low cell growth rate. In addition, NXR is the most key enzyme for nitrate production, but its abundance and activity pattern show contradictory results at decreased temperature. After a gradual decreasing temperature or cold shock, efficient NXR could be maintained in anammox sludge with dominated *Ca. Kuenenia* [73,77]. However, in the culture of *Ca. Brocadia fulgida*, NXR was downregulated after temperature reduction [73]. It is possible that the inconsistent NXR pattern could be attributed to different adaptive regimes of different AnAOB guilds.

The optimum pH for the growth and activity of denitrifiers is 7–8, and denitrification activity would fall off in out of the range [78,79]. Compared with dentrifiers, DNRA bacteria prefer to grow in mild alkaline environment, where nitrite reductase forming ammonium (NrfA) has an optimal of 8.0. Coincidentally, alkalinity production of anammox reaction supports the survive of dentrifiers and DNRA bacteria. Once pH is out of the optimal range, metabolic performance and community structure of anammox sludge would significantly be varied [80], resulting in nitrate formation disorder. Even so, stable anammox process still could be achieved in high pH of 9 or in low pH of 6.5 [81,82], where nitrate formation abnormal is possibly associated with dysfunction of AnAOB. This could be explained by the fact that AnAOB could be inhibited by high free ammonia or high free nitrous acid under inappropriate pH.

4. Influence of operating conditions on net nitrate formation

4.1. NLR

Substrate availability of AnAOB counts on NLR. Commonly, when AnAOB are enriched using conventional activated sludge, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) shows a decreasing pattern with stepwise NLR increase and its final value is always lower than or near to stoichiometric value, as shown in Table 2. Moreover, high performance of total nitrogen removal could be attributed to low nitrate formation due to endogenous denitrification [83]. While NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) is even lower than that in the related researches

on effects of exogenous biodegradable organic compounds on anammox process, endogenous denitrification should be not solely the contributor of low nitrate formation [84,85]. Inconsistent with the patterns, relatively stable nitrate formation with NLR increase indicates that nitrate overaccumulation may be eliminated by denitrifiers and DNRA bacteria [86]. Under no implementation of influent deoxygenation through inert gas flushing, the ratio of NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) published in Gutwinski et al. (2016) at steady state period was about 16.6 %, but the ratio of NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) published in Lu et al. (2018) was markedly reduced to about 4.5 %. The contradicting results indicate that the metabolism and function of fresh anammox sludge are not as excellent as mature anammox sludge. With stepwise NLR increase, nitrate formation kept relatively stable and NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) tends to be close to theoretical value when mature anammox sludge is inoculated [87,88]. Especially, as sharp gradient increase of NLR is performed, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) also showed a gradient increase. The increase in nitrate formation is associated with a good growth of AnAOB [51,82]. However, a persisting increase in NLR along with good nitrogen removal rate (NRR), leads to excessive EPS production, which is utilized by denitrifiers and DNRA bacteria. As a result, it is possible that NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) maybe have a decreasing tendency. In actual, though there are intensive researches on anammox process operation and the enrichment of AnAOB, comprehensive in-depth researches on effect of NLR on nitrate information are scarce. Because most of the above-mentioned experimental results are indirect evidences, final conclusions on this topic would be drawn in the further studies.

4.2. Substrate ratio

As known, the changes of FA and FNA are in line with ammonium and nitrite concentrations. At influent substrate ratio below 2, the variation patterns of NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) are inconsistent in different researches [89–91]. In order to minimize nitrate formation and maximize nitrogen removal, an appropriate influent substrate ratio within the range need to be selected. Notably, the optimized influent substrate ratio is possibly different from stoichiometric ratio, which could be attributed to the difference in operational conditions, the abundance of AnAOB and structure of microbial populations. At influent substrate ratio above 2, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) increases with the increasing influent NO_2^--N/NH_4^+-N ratio. And even nitrate overproduction could be aggravated when NO_2^--N/NH_4^+-N is further increased [92]. When NO_2^--N/NH_4^+-N increased from 2.5 to 4.5, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) increased significantly with a final peak value of 22.8 %, which was much higher than stoichiometric value [91]. It is unclear why AnAOB deal with high NO_2^--N/NH_4^+-N stress through nitrate overproduction and whether AnAOB are the solely participant in nitrate formation process. It is inevitable that high NO_2^--N/NH_4^+-N always brings about heavy nitrite inhibition on anammox process. A strategy of detoxifying the inhibition has been demonstrated by exogenous nitrate to alleviate nitrite toxicity [93]. Differently, a new hypothesis for self-detoxification of AnAOB is presented that anammox metabolism shifts towards nitrite oxidization to nitrate through NXR overexpression in the presence of high nitrite stress. Meanwhile, the abnormal increase in nitrate has been attributed to a response of AnAOB to relative high nitrite toxicity in recent researches [33,94,95]. For better understanding such behavior of AnAOB, it is needed to confirm the hypothesis in future studies.

4.3. Temperature

Temperature is regarded as the most important factor that substantially influences anammox activity and nitrogen removal efficiency. Below or above the optimal temperature, the related pattern of nitrate formation is different from that in the optimal temperature range (Table 3). When temperature is decreased from 30 °C to 20 °C in mainstream anammox process, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) gradiently decreases from 9.5 % to 4.6 % [96], indicating a synergic relationship among AnAOB, denitrifers and DNAR bacteria has been broken up. As temperature decreases below 20 °C, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) always shows an increasing pattern. However, two opposing mechanisms could be used to explain the increasing NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r). The increasing NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) with the decreasing temperature is close to or higher than stoichiometric value, indicating that AnAOB have well adapted to cold temperature [97–99]. The result is also supported by the fact that relatively high activity and growth rate of AnAOB could be detected. Besides, denitrifiers or DNRA bacteria coexisting with AnAOB are suppressed due to low temperature, which has been observed in activated sludge and partial denitrification-anammox process [69,100,101]. The potential reason is confirmed by an recent finding that nitrate reductase (Nar) expression does not markedly decrease at low temperature, while the activities of denitrification enzymes belonging to heterotrophs is reduced [73].

4.4. pH

The optimal pH for the activity and growth of AnAOB is inconsistent and ranges from 6.5 to 8.3 [71,102,103]. From the perspective of operation stability, a pH range of 7–8 is suggested to avoid substrate self-inhibition of free ammonia (FA), free nitrous acid (FNA) and nitrite [71]. Influence of pH on anammox process has been well summarized in recent reviews [71,104]. However, special researches on influence of pH on nitrate formation are still not conducted. In a study of evaluating importance of maintaining pH to stable anammox operation, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) at pH of 6.5 was always higher than that at pH of 7.5–8.1, but a better NRR was achieved at the acid pH [82]. The results show that maintaining low FA is critical for enhancing nitrogen removal than the control of FNA or nitrite, and these reactants (FA, FNA and nitrite) are connected with nitrate formation.

5. Rethinking current strategies of nitrate removal from perspective of native functional microorganisms

After achieving stable nitrogen removal through anammox process, further nitrate removal is key to enhance total nitrogen removal

Table 2
The researches published on the startup of anammox process. SBR - sequencing batch reactor; MBR - membrane bioreactor; FBR - fixed bed reactor; UASB - up-flow anaerobic sludge blanket; UBF - upflow biofilter; CSTR - continuous stirred-tank reactor.

Reactorconfiguration	Seedsludge	Wastewatertype	Temperature(°C)	pH	Duringofoperation(d)	Influent$NH_4^+ - N/NO_2^- - N(g - NL^{-1})$	Influent$NO_2^- - N/NH_4^+ - N$ratio	NLR($kg - Nm^{-3}d^{-1}$)	MaximumNRR($kg - Nm^{-3}d^{-1}$)	Totalnitrogenremoval(%)	Enrichedanammoxbacteria(Purity)	Remarkorpatternofnitrateformation	Reference
SBR	Mixed sludge from leachate and urban treatment plants[a]	Synthetic	36.0 ± 0.3	7.2–8.7	365	14.9–1268.0/ 9.6–1661.4	0.76–1.32	0.01–1.60	N.A.	N.A.	*Candidatus Brocadia anammoxidans* (85.0 ± 1.8 %)	With stepwise increase of NLR, nitrogen removal performance went up due to low nitrate formation	[110]
Up-flow column reactor with biofilm carrier material	Mixed sludge from aerobic, anoxic and anaerobic tanks	Synthetic	32 ± 2	7.1–7.8	About 160	35-112/35-112	N.A.	0.2–0.22	N.A.	91.0 %	N.A.	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) was markedly lower than the stoichiometric value.	[84]
MBR	Mixed aerobic activated sludge with nitrifying sludge	Synthetic	35	7.8–8.2	60	50-75/50-85	1–1.13	0.05–0.08	0.072	Over 90%[b]	N.A.	With stepwise increase of NLR, nitrogen removal performance went up due to low nitrate formation	[111]
MBR	Mixed aerobic activated sludge with digested sludge	Synthetic	32–35	7.5–8	277	37-152/39-134	0.88–1.05	0.063 ± 0.0074	N.A.	75 ± 8.51	N.A.	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) (16.6 %) was higher than the stoichiometric one (11.2 %).	[112]
SBR having polyurethane foam impregnated with activated carbon	Mixed sludge aerobic activated sludge and anaerobic sludge	Synthetic	37	7–8	212	35-200/ 45.5–264	1–1.32	0.016–0.15	0.133	85 %	*Candidatus Jettenia* (6.5 %)	Relatively stable effluent nitrate concentration with NLR increase.	[86]
UASB	Anaerobic granular sludge	Synthetic	35 ± 1	7–9	164	70-229/70-252	1–1.26	0.3–4.4	4.1	Above 95 %**	N.A.	With stepwise increase of NLR, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N) gradually decreased.	[113]
UBF with hollow bamboo balls	Activated sludge	Synthetic	35 ± 1	7	About 440	30-976/50-1280	1.2–1.4	0.21–33.9	N.A.	Above 98.1 %	*Candidatus Brocadia anammoxidans*	With stepwise increase of NLR, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) close to stoichiometric value kept stable.	[114]
UASB	Granular anammox sludge	Synthetic	35 ± 1	6.8–7.0	About 440	330-420/360	0.9–1.1	2.4–99	78.5	N.A.	N.A.	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) near to stoichiometric value.	[87]
CSTR	Granular anammox sludge	Synthetic	33 ± 1	N.A.	120	70-150/90-150	1–1.3	0.49–2.78	N.A.	N.A.	*Candidatus Brocadia caroliniensis*	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) near to theoretical value in spite of Variable NLR	[88]

a Mixed sludge has presented low anammox activity in previous cultivation.
b Nitrogen removal represented efficiencies of ammonia and nitrite.

Table 3
The researches published on anammox process performed at below optimal temperature. SBR - sequencing batch reactor; MBR - membrane bioreactor; up-flow anaerobic sludge blanket; CSTR - continuous stirred-tank reactor; CABR – continuous anammox bio-carrier reactor.

Reactor configuration	Seed sludge	Wastewater type	Oxygen elimination	Temperature	pH	During of operation (d)	Influent NH_4^+-N/ NO_2^--N (g-N L^{-1})	Influent NO_2^--N/ NH_4^+-N ratio	Nitrogen load rate (kg-N m^{-3} d^{-1})	Maximum NRR (kg-N m^{-3} d^{-1})	Total nitrogen removal (%)	Enriched anammox bacteria (Purity)	Remark or pattern of nitrate formation	Reference
SBR	Granular anammox sludge	Synthetic	No Oxygen elimination	Temperature shock of 35–46 °C in 8 days	7.5–8.5	About 170	N.A.	1.3 ± 0.1	01.-0.5	N.A.	N.A.	*Candidatus Brocadia anammoxidans* (44 %)	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) showed higher values than stoichiometric value after temperature shock.	[115]
MBR	Floc anammox sludge	Synthetic	Strict oxygen-free environments	Long term operation at 30 °C, 25 °C and 20 °C	7	About 1030	840/840	1	0.071–0.51	N.A.	N.A.	*Candidatus brocadia fulgida*	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) was at 30 °C, 25 °C and 20 °C 9.5 %, 7.0 % and 4.6 %, respectively.	[96]
SBR	Floc anammox sludge	Pretreated Or Synthetic	Strict oxygen-free environments	Progressive temperature decrease from 29 °C to 12.5 °C	7.3 ± 0.2	About 180	65/65	1	N.A.	N.A.	N.A.	N.A.	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) was 21 ± 26 %.	[76]
UASB	Floc anammox sludge	Real	No Oxygen elimination	Stepwise temperature decrease from 30 °C to 16 °C	N.A.	200	16.87 ± 2.09/ 20.57 ± 2.31	1.2	0.57–5.72	5.13	N.A.	N.A.	With decreasing temperature, NO_3^--N_p/ (NO_2^--N_r + NH_4^+-N_r) higher than stoichiometric value was increased	[116]
CSTR with gel carrier	Mature anammox sludge	Synthetic	Strict oxygen-free environments	Stepwise temperature decrease from 32 °C to 6.3 °C	7.2[a]	210	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	The nitrate production rate increased with the increasing temperature, reaching maxima at 37 °C, and then declining	[117]
Gas-lift reactor	Activated sludge	Synthetic and real	Strict oxygen-free environments	10 °C	7.5	722	30/30	1	N.A.	0.027	N.A.	*Candidatus Brocadia fulgida*	After enrichment of anammox bacteria, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) was significantly higher than stoichiometric value.	[99]
CABR	Suspended anammox sludge	Synthetic	Strict oxygen-free environments	Stepwise temperature decrease from 35 °C to 18 °C	7.4	125	25-83/30-100	1.2	0.2–0.4	0.3 ± 0.03	N.A.	*Candidatus jettenia* (10 %)	With decreasing temperature, NO_3^--N_p/ (NO_2^--N_r + NH_4^+-N_r) slightly higher than stoichiometric value was decreased.	[118]

(*continued on next page*)

Table 3 (*continued*)

Reactor configuration	Seed sludge	Wastewater type	Oxygen elimination	Temperature	pH	During of operation (d)	Influent NH_4^+-N/NO_2^--N (g-N L^{-1})	Influent NO_2^--N/NH_4^+-N ratio	Nitrogen load rate (kg-N m^{-3} d^{-1})	Maximum NRR (kg-N m^{-3} d^{-1})	Total nitrogen removal (%)	Enriched anammox bacteria (Purity)	Remark or pattern of nitrate formation	Reference
SBR	Granular anammox sludge	Synthetic	No Oxygen elimination	A gradient of decreasing temperature from 33 °C to 10 °C	7.0[a]	361	40-147/50/196		0.30–0.52	0.45	N.A.	*Candidatus Kuenenia* (5 %)	With decreasing temperature, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) decreased, reaching the minimum at 25 °C, and then increased.	[66]
UASB	Granular anammox sludge	Synthetic	No Oxygen elimination	A gradient of decreasing temperature from 30 °C to 5 °C		250	100/132	132	2.97 ± 0.13	N.A.	N.A.	*Candidatus Kuenenia* (25.48 %)	With decreasing temperature, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) increased from 0.203 ± 0.025 % (30 °C) to 0.269 ± 0.035 % (5 °C)	[97]
Gaslift reactor	Granular anammox sludge	Synthetic and real	Strict oxygen-free environments	20.0 ± 0.2 °C	7.5–8.2	253	30-40/30	0.75–1	0.31	0.26	N.A.	N.A.	NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) showed a slow increasing pattern and finally reached stoichiometric value.	[98]
Up-flow granular bed anammox reactor	Granular anammox sludge	Synthetic	No Oxygen elimination	A gradient of decreasing temperature from 35 °C to 15 °C	7.70	160	35-71/45.5–75	1.1–1.3	3.5–4.0	2.94	N.A.	*Candidatus Brocadia fulgida* (8.9 %)	With decreasing temperature, NO_3^--N_p/(NO_2^--N_r + NH_4^+-N_r) increased from 8.4 % to 28.3 %.	[73]

a. Influent pH was controlled to a constant value.
b. Nitrate production rate was determined by batch experiment.

efficiency. Based on organic and inorganic electron donor for nitrate reduction, the novel strategy of nitrate removal in anammox process could be categorized into: anammox process coupled with heterotrophic denitrification and anammox process coupled with autotrophic denitrification. The former consists of partial denitrification and anammox process (PD/A) and anammox coupled DNRA process (Fig. 3). The latter consists of sulfur-based or iron-based autotrophic denitrification and anammox process. Apparently, PD/A and anammox coupled DNRA process could be regarded as "bioaugmentation" of nitrate reduction during anammox process. Native denitrifiers, fermentative DNRA bacteria and DNRA pathway of AnAOB in anammox sludge could be used to startup the processes. On the other hand, effective anammox function is of first priority in the anammox-based processes. Thus, the synergic relationship of nitrate formation among AnAOB, denitrifers and DNAR bacteria should not be weakened in the anammox-based processes, but maintained or intensified.

Currently, PD/A has been developed as a promising alternate for partial nitrification/anammox (PN/A). In PD/A, NO_3^--N is reduced to NO_2^--N through partial denitrification, and then NH_4^+-N is oxidized using NO_2^--N by anammox process (Fig. 3(a)). Due to low investment and operation cost, one-stage PD/A seems to be more attractive than two-stage PD/A. The efficient and practical controlling strategies for long-term stable nitrite production via PD is still challenging when treating real municipal wastewater. The type of electron donor plays a crucial role in nitrite accumulation. The variety of common organic carbon sources including acetate, glucose, methanol and ethanol have been evaluated for the buildup of nitrite accumulation. A consistent conclusion could be drawn that acetate-driven partial denitrification display the most efficient performance among them [7]. However, in one-stage PD/A, non-fermentative acetate does not support fermentative DNRA bacteria, which would alter substantially anammox communities. Different in anammox process, nitrate formation only depends on AnAOB and denitrifiers in PD/A. Consequently, abundance of AnAOB is low than 1 %, while dentrifiers is in bloom [101], which intensify the competition between AnAOB and denitrifiers. The system is likely to be the brink of collapse. Instead of acetate, fermentable carbon source (glucose) could encourage the growth of fermentative *Anaerolineaceae* and *Ignavibacteria* over denitrifiers [105]. Another is addition of fermentation sludge to PD/A to enhance the hydrolyzation and fermentation of slowly biodegradable organics and offer organic acid for PD [106]. The strategy could be regarded as functional complement of fermentative DNRA bacteria. Moreover, the cooperation among AnAOB, denitrifers and DNAR bacteria could be sustained by adding fermentable carbon source or fermentation sludge.

As for anammox coupled DNRA process, nitrate is partly reduced to the end-product of ammonium by DNRA and partly to nitrite by partial DNRA (Fig. 3(b)), which are implemented by DNRA bacteria. Native fermentative DNRA bacteria should be enhanced with exogenous or endogenous organic carbon. The selected exogenous organic carbon could differentiate between DNRA bacteria and denitrifiers, where partial DNRA and complete denitrification are established. Another aspect is that DNRA pathway of AnAOB could be started by exogenous organic carbon, endogenous organic carbon and iron with different valence states (Fig. 3(c)) [34–36]. The strategies focus on exploring the metabolic versatility of AnAOB, and minimize the ill-effects on other functional microorganisms. Thus, anammox process coupled DNRA process should be recommended for addressing residual nitrate of anammox process.

6. Future perspectives

Even though comprehensive and in-depth researches on anammox process have been carried out, nitrate byproduct of anammox is commonly neglected. The current researches on the topic almost focus on nitrate removal process. We have highlighted that there are a

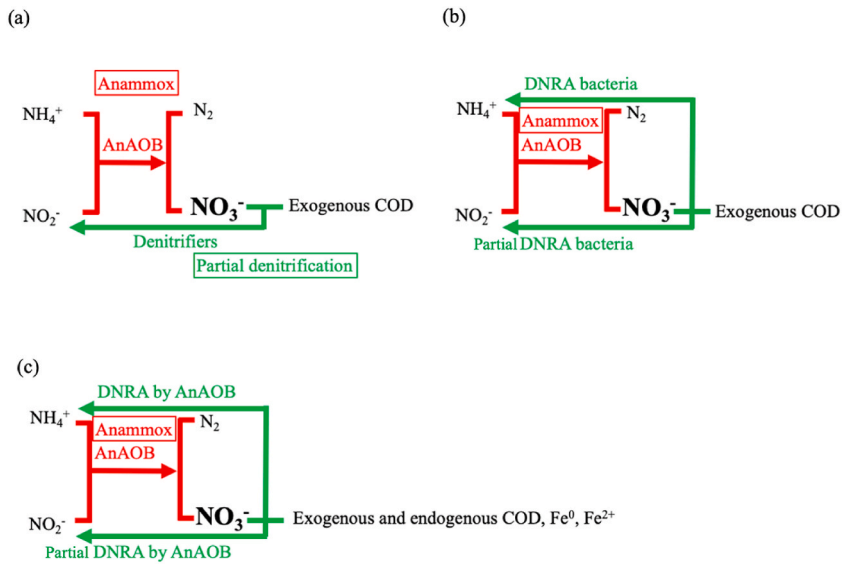

Fig. 3. Coupling of biological nitrate removal process with anammox process. (a) Partial denitrification and anammox process (PD/A); (b) Anammox process coupled with DNRA bacteria; (c) Anammox process coupled with DNRA pathway of AnAOB; AnAOB, anammox bacteria; DNRA, dissimilatory nitrate reduction to ammonium; Partial DNRA, dissimilatory nitrate reduction to nitrite.

lot of unknows and questions about nitrate formation mechanism. The key knowledge gaps are summarized for future research as follows:

- Clarification on how nitrate is produced or reduced by AnAOB, DNRA bacteria, and denitrifers is critical. AnAOB could be well enriched, but not purified until now. Although functional AnAOB in anammox sludge have been well identified, metabolic characteristics, spatial distribution and core community compositions of DNRA bacteria and denitrifers remain pooly understood. High-throughput molecular biology approaches, such as metagenomics and metatranscriptomics, can provide the information on community compositions, biochemistry and physiology. Furthermore, complete nitrate metabolic pathways between AnAOB, DNRA bacteria, and denitrifers could be mapped.
- Nitrate formation is regarded as an indicator of anammox growth. In different phases of enrichment, enhancement and maturation of anammox sludge, whether net nitrate formation is equivalent to better or worse anammox growth is still to be investigated. AnAOB are sensitive to heavy metals, salinity and nitrite involved in industrial wastewaters. Special attention to the factors influencing nitrate formation should be given. Unlike stable industrial wastewater quality, nitrate formation under the mainstream conditions (such as, wastewater quality and quantity fluctuations, low temperature and low NLR) would be further checked. Especially, whether deficient substrate and overdosed nitrite are potential contributors to nitrate overproduction still needs to be investigated.
- Variation patterns of net nitrate formation need to be further check. In the published anammox process researches, anammox activity is commonly monitored, but inherent denitrifying and DNRA activities are overlooked. It is difficult to explain nitrate formation patterns under without consideration of them. Thus, it is suggested that dynamic activity and population profiles of AnAOB, denitrifiers and DNRA bacteria during anammox process should be characterized for a better understanding of net nitrate formation.

7. Conclusions

In this review, mechanism, characteristics, operating conditions of nitrate formation in anammox process and its recent removal technologies have been critically summaries and analyzed. Nitrate formation is regulated by AnAOB, denitrifiers and DNRA bacteria, which are responsible for nitrate production and reduction, respectively. The discrepancy in physiological level and operational parameters (NLR, temperature, pH and substrate ratio) results in variable net nitrate formation. Especially, the cause of abnormal nitrate overproduction needs to be carefully traced. The strategies of nitrate removal from anammox process should not reverse microbial mechanism of nitrate formation, but maintain inherent community structure of anammox sludge.

CRediT authorship contribution statement

Heng Yu: Writing – original draft, Data curation. **Yue Dong:** Validation, Investigation. **Sike Wang:** Writing – review & editing, Funding acquisition. **Weiyi Jia:** Methodology, Investigation. **Yating Wang:** Investigation, Data curation. **Jiane Zuo:** Funding acquisition, Conceptualization. **Chengtun Qu:** Resources, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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