Review of characteristics of anammox bacteria and strategies for anammox start-up for sustainable wastewater resource management

Guangxue Wu, Tianqi Zhang, Mengqi Gu, Zhuo Chen and Qidong Yin

ABSTRACT

Wastewater management has experienced different stages, including pollutant removal, resource recovery, and water nexus. Within these stages, anaerobic ammonia oxidation-based biotechnology can be incorporated for nitrogen removal, which can help achieve sustainable wastewater management, such as reclamation and ecologization of wastewater. Here, the physiology, metabolism, reaction kinetics and microbial interactions of anammox bacteria are discussed, and strategies to start-up the anammox system are presented. Anammox bacteria are slow growers with a high doubling time and a low reaction rate. Although most anammox bacteria grow autotrophically, some types can grow mixotrophically. The reaction stoichiometric coefficients can be affected by loading rates and other biological reactions. Microbial interactions also contribute to enhanced biological nitrogen removal and promote activities of anammox bacteria. The start-up of the anammox process is the key aspect for its practical application, which can be realized through seed selection, system stimulation, and biomass concentration enhancement.

Key words | anammox, microbial interaction, microbial kinetics, start-up, sustainable wastewater management

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HIGHLIGHTS

- Sustainable wastewater management was proposed.
- Anammox process is the key for nitrogen removal from wastewater.
- High growth rate anammox bacteria could be selectively enriched.
- Microbial ecology of anammox bacteria was comprehensively summarized.
- Strategies for starting-up of the anammox process were concluded.

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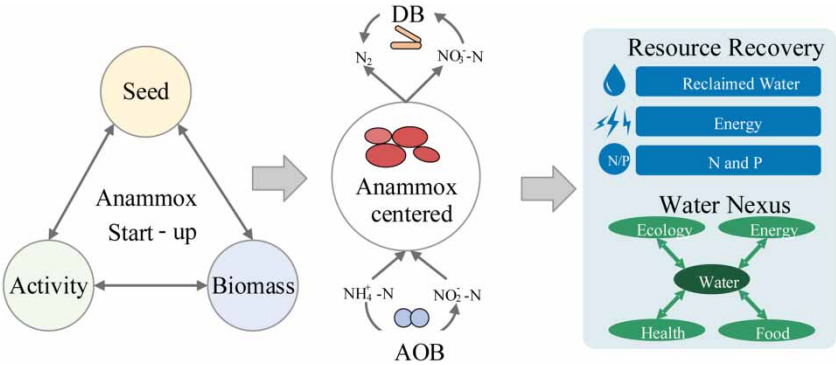
GRAPHICAL ABSTRACT



INTRODUCTION

Proper management of wastewater is vital for protecting our living conditions, which can support the sustainable development of our society and environment. Previously, three stages of wastewater management have been implemented: pollutant removal from wastewater, resource recovery from wastewater and water nexus (Figure 1) (Mo & Zhang 2013; Leck *et al.* 2015; Grasso 2019). Conventionally, pollutant removal from wastewater is the focus of wastewater treatment to meet the discharging standard, which can benefit the receiving water bodies. Wastewater contains pure water, organic carbon, nitrogen, phosphorus, and other elements. Currently, the concept of resource recovery, energy recovery, as well as water reclamation/ecologization has become the new trend in wastewater management. Through the application of this new concept, organic carbon in wastewater can be concentrated indirectly or converted directly by the anaerobic methanogenesis process to recover methane as renewable energy (Yin & Wu 2019). The phosphorus in wastewater can be recovered through precipitation as fertilizer, while nitrogen is mainly removed as a pollutant. Following compound recovery or removal, the water quality is significantly improved, and can be reclaimed for industrial and environmental applications. Furthermore, the wasted water and the treated water can be incorporated into the concept of nexus with the environment, ecology, energy, food, and other areas (Mo & Zhang 2013; Leck *et al.* 2015). To achieve the proposed purposes, green technology should be applied for nutrient removal or recovery, which can contribute to the sustainable management of wasted water.

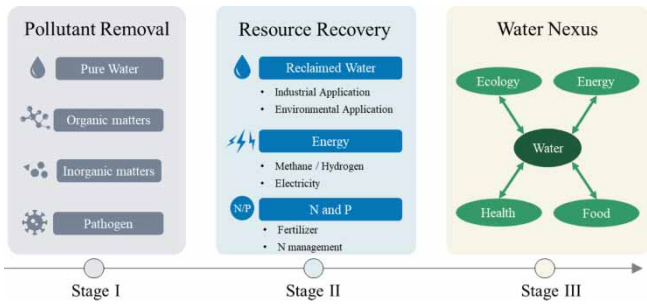


Figure 1 | Development stages for the wastewater management.

For nitrogen management in wastewater, conventional biological nitrogen removal is mainly achieved through full nitrification and denitrification processes (Figure 2) (Daims *et al.* 2006). Generally, for full nitrification, ammonia nitrogen (NH_4-N) in wastewater is oxidized to nitrite nitrogen (NO_2-N) by ammonia-oxidizing bacteria (AOB) and then to nitrate nitrogen (NO_3-N) by nitrite oxidizing bacteria (NOB) under aerobic conditions, with oxygen as the electron acceptor. Subsequently, NO_3-N is denitrified to nitrogen gas (N_2) through activities of denitrifiers with organic carbon as the electron donor. However, with the new concept of energy recovery, after organic carbon recovery from wastewater such as through anaerobic digestion, there is no adequate organic carbon for conventional denitrification. Therefore, the autotrophic nitrogen removal process is becoming a promising technology for energy neutral or even energy positive results in wastewater treatment (Wang *et al.* 2015). Autotrophic nitrogen removal is mainly achieved through partial nitrification and anaerobic ammonia oxidation (anammox) processes. For partial nitrification (or nitritation), only the first step carried out by AOB would occur, and the

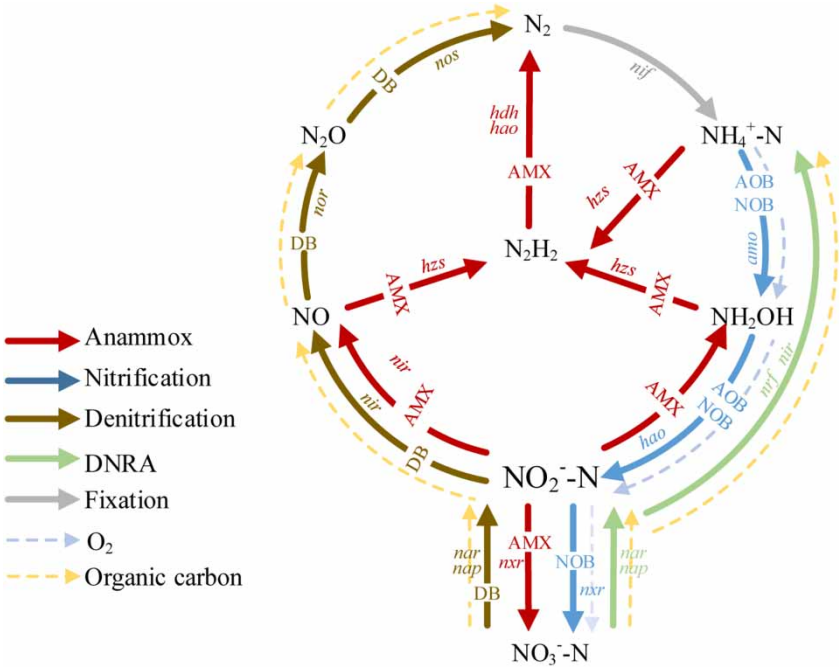


Figure 2 | Nitrogen metabolism of anammox bacteria (AMX), AOB, NOB and denitrifiers (DB).

second step by NOB would be inhibited. After nitritation, in the following anammox process, NH_4-N reacts with the produced NO_2-N, and both are converted to N_2.

The anammox process is carried out by anammox bacteria (Kartal *et al.* 2007a, 2007b), which are slow growers. Therefore, the key aspect for the anammox process is to enrich anammox bacteria. Lots of reviews have been conducted to discuss the physiology of anammox bacteria, the history, and technologies to start the process (i.e., Oshiki *et al.* 2011). However, till now, no reviews have been focusing on the systematic summary of the microbial characteristics of physiology, metabolism, kinetics and interactions, and strategies for start-up of the anammox process based on the microbial characteristics. In this review, the key clue for enhancing the growth of anammox bacteria based on microbiological characteristics and also strategies for enhancing system start-up will be focused on, which could provide a framework for practical application of the anammox process with the better management of wastewater resources.

ANAMMOX BACTERIA AND THEIR PHYSIOLOGY

Initially, the existence of anammox bacteria for removing ammonia with nitrite or nitrate as the electron acceptor was proposed by Broda (1977) based on the thermodynamic analysis. Later, the anammox phenomenon was discovered in a denitrifying bioreactor by Mulder *et al.* (1995). Thereafter, much research was conducted and practical applications were tested. After 3.5 years of project application, the first full-scale project was established for treating reject waters of anaerobic sludge digestion in Rotterdam (van der Star *et al.* 2007). Currently, more than 200 anammox facilities have been put into operation worldwide (Cao *et al.* 2017).

Six genera of anammox bacteria within the phylum Planctomycetes have been confirmed, including *Candidatus* Kuenenia, *Ca.* Brocadia, *Ca.* Anammoxoglobus, *Ca.* Anammoximicrobium, *Ca.* Jettenia, and *Ca.* Scalindua. Among these, the first five types are commonly found in wastewater treatment and freshwater systems, while the last one is commonly found in saline environments such as sea water and sediments (Kartal *et al.* 2013; Ali *et al.* 2015a; Guo *et al.* 2016; Lawson *et al.* 2017). Physiology and environmental factors affect the distribution of types of anammox bacteria significantly. For example, under low nitrogen loading rate (NLR) conditions, *Ca.* Brocadia anammoxidans, *Ca.* Jettenia, *Ca.* Anammoxoglobus, and *Ca.* Kuenenia are dominant (Li *et al.* 2017; Reino *et al.* 2018; Zhu *et al.* 2018), while under high NLR conditions, *Ca.* Brocadia sinica and *Ca.* Kuenenia stuttgartiensis dominate (Cho *et al.* 2018; Yang *et al.* 2018b). Furthermore, *Ca.* Brocadia and *Ca.* Brocadia fulgida are mainly observed at 6–15 °C (Hendrickx *et al.* 2014; Awata *et al.* 2015; Laureni *et al.* 2015; Lotti *et al.* 2015b), while *K. stuttgartiensis* was found at 25–45 °C (Isaka *et al.* 2008).

Although few anammox bacteria have been successfully isolated, they usually grow in the form of highly compact spheres, with diameters ranging from 0.6 to 1.0 μm (van Niftrik *et al.* 2004; Duan *et al.* 2012). So far, most studies have focused on the enriched anammox bacteria within biological reactors. Various proportions of anammox bacteria have been reported from different studies with varied reactor configurations, operational conditions and growth conditions (Ni *et al.* 2010). The purities of enriched anammox bacteria were 64 and 74% in the fluidized bed reactor (van de Graaf *et al.* 1997) and the sequencing batch reactor (SBR) (Strous *et al.* 1998), respectively. Anammox enrichment purity of 97.6% was achieved in a membrane bioreactor (MBR) inoculated with 60–80% purity granular anammox from the first full-scale anammox reactor (van der Star *et al.* 2008). Using cultured activated sludge with less than 10% anammox purity as the seed, the purity of enriched anammox bacteria could be up to 97.7% (van der Star *et al.* 2008).

NUTRIENT METABOLISM OF ANAMMOX BACTERIA

Nitrogen biotransformation

Generally, there are two types of nitrogen metabolic pathways for anammox bacteria. In anammox pathway I, NO_2-N is reduced to hydroxylamine and then combines with NH_4-N to form hydrazine, which is found in *Ca.* Brocadia anammoxidans (van de Graaf *et al.* 1997), while in anammox pathway II, NO_2-N is reduced to nitric oxide (NO) and then combines with NH_4-N to form hydrazine, which is found in *K. stuttgartiensis* (Strous *et al.* 2006). For the clarification of the metabolic pathways, the production of NO can be confirmed by using the NO scavenger PTIO (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide). For genes responsible for the nitrite reduction, neither nitrite reductases *nirS* nor *nirK* were detected in the *B. fulgida* and *B. sinica*; *nirS* encoding cytochrome *cd1*-type NO-forming nitrite reductase was observed in *K. stuttgartiensis* and *Ca. Scalindua profunda*, and *nirK* encoding copper-containing NO-forming nitrite reductase was found in *Ca. Jettenia caeni* (Gori *et al.* 2011; Oshiki *et al.* 2015).

CO_2 fixation

Anammox bacteria are autotrophic microorganisms, which use inorganic carbon such as CO_2 as the carbon source. There are various identified CO_2 fixation pathways, such as the pentose phosphate cycle (Calvin cycle), reductive acetyl coenzyme A pathway (acetyl-CoA), reductive tricarboxylic acid cycle, 3-hydroxypropionate bicycle, and 4-hydroxybutyrate cycles. Most anammox species, including *K. stuttgartiensis*, *Ca. Jettenia asiatica*, *B. fulgida*, and *Ca. Scalindua profunda*, possess the Wood-Ljungdahl pathway (also the reductive acetyl-CoA pathway) (Gori *et al.* 2011; Hu *et al.* 2012). In the anammox process, it is proposed that some nitrite is oxidized to nitrate to generate the electrons for CO_2 fixation, and the nitrite oxidation might be catalyzed by a nitrate oxidoreductase (NarGH) in *K. stuttgartiensis* (Strous *et al.* 2006). For the acetyl-CoA pathway, it should occur by electron transfer at very low redox potentials for NAD^+ reduction (−0.32 V), CO_2 reduction to formate (−0.44 V), and acetyl-CoA synthesis (−0.5 V). Therefore, the Wood-Ljungdahl pathway requires high energy input. Strous *et al.* (2006) proposed that the high reducing power electrons derived from the hydrazine oxidation to N_2 ($E_0' = -0.75$ V) could be channeled towards NAD^+ and CO_2 reduction to sustain the carbon fixation.

Organic carbon utilization

Because anammox bacteria are autotrophic, the presence of organic carbon can inhibit their growth. However, anammox species *Ca.* Ananimoxoglobus propionicus, *K. stuttgartiensis*, and *B. fulgida* are all shown to be able to co-metabolize fatty acids (Guven *et al.* 2005; Strous *et al.* 2006; Kartal *et al.* 2007b, 2008). Huang *et al.* (2014) found that *Ca.* Jettenia asiatica could grow at low acetate (≤120 mg/L)/propionate (≤200 mg/L) concentrations, while the acetate concentration of no more than 240 mg/L caused the decrease in ammonium consumption rate by 33% and by 29% for propionate with <400 mg/L. In addition, Kangwannarakul *et al.* (2018) found that the short term addition of 0.25 and 0.5 mM acetate did not affect the anammox activity, while the long term addition of 0.25 mM acetate could decrease the anammox activity.

Acetate could be activated by an acetyl-CoA synthetase-like protein (kustc1128) in a heterologous host, as well as whole cells of *K. stuttgartiensis* (Russ *et al.* 2012), which might lead to direct incorporation of acetate into cell biomass by anammox bacteria. However, based on $\delta^{13}C$ values of lipids and substrates, it was proposed that acetate might not be directly incorporated into the biomass, but would first degrade into CO_2 and then be fixed via the acetyl-CoA pathway (Kartal *et al.* 2008). By nanometer-scale secondary ion mass spectrometry (NanoSIMS) scanning, Tao *et al.* (2019) revealed that the enriched *J. asiatica* could utilize acetate

and propionate at a >10 times higher efficiency than bicarbonate incorporation, and acetate and propionate were likely not assimilated directly, but were first oxidized to CO_2 for the follow-up autotrophy. Both *B. sinica* and *J. caeni* possess AMP-Acs and ADP-Acs, which can both catalyze acetate into acetyl-CoA via different mechanisms. ADP-Acs catalyzes the synthesis of acetyl-CoA from acetate in a single step, while AMP-Acs synthesizes it in two steps (Starai & Escalante-Semerena 2004). The AMP-Acs route is a high affinity pathway, and the reaction occurred at a low acetate concentration (Krivoruchko *et al.* 2015). Russ *et al.* (2012) found that an AMP forming acetyl-CoA synthetase gene (*acs*) of *K. stuttgartiensis* could be functionally expressed to convert acetate to acetyl-CoA in *Escherichia coli*.

The utilization of organic carbon can benefit the growth of some anammox bacteria. For instance, *B. fulgida* cells had a high metabolic capability to oxidize acetate (Kartal *et al.* 2008), and they could outcompete other coexisting anammox bacteria with the addition of acetate, NH_4, NO_2, and NO_3 (Winkler *et al.* 2012; Jenni *et al.* 2014). The acetate addition could trigger the conversion of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) in *Ca.* Brocadia (Feng *et al.* 2018). In addition, the biomass of *B. fulgida* showed an increase under certain organic carbon to nitrogen (C/N) ratios (Jenni *et al.* 2014). On the contrary, *Ca.* Jettenia asiatica showed no superiority in growth under mixotrophic conditions (Huang *et al.* 2014).

Furthermore, organic carbon can be utilized as the electron donor for nitrate reduction in anammox bacteria, which is called the dissimilatory nitrate reduction to ammonium (DNRA) (Winkler *et al.* 2012). *A. propionicus* and *B. fulgida* were confirmed to oxidize propionate and acetate in NO_3-N reduction, and dominated in ecosystems where propionate or acetate are constantly available (Kartal *et al.* 2007b, 2008). Under low C/N ratios, anammox bacteria, such as *B. sinica*, could be promoted or even co-cultured with heterotrophs, which was due to the high rate of partial DNRA (Shu *et al.* 2016; Castro-Barros *et al.* 2017).

MICROBIAL KINETICS AND INTERACTIONS

Stoichiometric coefficients

Biological stoichiometry is very important for system design and optimization. Specifically, during anammox, the ratio of ammonia to nitrite is very important for system control and nitrogen removal. The anammox stoichiometry (Equation (1)) described by Strous *et al.* (1998) has been widely applied, and was revised (Equation (2)) by Lotti *et al.* (2014a). The stoichiometric equations were summarized by Guo *et al.* (2020), indicating that the loading rate could affect the equation coefficients.

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} + 2.002H_2O \quad (2)$$

Theoretically, under steady-state conditions, approximately 11% of the nitrogen load is converted to nitrate. Kowalski *et al.* (2019b) found that as much as 60% of the nitrogen load could be oxidized to nitrate in a non-aerated and lid-covered reactor, indicating that some nitrate would be produced by a shift in the anammox metabolism towards NO_3. The constitutive expression of nitrite oxidoreductase (NXR) in anammox bacteria might enable them to use NO_2 immediately when the energy source becomes available, causing the increased production of nitrate under anoxic conditions. In addition, it should be mentioned that NXR is not limited to obligate NOB (sNOB), but is also found in physiologically diverse bacteria (dNOB), and sNOB and dNOB might both contribute to the excess nitrate production in anammox systems (Sorokin *et al.* 2012; Daims *et al.* 2016; Li *et al.* 2020).

In addition, production of microbial composition such as extracellular polymeric substances (EPS) and soluble microbial products (SMP) requires investment of energy derived from the substrate utilization (Oshiki *et al.* 2011). The planktonic *B. sinica* cells could produce 109 μg extracellular protein/mg volatile suspended solids (VSS) and 29 μg extracellular carbohydrate/mg VSS at the stationary growth phase, which were higher than the production found during the exponential growth (Zhang *et al.* 2017b). Zhang *et al.* (2017b) found that the allocation of energy to the EPS production may hinder the planktonic anammox bacteria from growing at the maximum rate, whereas immobilized cells could use most of the energy for cellular growth.

Reaction kinetics

The doubling time of anammox bacteria directly affects their growth and the start-up of the anammox system (Ali & Okabe 2015). Anammox bacteria grow slowly, with typical doubling times ranging from one to several weeks (van der

Star *et al.* 2008; Lotti *et al.* 2014a). Zhang *et al.* (2017a) obtained the result showing that when free-living planktonic *B. sinica* and *J. caeni* cells were immobilized in polyvinyl alcohol and sodium alginate gel beads and cultivated in an up-flow column reactor with high substrate loading rates at 37 °C, the μ_{max} was determined to be 0.33 1/d and 0.18 1/d (corresponding the doubling time of 2.1 d and 3.9 d), respectively. For specific types of anammox bacteria, the doubling times of genus *Brocadia* varied from 1.6 to 7 d (Isaka *et al.* 2006; Tsushima *et al.* 2007a; Bae *et al.* 2010; Oshiki *et al.* 2011; Lotti *et al.* 2014b; Chi *et al.* 2018), depending on growing conditions (temperature and ammonium levels). However, other anammox bacteria might have high doubling times, such as *Ca.* Jettenia at 14.4 d, *Ca.* Scalindua at 14.4 d, and *Ca.* Anammoximicrobium at 32.1 d (Awata *et al.* 2013; Khramenkov *et al.* 2013; Ali *et al.* 2015a). Therefore, enrichment of *Ca.* Brocadia may result in a quick start-up of the anammox process. On the one hand, by applying a low sludge retention time (SRT), anammox bacteria with a low doubling time may be enriched. However, due to the relatively slow growth rates of the anammox bacteria and low biomass yield, maintaining a high SRT is important for system performance (Trigo *et al.* 2006; Chamchoi & Nitisoravut 2007). Therefore, cascade SRTs could be adopted, with initial low SRTs for selecting anammox bacteria with high growth rates and then high SRTs for biomass concentration enhancement (Miao *et al.* 2018).

The half-saturation constant (*Ks*) value for nitrite was 0.48 ± 0.29 mg N/L for *B. sinica*, 0.50 ± 0.013 mg N/L for *J. caeni*, and 0.0063 mg N/L for *Ca. Scalindua* sp. (Oshiki *et al.* 2016). Additionally, the inhibition constant (*Ki*) value for nitrite was <224 mg N/L for *Ca.* B. sinica, 154 mg N/L for *J. caeni*, and 105 mg N/L for *Scalindua* sp. (Oshiki *et al.* 2016). Based on the above kinetic coefficients, to obtain exponential growth of anammox bacteria, the nitrite concentration must be maintained at least in the range between two times its *Ks* value and half of the *Ki* value; for example, 0.96–112 mg N/L for *B. sinica*, 1.0–78 mg N/L for *J. caeni* and 0.0032–53 mg N/L for *Ca. Scalindua* sp. (Zhang *et al.* 2017b). This should be considered for enriching anammox bacteria or initiation of the anammox system.

Many environmental factors can inhibit the anammox reaction. Yang *et al.* (2018a) found that the anammox reactor could operate well with free ammonia (FA) at 13.65 ± 2.69 mg/L and free nitrous acid (FNA) at 39.49 ± 10.95 μg/L, while it was inhibited when FA and FNA concentrations reached 29.65 mg/L and 77.02 μg/L, respectively. After high substrate shocking, the abundance of *Ca.* Brocadia decreased while that of *Ca.* Jettenia increased (Yang *et al.* 2018a). In addition, Yang *et al.* (2018a) observed that overdoses of calcium or magnesium had adverse effects on the operation of anammox reactors by inhibiting anammox activity.

Microbial loading rates or activities

The applied loading rates or microbial activities affect the reactor size and the process footprint. Generally, low loading rates have been applied in the anammox process. For example, Zhang *et al.* (2016) reported specific anammox activity (SAA) values between 0.3 and 0.5 g N/g VSS·d. However, high loading rates or activities have been also obtained. For example, Xu *et al.* (2019) found that the SAA of anammox granules could reach up to 5.6 g N/g VSS·d, while the NLR and NRR (nitrogen removal rate) could be 76.7 kg N/m^3·d and 70.0 kg N/m^3·d, respectively. Tang *et al.* (2010) found that with dominant *Ca.* Brocadia, NRR of 11.7 kg N/m^3·d, SAA up to 0.7 g N/g VSS·d, and high biomass concentration of 28.4 g VSS/L were achieved with the efficient anammox sludge granulation. Jetten *et al.* (2009) proposed that the large membrane surface area serves to accommodate more respiratory proteins and leads to a higher maximum growth rate. Furthermore, anammox bacteria might adjust their activated membrane surface area to the substrate availability to cope with a large range in growth rates. By this means, when ample substrate is available, all membrane surface area would be activated, while when substrate is limited, only part of the internal membranes would be activated and the bacterium would still be capable of energy conservation (Jetten *et al.* 2009). In addition, Ni *et al.* (2019) found that in the expanded granular sludge bed reactor (EGSB) and the parent SBR, the NRR was 0.61 vs. 0.99 kg N/m^3·d, which caused the dominant anammox bacterial genus to shift from *Ca.* Kuenenia to *Ca.* Brocadia.

The temperature tolerance of anammox activity has been reported to be dependent on the species of the anammox bacteria (Magrí *et al.* 2013). For example, the marine anammox bacteria *Ca.* Scalindua favor lower temperatures for their growth compared to the wastewater anammox species (van de Vossenberg *et al.* 2008; Awata *et al.* 2013). Additionally, anammox bacteria have been observed to be able to alter their lipid membrane to adapt to temperature (Rattray *et al.* 2010). The maximum activity of the anammox reaction was observed from 35 to 40 °C; when the temperature was raised gradually, the anammox activity showed an irreversible decrease at 45 °C due to biomass lysis (Dosta *et al.* 2008). In addition, at a very low temperature (15 °C),

the anammox system became unstable due to nitrite accumulation, thereby affecting the anammox activity (Dosta *et al.* 2008). In addition, in a moving bed biofilm reactor (MBBR), the inhibition of anammox activity at 10 °C was observed (Persson *et al.* 2014). On the contrary, it was also reported that *B. fulgida* enriched from wastewater treatment plant sludge could survive at 10 °C (Hendrickx *et al.* 2014). Reino *et al.* (2018) successfully operated a *B. anammoxidans* anammox reactor at 11 °C, treating low-strength synthetic influent or nitrite-amended pre-treated real urban wastewater, and the achieved NLR was 1.8 ± 0.1 g N/L·d and 1.2 ± 0.5 g N/L·d, respectively. In the study of Lotti *et al.* (2014b), pre-treated municipal wastewater was treated by the anammox process with a dosage of nitrite; with the enrichment of *B. fulgida*, volumetric NLR higher than 0.4 g N/L·d and biomass specific NRR of 50 ± 7 mg N/g VSS·d were obtained at 10 °C (the doubling time was around 35, 77 and 132 d at 20, 15, and 10 °C). In particular, the up-flow anaerobic sludge blanket (UASB) reactor with plug-flow hydrodynamics and high sludge retention capabilities could improve the anammox activity at low temperatures considerably despite the mass transfer limitations (Reino *et al.* 2018).

Fernández *et al.* (2012) reported 50% inhibition in the SAA at the FA concentration of 38 mg NH_3-N/L. The optimal concentration of FA to maintain stable operation of a granular reactor was found to be less than 20–25 mg NH_3-N/L. Tang *et al.* (2014) found that in biofilm reactors, only concentrations as high as 57–187 mg NH_3-N/L caused inhibitory effects, suggesting that biofilm reactors are more resilient to FA inhibition. However, Jaroszynski *et al.* (2011) reported much higher anammox performance at a pH of 6.5 with the average FA concentration of 0.4 ± 0.3 mg NH_3-N/L than at a pH of 7.8 ± 0.2 with the bulk FA averaging 4 ± 3 mg N/L.

Microbial interactions and their contribution to enhanced nitrogen removal

In the anammox-based nitrogen removal systems, diverse microbial interactions exist. Generally, in oxygenated environments, AOB may provide nitrite for anammox bacteria, while NOB may compete for nitrite. Cooperation between anammox bacteria and AOB has been confirmed (Third *et al.* 2001; Schmidt *et al.* 2002a, 2002b; Sliekers *et al.* 2002; Vlaeminck *et al.* 2007). In anoxic environments, nitrate-reducing bacteria may produce nitrite for anammox bacteria under electron donor limitation conditions, while denitrifying microbes will also compete for nitrite when sufficient electron donors are available. The DNRA process could supply anammox bacteria with the necessary ammonium (Kartal *et al.* 2007a). Furthermore, Tao *et al.* (2013) found that some heterotrophs could compete with denitrifiers by mineralizing organic compounds faster than denitrifiers, which played a critical role in sponsoring anammox bacteria.

Ye *et al.* (2018) found that AOB could survive in the anammox reactor, but had extremely slow growth rates. For example, the anaerobic activity of *Nitrosomonas eutropha* was approximately 50-fold slower than the dedicated anaerobic ammonium oxidizer *Ca.* Brocadia anammoxidans, and more than 200 times slower than the aerobic activity of *Nitrosomonas eutropha* itself (Jetten *et al.* 2001). Since 2015, when Daims *et al.* (2015) demonstrated that *Nitrospira* sp. could perform both nitrification stages, this phylum has been observed to be relatively abundant in anaerobic ammonia oxidizing systems (Pinto *et al.* 2015; Ciesielski *et al.* 2018; Ziembińska-Buczyńska *et al.* 2019). All complete nitrifiers identified to date belong to sublineage II of the genus *Nitrospira* (Daims *et al.* 2001; Lebedeva *et al.* 2011). Under certain conditions, *Nitrospira* may not compete with the anammox bacteria, but supports anammox bacteria by providing nitrite through canonical ammonia oxidation (van Kessel *et al.* 2015; Ciesielski *et al.* 2018).

In the autotrophic nitrogen removal systems, washout of NOB is the key aspect for maintaining the anammox activity. Distribution between flocs and biofilm could be a good strategy to achieve the washout of NOB from systems. Laureni *et al.* (2019) found that floc removal is an effective operational strategy to achieve selective washout of NOB, and hybrid systems rather than solely biofilm systems would be more flexible in controlling NOB in mainstream nitritation and anammox (PN/A) applications. By separating NOB and anammox bacteria in flocs and biofilm, the direct competition for NO_2 between NOB and anammox bacteria was identified as key mechanism leading to a difference in the actual growth rates of AOB and NOB (mNOB < mAOB in flocs) and allowing the selective NOB washout over a broad range of simulated SRTs (6.8–24.5 d) (Laureni *et al.* 2019). In addition, by separating microbial communities between biofilm and flocs in the anammox system, denitrification activity present in flocs can protect anammox activity in biofilm, resulting in high ammonia removal efficiency and resistance to high organic loadings (Yang *et al.* 2019). In the study of Lotti *et al.* (2015a), the evaluation of the process in a plug-flow granular sludge-based pilot-scale reactor (4 m^3) was conducted at 19 ± 1 °C, which was continuously fed with the actual effluent of the A-stage of the wastewater treatment plant (WWTP), and anammox bacteria

were able to grow under mainstream WWTP conditions and new granules were formed and efficiently retained in the system, while heterotrophic biomass grew preferentially in flocs and was efficiently washed out of the system.

Microbial interactions could be incorporated into different processes to enhance nitrogen removal. For example, Xie *et al.* (2018) applied a novel technology integrating the anammox and denitrifying anaerobic methane oxidation (DAMO) reactions in a membrane biofilm reactor (MBfR), and an effluent total nitrogen (TN) concentration below 3.0 mg N/L and the TN removal rate of 0.28 kg $N/m^3 \cdot d$ could be achieved. In this process, 30–60% of the nitrate produced during anammox was reduced back to nitrite by DAMO archaea, and then the produced nitrite was removed by the anammox and DAMO bacteria with contributions of >90% and <10%, respectively (Xie *et al.* 2018).

START-UP OF THE ANAMMOX SYSTEM

The start-up of the anammox system is the bottleneck for its practical application, especially under situations without adequate seeding biomass. The first full-scale granular anammox reactor was started up successfully after 3.5 years' operation, which was longer than the planned 2 years, and the possible reasons were concluded (Abma *et al.* 2007). First, operational stability could have been affected by incidental biomass loss and freezing problems; second, microbial toxicity could have been caused by high nitrite concentrations, possible methanol slip from the Sharon reactor, and incidental discharges from chemical toilets to the digester; finally, mixing problems might have caused dead zones inside the reactor and inhibition from the formed sulfide (Abma *et al.* 2007). After start-up, the reactor operated stably even at the loading rate of 10 kg $N/m^3 \cdot d$, which could be due to the formation of dense anammox granules with settling velocities higher than 100 m/h (Abma *et al.* 2007).

Because anammox bacteria are slow growers with a doubling time of 11 days (Strous *et al.* 1998), the successful start-up and operation of an anammox system must maintain a high concentration of anammox bacteria and enhance their activities (stimulation or avoiding inhibition). During anammox reactor start-up, four consecutive phases of cell lysis, lag phase, activity elevation, and stationary occur (Tang *et al.* 2013). Many studies have been conducted to enhance the start-up of the anammox system through controlling the four phases. For example, signal substances can be dosed to enhance the microbial activity and also reduce the lag phase (Zhao *et al.* 2018). Generally, several types of strategies can be summarized, including microbial source and kinetic selection, stimulation with the addition of specific substances, and biomass retention with biofilm or membrane-based systems (Figure 3).

Start-up based on microbial selection and kinetic control

The slow growth rate of anammox bacteria in combination with inhibition and operational problems makes the start-up of the anammox process difficult. Therefore, suitable inoculum should be selected. In addition, suitable nutrient concentrations should be provided to maintain or enhance the microbial reaction activity, while simultaneously avoiding microbial inhibition. These can be achieved through choosing suitable microbial sources and selectively acclimating anammox bacteria with different microbial kinetics (such as the half saturation coefficients, inhibition coefficients and the maximum growth rate).

The inoculum can affect not only the amount of the anammox bacteria, but also the types of anammox bacteria. If an adequate amount of anammox biomass is available, it

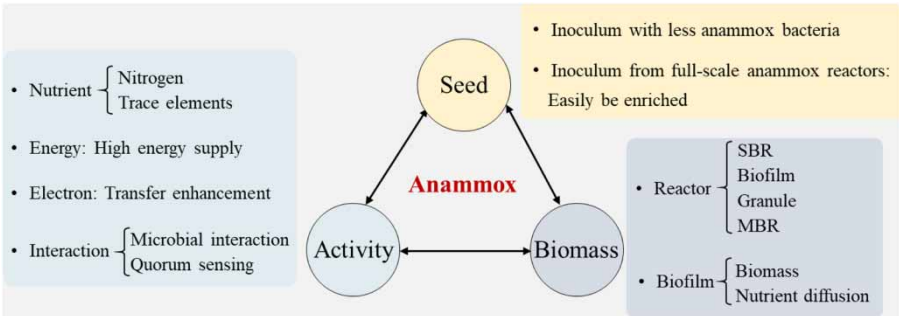


Figure 3 | Strategies to enhance the start-up of the anammox process.

can be used as the seed directly. On the contrary, without adequate anammox biomass sources, fast start-up of full-scale anammox process involves the stepwise cultivation of mature anammox biomass in laboratory- and pilot-scale reactors, which are subsequently switched to inoculate the full-scale reactor (Wett 2006; van der Star *et al.* 2007). Tang *et al.* (2013) proposed that the mixed inoculation with nitritation sludge as the main inoculum could be adopted as an efficient approach for full-scale anammox process start-up, where the obvious anammox activity appeared on 22 d when the reactor was seeded with nitritation sludge, which was only 1/3 of that inoculated with anaerobic granular sludge. Usually, *Ca.* Brocadia was the most dominant genus present in the enrichments transited from nitrifying sludge, denitrifying sludge, and anaerobic granular sludge, while *Ca.* Scalindua existed in the enrichment seeded with marine sediments and *Ca.* Jettenia from river sediments (Tang *et al.* 2010). By combining the anaerobic baffled reactor and MBR, starting up the cold-anammox process (13 °C) was achieved after 75 d, 45 d, and 90 d through inoculating flocculent nitrification sludge, anaerobic granular sludge and flocculent denitrification sludge, and the anammox species (*Ca.* Brocadia caroliniensis, B. sinica and *Ca*. Jettenia asiatica) with large maximum growth rates contributing to the rapid start-up of the cold-anammox process (Wu *et al.* 2018).

Anammox bacteria were partially inhibited at 50 mg NO_2-N/L and totally inactivated at 100 mg NO_2-N/L (Jetten *et al.* 2001). It is important to maintain the system NO_2-N concentration below the toxic level (Pyanert *et al.* 2004), especially during the start-up stage. To alleviate inhibition, mixing in the anammox reactor is a strategy to avoid inhibition induced by high nitrite levels or sulfide formed in dead zones (Strous *et al.* 1999b). The rapid increase in nitrite could inhibit anammox activity, and gradually increasing nitrite should be adopted as another strategy for the operation of full-scale reactors (Ni *et al.* 2011). The feeding strategy can also affect the selection of certain types of anammox bacteria. For example, the feeding strategy with a relatively low nitrite concentration to prevent nitrite inhibition is also a selective approach for *Ca.* Brocadia, which could not be enriched in high nitrite fed reactors (Gaul *et al.* 2005; van der Star *et al.* 2008). However, to increase the removal loading rate, increasing the influent nitrite concentration combined with the stepwise reduction in hydraulic retention time (HRT) could be adopted (Sliekers *et al.* 2003). Besides nitrite, high ammonium and nitrate concentrations of 25 and 50 mM could also inhibit anammox bacteria activity (Dapena-Mora *et al.* 2007), which should be considered during anammox system start-up, especially for treating ammonia-rich wastewater.

Start-up with stimulation

Microbial activities can be enhanced by increasing the activity of the electron transport chain, including the enzyme activity and electron transfer activity. As discussed below, this can be achieved through nutrient ensurance, intermediate enhancement, and electron transfer enhancement.

In addition to nitrogen macronutrient, trace element nutrients are very important for microbial activities. In particular, some trace elements are key co-factors of functional enzymes. Bi *et al.* (2014) found that the start-up time of the anammox process could be shortened from 70 to 58 d with 0.06 mM Fe^{2+} and 50 d with 0.09 mM Fe^{2+}. For anammox bacteria, insertion of a ferrous iron atom into the porphyrin macrocycle by the enzyme ferrochelatase creates heme, which can provide catalytic and electron transfer operations, and serve a critical role in the assembly of major enzyme complexes, including HDH (hydrazine dehydrogenase), HZS (hydrazine synthase), NirS (nitrite reductase), hydroxylamine oxidoreductase (HAO), and cytochrome bc_1 complex, among others. By dosing ferrous iron in the medium, the assembly of such enzymes could be enhanced for improving microbial activities directly.

During anammox, many intermediates are produced, and some intermediates can provide more energy for anammox bacteria. High energy supply is very important for the anammox bacteria due to the low energy production from anaerobic reactions. Ganesan & Vadivelu (2019) found that with the addition of 10 mg/L hydrazine, only 7 weeks were needed to stabilize and successfully operate the anammox process, whereas 12 weeks were necessary without the addition of hydrazine. Externally added hydrazine provides a greater energy source once metabolized, which can be used by the anammox bacteria (Yao *et al.* 2015).

Reduced graphene oxide (RGO) was reported to have a greater ability of electron transfer than graphene oxide by approximately three orders of magnitude, with the TN removal rate and enzyme activity increasing by 10.2% and 1.5–2 fold, respectively (Wang *et al.* 2013). By dosing RGO to the up-flow column reactor, the start-up period of the anammox process could be shortened from 67 to 49 d, which also enhanced anammox activity and stability even against the high NLR impacts (Yin *et al.* 2016). Based on the RGO biotransformation analysis, the applied stimulation may be strongly associated with the excellent electron transferability of RGO and its performance as a redox mediator (Yin *et al.*

2015, 2016). RGO could participate in the electron transfer from hydrazine dehydrogenase to cytochrome *bc*1 complex due to the faster electron transfer ability. Thus, with the addition of RGO, the enhancement of enzyme activity directly accelerated ATP synthesis and catabolism of anammox biomass. In addition, RGO could act as a scaffold for bacteria attachment (Ruiz *et al.* 2011), which can help anammox bacteria form macroflocs for enhancing the cell density, as anammox bacteria were not active until the cell concentration was higher than 10^{10}–10^{11} cells/mL (Strous *et al.* 1999a). In addition, by the dosage of 0.1 g/L of graphene oxide, greater EPS production was obtained accompanied with a maximum increase of 10.26% in anammox activity (Wang *et al.* 2013).

The observed microbial activity is the cooperation among all system microorganisms rather than just individual activity. Quorum sensing is a mechanism responsible for the microbial interactions within the microbial ecology, which can enhance microbial activity and gene expression. Quorum sensing is realized through the exchange of signal substances. By adding C_{12}-HSL-containing supernatant (signal substance) into the continuously stirred tank reactors, the start-up time of the anammox process was reduced from 80 to 66 days, and the NLR was also enhanced to 1.6 times that of the control reactor (Zhao *et al.* 2018). The possible reason is that signal substances could increase the secretion of EPS, resulting in better enrichment of anammox bacteria (Zhao *et al.* 2018).

Start-up with biomass retention

The limiting factor in fast start-up of biofilm reactors is not only the activity but also the ability to retain anammox biomass in the system and increase the attachment of anammox to the carrier material (Kowalski *et al.* 2019a). Many factors can affect the sludge settlement properties. For example, Wett (2007) found that nitrite concentration above 10 mg/L could deteriorate the settlement of suspended sludge, with the sludge volume index above 170 mL/g. In conventional reactors, strategies can also be adopted to enhance biomass settlement. For example, by seeding mixed activated sludge to start-up an anammox SBR with settling option (gradually reducing the setting time to 10 min), successful start-up of the anammox process was achieved, and the NLR was up to 506 g N/m^3·d and total NRR reached 433 g N/m^3·d by enriched *Ca.* Brocadia (Ye *et al.* 2018). In addition, MBR can also be adopted to retain biomass.

In biofilm systems, biofilm carriers and strategies to enhance microbial attachment should be developed to enhance biofilm growth. By comparing biofilm carriers of sponge, volcanic rock, and charcoal, Lu *et al.* (2018) found that using porous material as a carrier for biofilm development is an effective strategy for practical application of the anammox reactor, which can enhance biomass attachment and also create better anaerobic conditions for anammox bacteria. By using MBBR with a novel composite carrier, where the zeolites and floating materials were combined in the spherical shell and distributed evenly by the spherical polyhedron, the PN/A process could be realized in 53 days, and the TN removal efficiency reached around approximately 85% at an influent ammonium concentration of 50 mg/L (Lv *et al.* 2019). Kowalski *et al.* (2019b) discovered that rapid attachment of the anammox biomass was achieved in a reactor with media that had a predeveloped layer of a heterotrophic biofilm, and the SAA increased by almost 400% as compared to seed values. For the integrated PN/A process, anammox bacteria growth on biofilm carriers can be achieved first and then enrich nitrifiers, which can shorten the start-up duration (Zekker *et al.* 2013; Feng *et al.* 2019). In addition, the control strategy of the anammox process should be different for ammonia-rich wastewater and municipal wastewater. For example, FA could be applied for inhibiting NOB when treating ammonia-rich wastewater (Zekker *et al.* 2013). However, cascade oxygen supply can be applied when treating municipal wastewater with low ammonia concentrations (Feng *et al.* 2019).

For anammox granule formation, UASB reactors could be used. Even in UASB reactors, the problem of anammox sludge washout may occur due to the higher HRTs than the designed hydraulic loading (ca. HRT 4 h), which can be overcome by collecting and returning the washed out biomass (Li *et al.* 2012). Abma *et al.* (2007) found that fluctuations in the up-flow velocity caused incidental biomass losses, which should be removed efficiently through careful increments of the up-flow velocity to promote granule formation. In addition, inside the UASB reactor, floating granule accumulation and continuous adhesion on the edge of the three-phase separator are frequently encountered and block the gas vent and the effluent pipe, leading to severe sludge decay and loss, and even deterioration of reactor operation (Ni *et al.* 2019). To solve the biomass loss in conventional EGSB, a novel three-phase separator configuration was incorporated with an anammox granule circulating EGSB, and achieved stable operation of anammox processes by promoted granules circulation, retention, and reaction (Ni *et al.* 2019).

For membrane-based systems, suspended or biofilm-based biomass can be incorporated. Ni *et al.* (2010) used

the anammox nonwoven membrane reactor to form aggregates in the reactor and biofilm on the interior surface of the non-woven membrane, and the NLR and NRR reached 1,263 mg N/L·d and 1,047.5 mg N/L·d, respectively, with a maximum specific ammonium consumption of 51 nmol/mg protein·min after eight months of operation. In an up-flow fixed-bed biofilm column reactor with nonwoven fabric sheets as the biomass carrier, the anammox reaction (*Ca.* Brocadia anammoxidans was observed within 50 days, and a total NRR of 26.0 kg N/m^3·d (specific NRR of 1.6 kg N/m^3·d) obtained was attributed to the high anammox bacteria density (ca. 16 g VSS/L, more than 70% of total bacteria were anammox bacteria) (Tsushima *et al.* 2007b).

In biofilm-based anammox systems, nutrient diffusion is the key aspect for system performance, and the high NLR could prevent substrate transport limitation inside the biomass (Nicolella *et al.* 2000). For example, high anammox activity in up-flow column reactors was evenly observed throughout the immobilized gel beads due to faster and deeper substrate transport (Ali *et al.* 2015b). Due to the high relatively effective diffusivity, anammox reactors using artificially immobilized biomass tend to exhibit higher nitrogen removal performance than ones using naturally aggregated granules (Ali *et al.* 2015b). A reactor containing gel beads with biomass concentration of 0.33 g VSS/L achieved a NRR of 10.8 kg N/m^3·d and SAA of 278.5 ± 30.9 μmol-$^{29}N_2$/g VSS·h in just 35 days, whereas the reactor containing granular biomass of 2.5 g VSS/L could achieve only a NRR of 3.5 kg N/m^3·d and SAA of 184.7 ± 30.9 μmol-$^{29}N_2$/g VSS·h (Ali *et al.* 2015b). In biofilm systems, biomass concentration is important, and specific surface area is more important due to substrate transport limitation. This was also confirmed by Zhu *et al.* (2018), where the optimal granule sludge size was 0.5–0.9 mm for enhanced anammox bacteria abundance, activity and specific reaction rate.

CONCLUSIONS

Anammox-based biological processes play an important role in the sustainable management of wastewater, especially for nitrogen control. Anammox bacteria are slow growers with diverse phyla, which can be enriched from different environmental and operational conditions. The isolation of anammox bacteria should consider the relationship of microbial interaction from material metabolism, electron transfer and also information exchange. Suitable organic carbon contents may enhance activities of certain types of anammox bacteria, which should be carefully considered when treating real wastewater. The reaction stoichiometric coefficient can be affected by loading rates and other biological reactions. Microbial interactions also contribute to the enhanced biological nitrogen removal and promote activities of anammox bacteria, and functional microorganisms should be balanced for system stability. The start-up of the anammox process is the key aspect for its practical application, which can be realized through seed selection, system stimulation, and biomass concentration enhancement. Incorporation of the anammox process could achieve the sustainable management of wastewater, especially for the reclamation of water, recovery of energy and fertilizer, and also the control of pollutants.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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