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Increasing importance of anammox process: the present status and its development trend in municipal wastewater treatment system

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ABSTRACT

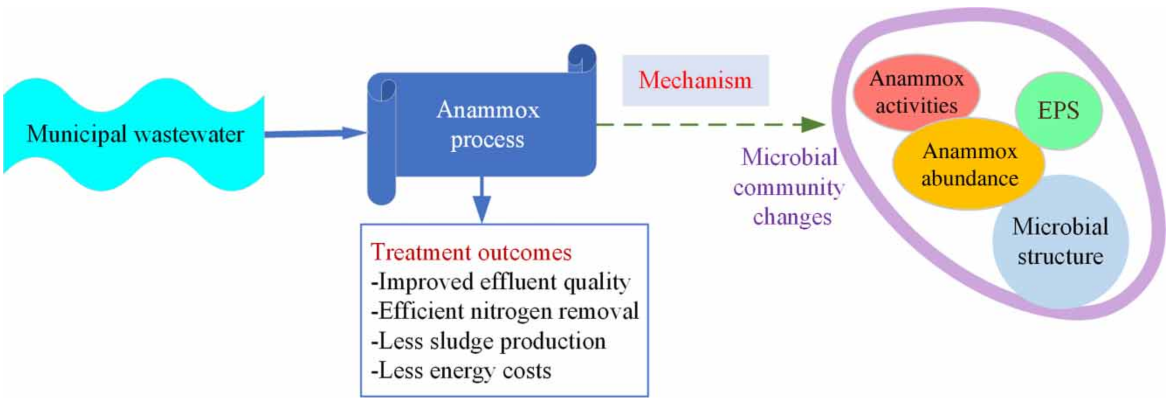
Anaerobic ammonium oxidation processes for the removal of nitrogen from municipal wastewater (known as 'mainstream anammox') are being involved in an on-going paradigm shift of the next generation of wastewater treatment plants due to their energy and resource efficiency. This review aims to present and summarize the recent research results of nitrogen removal performance and mechanism on mainstream anammox for its further successful application. The commonly encountered barriers are also discussed. It is proposed that the improvement of technology and the development of the mechanism for industrial application in municipal wastewater treatment are necessary. In particular, a framework of operable solutions to these difficulties and challenges is suggested and the partial denitratation/anammox process for treating municipal wastewater is specifically indicated as a significant research direction of new anammox development.

Key words: mainstream anammox, mechanism, municipal wastewater, nitrogen removal, performance

HIGHLIGHTS

- PNA and PDA were two feasible approaches for mainstream anammox.
- Nitrogen removal performance responses to influent water quality and operation conditions.
- Key microorganisms transformation including their activities, abundance and structure can result in changes of nitrogen metabolism mechanism.
- Fundamental and engineering solutions are needed for sustainable PNA, especially PDA at a large scale.

GRAPHICAL ABSTRACT



INTRODUCTION

Reducing nitrogen in the effluent of municipal wastewater (sewage) treatment plants is to prevent eutrophication and deoxygenation of receiving water bodies. The National Environmental Protection Agency of China calls for the implementation of discharge standards of pollutants for municipal wastewater treatment plants (WWTPs) (GB 18918-2002). According to this directive, a discharge limit of 5 mg NH_4^+-N/L and 15 mg TN/L is applicable for municipal wastewater treatment plants, even if for some sensitive areas and relatively developed areas, 1–1.5 mg N/L for ammonia-nitrogen and total nitrogen might be required. In the United States, 3 mg N/L for nitrate and nitrite has been discussed (TaskGroup 2009) and low effluent ammonium concentrations of $<$2 mg NH_4^+-N/L (Switzerland, WPO (1998)) or low total nitrogen concentrations of $<$10 mg TN/L are required (European Union, Council Directive 91/271/EEC (1991)), indicating the urgency of nitrogen pollution control worldwide.

For years, nitrogen removal in municipal wastewater has been treated by activated sludge technology, which comprises nitrification and denitrification (Gonzalez-Martinez *et al.* 2018). However, the high operational costs and energy requirements, as well as the greenhouse gas emissions, signify the current systems will not remain sustainable in the future (Hauck *et al.* 2016; Van Kessel *et al.* 2018). With the discovery of autotrophic anaerobic ammonium oxidation (anammox) bacteria in wastewater sludge in the early 1990s, anammox processes have become involved in the need for upgradation of conventional WWTPs since it will be possible to turn sewage into 'liquid gold' in an energy and resource efficient way. In recent years, growing number of researchers have paid close attention to the anammox process in municipal wastewater treatment (Kartal *et al.* 2010; Gu *et al.* 2018; Miao *et al.* 2020).

Anammox bacteria were first found to be widespread in traditional municipal WWTPs, such as observed in the anoxic zones of sewage treatment plants (Cao *et al.* 2013; Park *et al.* 2017; Wang 2018), which suggested that anammox bacteria enrichment could be possibly achieved in sewage treatment reactors and the contribution of anammox to nitrogen removal might be expected (Wang *et al.* 2015). To date, efforts and significant progress have been achieved in the mainstream for anammox process application, and system stability and nitrogen removal performance gradually improved by increasing experimental evidence (Li & Sung 2015; Zhang *et al.* 2016a, 2016b; Li *et al.* 2018a, 2018b).

Anammox represents its promise and bears the potential to bring municipal WWTPs close to energy autarky (63% less energy consumption and 90% less sludge production) and lower production of greenhouse gas (90% reduction in CO_2 emissions) (Joss *et al.* 2009; Lotti *et al.* 2014a; Yao *et al.* 2015), since anammox bacteria have the unique metabolic ability to combine ammonium and nitrite or nitrate to form nitrogen gas without consuming organic carbon (shown as Equation (1)) (Jetten *et al.* 1999):

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

According to Equation (1), the bicarbonate is the only source for the synthesis of cell biomass, nitrite and ammonium are the key substrates for anammox growth, so that providing this ratio of ammonia to nitrite to the anammox organisms is the key concern of operators in municipal wastewater treatment (Galí *et al.* 2007). While ammonium is directly available in sewage, nitrite needs to be produced in the treatment process. Today, the implementation of mainstream anammox processes in practical application face the first main barrier of unstable partial nitritation (low ammonium concentration (approximately 50 gN/m^3 instead of 500 gN/m^3) and the repression of nitrite-oxidizing bacteria (NOB)). Meanwhile, due to the preposterous nature of the anammox, the other barrier is extremely low growth rate of the anammox bacteria at low temperature (down to 10 °C instead of 30 °C) and in the presence of complicated substrate composition, such as comparatively high biodegradable organic matter (high BOD/N ratio) (Cao *et al.* 2017). Although the characteristics of municipal wastewater seem unfavorable to the mainstream anammox, the engineering application of anammox process to the mainstream wastewater treatment is expectant and has immense potential. Therefore, many researchers are attempting to surmount the barriers to prove its validity and feasibility for treating municipal wastewater (Liu *et al.* 2017a, 2017b; Ding *et al.* 2018; Miao *et al.* 2018).

This review presents and summarizes the recent research status of anammox technology applied in municipal wastewater treatment, which is mainly concerned with the performance and mechanism of nitrogen removal in municipal wastewater treatment in response to the characteristics of municipal wastewater or the instantaneous changes in water quality and operation conditions. Simultaneously, remaining existing problems are also discussed and future research and application directions of mainstream anammox technology are put forward.

BASIC NOTIONS ABOUT ANAMMOX BACTERIA AND ANAMMOX REACTION

The anammox bacteria are obligate anaerobic chemolithoautotrophic microorganisms (Erdim *et al.* 2018) belonging to the order *Planctomycetales* and having six identified genera: *Candidatus Brocadia, Candidatus Kuenenia, Candidatus Jettenia, Candidatus Scalindua, Candidatus Anammoxoglobus*, and *Candidatus Anammoximicrobium* (Ma *et al.* 2015a, 2015b; Li *et al.* 2018b). These genera carry the mutual cell morphology and metabolism despite the large phylogenetic distance (Ma *et al.* 2015a, 2015b). All the catabolic reactions take place in an anammoxosome (a unique intracytoplasmic compartment in anammox bacteria, Figure 1) based on the immunogold localization of hydrazine/hydroxylamine oxidoreductase (HAO), one of the key enzymes involved in the anammox process (Jetten *et al.* 2009; Li *et al.* 2018b).

There are two possible pathways proposed from advanced molecular studies. One of them is considered inaccurate because the biological disproportionation of hydroxylamine is observed. The truth in this case is that hydroxylamine can react with ammonium into dinitrogen gas in the absence of nitrite while a small amount of hydrazine accumulated during this process (Van De Graaf *et al.* 1997). The other one is accepted by the majority of the scientific community and is contradictory in the sense that nitric oxide (NO) acts as a precursor for the synthesis of the anammox intermediate called hydrazine (N_2H_4) (Kartal *et al.* 2011a, 2011b) (Strous *et al.* 2006; Van Niftrik *et al.* 2008; Erdim *et al.* 2018). In the most accepted pathway (Figure 2), nitrite (NO_2^-) is reduced to NO through heme protein nitrite reductase (NrfA/NIR) at the expense of one electron, then produces NO as a radical that directly attacks ammonium to produce hydrazine through hydrazine hydrolase (HH) with uptake of three low-energy electrons. The oxidation of hydrazine to nitrogen is by a variant of hydroxylamine oxidoreductase (HAO) and yields four high-energy electrons that drive the reduction in the preceding two steps (Strous *et al.* 2006; Van Niftrik *et al.* 2008; Irisa *et al.* 2014; Li *et al.* 2018b). NrfA/NIR and HAO are the key enzymes involved in this pathway, which is postulated to be directly proportional to nitrogen removal rate (NRR) and specific anammox activity (Xu *et al.* 2019). The electrons released from hydrazine oxidation are transferred to the cytochrome *bc1* complex (complex III) via ubiquinone (Q). The *bc1* complex transports the electrons towards nitrite reduction and hydrazine synthesis (van Niftrik *et al.* 2008). Associated with this electron transfer and chemolithoautotrophic nature of the bacteria, the protons are translocated from the riboplasm to anammoxosome resulting in the generation of electrochemical proton gradient routed from the anammoxosome to the riboplasm. The established electrochemical proton gradient, also called proton motive force (PMF), results in charge difference, rendering riboplasm negative and inside of the anammoxosome positive. PMF is used to energize ATPase localized in the anammoxosomal membrane to synthesize ATP (Van Niftrik & Jetten 2012). The synthesized ATP would then be released into the riboplasm (Van Niftrik *et al.* 2004).

With the anammox reaction, the anammox bacteria continually grow and enrich, which can be reified as the culture gradually turning red-colored due to the presence of the cytochrome (Zeng *et al.* 2016; Li *et al.* 2018b). It is estimated that about 50% of the total nitrogen gas present in the atmosphere is made by the anammox reaction (Kartal *et al.* 2012).

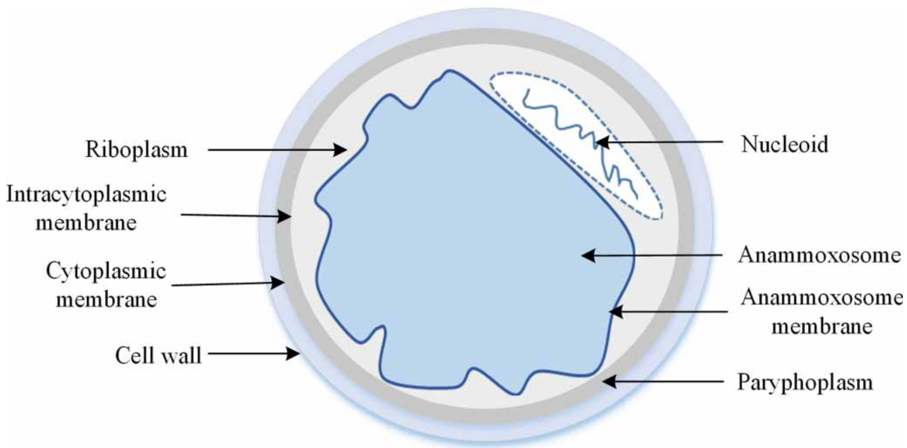


Figure 1 | Schematic representation of the structure of anammox unique compartment (anammoxosome) (Kuenen 2008; Van Niftrik *et al.* 2008; Fuerst & Sagulenko 2011; Kartal *et al.* 2011a; Peeters & Van Niftrik 2019).

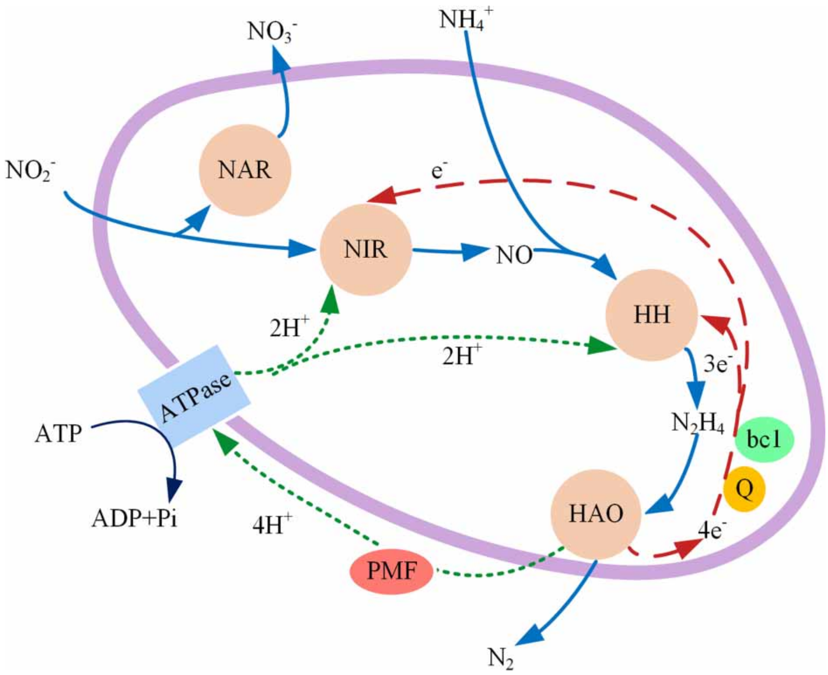


Figure 2 | Nitric oxide acting as a precursor for the synthesis of hydrazine (adapted from Kuenen (2008) and Kartal *et al.* (2011a, 2011b)).

THE APPLICATION OF ANAMMOX PROCESS IN MUNICIPAL WASTEWATER TREATMENT SYSTEM

Nitrogen removal performance of mainstream anammox process

Since the early 2010s, efforts have shifted from sidestream (1% of the volume flow of the WWTPs) to the mainstream with its great potential to solve the problems that many municipal WWTPs already face, such as rising investment costs and increasingly strict requirements of nutrient discharge (Kartal *et al.* 2010). Today, the anammox processes are considered as the most promising alternative for biological nitrogen removal in municipal wastewater applications (Van Loosdrecht & Brdjanovic 2014). Significant progress has been reported in bench-scale and pilot-scale studies, which mainly focus on the nitrogen removal performance response to influent substrates' concentrations and lowering temperature, more specifically related to (i) high biodegradable organic matter (higher COD/N ratio) in the sewage, (ii) low ammonium concentration, (iii) strict temperature requirements, (iv) presence of other undesired substrates (such as inorganic carbon (IC)), representing the current main challenges of anammox towards full-scale application in municipal WWTPs (Gilbert *et al.* 2014).

At present, studies on the anammox mainstream treatment of sewage are mainly focused on the partial nitritation (i.e., ammonium oxidation to nitrite)/anammox pathway (PNA) (Chen *et al.* 2021), including one-stage PNA process with partial nitritation and anammox reaction combined in one reactor (e.g., completely autotrophic nitrogen removal over nitrite (CANON) process), and two-stage PNA process with partial nitritation and anammox in two separate reactors (Clippeleir *et al.* 2011; Ma *et al.* 2011; Wett *et al.* 2013; Ødegaard 2016; Wang *et al.* 2016). Achieving partial nitritation by controlling the reaction process in a single reactor is more popular because of low capital costs and simple operation (Ma *et al.* 2011; Isanta *et al.* 2015; Kouba *et al.* 2017). Due to characteristics of municipal wastewater, several recent studies have demonstrated the partial denitratation (i.e., nitrate reduction to nitrite)/anammox (PDA) pathway (e.g., DNRA) could potentially provide an alternative pathway to produce nitrite even ammonium in a mainstream anammox system (Ji *et al.* 2017; Ma *et al.* 2017; Park *et al.* 2017; Xie *et al.* 2018; Cao *et al.* 2019; Du *et al.* 2019a, 2019b, 2020; Zhang *et al.* 2019).

Effects of influent COD (and or C/N ratios) on mainstream anammox performance

Generally, high COD concentration boosts the growth of heterotrophic bacteria and has a significant inhibition for ammonia oxidizing bacteria (AOB) and anammox bacteria. Therefore, the heterotrophic denitrification process has an advantage over the anammox process for treating urban sewage. It has been reported that the activities of AOB and anammox bacteria were reduced at a C/N ratio of 2.7–2.9 (Han *et al.* 2016a). Especially, a high COD concentration of 292 mg/L could entirely suppress the anammox bacteria (Molinuevo *et al.* 2009). The nitrogen removal efficiency reduced from 79% to 52% with increased influent C/N ratio from 0.5 to 0.75 (Chen *et al.* 2009). Therefore, the influent carbon source was believed to be a crucial factor for anammox performance (Jenni *et al.* 2014; Cao *et al.* 2017) and the COD removal will often be a treatment step prior to anammox treatment by a physico-chemical process or a high-rate activated sludge system (Jin *et al.* 2019; Zhang *et al.* 2020; Xiao *et al.* 2021).

However, recent studies have suggested that limited organic carbon (low amount of organic compounds) in PNA systems could promote the overall removal of nitrogen (Tao & Hamoud 2019). Bunse *et al.* (2020) operated MABRs with real municipal wastewater characterized by low concentrations of nitrogen (varying between 31 and 120 mg NH_4^+-N/L) and the presence of biodegradable organic carbon (soluble COD between 7 and 230 mg O_2/L). The achieved ammonium removal efficiency was between 70 and 90% and TN removal rate was between 60 and 80% on average.

PDA application was more reported in mainstream anammox for partial COD removal and nitrate to nitrite transformation. Partial heterotrophic denitrification (denitratation) was taken up by anammox organisms (Du *et al.* 2020; Pedrousoa *et al.* 2021). For instance, acetate could be used by anammox bacteria (Winkler *et al.* 2012a; Jenni *et al.* 2014). A high nitrogen removal efficiency of 84–95% could be obtained with glucose as carbon source and an influent C/N ratio of 1.2 (Jia *et al.* 2012). Du *et al.* (2016) investigated the synchronous treatment of nitrate (NO_3^--N of 50 mg/L) and domestic sewage (NH_4^+-N of 60.6 mg/L, COD of 166.3 mg/L) via PDA at low temperature (12.9–15.1 °C), with the results revealing that expected performance was achieved with average NO_3^--N, NH_4^+-N, and COD removal efficiencies of 89.5%, 97.6%, and 78.7%, respectively. Ma *et al.* (2017) demonstrated a TN removal efficiency of $80 \pm 4\%$ under a low influent C/N ratio of 2.6 and 42% of nitrate-to-nitrite transformation ratio (NTR). Xie *et al.* (2018) showed 0.28 kg N/(m^3·d) of TN removal rate and 30–60% of the nitrate removed in the absence of a low amount of COD. The nitrate was produced by the anammox reaction and then reduced back to nitrite by denitrifying anaerobic methane oxidation (DAMO) archaea. The anammox and DAMO archaea jointly contributed to nitrite removal of >90% and <10%. Bunse *et al.* (2020) investigated the membrane aerated biofilm reactors for mainstream PDA using real municipal wastewater characterized by low concentrations of nitrogen varying between 31 and 120 mg NH_4-N/L and the presence of biodegradable organic carbon (soluble COD (SCOD) between 7 and 230 mg O_2). The results showed that the achieved ammonium removal was between 70 and 90% and TN removal between 60 and 80% with average TN removal rates of approximately 1.2 g N/(m^2·d) and maximum TN removal rates of ~2 g N/(m^2·d). Thus, the PDA provided a great possibility to enhance overall nitrogen removal in the mainstream. However, these studies mainly concentrated on the achievement of PDA driven by the exogenous carbon substrate. Ji *et al.* (2017) reported the treatment of nitrate wastewater and stable nitrite production was achieved via endogenous partial denitrification (EPD) with a NTR of 87% and a suitable NO_2^--N/NH_4^+-N ratio of 1.20 for anammox reaction in the mainstream treatment of sewage. The results displayed an effluent TN of 5.8 mg N/L and a stable, high nitrogen removal efficiency (90%) at a low C/N of 2.9 by PDA. Anammox accounted for a 49.8% contribution for the overall nitrogen removal due to the steady nitrite supply from EPD (Ji *et al.* 2018). Miao *et al.* (2018) indicated that the TN removal efficiency increased steadily from 30.8% to 77.3% with the increasing C/N ratio (1.1, 1.5, 2.0, and 2.5) due to nitrate reduction using internal carbon. Due to the abundance and activity of anammox bacteria remaining stable at the C/N ratio of 2.5, TN removal efficiency still increased.

One recent study proposed the simultaneous partial nitritation, anammox, denitrification and COD oxidization (SNADCO) process for mainstream anammox (Zhou *et al.* 2020). Guo *et al.* (2020) investigated the performance of the SNADCO process with the stable nitrogen loading rate of 0.5 kg N/(m^3·d) and the variation of influent COD/NH_4^+-N (C/N) ratio from 0.0 to 1.6 in a one-stage carrier-packing airlift reactor with continuous mode for the first time. The results showed that at the C/N ratio of 0.8, the average nitrogen removal efficiency (NRE) of 80.9% was achieved due to both the anammox and denitrification pathways and the achieved average COD removal efficiency of 94.6% was attributed to both the denitrification and COD oxidization pathways. Zhao *et al.* (2021) illustrated the performance of the SNADCO process in a novel one-stage plug-flow microaerobic sludge blanket with membrane aerated for treating municipal sewage. The removal efficiencies of 93.2% ammonium and 87.1% TN were observed with a C/N ratio of 4. Similarly, the SNADCO process was operated in a modified two-stage anammox process for treating municipal wastewater. Nitrite accumulation rate was over

95% by partial nitrification (PN), then simultaneous anammox and denitrification occurred in the second stage, in which the total inorganic nitrogen (NH_4^+-N, NO_2^--N and NO_3^--N) of effluent was only 1.6 ± 0.8 mg N/L and the nitrogen removal efficiency reached 97.1% (Deng *et al.* 2020). When an innovative PNA and endogenous PDA process were developed in a single-stage integrated fixed film activated sludge sequencing batch reactor treating real municipal wastewater with C/N ratio below 3.2, the enhanced efficiency of TN removal reached 90.1% (Cui *et al.* 2021).

Effects of low influent ammonia on mainstream anammox performance

For especially low ammonia in influent of municipal wastewater, unstable supply of nitrite was the primary bottleneck (Du *et al.* 2016, 2019a, 2019b). Usually, the rapid nitritation of municipal wastewater for anammox was supplied by nitritation, partial denitrification, or dissimilatory nitrate reduction to ammonium (DNRA) (Cui *et al.* 2020; Wang *et al.* 2021a, 2021b).

Laureni *et al.* (2015) operated anammox-based sequencing batch reactors at total N concentrations in the range 5–20 mg N/L, as expected for municipal wastewater. Nitritation was established by PN and synchronous anammox activities that reached the peak of 465 mg N/(L·d) at 29 °C on the operation days of 18 d. Moralesa *et al.* (2016) determined the stable performance of the PNA process with the nitrogen removal efficiency of 70% in a single-stage reactor operated at a low ammonium concentration for 60 days. Zhang *et al.* (2020) established two lab-scale bioreactors to treat domestic wastewater. In the first bioreactors, the stable nitrogen removal efficiency of 51.1% was achieved by simultaneous PN and denitritation (SPND) and NOB was successfully outselected under low-DO of 0.1 mg/L, resulting in an average effluent NO_2^--N/NH_4^+-N ratio of 1.04. In the second bioreactors, the effluent total inorganic nitrogen (TIN) was only 8.4 ± 1.1 mg N/L and the NRE reached 88.24%. With a short aerobic HRT of 5.7 h, a modified two-stage PNA process was established for advanced nitrogen removal from domestic wastewater. The initiation and stable maintenance of PN was achieved by pre-anaerobic treatment without inoculation or addition of inhibitor; nitrite accumulation rate was over 90% in the first process and, meanwhile, pre-anaerobic treatment improved the storage of endogenous carbon sources to enhance the NRE. For the anammox in the second process which was fed with the effluent of the first process, the total inorganic nitrogen in the effluent was below 5 mg N/L, and the NRE was 92.06% (Xiao *et al.* 2021). Chen *et al.* (2020) provided an easy operation strategy to achieve 100% conversion of ammonium to nitrite by intermittent aeration, without coupling with low dissolved oxygen or short sludge retention time in a PA reactor, the effluent of which was directly mixed with the raw municipal wastewater in an anaerobic sequencing batch reactor with the mixture ratio of 2.5 and the effluent ammonium and TIN of only 0.97 and 2.52 mg N/L, respectively. Additionally, carbon-based pollutants were also removed in the proposed system without any pre-treatment, which made the process easier to operate in practice.

Although low ammonium concentration was unfavorable to the growth of anammox bacteria, many studies showed that, after achieving stable maintenance of nitrite concentration, the efficacy of mainstream anammox in removing nitrogen appears to be consistent. Ding *et al.* (2018) focused on the nitrogen removal performance treating real domestic sewage of an averagely 64.6 mg/L of ammonium based on the suspended activated sludge and obtained a TN removal rate of 86.1% while only 1.02 mg/L of nitrate in the effluent. Jin *et al.* (2019) evaluated the long-term stability of the PNA process to treat real municipal wastewater with influent ammonium concentration varying from 32 to 79 mg/L at an average hydraulic retention time of 3.39 h. It was seen that the PNA could obtain a removal efficiency of 93% for ammonium and the effluent TN was 4.8–11.8 mg/L. Wu *et al.* (2020) showed the single anammox (CANON) and two-stage anammox processes could be successfully started up for real low-strength wastewater treatment. The total nitrogen removal efficiencies were around 86–92% and 81–87% in these two systems, respectively. Yang *et al.* (2020) obtained an average effluent TN of 13.7 mg/L and a removal efficiency of 72.0% with an influent of 47.0 mg/L ammonium in the anaerobic/aerobic/anoxic/aerobic (AOAO) reactor processes.

Effects of temperature on anammox performance

Low temperature is a challenge for the typical anammox process (Gilbert *et al.* 2014; Qiu *et al.* 2020). Anammox bacteria prefer a medium temperature and have highest activity at around 35 °C. However, the actual temperature of most municipal wastewaters is frequently low (10–25 °C), even lower than 10 °C at higher latitudes. As a result, several recent studies have focused on achieving adequate nitrogen removal performance in the anammox process at low temperatures (Isaka *et al.* 2008; Van de Vossenberg *et al.* 2008; Hu *et al.* 2013; Ma *et al.* 2013; Lotti *et al.* 2015a). According to the findings of many studies, acceptable NRR in the anammox process could be achieved even at low temperatures (Vazquezpadin *et al.* 2009; Hendrickx *et al.* 2014; Lotti *et al.* 2014b). Combined PNA reactors could run stably at both 12 °C and 10 °C (Hu *et al.* 2013; Gilbert *et al.* 2014), although lowering the temperature to 12.5 °C brought about a noticeable decrease of anammox activity to 46 mg N/(L·d); however, it is still in the reasonable range (79 days doubling time) for autotrophic nitrogen removal from municipal wastewater (Laureni *et al.* 2015). The NRE was around 97.6% at 12.9 –15.1 °C (Du *et al.* 2016).

Laureni *et al.* (2016) assessed the stability of partial nitritation/anammox processes over a year, including more than five months at 15 °C. The results of the effluent concentration of 21 ± 5 mg NH4+-N/L and residual 69 ± 19 mg $CODto_t$/L showed that the PNA was able to meet nitrogen concentrations of typical discharge limits. Over 90% of ammonium and 70% of total nitrogen removal efficiencies were stably achieved, respectively. Zhang *et al.* (2018a) also showed the steady nitrogen removal efficiency of 88.2% at lower temperature from 27.0 to 12.8 °C. Akaboci *et al.* (2018) indicated NRR of 330.24 ± 25.36 mg N/(L·d) was obtained at 25 °C and no instability was experienced in a granular one-stage PNA by dropping the temperature to 15 °C for domestic sewage treatment. The anammox system had a higher substrate tolerance at 13 °C than at 18 °C and could be restored when the adverse effects occurred at a lower temperature of 8 °C (He *et al.* 2018). The NRR decreased with decreasing *in situ*-specific anammox activity (SAA), and, interestingly, the *ex situ* SAA acclimated higher at 23 °C than those at 33 and 28 °C. Yuan *et al.* (2021) investigated the feasibility of treating real municipal wastewater from summer to winter (28.1–15.3 °C) with a novel simultaneous enhanced biological phosphorus removal and semi-nitritation plus anammox process, respectively, in two lab-scale sequential reactors. Long-term operation suggested that reliable nutrient removal was still maintained in winter (16.4 ± 0.7 °C), i.e., the removal efficiencies for nitrogen and phosphorus were 80.0 ± 3.5% and 95.4 ± 5.2%, respectively, with short aerobic HRT (6.4 h) and low dissolved oxygen (0.2–1.5 mg/L). The effect of common seasonal temperature variation (15.1 °C–22.2 °C) on nitrogen removal performance was studied by Gong *et al.* (2020). The results showed that in autumn and winter, decrement of the NRR was 0.038 kg N/(m^3·d) (17.9 °C→15.1 °C), three-fold higher than 0.014 kg N/(m^3·d) (22.2 °C→17.9 °C), indicating that lower temperature placed a more negative impact on nitrogen removal. ^{15}N isotope tracing tests confirmed that the contribution of denitrification to nitrogen removal was far less than anammox, and anammox contributed more at 15.1 °C (91.7%) than 21.9 °C (78.9%). Li *et al.* (2021) obtained high-rate nitrogen removal at 86.42% accompanied with a total biomass concentration of 26.02 g SS/L under nitrogen loading rate of 4.25 ± 0.10 kg N/(m^3·d), HRT of 20 min, and 25 °C in a continuous biofilter anammox reactor. Pedrousoa *et al.* (2021) evaluated the long-term performance and stability of a granular anammox reactor operated at mainstream conditions, 15 °C and 50 mg TN/L, and inoculated with biomass which was previously not acclimated to low nitrogen and lower temperature. The results showed that an acclimation time (usually applied) was not required to gradually attain mainstream conditions, and a TN effluent content of less than 10 mg TN/L was obtained.

Impacts of influent IC on anammox performance

Inorganic carbon (IC) has an important role in maintaining the stability of the anammox system. As an assimilation carbon source for chemoautotrophic bacteria, many types of studies have recorded that long-term addition of IC could remarkably improve the NRE by a significant influence on the nitrogen-transforming microorganisms in a single anammox or some anammox-based systems (Kimura *et al.* 2011; Jin *et al.* 2014; Ma *et al.* 2015a, 2015b; Zhang *et al.* 2016a; Yue *et al.* 2018). Furthermore, recent studies have shown that IC concentrations were positively associated with N_2O emissions, and can affect the tolerance of anammox systems (Daguerre Martini *et al.* 2018; Zhang *et al.* 2018b). The IC/N ratios in actual engineering applications were often found in the range of 0.1:1 to 4:1; however, to avoid nitrogen removal effectiveness decreasing with the IC deficit, IC/N ratios of more than 1.5:1 are recommended (Ma *et al.* 2015a). Shu *et al.* (2018a, 2018b) indicated that insufficient IC had adverse effects on anammox process performance, while adding IC could recover the anammox activity. In the absence of IC, the TN removal efficiency dropped to 74.16 ± 1.41% and increased gradually to 80.41 ± 0.60% with the increase of IC/TN ratios from 0 to 0.31, but increasing IC/TN from 0.62 to 1.24 the TN removal efficiencies fell to 66.36 ± 1.62%. However, the calculated NLR and NRR were only marginally decreased to 0.33 and 0.23 kg N/(m^3·d), respectively. The results indicated that the nitrogen transforming microorganism had been steadily adapting to the new conditions of the IC/TN ratios (0.62 to 1.24) although anammox activity was slightly suppressed at first. With the increased IC/TN ratio of 1.87, the NLR and NRR were recovered as expected, and the increased NH_4^+-N (74.30 ± 1.65%) and NO_2^--N (84.77 ± 0.62%) removal efficiencies were also observed (Shu *et al.* 2018b).

Effects of control strategies on anammox performance

In fact, the potential of anammox processes has not yet been fully confirmed for the direct treatment of municipal wastewater despite increasing experimental evidence. The long-term stability of anammox processes used to treat municipal wastewater is still under investigation. Apart from the challenges discussed above, there are also other problems, mainly operational problems, that remain over the anammox process affecting anammox performance, such as pH, dissolved oxygen (DO) concentration, free ammonia and nitrite inhibition, the slow growth rates of anammox bacteria and retention of anammox bacteria, which play a decisive role for the smooth operation of the mainstream and also sidestream anammox process.

The first bottleneck we usually encounter in the anammox process is the initiation of the process, as the functional bacteria involved in anammox process, anammox bacteria, exhibits a tremendously slow growth rate of 0.0027 h^{-1} (Lin *et al.* 2019). If the anammox bacteria is coupled with unfavorable low temperature and high oxygen input (slow oxygen probe), the bacteria would form nitrite by grafting (rapid enough), not by reduction of ammonium. The nitrite concentration ran to a toxic concentration and caused irreversible persecution to the inoculated anammox bacteria. To reduce the start-up time of the process, methanogenic granules or some packing carrier was usually added that acted as a preserving material to keep the anammox biomass attached and ensure continuous operation at a high nitrogen loading rate without nitrite inhibition (Tokutomi *et al.* 2011; Ali & Okabe 2015). For example, Kowalski *et al.* (2019) accelerated the start-up time of anammox reactors using a pilot moving bed biofilm reactor at a dewatering facility of a municipal WWTP by combining bio-primer technique with seed carriers. In addition, control strategies of reactor parameters are also very important. Cai *et al.* (2020) initiated a single-stage PNA process in a sequencing batch biofilm reactor with conventional activated sludge within 46 days, which could be attributed to biomass retention provided by biofilm and regulation of parameters including dissolved oxygen (DO), pH, temperature, organic matter, IC, free ammonium (FA), and free nitrite acid (FNA).

In the anammox process, DO control is crucial for balancing AOB against anammox bacteria activities and achieving nitrite accumulation by PN. Therefore, the real-time control methods are generally used to regulate DO concentration in the reactors, but that can lead to serious consequences when DO sensors fault or react too slow due to getting dirty too quickly. The anammox activity was inhibited when DO concentration was higher than 0.5 mg/L or lower than 0.032 mg/L O_2 (Gao *et al.* 2014). Hence, the proper control of DO concentration is crucial for successful operation of the anammox process. Maybe monitoring DO volume instead of concentration can provide a more reliable solution, especially at lower DO concentration.

The pH fluctuation can have serious negative effects in some municipal WWTPs, since a high pH provides a large amount of free ammonia (NH_3) and a low pH causes an increase in the quantity of nitrous acid (HNO_2). Anthonisen *et al.* (1976) mentioned AOB inhibition of free ammonia at concentrations of 10 to 150 mg N/L, and inhibition of NOB from 0.1 to 1 mg N/L. Kowalski *et al.* (2019) thought a minimum FA concentration of 2 mg NH_3-N/L should be suggested for the optimal performance of PN and to prevent the build-up of nitrates in a municipal wastewater treatment process. The anammox process increased the pH whereas nitrification reduced pH. Therefore, the pH was frequently maintained by turning on/off the aeration system and changing the influx rate (Li *et al.* 2018c). However, Pedrousoa *et al.* (2021) reported anammox activity could be highly influenced by pH in short-term operation, but a long-term stable anammox process was maintained despite the low pH value of 6.2 of municipal wastewater. Zhang *et al.* (2021) proposed that the nitrogen removal performance improved under extreme pH shock (pH 5.0 and pH 10.5) by fluctuating the carbon and nitrogen ratio (C/N) cultivation strategy for denitrification sludge extracellular polymeric substances (EPS)-enhanced (DS-EPSCN) on the properties of anammox granules.

In addition, the practical application of the mainstream anammox process has been often hindered by the complicated ingredients of municipal wastewater, such as some heavy metals, suspended solids that contain many inhibitors (e.g., sulfides) and have suppressive effects on the performance of the anammox system. Generally, suppressive effects will last for some time since it is difficult to determine the specific inhibiting component, but possible countermeasures would be increasing sludge discharge or just waiting for recovery.

Anammox technology for municipal wastewater treatment implemented at pilot/full-scale

Considerable efforts have been made for applying anammox processes at mainstream wastewater. Significant progress has been achieved in bench studies for treating municipal wastewater, and some pilot and full-scale demonstration investigations have been also ongoing in Europe, the United States, and Asia. From a basic economical point of view, pilot and full-scale demonstration investigations are popular and have great potential for the removal of undesired nitrogen from wastewater. Table 1 shows some examples of anammox technologies for municipal wastewater treatment implemented at pilot/full-scale.

Mechanism of affecting nitrogen removal performance in municipal wastewater treatment

The viability of the mainstream anammox process has been validated by the performance studies, and the mechanism of affecting nitrogen removal performance is also investigated because it is important to look at the technology's key focal points for future optimization. A stable anammox process requires an appropriate balance among key functional microbes. Due to the characteristics of municipal wastewater or the instantaneous changes in water quality and operation conditions, key microorganisms can be possibly transformed, including their activities, abundance, and structure, which might result in changes of nitrogen metabolism and affecting nitrogen removal performance. It is essential to obtain a clear understanding of the nitrogen removal mechanism, to benefit the functional stability and facilitate the engineering application of the anammox system under abiotic stresses (Ma *et al.* 2011; Regmi *et al.* 2015; Liu *et al.* 2018). Anammox bacteria, as a discrete unit of the bacterial community, plays a particular role as a member of the community and contributes to the ecological function as it possesses a specific metabolic ability (Kang *et al.* 2019). Several reports provide some insights into the effect of nanoparticles (NPs), nitrite and ammonium, inorganic or organic, and temperature on the anammox mechanism. Anammox bacteria require a defined concentration of ammonium and nitrite as substrate. The presence of

Table 1 | Description of some examples of anammox technologies for municipal wastewater treatment implemented at pilot/full-scale

Country	Technology	Volume (m^3)	Bioreactor	COD_T fed (mg/L)	Nitrogen fed (mg N/L)	NLR (kg N/(m^3·d))	NRE (%)	References
France	Deammonification	50	Fxed-film fctivated sludge reactor	60–90	30–60 NH_4^+-N	0.2–0.3	60	Lemaire *et al.* (2014)
Austria	Deammonification	40	Carousel-tank	–	–	–	–	Wett *et al.* (2013)
Netherlands	One-stage PNA	4	Plug-flow reactor	62 ± 17	27 ± 5 NH_4^+-N	0.4	39	Lotti *et al.* (2015b)
Singapore	Two-stage PNA	–	Step-feed activated sludge	–	–	0.13	37.5	Cao *et al.* (2015)
Sweden	Deammonification	0.2	Fixed-film activated sludge reactor	49–106	35–50 NH_4^+-N	0.1	52	Malovanyy *et al.* (2015)
USA	Deammonification	0.2	Plug-flow reactor	41 ± 10	62 ± 4 TN	0.14	70	Han *et al.* (2016b)
Sweden	Deammonification	0.2	Moving bed biofilm reactor	44–71	45 ± 2 NH_4^+-N	0.05	44	Trojanowicz *et al.* (2016)
Netherlands	One-stage PNA	7	Upflow new activated sludge reactor	120	34–41 TN	0.1	51	Seuntjens *et al.* (2016)
China	Two-stage PNA	0.135	Sequencing batch reactor	300 ± 100	65 ± 15 NH_4^+-N	0.11	–	Jiang *et al.* (2018)
Spain	One-stage PNA	0.6	Sequencing batch reactor	16–197	6–25 TN	0.067	50	Pedrousoa *et al.* (2018)
China	PNA	–	Moving bed biofilm reactor	–	–	–	–	Wang *et al.* (2018)
Canada	Two -stage PNA	0.1	Fixed-film activated sludge reactor	347 ± 53	38 ± 7	0.055	67	Kowalski *et al.* (2019)
Sjölunda	One-stage PNA	2.6	Moving bed biofilm reactor	70	–	0.13–0.14	–	Gustavsson *et al.* (2020)

undesired substrates can result in a decrease in key enzymatic activity of NrfA/NIR and HAO involved in anammox metabolism, and impair the system nitrogen removal performance.

Zhang *et al.* (2018c) investigated the individual effects of four microporous organic nanoparticles (TiO_2, Al_2O_3, SiO_2, and CeO_2) and indicated the functional gene level of HzsA and the activities of three key enzymes (NIR, NXR, and HDH). No obvious inhibition effects on specific anammox activity were detected; however, the enzymatic activity of NIR and HAO was significantly inhibited after long-term exposure to NPs (Li *et al.* 2019). These NPs (Ni, Ag) retarded the normal metabolism and might displace the divalent cations in these pivotal enzymes (Xu *et al.* 2019). The HAO activity was critically reduced to 53% after long-term exposure to AgNPs at a concentration of 10 mg/L (Li *et al.* 2019).

Nitrite and ammonium are essential substrates of anammox metabolism, and they may act as potent inhibitors to the metabolic energy system. Insufficient ammonium in municipal wastewater treatment may cause anammox to grow slowly and alter the microbial community, such as the dominant bacteria changing from *Ca.* Brocadia to *Ca.* Anammoxoglobus (Gonzalez-Martinez *et al.* 2016). High pH is favorable for the formation of free ammonia and inhibits the HH activity (Maalcke *et al.* 2016). Based on metagenomics, Sun *et al.* (2018) deciphered that different ammonium concentrations in influent affected the balance between quorum quenching (QQ) and quorum sensing (QS) activities, resulting in different anammox activity and biofilm morphology.

Nitrite causes the inhibition of normal functions of cell organelles for different types of microorganisms (Meijer *et al.* 1979; Rowe *et al.* 1979; Yarbrough *et al.* 1980), which explains its importance to avoid anammox culture being exposed to NO_2^- with the absence of NH_4^+. Strous *et al.* (1999) reported that anammox deactivated at higher NO_2^- concentrations (>7.1 mM). However, the activity could be recovered when adding trace amounts of hydrazine or hydroxylamine to the culture. Dapena-Mora *et al.* (2007) showed that the 50% inhibition (IC_{50}) of anammox activity was 25 mM NO_2^- in a batch experiment. NO_2^- concentrations of more than 3.3 Mm could cause short-term inhibition to anammox bacteria with anammox activity dropping by more than 25% (Bettazzi *et al.* 2010). If pre-exposure to NO_2^- lacked NH_4^+, the IC_{50} of the anammox activity was 6.25 mM NO_2^- (Hernández *et al.* 2013).

Municipal wastewater is a source of nutrients and contains different types of organic and inorganic wastewater pollutants, which usually inhibit anammox activity. Taking the example of phenol, an organic pollutant, upon its entry into the anammoxosome, phenol molecule binds to the active sites of HZSα-haemαI and HZSγ-haemγI competing with haemαI and γI (Lyu *et al.* 2018). Binding to the active sites causes the inactivation of haemαI and γI, blocking the hydrazine synthetic reaction, further affecting the anammox metabolism and activity (Lyu *et al.* 2018). Methanol may cause different degrees of anammox activity inhibition (Baun *et al.* 2004; Guven *et al.* 2005). Toluene, with its toxic and carcinogenic potential, is related to many damages that happen on microorganisms, mainly at the level of cytoplasm membrane (Sikma *et al.* 1994). Hernández *et al.* (2013) investigated the inhibition synergistic effect of toluene and nitrite on anammox activity and demonstrated that anammox reaction was suppressed by toluene and the extent was related to its concentration, and more, the pre-exposure to toluene and NO_2^- or NO_2^- alone without NH_4^+ had a more severe inhibition than that in toluene alone.

Sometimes, the changes of organic carbon compounds in municipal wastewater can impact the biomass concentration and sludge characteristics, which is important to the enrichment of anammox bacteria. The dense structure and good settling ability of biomass could provide a long biomass retention time and a good internal anoxic environment, and then promote the growth of anammox bacteria (Ni *et al.* 2012; Winkler *et al.* 2012b). For example, anammox granular sludge exhibits a remarkable advantage of tolerance over floc sludge to fluctuation in external conditions (Ni *et al.* 2012). The anammox bacterial activity decreased as the particle size reduced (Jenni *et al.* 2014). COD affected EPS production (Li & Yang 2007), which could facilitate cells' aggregation and floc formation (Li & Yang 2007; Miao *et al.* 2016). Furthermore, some organic carbon compounds such as volatile fatty acids (VFA) in the wastewater, were discovered that are able to perform DNRA with anammox bacteria using nitrite as intermediate and VFA as electron donor (Guven *et al.* 2005; Kartal *et al.* 2007a, 2007b; Winkler *et al.* 2012a, 2012b; Shu *et al.* 2015). The VFA is not assimilated into biomass but oxidized to CO_2 via acetyl-CoA (Guven *et al.* 2005; Kartal *et al.* 2007a, 2007b; Russ *et al.* 2012), hence, low sludge production. Several studies indicated that the nitrogen removal performance of anammox was badly affected when the influent carbon source decreased to a low level. The measurement of anammox abundance and activity was strongly suggested for long-term stability at high influent organic carbon even if a stable removal performance was still obtained, since anammox bacterial abundance might be gradually decreased

(Miao *et al.* 2018). Cui *et al.* (2021) found the anammox bacteria (*Ca.* Brocadia) and endogenous denitrifying bacteria (*Candidatus Competibacter*) were abundant in a single-stage integrated fixed film activated sludge sequencing batch reactor treating real municipal wastewater with C/N ratio below 3.2. Detailed nitrogen removal mechanism analysis of a typical cycle revealed that 89.9% of TN was eliminated through the anammox pathway, meanwhile, AOB has outcompeted NOB, which all favored the synergistic effect of anammox with PN and EPD and contributed to the improvement of nitrogen removal and was a reliable and efficient alternative for mainstream anammox process.

As for IC in municipal wastewater, Shu *et al.* (2018a, 2018b) indicated that the influence of IC stresses the assemblage patterns and metabolic pathways of keystone microorganisms in the anammox system. Results confirmed that nitrogen transformation pathways were mediated by different bacterial sub-communities surviving under different levels of IC constraints. Namely, AOB and *Ca.* Brocadia plays its dominant role for nitrogen removal at lower IC/TN ratio (<0.31) situations, NOB, heterotrophic microorganism, and *Ca.* Jettenia are active at IC/TN ratio of 0.62–1.24, and *Ca.* Kuenenia prefer a higher IC/TN ratio (1.56–1.87) (Shu *et al.* 2018b).

As discussed earlier, the anammox system had a higher substrate tolerance at low temperatures (He *et al.* 2018). That is because more EPS were produced in response to higher or lower temperatures. The higher temperature accelerates enzymatic reactions while lower temperature stimulates microorganisms to secrete more EPS, which helps microbial aggregation and protect the sludge microbes. *Ca. Kuenenia* accounted for 55.18% of the microbial community and had its competitive edge against other anammox bacteria at 13 °C. However, microbial community structure changed with temperature decrease (Fernandesa *et al.* 2018). Anammox bacteria (*Ca.* Brocadia and *Ca.* Anammoximicrobium) and denitrifiers (*Burkholderiales, Myxococcales, Rhodocyclales, Xanthomonadales*, and *Pseudomonadales*) were favored when the temperature was lowered from 35 °C to 25 °C, while *Anaerolineales* and *Clostridiales* were negatively affected. Some anammox bacteria could adapt to lower temperature after short-term acclimatization, especially the dominant genus *Ca.* Brocadia which increased from 1.8% to 2.5% and its abundance was significantly correlated with nitrogen consumption ($p < 0.05$). The above findings suggest that the adaptability of *Ca.* Brocadia could provide the possibility to maintain nitrogen removal performance at a lower temperature. In spring, the improved maximum anammox activity from 2.85 to 3.23 mg NH_4^+-N/(g VSS·h) indicated the recovered removal capacity (Gong *et al.* 2020).

Different size-fractionated sludge has different microbial characterization (Liu *et al.* 2017a, 2017b). The spatial variability in the relative abundance of microorganisms was involved in nitrogen metabolism. Population segregation occurred in different size-fractionated sludge and the genus Nitrotoga was enriched only in large granules (>400 lm). *Candidatus* Brocadia and Kuenenia preferred to grow in large-sized granules (>400 lm), whereas *Ca.* Jettenia dominated in small and moderate-sized sludge (<400 lm). The members of genus *Ca.* Jettenia appeared to play a vital role in nitrogen removal since sludge with diameters smaller than 400 lm accounted for 81.55% of the total biomass.

OUTLOOK FOR ANAMMOX PROCESS RESEARCH

The anammox processes have attracted worldwide attention from diverse aspects, such as their molecular metabolism, their activities impact on the environment, and applications in wastewater treatment. Knowledge obtained from many studies will be critical for developing anammox processes for municipal wastewater treatment. The information on nitrogen removal performance and mechanism can help more and more municipal wastewater treatment operators to decide funding the technology or point out the focus points for more optimization.

In terms of wastewater treatment, the application of anammox bacteria, particularly for the elimination of municipal wastewater containing low contaminated nitrogen, relatively high organic content, and low temperature, is currently popular and promising. However, in many cases, its engineering application is confronted by many challenges, so tailor-made solutions based on insight into the technological knowledge of the metabolic mechanism of anammox bacteria are necessary.

Several important fundamental mechanism questions need to be answered in further investigations. (1) The pure cultures of anammox bacteria are an important issue, and some research will be delayed due to the lack of pure cultures. For example, when the daily treated wastewater in mainstream is too large, this would inevitably need mass biomass. This challenge could be addressed by currently existing culture methods, however, the process is still very limited due to the growth of anammox organisms, so it is necessary to conduct further research. When pure anammox bacteria cultivation, especially at room or low temperatures, is developed, the faster growth of anammox bacteria will open a new chapter into the possibilities for new designs of anammox treatment. (2) The difficulty of training anammox bacteria indicates much remains unknown about the anammox organisms. The current research on the ultrastructure of anammox organisms is limited to electron microscopy technology. It is recommended to study the ultrastructure and function of anammox combined with metagenomics and metproteomics in the future. At the same time, the difference of anammox ultrastructure and function under different water quality and operating conditions should also be examined, which will guide the application of the anammox process in sewage biological treatment. The energetics (such as bending mechanism), enzymology, and cell biology characteristics of anammoxosome require more attention and the development of greater understanding. (3) Evaluating anammox activity and identifying the responsible organism in the various ecological niches present a complex challenge in municipal wastewater treatment. New technology or highly sensitive sensors should be developed and implemented for measuring anammox activity in municipal wastewater treatment. Meantime, it remains necessary to gain an in-depth understanding of the interactions of functional bacteria, such as anammox organisms and nitrifiers, denitrifiers and dissimilatory nitrate reducers, as our current understanding of these relationships is still lacking. (4) The important orders of different factors on the microbial community assemblage and metabolic pathways of key microorganisms in anammox systems are still elusive, since it is crucial to have a clear understanding for anammox in response to different factors to benefit the functional stability and facilitate the engineering application under abiotic stresses as well.

Applied research is needed for anammox process systems into industrial use. (1) Achieving stable operation performance and process control methods for PN together with anammox is still one of the main topics in engineering applications for mainstream anammox and need to be developed or optimized, while further verification of practical applications is required. (2) For the obstacles discussed above, the evaluation of anammox performance and conventional nitrification should also be carefully tracked, not merely concentrated on nitrogen removal and energy savings, for it is necessary to determine the removal of micropollutants in anammox treatment because it is rarely known, while conventional nitrification can effectively degrade micropollutants. (3) To further increase the efficacy of the anammox process applied in municipal wastewater, its robustness against shock loads and potentially negative effects of municipal wastewater compositions must be explored.

Anyway, while challenges remain, anammox treatment has received a great deal of attention and offered great possibility for municipal wastewater treatment with desired nitrogen removal and significant energy savings. For now, besides PNA, sustainable PDA seems a more advantageous option towards large-scale mainstream anammox treatment of municipal wastewater from the perspectives of municipal wastewater complexity. Considering the implementation of the PDA process, which is challenging, the application of PDA in municipal WWTPs is proposed to be realized by retrofitting or upgrading the A^2O process. A^2O is a ubiquitous process in Chinese municipal WWTPs and the configuration is similar to PDA. Accordingly, to reduce energy input and operational cost as well as improve nitrogen removal performance, the current solution, integrating PDA with upgraded A^2O system and implementing PDA is proposed. PDA was recently reported to contribute to the nitrogen removal (15–30%) in a full-scale A^2O process for municipal wastewater treatment (such as the Fourth Municipal Wastewater Plant, Xi'an, China), suggesting a possibility of retrofitting the A^2O process to PDA. In the future, more studies on PDA will lead to a more delicate and successful application of mainstream PDA treating municipal wastewater.

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DATA AVAILABILITY STATEMENT

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

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