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Realizing stable operation of anaerobic ammonia oxidation at low temperatures treating low strength synthetic wastewater

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ABSTRACT

The low activity of Anammox bacteria at low temperatures and competition from nitrite oxidation bacteria (NOB) when treating low strength wastewater have been major bottlenecks in implementing Anammox in mainstream wastewater treatment. By intermittent high strength feeding (IHSF) and stepwise temperature reduction, stable operation of a granular Anammox reactor was realized at low temperatures (down to 15°C) for 28 days when treating low strength synthetic wastewater. The nitrogen loading rate reached 1.23–1.34 kg N/m^3/day, and the total nitrogen removal rate reached 0.71–0.98 kg N/m^3/day. The IHSF enriched the Anammox sludge in high strength cycles and compensated for sludge loss in low strength cycles, and the high concentration of ammonium in high strength cycles inhibited NOB. The 16SrRNA gene sequencing results revealed that *Candidatus Kuenenia* was predominant in the reactor at low temperatures.

Introduction

Nitrogen pollution can lead to serious eutrophication of water bodies. Along with gradual implementation of stricter pollution standards, treating urban sewage to meet the standards will encounter more difficulties and the cost will be increased accordingly, and the control of nitrogen is the key to influencing the quality of effluent.

The traditional biological nitrogen removal process for wastewater treatment was developed from nitrification and denitrification processes in the 1960s and further developed and derived in the past few decades, and has become the current mainstream process because of its stable and reliable nitrogen removal performance. At the same time, however, it consumes a vast amount of energy; whereas the anaerobic ammonium oxidation (Anammox) process, found in the 1990s, can remove both ammonia nitrogen (NH_4^+-N) and nitrite nitrogen (NO_2^--N) simultaneously along with the generation of nitrogen (Strous et al., 1999). Anammox, in the current climate of promoting resourceful and smart production, is regarded by the industry as one of the most promising technologies to solve nitrogen pollution in the future because of its advantages such as small aeration volume, lack of need for an external carbon source, and small amount of sludge generation. At the first decade of the 21st century, Anammox has been applied at full scale for dissolved nitrogen treatment on reject water, landfill leachate and the supernatant of anaerobic digestion. However, the application of the Anammox process in the main stream of municipal wastewater is considered to be a major challenge due to the low temperature, low substrate concentration and consequently low sludge enrichment (Stewart et al., 2017). The Strass wastewater treatment plant became

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known as a net energy positive plant because of its application of mainstream Anammox. A hydrocyclone classifier was used to separate biomass from the supernatant of anaerobic digestion treatment, to seed it into the mainstream. This method enhanced the mainstream biological nitrogen removal system efficiently (Wett et al., 2013). Despite some still-remaining challenges to extending the application of Anammox to more wastewater treatment plants (WWTPs) for biomass retention and removal efficiency in the mainstream environment, Anammox showed its strong potential for mainstream nitrogen removal.

The optimum temperature of Anammox is between 30 and 37°C (Strous et al., 2006). Therefore, currently, Anammox is mainly applied under high temperature (32–37°C) for treatment of anaerobic digestion supernatant and other high ammonia nitrogen wastewater (800–3000 mg/L) (Ma et al., 2011) to achieve stable operation of anaerobic ammonium oxidation. Nowadays, mainstream Anammox has attracted wide attention due to its highly efficient nitrogen removal and low operating costs. However, the low activity of Anammox bacteria at low temperatures and competition from nitrite oxidation bacteria (NOB) when treating low strength wastewater have been major bottlenecks in implementing Anammox in mainstream treatment (Cao et al., 2017).

The sequencing batch reactor (SBR) has been recognized as a promising technique for full-scale Anammox, according to a study (Lackner et al., 2014) that surveyed over 100 Anammox treatment plants and concluded that more than half of them were using SBR. Among them, 75% of these practical treatment plants realized granular systems during operation. In another study (Lackner et al., 2014), the Specific Anammox Activity (SAA) of eight surveyed full-scale Anammox SBRs ranged from 71 to 155 g-N/kg-Volatile Suspended Solid (VSS)/day. Under a high strength atmosphere for Anammox inoculum, Up-flow Anaerobic Sludge Bed (UASB) may lead to more impairment of the sludge system than SBR due to its limitation on the range of concentration. Normally, the higher the SAA of inoculum that can be reached, the better removal capacity and more stable operation will be realized. A study (Malovanyy et al., 2015) investigated the impact of differences in influent strength. During 157 days of operation at 20°C, due to the switch of influent, the SAA in the moving bed biofilm reactor (MBBR) was diminished from 0.062 to 0.009 g-N/g-VSS/day, which means only one fifth the strength of the initial influent. As a result, part of the Anammox biomass died off because the substrate, which was the nutrition for the biomass, was not sufficient.

The impact of even lower temperatures on Anammox was also studied with low strength influent. Some researchers (Lotti et al., 2014) studied one-step nitritation-Anammox at 10°C and 60 mg-NH_4^+-N/L using SBR and reached 39% total nitrogen removal efficiency. Another researcher (Gilbert et al., 2014) realized similar reactor performance by using a one-step nitritation-Anammox MBBR at 50 mg-NH_4^+-N/L with the temperature being decreased from 20 to 10°C. The nitrogen removal efficiency was only approximately 14% even when doubling the hydraulic retention time (HRT) to ensure no accumulation of nitritation, which indicated that the conventional treatment techniques are still not mature to realize mainstream Anammox under low temperature and low strength nutrition.

In this study, two strategies *i.e.*, intermittent high strength feeding (IHSF) and stepwise temperature reduction (STR) were applied to acclimate the sludge to lower temperature and low strength wastewater. The main objective of this study was to investigate the feasibility of these two acclimation strategies and to reveal how the microbial communities respond to lower temperatures and low strength wastewater. This study provided useful strategies to improve the performance of the mainstream Anammox process.

1. Materials and methods

1.1. Synthetic wastewater influent

The synthetic wastewater influent consisted of NH_4Cl 360 mg N/L, $NaNO_2$ 440 mg N/L, KH_2PO_4 15 mg P/L, $CaCl_2$ 300 mg/L, $MgSO_4{\cdot}7H_2O$ 200 mg/L, $NaHCO_3$ 400–800 mg/L. The trace element solutions I and II were both 1.25 mL/L (Li et al., 2016), and composed of: 0.05 g $NaWO_4{\cdot}2H_2O$; 0.25 g $CuSO_4{\cdot}5H_2O$; 0.22 g $Na_2MoO_4{\cdot}2H_2O$; 0.43 g $ZnSO_4{\cdot}7H_2O$; 0.24 g $CoCl_2{\cdot}6H_2O$; 0.99 g $MnCl_2{\cdot}4H_2O$; 0.19 g $NiCl_2{\cdot}6H_2O$; 0.1076 g Na_2SeO_4; 0.014 g H_3BO_3; 15 g EDTA. The proportion of NH_4^+-N to NO_2^--N was 1:1.22 (360 mg N/L: 440 mg N/L), to ensure that NH_4^+-N was in excess and to avoid NO_2^--N inhibition. Considering that the research was carried out in an SBR, IHSF could be controlled by adjusting the discharge ratio with the same influent. During high strength cycles, the discharge ratio was kept at 0.3. This ensured that the initial substrate concentration of total nitrogen (TN) was larger than 240 mg N/L. In low strength cycles, however, the discharge ratio was 0.08 and the initial substrate concentration was at least 64 mg N/L. The influent was aerated with nitrogen gas for 10 min to remove dissolved oxygen in the water before use.

1.2. Reactor configuration and operational conditions

A granular Anammox SBR with working volume of 5 L, as shown in Fig. 1, was used to conduct this experiment. The inner diameter and height of the reactor were 10 and 70 cm, respectively. The reactor was wrapped with a thermostatic water jacket to control the temperature. The reactor was operated in cycles consisting of 4 phases: feeding (2–7 min), reaction (30–80 min), settling (3–7 min) and decanting (3–9 min). IHSF was realized by adjusting the discharge ratio, which is described in Section 2.1. After 15–27 cycles of low strength cycles, the influent was switched to high strength for one cycle and ended at the decanting period of the high strength cycle. To maintain the volume of the reactor, the decanting period was precisely controlled to ensure that the total volume was 5 L after the next feeding phase. Then the whole cycle was repeated starting with a low-strength cycle.

The granular Anammox SBR had already run successfully for 93 days before the experiment and its sludge was in granular form. The operating temperature was 25 ± 1°C and the draining ratio was 0.3 (Zhao et al., 2014a). The average total nitrogen removal rate was 7.33 kg/m^3/day and the average total nitrogen removal efficiency was 84.9%. Considering that NH_4^+-N

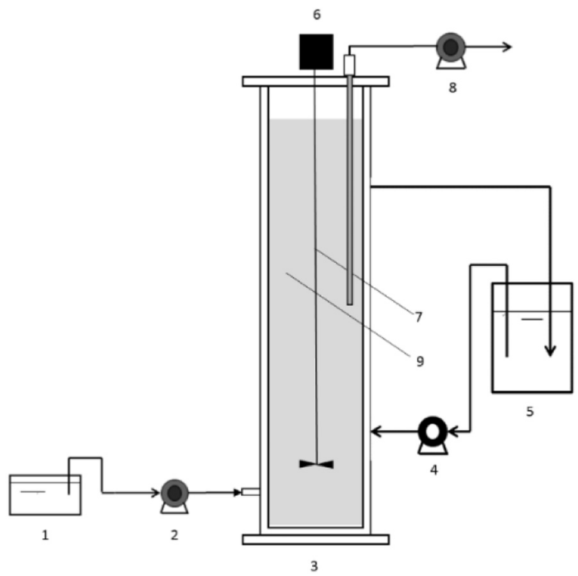

Fig. 1 – Schematic diagram of the granular Anammox Sequencing Batch Reactor. (1) inlet tank; (2). inlet peristaltic pump; (3) reactor; (4) water bath peristaltic pump; (5) water bath pot; (6) mixer; (7) stirring paddle; (8) outlet peristaltic pump;(9) reaction zone.

was in excess in the water, the removal rate was close to the theoretical maximum value. For 101 days of operation, STR was realized by gradually decreasing the temperature from 21 to 15°C, at a rate of 2°C approximately every 3 weeks. During the experiment, the nitrogen loading rate (NLR) was increased by adjusting the hydraulic retention time to ensure that the NO_2^--N in the reactor was not insufficient or excessive.

1.3. Analysis and testing methods

In this study, NLR (kgN/m^3/day) was defined as

$$NLR = \frac{24\Delta VC_{TN,inf}}{1000TV} \quad (1)$$

Nitrogen removal rate (NRR, kgN/m^3/day) was defined as

$$NRR = \left(\frac{\Delta VC_{TN,inf}}{V} - C_{TN,eff}\right)\frac{24}{1000T} \quad (2)$$

SAA (g-N/g-VSS/day) was defined as

$$SAA = \frac{NRR}{VSS} \quad (3)$$

where ΔV (L) refers to the draining volume, V (L) refers to the working volume of the granular Anammox SBR, $\frac{\Delta V}{V}$ refers to the discharge ratio, which is 0.08 under a low strength cycle and 0.3 under a high strength cycle, $C_{TN,inf}$ (mg N/L) refers to the total nitrogen concentration of the influent, $C_{TN,eff}$ (mg/L) refers to the total nitrogen concentration of the effluent, T (hr) refers to the SBR period, VSS (g/L) refers to volatile suspended solid.

The biomass samples were taken from a depth of 30 cm (from the bottom to the top) of the previously introduced reactor during its stirring, to ensure the homogeneity of the sample. The concentrations of ammonia, nitrite, nitrate and VSS were measured according to the standard methods (Clesceri et al., 2005). VSS was tested twice a week. SAA of high strength cycles and low strength cycles were determined as the average sludge loading rate. Temperature and pH values were measured using a HACH HQ30d instrument. The high-throughput sequencing of PCR amplicons was performed on the Illumina MiSeq platform. Primers 515F(5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) of the V4 amplification area were used. Comparative analysis of the COGs (Cluster of Orthologous Groups of functional genes) was performed by using the 16S sequencing approach to detect the microbial community structure and predict potential functional genes on clusters according to the functional gene database FunGene from RDP (The Ribosomal Database Project) (Fish et al., 2013).

2. Results and discussion

2.1. Performance of granular Anammox SBR at low temperatures

The nitrogen removal performance of the granular Anammox SBR was investigated for 101 days. As shown in Fig. 2, in the first period of each stage, the nitrogen removal was not stable. The duration of the stabilization period ranged from 3 days at 21°C to nearly 3 weeks at 15°C. The signal of stabilization was that the nitrogen removal rate would not drastically change. The removal rates of NH_4^+-N, NO_2^--N and TN at 21°C were 85.2% ± 5.0%, 91.1% ± 5.5% and 75.8% ± 4.4%. When the temperature dropped to 19°C, the system showed stability as the removal rates were maintained at 84.7% ± 4.4%, 90.8% ± 5.0% and 76.0% ± 5.3% correspondingly. After 23-day operation, the temperature was dropped to 17°C, and the removal rates of NH_4^+-N, NO_2^--N and TN were 81.7% ± 5.6%, 88.7% ± 6.2% and 72.8% ± 6.1%. It should be noticed that the drop in temperature resulted in nearly 60% diminishment in removal before stabilization. To avoid nitrite accumulation, HRT was doubled compared to that to 20°C. The decline of the removal rate was acceptable, but it took more than 4 weeks to stabilize. The last phase was at 15°C. The removal rates dropped to 77.4% ± 5.5%,

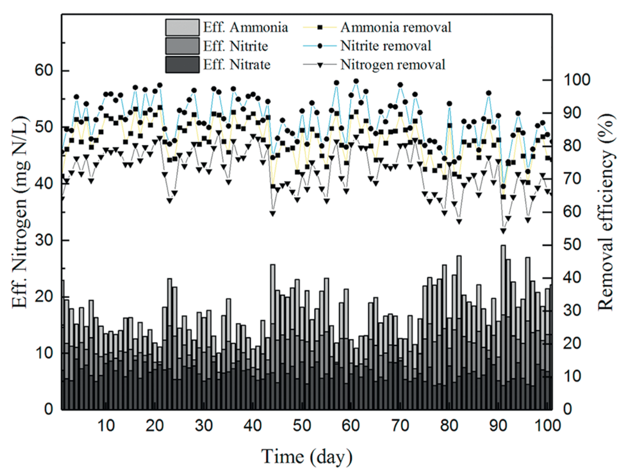

Fig. 2 – Effluent concentration of ammonia, nitrite, and nitrate and nitrogen removal rate of the reactor.

82.8% ± 6.2% and 66.4% ± 5.6%. Compared with the performance of 21°C, the TN removal rate dropped 12%.

Volatility of the removal rate after sudden decreases in temperature was found by other researchers (De Cocker et al., 2018; Park et al., 2017). The common view of the reason for this phenomenon was stabilization at the very beginning. When the stage lasted for more than three weeks, however, the nitrogen removal rate gradually returned to normal levels. The NH_4^+-N, NO_2^--N and TN removal rate were stable at 78.9%± 2.5%, 83.5%±1.9% and 67.0%±1.5% at 15°C, respectively. Compared with the above-mentioned reported research studies (De Cocker et al., 2018; Park et al., 2017), the stabilization period of which ranged from day 63 to day 151, this study showed superiority in terms of the relatively high efficiency of stabilization. This indicated that the microbial community gradually acclimated to lower temperatures.

The sudden reduction in removal rate could be explained by the reduced enzyme activity at low temperature. The activation energy of the Anammox reaction was 93–94 kJ/mol, while the activation energy in water treatment is usually between 8.37–83.68 kJ/mol (Isaka et al., 2008). Similar results were also reported (Lotti et al., 2015) at 20°C. The growth temperature and optimal temperature of different species of Anammox vary (Gilbert et al., 2014; Tomaszewski et al., 2017). The difference in the observed removal rate could be explained by shifting of the microbial community structure (Isanta et al., 2015), which is discussed in Section 2.3.

Fig. 3 shows that, in low strength cycles, the SAA decreased from 0.191 ± 0.018 to 0.112 ± 0.011 g-N/g VSS/day when the temperature dropped from 21 to 15°C. The approximately 42% decrease in activity could be attributed to the low temperature. However, the SAA increased a considerable amount in the high strength cycle. For instance, at 15°C, the SAA of the high strength cycle was 0.190 ± 0.015 g-N/g VSS/day, which was 73% higher than the SAA of the low strength cycle. Anammox biomass remained stable above 17°C. When the temperature dropped to 15°C, biomass loss was observed. The Anammox biomass declined from 8.74 to 7.01 g/L when the temperature dropped from 17 to 15°C for 28 days. This indicated that, above 17°C, the system was sustainable due to its gradually increasing biomass. However, when the temperature was lower than 15°C, the system tended to fail eventually. By using the IHSF operation strategy, the high strength cycle could enhance the biomass growth and enable sustainable operation under 17°C.

For comparison, Fig. 4 shows the NLR and NRR of high strength cycles and low strength cycles at different temperatures. At 15°C, the reactor was operated for 28 days, and the NLR under low strength reached 1.23–1.34 kg N/m^3/day. The NRR reached 0.71–0.98 kg N/m^3/day, and it could increase to 1.59 kg N/m^3/day in the high strength cycle. Other reported studies of Anammox at moderate temperatures or under low strength operation have been listed in Table 1. A review (Tomaszewski et al., 2017) comparing several research studies reported stable operation treating low strength wastewater under moderately low temperature with relatively high nitrogen removal performance. A remarkable NRR value of 8.1 kg N/m^3/day at 20–22°C was reported in an anaerobic biological filtered reactor with 20 g VSS/L (Isaka et al., 2007). A recent study (Guillén et al., 2016) reached 0.038 kg N/m^3/day with SAA of 0.029 g-N/g VSS/day with an SBR after more than 959 days of operation of STR from 30.5°C to 14.7°C, which indicated the significance of IHSF applied in this study on SAA (0.112 g-N/gVSS/day), NRR (0.98 kg N/m^3/day) and stabilization period (101 days). Another research study (Laureni et al., 2015) treated low strength wastewater at even lower temperature, 12.5°C, with NRR around 0.04 kg N/m^3/day by SBR. The record for highest NRR, 6.6 kg N/m^3/day, was made at 9.1°C with Anammox addition once every two days (Jin et al., 2013), and was impossible to stably operate alone. The lowest temperature was reported by Isaka et al. (2008) at 6.3°C by using biomass entrapped on a carrier to reach 0.36 kg N/m^3/day, which illustrated the superiority of biofilm in terms of tolerance and resistance.

Even though the performance in this study approaches the best reported performance in an SBR, it is still inferior to performance achieved with an Membrane Bio-Reactor (MBR) (Awata et al., 2015) and UASB (Ma et al., 2013). The limitation to raising the NRR of Anammox in the SBR is the retention of

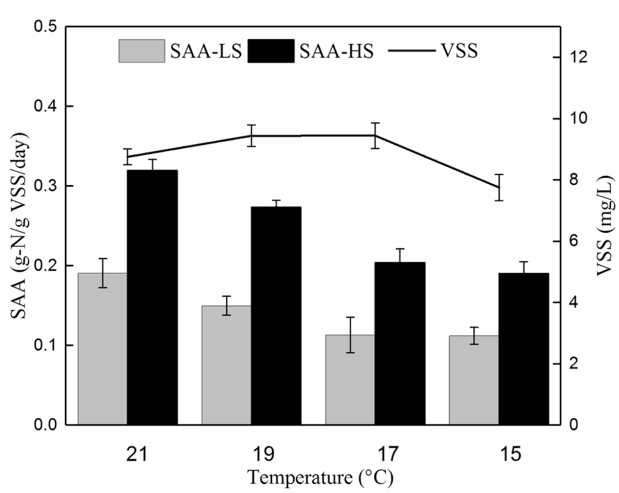

Fig. 3 – Specific Anammox activity (SAA) and volatile suspended solids (VSS) in high strength cycles and low strength cycles under different temperatures.

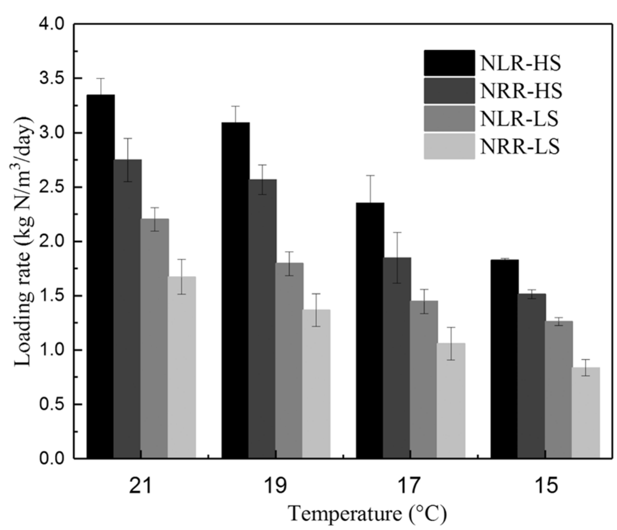

Fig. 4 – the nitrogen loading rate and nitrogen removal rate of high and low substrate periods under various temperatures.

Table 1 – Research development of Anammox under moderate temperature and low substrate.

Biomass	Reactor	Temperature (°C)	Initial substrate concentration TN (mg N/L) [a]	Biomass concentration (g VSS/L)	NLR (kg N/m^3/day)	NRR (kg N/m^3/day)	Reference
Entrapped on carrier	ABF	20–22	170–300	20	nd	8.1	(Isaka et al., 2007)
Granular	UASB	27–30	~46	4	0.47	0.4	(Ma et al., 2011)
Granular	SBR	13.2	~25	~1.3	0.032	0.029	(Guillén et al., 2016)
Granular	Gas-lift	20	69	1.0–2.6	0.31	0.29	(Hendrickx et al., 2012)
Suspended	Gas-lift	10	61	0.51	nd	0.02	(Hendrickx et al., 2014)
Suspended	SBR	12.5	5–20	~0.5	nd	0.046	(Laureni et al., 2015)
Suspended	MBR	15	123	8.0	2.2	1.1	(Awata et al., 2015)
Granular	UASB	16	51	4.89	3.2	2.28	(Ma et al., 2013)
Granular	UASB	10	210–280	15.05	13.1	5.7	(Jin et al., 2013)
Granular	SBR	15	64	7	1.23	0.98	this study

ABF: anaerobic biological filtrated reactor; MBR: membrane bioreactor; SBR: sequencing batch reactor; UASB: up-flow anaerobic sludge blanket; nd-not discussed.

[a] Here "Initial substrate concentration" was the lowest practical concentration of TN where Anammox survived.

biomass. With membrane isolation or a triphase separator, MBR and UASB can effectively discharge without any influence on the reaction. However, for biomass accumulation, the settling period significantly increased from 2 to 7 min when decreasing the temperature from 25 to 15°C. Thus, the durations of the non-reaction phases were non-negligible for low strength cycles, whose reaction period was short but still needed a relatively fixed time for feeding, settling and decanting. Moreover, the fixed non-reaction phases would have more significant negative influence on reactor performance at low temperature and low strength. It could be expected that IHSF and STR strategies will be useful for other kinds of reactors or biomass.

2.2. Inhibition of NOB in high strength cycles

NOB can compete with Anammox for NO_2^--N substrates. Thus, selective inhibition of NOB is another key success factor for stable operation. The effect of NOB inhibition in this study is shown in Fig. 5. In low strength cycles, nitrate significantly accumulated and the nitrate production rate (NPNR) was slightly above the theoretical value of 0.11 according to the Anammox process stoichiometry (Strous et al., 1998); while in high strength cycles, the NPNR was close to 0.11. This indicated that the high influent concentration in high strength cycles provided a short-term high ammonium concentration and inhibited the NOB, a major competitor of Anammox.

Free ammonia has been commonly used to inhibit NOB in Anammox reactors to avoid competition between Anammox and NOB. The inhibitory concentration of Free Ammonia (FA) for NOB was found to be 0.1–1.0 mg/L (Cho et al., 2011) while Anammox did not show inhibition in the presence of 1000 mg/L of NH_4^+-N (Strous et al., 1999). A theoretical calculation showed that under the conditions of medium-low temperature (15°C) (Anthonisen et al., 1976) and pH in the range 6–8, FA accounted for about 2.64%–2.71% of the total NH_4^+-N. In other words, when the concentration of NH_4^+-N was 10–40 mg/L in the low strength cycle, FA would not be adequate to inhibit NOB activity, but the concentration of NH_4^+-N in the high strength cycle, which was larger than 108 mg/L, could provide a selective inhibitory environment against NOB once per day. Considering that the reactor was at a high substrate concentration period every day, over a period of more than 100 days, no NOB adaptation effect was detected, and it can be considered that IHSF is a promising method to inhibit NOB in long-term operation.

2.3. Dynamics of microbial community

16S rRNA gene sequence information for the bacteria in granular sludge was determined by high-throughput sequencing to study the changes in the microbial community structure in the process of reactor operation. A total of 26 categories of bacteria were detected. Among these, the ones with relative abundance more than 1% included Proteobacteria, Chloroflexi, Ignavibacteriae, Bacteroidetes, Planctomycetes and Acidobacteria,

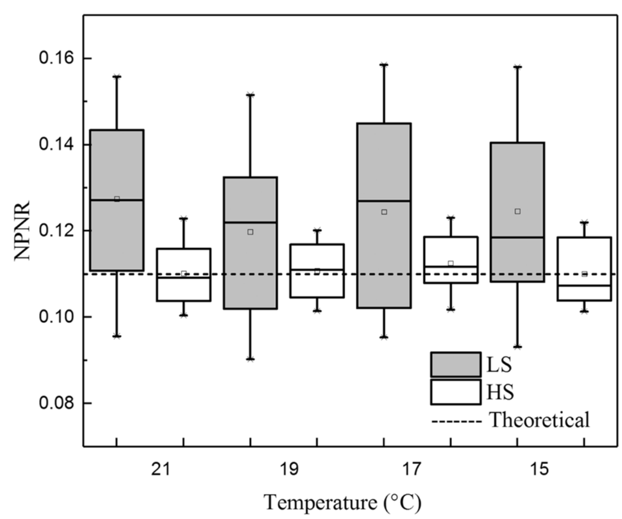

Fig. 5 – The molar ratio of nitrate production to ammonia and nitrite removal (NPNR) in high strength cycles and low strength cycles under different temperatures.

as shown in Fig. 6. Overall, the composition at the phylum level was stable during the whole experiment period.

In the phylum Planctomycetes, which Anammox is assigned to, only *Candidatus Brocadiaceae* was found, which is one of the 5 species of Anammox. In such genus, the relative abundance of *Candidatus Kuenenia* was above 99% at all temperatures. The other Anammox detected was *Candidatus Brocadia*, whose relative abundance within the genus was no more than 0.5%, indicating that the community structure of Anammox bacteria did not change with decreasing temperature. As temperature decreased, the relative abundance of Planctomycetes showed a decreasing trend.

Fig. 7 shows the relative abundance of Anammox (*Candidatus Kuenenia*), AOB (*Nitrosomonas*) and NOB (*Nitrospirae*) at each temperature. The relative abundance of *Candidatus Kuenenia* decreased from 9.83% to 4.91%, which was a 50.1% drop, as the temperature was reduced from 25 to 15°C, more than the decrease of SAA under the two different nutrient strength cycles, which were 40.6% and 41.1% respectively. NOB has higher activity than AOB under low temperature (Hellinga et al., 1998). Compared to Anammox, the sequence number of AOB was not significantly affected by the temperature change and NOB was constantly kept at a low level, which proved that the IHSF strategy has a significant effect on NOB inhibition.

Within the genus level of Anammox, *Candidatus Brocadia* and *Candidatus Brocadia fulgida* have been mainly reported at low temperature (6–15°C) (Awata et al., 2015; Hendrickx et al., 2014; Laureni et al., 2015; Lotti et al., 2015), while *Candidatus Kuenenia stuttgartiensis* was observed at 25–45°C (Isaka et al., 2008). Gradual adaptation may take place at low temperatures simultaneously. Though it was not verified here, Anammox has been observed to be able to alter its lipid membrane to adapt to temperature (Rattray et al., 2010). Other research (Gilbert et al., 2015; Isaka et al., 2008; Mancuso Nichols et al., 2004) also indicated that the dominant species of Anammox may have the ability to adapt to extreme temperature within a reactor.

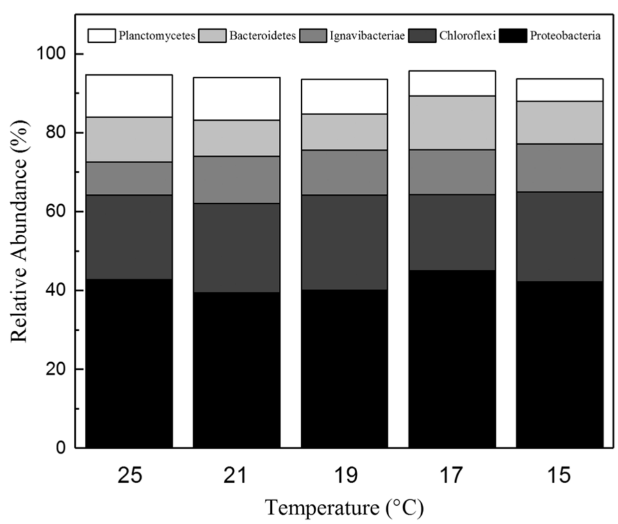

Fig. 6 – Relative abundance of major phyla in the reactor under different temperatures.

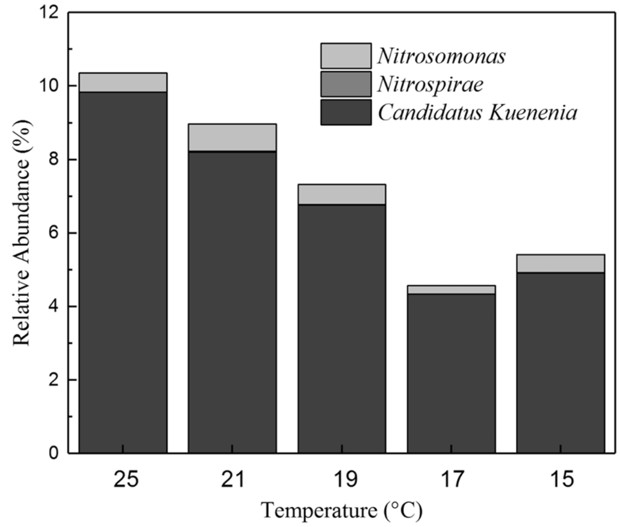

Fig. 7 – Relative abundance of *Candidatus Kuenenia* (Anammox), Nitrosomonas (AOB) and Nitrospirae (NOB) under different temperatures.

Fig. 8 shows a predicted cumulative function curve based on COGs (Cluster of Orthologous Groups), which can be used to analyse functional gene abundance. Here it should be noticed that the results were based on predictions mentioned in the Material and Methods section. The horizontal axis represents the number of samples, which is incremented stepwise from 25 to 15°C. The vertical axis indicates the number of sampled COG ID. The blue line in the figure represents the union set of the samples and the green line means the intersection of the samples. Within a certain range, as the sample size increases, if the blue curve indicates a sharp increase, and the green curve indicates a sharp decrease, then this indicates that a large number of new functional genes could be found in the microbial community of the newly-added sample. When the two curves are close to being smooth, this indicates that the functional genes in this experiment do not increase significantly with additional sample added.

When the temperature was reduced from 25 to 19°C, new functions of the microbial community appeared in large

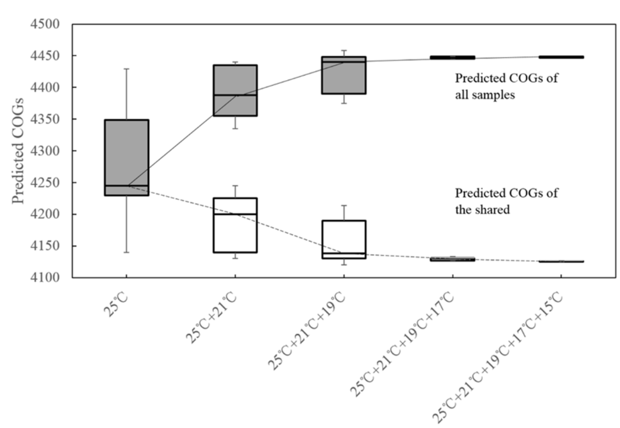

Fig. 8 – Predicted Cluster of Orthologous Group (COG) function accumulation curves

numbers. However, when the temperature gradually reduced from 19 to 15°C, the new functional gene growth trend was slow. It could be speculated that comparing operation at 19°C with operation at 25°C, a significant difference was shown in microbial metabolism, and more new functional genes were needed by the microbial community for adapting to the low temperature. However, in terms of operating at 15°C, no significant increase in functional genes was detected. In other words, the adaptability of the microbial community did not sufficiently react anymore when temperature dropped further, and its metabolism would be similar to operation at 19°C. It could be inferred that microorganisms need corresponding functional genes to adapt to low temperatures when facing a moderate temperature decrease, which occurs when the temperature is reduced from 25 to 19°C. However, when the temperature dropped beneath the limit of tolerance or adaptation, no more functional genes would emerge. This result was similar to the research results on the resistance of Anammox toward heavy metals and other harmful substances (Zhao et al., 2014b). When the community was no longer evolving to become more versatile, the sludge load tended to be stable, which was in accordance with the SAA tendency under different temperatures shown in Fig. 3.

3. Conclusions

Using intermittent high influent concentration feeding and stepwise temperature decrease, stable operation of granular Anammox was realized at low temperatures (down to 15°C) in the treatment of low strength synthetic wastewater. After 3-week acclimation at 15°C, the NRR reached 0.82 ± 0.03 kg N/m^3/day under the NLR of 1.23 kg N/m^3/day. The NOBs were successfully inhibited and did not show adaptation in a low FA environment in long-term operation. The 16SrRNA gene sequencing results revealed that *Candidatus Kuenenia* was predominant in the reactor and gradually adapted to the lower temperatures.

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