Nitrogen removal performance and operation strategy of anammox process under temperature shock

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Abstract Sequencing batch reactors were used to study anaerobic ammonium oxidation (anammox) process under temperature shock. Both long-term (15–35 °C) and short-term (10–50 °C) temperature effects on nitrogen removal performance were performed. In reactor operation test, the results indicated that ammonium removal rate decreased from 0.35 kg/(m³ day) gradually to 0.059 kg/(m³ day) when temperature dropped from 35 to 15 °C. Although bacteria morphology was not modified, sludge settling velocity decreased with decreasing temperature. In batch test, apparent activation energy (E_a) increased with decreasing temperature, which suggested the activity decrease of anaerobic ammonium oxidizing bacteria (AAOB). Low temperature inhibited AAOB and weakened nitrogen removal performance. The cardinal temperature model with inflection was first used to describe temperature effect on anammox process.

Simulated results revealed that anammox reaction could occur at 10.52–50.15 °C with maximum specific anammox activity of 0.50 kg/(kg day) at 36.72 °C. The cold acclimatization of AAOB could be achieved and glycine betaine could slightly improve nitrogen removal performance at low temperature.

Keywords Anammox · Temperature shock · Kinetics · Apparent activation energy (E_a) · Operation strategy · Glycine betaine

Introduction

With unique cell structure and ability to remove ammonium and nitrite simultaneously, anaerobic ammonium oxidation bacteria (AAOB) have broadened biological nitrogen cycle process (Schleper 2008). However, as an efficient and cost-effective technology to treat wastewater, anaerobic ammonium oxidation (anammox) still faced some problems like low temperature. Most of full-scale anammox reactors reported in literatures were operated at a temperature exceeding 25 °C (Dosta et al. 2008; van der Star et al. 2007; Zhang et al. 2016). As a result, wastewater must be heated to required temperature. This increased treatment cost undoubtedly and weakened its commercial application.

As one of the most important factors, temperature affected activity and growth of AAOB. Although anammox process could occur in a wide range of temperatures (6–43 °C) (Kartal et al. 2010), the

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optimal temperature for AAOB was acquired not the same, e.g., 40 °C acquired by Jetten et al. (1998), 35–40 °C acquired by Dosta et al. (2008) and 37 °C acquired by Isaka et al. (2008). Still, anammox process would perform very well at 30–40 °C (Egli et al. 2001; Strous et al. 1999), in which anammox activity would not be affected by temperature. However, the temperature of wastewater was generally below 25 °C and varied with outer surroundings (de Graaff et al. 2011; Guo et al. 2015). This was much lower than optimum temperature of AAOB. As a result, the main challenge to apply anammox process widely was to achieve high nitrogen removal performance at low temperature.

Recently, AAOB was identified in some harsh environments, such as deep-sea hydrothermal vents (Byrne et al. 2009) and Arctic sea ice (Rysgaard and Glud 2004). This proved that AAOB had activity among a wide temperature range. Although good nitrogen removal performance has been reported under moderately low temperatures of 20–12 °C (Isaka et al. 2007), significant drop of specific anammox activity (SAA) was found (Dosta et al. 2008; Lotti et al. 2014a, b).

The efficient and stable anammox process at low temperature would extend its application potential to treat municipal sewage and other industrial wastewater (Lotti et al. 2014a, b). Therefore, it was significant to study operation strategy at low temperature. Compatible solute was defined as small and soluble organic molecule which could occur at high intracellular concentration without hindering normal cellular metabolism (Brown 1976). As an ordinary compatible solute, glycine betaine was typically used to reduce toxicity resulting from high salinity (Vyrides and Stuckey 2009). As a kind of cryoprotectant, glycine betaine also had protective effect to against freeze thaw treatment of cell and enzyme (de Costa et al. 1998). Vyrides et al. (2010) indicated that 1 mM glycine betaine could be beneficial anaerobic biomass performance (CH_4 production) at 20 °C. Cleland et al. (2004) also found that glycine betaine was an effective cryoprotectant for prokaryotic organism through holding cell membrane fluidity and protecting cellular protein from cold aggregation. However, addition of glycine betaine on nitrogen removal performance of anammox under low temperature was studied little.

In this work, short-term effect of temperature on anammox was studied through batch test while long-term effect of temperature on anammox process was evaluated by continuous-flow test. Besides, four

kinetic models were used to simulate the effect resulting from temperature shock. Some of them were first used in anammox process. In addition, operation strategy to improve anammox activity under low temperature was also investigated.

Materials and methods

Inoculation and synthetic wastewater

The seeding sludge was inoculated from a lab-scale sequencing batch reactor (SBR) operated over 1 year at 35 °C. The digital and microscopic pictures of the sludge were shown in Fig. 1. Influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations of lab-scale SBR were 80 and 105 mg/L, respectively. Both removal efficiencies were more than 99%. The sludge was bright red with good settling property. The mixed liquid suspended solid (MLSS) concentration inoculated in SBR was 1.61 g/L. The diameter of granular sludge was 0.5–5.0 mm with a mean value of 1.72 mm. Synthetic wastewater was used in this work and its composition was presented in Table 1.

Long-term effect of temperature

Three SBRs (R1, R2 and R3) were operated in parallel during long-term operation. The working volume of each reactor was 1.5 L. Each reactor was inoculated with 1 L anammox sludge. All reactors were covered completely with aluminum caps to avoid the growth of photosynthetic microorganisms. The produced gas was discharged via porthole on the top of the reactor. The complete mixture inside the reactor was achieved through mechanical stirrer with rotating speed of 100 rpm. N_2 (99.99%) was purged into influent in order to maintain anaerobic condition. Each reactor was sparged by N_2 for 10 min before the sludge was inoculated into it. The initial pH was 7.5 by adding 1.0 mol/L hydrochloric acid or sodium hydroxide. The ammonium loading rate (ALR) and nitrite loading rate (NLR) were kept at $0.36 \pm 0.04 \text{ kg}/(\text{m}^3 \text{ day})$ and $0.44 \pm 0.05 \text{ kg}/(\text{m}^3 \text{ day})$, respectively.

Temperature of the reactor decreased gradually from 35 to 15 °C (35, 30, 25, 20 and 15 °C) during the operation. The reactors were operated at least 9 days at each temperature. Hereafter, R1 was analyzed for recovery performance of anammox process. R2 was

Fig. 1 Image of inoculated granular sludge

Table 1 Composition of synthetic wastewater

Compound	Concentration (g/L)	Compound	Concentration (g/L)
KH ₂ PO ₄	0.028	NH ₄ Cl	0.080
CaCl ₂	0.14	NaNO ₂	0.105
KHCO ₃	2.0	Trace element solution I	1.1 mL/L
MgSO ₄ ·7H ₂ O	0.2	Trace element solution II	1.1 mL/L

Trace element solution I: EDTA 5 g/L, FeSO₄·7H₂O 5 g/L

Trace element solution II: EDTA 15 g/L, H₃BO₃ 0.011 g/L, MnCl₂·4H₂O 0.99 g/L, CuSO₄·5H₂O 0.25 g/L, ZnSO₄·7H₂O 0.43 g/L, NiCl₂·6H₂O 0.19 g/L, (NH₄)₆Mo₇O₂₄·4 H₂O 0.16 g/L, CoCl₂·6H₂O 0.24 g/L, Na₂SeO₃·5H₂O 0.1596 g/L

continuously operated at 15 °C for more than 50 cycles. R3 was carried out at 20 °C to analyze the effect of glycine betaine under low temperature.

Short-term effect of temperature

Batch tests between 15 and 50 °C (15, 20, 25, 30, 35, 40, 45 and 50 °C) were carried out to investigate the short-term effect of temperature on SAA. Completely closed vials with a liquid volume of 100 mL were used and influent NH₄⁺-N and NO₂⁻-N concentrations were 80 mg/L and 105 mg/L, respectively, with NH₄Cl and NaNO₂ as the sources. At the initial of the test, the sludge concentration was 1.61 g/L. Influent pH was set at 7.5 and N₂ was purged into reactors to remove O₂. NH₄⁺-N and NO₂⁻-N were determined at regular intervals, and SAA was calculated by the ratio of maximal substrate consumption rate to sludge concentration (Qiao et al. 2014).

Kinetic analysis

Arrhenius model (Eq. 1) has been widely used to describe the effect of temperature on biological

activity, and the apparent activation energy (E_a) was calculated through the slope of the regression line according to the modified Arrhenius model (Eq. 2) (Isaka et al. 2008; Jin et al. 2013). However, the Arrhenius model had the drawback that it could not model the decrease of SAA at the temperature above optimal one (Wang and Wan 2009). In order to overcome this limitation, the Hinshelwood model (Eq. 3) (Rosso et al. 1993) which was dependent on the Arrhenius model was put forward to simulate the effect of temperature on anammox activity.

$$\mu = A \exp\left(-\frac{E_a}{RT}\right) \quad (1)$$

$$\ln \mu = -\frac{E_a}{RT} + \ln A \quad (2)$$

$$\mu = k_1 \exp\left(\frac{-E_a}{R \times T}\right) - k_2 \exp\left(\frac{-E_b}{R \times T}\right) \quad (3)$$

where μ is SAA at certain temperature, kgNH₄⁺-N/(kgMLSS day); E_a is apparent activation energy, kJ/mol; E_b is high-temperature denaturation, kJ/mol; R is ideal gas constant, 8.314 J/(kg K); T is

thermodynamic temperature, K; k_1 , k_2 are frequency factors without biological basis; and A refers to Arrhenius constant.

The Ratkowsky model (Eq. 4) (Ratkowsky et al. 1982) and the Zwietering model (Eq. 5) (Zwietering et al. 1991) were used to simulate the effect of temperature on bacterial growth. The formations of both models were quite similar. In addition, the cardinal temperature model with inflection (CTMI) was developed empirically (Eq. 6) (Rosso et al. 1993). It revealed that the growth rate changed as a function of cardinal temperature parameter. To our knowledge, this was the first time that CTMI was used in anammox process. In order to describe the effect of temperature on anammox activity throughout the entire bio-kinetic temperature range precisely, three models were analyzed.

$$\mu = [A(T - T_{\min})]^2 \{1 - \exp[B(T - T_{\max})]\}^2 \quad (4)$$

$$\mu = [A(T - T_{\min})]^2 \{1 - \exp[B(T - T_{\max})]\} \quad (5)$$

$$\mu = \text{Sup} \left\{ 0.0, \frac{\mu_{\text{opt}}(T - T_{\max})(T - T_{\min})^2}{(T_{\text{opt}} - T_{\min})[(T_{\text{opt}} - T_{\min})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max})(T_{\text{opt}} + T_{\min} - 2T)]} \right\} \quad (6)$$

where $T_{\min}$ and $T_{\max}$ are the minimum and maximum temperature at which nitrogen removal performance is observed, °C; T_{opt} is the temperature at which the maximum SAA equals its optimum value μ_{opt} ; and A , B are two parameters without biological basis.

Analytical methods

The supernatants were analyzed for NH_4^+ -N, NO_2^- -N and NO_3^- -N concentrations according to the standard methods (APHA 1998). Temperature and pH were measured by the WTW 340i/SET pH/Oxi portable multi parameter measuring meter. Microscopic images were taken with the light microscope (Olympus, CX31) and photos were taken with the digital camera (Canon, IXUS 500 HS). The morphological properties of the granules were observed by scanning electron microscopy (SEM) according to the

methods described by Tang et al. (2011). Sludge concentration was measured after filtering the washed samples through 0.45 μm membrane filter and drying at 105 °C to constant weight. The size of granular sludge was performed according to the methods described by Tang et al. (2011). The measure of settling velocity was based on the methods of Mu et al. (2008). Each data point presented in present research was the average of three replicates, and standard deviation was lower than 10% of mean value.

Results and discussion

Long-term temperature effect on anammox process

Nitrogen removal performance

The concentrations of three nitrogen species in influent and effluent at different temperatures are

presented in Fig. 2a. When temperature decreased to 30 °C, three nitrogen species in effluent varied little. At 25 °C, effluent ammonium and nitrite increased to 3 ± 1 and 5 ± 1.5 mg/L, respectively, and then both increased with reduction of temperature. Effluent ammonium and nitrite increased to 80.2 ± 2 and 106.1 ± 2.2 mg/L, respectively, when temperature was 15 °C. The sharp increase of effluent ammonium and nitrite indicated the deterioration of anammox process. As presented in Fig. 2b, nitrogen removal efficiencies decreased with dropping temperature. Under low temperature, nitrite could not be completely removed through anammox reaction and it could accumulate in the reactor. Nitrite was an inhibitory factor for anammox process (Jung et al. 2007). As a result, poor anammox performance at low temperature could be partly attributed to nitrite accumulation. In addition, effluent nitrate decreased

Fig. 2 Performance of Anammox process with different temperatures: **a** concentrations of three nitrogen species in influent and effluent; **b** nitrogen removal efficiency; **c** loading and removal rates of ammonium and nitrite; **d** stoichiometric ratio

with dropping temperature, and only small amount of nitrate was produced at 15 °C.

Nitrogen removal rates with temperature are presented in Fig. 2c. Ammonium removal rate (ARR) was slightly affected when temperature was not lower than 25 °C. ARR was about 0.35 $\text{kg}/(\text{m}^3\text{ day})$ at 35 °C. However, ARR was only around 0.059 $\text{kg}/(\text{m}^3\text{ day})$ when temperature was 15 °C. In terms of nitrite removal rate (NRR) and total nitrogen removal rate (TNRR), they were 0.44 and 0.70 $\text{kg}/(\text{m}^3\text{ day})$ at 35 °C, respectively. Then both decreased with

dropping temperature. When temperature was 15 °C, they were around 0.029 and 0.058 $\text{kg}/(\text{m}^3\text{ day})$, respectively. The poor nitrogen removal performance could be attributed to adverse effect resulting from low temperature on enzyme activity and mass transfer rate (Xing et al. 2015). In addition, substrate affinity of AAOB decreased under low temperature (Guo et al. 2015).

The relationship between TNRR and temperature was simulated by the modified Logistic model (Wang and Wan 2009), as shown in Fig. 3. The correlation coefficient (R^2) acquired was 0.9942, which suggest good appropriateness to describe nitrogen removal performance. Besides, there was little variation among 25–35 °C. TNRR approached 0.71 $\text{kg}/(\text{m}^3\text{ day})$ at 35 °C. Then it decreased sharply with dropping temperature, and arrived at 0.055 $\text{kg}/(\text{m}^3\text{ day})$ under 15 °C. Evaluation and validation of the modified Logistic model indicated that the predicted values were in line with experimental data. The modified Logistic model was recommended to simulate and analyze the TNRR under different temperatures.

Stoichiometric ratio was the calculation of quantitative relationships between reactant and product, which was an important indicator to evaluate reaction proceeding (Du et al. 2016). As a result, the stoichiometric ratio is analyzed and presented in Fig. 2d. At high temperature (25–35 °C), $\Delta\text{NO}_2^-\text{-N}/\Delta\text{NH}_4^+\text{-N}$ and $\Delta\text{NO}_3^-\text{-N}/\Delta\text{NH}_4^+\text{-N}$ were both stabilized at around 1.2 and 0.25, respectively. Then, $\Delta\text{NO}_2^-\text{-N}/\Delta\text{NH}_4^+\text{-N}$ decreased and $\Delta\text{NO}_3^-\text{-N}/\Delta\text{NH}_4^+\text{-N}$ increased. The transformation of stoichiometric ratios

Fig. 3 Regression curve between TNRR and temperature simulated by the modified Logistic model

could be related to change of AAOB metabolism in response to change of external environment (Kartal et al. 2007). The theoretical values of $\Delta\text{NO}_2^-/\text{N}/\Delta\text{NH}_4^+/\text{N}$ and $\Delta\text{NO}_3^-/\text{N}/\Delta\text{NH}_4^+/\text{N}$ for anammox process were calculated to be 1.32 and 0.26, respectively (Strous et al. 1998). However, both deviated from theoretical values greatly when temperature was 15 °C. The decrease of $\Delta\text{NO}_2^-/\text{N}/\Delta\text{NH}_4^+/\text{N}$ were consistent with previously reports (Dalsgaard and Thamdrup 2002; Dosta et al. 2008). Ammonification of organic matter resulting from cell decay or lysis might occur in anammox process. This was also similar with Guo et al. (2015) that the lower value of $\Delta\text{NO}_2^-/\text{N}/\Delta\text{NH}_4^+/\text{N}$ might be resulting from denitrification. In addition, $\Delta\text{NO}_3^-/\text{N}/\Delta\text{NH}_4^+/\text{N}$ increased at 15 °C agreed with Ma et al. (2013) and Jin et al. (2013) that there might be other reaction devoting to nitrate production.

After 134 cycles' operation, the operational temperature of R1 was directly restored to 35 °C. TNRR recovered to 0.70 kg/(m³ day) only after five cycles (data not shown). This agreed with Isaka et al. (2008) and Kawagoshi et al. (2012) that anammox activity would recover immediately when temperature was back to previous value. However, Dosta et al. (2008) found an irreversible loss of activity at 15 °C. The difference might result from varied nitrogen loading rate and duration time. In addition, sludge floating was observed in recovery period. This might be attributed to the trapping of N₂ bubbles (Quan et al. 2011). The floating sludge had poor settleability which led to sludge loss.

Biomass characteristics

Sludge settling velocity is a key factor in anammox process which was associated with wash-out of biomass. Granular sludge taken from three reactors was determined settling velocity at different temperatures and the results are presented in Fig. 4. The settling velocity was 2.15 cm/s at 35 °C, and then it decreased with dropping temperature. When temperature was 15 °C, the settling velocity was only 1.78 cm/s. The results indicated that settling velocity of anammox sludge was positively related to temperature at the range of 15–35 °C. The poor settleability suggested high biomass loss, which led to poorer performance of anammox process. This agreed with Guo et al. (2015) that bad settleability would

Fig. 4 Sludge settling velocity at different temperatures

contribute to poor performance of anammox system. Winkler et al. (2012) revealed that when temperature decreased from 40 to 5 °C, settling velocity decreased 50%. Low temperature increased the viscosity and density of wastewater (Jin et al. 2013; Winkler et al. 2012), which would inhibit the movement of biomass. As a result, low temperature had a significant effect on sludge settleability.

SBRs were inoculated with granular anammox biomass and granular sludge was kept throughout the whole period of operation. SEM images presented in Fig. 5 were acquired at 35 and 15 °C. The bacteria at both temperatures were round and had the smooth surface. The morphology of bacteria did not change when temperature decreased from 35 to 15 °C. This agreed with Ma et al. (2013) that anammox biomass did not change when temperature decreased from 30 to 16 °C. Low temperature had little effect on bacteria morphology.

Short-term temperature effect on SAA

Batch test

Short-term effect of temperature on SAA was performed by batch test at temperature range of 10–50 °C. Figure 6 indicated that there was no biomass activity when temperature was 10 °C. This did not agree with Hendrickx et al. (2014) that SAA was 0.044 kg/(kg day) at 10 °C. SAA increased when temperature increased from 10 to 35 °C. Then it decreased with further enhancement of temperature. These results were similar with previously studies

Fig. 5 SEM observation of granular sludge: **a** sludge withdrawn at 35 °C; **b** sludge withdrawn at 15 °C

Fig. 6 Microbial activity measured at different temperatures

(Dosta et al. 2008; Isaka et al. 2008; Lotti et al. 2014a, b). As a result, inhibition on SAA would occur at extreme temperature (lower than 25 °C and higher than 40 °C). Anammox activity observed at 50 °C was 0.021 kg/(kg day), which was higher than previous reports (Egli et al. 2001; Dosta et al. 2008). Different anammox biomass and nitrogen loading rate might devote to the discrepancy. Besides, biomass lysis might occur at high temperature, which would be another explanation for SAA decrease (Dosta et al. 2008).

E_a indicated necessary energy that substrate changed from normal state to active state. It was determined graphically by taking natural logarithm of the Arrhenius model (Eq. 2). As shown in Fig. 7a, the values of E_a were calculated for each temperature intervals. The results showed that E_a increased with decreasing temperature, which suggested the decrease of AAOB activity. This agreed with Lotti et al. (2015) that lower temperature would result in a sharp increase

of E_a , and SAA seemed to decrease more on a 5 °C temperature descent. During wastewater treatment process, temperature coefficient (θ) was usually preferred (Lotti et al. 2015). The results revealed that θ also increased with decreasing temperature. As a result, low temperature inhibited anammox process, and occurrence of anammox reaction became much more difficult.

Based on the findings above, temperature effect on SAA could be described by a single E_a (presented in Fig. 7b). The low value of correlation coefficient ($R^2 = 0.93$) of linear regression indicated that a single E_a could not demonstrate temperature inhibition satisfactorily. As a result, two Arrhenius regression lines of data were plotted. The high correlation coefficient ($R^2 > 0.98$) of each regression line suggested good simulation. From these plots, E_a of AAOB could be calculated by the Arrhenius equation for each temperature range. Different slope of the regression line for different temperature interval (35–25 and 25–15 °C) was acquired. Each slope deviated from regression linear which was plotted in the whole temperature interval (35–15 °C). The smaller value of E_a (69.5 kJ/mol) in temperature interval 25–15 °C indicated lower biomass activity at low temperature.

According to Fig. 7b, there is a flexion point existing between two regression lines at 20–30 °C. Two rate-determining enzymes with different optimum temperatures might exist in AAOB (Isaka et al. 2008). In addition, there might be other types of microorganisms in anammox system, and their relative activities were not the same as AAOB. A shift of relative activity during different microorganisms was possible in reactors. When external temperature decreased, anammox biomass was affected, but other bacteria activity with lower optimum temperature was enhanced (Hendrickx et al. 2012).

Fig. 7 a E_a and θ plotted by Arrhenius equation; b E_a relative to two temperature intervals

Table 2 Comparison of E_a in this work and previous studies

Temperature interval (°C)	Optimum temperature (°C)	Biomass type	E_a (kJ/mol)	Reference
6–22	37	Entrapped biomass	94	Isaka et al. (2008)
22–28	37	Entrapped biomass	93	Isaka et al. (2008)
28–37	37	Entrapped biomass	33	Isaka et al. (2008)
20–43	–	Aggregated biomass	70	Strous et al. (1999)
–2–13	12	Marine sediment	51	Rysgaard et al. (2004)
10–20	–	Granule sludge	72	Hendrickx et al. (2012)
10–40	35–40	Bio-film biomass	63	Dosta et al. (2008)
10–40	35–40	Granular sludge	63	Dosta et al. (2008)
6.5–37	15	Marine sediments	61	Dalsgaard and Thamdrup (2002)
15–35	>35	Granular sludge	43	Jin et al. (2013)
5–30	25	Marine sediments	54.6	Kawagoshi et al. (2012)
15–30	–	Granular sludge	83.1	Lotti et al. (2015)
15–30	–	Free-cell suspension	83.1	Lotti et al. (2015)
9–25	–	Granular sludge	54.9	Xing et al. (2015)
5–17	20–30	Activated sludge	66	Hendrickx et al. (2014)
15–25	35–40	Granular sludge	95.9	This study
25–35	35–40	Granular sludge	43.2	This study
15–35	35–40	Granular sludge	69.5	This study

The comparison of E_a in different studies is shown in Table 2. E_a acquired in this work was 95.9 and 69.5 kJ/mol at 15–25 and 25–35 °C, respectively. The result was not consistent with previous reports. Lotti et al. (2015) reported that the average E_a value for granular anammox biomass was 83.1 kJ/mol at

15–30 °C. Jin et al. (2013) analyzed granular biomass at 15–35 °C, and the value of E_a obtained was 43 kJ/mol. Besides, E_a of marine AAOB was generally smaller than that of fresh ones. In addition, the immobilized or free-cell biomass and the type of microorganisms were also important factors which affected E_a .

Kinetic analysis

The Arrhenius model was used most widely to simulate temperature effect on anammox activity and AAOB growth (Jin et al. 2013; Lotti et al. 2015; Xing et al. 2015). However, it could not model the effect of temperature which was higher than optimal one (presented in Fig. 6) (Wang and Wan 2009). The Hinshelwood model which was based on the Arrhenius model was put forward to solve this problem and it reached good simulation result ($R^2 = 0.9897$) (data not shown). However, the parameters in equation were strongly correlated with each other and were very difficult to estimate. This aspect limited its further application. Besides, the simulated activation energies were unrealistic (Zwietering et al. 1991) and had little

benefit to engineering application. As a result, it was not analyzed in this work.

The simulation of Ratkowsky model is presented in Fig. 8a. High correlation coefficient ($R^2 = 0.9949$) indicated that the Ratkowsky model was able to simulate SAA under different temperatures. In addition, the values of $T_{\min}$ and $T_{\max}$ were 7.8 °C and 51.14 °C (shown in Table 3), respectively, which suggested anammox reaction in present research only occurred at 7.8–51.14 °C. However, experimental result showed that AAOB had no activity at 10 °C in this work. This was not consistent with simulated value. The Zwietering model appeared to fit the data very well ($R^2 = 0.9886$) (shown in Fig. 8b) and the simulated results indicated that $T_{\min}$ and $T_{\max}$ were 10.21 and 50.13 °C, respectively. The predicted

Fig. 8 The simulation of three models: **a** the Ratkowsky model; **b** the Zwietering model; **c** CITM

Table 3 Simulated parameters of three kinetic models

Model	Parameter						R ²
	T _{min} (°C)	T _{max} (°C)	T _{opt} (°C)	A	B	$\mu_{\max}/[\text{kgNH}_4^+-\text{N}/(\text{kgMLSS}\cdot\text{d})]$	
Ratkowsky model	7.80	51.14	–	0.03	0.09	–	0.9949
Zwietering model	10.21	50.13	–	1.15	4.06	–	0.9886
CTMI	10.52	50.15	36.72	–	–	0.50	0.9887

values were in good agreement with experimental data. Similar with Ratkowsky model, only two practical parameters were fitted by Zwietering model. As a result, the cardinal temperature model with inflection (CTMI) was used in anammox process. It was first used in the analysis of anammox process.

As presented in Fig. 8c, CTMI had high correlation coefficient ($R^2 = 0.9887$) which indicated it was able to simulate SAA with temperature shock. Compared to the two models described above, CTMI could simulate T_{opt} and $\mu_{\max}$. For the experimental data, anammox process could occur within 10–50 °C with maximum SAA at 35–40 °C. According to the simulation of CTMI, anammox reaction could occur at 10.52–50.15 °C with maximum SAA of 0.50 kg/(kg day) at 36.72 °C (shown in Table 3). The value of simulated parameters (e.g., T_{min} , T_{max} , T_{opt} and $\mu_{\max}$) were similar with experimental data. This was beneficial to engineering application of anammox process. In conclusion, CTMI could describe temperature effect on anammox activity throughout the entire bio-kinetic temperature range precisely.

The speculative mechanisms resulting temperature effect on anammox process were as follows. Firstly, as thermophilic bacteria, temperature was an important factor to some essential enzyme in cell (Wang and Wan 2009; Xing et al. 2015). The enzyme-catalyzed rate was affected greatly at low or high temperature which resulted in the inhibition of anammox process. Secondly, the fluidity of cytoplasm and cell membrane were also affected by temperature, as well as liquidity of cytoplasm and mass transfer rate (Jin et al. 2013; Xing et al. 2015). Protein was the carrier in cell membrane. It would denature at high temperature and lose activity at low temperature. Both affected trans-membrane transport and worsened nitrogen removal performance. Thirdly, nitrite was accumulated in anammox reactor at extreme temperature. As nitrite was inhibitory for AAOB, even at moderate

concentrations (Jung et al. 2007; Strous et al. 1999), it could cause a decrease of anammox activity. Fourthly, anammox process was also affected by free ammonia (FA) and free nitrous acid (FNA) at extreme temperature (Anthonisen et al. 1976). A temperature rise increased FA concentration, which had been reported to inhibit anammox activity at 1.7–8.3 mg/L (Jaroszynski et al. 2011; Jung et al. 2007). In addition, a decrease in substrate affinity under low temperature could also result in poor anammox performance (Guo et al. 2015).

Operation strategy under low temperature

Acclimatization to low temperature

As presented in Fig. 2c, TNRR increased from 0.02 ± 0.01 to 0.072 ± 0.01 kg/(m³ day) after 50 cycles' operation at 15 °C. This indicated that the activity of AAOB could be enhanced with cold acclimatization. In view of slow growth rate of AAOB (Strous et al. 1999), the acclimatization duration should be long enough.

In order to explore cold acclimatization, R2 was continuously operated at 15 °C. TNRR increased to around 0.2 kg/(m³ day) with another 50 cycles' operation. It restored to about 30% of TNRR at 35 °C (data not shown). Anammox biomass could progressively adapt to low temperature. Hu et al. (2013) found the optimum temperature for AAOB decreased from 35 to 25 °C after long-term cultivation at 12 °C. Similar result was acquired by Dosta et al. (2008). Acclimatization at low temperature depended on duration time and longer acclimatization period was needed at lower temperature. Therefore, low temperature could result in the enrichment of other anammox bacteria which had a lower optimum temperature (Hendrickx et al. 2012).

Ma et al. (2013) obtained a high TNRR of 2.28 kg/(m³ day) in an anammox reactor at 16 °C, which was

much higher than the present work. Sludge concentration might also result in the discrepancy. Even though a much higher biomass concentration was required to treat wastewater at low temperature (Hendrickx et al. 2014), it was also meaningful to study the acclimatization of anammox bacteria to low temperature. Due to the low growth rate of anammox bacteria, it needed a long time to start-up anammox system and the sludge concentration usually was low at the beginning. Adding sludge into reactor was an effective method to enhance nitrogen removal performance at low temperature (Yang and Jin 2013; Jin et al. 2013).

Fig. 9 Nitrogen removal performance with glycine betaine addition at low temperature

Addition of glycine betaine

In order to explore glycine betaine effect on anammox at low temperature, R3 was continuously operated at 20 °C. As presented in Fig. 9, when glycine betaine dose was 0.10 mM, ARR was 0.121 ± 0.008 kg/(m³ day), slightly higher than that without glycine betaine addition. NRR and TNRR were also slightly improved. When glycine betaine dose was 0.5 mM, ARR decreased to 0.095 ± 0.02 kg/(m³ day). However, NRR and TNRR increased to 0.198 ± 0.015 and 0.274 ± 0.008 kg/(m³ day), respectively. As a kind of organic matter, glycine betaine could promote the growth of heterotrophic denitrifying bacteria. Glycine betaine addition could slightly promote anammox activity at low temperature. However, as a small molecular organic matter accumulated in bacteria, it might lead to denitrification in reactor at high concentration. As a result, intermittent addition of glycine betaine might be more suitable for anammox process than continuous addition.

The slight enhancement of anammox performance could be attributed to the role of glycine betaine in maintaining membrane fluidity at low temperature and the ability to prevent cold-induced aggregation of cellular proteins (Chattopadhyay 2002; Jones et al. 2002). In this way, glycine betaine increased the permeability of cell membrane. Chattopadhyay (2002) found that inductive synthesis of some heat shock

Table 4 Comparison of different operation strategies at low temperature

Method	Temperature (°C)	Description ^a	Effect	Reference
pH	15	pH 6.5 → 7.5	SAA 0 → 0.0025 ^b	Daverey et al. (2015)
pH	15	pH 6.5 → 8.0	Doubled SAA	Daverey et al. (2015)
Ultrasound irradiation	15	low-strength	258% more NRR	Yu et al. (2013)
HRT	20–22	shorter HRT	higher TNRR	Isaka et al. (2007)
Extra calcium	25	addition	higher TNRR	de Graaff et al. (2011)
Polymeric matrix	–	addition	cold tolerance enhancement	Heylen et al. (2012)
Biomass	9.1	addition ^c	20% higher TNRR	Jin et al. (2013)
Biomass acclimatization	12	120 days operation	SAA 12 → 18 ^d	Hu et al. (2013)
Glycine betaine addition	9.2 ± 0.5	0.15 mM	effective	Jin et al. (2013)
Biomass acclimatization	15	>50 days operation	effective	This study
Glycine betaine addition	20	0.1 mM	effective	This study

^a Description of method in detail

^b kgNH₄⁺-N/(kgMLSS day)

^c Added 10 mL sludge every other day

^d nmol N/(mg total protein min)

proteins (HSPs) was remarkably increased. In addition, glycine betaine helped to maintain membrane fluidity by promoting synthesis of specific fatty acids at low temperature (Chattopadhyay 2002; Jones et al. 2002). Still, previous studies reported that low concentration of organic matter could promote anammox performance (van de Graaf et al. 1996; Güven et al. 2005). As a result, anammox performance was improved upon glycine betaine addition. Jin et al. (2013) demonstrated that glycine betaine addition was a strategy to improve bioprocess performance at 10 °C. However, glycine betaine might increase anammox activity transiently just as reported in the previous study on anaerobic biomass (Vyrides et al. 2010). The contact time and feed mode might also affect process performance and further study was needed.

Compared with other enhancing strategies listed in Table 4, ultrasound irradiation, addition of extra calcium and polymeric matrix, biomass addition were all effective to strengthen anammox performance. Among them, intermittent ultrasound could enhance bio-activity with a short time through accelerating substance exchange and membrane permeability (Xie et al. 2009). However, continuous exposure should be avoided because cell structure would be destroyed. Therefore, it is important to evaluate different strategies and achieves efficient and stable nitrogen removal at low temperature.

Conclusions

Both long-term and short-term temperature effects on anammox process were studied in this work. Nitrogen removal performance worsened when temperature was lower than 25 °C. Besides, low temperature reduced settling velocity of biomass although the morphology of bacteria was not modified. In batch test, E_a decreased with dropping temperature. The inhibition resulting from low temperature could be relieved through cold acclimatization of bacteria. In addition, the addition of glycine betaine could slightly improve nitrogen removal performance. CTMI could describe temperature effect on anammox activity throughout the entire bio-kinetic temperature range precisely. The simulated results revealed that anammox reaction could occur at 10.52–50.15 °C with maximum SAA of 0.50 kg/(kg day) at 36.72 °C.

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