Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2020.25025

179 (2020) 54–62
March

Effect of nitrite concentration on the growth and microbial diversity of anaerobic ammonia oxidation (anammox) sludge

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Received 7 June 2019; Accepted 12 October 2019

ABSTRACT

The effect of influent NO_2^-–N concentration on the growth and microbial diversity of anaerobic ammonia oxidation (anammox) sludge is unclear. In this study, influent NO_2^-–N concentrations of 25.53, 51.63, and 102.99 mg/L were used to study the effect of the anammox sludge growth and microbial diversity in an up-flow anaerobic sludge blanket reactor. Influent concentration had a substantial influence on anammox microorganisms. When the NO_2^-–N concentration was 51.63 mg/L, the nitrogen removal efficiency was 12.96% less than that at low concentration and 8.50% higher than that at high concentration. However, the specific anammox activity, total solids, and carbon sequestration were at maximum levels. Therefore, this concentration was most suitable for the growth of anammox microorganisms, with a growth rate of 1.31 g-VSS/g-N, which was 15 times higher than the theoretical growth rate, and *Candidatus Kuenenia* comprising 75.11% of the dominant bacteria in the reactor.

Keywords: Anammox sludge; NO_2^-–N; Growth rate; SAA; High-throughput sequencing

1. Introduction

In recent years, a new pathway has been discovered for ammonium removal, anaerobic ammonium oxidation (anammox), which has been widely studied for its potential engineering applications [1–3]. Compared with the traditional nitrification–denitrification process, the anammox process can shorten the redox process of NH_4^+–N to N_2 without an excessive organic carbon source [4] and reduce the excess sludge yield by 90%. In the field of environmental engineering, anammox has been successfully applied to the biological denitrification of different types of industrial wastewater, including landfill leachate [5], sludge digestion liquid [6], monosodium glutamate wastewater [7], and semiconductor industrial wastewater [8], which has good industrial application prospects [9]. To date, there are more than 100 full-scale partial nitrification-anammox plants in the world, and this number is growing rapidly [10]. However, due to the slow growth of anammox bacteria, the start-up period of the anammox process is very long [11]. Although many culture experiments have been conducted, the low yield and slow growth of anammox bacteria and the sensitivity of reactor operating conditions remain key challenges to the industrial application of anammox [12,13], limiting its stability and practical application potential.

NO_2^- is a key parameter for maintaining the stability of the anammox process because it acts as an electron acceptor. It can be transformed by anammox bacteria [14] and can also

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inhibit the reaction [15,16]. An appropriate concentration of NO_2^-–N in the reaction process can maintain a high total nitrogen (TN) removal rate [17]; however, ammonium oxidizing bacteria grow faster than anammox bacteria, which can lead to high and inhibitory NO_2^-–N concentrations [14]. The maximum threshold of influent NO_2^-–N concentration is still under debate. Some researchers have reported that anammox activity is completely inhibited at a NO_2^-–N concentration of 100–280 mg-N/L [18,19]. Hendrickx et al [20] and Zekker et al [21] suggested that 100 mg of NO_2^-–N could act as a barrier and inhibit the growth of anammox bacteria. Qi et al [22] studied the effect of an influent NO_2^-–N concentration more than 150 mg-N/L ($NO_2^-N_{inf}/NH_4^+N_{inf} = 1.37 \pm 0.02$) on the denitrification performance of an anammox reactor under 100% seawater conditions. Qiao et al [23] found that a NO_2^-–N concentration of 300 mg/L inhibited anammox bacteria.

In addition, previous studies have predominantly focused on the effect of NO_2^-–N concentration on anammox bacteria activity, whereas few studies have analyzed the growth rate of anammox bacteria. Therefore, the effects of influent NO_2^-–N concentration on the activity and growth rate of anammox sludge were studied and evaluated particularly in this study. Specifically, using the successful start-up of an up-flow anaerobic sludge blanket (UASB) anammox reactor [24,25]. Under the condition of maintaining the nitrogen removal capacity of anammox sludge, the effects of influent NO_2^-–N concentration on specific anammox activity (SAA), growth rate and carbon sequestration ability in the anammox reactor were studied. Moreover, using high-throughput sequencing, the microbial community structure of anammox sludge under different influent NO_2^-–N concentrations were analyzed to determine the inhibitory effect of NO_2^-–N on microorganisms in anammox sludge. These findings provide a theoretical basis for engineering applications of the anammox process.

2. Materials and methods

2.1. Anammox reactor

An integrated anammox process reactor with independent intellectual property rights was developed in our laboratory [26]. The reaction system comprises an intake bucket, outlet pipe, main reactor, and temperature control system. The main body of the reactor is made of plexiglass with a length of 9 cm, a width of 9 cm, a height of 74 cm, an inner diameter of 12.5 cm, an effective volume of 5.5 L, and anammox bacteria inoculated in the main reactor. An insulation interlayer is installed on the outer layer of the main reactor. When the indoor temperature is low, the temperature control system provides continuous circulating water for the reactor. The circulating water enters the interlayer from the bottom of the reactor, then returns to the temperature control system from the top of the reactor. There are four sampling ports on the side of the main reactor for collecting sludge samples. A three-phase separator is installed in the reactor to prevent the loss of anammox sludge with increasing water flow and achieve sludge separation. When the reactor is running, the influent pump pumps the wastewater from the distribution bucket into the bottom of the main reactor. The water from the bottom of the reactor enters the reactor and the system outflows from the top outlet.

The reactor was used to conduct three stages of experiments (group A, group B, and group C), lasting 35 d each. It was placed in the dark to prevent the adverse effects of light on bacterial metabolism [27]. Using a 0.4 mol/L H_2SO_4 solution as an acid solution to adjust the influent pH to 7.5, the operating temperature was controlled at 32°C ± 1°C.

2.2. Inoculate sludge and wastewater compositions

The inoculated sludge from this experiment was derived used in anammox bacteria cultured in the sewage station of Guilin University of Technology, which exhibited good activity [28]. It was red, viscous, and granular sludge. 200 mL of anammox bacteria were selected for the starting sludge in each stage.

The influent of this experiment was artificially simulated wastewater. The NH_4^+–N and NO_2^-–N in the influent was supplied by NH_4HCO_3 and $NaNO_2$, respectively. The components of artificial wastewater include NH_4HCO_3 and $NaNO_2$, which were added according to the required water concentration. The ratio of nitrate to ammonium was 1.1–1.3 [29]. According to the literature, when the concentration of NO_2^-–N is 98 mg/L, anammox activity is completely inhibited [15]. Therefore, three groups of NO_2^-–N concentration gradients were set up in this experiment: 25.53, 51.63, and 102.99 mg/L. The corresponding NH_4^+–N concentration gradients were approximately 20.19, 39.23, and 77.83 mg/L (as shown in Table 1). The following concentrations were used: KH_2PO_4 50 mg/L, $CaCl_2{\cdot}2H_2O$ 100 mg/L, $MgSO_4{\cdot}7H_2O$ 100 mg/L, anhydrous sodium sulfite (Na_2SO_3) 50 mg/L, microbial promoter 1 mL/L, and $NaHCO_3$ 1000 mg/L. Firstly, high purity (99%) N_2 was used to strip the simulated wastewater for approximately 45 min, then a small amount of Na_2SO_3 was added to control the dissolved oxygen (DO) below 0.5 mg/L.

Selective pressure is an important condition for the formation of granular sludge; therefore, this experiment maintained the basic conditions of stable water quality in the anammox reactor. The inflow rate was increased gradually to increase the upwelling velocity. With the increase of up-flow velocity, the hydraulic retention time (HRT) of the reactor decreased gradually and the selective pressure increased, which promoted the formation of granular sludge. The reduction of the reactor was different for each group. When the concentration of NO_2^-–N in the effluent of the reactor is less than 10 mg/L, it is considered that the substrate of anammox bacteria in the reactor has been consumed and the inflow of the reactor should be increased.

2.3. Analytical methods

The collected water samples were placed in the sampling bottle after passing through a 0.45 μm filter membrane. After sampling, the samples were detected immediately or placed in a 4°C refrigerator for uniform sampling. The conventional water quality indexes determined in this experiment were: NH_4^+–N, NO_2^-–N, and TN. NH_4^+–N was determined by Nessler's reagent spectrophotometry, NO_2^-–N was determined by N-(1-Naphthyl)ethylenediamine spectrophotometry, and

Table 1
NH_4^+–N and NO_2^-–N concentration required by each group

Influent matrix concentration (mg/L)	Group A	Group B	Group C
NH_4^+–N	20.19	39.23	77.83
NO_2^-–N	25.53	51.63	102.99

TN was determined by alkaline potassium persulfate ultraviolet spectrophotometry [30]. The pH/temperature was determined by PHS-3C acidity meter and DO was determined by portable dissolved oxygen meter HQ30d (Hach, USA). The data were analyzed the one-way analysis of variance by SPSS (IBM SPSS Statistics (V24.0), New York, USA).

The methods for determining total solids (TS) and volatile solids (VSS) can be found in "Anaerobic Biotechnology for Bioenergy Production: Principles and Applications" [31]. Because a small amount of anammox sludge stuck to the inside surface of the dismantled reactor, the inner surface of the reactor was washed with influent water and wiped with non-woven fabric. Collect flushing water and use it for cell quantification. All effluent water was collected in a tank and concentrated using a filter with a 0.45 μm mesh at the end of a phase. Then, the concentration of anammox bacteria in the concentrated effluent water was measured. The growth rate of anammox bacteria was expressed by g-VSS/g-N. SAA was calculated from typical one-cycle experimental data. The specific calculation is shown in Eq. (1):

$$\mathrm{SAA} = \frac{\left(\frac{\Delta N}{\Delta t}\right)_{\max}}{\mathrm{VSS}} \tag{1}$$

where ΔN is the degradation amount of matrix (NH_4^+–N, NO_2^-–N), mg-N/L; Δt is the reaction time, d; $\Delta N/\Delta t$ is the maximum slope of the linear fitting of matrix degradation time; VSS is the concentration of sludge in the reactor, g-VSS/L, and the units for SAA are g-N/(g VSS d).

2.4. Carbon sequestration

Inorganic carbon (IC) was analyzed by a total organic carbon analyzer (Multi N/C 3100, Analytik Jena, Germany). The CO_2 gas was collected in a gas collecting bag at the top of the main reactor by an exhaust valve. Gas analysis was performed by gas chromatography (GC-112A, Shanghai Jingke) using an N (VI) 2000 chromatographic data workstation (developed by the Intelligent Information Research Institute of Zhejiang University) to determine the content of CO_2 in the samples. The concentration of CO_2 standard gas (produced by Shanghai Shenkai Gas Co. Ltd.) was 4.92% and the equilibrium gas was argon. Chromatographic conditions were as follows: the carrier gas flow rate was 4.0 mL/min, the injector temperature was 70°C, the column box temperature was 35°C, the detector temperature was 120°C, the detector current was 110 mA, and the injection volume was 1 mL. The content of CO_2 in the sample was calculated by Eq. (2). Each gas sample was measured at least three times to obtain an average value.

$$C_{\mathrm{sample}} = \frac{C_{\mathrm{standard\ sample}} \times A_{\mathrm{sample}}}{A_{\mathrm{standard\ sample}}} \tag{2}$$

where C_{sample} is the CO_2 content in the gas sample; A_{sample} is the peak area of CO_2 in the gas sample; C_{standard} sample is the CO_2 content in standard gas; A_{standard} sample is the peak area of CO_2 in standard gas.

Carbon sequestration was calculated by Eq. (3).

$$\text{Carbon sequestration} = \frac{\mathrm{IC}_{\mathrm{inf.}} - \mathrm{IC}_{\mathrm{eff.}} - \mathrm{CO}_2 \times \rho_{\mathrm{CO}_2}}{N} \tag{3}$$

where $\mathrm{IC}_{\mathrm{inf.}}$ is the IC in the influent water, mg; $\mathrm{IC}_{\mathrm{eff.}}$ is the IC in the effluent, mg; CO_2 is the CO_2 production per unit time, mL; ρ_{CO_2} is the density of CO_2, g/L; and N is the nitrogen content per unit time entering the reactor, mg. The CO_2 density is approximately 1.7738 g/L under conditions of 30°C and 101 kPa. IC refers to the IC existing in various forms in the water. Because there are no methanogens in the reactor, the production of CH_4 is neglected in the calculation.

2.5. Microbial diversity

The start and end sludge of group A and the end sludge of group B and C were placed in the refrigerator at –20°C for later use. The deoxyribonucleic acid (DNA) samples were subjected to DNA extraction using a soil DNA kit (DNeasy Power Soil DNA Isolation Kit 12888-50, Mo Bio, USA). The procedure was performed according to the manufacturer's instructions. Two rounds of polymerase chain reaction amplification were conducted on the qualified DNA after extraction. The amplified region was V3-V4. After amplification, agarose gel electrophoresis was used to identify the DNA. After being qualified, the purified DNA was quantified and sequenced. Sequencing was conducted by Bioengineering (Shanghai) Co. Ltd.

Prinseq (v.0.20.4) software was used to control the original sequence data. After discarding the low-quality sequence, the sequence length was distributed between 400 and 600, the average length was more than 440, and the number of sample sequences was less than 500. Species were classified by ribosomal database project classifier (v.2.12). Operational taxonomic units were classified according to 97% similarity criteria [32]. The summary single command was run in the software mothur (v.1.30.0) (http://www.mothur.org/) to calculate commonly used biodiversity indices, such as the Chao/ACE index and Shannon index. The species abundance thermogram was made by using the gplots package to reflect the similarities and differences of community distributions at different taxonomic levels.

3. Results and discussion

3.1. Nitrogen removal performance

In this study, three groups of UASB reactors were compared with different influent NO_2^-–N concentrations. The removal rate of various nitrogen decreased gradually with increasing NO_2^-–N concentration in the influent (Fig. 1). The numbers in the figure represent the average of the 35 d after the end of each phase. When the concentration

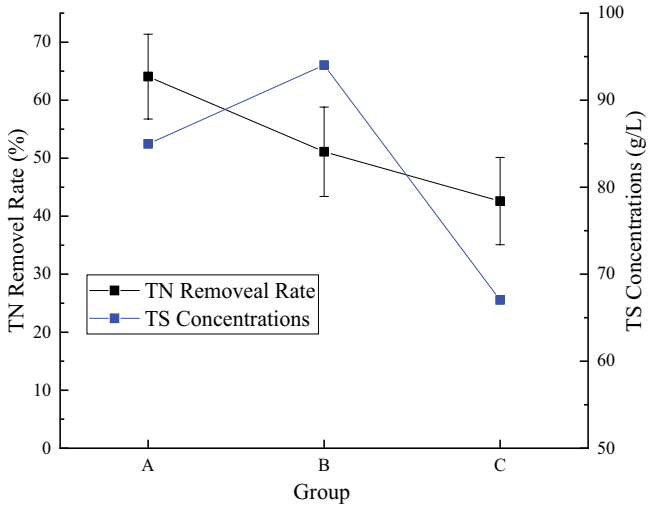

Fig. 1. TN removal rate and TS concentration at different influent NO_2^-–N concentrations.

of NH_4^+–N and NO_2^-–N in the influent of group A was 20.19 and 25.53 mg/L, the removal rate of NH_4^+–N and NO_2^-–N in the effluent were the highest. The effluent average removal rate of NH_4^+–N is 96.91% and the removal rate of NO_2^-–N is 59.45%. The average removal rate of TN was approximately 64.06%. When the concentration of NH_4^+–N and NO_2^-–N increased to 39.23 mg/L and 51.63 mg/L in group B, the removal rate of NH_4^+–N and NO_2^-–N in the effluent was 73.45% and 44.05%, respectively, and the average removal rate of TN was approximately 51.10%. When the concentration of NH_4^+–N and NO_2^-–N in the influent increased to 77.83 and 102.99 mg/L in group C, the removal rate of NH_4^+–N and NO_2^-–N in the effluent was the lowest, with an average of 71.40% and 25.76%, respectively, and the average removal rate of TN of approximately 42.60%.

Under the condition of stable influent matrix, the influent is gradually adjusted according to the effluent of NO_2^-–N, so the HRT of each reactor group is different. The HRT of group A decreased from 24 h to 8 h, the volumetric TN loading rate (TNLR) increased from 0.049 to 0.152 kg-N/m^3/d, and the TS concentration was 84.97 g/L. The HRT of group B decreased from 24 to 16 h, TNLR increased from 0.088 to 0.139 kg-N/m^3/d, the TS concentration increased significantly to 94.03 g/L ($P < 0.01$). The HRT of group C was maintained for 24 h, the TNLR was maintained at approximately 0.186 kg-N/m^3/d, and the TS concentration was 67.04 g/L. This is because reducing the HRT of each reactor group can increase the up-flow rate, promote the formation of granular sludge [33], and change the TS concentration of each reactor. Group B had the highest TS concentration, followed by group A. This may be due to the impact of too short HRT on anaerobic ammonia oxidation microorganisms in group A. As a result, many anaerobic ammonia oxidation sludge is not attached to the carrier, and some of it may be lost with the effluent. However, the removal ability of group A was the highest because the matrix concentration in group A was too low for the growth and utilization of microorganisms. The TS concentration of microorganisms in group B was the highest, resulting in a far greater removal effect than that in group C.

The removal rate of nitrogen in the reactor decreases with the increase of NO_2^-–N concentration in the influent. Because the influent concentration of group A was low, the influent matrix concentration did not reach the optimum concentration required by microorganisms. Sludge in starvation can directly consume the low concentration influent matrix, so the removal rate of group A is the highest. Compared with group A, the TN removal rate in group B decreased by 12.96%, but TS concentration in group B was the highest. Anammox bacteria exist in the form of granular sludge, and the biomass of granular sludge is large, which helps to alleviate the inhibition of high concentration substrate on anammox bacteria [34,35], so group B shows high activity and removal rate. The removal rate of group C was the lowest because the high concentration of the influent matrix inhibited the anammox sludge. According to the concentration of HRT and TS in the reactor, group B was the most suitable for the growth of microorganisms. The high concentration of NO_2^-–N in the influent of group C could inhibit the anammox process and the formation of anammox granular sludge, and reduce the concentration of sludge and the removal rate of nitrogen in the reactor.

3.2. Growth rate of anammox sludge

The matrix concentration is very important for the growth rate of anammox granular sludge. Increasing matrix concentration can promote the growth and metabolism of microorganisms [36]. However, many studies [37,38] have shown that excessive concentration of NO_2^-–N and NH_4^+–N can inhibit the anammox process and obtained different NO_2^- inhibition thresholds. However, there have been no previous reports on the growth rate of anammox sludge.

In the experiment, the growth rate of anammox sludge in group A was approximately 1.91 g-VSS/g-N and the SAA was 0.22 g-N/(g-VSS d). A low concentration of the influent matrix can be directly consumed by anammox sludge, which can prolong the cultivation time of granular sludge [39]. Therefore, the removal rate of group A was the highest, so the growth of anammox sludge would be faster. The growth rate of anammox in group B was approximately 1.31 g-VSS/g-N and the SAA increased significantly was 0.31 g-N/(g-VSS d) ($0.05 > P > 0.01$). This was the highest SAA and the growth rate was relatively fast. This is because the anammox sludge TS concentration in the reactor was high and the biomass of the granular sludge was large, which can alleviate the inhibitory effect of high substrate concentration on anammox sludge and result in high activity. In the growth stage of anammox sludge, it is necessary to maintain a certain matrix pressure [39]. A higher matrix concentration is conducive to substrate transfer in the granular sludge and the growth of anammox sludge. At the same time, extracellular polymeric substances secreted by microorganisms are conducive to cell adhesion and aggregation and the formation of granular sludge [40,41]. Compared with the former two groups, the growth rate of group C decreased by only 0.94 g-VSS/g-N, the SAA was 0.28 g-N/(g-VSS d), and anammox sludge growth was minimal. Moreover, the concentration of SAA decreased significantly, but there was no significant difference in group B ($P > 0.05$). In addition, the removal rate of NO_2^-–N was only half that of group A

and B. This is because an excessive concentration of NO_2^-–N in the influent can inhibit the anammox reaction, inhibit the growth of anammox sludge, and interfere with sludge metabolism. Nevertheless, the experimental results of group C showed that the growth rate of anammox sludge was 10 times higher than the reported theoretical value (0.08–0.11 g-VSS/g-N). Group B has the highest activity and maximum TS concentration of anammox, which indicates that the anammox reactor can operate steadily and efficiently under the conditions of group B.

Additionally, differences were observed in the nitrate conversion under different influent NO_2^-–N concentrations. The nitrate conversion rate in group B was 12.93 % lower than that in group A, and that in group C was 8.6% lower than that in group B. Therefore, the activities of nitrogen conversion may be related to the growth characteristics of anammox sludge. The growth rate of nitrogen conversion activity and anammox sludge was compared by SPSS statistical analysis method [42]. The results showed that there were statistical differences [43,44]. Fig. 2 clearly shows that the nitrogen conversion rate had a good linear relationship with anammox sludge (R^2 = 0.9998) and had a significant positive correlation (P = 0.001 < 0.01), and the results are similar to those of Isaka et al [3]. This confirms the previous conjecture.

For a smooth anammox reaction, the concentration of the matrix must be controlled below the inhibitory concentration.

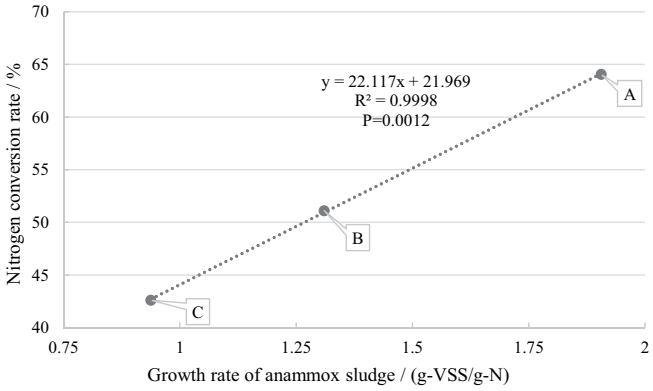

Fig. 2. Relationship between the growth rate of anammox bacteria and nitrogen conversion rate.

Table 2 shows the effect of influent NO_2^-–N concentration on anammox according to different studies. The results obtained by different researchers indicate different inhibitory concentrations of influent NO_2^-–N; however, a high concentration of NO_2^-–N always inhibits the anammox process. According to Strous et al. [19] research results, it appeared that the anammox activity decreased with increasing nitrate concentration. In the presence of more than 0.1 g of nitrate-nitrogen per liter, the process was completely inhibited. Similar to their results, when the maximum influent concentration of NO_2^-–N was 102.99 mg/L, the high concentration of the influent matrix could neither promote the anammox reaction nor inhibit the activity of anammox. However, unlike their research results, the effect of influent NO_2^-–N on the growth rate of anammox sludge was analyzed for the first time in this study. When the maximum concentration of influent NO_2^-–N is 102.99 mg/L, the growth of the anammox sludge will be seriously inhibited, resulting in minimal growth. Anammox sludge growth rate is only 0.94 g-VSS/g-N, but this is 10 times the theoretical value. When the influent concentration of NO_2^-–N in group B was 51.63 mg/L, the maximum SAA was observed and the growth rate was faster than group A. In conclusion, anammox sludge growth was optimal at these conditions.

3.3. Effect on carbon sequestration capacity

Anammox bacteria are chemoautotrophic bacteria and IC is the carbon source of metabolism. According to the anammox reaction (Eq. (4)) [26], approximately 0.066 mol of IC per mol of anammox bacteria is metabolized into new cells by anammox bacteria.

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (4)$$

So far, there are five pathways of IC fixation: the Calvin cycle, anaerobic acetyl-coenzyme A pathway, reductive tricarboxylic acid pathway, 3-hydroxy propionic acid pathway, and succinyl coenzyme A pathway [45,46]. Among them, the anaerobic acetyl coenzyme A pathway is a newly discovered IC fixation pathway for autotrophic microorganisms in strictly anaerobic bacteria. Laboratory or engineering studies have shown that the carbon sequestration capacity

Table 2
Inhibition of anammox by NO_2^-–N concentration in different influent waters

No.	NO_2^- inhibition thresholds $C_{(inf.\ NO_2^-N)}$/(mg/L)	Effect	References
1	≥150 (100% seawater)	Reduced denitrification performance	Qi et al. [22]
2	≥100	Total inhibition activity	Strous et al. [19]
3	350	50% inhibition concentration (IC_{50})	Dapena-Mora et al. [38]
4	53 (No ammonium) 384 (Add to ammonium)	IC_{50} IC_{50}	Carvajal-Arroyo et al. [50]
5	400	IC_{50}	Lotti et al. [14]
6	≥100	Inhibited anammox growth	This research

of anammox bacteria is related to the microbial metabolism and the process of biochemical reaction. It was also confirmed for the first time in our laboratory that anammox bacteria have functional genes that follow the Calvin cycle carbon sequestration pathway and exhibit a certain carbon sequestration capacity [47].

In this experiment, the IC of the effluent NH_4^+–N and NO_2^-–N of each reactor group was detected, and the CO_2 produced by the reactor was collected to detect its concentration. The final carbon sequestration of microorganisms should be the difference between the IC of the influent and the IC of the effluent minus the IC in the form of CO_2 in the tail gas. Fig. 3 shows the relationship between influent NO_2^-–N and the average consumption and carbon sequestration of IC. With increasing influent NO_2^-–N, the average consumption of IC also increased significantly ($P < 0.01$), but the carbon sequestration first increased then decreased. Under three different NO_2^-–N influent conditions, the CO_2 release and IC consumption of group A were 9.15 mL/g-N and 14.43 mg/L, respectively. In group B, the CO_2 release was 12.88 mL/g-N and the average IC consumption was approximately 30.16 mg/L. In group C, the release of CO_2 was 14.99 mL/g-N and the average consumption of IC was 49.1 mg/L. The average carbon sequestration of groups A, B, and C was approximately 0.66, 0.71, and 0.59 mg/mg-N, respectively. Anammox sludge has the largest carbon sequestration under the concentration of NO_2^-–N in the influent of group B. It can carry out carbon metabolism well, and the more carbon is transformed into microbial cells. Therefore, anammox sludge has the best carbon sequestration ability at this stage and can make the anammox sludge grow rapidly under this condition with high activity. When the influent concentration of NO_2^-–N was approximately 51.6 mg/L, the carbon sequestration capacity was the strongest is 0.71 mg/mg-N.

3.4. Effects on microbial changes

High throughput sequencing analysis was conducted by collecting the sludge from the start and end of group A reactor (A1 and A2) and the sludge from groups B and C (B and C). High throughput analysis showed that *Candidatus Kuenenia* was the dominant bacteria in this experiment. Moreover, the microbial community changed gradually with increasing NO_2^-–N concentration in the influent water. Table 3 reflects the diversity of microbial communities through a sample diversity analysis (Alpha diversity). In Table 3, a larger Shannon value indicates higher community diversity and a higher Simpson index indicates lower community diversity. The coverage rates of the samples were 0.96 and 0.97, which indicates a lower probability that the sequences in the samples were not detected. The index reflects the authenticity of the sequencing results; therefore, the same rules can be observed in Table 3. The community diversity of group A and group B was approximately similar, while the community diversity of group C was higher because of the high concentration of NO_2^-–N in the influent water.

As shown in Table 4, the genus abundance of microbial populations at different stages of the reactor. *Candidatus Kuenenia* was the main genus of bacteria in each group of reactors. Among them, it accounted for 76.03% of the initial samples and 75.29% of the final samples in group A, exhibiting a slight decrease. In group B it accounted for 75.11% of the total amount of sludge at the end, which was almost unchanged from that at the beginning. It only accounted for 28.64% of the sludge samples at the end of group C, which was 47.39% lower than that at the beginning of group C, and only 1/3 of the major bacterial genera survived. The decrease in the number of *Candidatus Kuenenia* in group C also explains why the growth rate of anammox sludge was minimal when the influent concentration of NO_2^-–N

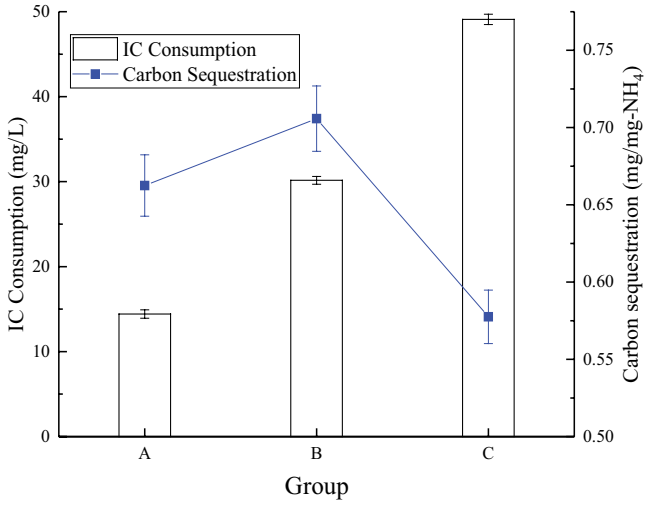

Fig. 3. Changes of IC consumption and carbon sequestration under different influent NO_2^-–N concentrations.

Table 3
Alpha diversity index statistics

Sample ID	Shannon index	Simpson	Coverage
A1	1.79	0.54	0.96
A2	1.79	0.54	0.97
B	1.78	0.53	0.97
C	3.53	0.11	0.96

Table 4
Microbial abundance in different groups of samples at genus level

Group	A1 (%)	A2 (%)	B (%)	C (%)
Candidatus Kuenenia	76.03	75.29	75.11	28.64
Unclassified	15.54	14.79	15.31	44.92
Armatimonadetes_gp5	3.7	4.85	5.13	11.06
Rhodoplanes	0.76	0.79	0.72	2.51
Phycisphaera	0.41	0.45	0.45	0.42
Limnobacter	0.34	0.25	0.19	0.76
Kofleria	0.33	0.27	0.37	1.43
Nitrosomonas	0.24	0.39	0.22	0.78
Oceanibaculum	0.18	0.16	0.14	0.63
Hyphomicrobium	0.18	0.19	0.16	0.45
Other	1.32	1.5	1.04	3.99

was 102.99 mg/L (section 3.2). The community structure of group C is also clearly observed in the distribution map of all samples at the genus level. Compared with the beginning of group C, *Candidatus Kuenenia* decreased by approximately 62%, *Armatimonadetes GP5* approximately doubled, *Rhodoplanes* increased by approximately three times, and *Nitrosomonas* also increased by three times, although the number was small. It is clear that the decrease of *Candidatus Kuenenia's* relative abundance at the end of period C strongly suggests that the anammox reaction activity was inhibited and degradation of anammox sludge at high concentration of nitrite [48].

Fig. 4 shows the species abundance heat map at the generic level, which shows the species changes in different reactors. Each column in the graph represents a sample, each row represents the community structure, and the color bar represents the relative species abundance value; the redder the color, the higher the relative abundance but the bluer the color, the lower the relative abundance. The community structure of the genus in group C reveals a clear change. The red rectangle in the picture shows a more obvious increase in the genus of bacteria, while the blue rectangle exhibits a more obvious decrease in the genus of bacteria. These changes indicate that the high concentration of NO_2^-–N in the influent affected the microbial community structure in the group C reactor. It inhibited the growth of anammox microorganisms and stimulated the growth of other bacteria, resulting in a reduction of anammox sludge

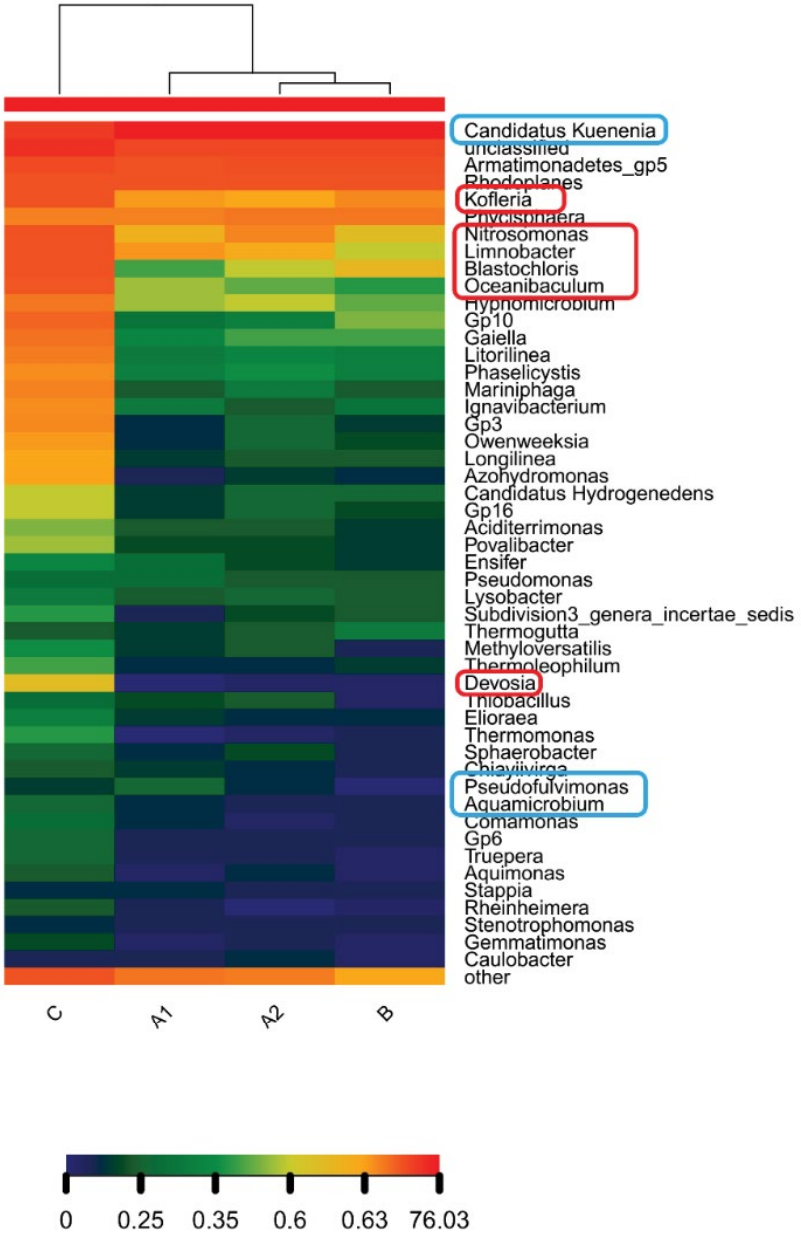

Fig. 4. Genus level species abundance heat map.

concentration and SAA. By comparing the three groups of reactions, it was found that group B could screen out the important bacteria, in that all bacteria except anammox bacteria decreased to a certain extent. Therefore, group B is suggested as the best substrate for anammox.

4. Conclusion

Three groups of UASB reactors were used to culture anammox sludge. Influent NO_2^-–N concentration was negatively correlated with nitrogen removal efficiency. Our results show for the first time that influent NO_2^-–N affects the growth rate of anammox sludge. When the NO_2^-–N influent concentration was high about 102.99 mg/L, anammox activity was inhibited, the carbon sequestration ability of anammox sludge was reduced, the proliferation rate was greatly affected, and anammox sludge growth was minimal. High throughput sequencing indicated that microbial diversity changed at this concentration. *Candidatus Kuenenia* decreased significantly, anammox bacteria growth was inhibited, and optimum conditions occurred for cultivating anammox sludge. When the influent NO_2^-–N concentration was low about 51.63 mg/L, maximum anammox activity and carbon sequestration ability were observed. This allowed more IC to be utilized by anammox sludge, facilitating rapid anammox granular sludge growth. The dominant bacteria in each reactor was *Candidatus Kuenenia*, and these conditions enabled the screening of these bacteria to promote anammox sludge growth.

In addition, ammonia and nitrite have been widely recognized as barriers to their application and industrialization, but there are other inhibitors in the anammox process. For example, different matrix concentrations, nutrients, and intermediates in the reaction process. They will directly or indirectly affect the growth and activity of anaerobic ammonia-oxidizing bacteria, reactor stability, autotrophic denitrification performance, and bacterial community structure. However, the inhibitory impacts of these inhibitors are linked to the anammox species, exposure dosage and operating conditions of the anammox process. At a moderate dose range, some so-called inhibitors (such as NaCl and some species of organic matter) paradoxically promote nitrogen elimination [49]. Therefore, each of these factors could be effectively controlled by appropriate measures. In the experiment, only the external reasons for inhibiting the proliferation of anammox microorganisms were studied. However, the mechanism of anammox microorganism self-proliferation and the effect of hydrazine and hydroxylamine on it have not been studied, which will be the next research direction. Fortunately, the existing research provides a theoretical basis for the rapid cultivation of anaerobic ammonia oxidation sludge and various engineering applications of anaerobic ammonia oxidation process.

Symbols

SAA	—	Specific anammox activity, g-N/(g-VSS d)
ΔN	—	Degradation amount of matrix, mg-N/L
Δt	—	Reaction time, d
$\Delta N/\Delta t$	—	Maximum slope of the linear fitting of matrix degradation time
VSS	—	Concentration of sludge, g-VSS/L
C_{sample}	—	CO_2 content in the gas sample, mg/L
A_{sample}	—	Peak area of CO_2 in the gas sample, Vs
$C_{standard}$	—	CO_2 content in standard gas, mg/L
$A_{standard}$	—	Peak area of CO_2 in standard gas, Vs
$IC_{inf.}$	—	IC in the influent water, mg
$IC_{eff.}$	—	IC in the effluent, mg
CO_2	—	CO_2 production per unit time, mL
ρ_{CO_2}	—	Density of CO_2, g/L
N	—	Nitrogen content per unit time e, mg

Acknowledgements

This research was supported by the Open Project Program of State Key Laboratory of Petroleum Pollution Control (Grant No. PPC2018010), CNPC Research Institute of Safety and Environmental Technology; the National Natural Science Foundation of China (Grant No. 51668013, 51638006); Guangxi Science and Technology Planning Project [grant number GuiKe-AD18126018]; and Special Funding for a Guangxi "BaGui Scholar" Construction Project.

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