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Recovery of nitrogen removal by N_2H_4 after nitrite inhibited anammox reaction

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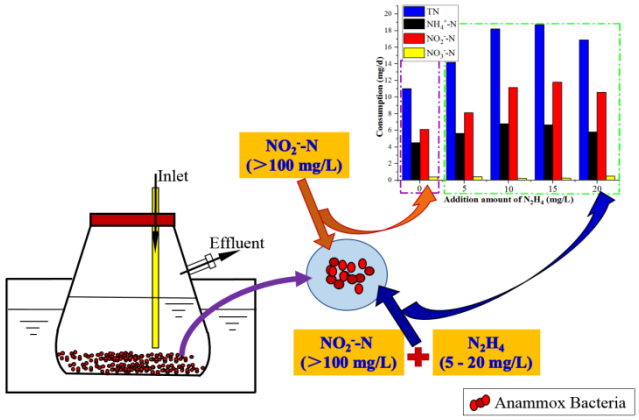
Graphical abstract



Abstract

A pilot-scale sequencing batch reactor (SBR) for anaerobic ammonium oxidation (anammox) of bacteria culture was used along with a batch experimental reaction device to study the effect of NO_2^--N concentration on the activity of anammox bacteria and the recovery of N_2H_4 on anammox bacteria after inhibition by high concentrations of NO_2^--N. The optimal influent NO_2^--N concentration in the pilot-scale reactor was 72.0 mg/L, with its total nitrogen consumption being approximately 40.0 g/d. Influent water NO_2^--N concentrations greater than 100 mg/L had a serious inhibitory effect on the anammox bacteria. At an influent NO_2^--N concentration of 120.35 mg/L, the addition of 10.0-15.0 mg/L of N_2H_4, restored the activity of granular anammox bacteria; the total nitrogen consumption was increased by 69.96%. Microbiological analysis showed that a change in NO_2^--N concentration within the range of 18.87-115.39 mg/L did not affect the microbial population structure of the pilot-scale reactor, wherein *Candidatus Kuenenia* was the dominant bacterial species. In samples collected at stages A0 (sludge inoculation), A20 (the number indicates the NO_2^--N concentration, which, in this stage, was 20 mg/L), A40, A60, A80, and A100, the proportion of *Candidatus Kuenenia* was 27%, 23%, 36%, 26%, 34%, and 33%, respectively.

Keywords: Anammox, pilot-scale, sequencing batch reactor, nitrite, N_2H_4.

1. Introduction

Ammonia nitrogen (NH_4^+-N) wastewater can be obtained from a wide range of human sources. However, if left untreated or below standard, it is discharged into the environment, which can cause serious eutrophication of water (Brase *et al.*, 2018) this leads to significant changes in the aquatic community structure, thereby influencing the safety of human life and production. Therefore, the development of an efficient and low-cost ammonia nitrogen wastewater treatment process is of great significance for environmental protection and social development.

Anaerobic ammonium oxidation (anammox) is a biological reaction process in which anammox bacteria directly reduces NH_4^+-N into N_2 using NO_2^--N as the electron acceptor under anaerobic or anoxic conditions (Kartal *et al.*, 2011; Strous *et al.*, 1999). According to Jetten et al. (2001), in anammox reaction, NO_2^--N and NH_4^+-N were respectively located on the cytoplasmic side of anammox cell membrane and the anammoxosome side of cell membrane. When NO_2^--N on the cytoplasmic side is reduced to NH_2OH, it was transported to the anammoxosome side by hydrazine hydrolase, condensed with NH_4^+-N to form N_2H_4 under its catalysis, and finally reduced to N_2 by hydrazine-oxidising enzyme on the anammoxosome side. And this process is different from nitrification and denitrification (Azhdarpoor *et al.*, 2015; Azhdarpoor *et al.*, 2018). Compared with the traditional nitrogen removal process, the anammox process has the advantages of a high nitrogen removal efficiency, eliminating the need for additional organic carbon sources, low residual sludge outputs, and low operating costs (Chen

et al., 2019; Kuenen, 2008; Lackner *et al.*, 2014). Anammox technology has been applied in many fields for ammonia nitrogen wastewater treatment, such as livestock and poultry farming with wastewater (Wang *et al.*, 2019), landfill leachate (Wang *et al.*, 2016), and monosodium glutamate production wastewater (Shen *et al.*, 2012), and has become a research hotspot in the field of ammonia nitrogen wastewater treatment in China (Wen *et al.*, 2020). Although NH_4^+-N and NO_2^--N are both substrates of the anammox process, anammox bacteria only have a high load capacity for NH_4^+-N; further, high concentrations of NO_2^--N inhibit the activity of anammox bacteria, which is slow to recover. For instance, Bettazzi *et al.* found that the inhibition threshold of NO_2^--N against anammox bacteria in batch experiments was 60 mg/L (Bettazzi *et al.*, 2010). Liu et al. conducted a research in a small integrated anammox process reactor (Zhang *et al.*, 2018) and found that influent NO_2^--N concentrations higher than 100 mg/L had an inhibitory effect on anammox bacteria (Liu *et al.*, 2020). Egli et al. found that anammox bacteria were completely inactivated when the concentration of NO_2^--N in the influent reached 185 mg/L (Egli *et al.*, 2001). According to Dietl *et al.*, N_2H_4 is an important intermediate product in the metabolic process of anammox bacteria (Dietl *et al.*, 2015). N_2H_4 can not only promote the anammox process, but also restore the activity of anammox bacteria inhibited by high concentrations of NO_2^--N. For example, Yao *et al.* (2013) studied anammox bacteria in a sequencing batch reactor (SBR), and found that N_2H_4 increased the specific growth rate of anammox bacteria and inhibited aerobic ammonia oxidation. The optimal concentration of N_2H_4 in the influent was 3.99 mg/L, and the corresponding total nitrogen (TN) removal increased from 0.202 ± 0.011 kg N/m^3/d to 0.370 ± 0.016 kg N/m^3/d. Moreover, Bettazzi *et al.* found that adding 2 mg/L of N_2H_4 could restore 20% activity for anammox bacteria inhibited by high concentrations of NO_2^--N (Bettazzi *et al.*, 2010).

However, most of the above-mentioned studies were based on the results of small-scale tests obtained from sequential batch experiments, and pilot tests or engineering application data for continuous water inflow have rarely been reported. Anammox bacteria are extremely sensitive to their living environment (Khramenkov *et al.*, 2013; Miodoński *et al.*, 2019; Strous *et al.*, 1998). In pilot-scale and engineering applications, due to changes in the reactor and its operating conditions, relevant research results would likely differ somewhat, or even significantly, from those of small-scale tests. Therefore, to support engineering applications of anammox, in view of the problem that anammox bacteria are inhibited by high concentrations of NO_2^--N, the recovery effect of N_2H_4 on the activity of anammox bacteria was tested in a pilot-scale continuous water inflow experiment. Herein, the research object was anammox bacteria cultivated in the laboratory (Jin *et al.*, 2016; Wei *et al.*, 2020), and the concentration gradient was set based on the NO_2^--N concentration of the influent water. Anammox activity was studied by detecting the influent and effluent concentrations of NH_4^+-N and NO_2^--N, as well as the effluent concentration of TN. A concentration gradient of N_2H_4 was set in the influent to study the recovery of different concentrations of N_2H_4 on the activity of anammox bacteria inhibited by high concentrations of NO_2^--N. High-throughput sequencing and field emission scanning electron microscopy (FE-SEM) were used to analyze the sludge samples of anammox bacteria. The pilot experiment provided a more accurate NO_2^--N inhibition threshold for the engineering application of anammox. And the batch experiment provided a concentration reference and theoretical basis for N_2H_4 to restore the activity of anammox when it is inhibited by high concentration NO_2^--N.

2. Materials and methods

2.1. Experimental setup

In this study, a pilot-scale SBR for anammox of bacteria culture was used (Figure 1) along with a batch experiment reactor (Figure 2) (Liu *et al.*, 2019).

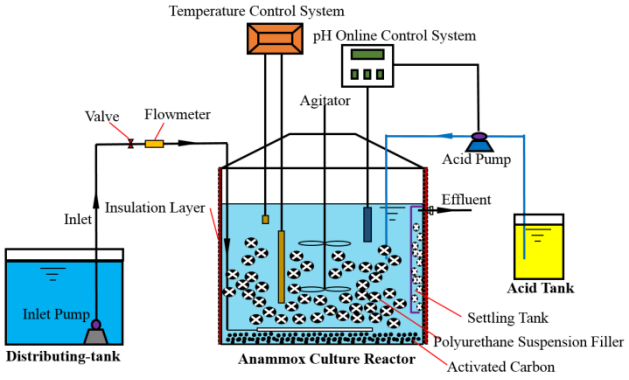


Figure 1. Anammox culture reactor

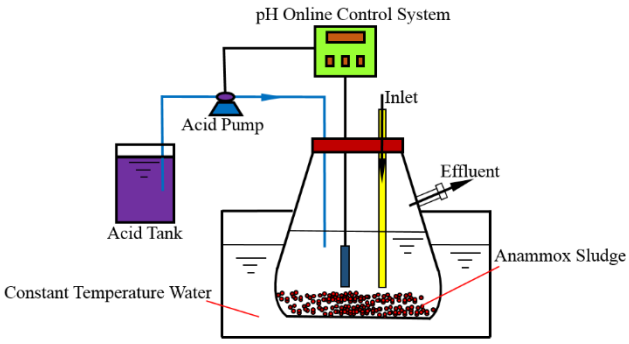


Figure 2. Batch experiment reaction device

The pilot-scale SBR included a distribution tank, an anammox culture reactor, a temperature control system, and an online pH control system. The reaction process is influent, reaction, precipitation and effluent. The influent time was controlled to 40 min, and the reaction time depended on the concentration of NO_2^--N in effluent. The effluent from the reaction was precipitated in the sedimentation tank for 30 min and then discharged. The reactor was basically a cylindrical barrel with a radius of 60 cm, a height of 150 cm, and an effective volume of 1.5 t. It was wrapped with an insulating cotton layer on the outside to keep the reactor warm and provide a dark environment for the anammox bacteria. The dissolved oxygen (DO) in

the water inlet bucket was controlled below 0.6 mg/L by aeration of nitrogen generator (Strous et al., 1998; Yin *et al.*, 2016). The inlet water was pumped into the bottom of the reactor through the inlet pump. Flow control valves and flow meters were installed in the inlet pipe to monitor and control the inlet water flow in real time. The reactor was filled with 15 cm of activated carbon (The activated carbon was soaked in experimental water before adding) and 0.4 m^3 of polyurethane suspension filler to provide an environment for the attachment and growth of anammox bacteria. The speed of the agitator was controlled at 30.0-40.0 r/min. A sedimentation water tank filled with a polyurethane suspension filler was installed on the inner wall of the reactor. Effluent flowed into the sewage treatment system after sedimentation. The pH online control system pumped 5% H_2SO_4 solution into the anammox culture reactor by controlling the acid feed pump, keeping the pH of the reactor between 7.0 and 8.0 (Li *et al.*, 2017; Strous *et al.*, 1999). The temperature control system controlled the temperature within the range of 28.0-31.0 with a temperature sensor and heating rod (deGraaf *et al.*, 1996; Isaka *et al.*, 2007).

The reaction device of the batch experiment was designed in the laboratory; it was made of plexiglass, and the volume was 0.5 L (Liu *et al.*, 2019). During the experiment, the device was protected from light to prevent the adverse effects of light on bacteria (Ni *et al.*, 2011). Aeration of nitrogen generator was used to control the DO in the water tank to below 0.6 mg/L (Strous *et al.*, 1998; Yin *et al.*, 2016). pH was controlled between 7.0 and 8.0 using a pH online control system (Li *et al.*, 2017; Strous *et al.*, 1999). Temperature was maintained at 30.0 ± 1.0°C by heating in a water bath (deGraaf *et al.*, 1996; Isaka *et al.*, 2007). The inlet and outlet water were sampled with a peristaltic pump to avoid the DO influence during operation. When water entered, one end of the inlet pipe (silicone hose) was connected to the inlet of the device, where the bottom end of the inlet was placed deep in the sludge layer to prevent oxygen in the air from mixing in the reactor, and the other end was connected to the prepared artificial simulated wastewater. When out of water, the soft silicone tube was gradually moved downward along the side wall of the reactor to take water, and the water outlet was kept sealed during the operation of the reaction device. There were five groups in the batch experiment, each group had three parallel samples, and the running period of each group was 1 d.

2.2. Operational strategies and inoculated sludge

Artificial wastewater was used as the experimental water; its composition is shown in Table 1. The NH_4^+-N content of the wastewater was provided by NH_4HCO_3, and NO_2^--N was provided by $NaNO_2$. In addition, given amounts of $NaHCO_3$, KH_2PO_4, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, and microbial accelerant (Ruicheng Environmental Protection, Shandong, China) (Liu *et al.*, 2019) were added. The experimental process was divided into two phases. Phase I mainly studied the effect of different influent NO_2^--N concentrations on anammox bacteria activity. Phase II mainly aimed to study the recovery effect of different concentrations of N_2H_4 on anammox bacteria inhibited by high NO_2^--N concentrations. The NO_2^--N concentration in the influent of phase I was divided into five concentration gradients of A20, A40, A60, A80, and A100, where the concentration of NO_2^--N was 20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L, and ≥ 100 mg/L, respectively. Each phase run for 20 days. Phase II influent N_2H_4 was divided into five concentration gradients of B0, B5, B10, B15, and B20, where the concentration of N_2H_4 was 0 mg/L, 5.0 mg/L, 10.0 mg/L, 15.0 mg/L, and 20.0 mg/L, respectively. Phase I was carried out in the pilot-scale SBR, and phase II was carried out in the batch experimental reaction device. According to the experimental results of phase I, when the concentration of NO_2^--N in the influent was 103.45-115.39 mg/L (A100), anammox bacteria would be severely inhibited. Therefore, in order to study the recovery effect of N_2H_4 on anammox bacteria inhibited by high concentration of NO_2^--N, the concentration of NO_2^--N in the influent in phase II was set to 120 mg/L.

The inoculated sludge was taken from anaerobic ammonia oxidation sludge cultured in the laboratory of Kumamoto University for more than 1 year for the inoculation of this study, and the load of anaerobic ammonia oxidation bacteria was 0.3 kg $N/m^3/d$, and its shape was red granules. In phase I, the sludge inoculation amount and sludge concentration of the pilot-scale SBR of anammox bacteria culture were 3 L and 3000 mg/L, respectively. In phase II, the batch experimental device was inoculated with anammox sludge taken after the completion of the phase I experiment; the inoculation amount and concentration were 100 mL and 2000 mg/L, respectively.

2.3. Quantification methods

During the experiment, DO was measured using a HQ30d portable dissolved oxygen meter (Hach Company, Loveland, CO, USA). The online pH control system was a DPH10AC + DPH-SOC10 instrument (Tianjian Innovation Environmental Technology Co., Ltd., Beijing, China). The temperature control system was a WK-05 + HW-3000 instrument (Hai Cube Refrigeration Equipment Co., Ltd., Shandong, China). NH_4^+-N and NO_2^--N were determined according to the standard colorimetric method (Jin *et al.*, 2016). NO_3^--N was calculated by subtraction method. TN via alkaline potassium persulfate ultraviolet spectrophotometry (Zhang W. *et al.*, 2015). The concentration of biomass is calculated according to the following method: The anaerobic ammonia oxidizing bacteria mixture was taken from the reactor species, 100 ml of the mixture was centrifuged at 2000 *g* for 5 min, the supernatant was discarded, the remaining sludge was weighed, and 2 other parallel groups were made, and the average value was taken as the inoculated sludge concentration.

2.4. Microbiological analysis

The sludge samples of anammox bacteria were analyzed by high-throughput sequencing and FE-SEM technology. A soil DNA kit (DNeasy Power Soil DNA Isolation Kit 12888-50, Mo Bio, USA) was used to extract DNA from all the sludge after phase I experiment samples(A0-A100) according to the instructions. The v3-v4 region of the qualified DNA was

amplified by PCR using 341F (5'-CCCTACACGACGCTCTTCCGATCTG-3') and 805R (5'-GACTGGAGTTCCTTGGCACCCGAGAATTCCA-3') primers. After amplification, agarose gel electrophoresis, purification, and quantitative mixing were performed. Shanghai Sangon Biotech (Shanghai) Co., Ltd. was entrusted to complete the subsequent sequencing of the microorganisms. Finally, the gene sequence was compared in the GenBank to analyze the diversity of samples (Liu X. *et al.*, 2020). Sludge samples taken after completing the B10 experiment were analyzed by FE-SEM. In the FE-SEM experiment, the sludge samples were cleaned and then vacuum-dried for 2 h. After adding a gold-plated conductive film, the sludge samples were observed using a Sigma 500 scanning electron microscope (Carl Zeiss, Jena, Germany).

Table 1. Composition of experimental wastewater used in each stage of the experiment

Phase	Stage	NO_2^-N (mg/L)	NH_4^+-N (mg/L)	N_2H_4 (mg/L)	$NaHCO_3$ (mg/L)	KH_2PO_4 (mg/L)	$CaCl_2 \cdot 2H_2O$ (mg/L)	$MgSO_4 \cdot 7H_2O$ (mg/L)	Microbial accelerant (mg/L)
I	A20	18.87-22.95	14.24 - 17.56	--	1000	25	113	100	0.5
	A40	41.31-43.61	30.12 - 35.84	--					
	A60	61.84-68.34	44.85 - 48.35	--					
	A80	79.61-86.34	58.96 - 64.35	--					
	A100	103.45-115.39	71.34 - 90.35	--					
II	B0	120.35	91.74	0					
	B5			5.0					
	B10			10.0					
	B15			15.0					
	B20			20.0					

Table 2. A20-A100 pearson correlation analysis

Stage		Inf- NO_2^--N	NH_4^+-N	NO_2^--N	NO_3^--N	TN
A20-A60	Inf- NO_2^--N	-	0.955*	0.947**	0.088	0.952**
A80-A100	Inf- NO_2^--N	-	-0.837**	-0.869**	0.086	-0.896**

*At the 0.05 level (two-tailed), the correlation is significant.

**At the 0.01 level (two-tailed), the correlation is significant.

2.5. Statistical methods

All statistical analyses were performed using SPSS project software. Pearson correlation analysis was performed using Pearson analysis for N_2H_4 and NH_4^+-N, NO_2^--N, NO_3^--N, TN. The main purpose of stage I was to study the effect of different influent NO_2^--N concentrations on the activity of anammox bacteria, which was divided into two stages for correlation analysis according to the experimental results in order to better reflect the realized results; the main purpose of stage II was to study the recovery effect of different concentrations of N_2H_4 on anammox bacteria inhibited by high concentrations of NO_2^--N, and correlation analysis was performed on B0-B15 data.

3. Results and discussion

3.1. Effect of NO_2^--N on anammox

The results of Pearson's correlation analysis between the concentration of NH_4^+-N, NO_2^--N and TN consumption and NO_3^--N production are shown in Table 2 for the stages A20-A60 and A80-A100. In the A20-A60 stage, there was a significant positive correlation between the influent NO_2^--N concentration and the consumption of NH_4^+-N, NO_2^--N and TN. In A80-A100 stage, the influent NO_2^--N concentration and the consumption of NH_4^+-N, NO_2^--N and TN were significantly negatively correlated. From the perspective of correlation analysis, the nitrogen consumption increased significantly with the increase of influent NO_2^--N concentration in the A20-A60 stage. But decreased with the increase of influent NO_2^--N concentration in the A80-A100 stage, indicating that the influent NO_2^--N inhibited the activity of anammox reaction in the A80-A100 stage, resulting in the decrease of nitrogen consumption.

Figure 3 shows the consumption of NH_4^+-N, NO_2^--N, and TN in the pilot-scale SBR of anammox bacteria culture under different influent NO_2^--N concentrations. According to the experimental results, in A20, A40, A60, A80, and A100, the average consumption of TN was 7.43 g/d, 23.56 g/d, 39.02 g/d, 37.51 g/d, and 21.31 g/d, respectively; the average consumption of NH_4^+-N was 2.87 g/d, 9.73 g/d, 15.83 g/d, 16.62 g/d, and 8.81 g/d, respectively; and the average consumption of NO_2^--N was 4.12 g/d, 23.34 g/d, 22.73 g/d, 21.68 g/d, and 12.06 g/d, respectively. The average production of NO_3^--N was less than 0.5mg/d. During the A20 to A60 stage, when the NO_2^--N concentration of the influent increased from 18.87-22.95 mg/L to 61.84-68.34 mg/L, the consumption of TN in the pilot-scale SBR continuously increased. The average consumption of TN

during A40 and A60 relative to A20 increased by 217.09% and 425.17%, respectively, indicating that the increase in the concentration of NO_2^--N in the influent at this time provided more substrate for anammox bacteria in the reactor, which helped to improve anammox activity. During the A80 to A100 stage, when the NO_2^--N concentration in the influent increased from 79.61-86.34 mg/L to 103.45-115.39 mg/L, the TN consumption of the reactor was significantly reduced. The average consumption of TN in the A100 stage was only 56.81% that of the A80 stage, indicating that the concentration of NO_2^--N in the influent was too high, and the activity of anammox bacteria was inhibited by high NO_2^--N concentrations.

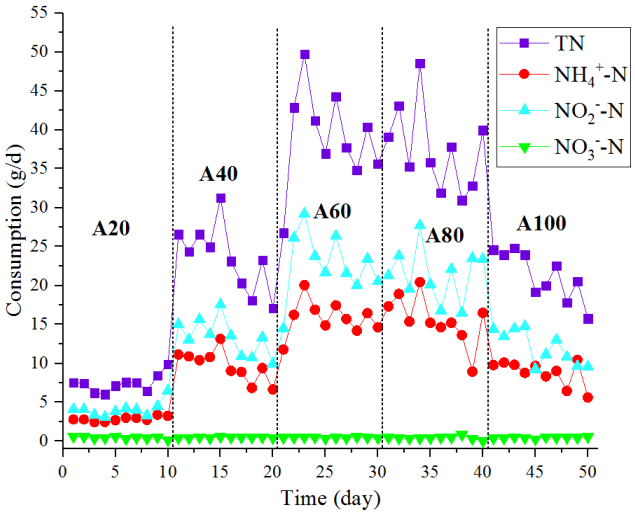


Figure 3. Consumption of NH_4^+-N, NO_2^--N, NO_3^--N, and TN in the reactor under different influent NO_2^--N concentrations

Figure 4 plots the relationship of NO_2^--N concentration in the influent of A20, A40, A60, A80, and A100 with the corresponding average consumption of TN. Accordingly, the optimal influent NO_2^--N concentration of the pilot-scale SBR was approximately 72.0 mg/L, and the corresponding TN consumption was about 40.0 g/d. The NO_2^--N concentration inhibition threshold obtained in this study was 12.0 mg/L higher than the inhibition threshold (60 mg/L) obtained by Bettazzi *et al.* in batch experiments (Bettazzi *et al.*, 2010). This may be because the culture mode of the SBR at the pilot scale was more conducive to anammox bacteria, or it may be that the load of NO_2^--N of the granular anammox sludge (Jin *et al.*, 2016; Wei *et al.*, 2020) inoculated in this experiment was greater than that of the liquid anammox sludge inoculated by Bettazzi *et al.* (Bettazzi *et al.*, 2010). Lopez *et al.* used granular anammox sludge in a SBR to treat high-nitrogen wastewater. When the concentration of NO_2^--N exceeded 100 mg/L, the inhibitory effect of NO_2^--N on anammox was observed (Lopez *et al.*, 2008), which indicated that granular anammox had a stronger tolerance to changes in the growth environment than liquid anammox.

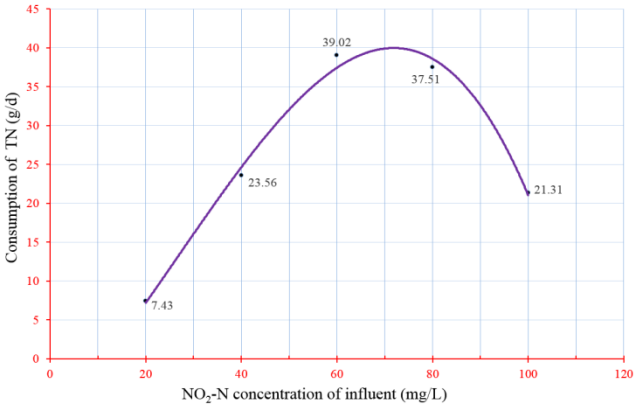


Figure 4. Relationship between NO_2^--N concentration in influent and TN consumption

3.2. Effect of N_2H_4 on anammox inhibited by high concentrations of NO_2^--N

According to the experimental results of phase I, to study the recovery effect of N_2H_4 on anammox bacteria inhibited by high concentrations of NO_2^--N, the influent NO_2^--N of phase II was set around 120 mg/L.

The results of Pearson correlation analysis of N_2H_4 concentration on NH_4^+-N, NO_2^--N and TN consumption and NO_3^--N production are shown in Table 3. In the B0-B15 stage, the concentration of N_2H_4 in the feed water and nitrogen consumption showed a significant positive correlation. It can be seen that in a certain concentration range, increasing N2H4 concentration is beneficial to the recovery of anammox bacteria activity after being inhibited by NO_2^--N, among which the recovery of NO_2^--N consumption is more obvious.

Table.3 B0-B15 pearson correlation analysis

	N_2H_4	NH_4^+-N	NO_2^--N	NO_3^--N	TN
N_2H_4	-	0.925	0.975*	-0.804	0.965*

*At the 0.05 level (two-tailed), the correlation is significant.
**At the 0.01 level (two-tailed), the correlation is significant.

Figure 5 shows the average daily consumption of NH_4^+-N, NO_2^--N, and TN by each batch of reactors. According to the experimental results, the average NH_4^+-N removal amounts of B0 (0 mg/L), B5 (5.0 mg/L), B10 (10.0 mg/L), B15 (15.0 mg/L) and B20 (20.0 mg/L) were 4.50 mg/d, 5.63 mg/d, 6.81 mg/d, 6.67 mg/d and 5.81 mg/d, respectively. The average removal of NO_2^--N was 6.13 mg/d, 8.11 mg/d, 11.17 mg/d, 11.82 mg/d, and 10.59 mg/d, respectively; The average removal of NO_3^--N was kept at 0.23-0.49 mg/d. The average TN removal was 11.02 mg/d, 14.18 mg/d, 18.21 mg/d, 18.73 mg/d, and 16.89 mg/d, respectively. Thus, B0 showed the lowest nitrogen removal efficiency. Combined with the data obtained in phase I, the consumption of the substrate by anammox bacteria is greatly reduced in environments with a high concentration of NO_2^--N (concentration > 100 mg/L); therefore, its activity was inhibited by high concentrations of NO_2^--N. After the addition of N_2H_4, the consumption of NH_4^+-N and NO_2^--N in B5, B10, B15, and B20 were significantly greater than that of B0, indicating that N_2H_4 had a restorative effect on anammox bacteria activity (Xiao *et al.*, 2015; Zekker *et al.*,

2012). As shown in Figure 5, after adding N_2H_4, the consumption of TN by B5, B10, B15, and B20 increased by 28.68%, 65.24%, 69.96%, and 53.27% relative to B0, respectively. When $N_2H_4 \leq 15.0$ mg/L, the consumption of TN increased, but when $N_2H_4 \geq 20.0$ mg/L, it decreased. The reason for this result may be that excessive N_2H_4 causes hydrazine oxidase to directly oxidize N_2H_4 into N_2, reducing the demand for nitrite oxidoreductase for NO_2^--N; alternatively, an excessively high concentration of N_2H_4 might have poisoned the anammox bacteria. According to the experimental results, addition of $10.0 \leq N_2H_4 \leq 15.0$ mg/L recovered the activity of anammox bacteria inhibited by high concentrations of NO_2^--N.

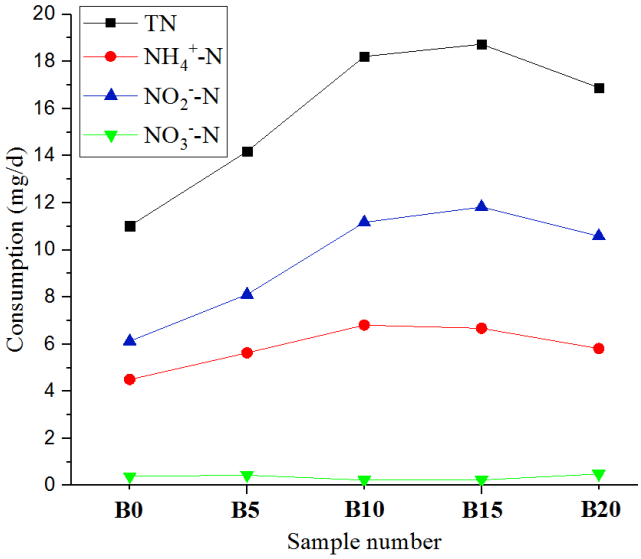


Figure 5. Recovery effect of N_2H_4 on anammox bacteria

3.3. Microbiological analysis

FE-SEM images of the sludge samples of B10 (N_2H_4 = 10.0 mg/L) in phase II are shown in Figure 6. In Figure 6, a large number of spheroid bacteria with a diameter of approximately 1-2 μm can be observed. This was consistent with the observation results of the external morphological characteristics of mature and highly active anammox bacteria by Kartal *et al.* (Kartal *et al.*, 2007) and Van *et al.* (van Niftrik *et al.*, 2008). Combined with the experimental results of Phase II, after adding 10.0 mg/L of N_2H_4, TN consumption by anammox bacteria in B10 increased by 65.24% relative to B0 (N_2H_4 = 0 mg/L), indicating that the anammox bacteria could maintain high activity and good metabolism and reproduction activities.

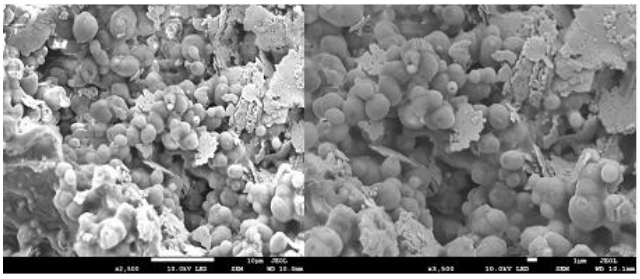


Figure 6. Anammox morphology observed by FE-SEM

In order to find out the effect of different influent concentrations (A20, A20) on the structure of anaerobic sludge, the influent concentrations of A2 and A2 were further investigated Kuenenia was the main dominant strain in our experiment. In the experiment of stage I, with the increase of NO_2^-- N concentration in influent water, anammox flora also changed gradually. Table 4 reflects the diversity of microbial community through alpha diversity analysis (5 species). The Shannon value showed that the diversity of A60 community was the highest, followed by A20. According to Simpson index, A20 community diversity was the highest, followed by the initial sludge A0 and A60. The coverage rates of all samples were 0.996 and 0.997 respectively, which indicated that the probability of no sequence detected in the samples was low, which also reflected the authenticity of the sequencing results of this experiment.

Table 4. Statistical table of alpha diversity index (5 species)

Sample ID	ACE	Chao1	Shannon	Simpson	Coverage
A0	1106.70	1084.17	3.82	0.09	0.9964
A20	1331.79	1155.92	3.94	0.07	0.9962
A40	1087.27	1058.90	3.57	0.15	0.9974
A60	1225.42	1210.92	4.04	0.09	0.9974
A80	1082.77	1101. 70	3.88	0.11	0.9968
A100	1139.18	1119.39	3.68	0.13	0.9973

Figure 7 shows the changes of N consumption and microbial community under different concentrations in stage a. At the concentration of A40, anammox bacteria accounted for the highest proportion. This also explains the increase in TN consumption compared to the initial sludge (Section 3.1). It can be seen from Figure 7 that with the increase of NO_2^--N concentration and incubation time, the proportion of anammox bacteria also changed, from 27.36% of initial sludge to 34.11% of final sludge. However, during this period, the consumption of TN changed differently, A40 (36%) and A80 (34%) accounted for more than anammox bacteria under A60 (26%), but the TN consumption was the highest under A60 conditions. It can be seen that anammox bacteria are not the only bacteria involved in nitrogen removal in the reactor, and the proportion of armimonadetes-gp5 strain under A60 condition is 2.99%, which is second only to anammox bacteria abundance, and combined with A20 (2.81%), a40 (2.75%), A80 (2.24%) and A100(72%) showed that *Armatimonadetes-gp5* strain had a certain contribution rate of denitrification to the reactor. From Figure 7, it can also be found that the proportion of *Armimonadetes-gp5*

bacteria in A80 and A100 sludge samples is decreased to a certain extent compared with A20, A40 and A60. Combined with the experimental data of nitrous acid substrate, the nitrite concentrations in A80 and A100 are 80 mg/L and 100 mg/L, respectively. The results showed that the anammox bacteria in the reactor were inhibited to a certain extent, which was consistent with the decreasing trend of the proportion of *Armatimonates-gp5*. Sample A100 represents that the concentration of nitrite in the reactor where the sludge is located is the highest (above 100 mg/L), while the proportion of *Armimonadetes-gp5* is the lowest. Therefore, the change of the specific gravity of *Armimonadetes-gp5* can be used as one of the reference points for the inhibition of anammox.

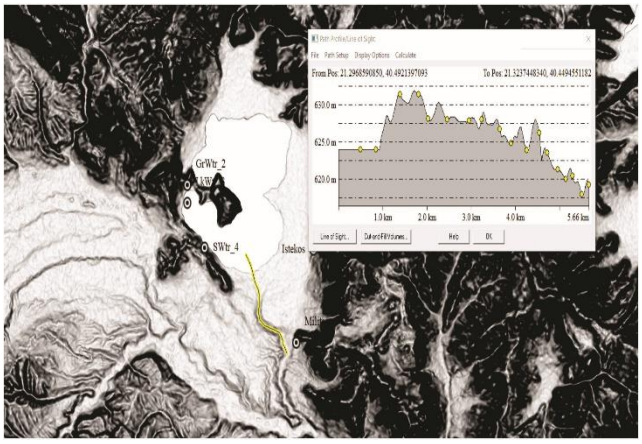


Figure 7. Changes of N consumption and microbial community under different concentrations

With the influent nitrite concentration reaching 100mg/L, anammox bacteria accounted for 33%, higher than A60 (26%), which was basically the same as A80 (34%). However, the TN consumption decreased sharply. It can be seen that when the influent nitrite concentration reaches 100mg/L, anammox bacteria account for 33%, which is higher than A60 (26%), which is basically the same as A80 (34%), the activity of anammox will be inhibited, which will affect the overall denitrification rate of the reactor.

In this study, the inhibitory effect of NO_2^--N on anammox bacteria and the recovery effect of N_2H_4 on anammox bacterial activity inhibited by high concentrations of NO_2^--N were studied in a pilot-scale SBR of anammox bacterial culture and a batch experimental device. This study helps address the lack of experimental results for actual engineering applications, and provides a reference and theoretical basis for accelerating engineering applications of the anammox process.

4. Conclusions

The optimal influent NO_2^--N concentration of the pilot sequencing batch anammox bacteria culture reactor was 72.0 mg/L, which yielded a TN consumption rate of approximately 40.0 g/d. Influent NO_2^--N concentrations exceeding 100 mg/L had a serious inhibitory effect on the anammox bacteria. However, at an influent NO_2^--N concentration of 120.35 mg/L, adding 10.0-15.0 mg/L of N_2H_4 was observed to restore the anammox bacteria activity, which increased the TN consumption by 69.96%. According to the results of the microbiological analysis, the anammox bacterium *Candidatus Kuenenia* was the dominant strain in the pilot sequencing batch anammox bacteria culture reactor.

Author contributions

Conceptualization, W.Z.; methodology, R.W. and W.Y.; software, R.W. and W.Y.; validation, W.Z.; formal analysis, R.W.; investigation, R.W.; resources, Y.W.; data curation, Y.W.; writing—original draft preparation, Y.W.; writing—review and editing, R.W.; visualization, R.W.; supervision, W.Z.; project administration, W.Z.; funding acquisition, W.Z.

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Conflicts of interest

The authors declare no conflict of interest.

References

Azhdarpoor A., Abbasi L. and Samaei M.R. (2018), Investigation of a new double-stage aerobic-anoxic continuous-flow cyclic baffled bioreactor efficiency for wastewater nutrient removal, *Journal of Environmental Management i,* **211**, 1–8.

Azhdarpoor A., Mohammadi P. and Dehghani M. (2015), Simultaneous removal of nutrients in a novel anaerobic–anoxic/aerobic sequencing reactor: removal of nutrients in a novel reactor, *International Journal of Environmental Science and Technology,* **13**, 543–550.

Bettazzi E., Caffaz S., Vannini C. and Lubello C. (2010), Nitrite inhibition and intermediates effects on Anammox bacteria: A batch-scale experimental study, *Process Biochemistry,* **45**, 573–580.

Brase L., Sanders T. and Dahnke K. (2018), Anthropogenic changes of nitrogen loads in a small river: external nutrient sources vs. internal turnover processes, *Isotopes in Environmental and Health Studies,* **54**, 168–184.

Chen Z.J., Meng Y.B., Sheng B.B., Zhou Z.B., Jin C. and Meng F.G. (2019), Linking Exoproteome Function and Structure to Anammox Biofilm Development, *Environmental Science & Technology,* **53**, 1490–1500.

deGraaf A.A.V., deBruijn P., Robertson L.A., Jetten M.S.M. and Kuenen J.G. (1996), Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor, *Microbiology-Uk,* **142**, 2187–2196.

Dietl A., Ferousi C., Maalcke W.J., Menzel A., de Vries S., Keltjens J.T., Jetten M.S.M., Kartal B. and Barends T.R.M. (2015), The inner workings of the hydrazine synthase multiprotein complex, *Nature,* **527**, 394–397.

Egli K., Fanger U., Alvarez P.J.J., Siegrist H., van der Meer J.R. and Zehnder A.J.B. (2001), Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate, *Archives of Microbiology,* **175**, 198–207.

Isaka K., Sumino T. and Tsuneda S. (2007), High nitrogen removal performance at moderately low temperature utilizing anaerobic ammonium oxidation reactions, *Journal of Bioscience and Bioengineering,* **103**, 486–490.

Jetten M.S.M., Wagner M., Fuerst J., van Loosdrecht M., Kuenen G. and Strous M. (2001), Microbiology and application of the anaerobic ammonium oxidation ('anammox') process, *Current Opinion in Biotechnology,* **12**, 283–288.

Jin Y., Wang D.Q. and Zhang W.J. (2016), Use of bamboo charcoal reduced the cultivated anammox seed sludge dosage during the start-up period, *Desalination and Water Treatment,* **57**, 20248–20253.

Kartal B., Maalcke W.J., de Almeida N.M., Cirpus I., Gloerich J., Geerts W., den Camp H.J.M.O., Harhangi H.R., Janssen-Megens E.M., Francoijs K.J., Stunnenberg H.G., Keltjens J.T., Jetten M.S.M. and Strous M. (2011), Molecular mechanism of anaerobic ammonium oxidation, *Nature,* **479**, 127–U159.

Kartal B., Rattray J., van Niftrik L.A., van de Vossenberg J., Schmid M.C., Webb R.I., Schouten S., Fuerst J.A., Damste J.S.S., Jetten M.S.M. and Strous M. (2007), Candidatus "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria, *Systematic and Applied Microbiology,* **30**, 39–49.

Khramenkov S.V., Kozlov M.N., Kevbrina M.V., Dorofeev A.G., Kazakova E.A., Grachev V.A., Kuznetsov B.B., Polyakov D.Y. and Nikolaev Y.A. (2013), A novel bacterium carrying out anaerobic ammonium oxidation in a reactor for biological treatment of the filtrate of wastewater fermented sludge, *Microbiology,* **82**, 628–636.

Kuenen J.G. (2008), Anammox bacteria: from discovery to application, *Nature Reviews Microbiology,* **6**, 320–326.

Lackner S., Gilbert E.M., Vlaeminck S.E., Joss A., Horn H. and van Loosdrecht M.C.M. (2014), Full-scale partial nitritation/anammox experiences - An application survey, *Water Research,* **55**, 292–303.

Li J., Zhu W.Q., Dong H.Y. and Wang D. (2017), Performance and kinetics of ANAMMOX granular sludge with pH shock in a sequencing batch reactor, *Biodegradation,* **28**, 245–259.

Liu X., Jin Y. and Zhang W. (2020), Effect of nitrite concentration on the growth and microbial diversity of anaerobic ammonia oxidation (anammox) sludge, *Desalination and Water Treatment,* **179**, 54–62.

Liu X.N., Wang H.Q., Li H.X., Jin Y. and Zhang W.J. (2019), Carbon sequestration pathway of inorganic carbon in partial nitrification sludge, *Bioresource Technology,* **293**

Lopez H., Puig S., Ganigue R., Ruscalleda M., Balaguer M.D. and Colprim J. (2008), Start-up and enrichment of a granular anammox SBR to treat high nitrogen load wastewaters, *Journal of Chemical Technology and Biotechnology,* **83**, 233–241.

Miodoński S., Muszyński-Huhajło M., Zięba B., Ratkiewicz K. and Łagocka M. (2019), Fast start-up of anammox process with hydrazine addition, *SN Applied Sciences,* **1**, 523.

Ni S.Q., Gao B.Y., Wang C.C., Lin J.G. and Sung S.W. (2011), Fast start-up, performance and microbial community in a pilot-scale anammox reactor seeded with exotic mature granules, *Bioresource Technology,* **102**, 2448–2454.

Shen L.D., Hu A.H., Jin R.C., Cheng D.Q., Zheng P., Xu X.Y. and Hu B.L. (2012), Enrichment of anammox bacteria from three sludge sources for the startup of monosodium glutamate industrial wastewater treatment system, *Journal of Hazardous Materials,* **199**, 193–199.

Strous M., Heijnen J.J., Kuenen J.G. and Jetten M.S.M. (1998), The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms, *Applied Microbiology and Biotechnology,* **50**, 589–596.

Strous M., Kuenen J.G. and Jetten M.S.M. (1999), Key physiology of anaerobic ammonium oxidation, *Applied and Environmental Microbiology,* **65**, 3248–3250.

van Niftrik L., Geerts W.J.C., van Donselaar E.G., Humbel B.M., Webb R.I., Fuerst J.A., Verkleij A.J., Jetten M.S.M. and Strous M. (2008), Linking ultrastructure and function in four genera of anaerobic ammonium-oxidizing bacteria: Cell plan, glycogen storage, and localization of cytochrome c proteins, *Journal of Bacteriology,* **190**, 708–717.

Wang X., Yang R., Zhang Z., Wu J. and Chen S. (2019), Mass balance and bacterial characteristics in an in-situ full-scale swine wastewater treatment system occurring anammox process, *Bioresource Technology,* **292.**

Wang Z., Peng Y.Z., Miao L., Cao T.H., Zhang F.Z., Wang S.Y. and Han J.H. (2016), Continuous-flow combined process of nitritation and ANAMMOX for treatment of landfill leachate, *Bioresource Technology,* **214**, 514–519.

Wei Y., Jin Y. and Zhang W. (2020), Domestic Sewage Treatment Using a One-Stage ANAMMOX Process, *International Journal of Environmental Research and Public Health,* **17**, 3284.

Wen R., Jin Y. and Zhang W. (2020), Application of the Anammox in China-A Review, *International Journal of Environmental Research and Public Health.,* **17.**

Xiao P.Y., Lu P.L., Zhang D.J., Han X.K. and Yang Q.X. (2015), Effect of trace hydrazine addition on the functional bacterial community of a sequencing batch reactor performing completely autotrophic nitrogen removal over nitrite, *Bioresource Technology,* **175**, 216–223.

Yao Z.B., Cai Q., Zhang D.J., Xiao P.Y. and Lu P.L. (2013), The enhancement of completely autotrophic nitrogen removal over nitrite (CANON) by N2H4 addition, *Bioresource Technology,* **146**, 591–596.

Yin Z.X., dos Santos C.E.D., Vilaplana J.G., Sobotka D., Czerwionka K., Damianovic M.H.R.Z., Xie L., Morales F.J.F. and Makinia J. (2016), Importance of the combined effects of dissolved oxygen and pH on optimization of nitrogen removal in anammox-enriched granular sludge, *Process Biochemistry,* **51**, 1274–1282.

Zekker I., Kroon K., Rikmann E., Tenno T., Tomingas M., Vabamae P., Vlaeminck S.E. and Tenno T. (2012), Accelerating effect of hydroxylamine and hydrazine on nitrogen removal rate in moving bed biofilm reactor, *Biodegradation,* **23**, 739–749.

Zhang W., Wang H., Yue J. and Joseph D.R. (2015), Granular activated carbon as nucleus for formation of Anammox granules in an expanded granular-sludge-bed reactor, *Global NEST Journal,* **17**, 508–514.

Zhang W.J., Wang D.Q. and Jin Y. (2018), Effects of inorganic carbon on the nitrous oxide emissions and microbial diversity of an anaerobic ammonia oxidation reactor, *Bioresource Technology,* **250**, 124–130.