# Recovery of nitrogen removal by $N_2H_4$ after nitrite inhibited anammox reaction

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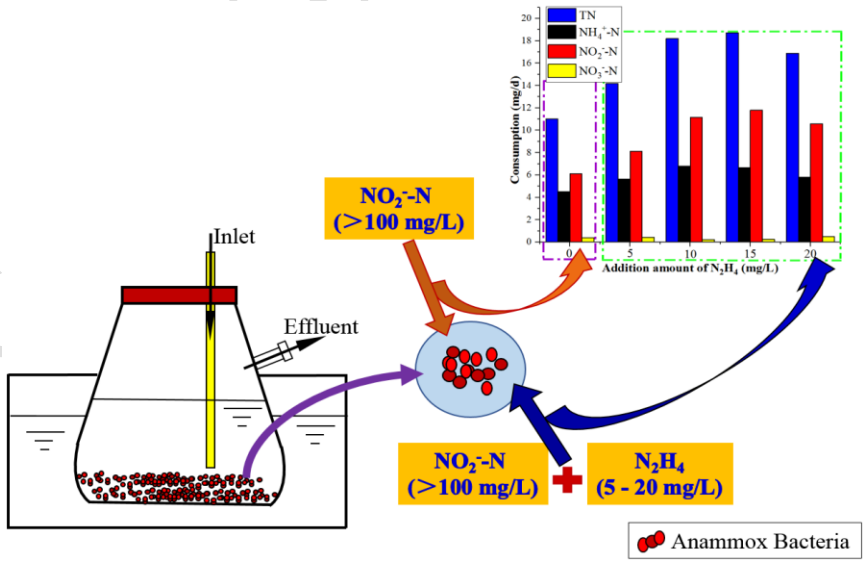
**Graphical abstract：**



**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.*, 2018; Azhdarpoor *et al.*, 2015). 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 X. *et al.*, 2019), landfill leachate (Wang Z. *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 W. J. *et al.*, 2018) and found that influent $NO_2^-$-N concentrations higher than 100

mg/L had an inhibitory effect on anammox bacteria (Liu X. *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 X. N. *et al.*, 2019).

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).

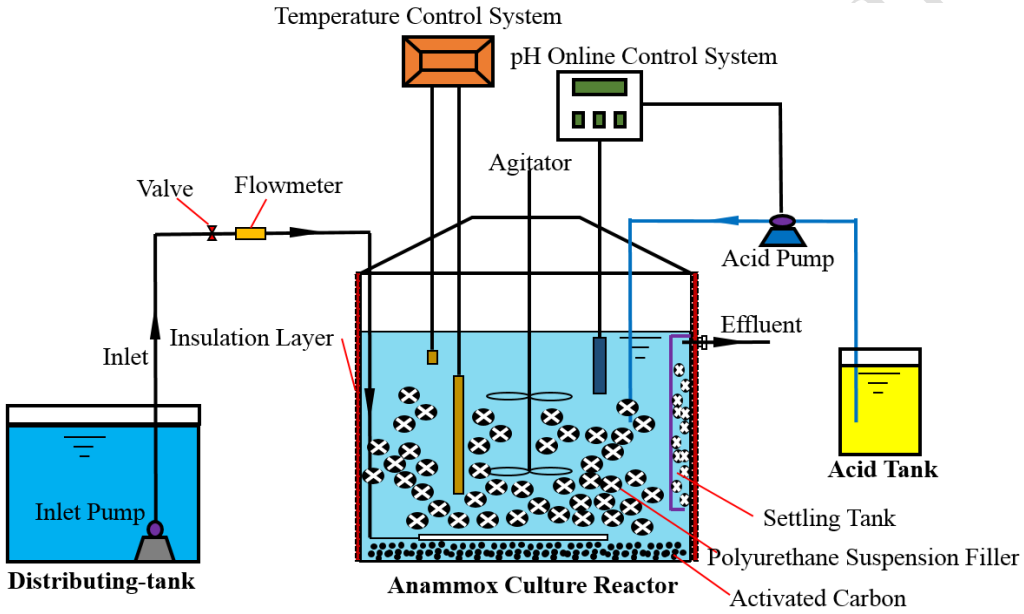



**Figure 1.** Anammox culture reactor

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.

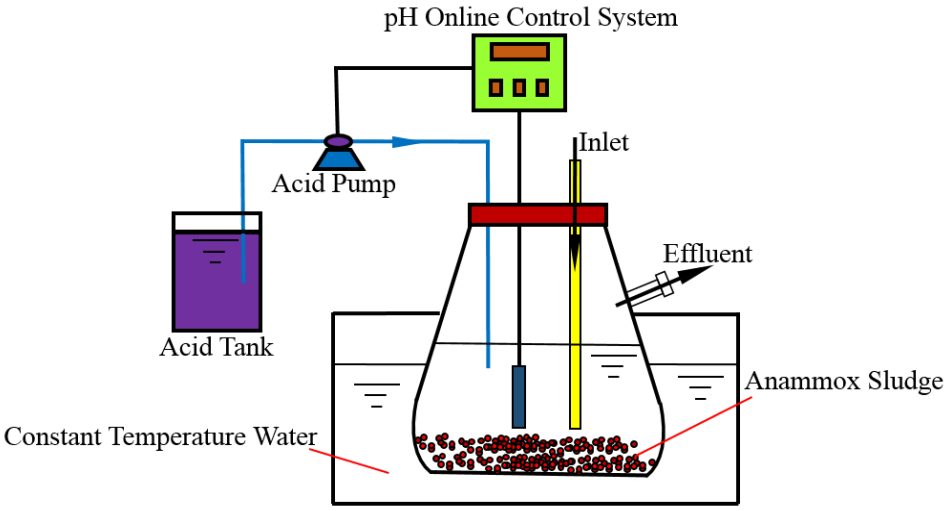



**Figure 2.** Batch experiment reaction device

*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.

**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 | | | | | |

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

198 experiment were analyzed by FE-SEM. In the FE-SEM experiment, the sludge samples were
199 cleaned and then vacuum-dried for 2 h. After adding a gold-plated conductive film, the
200 sludge samples were observed using a Sigma 500 scanning electron microscope (Carl Zeiss,
201 Jena, Germany).

202 *2.5. Statistical methods*

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

211 **3. Results and Discussion**

212 *3.1. Effect of $NO_2^-$-N on anammox*

213 The results of Pearson's correlation analysis between the concentration of $NH_4^+$-N, $NO_2^-$-N
214 and TN consumption and $NO_3^-$-N production are shown in **Table 2** for the stages A20-A60
215 and A80-A100. In the A20-A60 stage, there was a significant positive correlation between
216 the influent $NO_2^-$-N concentration and the consumption of $NH_4^+$-N, $NO_2^-$-N and TN. In
217 A80-A100 stage, the influent $NO_2^-$-N concentration and the consumption of $NH_4^+$-N, $NO_2^-$-N
218 and TN were significantly negatively correlated. From the perspective of correlation
219 analysis, the nitrogen consumption increased significantly with the increase of influent
220 $NO_2^-$-N concentration in the A20-A60 stage. But decreased with the increase of influent
221 $NO_2^-$-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.

**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.

**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.

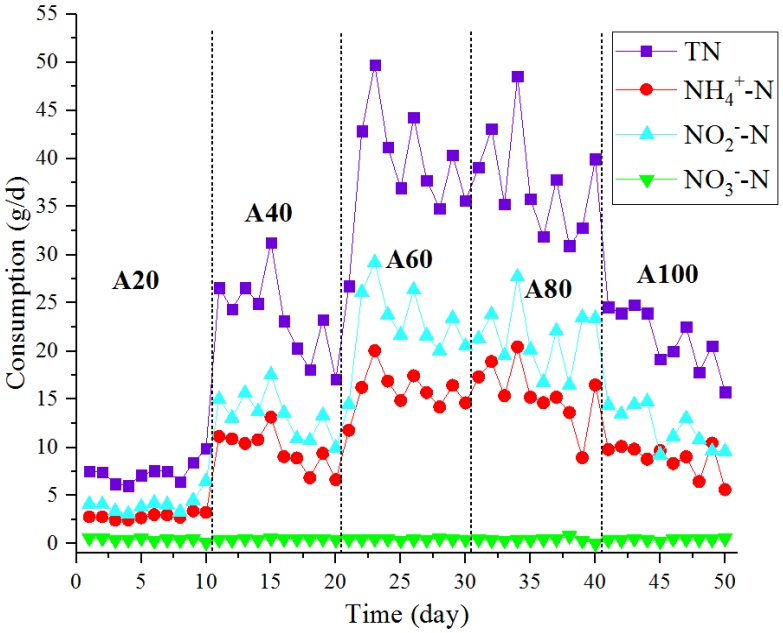



**Figure 3.** Consumption of $NH_4^+$-N, $NO_2^-$-N, $NO_3^-$-N, and TN in the reactor under different influent $NO_2^-$-N concentrations

**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.

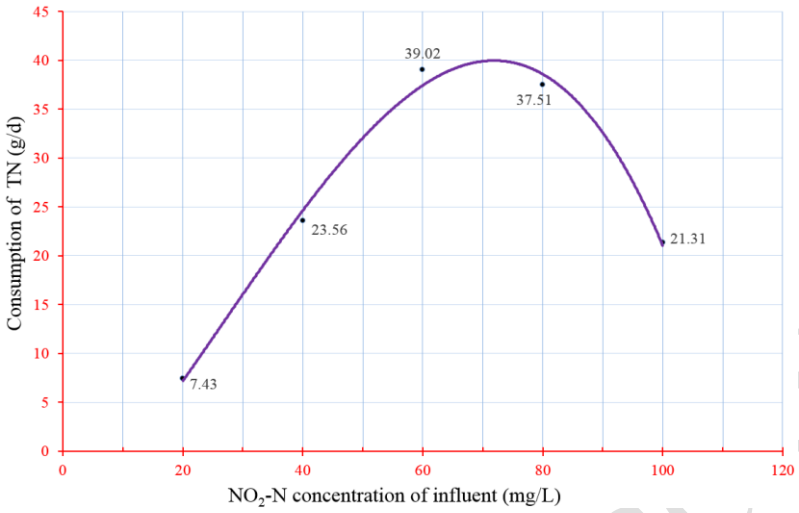



**Figure 4.** Relationship between $NO_2^-$-N concentration in influent and TN consumption

*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.

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

277 **Figure 5** shows the average daily consumption of $NH_4^+$-N, $NO_2^-$-N, and TN by each batch of
278 reactors. According to the experimental results, the average $NH_4^+$-N removal amounts of B0
279 (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
280 mg/d, 5.63 mg/d, 6.81 mg/d, 6.67 mg/d and 5.81 mg/d, respectively. The average removal of
281 $NO_2^-$-N was 6.13 mg/d, 8.11 mg/d, 11.17 mg/d, 11.82 mg/d, and 10.59 mg/d, respectively;
282 The average removal of $NO_3^-$-N was kept at 0.23-0.49 mg/d. The average TN removal was
283 11.02 mg/d, 14.18 mg/d, 18.21 mg/d, 18.73 mg/d, and 16.89 mg/d, respectively. Thus, B0
284 showed the lowest nitrogen removal efficiency. Combined with the data obtained in phase I,
285 the consumption of the substrate by anammox bacteria is greatly reduced in environments
286 with a high concentration of $NO_2^-$-N (concentration > 100 mg/L); therefore, its activity was
287 inhibited by high concentrations of $NO_2^-$-N. After the addition of $N_2H_4$, the consumption of
288 $NH_4^+$-N and $NO_2^-$-N in B5, B10, B15, and B20 were significantly greater than that of B0,
289 indicating that $N_2H_4$ had a restorative effect on anammox bacteria activity (Xiao *et al.*, 2015;
290 Zekker *et al.*, 2012). As shown in **Figure 5**, after adding $N_2H_4$, the consumption of TN by B5,
291 B10, B15, and B20 increased by 28.68%, 65.24%, 69.96%, and 53.27% relative to B0,
292 respectively. When $N_2H_4 \leq 15.0$ mg/L, the consumption of TN increased, but when $N_2H_4 \geq$
293 20.0 mg/L, it decreased. The reason for this result may be that excessive $N_2H_4$ causes
294 hydrazine oxidase to directly oxidize $N_2H_4$ into $N_2$, reducing the demand for nitrite
295 oxidoreductase for $NO_2^-$-N; alternatively, an excessively high concentration of $N_2H_4$ might
296 have poisoned the anammox bacteria. According to the experimental results, addition of 10.0
297 $\leq N_2H_4 \leq$ 15.0 mg/L recovered the activity of anammox bacteria inhibited by high
298 concentrations of $NO_2^-$-N.

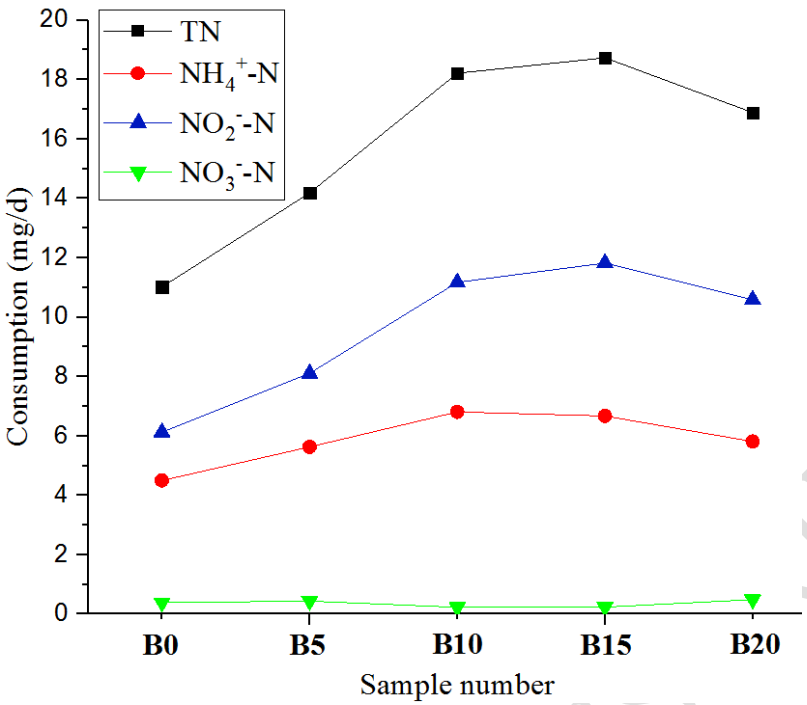



**Figure 5.** Recovery effect of $N_2H_4$ on anammox bacteria

*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.

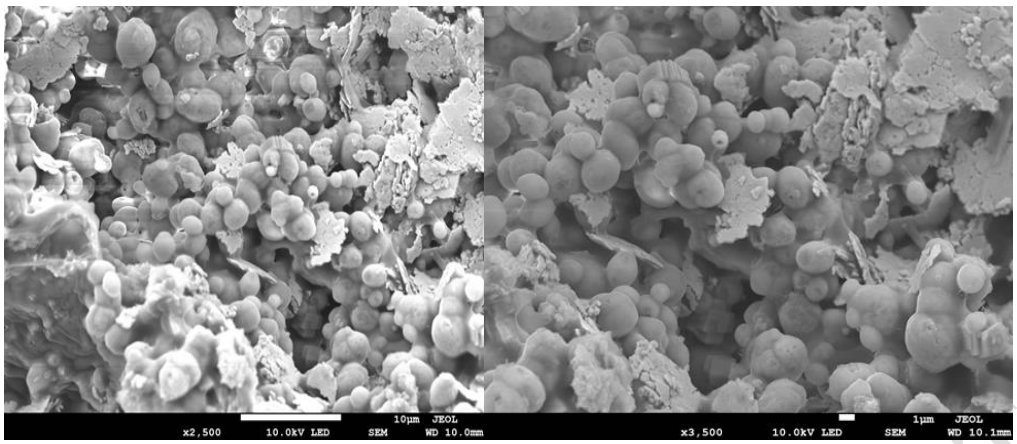



**Figure 6.** Anammox morphology observed by FE-SEM

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 |

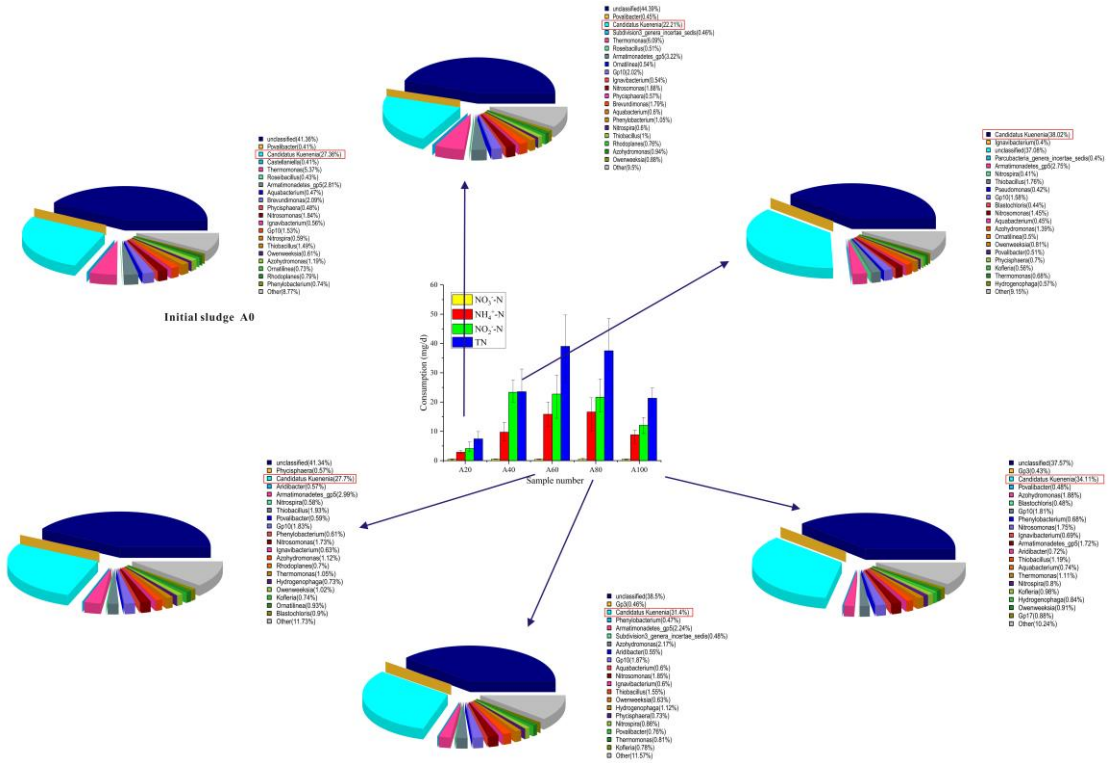



**Figure 7.** Changes of N consumption and microbial community under different concentrations

**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.

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.

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