Nitrogen removal performance using anaerobic ammonium oxidation considering variable conditions

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Abstract
The anaerobic nitrogen removal performance of anammox at 30°C, 25°C, and 16°C were studied by using the UASB (Up flow Anaerobic Sludge Blanket) reactor and the influent concentration of NH$_4^+$-N and NO$_2^-$-N were 16.9 and 20.6 mg L$^{-1}$ respectively. Experimental results showed that high-efficiency anammox nitrogen removal could be achieved at 30°C, when hydraulic retention time (HRT) was 0.14 h, the nitrogen removal rate (NRR) was 5.73 kg N m$^{-3}$ d$^{-1}$. The anammox reactor operated stably for more than 80 days under the condition of 16°C–20°C, and the high NRR of 2.78 kg N m$^{-3}$ d$^{-1}$ was obtained. In this experiment, DO had little effect on the activity of anammox granular sludge, and the nitrogen removal performance could be quickly recovered in a short period of time after being affected by DO. Moreover, the stoichiometric ratio of NO$_2^-$-N and NH$_4^+$-N consumption ($\Delta$NO$_2^-$-N/$\Delta$NH$_4^+$-N) and the stoichiometric ratio of NO$_3^-$-N production and NH$_4^+$-N conversion ($\Delta$NO$_3^-$-N/$\Delta$NH$_4^+$-N) were 1.21 ± 0.11 and 0.25 ± 0.06 respectively at 30°C, which were very close to the theoretical value, it indicated that anammox bacteria were the dominant bacteria at 30°C.

Keywords
Moderate and low temperature, low strength substrate, Anammox, nitrogen removal performance, stoichiometric ratio

Introduction
Anammox was a biological reaction process in which anammox bacteria took NO$_2^-$-N as electron acceptor to oxidize NH$_4^+$-N to N$_2$ under anaerobic conditions. Because of
its advantages of saving energy and carbon source, reduction of sludge yielded,\textsuperscript{1–3} it had attracted extensive attention from researchers. At present, the application of Anammox technology was mainly focused on the field of high temperature and high ammonia nitrogen wastewater treatment. For example, the world’s first practical project of Anammox was used to treat sludge digestion liquid, moreover, there were practical projects for treating tannery wastewater and potato wastewater.\textsuperscript{4} However, Kartal et al.\textsuperscript{5} proposed the idea of using anaerobic ammonium oxidation process to treat urban sewage, Ma et al.\textsuperscript{6} studied the feasibility of two-stage autotrophic nitrogen removal process to treat municipal sewage. All these indicated that it was feasible to use Anammox process for the treatment of municipal sewage.

Most studies believed that the optimal operating temperature of Anammox process was 30°C–40°C,\textsuperscript{7,8} in order to keep the high state activity of Anammoxic bacteria, it was necessary to heat municipal sewage, which had the characteristics of large amount of water, so the application of heating in practical engineering field was infeasible. Therefore, it was imperative to study the nitrogen removal performance of Anammox at moderate and low temperature. Recently, Cema et al.\textsuperscript{9} used the rotating biological contactor (RBC) to operate the anaerobic ammonia oxidation successfully at 20°C. Isaka et al.\textsuperscript{10} treated the wastewater which the influent concentration of NO\textsubscript{2}^-N was less than 280 mg L\textsuperscript{-1} with an anaerobic biological filter at 20°C–22°C, and the nitrogen removal rate (NRR) was 8.1 kg N m\textsuperscript{-3} d\textsuperscript{-1}. Isaka et al.\textsuperscript{11} also found that anammox bacteria still had relatively high activity at 6°C, and its NRR was 0.36 kg N m\textsuperscript{-3} d\textsuperscript{-1}, moreover, through long-term operation (>130 d) under the condition of 20°C it was confirmed that anammox bacteria had stable activity at low temperature. In addition, scientists also measured the activity of anammox bacteria under low temperature conditions in the ocean, for example, Rysgaard et al.\textsuperscript{12} detected the seafloor sediments on the east and west coasts of Greenland, and found that the anammox bacteria showed activity between −2°C and 30°C, and the most appropriate temperature was 12°C. These results provided a lot of support for us to study the nitrogen removal performance of anammox bacteria at low temperature treating low strength wastewater.

In order to promote the application of anammox process in municipal wastewater treatment, Ma et al.\textsuperscript{6} first proposed the partial nitritation coupled with anammox process for treatment of municipal wastewater. In this paper, it chose the secondary effluent of municipal wastewater treatment plant with adding NH\textsubscript{4}Cl and NaNO\textsubscript{2} as the influent, aimed to mimic the effluent of partial nitritation system of municipal sewage, on the basis of successful start-up of anammox reactor, the nitrogen removal performance with low strength substrate at 16°C–30°C and the stability at low temperature were studied.

**Materials and methods**

**Wastewater**

The influent of the anammox-UASB was the effluent from a secondary clarifier from Gaobeidian wastewater treatment plant adding NH\textsubscript{4}Cl and NaNO\textsubscript{2}, aiming
to mimic the effluent of partial nitritation system of municipal sewage. The influent of the anammox-UASB were shown in Table 1.

**Reactor and experimental setup**

The up-flow anaerobic sludge blanket (UASB) reactor was used in this study as shown in Figure 1. It was made of organic glass with an effective volume of 8 L and an inner diameter of 8 cm. It was wrapped with black rubber insulation material so as to avoid the negative effects of light on the anammox bacteria and keep the reactor warm. The reactor temperature was controlled by water bath heating.

**Anammox sludge characteristics**

When the experiment was carried out, the UASB reactor had been successfully started. When the average influent concentration of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ were

| Parameter   | $\text{NH}_4^+\text{-N}$ | $\text{NO}_2^-\text{-N}$ | $\text{NO}_3^-\text{-N}$ | COD  |
|-------------|--------------------------|--------------------------|--------------------------|------|
| Unit        | mg L$^{-1}$              | mg L$^{-1}$              | mg L$^{-1}$              | mg L$^{-1}$ |
| Concentration | 16.9 ± 2.1               | 20.6 ± 2.3               | 13.7 ± 2.7               | 25.5 ± 6.9   |

![Figure 1. Anammox-UASB reactor setup.](image)
125 and 131 mg L\(^{-1}\) respectively, the average NRR was 1.15 kg N m\(^{-3}\)d\(^{-1}\). DNA extraction and gene sequencing showed that the main anammox bacteria in the reactor was *Candidatus Kuenenia stuttgartiensis*.\(^{13}\) The sludge was flocculated and no granular sludge was found, and the initial mixed liquor suspended solids (MLSS) was 9800 mg L\(^{-1}\).

### Experimental procedure

As shown in Table 2, the experiment lasted a total of 192 days and was mainly divided into three phases. The first phase (1–70 days) was the load increasing stage. At this stage, the temperature was maintained at 30°C, and the influent concentration of NH\(_4^+\)-N or NO\(_2^-\)-N were kept stable, the concentration of effluent NH\(_4^+\)-N or NO\(_2^-\)-N was taken as the control index. When the effluent concentration of NH\(_4^+\)-N or NO\(_2^-\)-N was less than 1 mg L\(^{-1}\), it proved that the substrate in the reactor was almost consumed, so increased the influent flow rate gradually. The second phase (71–110 days) was the load stabilization stage. At this stage, the temperature dropped from 30°C to 20°C gradually, and the concentration and flow rate of the influent remained unchanged. The third phase (111–192 days) was the load reduction stage. At this stage, the temperature decreased from 20°C to 16°C gradually, and kept the concentration of influent stable, reduced the flow rate according to the effluent concentration of NO\(_2^-\)-N.

### Analytical methods

Ammonium concentration (NH\(_4^+\)-N), nitrite concentration (NO\(_2^-\)-N), and nitrate concentration (NO\(_3^-\)-N) were measured according to standard methods,\(^{14}\) COD was measured using a COD quick-analysis apparatus (Lianhua, 5B-3C). All samples were filtered through a 0.45 μm filter before analysis. DO concentration, pH, and temperature were measured by oxygen, pH, and temperature probes (WTW 340i, Germany). DNA extracted from about 0.25 g of sludge using the Fast DNA spin kit for Soil (QBIOgene INC., Carlsbad, CA, USA).

| Phase | Time of duration | Average temperature | Operation method |
|-------|-----------------|---------------------|------------------|
| I     | 1–70 days       | 29.9°C ± 0.2°C      | Load increasing stage: kept the influent concentration stable and increased the influent flow rate. |
| II    | 71–110 days     | 25.1°C ± 2.2°C      | Load stabilization stage: maintained the influent concentration and flow rate stability. |
| III   | 111–192 days    | 17.4°C ± 1.5°C      | Load reduction stage: maintained the influent concentration stability and reduced the influent flow rate. |

**Table 2**: Experimental procedure of the anammox-UASB reactor.
Results and discussion

**Nitrogen removal performance at different temperatures**

Phase I (1–70 days) was the load increasing stage, the influent concentration of NH$_4^+$ -N and NO$_2^-$ -N were 16.6 and 20.0 mg L$^{-1}$ respectively at 30°C, with the increasing of influent flow rate, HRT decreased gradually, and the NRR increased gradually. When the HRT decreased from 1.26 to 0.14 h, the NRR of the reactor increased from the 0.57 kg N m$^{-3}$ d$^{-1}$ to the highest 5.73 kg N m$^{-3}$ d$^{-1}$ as shown in Figure 4. In this process, the average TN removal efficiency was 62% and the average NO$_3^-$ -N/TN in effluent was 87%.

During phase II (71–110 days), the temperature decreased from 30°C to 20°C, keeping the influent concentration and the influent flow rate stable. As can be seen from Figure 2, when the influent concentration of NH$_4^+$ -N and NO$_2^-$ -N were 16.6 and 21.6 mg L$^{-1}$ respectively, the effluent concentration of NH$_4^+$ -N and NO$_2^-$ -N began to increase with the decrease of temperature, and the removal efficiency of NH$_4^+$ -N and NO$_2^-$ -N began to decrease correspondingly. In this process, the NRR of the reactor slightly decreased, with an average value of 4.25 kg N m$^{-3}$ d$^{-1}$, the average TN removal efficiency was 52% and the average NO$_3^-$ -N/TN in effluent was 79%.

Phase III (111–192 days) was the process of reducing temperature from 20°C to 16°C, the influent concentration of NH$_4^+$ -N and NO$_2^-$ -N were 17.3 and 20.5 mg L$^{-1}$ respectively, with the effluent concentration of NO$_2^-$ -N as the control index, then reduced the flow rate gradually. As can be seen from the Figure 2, compared to the phase II, the effluent concentration of NH$_4^+$ -N remained unchanged, and the effluent concentration of NO$_2^-$ -N decreased slightly in phase III. Because of the decrease of influent flow rate, HRT increased from 0.15 to
0.24 h, the corresponding average NRR was 2.78 kg N m\(^{-3}\) d\(^{-1}\). In this process, the average TN removal efficiency was 49% and the average NO\(_3^-\)-N/TN in effluent was 82%, as shown in Figure 4.

It can be seen from Figure 4 that the anammox reactor had a good nitrogen removal performance, with the maximum NRR of 5.73 kg N m\(^{-3}\) d\(^{-1}\) at 30°C, and gradually decreased to 2.78 kg N m\(^{-3}\) d\(^{-1}\) at 16°C. However, the removal efficiency of TN was not very high, with an average of 55%. The main component of effluent TN was NO\(_3^-\)-N, and the average NO\(_3^-\)-N/TN reached 84%. Because of the low concentration of COD in anammox reactor, and the main component of COD was difficult to degrade organic matter, it was difficult to remove NO\(_3^-\)-N from effluent by denitrification. In order to make the effluent TN concentration of the autotrophic denitrification system of municipal sewage meet the national or local requirements, the effluent of the anammox system could be recycled to the anoxic section at the front end of the A/O reactor, and part of the remaining carbon sources in the effluent of the high-load activated sludge could be used for denitrification to remove NO\(_3^-\)-N.

**The stoichiometric ratio of anammox at different temperatures**

Kuenen et al.\(^{15}\) put forward the reaction equation of anammox, where the \(\Delta NO_2^-\)/\(\Delta NH_4^+\)-N was 1.32 and the \(\Delta NO_3^-\)/\(\Delta NH_4^+\)-N was 0.26 (the \(\Delta NO_2^-\)-N, \(\Delta NH_4^+\)-N were the consumption of NO\(_2^-\)-N and NH\(_4^+\)-N, the \(\Delta NO_3^-\)-N was the production of NO\(_3^-\)-N). By comparing the stoichiometric ratio in the experiment with the theoretical stoichiometric ratio, we can judged the Anammox reaction effect and the accompanying reaction. The stoichiometric ratio in anammox...
reactor were shown in Figure 5. The $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ was 1.21 ± 0.11, and the $\Delta \text{NO}_3^-/\Delta \text{NH}_4^+$ was 0.25 ± 0.06 in phase I, the $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ was 1.59 ± 0.29, and the $\Delta \text{NO}_3^-/\Delta \text{NH}_4^+$ was 0.49 ± 0.18 in phase II, the $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ was 1.45 ± 0.27, and the $\Delta \text{NO}_3^-/\Delta \text{NH}_4^+$ was 0.48 ± 0.18 in phase III. Among them, the $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ and $\Delta \text{NO}_3^-/\Delta \text{NH}_4^+$ in phase I were closed to the theoretical values reported in literature (equation (1)), while the $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ and $\Delta \text{NO}_3^-/\Delta \text{NH}_4^+$ in phase I and III were larger than the theoretical values reported in literature.

$$\text{NH}_4^+ + 1.31\text{NO}_2^- + 0.066\text{HCO}_3^- + 0.13H^+ \rightarrow 1.02\text{N}_2 + 0.26\text{NO}_3^- + 0.066\text{CH}_2\text{O}_{0.5}\text{N}_{0.15} + 2.03\text{H}_2\text{O} \quad (1)$$

Dosta et al.\textsuperscript{16} studied the long-term influence of temperature on anammox and found that $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ was 1.38 ± 0.10 at 30°C, but when the temperature dropped to 18°C, $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ decreased to 1.05 ± 0.01. Dalsgaard and Thamdrup\textsuperscript{17} studied the anammox reaction in Marine sediments at 15°C, and obtained the $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ equal to 1. Their experimental results showed that with the decrease of temperature, the $\Delta \text{NO}_2^-/\Delta \text{NH}_4^+$ also decreased, however the results of this experiment presented a change rule contrary to their experimental results. This difference may be related to the structure of the microbial community in the reactor. In this experiment, the effluent from the secondary sedimentation tank of the municipal sewage treatment plant was used as the influent, so in addition to the anammox bacteria, there were ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), and denitrifying bacteria in the reactor.
Considering that the average COD concentration of influent was 25.5 mg L\(^{-1}\) and the average COD concentration of effluent was 26.6 mg L\(^{-1}\) in this experiment, it could be seen that no COD was consumed in the UASB reactor, indicating that no obviously heterotrophic denitrification occurred in the reactor. In addition, the amount of NOB in the secondary sedimentation tank of the municipal sewage treatment plant was greater than the amount of AOB, so the amount of NOB in the reactor was larger. In phase I, when the temperature was higher, the activity of anammox bacteria was higher too, so anammox process played a dominant role in the reactor. With the decrease of temperature, the activity of anammox bacteria decreased, while the activity of other strains (such as AOB and NOB) in the reactor did not decline as fast as that of anammox bacteria. In the presence of DO (the concentration of DO in the influent in this experiment was about 5 mg L\(^{-1}\)), part of NO\(_2^-\) was directly oxidized to NO\(_3^-\). And it also showed that the \(\Delta\text{NO}_3^-/\Delta\text{NH}_4^+\) was greater than that at 30°C. In addition, it may be related to the stress of the metabolic performance of anaerobic ammonia on environmental pressure.\(^{18,19}\)

### Analysis of anammox nitrogen removal performance

**Effect of temperature on NH\(_4^+\) and NO\(_2^-\) removal efficiency.** It can be seen from Figure 3, in phase I the removal efficiency of NH\(_4^+\) and NO\(_2^-\) were maintained at a higher level, with an average of 93.3% and 94.6% respectively, and in phase II, the removal efficiency of NH\(_4^+\) decreased to 77.2% and
90.9% respectively, in phase III, the removal efficiency of NH$_4^+$-N and NO$_2^-$-N increased slightly, which were 78.7% and 92.7% respectively. It can be seen that, with the decrease of temperature, the removal efficiency of NH$_4^+$-N and NO$_2^-$-N in the reactor showed a downward trend. However, the removal efficiency of NO$_2^-$-N was not significantly affected by temperature and could maintain over 90% at 16°C. The removal efficiency of NH$_4^+$-N decreased by about 16% when the temperature dropped from 30°C to 20°C. Strous et al.\textsuperscript{7} reported that the conversion of NH$_4^+$-N and NO$_2^-$-N was not proportional with the change of temperature, the consumption of NH$_4^+$-N stopped at high temperature, and only observed the consumption of NO$_2^-$-N. With the decrease of temperature, it would a similar phenomenon be shown, the consumption of NH$_4^+$-N decreased and the consumption of NO$_2^-$-N remained unchanged. If this hypothesis was true, could explain this phenomenon, but it needed further research. In addition, it was also related to the structure of the bacterial in the reactor, there were AOB and NOB except anammox bacterial. With the decreasing of temperature, the activity of anammox bacteria decreased, while the activity of AOB and NOB did not decrease so fast, and the amount of NOB was larger than that of AOB, so part of NO$_2^-$-N was directly oxidized to NO$_3^-$-N, which shows that NO$_2^-$-N had a higher removal rate.

In order to investigate the succession of anammox bacteria in the temperature decreasing process, this paper conducted sampling analysis on the granular sludge at the end of operation at 16°C (192 days), and it could be seen that with the decrease of temperature, anammox bacteria in granular sludge evolved from Candidatus ‘‘Kuenenia Stuttgartiensis’’ to Candidatus ‘‘Kuenenia Stuttgartiensis’’ and Candidatus ‘‘Brocadia Fulgida’’.

**Stability of nitrogen removal performance at low temperature.** In order to investigate the stability of the anammox at low temperature, the reactor was operated continuously at 16°C–20°C more than 80 days (Phase III). Figure 2 showed the change of effluent and influent substrate concentration at low temperature, under this conditions, the removal efficiency of NH$_4^+$-N and NO$_2^-$-N were 78.7% and 92.7% respectively, and the NRR was 2.78 kg N m$^{-3}$ d$^{-1}$, which was equivalent to 2.8 kg N m$^{-3}$ d$^{-1}$ obtained by Isaka et al.\textsuperscript{11} at 22°C. The average NRR was 3.56 kg N m$^{-3}$ d$^{-1}$ and the corresponding HRT was 0.18 h during the 111–138 days at 19°C. On the 139th day, when the temperature dropped to 16°C, the effluent concentration of NO$_2^-$-N increased from 1.08 to 6.78 mg L$^{-1}$, therefore, the influent flow rate decreased on the 140th day, and the HRT increased to 0.30 h, when the effluent concentration of NO$_3^-$-N was significantly improved, was about 1 mg L$^{-1}$. At 16°C the average NRR was 2.78 kg N m$^{-3}$ d$^{-1}$, and the removal efficiency was kept stable. It could be clearly seen from these results that the anammox reaction could be maintained stably under the condition of less than 20°C, and a higher NRR was obtained, which provided a reliable experimental basis for the application of anammox process in the treatment of low temperature and low concentration municipal sewage.
Effect of DO on anammox performance. Strous et al.\textsuperscript{20} studied the effects of DO on the performance of anammox bacteria in a sequencing batch reactor (SBR). The results showed that DO could inhibit the activity of anammox bacteria, but the activity of anammox bacteria could be restored after DO removal. On the 10th and 42nd day of operation in this experiment, due to the lack of water in the inlet tank, a large amount of air (about 100 L) was injected into the anammox reactor, leading to the increase of DO concentration in the reactor. The increase of DO concentration inhibited the activity of anammox bacteria to a certain extent, but this inhibition could be immediately relieved after normal water inflow and its nitrogen removal performance showed a certain upward trend. On the 12th day, the NRR was 0.96 kg N m\(^{-3}\) d\(^{-1}\), which was slightly higher than that of 0.91 kg N m\(^{-3}\) d\(^{-1}\) on the 9th day. On the 43rd day, the NRR was 2.66 kg N m\(^{-3}\) d\(^{-1}\), which was 8.6\% higher than that of 2.45 kg N m\(^{-3}\) d\(^{-1}\) on the 40th day. Therefore, in this experiment, DO had little effect on the activity of anammox granular sludge, and the nitrogen removal performance of anammox-UASB could be quickly recovered in a short period of time after being affected by DO. This may be related to the presence of DO in the influent of this experiment (DO concentration was about 5 mg/L), because the water was not deliberately deoxygenated, the anammox bacteria in the reactor had certain adaptability to DO, so that the inhibition of oxygen on anammox bacteria was weakened, and the activity of anammox bacteria could be quickly recovered after the elimination of DO. This explanation needed to be further tested by experiments.

Conclusion

High efficiency anammox nitrogen removal could be achieved under the condition of 16°C–30°C with low concentration substrate. At 30°C, the NRR increased gradually by decreasing the HRT. When the HRT was 0.14 h, the corresponding NRR was increased to 5.73 kg N m\(^{-3}\) d\(^{-1}\). The anammox reactor operated stably for more than 80 days under the condition of 16°C–20°C, and the high NRR of 2.78 kg N m\(^{-3}\) d\(^{-1}\) was obtained, which provided a reliable experimental basis for the application of anammox process in the treatment of low temperature and low concentration municipal wastewater.

In this experiment, DO had little effect on the activity of anammox granular sludge, and the nitrogen removal performance of Anammox-UASB could be quickly recovered in a short period of time after being affected by DO. At 30°C, $\Delta$NO\(_2^\cdot\)N/$\Delta$NH\(_4^+\) -N was 1.21 ± 0.11, and $\Delta$NO\(_3^\cdot\)N/$\Delta$NH\(_4^+\) -N was 0.25 ± 0.06. The stoichiometric relationship was very close to the theoretical value, which indicated that the anammox bacteria were the dominant bacteria in the reactor.

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