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Long-term storage and subsequent reactivation of Anammox sludge at 35℃

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ABSTRACT

The feasibility of a long-term storage and subsequent reactivation of Anammox sludge was investigated. The storage strategy was improved for a long-term storage. The storage at an optimized temperature (35℃) for growth and metabolism of Anammox bacteria was attempted to retain Anammox activity for acceleration of the reactivation process after the storage. During the storage, a little of Anammox nutrient medium containing 50 mg-N l^{-1} $(NH_4)_2SO_4$ and $NaNO_2$ was added and periodically (every week) refreshed to keep the survival of Anammox bacteria. After the 6-month storage, there was still an Anammox activity of 0.07 g $(NH_4^+$-N + NO_2^--N) $(gVSS \times d)^{-1}$, even though most Anammox bacteria were in dormant state. The reactivation of the stored Anammox sludge was achieved in an SBR after a 2-month operation, and the final nitrogen removal rate of 0.32 g $(NH_4^+$-N + NO_2^--N) L^{-1} d^{-1} was gained. Compared with the fresh activated sludge, the stored Anammox sludge could realize a quick start-up of Anammox process. The results demonstrated that the improved storage strategy was suitable for a long-term storage and subsequent reactivation of Anammox sludge.

Keywords: Anammox sludge; Storage; Reactivation; Start-up

1. Introduction

At present, ammonium removal from wastewater has become one research hotspot in the field of urban water environment protection, since nitrogen in municipal and industrial wastewater is mainly present in the form of ammonium. Traditionally, ammonium is removed by two combined processes, namely nitrification and denitrification. However, the traditional biological nitrogen removal process is usually expensive and difficult for treating various kinds of wastewater with a low COD/TKN ratio because supplying massive oxygen for nitrification, adding external organic carbon donors for denitrification, and adjusting pH for both the processes are necessary to guarantee effluent quality [1,2].

Anaerobic ammonium oxidation (Anammox) process is applied as a novel and sustainable biotechnology for nitrogen removal from ammonium-rich wastewater. This is an anaerobic and chemoautotrophic bioprocess, in which nitrite and ammonium are directly converted to nitrogen gas by Anammox bacteria. The process provides remarkable advantages, such as no demand of oxygen and external organic

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carbon source, high nitrogen removal rate, and low sludge production rate [3–5]. Nevertheless, Anammox bacteria grow extremely slowly with the generation time of 7–22 d, leading to a long start-up period of Anammox process so as to challenge its wide applications [6,7]. Moreover, Anammox bacteria are scarce and difficult to be cultivated [7]. For rapid and effective start-up of an Anammox reactor, storage and reactivation of Anammox sludge is a possible and promising approach, as the stored Anammox sludge can be used as a kind of ready-to-use inoculum to provide enough original Anammox bacteria.

Up to now, there have been limited researches focusing on storage of Anammox biomass. The practicable techniques used to preserve microorganism, including freezing, lyophilization, and refrigeration, have been tested for preserving Anammox sludge or Anammox bacteria. Vlaeminck et al. discovered that 2-month storage at –20˚C led to irrevocable inactivation of Anammox biomass [8]. Rothrock et al. reported that Anammox biomass preserved in liquid nitrogen (–200˚C) via lyophilization in skim milk media (without glycerol) achieved the recovery of Anammox activity after 4-month storage and the similar stoichiometric ratios to those of fresh Anammox sludge harvested from the parent bioreactor [9]. Ji and Jin demonstrated that the activity of the refrigerated Anammox sludge could be recovered after 2-month storage at 4˚C and the nitrogen removal rate attained 4.41 kg m^{-3} d^{-1} [10]. However, the storage at a low temperature (–200~4˚C) may cause high-energy consumption and difficulties in recovery of Anammox activity due to the freezing or cold damage to Anammox cells.

To solve the problems, this study aimed to develop a simple and cost-effective storage strategy for a long-term preservation of Anammox sludge and investigate the subsequent reactivation. The storage at an optimized temperature (35˚C) of Anammox bacteria was attempted to retain Anammox activity for acceleration of the reactivation process after the storage. During long-term storage period of Anammox sludge, Anammox nutrient medium with 50 mg L^{-1} ammonium and nitrite was refreshed periodically (every week) to further retain Anammox activity. Before and after long-term storage, the activity of Anammox bacteria was determined by batch experiments and their occupying percentage of total bacteria was analyzed by FISH technique. The long-term stored Anammox sludge was used as the seed sludge to start up Anammox process, and reactivation performance of the stored sludge was investigated. The study would provide useful information for quick start-up and engineering applications of Anammox.

2. Materials and methods

2.1. Anammox sludge and storage strategy

Anammox sludge was harvested from an Anammox reactor of 9-months operation, which was the parent reactor in this study. The sludge was washed by pure water, and the supernatant was discarded after sedimentation. The procedure was repeated three times. Then, 700 mL of Anammox sludge was transferred into a 2-L glass bottle. The Anammox nutrient medium with 50 mg-N L^{-1} $(NH_4)_2SO_4$ and $NaNO_2$ was added to the bottle. The medium compositions specially described by van de Graaf et al. are shown in Tables 1 and 2 [11]. N_2 gas was purged for 15 min to dispel the dissolved oxygen (DO). The bottle was sealed with a rubber stopper. The Anammox sludge was then stored in a constant temperature cabinet at 35˚C for 6 months. During the storage, the Anammox nutrient medium was refreshed weekly.

2.2. Batch test of Anammox activity

The Anammox activity was determined on the first day and the last day of the storage. To determine the Anammox activity, 0.1 gVSS L^{-1} of the stored Anammox sludge was added to 100-mL flasks that were filled with the Anammox nutrient medium. The Anammox nutrient medium contained 50 mg-N L^{-1} $(NH_4)_2SO_4$ and $NaNO_2$. The headspace and liquid phase were purged with N_2 to remove DO. The pH was adjusted to 7.5. The flasks sealed tightly with butyl rubber caps were incubated at 35˚C in the dark. Liquid samples were collected using syringes with needles for monitoring the concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N.

Table 1
Compositions of the synthetic wastewater fed to the reactors

Compound	Concentration (g/L)
$(NH_4)_2SO_4$	RA[a]
$NaNO_2$	RA
$KHCO_3$	0.8
KH_2PO_4	0.025
$CaCl_2$	0.2
$MgSO_4{\cdot}7H_2O$	0.2
$FeSO_4$	0.00625
EDTA	0.00625
Trace elements solution	1.25 ml/L

[a]The required amount.

Table 2
Compositions of trace elements solution

Compound	Concentration (g/L)
EDTA	15
$ZnSO_4{\cdot}7H_2O$	0.43
$CoCl_2{\cdot}6H_2O$	0.24
$MnCl_2{\cdot}4H_2O$	0.99
$CuSO_4{\cdot}5H_2O$	0.25
$NaMoO_4{\cdot}2H_2O$	0.22
$NiCl_2{\cdot}2H_2O$	0.19
$NaSeO_4{\cdot}10H_2O$	0.21
H_3BO_4	0.014
$NaWO_4{\cdot}2H_2O$	0.050

2.3. Chemical analysis

The water quality was monitored according to the standard methods [12]. NH_4^+-N and NO_2^--N were measured colorimetrically, while NO_3^--N was measured spectrophotometrically. The pH and DO levels were analyzed by a digital portable pH meter and a DO meter (YSI, Model 55, US), respectively. MLSS and MLVSS were determined to demonstrate some of the sludge characteristics.

2.4. FISH analysis

FISH analysis was used to detect Anammox bacteria and investigate the evolutions of microbial community structure in Anammox sludge during the storage. Paraformaldehyde cell fixation and FISH analyses were performed according to the standard hybridization protocol [13–15], using probe AMX825 and EUB338. AMX825 was used to detect Anammox bacteria, while EUB338 was used to detect all bacteria. Both probes were purchased from TaKaRa Company (Dalian, China). Hybridizations were performed on 4% (w/v) paraformaldehyde-fixed sludge samples as described previously. A Leica TCS-SP2 confocal scanning laser microscope (CSLM) (Leica, Germany) was employed for image acquisitions. FISH images were analyzed by Software Image-Pro Plus 6.0 to evaluate the content of Anammox bacteria preliminarily. In the FISH images, the blue area represents Anammox bacteria, while the green area represents total bacteria. The size of blue area or green area was analyzed by Software Image-Pro Plus 6.0. So, the ratio of Anammox bacteria to total bacteria could be reflected by the ratio of blue area size to green area size.

2.5. Reactivation

After 6-month storage, the Anammox sludge was used as the seed sludge to start up the Anammox process for investigating the reactivation performance of the Anammox sludge. A 2-L SBR worked as the Anammox reactor with 0.1 gVSS L^{-1} of the stored Anammox sludge in the reactor. The initial hydraulic retention time (HRT) was set at 2 d. The time sequence in a cycle was maintained on the following time steps: feed and reaction 630 min, settling 80 min, discharge 10 min. The time of a cycle could be adjusted to change HRT. In feed and reaction phase, the reactor was flushed with N_2/CO_2 (95/5%) gas mixture to maintain anaerobic condition and provide CO_2 for Anammox bacteria [16], and the stirrer worked at the speed of 100 rpm to keep the biomass suspended as free cells. The reactor equipped with a thermostatic jacket was maintained at 35°C. In the reactor, the pH was kept at the range from 7.8 to 8.2 and the DO was controlled below 0.05 mg L^{-1}. The feed vessels were sealed to maintain anaerobic condition and the reactor was covered to protect the bacteria from disturbance of light and algal growth Fig. 1.

The synthetic wastewater fed to the reactor was prepared according to Anammox nutrient medium, whose compositions are listed in Tables 1 and 2. The medium concentrations of $(NH_4)_2SO_4$ and $NaNO_2$ were both initially set to around 60 and 72 mg N L^{-1}, respectively. The N-loading rate (NLR) was enhanced by increasing the concentrations of $(NH_4)_2SO_4$ and $NaNO_2$ in the feed vessel as the inhibiting substrate ($NaNO_2$) for Anammox bacteria was almost consumed. The synthetic wastewater was replaced daily to avoid changes in feed compositions due to the biological activity or other influencing factors.

3. Results and discussion

3.1. Anammox activity

Anammox activity of the Anammox sludge stored at 35°C was tested on day 1 and day 180, as described in Fig. 2. On day 1 of the storage, the Anammox activity was obvious. The Anammox sludge consumed 47.8 mg-N L^{-1} NH_4^+ and 50 mg-N L^{-1} NO_2^- with the concomitant generation of 8.4 mg-N L^{-1} NO_3^- within 4 d. It was calculated that the specific Anammox activity was 0.24 g (NH_4^+-N + NO_2^--N) $(gVSS \times d)^{-1}$. On day 180 of the storage, the sludge took 14 d to consume 41.8 mg-N L^{-1} of NH_4^+ and 50 mg-N L^{-1} of NO_2^- with generation of 10.6 mg-N L^{-1} NO_3^-. The results implied that the Anammox activity was weak but identifiable. The specific Anammox activity was 0.07 g (NH_4^+-N + NO_2^--N) $(gVSS \times d)^{-1}$. Interestingly, the results showed that even after 6-month storage, Anammox bacteria were still active to some extent and 29.2% of the Anammox activity was persisted,

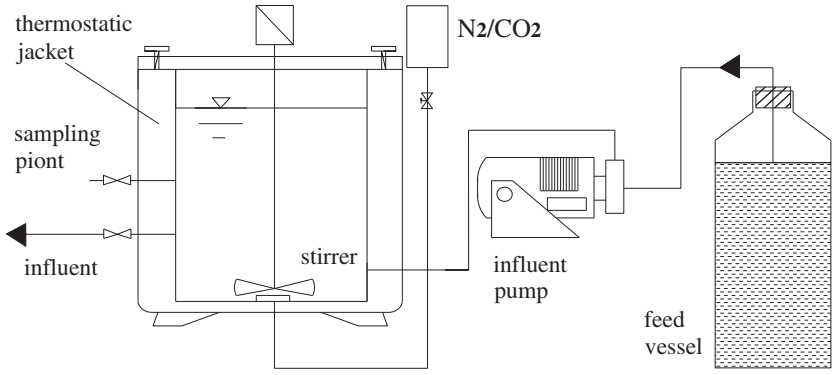

Fig. 1. Schematic diagram of the SBR for reactivation of the stored Anammox sludge.

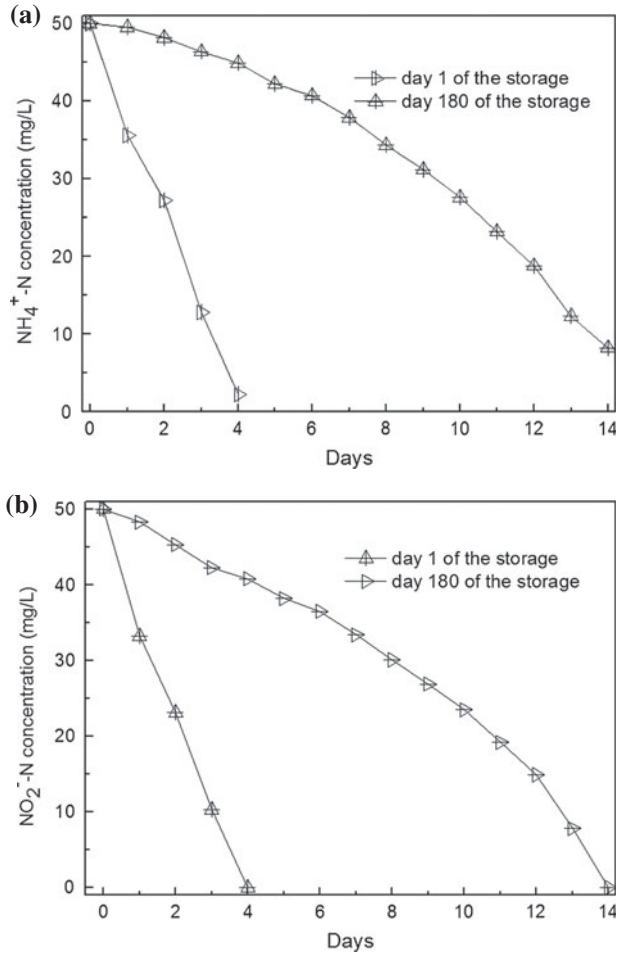

Fig. 2. The variations of ammonium and nitrite concentrations in the test of Anammox activity on day 1 and day 180 of the storage: (a) the variations of ammonium in the test of Anammox activity and (b) the variations of nitrite in the test of Anammox activity.

even though the Anammox activity decreased greatly. The maintenance of 29.2% Anammox activity could be attributed to the improved storage strategy, in which addition of a little of Anammox nutrient medium once a week was necessary to let Anammox bacteria survive at 35˚C. Previously, it was reported that the storage of 2 months at –20˚C or 4 months at –60˚C caused irreversible inactivation of Anammox bacteria or Anammox sludge [8,9]. It was also reported that, Anammox activity of Anammox biomass could be severely damaged after the Anammox biomass immobilized in polyvinyl alcohol gel were stored at –8˚C for only 17 h, so that only 10% of the Anammox activity was retained [17]. These phenomena demonstrated that storage at a low temperature below zero could lead to damage of Anammox cells. The cold damage became serious with time. When the cold damage reached a threshold extent, the Anammox activity could not be reactivated. In this case, long-term preservation could make the persisted Anammox activity become much lower and weaker so that recovery of Anammox activity would be extremely hard. Comparing the results on the activity and reactivation of Anammox biomass from different literatures as discussed above, it was inferred that storing temperature was always one of the key factors determining the preservation performance. Low storing temperatures probably lead to cold or freezing damage of the Anammox cells. Thus, to avoid these problems arising from the storage at low temperatures, an optimized temperature (35˚C) for growth and metabolism of Anammox bacteria was selected to be an alternative to low storing temperatures and was adopted in the study. The results of Anammox activity test showed that the preservation performance of Anammox sludge was improved by storing at 35˚C and periodically supplying a few substrates.

It was calculated that the ratio of NO_2^--N conversion to NH_4^+-N conversion was 1.05 on day 1 of the storage, and the ratio increased to 1.20 on day 180 of the storage. The phenomenon may be caused by some aerobic nitrifying bacteria (AOB) in the Anammox sludge, living on the leakage of oxygen into the Anammox reactor [18,19]. The AOB could consume external NH_4^+-N. So, on day 1 of the storage, the ratio of NO_2^--N conversion to NH_4^+-N conversion was 1.05, which was evidently lower than the stoichiometric ratio of 1.32 presented by Strous et al. [20]. Most of the AOB would die and be eliminated in the storage for the strict control of oxygen. Therefore, after 180 d of storage, the ratio of NO_2^--N conversion to NH_4^+-N conversion reached 1.2, which was close to the stoichiometric ratio of 1.32. On the other hand, the ratio of NO_3^--N production to NH_4^+-N conversion was 0.18 on day 1 of the storage, lower than the stoichiometric ratio of 0.26. The reason could be that some denitrifying bacteria still existed in the Anammox sludge. The denitrifying bacteria could live on the degradable substrate such as proteins and polysaccharides from decay and metabolism of biomass and consumed the NO_3^--N generated by Anammox bacteria [21,22]. After 6-month storage, the biological activity decreased greatly, so the degradable substrate was hardly produced. Then, the denitrifying bacteria were killed in the strict autotrophic conditions. As a result, on day 180 of the storage, the ratio increased to 0.25, which was extremely close to the stoichiometric ratio of 0.26. Experiencing the storage, the ratio of NO_2^--N conversion to NH_4^+-N conversion and that of NO_3^--N production to NH_4^+-N conversion were both close to the theoretical value. The variations of the ratios implied that Anammox bacteria had a greater advantage in the bacterial community of the Anammox sludge after the long-term storage at 35°C. The deduction should be further confirmed by FISH analysis.

3.2. FISH analysis

The FISH image (Fig. 3) showed that, no matter in the first day or in the last day of the storage, most bacteria detected with EUB338 were hybridized with AMX820 simultaneously, presumably Anammox bacteria. The percentage of Anammox bacteria in total bacteria was analyzed by Software Image-Pro Plus 6.0. For the fresh Anammox sludge on day 1 of the storage, Anammox bacteria comprised 67% of the total bacteria population. It implied that Anammox bacteria had an overwhelming advantage in competition with other bacteria and predominated in the microbial community structure of the fresh Anammox sludge harvested from the parent reactor. However, after 6-month storage, Anammox bacteria occupied a larger percentage (81%) of the total bacteria population in the stored Anammox sludge. The results of FISH analysis were consistent with the ratio of NO_2^--N conversion or NO_3^--N production to NH_4^+-N conversion, implying that most other bacteria were eliminated after the storage. By comparing Fig. 3(a) and (b), it was noticed that the distribution of Anammox bacteria was more concentrated in the Anammox sludge after the storage of 6 months and Anammox bacteria formed more clusters in the stored sludge. It was pointed out that Anammox bacteria tended to grow in the natural state of forming clusters [6]. Similarly, in an Anammox-denitrifying system reported by Gao et al. [1], the aggregated growth of Anammox bacteria was observed, which was caused by the substrate and space competition between Anammox and denitrification. In our study, since the Anammox medium with only 50 mg-N L^{-1} of both NH_4^+ and NO_2^- was supplied once a week during the storage, substrate deficiency could promote formation of the clusters of Anammox bacteria.

From the above discussion, there was still some Anammox activity in the sludge on the last day of the storage. The images of FISH analysis combined with the results of Anammox activity test indicated that: (1) most Anammox bacteria were in dormant state under the modified storing condition at 35°C and could be retained after 6-month storage; (2) other bacteria, such as AOB and denitrifying bacteria, autolyzed and thus decreased in the strict autotrophic and anaerobic conditions; (3) the storing temperature was so high that the activity of most other bacteria could be hindered.

3.3. Reactivation

Fig. 4 describes nitrogen removal performances of the SBR reactor during reactivation of the stored Anammox sludge. The initial HRT was set at 2 d. The temperature, pH and DO concentration in the reactor was kept at 35°C, 7.8–8.2, <0.05 mg l^{-1}, respectively. The NLR was enhanced by means of the increment of the $(NH_4)_2SO_4$ and $NaNO_2$ concentrations in the feed vessel as the inhibiting substrate ($NaNO_2$) was almost consumed. The whole process of the reactivation could be divided into three phases: preliminary phase (Anammox activity appearance phase), NLR-increase phase (Anammox activity elevation phase) and steady phase. In the preliminary phase (days 1–9), $(NH_4)_2SO_4$ and $NaNO_2$ in the influent were set at 60 and 72 mg-N L^{-1}, and the removal efficiency of $(NH_4)_2SO_4$ and that of $NaNO_2$ increased gradually from 28.8 and 29.5% to

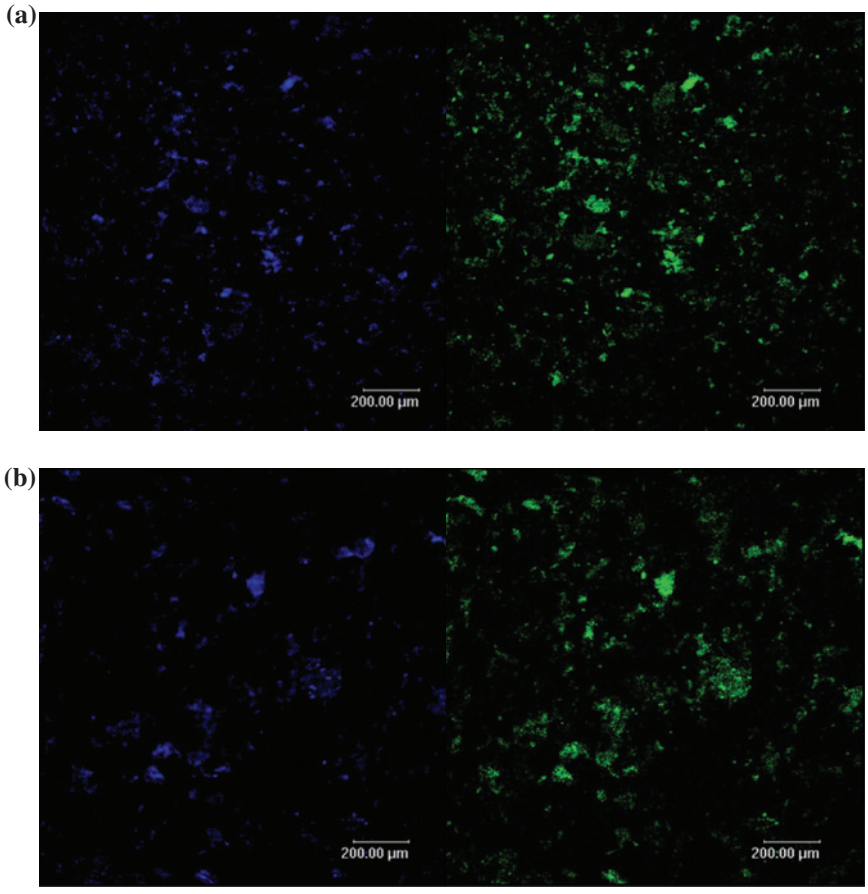

Fig. 3. FISH analysis of the Anammox sludge. Blue color indicates Anammox bacteria hybridized with AMX820 probe, and green color indicates all bacteria hybridized with EUB338 probe: (a) FISH analysis of the Anammox sludge on day 1 of the storage and (b) FISH analysis of the Anammox sludge on day 180 of the storage

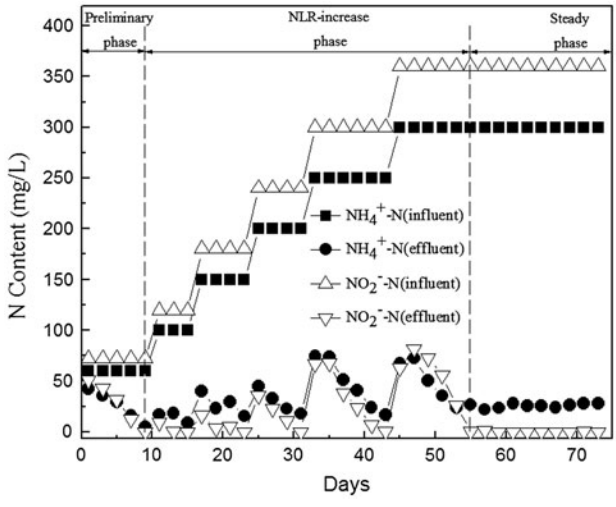

Fig. 4. Variations of nitrogen concentrations in the SBR reactor during reactivation of the Anammox sludge.

91.8 and 99.9%. In the NLR-increase phase (day 9–55), the concentrations of $(NH_4)_2SO_4$ and $NaNO_2$ in the influent increased stepwise from 60 and 72 mg-N L^{-1} to 300 and 360 mg-N L^{-1}, respectively. Every time the concentrations of $(NH_4)_2SO_4$ and $NaNO_2$ increased, the effluent NH_4^+-N concentration slightly fluctuated with the effluent NO_2^--N concentration, and NH_4^+-N consumption rate and NO_2^--N consumption rate seemed to exhibit a good correlation. The interesting phenomenon demonstrated that Anammox reaction was a dominant process in the SBR. The average removal efficiency of $(NH_4)_2SO_4$ and that of $NaNO_2$ in this phase was 83.1 and 89.4%, respectively. The results showed that an excellent start-up performance of Anammox process was gained using the stored Anammox sludge as an inoculum. Ni et al. discovered that the mixture of Anammox sludge and inactive methanogenic granules was suitable for fast start-up of Anammox process and $(NH_4)_2SO_4$ and $NaNO_2$ in the influent increased to 245 and 335 mg-N L^{-1} after 90 d

operation [23]. In this study, a faster start-up of Anammox process was realized as $(NH_4)_2SO_4$ and $NaNO_2$ in the influent increased to 300 and 360 mg-N L^{-1} only after 47-d operation. In the steady phase (day 55–60), $(NH_4)_2SO_4$ and $NaNO_2$ in the influent were maintained at 300 and 360 mg-N L^{-1} and the average removal efficiency of NH_4^+-N and that of NO_2^--N reached 91.4 and 99.9%, respectively. After about 2-month operation, the N (NH_4^+-N plus NO_2^--N) removal rate (NRR) reached 0.32 g L^{-1} d^{-1}.

The Anammox start-up process with the stored Anammox sludge was different from those seeded with conventional activated sludge, which were typically divided into five phases: endogenous denitrification phase, lag phase, appearance phase, elevation phase, and steady phase of Anammox activity [16,19,22]. Compared with those seeded with the activated sludge, this process of Anammox start-up was simplified and accelerated, because the first two phases of endogenous denitrification and lag of Anammox activity were saved using the stored Anammox sludge as the inoculum. That is to say, the Anammox activity appeared as soon as the reactivation of the stored Anammox sludge was initiated and afterward the Anammox activity was gradually enhanced as Anammox bacteria grew and reproduced. The similar phenomenon was also reported by Zhang et al., who discovered that Anammox bacteria could soon predominated in an expanded granular sludge bed by increasing nitrogen loading after the reactor was inoculated with the stored Anammox sludge, which had been kept at 4°C for 2 years [24]. Previously, it was pointed out that mature Anammox granule was an ideal inoculum for accelerating Anammox start-up process [25]. This study demonstrated that the stored Anammox sludge was also an ideal inoculum for quick start-up of Anammox as saving of the endogenous denitrification and lag phase was beneficial to the quick start-up process.

In a word, the period of Anammox start-up in this study was distinctly shortened compared with the periods of those seeded with the fresh activated sludge (usually 3 months or more) [18,21,26,27]. The stored Anammox sludge was successfully reactivated and Anammox process was quickly started up within 2 months.

4. Conclusion

In this study, the long-term storage strategy of Anammox sludge was improved and subsequent quick and effective reactivation of the stored Anammox sludge was achieved. Anammox sludge immersed in Anammox nutrient medium containing 50 mg-N L^{-1} $(NH_4)_2SO_4$ and $NaNO_2$ was stored at 35°C. During the storage, periodical (every week) refreshing of the Anammox nutrient medium could keep the survival of Anammox bacteria. After 6-month storage, there was still an Anammox activity of 0.07 g (NH_4^+-N + NO_2^--N) (gVSS × d)$^{-1}$, and Anammox bacteria occupied 81% of the total bacteria in the Anammox sludge. The stored Anammox sludge was used as seed and successfully started up Anammox process within 2 months. The period of the start-up was evidently shorter than those in other Anammox start-up processes seeded with the fresh activated sludge. The results demonstrated that the improved storage strategy was suitable for the long-term storage and subsequent reactivation of Anammox sludge.

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