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# Effect of inorganic carbon on anaerobic ammonium oxidation enriched in sequencing batch reactor

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**Abstract**

The present lab-scale research reveals the enrichment of anaerobic ammonium oxidation microorganism from methanogenic anaerobic granular sludge and the effect of inorganic carbon (sodium bicarbonate) on anaerobic ammonium oxidation. The enrichment of anammox bacteria was carried out in a 7.0-L sequencing batch reactor (SBR) and the effect of bicarbonate on anammox was conducted in a 3.0-L SBR. Research results , especially the biomass, showed first signs of anammox activity after 54 d cultivation with synthetic wastewater, when the pH was controlled between 7.5 and 8.3, the temperature was 35°C. The anammox activity increased as the influent bicarbonate concentration increased from 1.0 to 1.5 g/L, and then, was inhibited as the bicarbonate concentration approached 2.0 g/L. However, the activity could be restored by the reduction of bicarbonate concentration to 1.0 g/L, as shown by rapid conversion of ammonium, and nitrite and nitrate production with normal stoichiometry. The optimization of the bicarbonate concentration in the reactor could increase the anammox rate up to 66.4 mgN/(L·d).

**Key words**: inorganic carbon; anaerobic ammonium oxidation (anammox); sequencing batch reactor (SBR); nitritation; methanogenic granular sludge

## Introduction

The anaerobic ammonium oxidation (anammox) process is a novel, promising, and cost-effective alternative method to remove nitrogen compounds in place of the traditional denitrification strategy (Pynaert *et al*., 2004; Strous *et al*., 2004; Van Loosdrecht *et al*., 2004). This process is carried out by some autotrophic bacteria (Van de Graaf *et al*., 1996), which form a monophyletic cluster branching-off deep in the Order Planctomycetales. These bacteria belong to three genera: *Brocadia* including *Brocadia anammoxidans* (Strous *et al*., 2002) and *Brocadia fulgida* (Kartal *et al*., 2004), *Kuenenia* including *Kuenenia stuttgartiensis* (Schmid *et al.*, 2000), and *Scalindua* including *Scalindua wagneri*, *Scalindua brodae*, and *Scalindua sorokinii* (Kuypers *et al.*, 2003; Schmid *et al*., 2003; Jetten *et al*., 2005). Recently, a new anammox species tentatively named Candidatus *Anammoxoglobus propionicus* was enriched in a laboratory scale bioreactor in the presence of ammonium and propionate (Kartal *et al*., 2007). Nitrite is the electron acceptor for the anaerobic oxidation of ammonia to dinitrogen gas, and hydrazine is an important intermediate (Van de Graaf *et al*., 1996). The overall nitrogen balance gives a ratio of ammonium conversion to nitrite conversion to nitrate production of 1:1.32:0.26 (Strous *et al*., 1998). The overall anammox reaction is presented in the following reaction.

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

However, the anammox microorganisms have a very slow growth rate and low yield constant, and activity is inhibited by low (0.5% air saturation) concentrations of oxygen (Van de Graaf *et al*., 1996; Strous *et al*., 1997); thus, it is believed that they are difficult to cultivate. For the industrial use of the slow-growing bacteria, it is essential to find appropriate seed sludge and an adequate reactor configuration to enrich the anammox microorganism. The sequencing batch reactor (SBR) was proved to be a suitable system for the isolation of a microbial community with an extremely slow growth rate (Strous *et al*., 1998; Dapena-Mora *et al*., 2004a). Dapena-Mora *et al*. (2004a, 2004b) enriched anammox biomass obtained from municipal activated sludge in SBR. The nitrifying granular sludge taken from an airlift nitrifying granular sludge bed reactor was used as inoculum for anammox reactor (Zheng *et al*., 2004).

It is common knowledge that the chemolithoautotrophs mainly utilize inorganic carbon as carbon source. Therefore, the influent bicarbonate concentration is an important factor to affect the anammox enrichment. Some studies can be identified in the published work investigating the inorganic carbon limitation in some chemolithoautotrophic

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bacteria. Wickins (1993) and Furukawa *et al*. (1993) observed that nitrification rate was limited by the lack of inorganic carbon source in acclimated marine nitrifying sludge. Inorganic carbon limitation has been observed in nitrifying biofilms (Flora *et al*., 1999) and a stimulation of nitrifier growth has been achieved by bicarbonate addition (Byong-Hee *et al*., 2000). Wett and Rauch (2003) pointed out that carbon limitation might be the main cause that growth and activity of nitrifying bacteria decreased dramatically below neutrality. However, no study, to our knowledge, has reported the effects of inorganic carbon on the anammox process.

On the basis of these considerations, this study focuses on the enrichment of anammox bacteria from anaerobic granular sludge, and the effects of influent bicarbonate concentration on the anammox process was also discussed.

# 1 Materials and methods

## 1.1 Wastewater

The composition of the mineral medium was (g/L): $NH_4Cl$ 0.268, $NaNO_2$ 0.345, $KH_2PO_4$ 0.027, $MgSO_4 \cdot 7H_2O$ 0.3, $CaCl_2$ 0.05, and 1.0 ml of trace element solution (Strous *et al*., 1998). Inorganic carbon concentration was added as specified in the results section. No organic substrates were added to the biomass to favor the enrichment of autotrophic anammox bacteria.

## 1.2 Operation of the reactors

Two different SBR reactors were used in these experiments. Enrichment and breeding of the anammox sludge was conducted in a 7.0-L SBR reactor (height 74 cm, diameter 11 cm). The study related to the effect of inorganic carbon on anammox was carried out in another 3.0-L SBR reactor (diameter 9 cm). Both reactors were operated at a fixed temperature of 35°C by means of a thermostatted jacket. The 7.0-L SBR reactor was seeded with methanogenic anaerobic activated sludge obtained from an Internal Circulation (IC) reactor of a paper mill wastewater treatment plant. The synthetic feeding solution containing 1.0 g/L bicarbonate was fed to the reactor after deoxygenation by stripping with nitrogen gas 4–5 times in a water tank. Before feeding, the sludge was allowed to settle for 1 h. Approximately 5 L of the supernatant was removed, and 5 L of the freshly prepared synthetic wastewater was fed. The pH of the reactor was controlled between 7.5 and 8.3 with 1 mol/L HCl and NaOH stock solutions.

The anammox sludge was taken out from the breeding reactor and seeded into the 3.0-L SBR at a concentration of 1.24 g VSS/L. Sequencing batch experiments concerning the effect of inorganic carbon on anammox were carried out with the sodium bicarbonate addition at concentrations 1.0, 1.25, 1.5, 1.75, and 2.0 g $NaHCO_3$/L during 20 d, respectively. Mineral medium (80 ± 10 mg/L ammonium, 80 ± 10 mg/L nitrite) was fed to the 3.0-L reactor after deoxygenation by stripping with nitrogen. The pH was measured. The hydraulic retention time (HRT) was maintained between 3 and 5 d according to the removal efficiency of ammonium and nitrite.

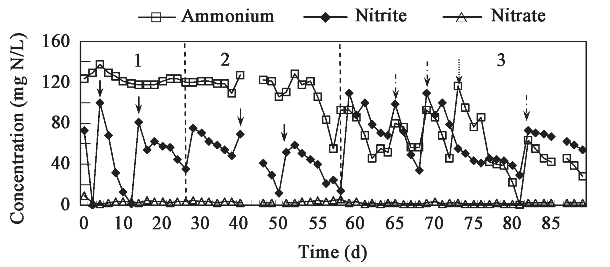


**Fig. 1** Startup of anammox reactor in the SBR. 1, 2, 3 means three experiment cycles. (1) adding ammonium; (2) adding nitrite; (3) adding ammonium and nitrite.

The 7.0-L SBR and 3.0-L SBR were sparged with nitrogen gas at a maximum gas flow of 40 and 30 ml/min, respectively, for the fluidization of the biomass and the maintenance of anaerobiosis of the reactor.

## 1.3 Analytical procedures

Concentrations of ammonium, nitrite, and nitrate were measured by colorimetric methods according to standard methods (EPBC, 1997). The chemical oxygen demand (COD) measurement was on the basis of digestion with potassium dichromate in concentrated sulphuric acid for 2 h at 150°C. Sludge concentration in terms of total suspended solids (TSS) and volatile suspended solids (VSS) was evaluated every 20–30 d. Dry weight was determined after drying the sample at 105°C for at least 12 h. Subsequently. the sample was ashed in a furnace for 2 h. The dry weight minus the ashed weight is termed VSS.

# 2 Results and discussion

## 2.1 Enrichment

The anammox population was enriched in the 7.0-L SBR, which was inoculated with a high concentration of VSS (8.913 g/L) because of the decay of the original biomass. Initially, the medium (70 mg/L $NH_4^+$, 70 mg/L $NO_2^-$) was supplied with HRT of 30 d for the low growth rate of anammox bacteria, according to Strous *et al*. (1998).

Figure 1 shows that no reduction of ammonium was observed in the reactor and only nitrite decreased rapidly in the initial 54 d. The maximum nitrite removal rate reached 36.5 g nitrite/($m^3 \cdot d$) and declined gradually to 1.66 g/($m^3 \cdot d$) on day 38. At the same time, COD also decreased from 920 to 540 mg/L (Fig.2). Nitrite was added to the reactor after its concentration was below the detection limit. These results indicated that heterotrophic denitrifying activity was the favored process in the reactor. The medium did not contain organic carbon source, and the COD in the reactor must be produced by the lysis of biomass. This view was supported by an initial consumption of nitrite together with a significant decrease in biomass concentration during the first few days (Table 1).

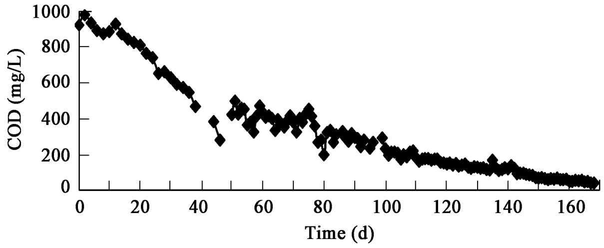


**Fig. 2** COD concentration as a function of time in the reactor.

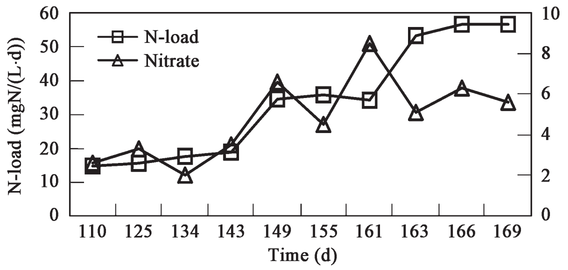


**Fig. 3** Increasing nitrogen-conversation in the 7.0-L SBR.

After fed with $NH_4^+$ and $NO_2^-$ for 54 d, the biomass showed the first signs of ammonium consumption. On day 90, the maximum removal rates reached 14.6 g $NH_4^+$-N/($m^3$·d) and 6.67 g $NO_2^-$-N/($m^3$·d), respectively. At this point, the nitrogen load could be increased gradually by gradually decreasing the HRT to 3 d between the day 91 and day 169 (Fig.3), and reached up to 56.7 mgN/(L·d) on day 169. At the end of the culture period, the anaerobic sludge granules gradually changed from grey to reddish colour, probably because of a high cytochrome content (Jetten *et al.*, 1999).

**Table 1** Variation of sludge characteristics during enrichment period

| Inoculation time (d) | TSS (g/L) | VSS (g/L) | VSS/SS | SVI (ml/g) |
|---|---|---|---|---|
| 0 | 9.482 | 8.913 | 0.94 | 99 |
| 58 | 8.155 | 7.095 | 0.87 | 106.7 |
| 156 | 5.432 | 4.554 | 0.84 | 73.6 |

TSS: total suspended solids; VSS: volatile suspended solids; SVI: sludge volumetric index.

## 2.2 Effects of inorganic carbon (bicarbonate) on the anammox process

The $NH_4^+$-N and the $NO_2^-$-N concentrations in the influent both were remained at 80 ± 10 mg/L during the whole experimental period. However, the performance of the anammox process was different with different influent bicarbonate concentration. The changes of ammonium, nitrite, nitrate concentrations, and pH in the reactor for different influent bicarbonate concentration are illustrated in Fig.4.

The anammox efficiency was inhibited (only 41.6% ammonium and 46.2% nitrite were removed with a HRT of 3 d) when the influent bicarbonate concentration was 1.0 g/L. At the same time, a decrease of pH from 7.5 to 6.8 occurred during the reaction period. When the influent bicarbonate concentration increased to 1.5 g/L, the buffer capability was increased and the pH in the reactor was maintained between 7.6 and 8.1. The response of the process at this bicarbonate concentration was very fast, and the removal percentages of ammonium and nitrite increased to 83.6% and 100%, respectively. Similar performance was also observed when the inorganic carbon increased to 1.75 g/L. The percentage of removal dropped sharply as the influent bicarbonate concentration increased from 1.5 to 2.0 g/L. The anammox activity was inhibited by high concentrations of bicarbonate. However, the activity was restored by the reduction of bicarbonate concentration to 1.0 g/L, as rapid conversion of ammonium and nitrate production with normal stoichiometry (Fig.5).

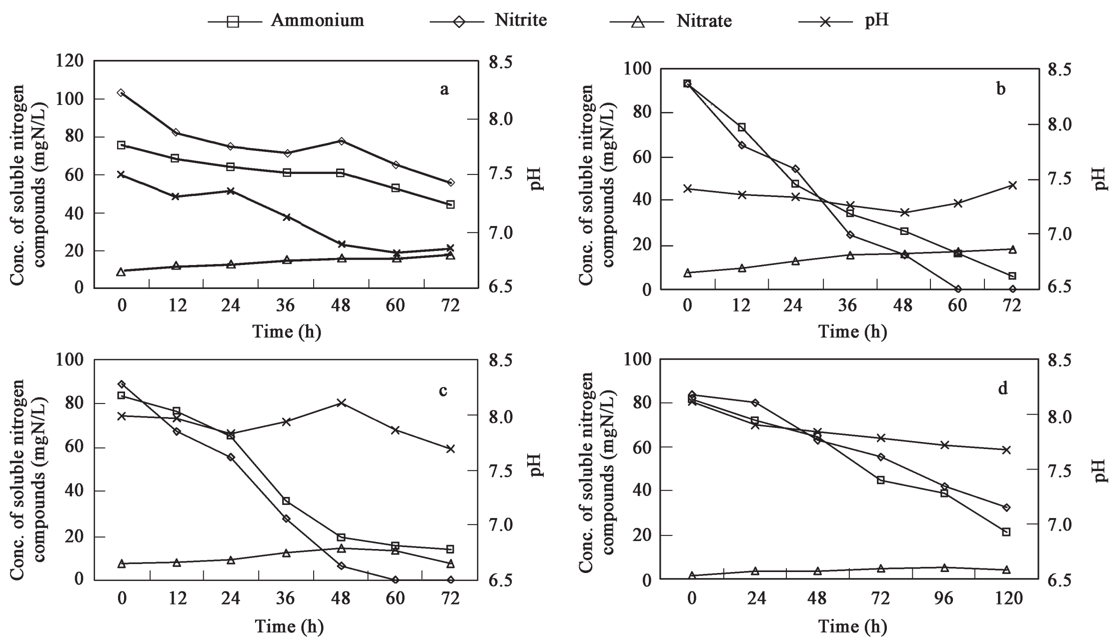


**Fig. 4** Concentration profiles of soluble nitrogen compounds and pH in the anammox reactor at different influent bicarbonate. Bicarbonate concentration: (a) 1.0 g/L; (b) 1.5 g/L; (c) 1.75 g/L; (d) 2.0 g/L.

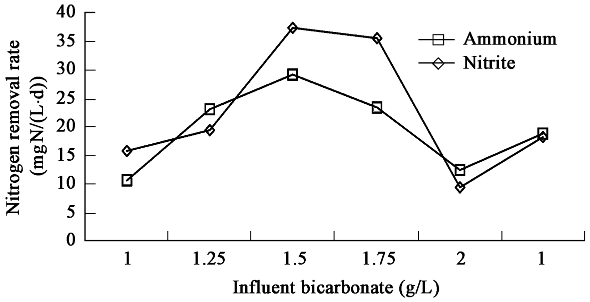


**Fig. 5** Removal rates of ammonium and nitrite versus influent bicarbonate.

Figure 5 shows the removal rates of ammonium and nitrite at different influent-bicarbonate concentrations. The ammonium removal rate reached its highest value of 29.1 mg $NH_4^+$/(L·d) at 1.5 g/L bicarbonate but decreased to 12.3 mg $NH_4^+$/(L·d) at 2.0 g/L bicarbonate. The nitrite removal rate increased from 15.9 to 37.3 mg $NO_2^-$/(L·d) as bicarbonate increased from 1.0 to 1.5 g/L and dropped sharply to 9.5 mg $NO_2^-$/(L·d) at 2.0 g/L bicarbonate. When the influent bicarbonate concentration decreased to 1.0 g/L, the anammox activity was restored, and both the ammonium and nitrite removal rate increased, and reached 18.7 mg $NH_4^+$/(L·d) and 18.3 mg $NO_2^-$/(L·d), respectively. The optimization of the influent bicarbonate concentration was 1.5 g/L, and the total nitrogen removal rate was 66.4 mgN/(L·d).

The low anammox activity at the bicarbonate concentration of 1.0 g/L might cause limitation effects because of a lack of $CO_2$ as the actual substrate for anammox. The concentration of inorganic carbon resulted from a balance of assimilation, respiration, and $CO_2$ stripping because of dinitrogen gas aeration. When the influent bicarbonate concentration was 1.0 g/L, the pH in the 3.0-L SBR was decreased to 6.8. At low pH, the aeration process caused $CO_2$ stripping and consequently a decrease of the available inorganic carbon. Thus, a limitation of bicarbonate occurred. The deduction was confirmed by Wett and Rauch (2003). The portion of $CO_2$ reduced from 19.0% to 10.5% of the total inorganic carbon when the pH value increased from $7.0 < pH < 7.3$ to $7.3 < pH < 7.6$.

An inhibition effect occurred at high bicarbonate concentration (2.0 g/L), which might cause high free ammonia ($NH_3$) concentration. The free ammonia concentration in reactor can be estimated by the expression proposed by Ford *et al*. (1980). Free ammonia is related with the pH, ammonium concentration, and reaction temperature, whereas the pH in the 3.0-L SBR reached 8.1 when bicarbonate increased to 2.0 g/L. The $NH_3$ concentration in the reactor increased from 3.3 to 13.3 mg/L as the influent bicarbonate increased from 1.5 to 2.0 g/L. Free ammonia can inhibit the activities of *Nitrosomonas*, *Nitrobacter*, and other microbial species.

## 3 Conclusions

The present results have shown that the anaerobic methanogenic granular sludge was a very suitable inoculum for the enrichment of anammox bacteria.

The SBR is a suitable system for the enrichment of a microbial community with an extremely slow growth rate. Both, a long HRT during initial cultivation period and a high concentration of inoculum, were beneficial to the fast enrichment of anammox bacteria.

The anammox process underlies limitations at low inorganic carbon concentrations. When the influent bicarbonate concentrate was 1.0 mg/L, a deficit of carbon source occurred and resulted in a decrease of the process rate. When the influent bicarbonate increased to 1.5–1.75 mg/L, the anammox activity increased and reached 66.4 mgN/(L·d). However, as the influent inorganic carbon further increased to 2.0 mg/L, the anammox rate decreased sharply.

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