Nitrogen Removal by Simultaneous Anammox and Denitrification under Low Temperature: Preliminary Batch Trials

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Abstract

The simultaneous anaerobic ammonium oxidation (anammox) and heterotrophic denitrification (SAD) process has been proposed for the nitrogen removal from domestic wastewater. In the SAD process, anammox bacteria are inoculated into the denitrification reactor. Nitrate is reduced to nitrite by heterotrophic denitrifiers. Then, nitrite is partly intercepted by anammox bacteria from heterotrophic denitrifiers and is reduced to N₂ using ammonium. The purpose of this study was to investigate the possibility of nitrogen removal by the SAD process in the 24-h batch mode at 20°C. Both samples of anammox sludge acclimated at 20°C and activated sludge collected from a wastewater treatment plant were inoculated to 20 mL synthetic wastewater at 1500 mg-MLSS/L, respectively. The nitrogen removal of 49–80% in the SAD process was demonstrated in a wide range of the C/N ratio of 0.4–1.5 with synthetic wastewater containing 70 mg-N/L nitrate, 50 mg-N/L ammonium, and acetate as the sole carbon source. The nitrogen removal in the SAD process was higher than the typical denitrification process at lower C/N ratio. However, the nitrogen removal in the SAD process at 20°C was much slower than that obtained at 33°C in a previous study (Park et al., J. Biosci. Bioeng., 123, 505–511) and highly depended on the activity of the heterotrophic denitrifiers. The high nitrite-producing activity of heterotrophic denitrifiers will be needed for developing the efficient SAD process at low temperature.

Keywords: low temperature, anammox, heterotrophic denitrification, C/N ratio

INTRODUCTION

The nitrogen removal process using anaerobic ammonium oxidation (anammox) has advantages over conventional nitrification-denitrification processes, such as its lower need for a supply of oxygen and external carbon source and less sludge production⁹. However, the partial nitritation process, which supplies two substrates for anammox bacteria, ammonium and nitrite, has technical difficulties, especially at low ammonium concentrations. Therefore, anammox has been applied rarely to domestic wastewater with low ammonium concentrations also because of its high concentration of organic matters, which inhibit anammox bacteria.

Therefore, the simultaneous anammox and heterotrophic denitrification (SAD) process has been proposed for nitrogen removal from domestic wastewater⁸,⁹, as presented in Fig. 1. In the SAD process, anammox bacteria are inoculated into the conventional denitrification reactor. Nitrate returned from the nitrification reactor is reduced to nitrite by heterotrophic denitrifiers with the carbon

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source fed as the influent in the denitrification reactor. Then, nitrite is intercepted by anammox bacteria from heterotrophic denitrifiers and is reduced to N₂ using ammonium (bold lines). Therefore, the SAD process can remove nitrogen from wastewater without the nitritation process which is difficult to be controlled. Moreover, nitrogen removal in the SAD process can be performed at lower C/N ratios than those used for the conventional denitrification process. Ideally, nitrate produced by anammox would be removed by SAD also. Another benefit of this process is that inhibition of the anammox activity by organic matter can be moderated because heterotrophic bacteria consume it.

The SAD process will be successful only when slow-growing anammox bacteria win the competition for nitrite as the common substrate against heterotrophic denitrifiers. Our previous study has demonstrated that the C/N ratio of 1.5–2.0 and the population ratio of heterotrophic denitrifiers to anammox bacteria within 0.3–0.4 were suitable conditions for the batch SAD process at 33°C. However, the temperature of sewage flowing to domestic wastewater treatment plants (WWTPs) is generally 20–30°C in the south of central Honshu in Japan. Actually, the activity of the anammox sludge enriched at 33°C decreased at low temperature. Therefore a heating system to maintain water temperatures of 30–35°C is necessary to achieve efficient treatment by SAD in WWTPs, lowers its energy efficiency. Namely, development of the SAD process which is applicable at lower temperatures (< 20°C) in winter without a heating system is desired for domestic wastewater treatment.

Therefore, our research group has enriched anammox bacteria in a continuous treatment reactor operated at 20°C. As shown in Fig. 2, the anammox sludge enriched at 20°C maintains the high activity at 10–30°C, although its activity drops at temperatures higher than 30°C, which may enable efficient SAD at lower temperature. The purpose of this study was to investigate the possibility of nitrogen removal by the SAD process at 20°C with various C/N ratios in the batch mode utilizing the anammox sludge enriched at 20°C.

**MATERIARLS AND METHODS**

**Anammox sludge and activated sludge**

The anammox sludge originated from a livestock wastewater treatment plant in Hokkaido Prefecture was used in this study. The anammox sludge enriched at 20°C had the highest specific anammox activity 4.2 mg-N/g-VSS/h at 30°C. The temperature coefficient for the activity of the anammox sludge enriched at 20°C and 33°C was 0.0928°C⁻¹ (10–30°C) and 0.104°C⁻¹ (10–35°C), respectively.

As the source of heterotrophic denitrifiers, activated sludge samples were collected from
an anoxic tank of a recycled nitrification-denitrification process in a WWTP for domestic wastewater in Osaka Prefecture in October, November, and December 2015.

**Batch experiments for nitrite and nitrate removal**

Temperature dependences of the removal activity for nitrate and nitrite were examined by using activated sludge samples collected in October (water temperature 24.0°C) and December (20.0°C) 2015, respectively. The activated sludge sample was inoculated to 20 mL synthetic wastewater in a 30-mL vial at 1500 mg/L as mixed liquor suspended solids (MLSS). Synthetic wastewater contained 50 mg-N/L nitrate and 50 mg-C/L acetate for the nitrite removal test and 25 mg-N/L nitrite and 25 mg-C/L acetate for the nitrite removal test, in addition to 54 mg/L KH₂PO₄, 5 mg/L EDTA, 9 mg/L FeSO₄·7H₂O, and 1 mL/L trace element solution. The vial was sealed with a butyl rubber septa and aluminum crimp seal. The headspace of the sealed bottle was purged with N₂ gas. The bottles were set on a rotary shaker at 100 rpm at 20, 28, and 35°C for 8 h. Water samples were taken from the vials periodically for chemical analysis. These experiments were performed in triplicate.

**Batch SAD experiments simulating nitrogen removal from domestic wastewater**

The first (Exp. 1) and the second SAD experiments (Exp. 2) were carried out by using activated sludge samples collected in November (water temperature 22.3°C) and December (20.0°C) 2015, respectively. Both anammox sludge and activated sludge samples were inoculated to 20 mL synthetic wastewater in a 30-mL vial at 1500 mg-MLSS/L, respectively, 3000 mg-MLSS/L in total. Synthetic wastewater contained 70 mg-N/L nitrate, 50 mg-N/L ammonium, and acetate as the sole carbon source, in addition to 54 mg/L KH₂PO₄, 5 mg/L EDTA, 9 mg/L FeSO₄·7H₂O, 84 mg/L NaHCO₃ and 1 mL/L trace element solution. The initial C/N ratio defined by the total organic carbon (TOC)/NO₃-N concentration was set at 0.4, 0.8, 1.0, 1.2, and 1.5. All vials were anaerobically incubated on a rotary shaker at 100 rpm at 20°C. The anammox process without activated sludge was also investigated using synthetic water containing 45 mg-N/L ammonium and 30 mg-N/L nitrite, and the denitrification process without the anammox sludge was also investigated 50 mg-N/L nitrate and 50 mg-C/L acetate for comparison with SAD. Water samples were taken from the vials periodically for chemical analysis. These experiments were performed in triplicate.

**Estimation of nitrogen removal by annamox in the SAD process**

The anammox process typically removes nitrite of 1.32 mol with ammonium of 1.0 mol. In the SAD process, 1.32 mol of nitrite reduced from the equivalent molar of nitrate is removed with 1.0 mol of ammonium, but with production of 0.26 mol of nitrate. Therefore, it was assumed that the anammox in the SAD process removes 1.06 mol (=1.32−0.26) of nitrate with 1.0 mol of ammonium without nitrite accumulation. Based on this assumption, the contribution of the anammox process to the total nitrogen removal in the SAD process was estimated.

**Analytical Procedures**

The MLSS and volatile suspended solids (VSS) were measured according to the methods set out in JIS (Japan Industrial Standards) K0102. The ammonium, nitrite, and nitrate concentrations were measured using an ion-chromatography system (HIC–SP system; Shimadzu Corp., Kyoto) as previously reported. The TOC concentration was measured using a TOC analyzer (TOC–5000A; Shimadzu Corp., Kyoto). The total nitrogen (T–N) concentration was defined as the sum of the ammonium, nitrite, and nitrate concentrations in this study.

**RESULTS AND DISCUSSION**

**Temperature dependence of nitrogen removal activity of sludge samples**

The specific removal activity for nitrite and nitrate of the activated sludge sample was shown in Fig. 2. In the nitrite removal test, the activated sludge sample collected in December removed only about 5 mg/L of TOC and 5–20 mg/L of nitrite within 8 h, suggesting inhibition by high nitrite concentrations. The specific nitrite removal
rate of the sludge sample slightly decreased from 1.8 mg-N/g-VSS/h at 35°C to 0.5 mg-N/g-VSS/h at 20°C.

In the nitrate removal test, the activated sludge samples collected in October completely removed TOC within 4–6 h and 20–45 mg/L of nitrate in 8 h with only slight nitrite accumulation (1.8 mg-N/L). This suggests that nitrite reduction at low concentrations was not a rate-limiting step in the nitrate removal test. The specific nitrate removal rate of the sludge sample from nitrate dropped from 5.1 mg-N/g-VSS/h at 35°C to 2.3 mg-N/g-VSS/h at 20°C.

The temperature coefficient for the nitrite and nitrate removal was respectively estimated to be 0.0518°C⁻¹ and 0.0894°C⁻¹, which was comparable to the typical value (0.07°C⁻¹) of heterotrophic bacteria. These results suggest that the nitrogen removal rate in the SAD process would decrease by almost half if temperature decreases from 33°C to 20°C.

**Nitrogen removal in the SAD process at 20°C**

Figure 3 shows nitrogen and TOC removal in the batch SAD processes (Exp. 1). The anammox sludge almost completely removed 30 mg-N/L of nitrite and 45 mg-N/L of ammonium within 24 h (Fig. 3a). The activated sludge sample collected in November from the WWTP completely removed 50 mg/L of TOC within 6 h, consequently nitrate from 50 mg–N /L to 10 mg/L within 10 h with temporal accumulation of nitrite up to 5.5 mg–N/L (Fig. 3b). The decrease in the nitrate and nitrite concentrations under carbon depletion suggests that acetate in wastewater was converted to intracellular storage products in heterotrophs for denitrification. In the SAD process, the rapid depletions of nitrate and TOC were observed within the first 4–6 h (Fig. 3c–3e). In each batch run of the SAD process, about 20 mg/L of ammonium was removed without nitrite accumulation, reflecting that nitrite produced by heterotrophic bacteria was partly intercepted by anammox bacteria. The T-N removal in the SAD process increased from 63% to 80% with the C/N ratio from 0.4 to 0.8.

Figure 4 shows nitrogen and TOC removal in the batch SAD processes (Exp. 2). The activated sludge collected in December from the WWTP reduced the TOC concentration from 50 mg/L to 20 mg/L within 8 h, consequently nitrate from 50 mg–N /L to 0 mg/L within 6 h with temporal accumulation of nitrite up to 9.7 mg–N/L (Fig. 4a). These results indicate that the denitrification
activity from nitrate to nitrite of activated sludge in December was much higher than that in November. In the SAD process, the rapid removal of nitrate was observed at the high C/N ratio (Fig. 4b–4e). Nitrite reduced from nitrate by heterotrophic denitrification was temporarily accumulated, and subsequently removed with ammonium by anammox with all C/N ratios in the SAD process. The T-N removal in the SAD process increased from 49% to 70% with the C/N ratio from 0.4 to 1.5. These results obtained at 20°C suggest that the SAD process would remove nitrogen from domestic wastewater to a certain degree at low temperature in winter, depending on the C/N ratio.

**Effects of C/N ratio on the SAD process at 20°C**

Figure 5 summarizes the T-N removal and contribution of anammox and heterotrophic denitrification in the SAD process. In Exp. 1 and Exp. 2, nitrogen removal was well performed in the SAD process than the typical denitrification process at lower C/N ratio. The contribution of anammox to the T-N removal was estimated to be 40–52%, suggesting heterotrophic denitrifiers and anammox bacteria had comparable high affinities to nitrite. Without the enrichment process at 20°C for the anammox sludge, the contribution of anammox to the T-N removal would be much lower because the anammox sludge enriched at 33°C showed the lower activity at 10–30°C. It was reported that the denitrification efficiency from nitrate was only 30% by activated sludge fed with acetate at the C/N (TOC/NO₃-N) ratio of 0.3. Thus, the C/N ratio over 1.9 was needed for more than 90% denitrification by heterotrophic bacteria

Although the optimum C/N ratio for the

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**Fig. 4** Time courses of nitrogen and TOC removal in the batch-mode process (Exp. 2). Denitrification process by activated sludge (a), SAD with the C/N ratio = 0.4 (b), 0.8 (c) 1.0 (d), 1.2 (e) and 1.5 (f). Symbols represent the average of triplicate experiments, and error bars show the standard deviation.

**Fig. 5** Effects on the C/N ratio on the T-N removal in the SAD process in the batch mode. Exp.1 and Exp.2 were performed by using activated sludge collected from November and December, respectively. Symbols represent the average of triplicate experiments, and error bars show the standard deviation.
SAD process at 20°C was not found in this study, the optimal C/N ratio for the batch SAD process at 33°C was reported to be 1.5 –2.0. Deficient amounts of carbon supply at low C/N ratios decrease the nitrate removal, resulting in nitrite starvation for anammox bacteria. Conversely, an excessive high C/N ratio results in the predominance of heterotrophic denitrifiers over anammox bacteria in the SAD process, which decrease the ammonium removal as well as the T-N removal.

As shown in the results of Exp. 1 and Exp. 2, the SAD process highly depended on the activity of the heterotrophic denitrifiers. It is important to note that the SAD process at 20°C in this study needed longer time (10–24 h) than that at 33°C in the previous study (6–8 h) for achieving same levels of the T-N removal\(^2\). The most influential parameters in the SAD process are the ratio of half saturation constant for nitrite of heterotrophic bacteria to that of anammox bacteria and the ratio of anoxic reducing factors of heterotrophic bacteria for the nitrate-reducing rate to that for the nitrite-reducing rate\(^6\). The seasonal change of the composition and the activity of denitrifiers in activated sludge would significantly affect such parameters and the performance of the SAD process.

**CONCLUSIONS**

The nitrogen removal of 49–80% in the SAD process was demonstrated in the 24-h batch experiments at 20°C in a wide range of the C/N ratio of 0.4–1.5 by utilizing the anammox sludge enriched at 20°C. The nitrogen removal in the SAD process was higher than the typical denitrification process at lower C/N ratio. However, the nitrogen removal in the SAD process at 20°C was much slower than that at 33°C and highly depended on the activity of the heterotrophic denitrifiers. The high nitrite-producing activity of heterotrophic denitrifiers will be needed for developing the efficient SAD process without a heating system. Demonstration of the SAD process in the sequencing batch mode and the continuous mode for the long operation period at low temperature will be needed for further studies.

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