Anaerobic Ammonium Oxidation Process in an Upflow Anaerobic Sludge Blanket Reactor with Granular Sludge Selected from an Anaerobic Digestor

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Abstract The purpose of this work was to evaluate the development of the anammox process by the use of granular sludge selected from a digestion reactor as a potential seed source in a lab-scale UASB (upflow anaerobic sludge blanket) reactor system. The reactor was operated for approximately 11 months and was fed by synthetic wastewater. After 200 days of feeding with NH_4^+ and NO_2^- as the main substrates, the biomass showed steady signs of ammonium consumption, resulting in over 60% of ammonium nitrogen removal. This report aims to present the results and to more closely examine what occurs after the onset of anammox activity, while the previous work described the start-up experiment and the presence of anammox bacteria in the enriched community using the fluorescence *in situ* hybridization (FISH) technique. By the last month of operation, the consumed NO_2^--N/NH_4^+-N ratio in the UASB reactor was close to 1.32, the stoichiometric ratio of the anammox reaction. The obtained results from the influent-shutdown test suggested that nitrite concentration would be one key parameter that promotes the anammox reaction during the start-up enrichment of anammox bacteria from granular sludge. During the study period, the sludge color gradually changed from black to red-brownish.

Keywords: nitrogen removal, anaerobic ammonium oxidation (anammox), granular sludge, UASB reactor, ammonium, nitrite

INTRODUCTION

Conventional nitrogen elimination is normally implemented in two sequential steps, referred to as a whole as autotrophic nitrification/heterotrophic denitrification, by nitrifying and denitrifying bacteria respectively [1]. Based on these microbial nitrogen conversions, a wide spectrum of approaches for improving treatment efficiency has been reported and reviewed both at the whole cell and the enzyme level [2-5]. However, it is evident that this classical process is less suitable for nitrogen removal from ammonium-rich effluents with a very unfavorable C/N ratio, such as livestock and landfill leachate wastewaters, because an external carbon source must be added to complete denitrification. Therefore, the development of efficient and cost-effective treatment processes for such effluents has become an important part of the overall wastewater treatment process.

Several novel technologies for nitrogen removal have recently been discovered and applied using new microbial conversions, which are often addressed with highly specific abbreviations. Among them, the anaerobic ammonium oxidation process has been proved to be a promising alternative to autotrophically treat ammonium in wastewater. Ammonium can be biologically oxidized under both aerobic and anaerobic conditions [1]. Ammonium oxidation carried out by the use of nitrite (or nitrate) as an electron acceptor in anaerobic conditions is referred to as the anammox (*an*aerobic *amm*onium *ox*idation) process [6]. The free energy for this reaction (−358 kJ/mol) is favorable compared to that of aerobic ammonium oxidation [7]. The evidence for the occurrence of anaerobic ammonium oxidation was discovered by Mulder *et al.* in a denitrifying fluidized bed reactor treating effluent from a methanogenic reactor in Delft, the Netherlands [8]. During the experiment, large quantities of ammonium disappeared together with nitrate, and dinitrogen gas was concomitantly produced. However, after a long process of trial and error, comparison of the labeling pattern of the $^{14,15}N_2$ product indicated that nitrite might be the preferred electron acceptor of the anammox process rather than nitrate [9]. In other words, the anammox process represents the denitritation of nitrite with ammonium as the electron donor.

In follow-up studies, the biological nature of the anammox reaction and the identification of the responsible bacteria were investigated in great detail [10,11]. The anammox bacteria grow autotrophically with CO_2 as the

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carbon source, and nitrite is not only the electron acceptor in the reaction, but it is also the electron donor for carbon fixation [12]. On the basis of experiments in a sequencing batch reactor (SBR), Strous *et al.* reported the most comprehensive stoichiometry for anaerobic ammonium oxidation [13]:

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

During the past decade, several reports showing the presence and activity of anammox bacteria in engineered systems, sediment, or enrichment cultures have been published. Anammox biomass has already been detected in wastewater treatment plants and in a non-artificial ecosystem [8,14,15]. Based on these findings, phylogenetic analyses of the 16S rRNA gene revealed that the anammox reaction is catalyzed by new chemolithoautotrophic members of the order *Planctomycetales*, one of the major distinct divisions of bacteria. Recently, some completely autotrophic nitrogen elimination processes have been developed as the realization of partial nitrification (nitritation) combined with the anammox mechanism. These processes offer new opportunities for efficient and sustainable nitrogen removal in wastewater treatment and were named accordingly, as the combined Sharon/Anammox, CANON (Completely Autotrophic Nitrogen removal Over Nitrite) and OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) processes [16-18].

For the case of anammox process, one of the main challenges is decreasing the long start-up time due to the extremely low growth rate of anammox bacteria (doubling time: 11 days) [13]. Although a large range of bioreactors have been successfully applied for the cultivation of anammox microorganisms in different laboratories, many others who have conducted studies on this process agree that the detection and enrichment of anammox bacteria still remain significant obstacles because the bacteria cannot be cultivated using classical microbiological techniques [7,12]. Therefore, it is not surprising that these kinds of microorganisms are not abundantly found in nature.

Considering previous reports on the anammox process, reactors containing granular sludge were considered to be suitable for the enrichment of anammox bacteria with low growth rates. As anaerobic *Planctomycetes* have been found in anaerobic granular sludge [19], granular sludge resulting from operation over 10 years was considered to be suitable for the detection and the enrichment of anammox bacteria in this study. Unlike other studies in which the reactors were inoculated by repeat incubation of enriched anammox bacteria, this work focused on the use of granular sludge selected from a digestor treating brewery wastewater as a potential inoculum for enriching anammox microorganisms. This report aims to present long-term experimental results and to more closely examine what occurs after the onset of anammox activity, while the previous report described the start-up experiment and the presence of anammox bacteria using the FISH technique [20].

MATERIALS AND METHODS

Seed Sludge

Granular sludge used as inoculum was obtained from a full-scale UASB reactor used for treating brewery wastewater, which had been operated at 30 ± 2°C and neutral pH. As mentioned in the Introduction section, this sludge was used because anaerobic *Planctomycetes* have been detected in such an environment, thus increasing the possibility that anammox bacteria would be present. Granular sludge obtained from the reactor was characterized as 18.6 g VSS/L (65% VSS/TS), ranged between 0.8 and 3 mm in diameter, and was black in color.

Experimental Set-up

In this study, the UASB reactor (total volume: 6.25 L; working volume: 6 L) was employed and details of the experimental apparatus were described in Tran *et al.* [20]. Recycling was applied to improve the mixing condition and to dilute the influent because high nitrite concentrations could be toxic to anammox bacteria [21]. This resulted in a recycling ratio of about 3Q during the operating periods as a whole. Granular sludge (4.5 L) was inoculated as seed biomass, giving a total of 84.2 g VSS in the UASB reactor. After seeding, the reactor was covered completely in order to avoid oxygen diffusion.

During the first period of this work (0~225 days), due to a prediction of low anammox activity, the reactor was conducted at hydraulic retention times (HRT) of 1 and 5 days, respectively, to avoid the possible accumulation of unacceptable nitrite levels in the system when high nitrite concentration was fed [20]. During the last 111 days, after the anammox activity steadily appeared, the HRT was maintained at 3.5 days.

Synthetic Wastewater

Synthetic wastewater mainly contained nitrite and ammonium for the support of anammox activity. The composition of mineral medium used as influent was also described in Tran *et al.* [20]. To minimize oxygen intrusion via the influent, every new synthetic feed batch was purged with N_2 for 15 min. Between days 75 and 225, the feed was composed of various nitrite nitrogen concentrations (50, 100, and 150 mg/L), and acetic acid was added to obtain COD_{Cr}/NO_2^--N = 1 (low C/N ratio). The addition of this organic compound as a carbon source was expected to restore the activity of facultative bacteria existing in granular sludge. This operation strategy was explained and shown in detail in the previous report produced from this work [20]. During the last period of the experiment (after 225 days), except between days 244 and 255, influent nitrite concentration was changed to 70 mg NO_2^--N/L in order to ensure that the ratio of NH_4^+-N/NO_2^--N was nearly the same as in

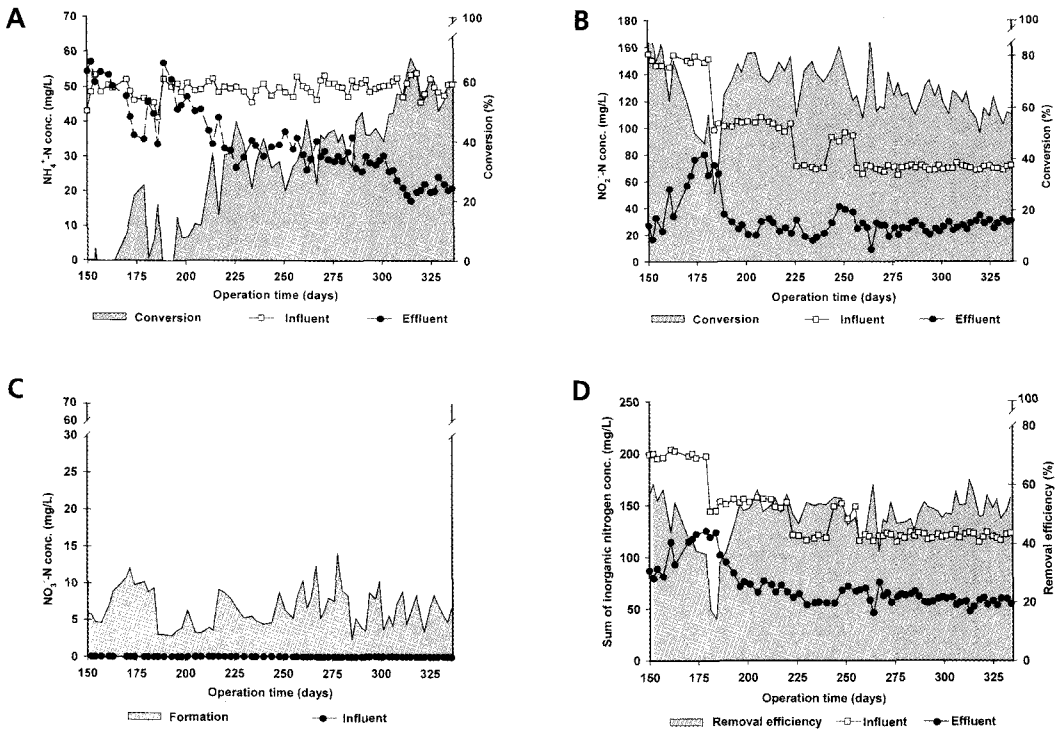


Fig. 1. Ammonium (A), nitrite (B), and nitrate (C) trends, and nitrogen removal efficiency (D) in the UASB reactor before and after the appearance of anammox activity on day 200. The effluent ammonium concentration was steadily decreasing with consumption of nitrite and low production of nitrate as by-product. By the end of the experiment, approximately 60% of the influent ammonium was converted.

the anammox stoichiometric reaction.

Sampling and Analysis

Both influent and effluent samples were measured two or three times a week. Samples were prepared by filtering through 0.45 μm of filter paper (GF/C-Whatman®). Ammonium was measured by selective electrode (Ammonia-Selective Electrode ORION®). Nitrite and nitrate concentrations were determined by ion-chromatography (Dionex, DX-120 Ion-Chromatography). COD_{Cr} was analyzed using a closed reflux method. pH was examined by pH meter (inoLAB WTW). Oxidation reduction potential (ORP) was measured using a Beckman Φ34 pH transmitter and a redox electrode Thermo Orion (Ag/AgCl reference system). Conventional and other parameters of interest, such as total suspended solid (TSS), volatile suspended solid (VSS), and alkalinity, were performed in accordance with the Standard Methods [22].

RESULTS AND DICUSSION

According to a number of published reports, it is known that several months of operating time was required to reach good anammox activity, although other investigators used the enhanced anammox biomass as inoculum [6,7,18]. Mulder *et al.* even reported in the first discovery that the occurrence of anaerobic ammonium oxidation was only observed after more than 400 days of operation [8]. In this study, the onset of ammonium reduction appeared after 200 days of granular sludge cultivation in the UASB reactor.

The nitrogen trends and percentage removal efficiencies before and after the observation of anammox activity are shown in Fig. 1. Between days 173~186, the effluent ammonium concentration dropped compared to its influent values. However, it later increased to almost same value of the influent concentration during the next 15 days. The decrease in effluent ammonium concentration on those days cannot be adequately explained, but it may result from unstable anammox reactions during adaptation periods. Strous *et al.* supposed that, after inoculation, the population of anaerobic ammonium-oxidizing bacteria grew, but a large portion of the other bacterial populations (not previously cultured with that type of substrate) were starved [7]. This could lead to a momentary increase in concentrations of organic nutrients (containing ammonium) and biofilm space, causing the transient growth of a heterotrophic, denitrifying bacterial population. When growth and starvation processes of the different populations of bacteria were not well attuned, sludge deterioration might have occurred. For other possible reasons, anammox bacteria could be inhibited by nitrite concentrations higher than 70 mg N/L [21], while nitrite accumulated in the reactor at approximately 80 mg NO_2^--N/L during such a period, Fig. 1B. This might inhibit anaerobic ammonium oxidation activity of enriched

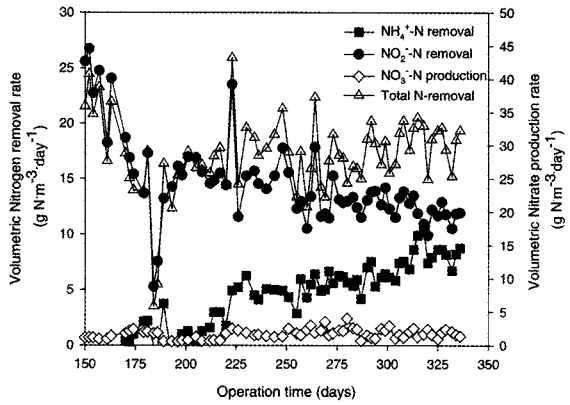
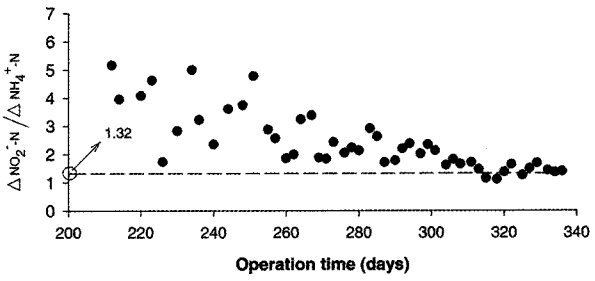


Fig. 2. Time course of the volumetric nitrogen removal rate in the UASB reactor before and after the appearance of anammox activity on day 200. The observed ammonium removal rate gradually increased and reached to 9 g NH_4^+-N·m^{-3}·day^{-1} at the end of the experiment.



△ indicates the difference between the concentrations of influent and effluent

Fig. 3. Ratio of consumed nitrite to consumed ammonium in the UASB reactor after the appearance of anammox activity on day 200. The ratios slowly decreased and were close to the stoichiometric ratio of the anammox reaction (1.32) during last month of the experiment.

anammox microorganisms. The temporary accumulation of nitrite could be explained by the decrease of facultative bacteria activity due to long-term operation under a high influent concentration of nitrite (150 mg/L), whereas a low C/N ratio was maintained [20]. After the observation of these phenomena, the influent composition was changed in terms of the reducing nitrite concentration, and the consequent values were 100 and 70 mg N/L, respectively, as shown in Fig. 1B.

After day 200, it became apparent that the ammonium concentration in the effluent was steadily decreasing. By the end of the experiment, approximately 60% of the influent ammonium was converted, as seen in Fig. 1A. For explaining this ammonium removal, the possibility of the occurrence of aerobic nitrification seemed unlikely because nitrite concentration had also decreased and a low nitrate concentration had been produced in the effluent, as shown in Figs. 1B and 1C. Additionally, the observed ammonium removal rate of 9 g NH_4^+-N·m^{-3}·day^{-1} (Fig. 2) would have required an oxygen supply corresponding to 40.5 g O_2·m^{-3}·day^{-1}. This was very unlikely in view of the precautions taken to limit oxygen diffusion to the reactor (see Materials and Methods section). Therefore, when these factors were considered, the results indicate that the ammonium conversion is most likely due to the anaerobic ammonium oxidation.

As shown in Figs. 1 and 3, after the anammox reaction was observed, the results obtained between 225 and 300 days of operation show that the ammonium and nitrite conversion were unlike the stoichiometric anammox reaction ratio (1:1.32) [13]. The electron donor for the uncoupled nitrite conversion might be a storage product or could be the biomass itself. It remains unclear which population is responsible for the ongoing nitrite conversion. It could be suggested that cell lysis was still occurring in the system under high temperature (33 ± 2°C). In previous study, Van Benthum *et al.* supposed that the large average diameter of the granules could be another negative factor affecting system stability, because granules can break due to the limitation of substrate by diffusion in the inner zones, causing the lysis of the cells inside the granule [23]. Additionally, working with a sequencing batch reactor (SBR) using anammox granule, Strous *et al.* postulated that 50% of the biomass was inactive (lysis) because of substrate limitations inside the granules [13]. The mean granule diameter in the SBR was reported to be 0.82 mm, lower than that measured in our study (0.8~3 mm). These results indicate that more inactive biomass was present in our study. A denitrifying population can use biomass as the electron donor and, consequently, can consume nitrite.

Interestingly, after steady disappearance of ammonium, the volumetric removal rate of ammonium gradually increased, while the nitrite value decreased in contrast, as shown in Fig. 2. This implied that the activity of facultative bacteria became trivial in the reactor system. Furthermore, a significant change was observed in the ratio of consumed NO_2^--N/NH_4^+-N (Fig. 3). The ratios obtained during last month of the operation were close to the stoichiometric ratio of the anammox reaction. This definitely indicates that the major nitrite removal mechanism had changed from classical denitritation to the anammox reaction.

To further our understanding of the occurrence of anaerobic ammonium oxidation inside the UASB reactor, the influent-shutdown test was performed at day 322. Trendlines of substrate consumption obtained during two days of monitoring without feeding are shown in Fig. 4. The results of this monitoring in terms of NO_3^--N production, pH, and alkalinity are also presented. As shown in this Fig. 4, the concentration of NH_4^+-N constantly decreased over 21 h. In this period, a decrease of NO_2^--N concomitantly occurred. During the last one day period, after the concentration of nitrite nitrogen was lower than 8 mg/L, no removal of ammonium was observed. Meanwhile, the nitrite trendline continuously dropped. No nitrite was detected after 33 h monitoring. The disappearance of nitrite is probably due to heterotrophic denitritation, even to insignificant levels, which could still occur in the system under high temperature, as discussed previously. It makes sense that anammox bacteria activity

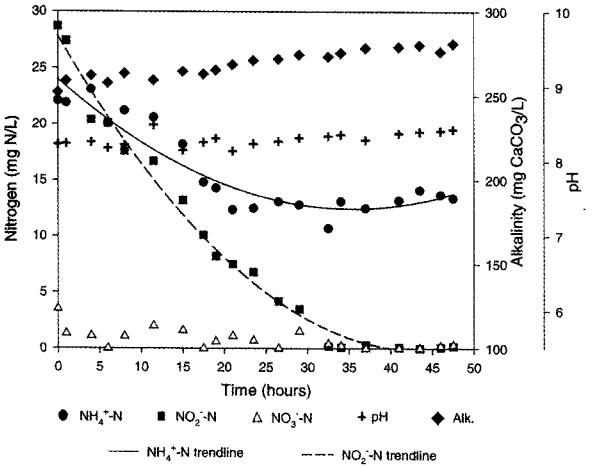


Fig. 4. Forty-eight hour monitoring of the UASB system, performed at day 322. After the feeding procedure, samples were taken every 1 to 4 h and analyzed immediately.

was depressed by or competed with the other heterotrophic bacteria under low nitrite concentration. Additionally, nitrate production was not detected during any of the test period. This result indicates that heterotrophic denitrifying bacteria simultaneously consume the nitrate produced by anammox bacteria. A slight change in terms of pH and alkalinity was observed in the reactor.

The granular sludge color gradually changed from black to a red-brownish during the study period. This phenomenon has been well documented in many previous studies on the enrichment culture system for the anammox process [6,14]. Cell samples were collected from the UASB reactor after ammonium removal, and the FISH technique was carried out to detect and characterize the anammox bacteria population existing in the reactor by the use of specific phylogenetic probes such as Amx820, Amx1240, Kst1273 [24]. It is interesting that two species of anammox bacteria were identified from granular sludge in the UASB reactor after long-term incubation. The FISH images were presented in a previous paper [20].

CONCLUSION

In this study, the anammox process for autotrophic nitrogen removal in a lab-scale UASB reactor was evalua ted. From the results reported here, it is apparent that the anammox process can be successfully developed u sing granular sludge selected from a digestion reactor for treating brewery wastewater. Although a lower rate of ammonium removal was observed, this study opened up a new opportunity for examination of the anammox biomass, which has been a big challenge in the development of anammox process in the past. The ratio of the converted NO_2^--N/NH_4^+-N obtained from the UASB reactor after the onset of anammox activity was close to the stoichiometric ratio of the anammox reaction. According to the results of influent-shutdown test, it makes sense that nitrite concentration seemed to be one key parameter for promoting the anammox reaction. In future work, the evaluation of specific activities of the enriched population should be investigated in various operating conditions in order to accelerate the growth rate of anammox bacteria in the system.

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