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Anammox Bacteria Enrichment in Upflow Anaerobic Sludge Blanket (UASB) Reactor

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Abstract We investigated the anaerobic ammonium oxidation (anammox) reaction in a lab-scale upflow anaerobic sludge blanket (UASB) reactor. Our aim was to detect and enrich the organisms responsible for the anammox reaction using a synthetic medium that contained low concentrations of substrates (ammonium and nitrite). The reactor was inoculated with granular sludge collected from a full-scale anaerobic digestor used for treating brewery wastewater. The experiment was performed during 260 days under conditions of constant ammonium concentration (50 mg NH_4^+-N/L) and different nitrite concentrations (50~150 mg NO_2-N/L). After 200 days, anammox activity was observed in the system. The microorganisms involved in this anammox reaction were identified as Candidatus *B. Anammoxidans* and *K. Stuttgartiensis* using fluorescence *in situ* hybridization (FISH) method.

Keywords: anaerobic ammonium oxidation (anammox), anaerobic granular sludge, UASB, ammonium, nitrite, fluorescence *in situ* hybridization (FISH)

INTRODUCTION

Due to stringent wastewater discharge limits for nitrogen compounds, it is essential to remove them to permissible levels. Biotreatment, being cost effective, is normally adopted for their removal from wastewater. Conventional biotreatment for ammonium removal involves nitrification and denitrification [1]. Biological nitrification is generally carried out by autotrophic nitrifying bacteria that oxidize ammonium to nitrite and nitrite to nitrate with molecular oxygen as an electron acceptor. Nitrite and nitrate are subsequently reduced to nitrogen gas by denitrifying bacteria, under anoxic conditions. However, this conventional biological nitrification/denitrification process is expensive and time-consuming. Nitrification demands a very efficient oxygen supply coupled with adjustment for changes in the alkalinity of the wastewater due to the formation of nitrate ions. Denitrifying bacteria essentially need a carbon source as an electron donor. These two requirements demand additional recurring expenditures. Therefore, it is necessary to develop a process for reducing oxygen and external carbon requirements for treating ammonium-rich wastewater with low C/N ratios. Wastewater of this type includes runoff from piggery wastewater, landfill leachate wastewater, and filtrate from anaerobic sludge digestion processes.

In 1977, Broda published a theoretical paper entitled 'Two kinds of lithotrophs missing in nature' describing the potential existence of chemolithotrophic bacteria able to oxidize ammonia to nitrogen gas with nitrate as the electron acceptor [2]. Over the past decade, it was observed that ammonium was disappearing from a denitrifying fluidized bed reactor treating effluent from a methanogenic reactor [3,4]. Ammonium conversion was associated with nitrate consumption and concomitant gas production. It was concluded that anaerobic ammonium oxidation was a new process in which ammonium was oxidized with nitrate serving as the electron acceptor under anaerobic conditions, producing dinitrogen gas. This biological process has been called as 'anammox' (*ana*erobic *amm*onium *ox*idation) process [4]. Experiments with ^{15}N-labelled NH_4^+ and unlabelled $^{14}NO_3^-$ were performed to confirm that the end product of the anammox reaction was dinitrogen gas. However, comparison of the labeling pattern of the $^{14,15}N_2$ product indicated that nitrite might be the preferred electron acceptor of the process rather than nitrate [5]. The anammox reaction is a complete autotrophic process and is catalyzed by at least new two autotrophic microorganisms of the order *Planctomycetales* [6,7]. The anammox bacteria grow autotrophycally with CO_2 as the only carbon source and the overall anammox process consists of the following reaction [7]:

Catabolic reaction: $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$ (1)

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Anabolic reaction: $CO_2 + 2NO_2^- + H_2O \rightarrow CH_2O$ (biomass) $+ 2NO_3^-$ (2)

Thus, NO_2^- is not only the electron acceptor in reaction (1) but also the electron donor for CO_2 fixation (2).

During the past decade, several publications have appeared showing the presence and activity of anammox bacteria in engineered systems, sediment, or enrichment cultures [5,8-12]. Discovering this group of anammox microorganisms opened new ways of nitrogen removal such as the combined Sharon/Anammox, CANON, and OLAND process [13-15]. However, process start-up could be hindered by the relatively low growth rate of anammox bacteria (doubling time; 11 days) that causes long incubation time [16]. Although some researchers have succeeded in detecting anammox microorganisms in biofilm systems [9,17], many others conducting studies on this process agree that the detection and enrichment of anammox bacteria still remain a big obstacle. It is therefore not surprising that these kinds of microorganisms are not abundantly found, especially since they cannot be cultivated using conventional microbiological techniques [6,18]. Considering earlier reports on anammox bacteria enrichment, it seems that the preferred niche conditions for the presence of anammox bacteria would be in treatment systems operated in combination with nitrification-denitrification process or those with long SRT. Reactors containing granular-sludge were considered to be suitable for enriching anammox bacteria because the anammox bacteria are distinguished as having low growth rates. Various bioreactors have been used for the enrichment of anammox microorganisms in different laboratories, including fluidized (or fixed) bed, SBR, and others [4,10,16]. Among these types of reactors, UASB reactors have been successfully used for development and retention of high concentrations of slow-growing, low- yield anaerobic microorganisms.

Base on these arguments, the objectives of this study are: (1) to assess the feasibility of detecting anammox bacteria in anaerobic granular sludge, (2) to experimentally enrich anammox bacteria in a UASB reactor with low substrate concentration, and (3) to characterize the enriched community by the use of FISH with known phylogenetic probes.

MATERIALS AND METHODS

Reactor Systems Set-up

A lab-scale upflow anaerobic sludge blanket (UASB) reactor with working volume of 6 L was used. The reactor was constructed from stainless steel and equipped with sampling ports that allowed the extraction of gas and liquid samples. In addition, the sidewalls were enclosed with a water jacket to maintain the reactor temperature at 35°C. Peristaltic pumps were used to control recirculation rate and influent feed rate to reactor. A recycle was applied to dilute the influent, because high nitrite concentrations could be toxic to anammox bacteria, resulting in a recycle ratio of about 3Q during the overall operating period. Fig. 1 shows a schematic diagram of the UASB reactor. All tubing and connectors were of butyl rubber and PVC to limit oxygen diffusion into the system.

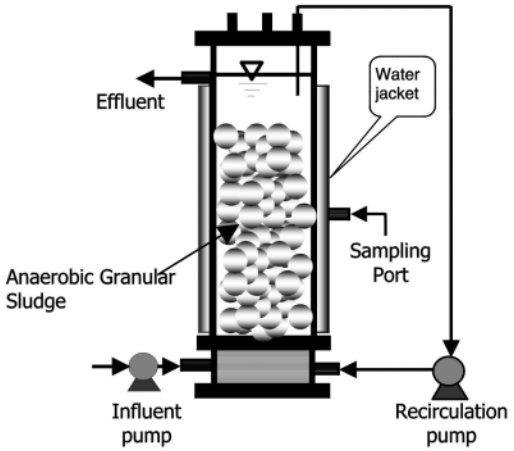

Fig.1. Schematic diagram of lab-scale upflow anaerobic sludge blanket (UASB) reactor for the start-up of anammox process.

The reactor was seeded with 4.5 L of anaerobic granular sludge taken from a full-scale UASB reactor used for treating brewery wastewater, which was operated at 30 ± 2°C and neutral pH. As mentioned in the introduction section, the use of this sludge increased the possibility of the presence of anammox bacteria. Granular sludge from this reactor was characterized by 18.6 g VSS/L (65% VSS/TS) and was black in color. After seeding it to the reactor, the lid was covered completely in order to avoid oxygen diffusion.

Throughout the whole experiment, the hydraulic retention time (HRT) was in the range of 1, 5, and 3.5 days. The operation time was also divided into various periods depending on the nitrite concentration of influent and the addition of acetic acid, respectively.

Synthetic Wastewater

Mineral medium was composed as described in Table 1. Synthetic wastewater contained mainly nitrite and ammonium to support anammox activity. Bicarbonate was added to this medium as an inorganic carbon source for cell growth. The influent pH was not adjusted. Oxygen intrusion *via* the influent was not completely prevented but the oxidation-reduction potential (ORP) was regulated to 215 ± 55 mV (Ag/AgCl reference) as a result of approximately 15 min of purging with N_2. Acetic acid was added between days 76 and 220 in order to achieve COD_{cr}/NO_2^--N = 1.

Analysis

Concentrations nitrogen compounds were measured two or three times a week in the influent, the effluent, and in the reactor itself. Samples were prepared by filtering through 0.45 μm of filter paper (GF/C-Whatman®).

Table 1. Composition of the synthetic wastewater used in this study period

Component	Unit	Value
• pH	-	7.8-8.5
• $(NH_4)_2SO_4$	mg/L	235.75
• $NaNO_2$	mg/L	246.5~739.5
• $KHCO_3$	mg/L	500
• KH_2PO_4	mg/L	27.2
• $CaCl_2 \cdot 2H_2O$	mg/L	180
• $MgSO_4 \cdot 7H_2O$	mg/L	120
• Fe solution (*)	mL/L	1
• Trace metal solution (*)	mL/L	1

(*) U. Imajo *et al.*, 2001 [8]

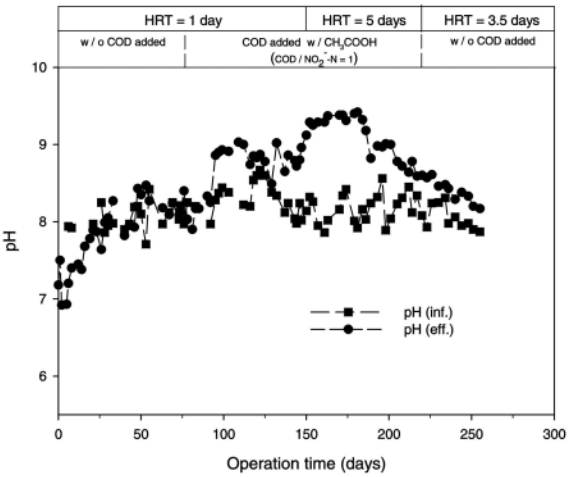

Fig. 2. Variations in pH in the influent and effluent of UASB reactor.

Table 2. 16S rRNA targeted oligonucleotide probes used for fluorescence *in situ* hybridization

Probe	Sequence (5′ → 3′)	Formamide concentration	Target organisms
Amx820	AAAACCCCTCTACTTAGTGCCC	25%	All anammox bacteria
Amx1240	TTTAGCATCCCTTGTACCAACC	60%	Candidatus *B. Anammoxidans*
Kst1273	TCGGCTTTATAGGTTTCGCA	25%	Candidatus *K. Stuttgartiensis*

Ammonium was measured by selective electrode. Nitrite and nitrate content was determined by ion-chromatography (Dionex, DX-120 Ion-Chromatography). COD_{Cr} was analyzed using a closed reflux method. Detecting of conventional and other parameters of interest such as pH, ORP, and alkalinity were also performed in accordance with the Standard Methods for the Examination of Water and Wastewater [19].

In situ Hybridization

Cell samples were fixed immediately by adding three volumes of 4% paraformaldehyde (in phosphate-buffered saline, or PBS). Samples were then mixed and incubated for 2~3 h on ice. FISH analysis was carried out as described in [9] and the used probes were shown in Table 2.

RESULTS AND DISCUSSION

During the whole experiment, the UASB reactor was operated without pH control and the influent pH was kept at 7.8~8.5 by using tap water for preparing the synthetic wastewater. Fig. 2 represents the pH profiles in both influent and effluent. Effluent pH was around 7.2 at initial operation time. This was because the reactor was seeded with anaerobic granular sludge taken from a full-scale UASB reactor, which was operated at neutral pH. During the first 90 days, the effluent pH gradually increased and remained at nearly the same value as the influent. But effluent pH continuously increased to around 8.8 for the next 50 days of operation time and reached up to 9.4 during the next 30 days after day 50. During these operation periods, the reactor was fed by influent containing various nitrite nitrogen concentrations between 50~150 mg/L and acetic acid (COD/NO_2^-- N =1) in order to restore the activity of facultative bacteria existing in granular sludge. Those increases of pH could be explained by conventional denitrification occurring in the UASB reactor, where nitrogen oxides were reduced to nitrogen gas.

According to the first anammox process study [3,4], A. Mulder *et al*. indicated that effluent pH decreased after ammonium consumption was detected. Lately, Strous *et al*. supposed that the pH in an anammox reactor might be greater than influent pH because bicarbonate (HCO_3^-) serves as the sole carbon source [18]. In this research, when the reduction of ammonium was observed in the reactor 200 days (Fig. 4), the pH of the effluent also decreased. Interestingly, at that time, even when the reactor was operated under the same feeding conditions (influent nitrite concentration, HRT and with adding of acetic acid), the decrease in pH was still observed. During overall experiment, it is evident that conventional denitrification (denitritation) and anammox reactions were simultaneously occurred in the reactor. The increase of pH in the effluent is essentially explained by the production of alkalinity due to denitritation reaction. As the anam-

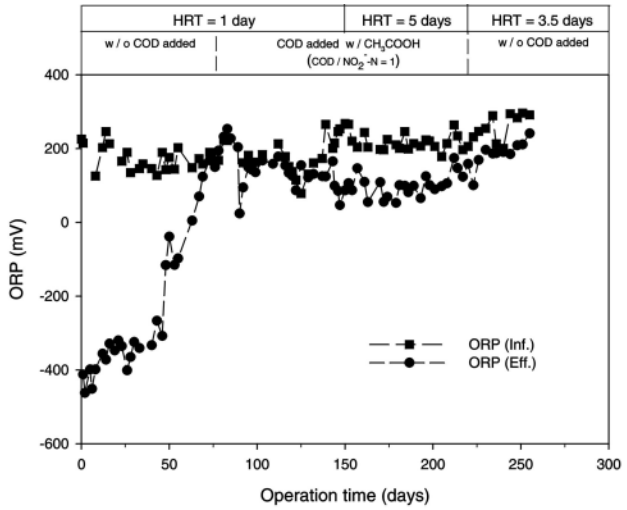

Fig. 3. Variations in ORP in the influent and effluent of UASB reactor.

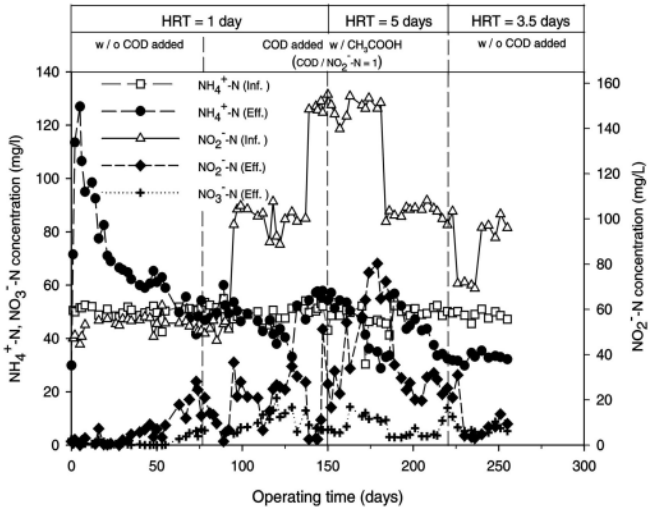

Fig. 4. Variations in nitrogen concentrations in the influent and effluent of UASB reactor.

mox reaction become dominant, the decline of nitrite source for facultative bacteria would be occurred and result in the decrease of denitritation rate. Furthermore, A. Mulder indicated that the decrease of pH was occurred when H^+ was formed by the ammonium ion oxidation [3]. Even if effluent pH was still 0.2~0.4 units higher than influent pH during the latter period of the experiment, effluent pH is predicted to continuously decrease and reach almost the same value as influent pH.

Variations in oxidation-reduction potential (ORP) were also measured and are shown in Fig. 3. During the first 50 days, ORP in the effluent showed very low values (from -450~-100 mV). In this period, we expected that cell lysis could be occurring in the reactor and organic compounds released from cells would be used as carbon sources, even if acetic acid was not added as a carbon source for facultative bacteria. It was also expected that facultative bacteria attached to granular sludge would use carbon sources from cell decay as electron donors to reduce all oxygen (free and boundary oxygen) to obtain their growth energy [20]. As shown in Fig. 3, the effluent ORP plot sharply increased and reached up to almost its influent values after 70 days of operation. As anoxic or anaerobic conditions were essential for detecting and enriching anammox bacteria, HRT was adjusted as 5 days and acetic acid equivalent to 150 mg COD/L (COD/NO_2^--N =1) was added to influent. This caused effluent ORP to decrease slightly to about 95 mV. During the last 45 days, ORP of effluent increased again while no acetic acid was added into the reactor. This result indicated that the addition of organic compound is a promising method for maintaining the activity of facultative bacteria in decreasing oxygen state in reactor. It still requires a better understanding and further study of the conditions under which organic compounds are added: C/N ratio, period of addition, *etc*.

Strous *et al*. [16] indicated that the anammox bacteria consume ammonium and nitrite in a ratio 1:1.3. In this study, we envisioned that a simultaneous denitrification process is unavoidable, even if the main purpose of the addition of organic compound is to consume free oxygen in the system. So the ammonium/nitrite ratio in the synthetic wastewater should be higher than its value for the anammox reaction. It is also expected that excess added nitrite will be left over due to incomplete denitrification (low C/N ratio) and will consequently be used by anammox bacteria.

A constant amount of ammonium (50 mg NH_4^+-N/L) and various nitrite compositions (50~150 mg NO_2^-- N/L) was fed to the UASB reactor during the entire experiment. Fig. 4 shows the nitrogen concentration in both influent and effluent. During the initial period, maximum ammonium production was achieved at 130 mg/L. After seven days, the effluent ammonium concentration gradually decreased and was virtually equal to its influent concentration by day 60. However, this decrease was not proportional to that due to the effects of dilution rate when using low influent ammonium concentration. It proved that the production of ammonium was still occurring in the reactor during this period. Experiments by Imajo *et al*. showed that ammonium was produced due to cell decay and starvation during adaptation time [8]. Akunna *et al*. quoted from other studies that dissimilatory nitrogen oxides to ammonium is the major nitrogen oxide reduction pathway in anaerobic digesters, because of the abundance of facultative and obligate anaerobes (ammonium formers) [20,21]. It was also found that ammonium production was higher in nitrite cultures than in nitrate cultures.

In the second period (60~200 days), the ammonium concentration in the effluent of the UASB reactor was almost the same as in the influent. It demonstrated that the production of ammonium that might be due to the decay of cells in the reactor was trivial. The effluent ammonium concentration dropped somewhat on day 127 and between days 173~186. By the last period (the last 55 days), however, it became apparent that the ammo-

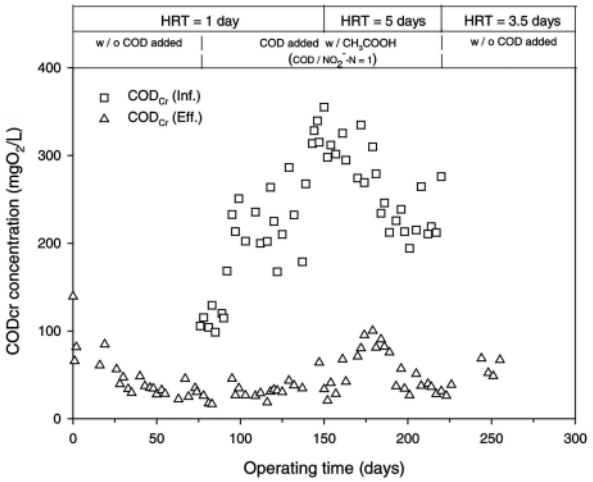

Fig. 5. Variations in COD_{Cr} concentration in the influent and effluent of UASB reactor.

nium concentration in the effluent had decreased and the ammonium reduction was steadily observed in the system. The possibility of the occurrence of aerobic nitrification resulting in nitrite or nitrate production seemed unlikely because nitrite concentration in the effluent had also decreased and nitrate showed low concentrations (Fig. 4). The nitrate concentration in the effluent was about 8 mg/L when the ammonium removal was observed as 15~20 mg/L. Consequently, it asserted that the removal of ammonium did not bring about a high production of nitrate in the whole experiment.

Variations in nitrite concentrations are also shown in Fig. 4. During the initial period, cell decay was the major reaction in the reactor, since nitrite could be removed by conventional denitrification. This process is usually carried out by facultative bacteria who use carbon from cell decay as an electron donor. On the other hand, as mentioned above, nitrite may be reduced to ammonium *via* dissimilatory pathways by abundant obligate anaerobes

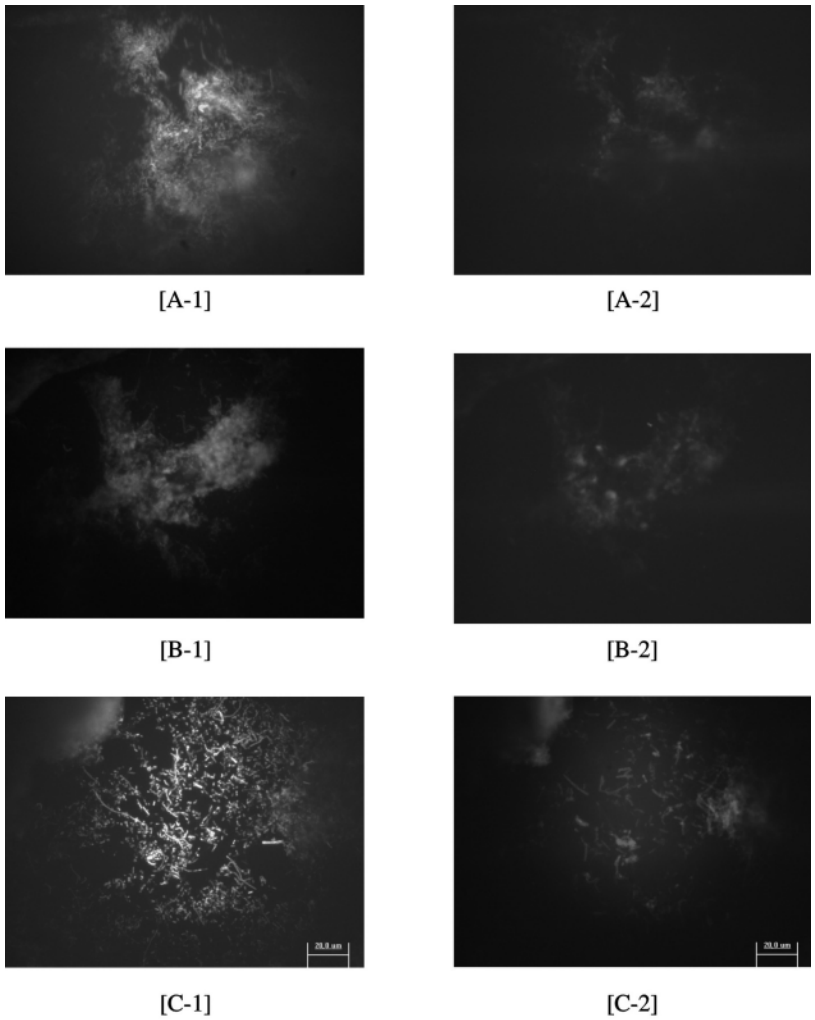

Fig. 6. FISH images of the granular sludge in UASB reactor. [A-1]; Amx 820 (DAPI), [A-2]; Amx 820 (Cy3), [B-1]; Amx 1240 (DAPI), [B-2]; Amx 1240 (Cy3), [C-1]; Kst 1273 (DAPI), [C-2]; Kst 1273 (Cy3).

(ammonium formers). These reasons can also be used to explain the low nitrite concentration in the effluent during first 60 days. The nitrite removal efficiency gradually decreased to about 50% after 75 days of operation (without adding acetic acid). In the next period, nitrite was still removed through denitrification by the addition of organic matter, but excess nitrite remained because of incomplete denitrification (low C/N). This excess nitrite remaining in the reactor could be used by anammox bacteria. After the anammox reaction was observed, the ammonium and nitrite concentration in the effluent concurrently decreased. But the results as shown in Fig. 4 indicate that ammonium and nitrite conversion were uncoupled even without the addition of external organic carbon, as it should be about 1:1.32 [14]. The electron donor for the uncoupled nitrite conversion might be a storage product or 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 (35°C). A denitrifying population could use biomass as the electron donor while a proportional fraction of ammonium from this cell lysis was removed by anammox reaction.

Fig. 5 describes variations in COD_{Cr} concentration in both influent and effluent. In this data, plots of the influent only showed changes when acetic acid was added, but the effluent plots showed changes throughout the whole experiment. The data on influent COD concentrations was higher than the calculated values since nitrite exerts a COD of 1.1 mg O_2/mg NO_2-N. This property should also be applied to the effluent COD concentration. At the beginning of the experiment, almost all COD concentration values can be attributed to organic compounds because nitrite concentration is being eligible. Considering the effluent concentration of nitrite in Fig. 4 and the results from Fig. 5, it seemed that acetic acid was used almost exclusively as the electron donor for reduction of nitrite to dinitrogen gas by conventional denitrification.

Cell samples were collected from the UASB reactor after ammonium removal occurred and FISH technique was carried out to detect the anammox bacteria population existing in the reactor. The cells were stained with known phylogenetic probes such as Amx820, Amx1240, and Kst1273. In a recent study, the ISRs (intergenic spacer region) of *Brocadia anammoxidans* and *Kuenenia stuttgartiensis* were sequenced and, subsequently, probes for the *in situ* detection of these ISRs were constructed [22]. As shown in Fig. 6, both *Brocadia anammoxidans* and *Kuenenia stuttgartiensis* were detected in UASB reactor. FISH with specific probes (Kst 1273) for *Kuenenia stuttgartiensis* demonstrated that these anammox bacteria dominated the microbial biofilm communities of the investigated plants [23]. Recent studies have indicated that *K. stuttgartiensis* is in many ways very similar to *B. anammoxidans* [9]. It is interesting that both anammox organisms were detected together in a UASB reactor in this experiment. Future research will be necessary to evaluate the differences in specific activity and nitrite tolerance of the two organisms.

CONCLUSION

The results of this work indicate that anammox bacteria can be successfully detected and enriched from anaerobic granular sludge even with low substrate concentrations using UASB reactors. Discovery of the anammox biomass may be the key to the development of anammox processes. Using the fluorescence *in situ* hybridization technique, it has been demonstrated that anammox cells from the sludge are related to Candidatus *B. Anammoxidans* and *K. Stuttgartiensis*.

However, the use of anaerobic granular sludge has one disadvantage in that the cells decay and ammonium formation from the dissimilatory of nitrogen oxides were major reactions in the reactor during the initial start-up period. Even if a small number of anammox microorganisms existed in source sludge, their activities in ammonium and nitrite removal could be completely masked due to ammonium production by decay and anaerobic reduction of nitrite. Therefore, to detect the ammonium removal by anammox bacteria, it was required to wait until ammonium production through cell lysis became negligible. To minimize this period, we suggest that the reactor should be operated with a high (sufficient) concentration of nitrate as an electron acceptor substance from the initial period until there is no detected ammonium formation. This is because the use of nitrate instead of nitrite or sulphate can prevent the inhibition of high concentrations of nitrite or prevent sulphate reduction.

Further studies on how to accelerate reaction rate and growth rate of anammox organisms should be investigated.

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