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Effect of salt concentration in anammox treatment using non woven biomass carrier

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Effect of high salt concentration on the anammox treatment was investigated to establish an acclimation strategy under high salt concentration conditions. An anammox fixed-bed reactor with non-woven biomass carrier was used and the salt concentration was gradually increased from 2.5 g L^{-1} to 33 g L^{-1}. The anammox reactor demonstrated stable nitrogen removal rate (NRR) of 1.7 kg-N m^{-3} d^{-1} for 65 days under a salt concentration of 30 g L^{-1}. However, the NRR sharply declined at a salt concentration of greater than 30 g L^{-1}. The bacterial community was examined by 16S rRNA gene analysis and DGGE after the acclimation of the anammox sludge to high salt conditions. Although the salt concentration was almost sea level, the freshwater anammox bacteria, KU2, were detected. In addition, the unidentified bacteria which perhaps belong to candidate division OP10 and *Lysobacter* sp. were found to coexist with anammox bacteria at a salt concentration of 30 g L^{-1}.

[**Key words:** Anammox; Salt concentration; Nitrogen removal; Biofilm reactor; Fixed-bed reactor]

Nitrification–denitrification process has been widely used for nitrogen removal from wastewater. However, not only a large amount of oxygen for nitrification and extra carbon sources (i.e., methanol) for denitrification are required but considerable amount of extra sludge is also produced. In order to overcome these factors, attempts have been made to develop alternate techniques. One of the processes recently proposed was the partial nitritation–anammox process that was shown to have many potential advantages over the conventional nitrogen removal process (1). In this process, 60% of the amount of ammonia is converted to nitrite in the aerobic partial nitritation treatment and then anammox bacteria oxidize ammonia to nitrogen gas using nitrite as an electron accepter under anoxic conditions, with their growth occurring by carbon dioxide fixation (2). For these reasons, the amounts of oxygen supply, extra carbon source, and extra sludge production can be reduced in the partial nitritation–anammox process (1).

Since partial nitritation–anammox process was successfully applied to the treatment of sewage sludge digester liquor in the Netherlands (3), it opened doors for application to many kinds of wastewater treatment such as industrial wastewater, livestock wastewater, and landfill leachate. However, these wastewaters contain high concentrations of salts which have been considered as an inhibition factor in biological nitrogen removal process. Thus, effect of high salt concentration on nitrification and denitrification has been previously investigated (4–6). It was reported in these studies that nitrification and denitrification activities were sustained by gradual acclimation of freshwater sludge to high salt conditions. Halophilic denitrifying bacteria were isolated from the long-term acclimated sludge, and higher denitrification performances were demonstrated when the long-term acclimated sludge was used as inoculum (7). Furthermore, Furukawa et al. (8) reported that nitrifying sludge taken from night soil treatment plant employing a sea-water dilution in summer season could adapt more smoothly to high salt condition than the case of freshwater sludge. In other studies, marine anammox bacteria belonging to *Scalindua* genus have been detected in natural surroundings (9–11) and very recently Nakajima et al. (12) enriched them from an enclosed coastal sea in Japan using a continuous culture system. These results suggested that the anammox bacteria inherently preferring to the culture containing high concentration of salts and living in the high salt habitats would be accumulated in the cultivations and available for industrial application. In other hand, there is an inconsistent experiment. Kartal et al. (13) adapted the anammox sludge which consisted of 50% of *Candidatus* Kuenenia stuttgartiensis and 50% of *Candidatus* Scalindua wagneri to the salt concentration of 30 g L^{-1}. Although it would be the culture condition suitable to the growth of *Candidatus* Scalindua. They reported that the major anammox bacteria after the acclimation were *Candidatus* Kuenenia stuttgartiensis enriched from freshwater condition. Because Kartal et al. (13) used the seed sludge containing marine anammox bacteria besides freshwater anammox bacterium, the result that major anammox bacteria at high salt condition were freshwater anammox specie is open to question. In addition, Kartal et al. (13) focused on only the population of anammox bacterium species without the evaluation of coexistent bacteria community.

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In this study, the effect of high concentration of sodium chloride on anammox treatment was investigated. We used the anammox fixed-bed reactor using non-woven biomass carrier with a seed containing only freshwater anammox bacteria, strain KU2 and KSU-1, differing from those used by Kartal et al (13). The salt concentration was increased stepwise from 2.5 g L^{-1} to 33 g L^{-1}. In addition, the bacterial community was also examined by 16S rRNA gene analysis after the acclimation of the anammox sludge to high salt condition.

MATERIALS AND METHODS

Set up and operational conditions of anammox reactor An up-flow fixed-bed column reactor with an inner diameter of 95 mm, and a height of 400 mm (an effective volume of 2.8 L) was used in this study as shown in Fig. 1. A porous polyester non-woven fabric carrier (1.0 L, Japan Vilene, Tokyo, Japan) was used as support material at a packing ratio of 35.7%. The reactor was inoculated with 1.0 g-volatile suspended solids (VSS) of freshwater anammox sludge, which was taken from another anammox reactor treating synthetic wastewater without salt addition (Qiao, S., Ph.D. thesis, Kumamoto University, Kumamoto, 2007). The reactor temperature was maintained at 25 °C during the entire operational period. The pH of the reactor was not adjusted, and the pH of influent and effluent were around 7.6 and 7.7, respectively. The composition of the synthetic wastewater used in this study is shown in Table 1. The synthetic wastewater was flushed with nitrogen gas to decrease the dissolved oxygen (DO) concentration below 1.0 mg L^{-1}. The hydraulic retention time (HRT) was varied from 36 h to 1.7 h depending on the nitrogen loading rate (NLR). The reactor was fed with synthetic wastewater without salt addition until the nitrogen removal rate (NRR) increased to 1.6 kg-N m^{-3} d^{-1} (day 102). After that, the NLR was maintained at 2.2 kg-N m^{-3} d^{-1} and the influent salt concentration was increased stepwise from 2.5 g L^{-1} to 33 g L^{-1}.

Analytical methods The concentrations of NO_2-N and NO_3-N were measured by the colorimetric method in accordance with the Standard Methods (14). NH_4-N concentration was measured by the modified phenate method using ortho-phenyl phenol (OPP) (15). Absorbance and pH values were measured using a spectrophotometer (U-2010; Hitachi, Tokyo, Japan) and a pH meter (B-211; Horiba, Kyoto, Japan), respectively.

DNA extraction and PCR amplification A sludge sample was taken from the reactor after its acclimation to high salt concentration (day 320). The sample was suspended in 1 mL of TE buffer with 1 μL of Ready-Lyse Lysozyme Solution (Epicentre, Madison, WI, USA) and incubated at 37 °C for 1 h. The solution was added with 1.5 mg of achromopeptidase (Wako, Osaka, Japan) and incubated at 37 °C for 30 min. Then, bacteriolysis was performed by addition of 100 μL of 10% sodium dodecyl sulfate solution. Proteins in the supernatant prepared by centrifugation were decomposed with Proteinase K (Wako, Osaka, Japan) treatment. The supernatant was prepared by centrifugation and meta-genomic DNA in it was purified by phenol/chloroform extraction. The DNA was recovered by ethanol. The meta-genomic DNA was dissolved in TE buffer, treated with RNase A, precipitated by ethanol with 13% PEG8000-1.6 M NaCl, and dissolved again in TE buffer. The amplification of 16S rRNA gene was performed with KOD-plus-DNA polymerase (TOYOBO, Osaka, Japan) using eubacterial primers 6F (forward primer: 5′-GGAGAGTTAGATCTTGGCTCAG-3′) and 1492R (reverse primer: 5′-GGTTACCTTGTTACGACT-3′) (16, 17). PCR was carried out according to the following thermocycling parameters: 2 min initial denaturation at 94 °C, 25 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 68 °C. The amplified products were electrophoresed on a 1% agarose gel and the DNAs in excided agarose gel were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

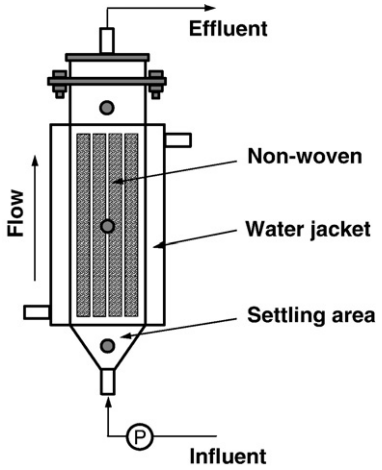

FIG. 1. Schematic diagram of fixed bed anammox reactor.

TABLE 1. Composition of synthetic wastewater

Composition	Concentration
$(NH_4)_2SO_4$	75 mg-N L^{-1}
$NaNO_2$	75 mg-N L^{-1}
$KHCO_3$	125 mg L^{-1}
KH_2PO_4	54 mg L^{-1}
T. element	0.5 mL L^{-1}
NaCl	0–33.0 g L^{-1}

T. element: $FeSO_4 \cdot 7H_2O$, 18 g L^{-1}; EDTA · 2Na, 10 g L^{-1}.

Cloning and sequencing of 16S rRNA The purified fragments were ligated into the Hinc II site of pBluescript II KS+ (Stratagene, La Jolla, CA, USA). *E. coli* DH10B was transformed using the constructed plasmids. The plasmids were extracted by the alkaline method from 33 clones carrying them. The nucleotide sequences of inserted DNA in them were determined with 3130*xl* genetic analyzer and BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences determined in this study were compared with those in nr database by the basic local alignment search tool (BLAST) program on the NCBI web site.

Denaturing gradient gel electrophoresis (DGGE) Partial 16S rRNA gene was amplified by PCR using a eubacterial primer set, 357F (forward primer: 5′-CCTACGGGAGG-CAGCAG-3′) and 534R (reverse primer: 5′-ATTACCGCGGCTGCTGG-3′) (18), and the extracted meta-genomic DNA as template. The amplified fragments were purified and added with the GC-clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) at the 5′ termini by second PCR using a primer set, 357F with GC-clamp and 534R. The products were resolved by DGGE for 16 h at 100 V at 60 °C using DCode system (Bio-Rad, Hercules, CA, USA). An 8% acrylamide gel with a 30-to-65% denaturing gradient was used, where 100% denaturant was defined as 7 M urea and 40% formamide. The gel was stained with SYBR-Gold solution (Invitrogen, Carlsbad, CA, USA), and visualized using FLA-2000 system (Fuji Photo Film, Tokyo, Japan). Five bands were excised from the gel to determine the sequences. The gel piece was crushed with disposable polypropylene pestles and soaked in the DNA extraction buffer (500 mM sodium acetate (pH 5.2), 1 mM EDTA). After overnight incubation at 4 °C, the mixture was centrifuged and the supernatant containing the extracted DNA was transferred into a new tube. The DNA was ethanol precipitated, dissolved in TE buffer and amplified by PCR with a primer set, 357F and 534R. The amplified fragment was directly sequenced.

Nucleotide sequence accession numbers The partial 16S rRNA gene sequences of Operational taxonomic unit (OTU) 1, OTU 2, OTU 3, OTU 4, OTU 5, OTU 6, and OTU 7 were submitted to the DDBJ database under accession numbers AB434253-AB434256, AB434257, AB434258, AB434259, AB434260, AB434261, and AB434262, respectively.

RESULTS AND DISCUSSION

Reactor performance under salt conditions After achieving a NRR of 1.6 kg-N m^{-3} d^{-1} (day 102) without salt addition, the NLR was maintained at 2.2 kg-N m^{-3} d^{-1} and salt addition was initiated. Fig. 2 shows the time courses of nitrogen concentrations under various salt concentrations. When the salt concentration was set at 5 g L^{-1}, the effluent NH_4^+ and NO_2^- concentrations increased. However, the effluent NH_4^+ and NO_2^- concentrations decreased by decreasing the salt concentration from 5 g L^{-1} to 2.5 g L^{-1}. Thereafter, although the salt concentration was increased to 5 g L^{-1}, the effluent NH_4^+ and NO_2^- concentrations did not increase. The salt concentration was then increased stepwise to 30 g L^{-1}. During this period, the effluent NH_4^+ and NO_2^- concentrations remained constant demonstrating successful performance of anammox treatment under high salt conditions. The anammox treatment was successfully maintained at the salt concentration of 30 g L^{-1} between days 195 and 203. However, when the salt concentration was further increased above 30 g L^{-1}, the effluent NH_4^+ and NO_2^- concentrations increased. Consequently, the salt concentration was decreased to 28 g L^{-1} until the efficiency of anammox treatment was recovered. After recovery, the salt concentration was again increased to 30 g L^{-1}. As shown in Fig. 2 the anammox treatment then became stable at the salt concentration of 30 g L^{-1} and the NRR of 1.7 kg-N m^{-3} d^{-1} was achieved for the ensuing 65 days (days 272–337).