(19) PRESENCE OF ANAMMOX PROCESS IN LOW AMMONIUM-FED SOLID-PHASE DENITRIFICATION BIOREACTOR

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Presence of anaerobic ammonium oxidation (anammox) process was investigated in low ammonium-fed solid-phase denitrification bioreactor. A promising application of solid substrates for denitrification has been appeared since last decade and this type of denitrification here termed as solid-phase denitrification. Bioreactor was operated for 300 days in a temperature-controlled incubator. An immobilized biomass of activated sludge and anaerobic granules was used as inocula in the bioreactor and solid biodegradable plastic as a carbon source. Nitrogen mass balance showed that the average nitrogen removal efficiency in the bioreactor was 80%. The combined results of nutrient profiles, ^{15}N-labelling techniques and qualitative fluorescence in situ hybridization (FISH) probe confirmed that anammox bacteria were active in the bioreactor. This clearly demonstrates that both anammox and denitrification processes work symbiotically in low ammonium concentrations under the support of solid substrates. It revealed that solid substrates not only serve as constant sources of reducing power for denitrification but also creates the favourable condition for anammox process by utilizing dissolved oxygen during the degradation of solid substrates.

Key Words : *Anammox, FISH, immobilized biomass, solid-phase denitrification and stable isotopes.*

1. INTRODUCTION

Freshwater often receives increased downward fluxes of nitrogen from agricultural runoff and points sources and upward fluxes from sediments by degradation of organic matters. Several possible processes such as simultaneous nitrification and denitrification[1)], anaerobic oxidation of ammonium (ANAMMOX)[2)], SHARON[3)], and completely autotrophic nitrogen removal over nitrite (CANON) [4)] have been employed to reduce the nitrogen concentration. However, some of these technologies are still uneconomical and have many demerits. Indeed, ANAMMOX process is promising and has been extensively used in ammonium-rich wastewater [5,6,7)]. However, applications of the anammox process in freshwater/drinking water have been still limited [8)]. Moreover, anammox process converts 10% of the influent ammonium nitrogen into nitrate nitrogen[9)] and thus post denitrification is required to eliminate the nitrogen completly. A promising application of solid substrates for denitrification has been appeared since last decade and this type of denitrification here termed as solid-phase denitrification. Therefore, the aim of this paper was to examine the presence of anammox processes in low ammonia-fed solid-phase denitrification single bioreactor.

2. MATERIALS AND METHODS

(1) Biopellet fabrication

Biomass from the activated sludge of a domestic wastewater treatment plant and anaerobic granules from full-scale upflow anaerobic sludge blanket (UASB) reactors treating brewery wastewater in Japan were collected separately. Then the collected activated sludge was entrapped in polyethylene glycol (PEG) prepolymer gel as biopellets [10)]. The composition of the immobilized material was 10%

(w/v) PEG prepolymer and 2% (w/v) activated sludge. The size of the cubic biopellets was 3 mm and was found to be stable under various hydraulic conditions [10].

(2) Bioreactor setup and operation

Upflow bioreactor had an effective volume of 100 ml was designed (Fig. 1). The bioreactor was inoculated with a mixture of biopellets and anaerobic granules to 20% of the total volume. A 20 g aliquot of poly-caprolactone $(C_6H_{10}O_2)n$ (solid biodegradable plastic produced by Nishi Nihon Company Japan) equivalent to 30% of the volume of the bioreactor was supplied as an additional carbon source. The shape of poly-caprolactone was hollow cylindirical mesh. Water samples with a total nitrogen content of 1.47 ± 1.00 mg/l (>95% in the form of nitrate nitrogen) were retrieved from Shiokawa Reservoir (Yamanashi, Japan). Ammonium nitrogen and nitrite nitrogen (concentrations reported in the 'Results and Discussion' section below) were added to understand the nitrification and denitrification processes more precisely under oxygen limiting conditions. The modified water was supplied to all the bioreactors continuously using peristaltic pumps without recirculation. All experiments were performed for 300 days in temperature-controlled bioreactor (Eyelatron FL1-301NH) and the pH in all bioreactor was 7.0 ± 0.5. The inflow rate was 200 ml/day; the hydraulic retention time was 12 hours and dissolved oxygen (DO) in the influents ranged from 0.5 to 5.0 mg/l.

(3) Identification of nitrogen removal pathway

Nutrient profiles, ^{15}N-labelling techniques and fluorescently labelled rRNA probes were used to identify the nitrogen removal pathway as described by Schmid et al. [11].

a) Nutrient analysis

Grab samples of influents and effluents were collected twice a week from the bioreactor for chemical analyses. Samples were filtered using Whatman GF/F filters (0.45 μm) and ammonium nitrogen (NH_4^+–N), nitrate nitrogen (NO_3^-–N) and nitrite nitrogen (NO_2^-–N) concentrations were measured colorimetrically as described in APWA [12]. Nitrification performance was calculated based on the change in ammonium concentration in the influents and effluents with respect to the initial NH_4^+–N concentration. Summation of the differences in NO_3^-–N and NO_2^-–N in the influents and effluents was used to determine the denitrification. The nitrogen removal efficiency was calculated as (Nin–Nout), where Nout and Nin are the nitrogen concentrations (mg/l) in the effluent and influent respectively. The nitrogen removal rate (g-N/m^3.day) was calculated as the daily nitrogen load removed. [daily flow rate × (Nin–Nout)] per unit volume of reactors. Dissolved organic carbon (DOC) concentration in the effluent was measured using a total organic carbon analyser (Shimadzu - TOC-VCSH). Available DOC inside the reactors and effluent DOC could be different, however, at equilibrium, it is expected that effluent DOC and available DOC would be nearly the same and comparable.

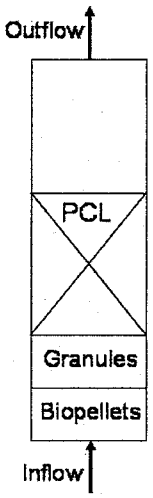

Fig.1 Schematic setup of an upflow bioreactor, PCL indicates the poly-caprolactone

b) ^{15}N-labelling techniques

To identify the nitrogen removal pathway in the bioreactor, influent samples retrieved from the reservoirs were modified using 99% $^{15}NO_2^-$–N (Cambridge Isotope Laboratories, USA) and native NH_4^+–N in equal concentrations. The modified water was applied to the bioreactor for t = 266–296 days. The gas produced in the bioreactor was collected using a syringe and transferred to a bottle fully containing saturated NaCl. Saturated NaCl solution prevents contamination of samples by atmospheric gases. The bottle was sealed and kept in an inverted position in a refrigerator until delivery to the laboratory at Shoko Co. Ltd (Saitama, Japan). The concentrations of $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ were determined using a Hitachi RM1-2 mass spectrometer and atmospheric air was used as standard. The experiments were repeated three times.

c) Microbial analysis using florescence in situ hybridization (FISH)

Granules as well as biopellets samples were collected at t = 300 days. The oligonucleotide probes, their target groups, formamide concentrations of the hybridization buffer and relevant references used in this study are given in Table 1. The combination of all EUB338 probes was mixed in equal proportion with ARC915, Amx820, and Pla46 separately. All of the probes were complementary to regions of

Table 1 Oligonucleotide probes for the detection of anammox and other organisms.

Probes	Specificity	Formamide (%)	Ref.
AMX820	Anammox bacteria	40	15
Pla46	Planctomycete	25	16
EUB338	General Bacteria	35	17
EUB338II	Bacterial lineages not covered by probes EUB338	35	17
EUBIII	Bacterial lineages not covered by probes EUB338 and EUB338II	35	17
ARC915	Methanogens	20	18

the small subunit of 16s rRNA molecules and were conjugated with the fluorescent dyes, fluorescein isothiocyanate (FITC) and Cy3. The synthesized and purified probes were purchased as Cy3 and FITC-labelled derivatives from Takara Bio Inc. (Shiga, Japan). Granules and biopellet sample fixation, slice preparation and hybridization were performed as reported by Amann and Saiki et al. [13, 14] Hybridized slices were viewed immediately under an Olympus FLUOVIEW FV300 laser microscope equipped with an Ar/HeNe laser unit and IX70 microscope (Olympus, Tokyo).

3. RESULTS AND DISCUSSIONS

(1) Nitrogen removal activities in the bioreactor

Influent NH_4^+–N, NO_3^-–N and NO_2^-–N were 2.3 ± 0.38, 1.47 ± 1.00 and 2.1 ± 0.63 mg/l, respectively. Nitrogen mass balance showed that the average nitrogen removal efficiency was 80% and the nitrogen removal rate was 9.3 ± 1.7 g-N/m^3.day. Nitrification and denitrification efficiencies were 75% and 92%, respectively. NO_2^-–N was almost completely removed (97 ± 6 %) and NH_4^+–N and NO_3^-–N removal efficiencies were 75 ±21 % and 67 ± 25 %, respectively. This clearly showed that NO_3^-–N removal efficiency was lower than NH_4^+–N removal efficiency. Moreover, effluent NO_3^-–N was always less than influent nitrogen (Fig. 2) and neither accumulated nor was eliminated entirely despite its low influent concentration (1.47 ± 1.00 mg/l). This showed the apparent sink of NH_4^+–N and the method of NH_4^+–N loss had to be explored. In order to explore the loss of NH_4^+–N, evidence for the anammox process in the bioreactor was considered based on its stoichiometry. The ratio of NO_2^-–N/ NH_4^+–N conversion was 1.29, which was very close to the stoichiometric relationship between nitrite and ammonium in the anammox process[9] (Fig. 3). The ratio agreed well with values obtained in a previous study [5]. However, this ratio could be similar even in normal denitrification and requires further verifications using conventional techniques. It revealed that solid substrates not only serve as constant sources of reducing power for denitrification but also creates the anaerobic condition by utilizing DO during the degradation of solid substrates. Moreover, the role of solid carbon substrates on nitrogen removal was evaluated by the authors in another study [19].

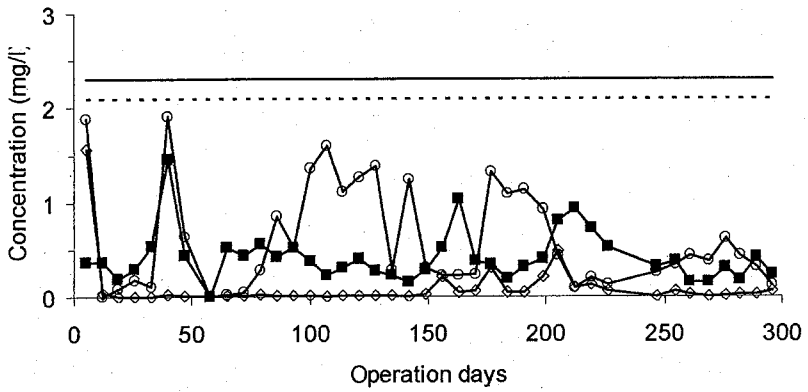

Fig.2 Nitrogen concentration in the bioreactor, effluent NH_4^+–N (white circle), effluent NO_3^-–N (Black Square), effluent NO_2^-–N (White diamond), average influent NH_4^+–N (solid line), and average influent NO_2^-–N (dotted line).

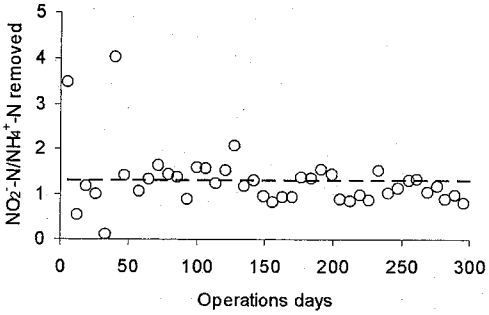

Fig.3 Ratio of NO_2^-–N / NH_4^+–N converted (white circle) in the bioreactor, stoichiometry of anammox process (NO_2^-–N / NH_4^+–N =1.31) as reported by van de Graaf [9] (dotted line).

(2) ^{15}N-labelling techniques

In order to examine the nitrogen removal pathway, the bioreactor was fed with $^{15}NO_2^-$–N and native NH_4^+–N. Anammox produced $^{14}N^{15}N$ and $^{15}N^{15}N$ whereas denitrification produced $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ through random isotope pairing when unlabelled ^{14}N and labelled ^{15}N were applied [2,20]. Because the anammox process combines 1 mole of (^{15}N) nitrite and 1 mole of (^{14}N) ammonium to form 1 mole of single-labelled dinitrogen gas ($^{14}N^{15}N$), the isotope fraction of $^{14}N^{15}N$ with reference to atmospheric air expresses the anammox potential activity. The $^{14}N^{15}N$ observed in this study was higher than in atmospheric air and production of $^{15}N^{15}N$ as a result of coupling of $^{15}NO_2^-$–N/$^{15}NO_2^-$–N via denitrification was one third of $^{14}N^{15}N$ (Table 2). This suggested that the anammox process is possible and further analysis using a FISH technique is required to confirm the existence of anammox bacteria.

Table 2 Isotope fractions of N_2 (%) with mass 28 ($^{14}N^{14}N$), 29 ($^{14}N^{15}N$) and 30 ($^{15}N^{15}N$) in the bioreactor after the addition of $^{15}NO_2^-$–N compounds, compared with reference atmospheric N_2. The results are mean values of three replicate.

Items	$^{14}N^{14}N$ (%)	$^{14}N^{15}N$ (%)	$^{15}N^{15}N$ (%)
Bioreactor	98.35 ± 0.25	1.25 ± 0.14	0.40 ± 0.12
Air	99.16 ± 0.15	0.83 ± 0.10	0.01 ± 0.00

(3)Distribution of microorganism in the biopellets and granules

The characterization of the microbial community in the bioreactor was examined using FISH technique.No signal was detected when granule slices taken from the bioreactor were hybridized with the ARC915 probe (pictures not shown). This may be due to the low oxidation reduction potential (ORP) condition inside the reactor which may be insufficient for methanogens. Furthermore, hybridizing cells of the biopellets taken from the bioreactor with AMX820 produced a bright signal and were active. Anammox bacteria mainly grew in the central part of the biopellets (200 μm from the surface) (Fig. 4). This indicated the unfavourable growth conditions for the anammox bacteria either due to the presence of dissolved oxygen or the production of methanol from anaerobic granules. Dissolved oxygen and methanol are inhibitors of anammox bacteria as reported by Third et al. and Guven et al. [4, 21]. In addition, cell hybridization with Pla46 probe showed weak signal, indicating the presence of low population of planctomycetes. Other bacteria were present in lower amounts on the surface and central part of the biopellets.

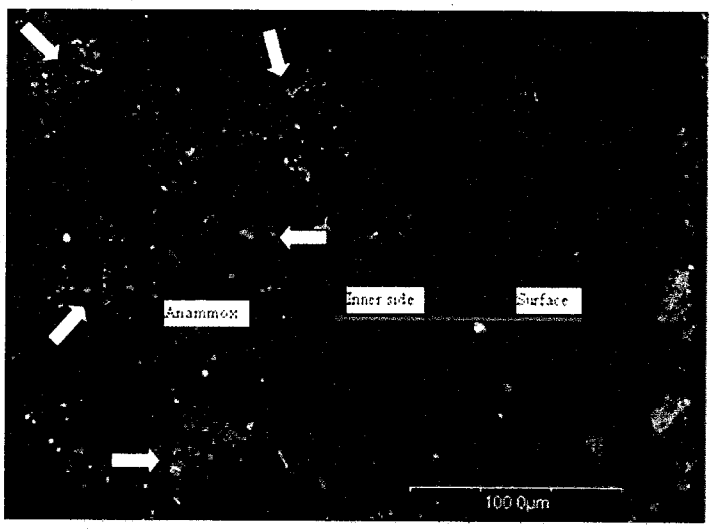

Fig.4 Fluorescence in situ hybridization of a biopellet with Amx820 probe (labelled with Cy3; red) and EUB probe mix[17] (FITC labelled; green), arrow indicates the position of anammox bacteria.

4. CONCLUSIONS

The presence anammox process in low ammonium-fed solid-phase denitrification bioreactor was assayed for 300 days in the laboratory. Nitrogen mass balance showed that the average nitrogen removal efficiency was 80%. The composition of the effluents generated in an upflow oxygen limited bioreactor was highly dependent on the solid carbon substrates. The combined results of nutrient profiles, ^{15}N-labelling techniques and FISH probe confirmed that anammox bacteria were found to be active. This clearly demonstrates that both anammox and denitrification processes work symbiotically in low ammonium concentrations under the support of solid organic substrates. Finally, nitrogen removal based on a mixture of an immobilized biomass of activated sludge and anaerobic granules under the support of solid carbon substrates elucidates a positive response to freshwater and drinkingwater.

ACKNOWLEDGMENT: Sponsorship by University of Yamanashi, 21st centuary center of excellence project, Japan is gratefully acknowledged. We sincerely thank Yuko Saiki and Yukiko Hiraga for their technical help.

REFERENCES

1) Neef, A., Zaglauer, A., Meier, H., Amann, R., Lemmer, H., Schleifer, K. H.: Population analysis in a denitrifying sand filter: conventional and in situ identification of Paracoccus spp. in methanol-fed biofilms, Appl. Environ. Microbiol. Vol. 62, pp.4329–4339, 1996.
2) Van de Graaf, A. A., Mulder, A., de Bruijn, P., Jetten, M. S.M, Robertson, L. A., Kuenen. J. G.: Anaerobic oxidation of ammonium is a biologically mediated process. Appl. Environ. Microbiol. Vol. 61, pp.1246–1251, 1995.
3) Hellinga, C., Schellen, A. A. J. C., Mulder, J. W, van Loosdrecht, M. C. M., Heijnen, J. J.:. The Sharon process: an innovative method for nitrogen removal from ammonium-rich waste water, Water Sci. Technol. Vol. 37, pp.135–142, 1998.
4) Third, K. A., Sliekers, A. O., Kuenen, J. G., Jetten, M. S. M.: The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: Interaction and competition between three groups of bacteria. Syst. Appl. Microbiol. Vol. 24, pp.588–596, 2001.
5) Guven, M., van de Pas-schoonen, K., Schmid, M. C., Strous, M., Jetten, M.S.M, Sozen, S., Orhon, D., and Schmidt, I.: Implementation of the anammox process for improved nitrogen removal. J. of Environ. Science and Health, Vol.39, No.7, pp.1729–1738, 2004.
6) Jetten, M.S.M., Cirpus, I., Kartal, B., van Niftrik, L., van de Pas-Schoonen, K.T., Sliekers, O.,. Haaijer, S., van der Star, W., Schmid, M., van de Vossenberg, J., Schmidt, I., Harhangi, H., van Loosdrecht, M., Gijs Kuenen, J., Op den Camp, H., Strous, M.: 1994–2004: 10 years of research on the anaerobic oxidation of ammonium, Biochemical Society Transaction Vol. 33, pp.119–123, 2005.
7) van Dongen, U., Jetten, M. S.M., van Loosdrecht, M. C.: The Sharon-anammox process for treatment of ammonium rich wastewater. Water Sci. Technol. Vol. 44, pp.153–160, 2001.
8) Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jøgensen, B. B., Kuenen, J. G. , Sinninghe Damste', J. S. , Strous, M., Jetten, M. S. M.: Anaerobic ammonium oxidation by anammox bacteria in the Black Sea, Nature, Vol. 422, pp. 608–611, 2003.
9) Van de Graaf, A.A., De Bruijn, P, Robertson, L.A., Jetten, M.S.M., Kuenen, J.G. : Autotrophic growth of anaerobic ammonium-oxidizing microorganisms in a fluidized bed reactor. Microbiology, Vol. 142, pp. 2187–2196, 1996.
10) Sumino, T., Nakamura, H., Mori, N., Kawaguchi, Y. : Immobilization of nitrifying bacteria by polyethylene glycol prepolymer, J Fermentation and Bioeng., Vol. 73, No. 1, pp.37–42, 1992.
11) Schmid, M. C., Maas, B., Dapena, A., van de Pas-Schoonen, K., van de Vossenberg, J., Kartal, B., van Niftrik, L., Schmidt, I. Cirpus, I., Kuenen, J. G., Wagner, M., Damste, J. S. S., Kuypers, M., Revsbech, N. P., Mendez,R., Jetten, M. S. M., Strous, M.: Biomarkers for in situ detection of anaerobic ammonium-oxidizing (Anammox) bacteria, Appl. Env. Microbiol., Vol. 71, No. 4, pp.1677–1684, 2005.
13) Amann, R.I.: In situ identification of microorganism by whole cell hybridization with rRNA targeted nucleic acid probes, in Akkermans, A.D.L and van Elas, J.D. (Ed.), Molecular microbial ecology manual, Kluwer academic publishers London, pp.1–15, 1995.
12) APHA, AWWA, WEF: Standard methods for water and wastewater examination, American Public Health Association 20th Edition, Washington DC, 1998.
14) Saiki, Y., Iwabuchi, C., Katami, A., Kitagawa, Y.: Microbial analyses by fluorescence in situ hybridization of well-settled granular sludge in brewery wastewater treatment plants, J. Biosci. Bioeng., Vol. 93, pp.601–606, 2002.
15) Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., Metzger. J. W., Schleifer, K.-H., Wagner, M.: Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation, Syst. Appl. Microbiol., Vol. 23, pp.93–106, 2000.
16) Neef, A., Amann, R. I., Schlesner, H., Schleifer, K.-H.: Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S-rRNA-targeted probes. Microbiology, Vol. 144, pp. 3257–3266,1998.
17) Daims, H., Bruhl, A., Amann, R., Schleifer, K.-H., Wagner M.:The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set, Syst. Appl. Microbiol., Vol. 22, pp. 434–44, 1999.
18) Stahl, D. A., Amann, R.: Development and application of nucleic acid probes, in Stackebrandt, E. and Goodfellow, M. (ed.), Nucleic acid techniques in bacterial systematic, John Wiley and Sons Inc., New York, pp.205–248, 1991.
19) Pathak, B.K., Sumino, T., Saiki, Y., Kazama, F.: Application of various carbon substrates for improved biological nitrification and denitrification using immobilized biomass as a removal carrier, Environmental Technology (submitted), 2006.
20) Dalsgaard, T., Canfield, D. E., Petersen, J., Thamdrup, B., Acun˜a- Gonza´lez J.: Anammox is a significant pathway of N_2 production in the anoxic water column of Golfo Dulce, Costa Rica. Nature, Vol.422, pp. 606–608, 2003.
21) Guven, D., Dapena, A., Kartal, B., Schmid, M.C., Mass, B., van de Pas-Schoonen, K., Sozen, S., Mendez, R., Op den Camp, H., Jetten, S.M., Strous, M., and Schmidt, I.: Propionate oxidation by and methanol inhibition of anaerobic ammonium-oxidizing bacteria, Appl. Environ. Microbiol., Vol. 71, pp. 1066–1071, 2005.

(Recived on May 26, 2006)