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ORIGINAL PAPER

Identification of bacteria coexisting with anammox bacteria in an upflow column type reactor

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Abstract Anammox process has attracted considerable attention in the recent years as an alternative to conventional nitrogen removal technologies. In this study, a column type reactor using a novel net type acrylic fiber (Biofix) support material was used for anammox treatment. The Biofix reactor was operated at a temperature of 25°C (peak summer temperature, 31.5°C). During more than 340 days of operation for synthetic wastewater treatment, the nitrogen loading rates of the reactor were increased to 3.6 kg-N/m^3/d with TN removal efficiencies reaching 81.3%. When the reactor was used for raw anaerobic sludge digester liquor treatment, an average TN removal efficiency of 72% was obtained with highest removal efficiency of 81.6% at a nitrogen loading rate of 2.2 kg-N/m^3/d. Results of extracellular polymeric substances (EPS) quantification revealed that protein was the most abundant component in the granular sludge and was found to be almost twice than that in the sludge attached to the biomass carriers. The anammox granules in the Biofix reactor illustrated a dense morphology substantiated by scanning electron microscopy and EPS results. The results of DNA analyses indicated that the anammox strain KSU-1 might prefer relatively low nutrient levels, while the anammox strain KU2 strain might be better suited at high nutrient concentration. Other types of bacteria were also identified with the potential of consuming dissolved oxygen in the influent and facilitating survival of anammox bacteria under aerobic conditions.

Keywords Anammox · Nitrogen removal · EPS · DNA analysis · Symbiotic relationship

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Introduction

Removal of nitrogenous compounds from wastewaters has historically been accomplished by conventional nitrification and denitrification processes. Due to the fact that these processes entail high energy and chemical costs, there has been a need to explore low-cost and efficient alternate technologies. As a potentially useful autotrophic biological process, anammox was first discovered about 13 years ago (Mulder et al. 1995). In this process, nitrite serves as an electron acceptor combining with ammonium to produce dinitrogen gas, the only environmentally friendly form of nitrogen. The species responsible for this nitrogen conversion have

been identified as deeply branching *planctomycete* with a doubling time of 11 days (Strous et al. 1999a).

Due to the long doubling time of anammox bacteria, a reactor that can retain biomass effectively and can provide a long solids retention time is desirable for successful and efficient operation of anammox process. Biofilm type reactors, such as fixed bed, fluidized bed and gas lift reactors have initially been applied for anammox treatment (Van de Graaf et al. 1996; Strous et al. 1997; Sliekers et al. 2003). Sequencing batch reactor (SBR) reactor has also been applied for anammox sludge cultivation, however, mechanical stirring could not adequately provide gas–solids separation and sludge floating became a serious problem, which resulted in deterioration of effluent quality (Strous et al. 1997; Strous et al. 1999b).

Although these systems could achieve good performances during laboratory studies, it was inevitable that valuable biomass would washout from the reactors. Furthermore, the relatively high operational temperature of over 30°C would result in high cost thus negating the purpose of anammox process. The objectives of this study were to investigate anammox treatment in a reactor that could retain the biomass effectively while providing high treatment level at a relatively lower temperature of 25°C. Characterization and identification of the anammox microorganisms was also undertaken by quantifying EPS and by using analytical techniques of scanning electron microscopy (SEM) and 16S rDNA.

Materials and methods

Biofix experiment set-up

An up-flow column type reactor equipped with Biofix as the support material was used for the anammox treatment in this study as shown in Fig. 1. The reactor had a square (15-cm by 15-cm) cross section and a height (to effluent port) of 102 cm. The volume of the reaction zone, including influent distribution and biomass retention sections, was 18.8 l. The Biofix biomass carrier was made of acrylic resin with a specific surface area of 113.8 m^2/m^3. Five bundles of the Biofix material were inserted in the reactor for a volume of 4.24 l (packing rate of 22.6%).

The system was operated at 25°C, controlled thermostatically in a water bath, however, the thermostat heater was not used in summer season due to the room temperature being in excess of 25°C (peak of 31.5°C). In addition, dark conditions were maintained with a black-vinyl sheet enclosure. The reactor was operated for approximately 340 days with synthetic wastewater and for 60 days with actual anaerobic sludge digester liquor. During synthetic wastewater treatment periods, middle influent was started in order to improve the proliferation of anammox bacteria in the reactor on day 89; the middle influent pump was changed for internal cycling by connecting the internal tube with influent tube in order to mitigate the severe effect of high nitrite concentration to anammox activity on day 236.

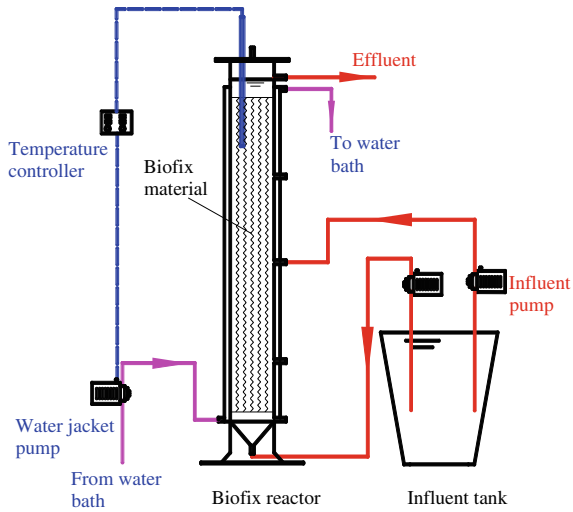

Fig. 1 Schematic diagram of Biofix reactor

Inocula and influent wastewater

The Biofix reactor was inoculated with sludge from another laboratory scale fix-bed anammox reactor operated at the Kumamoto University (Furukawa et al. 2003). The initial mixed liquor volatile suspended solids (MLVSS) concentration of the Biofix reactor was 1,700 mg/l. The reactor was first fed with synthetic media and later with actual digester liquor taken from the Kumamoto East Wastewater Treatment Plant (Kumamoto, Japan). Composition of the synthetic wastewater and the digester liquor is reported in Tables 1 and 2, respectively.

Analytical methods

NO_3–N and NO_2–N were measured by using UV spectrophotometric screening method and colorimetric

Table 1 Composition of synthetic medium

Compounds	Concentration (mg/l)
$(NH_4)_2SO_4$	50–300 (As N)
$NaNO_2$	50–300 (As N)
$KHCO_3$	125
KH_2PO_4	54
$FeSO_4 \cdot 7H_2O$	9
EDTA · 2Na	5

Table 2 Water quality of the anaerobic digestion liquor

Compounds	Concentration (mg/l)
BOD_5	150.1–200.8
COD	178.1–274.3
NH_4–N	428.0–1012.5
NO_2–N	0
NO_3–N	0
SS	40–100
pH	8.16–9.37

method, respectively (APHA, AWWA and WEF 1995). NH_4–N was quantified by the method described by Kanda, which using involved the use of *o*-phenylphenol as a substitute for liquid phenol (Kanda 1995). Dissolved oxygen (DO) was measured using a DO meter (HORIBA, pH/DO meter D-55).

In the determination of EPS, proteins were measured using the method of Lowry et al. (1951) and carbohydrates by the method of Dubois et al. (1956). Nucleic acids (combined RNA and DNA) were estimated by the UV absorption method (Experimental Guidelines for Biotechnology 1992) using the following equation:

$$\text{Nucleic acids (g/l)} = 30.98A/[10,000 \times (0.09)b] \quad (1)$$

where, 30.98 is the gram molecular weight of phosphorous, A is the absorbance of the sample solution at 260 nm, 10,000 is the constant of proportionality (*absorbtivity*) of phosphorous in nucleic-acid form (average of the RNA and DNA components), 0.09 is the weight fraction of phosphorous in nucleic acids, and b is the length of light path (1.0 cm in this study).

For SEM, samples were first washed in a 0.1 M phosphate buffer solution (pH 7.4) for 5 min each time. Then samples were hardened for 90 min in a 2.5% glutaraldehyde solution prepared with the buffer solution. Next, samples were washed in the buffer solution three times for 10 min each and then fixed for 90 min in a 1.0% OsO_4 solution prepared with the buffer solution. After washing samples three times for 10 min each in the buffer solution, they were dewatered for 10 min each in serially graded solutions of ethanol at concentrations of 10, 30, 50, 70, 90, and 95%. SEM observations were conducted using a scanning electron microscope (JEOL, JSM-5310LV).

The abundance of bacteria in the sludge was estimated from the image of denaturing gradient gel electrophoresis (DGGE). Metagenomic DNA was extracted from the sludge using ISOIL kit (Nippon gene Co., Ltd., Tokyo, Japan). Partial 16S rRNA genes in the metagenome were amplified by PCR with a primer set, 357F with a GC-clamp and 534R (Muyzer and Smalla 1998). The amplified fragments were resolved by DGGE for 16 h at 100 V at 60°C using DCode system (Bio-Rad Laboratories, 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 Corp., Carlsbad, CA, USA), and visualized as a 16-bit gray-scale image using FLA-2000 system (Fuji Photo Film Co., Ltd., Tokyo, Japan). The intensities of all bands were quantified using a software, Image Gauge v3.4, included in the system. KSU-1 population content was calculated from the corresponding band intensity and the sum of all band intensities (Muyzer and Smalla 1998).

Results and discussion

Synthetic wastewater treatment

The reactor was operated continuously for about 340 days with synthetic wastewater as the influent. The nitrogen loading rates (NLRs) were increased from 0.05 to 3.6 kg-N/m^3/d by means of increasing the concentrations of nitrogen compounds (NH_4^+ and NO_2^-) and shortening the HRT as shown in Fig. 2. In the first phase, NLRs were increased by increasing influent TN concentrations stepwise at a constant HRT of 23 h. Because the reactor was initially at an

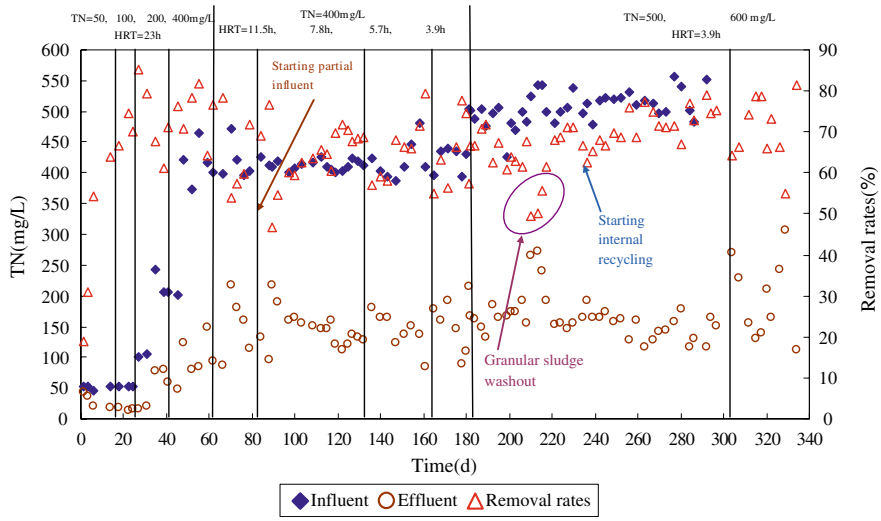

Fig. 2 TN removal performance for synthetic media

ambient temperature of about 25°C, the anammox biomass could not adapt well and had very low removal rates. It took 24 days for the TN removal efficiency to progress from 19% to 78%. Thereafter, stable anammox activities were maintained and an average TN removal rate of 75% was observed even when the influent TN concentration was increased to 400 mg/l (NLR of 0.4 kg-TN/m^3/d). Moreover, the removal efficiencies did not drop sharply even when the NLR was doubled. It was concluded that increasing the NLRs by increasing the influent TN concentration did not show severe adverse impact on anammox treatment performance. In phase II, occurring over a period of about 120 days, TN concentration was kept constant and HRT was shortened to increase the NLR. Towards the end, the NLR was increased to 2.5 kg-TN/m^3/d and the highest TN removal achieved at this loading rate was 77%. Compared with phase I, the change in the NLR by shortening the HRT resulted in negative impact on anammox treatment performance. For example, when the HRT was shortened from 11.5 h to 7.8 h and from 5.7 to 3.9 h, TN removal efficiencies decreased by 31% and 25%, respectively. From day 210 to day 220 in phase III, a relatively high influent TN of 500 mg/l was associated with unstable nitrogen removal performance. TN removal efficiencies declined due to washout of the granular sludge resulting from the detachment of anammox sludge. Internal recycling was applied to mitigate the problem on day 238, which resulted in the recovery of the treatment performance. During this period, the highest TN removal efficiency of 81.3% was achieved at a NLR of 3.6 kg-N/m^3/d (TN of 600 mg/l, HRT of 3.9 h).

Digester supernatant treatment

After the successful treatment of synthetic wastewater, raw anaerobic sludge digester liquor was introduced into the anammox reactor after a pretreatment by partial nitrification (PN) reactor. The NLRs of anammox reactor fluctuated between 1.1 and 2.2 kg-N/m^3/d since the flow rate was adjusted to produce the suitable NO_2–N/NH_4–N ratio for anammox reaction. However, high nitrite removal efficiencies (99%) and low nitrate production (effluent nitrate of 49.2 mg/l) were observed and average TN removal rate of 78% was obtained in the first 10 days. High nitrite concentrations and relatively long HRT initiated growth of nitrogen oxidizing bacteria (NOB) in the PN reactor, resulting in an increase in nitrate production in the system; e.g., the highest effluent nitrate concentration of anammox reactor was 312.1 mg/l. Due to this reason, the TN removal performance deteriorated gradually. As the performance in the PN reactor improved, TN removal efficiencies of anammox reactor also improved, which implied that TN removal of the whole system depended on the nitrogen conversion in the PN reactor. Despite high influent nitrate concentrations, fix-bed anammox reactor removed most of the influent ammonium and nitrite content and the average TN removal efficiency of 72% was achieved (Fig. 3).

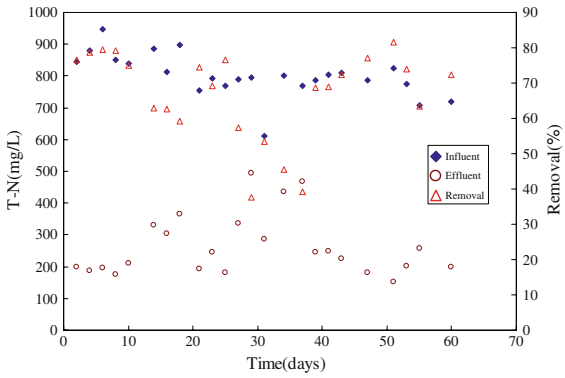

Fig. 3 TN removal performance during digester supernatant treatment

EPS analysis and SEM observation

EPS assists in the formation of microbial aggregates whether the biomass is in suspended or biofilm states. EPS is considered to support the metabolic cooperation among cells coexisting in aggregate form. Moreover, it benefits surface adhesion, cell aggregation in flocs and biofilms, stabilization of biofilm structure, etc. (Wingender et al. 1999). Figure 4 shows a comparison of EPS levels between the anammox granular sludge and the attached biomass of the reactor. From this figure, it was clear that protein was the predominated component in the EPS of anammox sludge. Furthermore, EPS content of the granular sludge was almost two times higher than that of the attached biomass, which suggests that high EPS levels would be beneficial for the formation and stabilization of granular sludge. Laspidou and Rittmann (2002) considered that due to the high content of negatively charged amino acids, protein was more involved than sugars in electrostatic bonds with multivalent cations, a key factor in stabilizing aggregate structure. The other significant function of extracellular protein is as enzymes performing the digestion of macromolecules and particulate material in the microenvironment of embedded cells, which could trap, bind, and concentrate organic materials in close proximity to the cells (Laspidou and Rittmann 2002).

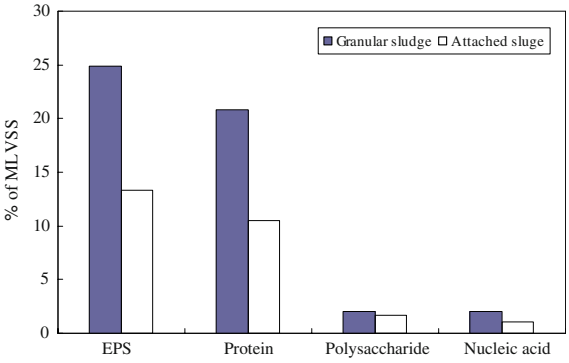

Fig. 4 EPS comparison of granular and attached biomass

The SEM photos of the granular sludge in the Biofix reactor show a high degree of compactness (Fig. 5). From the exterior view, each micro-element was tightly integrated with other parts and there was little interspace between them; while the interior showed drape-shape and the micro units inside could interlock with each other, which was in favor of the granular sludge joining tightly and existing stably. Based on the SEM observation of the granular anammox sludge, it was concluded that the micro-organization structure in the Biofix reactor presented high compactness. Figure 6 shows the SEM photos of the attached biomass on Biofix materials. The micro-organization structure exhibited sphericity from the microcosmic point of view. This structure may have been formed by the shear forces caused by the upward flow of gas bubbles and water currents in the spaces between support materials. The Biofix materials with its net type structure provided a favorable environment for the attached biomass allowing for effective contact with nutrients, gas bubbles and water current.

DNA analysis

Figure 7 illustrates comparative DGGE photos of the microorganisms in the anammox reactor operated with synthetic and actual wastewaters (samples taken on day 326 and day 55 during synthetic and actual periods, respectively). Samples were taken out of the reactor from the bottom, middle and upper parts of the Biofix media. During the period when synthetic wastewater was treated, the KU2 strain (AB054007, Furukawa et al. 2001) was dominant and the three KU2 bands of the different parts demonstrated almost identical concentrations; while the KSU-1 concentrations showed much lower levels relative to KU2; however, an increasing trend from the bottom upward of the KSU-1 strain (AB057453, Fujii et al. 2002) could be distinguished based on the thickness of the

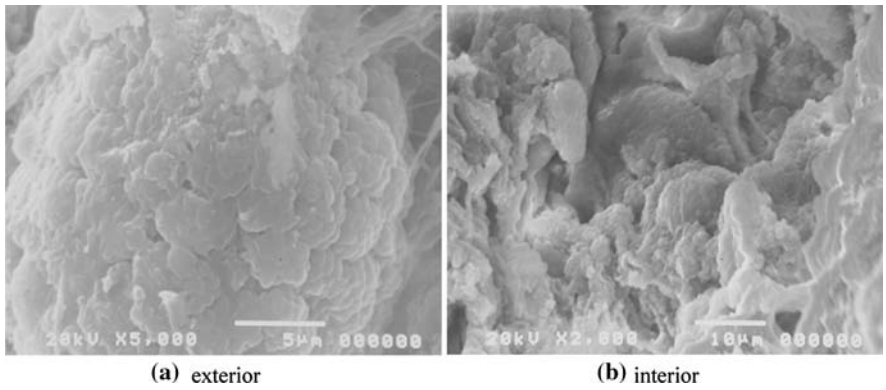

Fig. 5 SEM comparison of exterior and interior condition of granular sludge

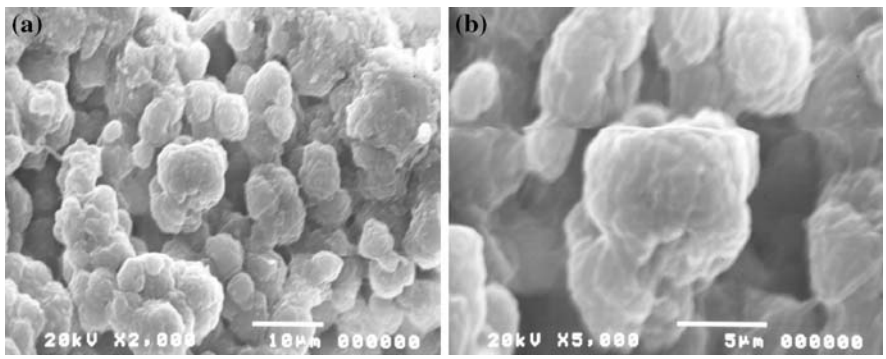

Fig. 6 SEM photos of attached biomass on Biofix

bands. After actual wastewater was introduced into the reactor, there were some varieties that appeared to occur in the reactor, e.g., the responsible anammox bacteria KSU-1 and the new band of B1 almost faded away according to the bands thickness. On the other hand, a new band B3 was discovered during the period when actual wastewater was treated.

Among the new discovered bands, band B1 was identified as a close match to the uncultured bacterium PHOS-HE36 (identity of 97%, accession: AF314435). This type of bacteria could consume DO in the influent and survive under anaerobic and anoxic conditions. Band B2 was identified as *Chloroflexi* belonging to the green non-sulfur bacteria (AB113620, identity of 100%; AB113606, identity of 100%). *Chloroflexi* bacteria are facultative anaerobes frequently found in UASB reactors that are filamentous and often associated with sludge granulation and bulking (Yamada et al. 2005). Recently, this type of bacteria was also found in a methane fermentation process where it consumed organic compounds in the reactor (Sekiguchi et al. 2001). Due to its anaerobic respiration metabolism, it was postulated that this

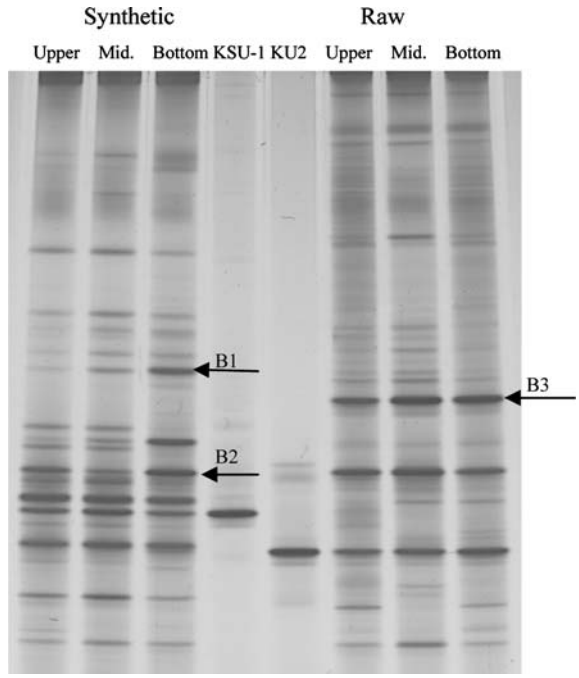

Fig. 7 Comparison DGGE results of synthetic and raw wastewater treatment periods

bacteria could consume nitrite or nitrate. The details of band B3 are unknown and are under investigation.

Both anammox bacteria (*B. anammoxidans*) and ammonium oxidizing bacteria (*N. eutropha*) have the ability of carrying out anammox reaction. When ammonium becomes the limited nutritent, both kinds of bacteria will compete for ammonium. Simultaneously, *N. eutropha* could supply nitrite for *B. anammoxidans* and facilitate the anammox reaction under anoxic conditions. In this case, both kinds of bacteria could coexist together well and the reaction rate is mainly dependent on the production of nitrite by *N. eutropha* (Schmid et al. 2002). Pathak et al. also reported that anammox and denitrificaiton processes could coexist in the same environment and that anammox bacteria were less competitive than denitrifying bacteria, especially in an organic carbon rich environment (Pathak et al. 2007). In this study, anammox bacteria exhibited capacity to coexist not only with nitrifiers, but also with other kinds of bacteria which could consume the residual DO in the liquid. Some of these bacteria presented the possibility to consume nitrite or nitrate in the reactor, which could assist further TN removal performance. This kind of symbiotic relationship will be in favor of the survival of anammox biomass in some macro-aerobic environments and the development of anammox process.

Conclusions

TN removal efficiency of 81.3% was observed at a NLR of 3.6 kg-N/m^3/d and a temperature of 25°C for synthetic wastewater treatment, while for actual digester supernatant treatment average TN removal efficiency reached 72% after about 60 days of operation and the highest removal efficiency of 81.6% was observed at a loading rate of 2.2 kg-N/m^3/d. High EPS levels, especially high extracellular protein levels, were shown to be related to the formation of anammox granular sludge. Based on the analysis of EPS and SEM observation, the anammox granular sludge formed in the reactor was revealed to have a compact morphology. The microstructure of the anammox sludge attached on the Biofix material exhibited a spherical form as observed in SEM photos, which may be due to shear forces caused by the upward flow of gas bubbles and water through the support material. DNA analyses revealed that the identified bacteria, except the anammox strains, could serve the function of consuming DO and nitrite or nitrate in the water solution, thereby potentially assisting the anammox microorganisms to function under macro-aerobic conditions. Although this study provided some insight, further research is recommended to investigate the establishment of stable treatment performance of municipal sludge digester supernatant by applying the phenomenon of coexistence of anammox and other kinds of bacteria. Moreover, the effect of digester supernatant on the bacterial changes in the reactor also needs further investigation.

References

APHA, AWWA, WEF (1995) Standard methods for the examination of water and wastewater, 19th edn. American Public Health Association, Washington DC, USA

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28(3):350–356. doi:10.1021/ac60111a017

Experimental Guidelines for Biotechnology (1992) Society of Fermentation and Bioengineering, Osaka, Japan, pp 98–99 (in Japanese)

Fujii T, Sugino H, Rouse JD, Furukawa K (2002) Characterization of the microbial community in an anaerobic ammonium-oxidizing biofilm cultured on a nonwoven biomass carrier. J Biosci Bioeng 94:412–418

Furukawa K, Rouse JD, Imajo U, Nakamura K, Ishida H (2001) Anaerobic oxidation of ammonium confirmed in continuous flow treatment using a non-woven biomass carrier. Jpn J Water Treat Biol 38(2):87–94

Furukawa K, Rouse JD, Yoshida N, Hatanaka H (2003) Mass cultivation of anaerobic ammonium-oxidizing sludge using a novel nonwoven biomass carrier. J Chem Eng of Jpn 36(10):1163–1169. doi:10.1252/jcej.36.1163

Kanda J (1995) Determination of ammonium in seawater based on the indophenol reaction with *o*-phenylphenol (OPP). Water Res 29:2746–2750. doi:10.1016/0043-1354(95)00149-F

Laspidou CS, Rittmann BE (2002) A unified theory for extracellular polymetic substances, soluble microbial products, and active and inert biomass. Water Res 36:2711–2720. doi:10.1016/S0043-1354(01)00413-4

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Mulder A, van de Graaf AA, Robertson LA, Kuenen JG (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol Ecol 16:177–184. doi:10.1111/j.1574-6941.1995.tb00281.x

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie

Van Leeuwenhoek 73:127–141. doi:10.1023/A:1000669317571

Pathak BK, Kazama F, Saiki Y, Sumino T (2007) Presence and activity of anammox and denitrification process in low ammonium-fed bioreactors. Bioresour Technol 98:2201–2206. doi:10.1016/j.biortech.2006.08.014

Schmid I, Schmid M, Cirpus I, Strous M, Bock E, Kuenen JG et al (2002) Aerobic and anaerobic ammonia oxidizing bacteria-competitors or natural partners? FEMS Microbiol Ecol 39:175–181

Sekiguchi Y, Takahashi H, Kamagata Y, Ohashi A, Harada H (2001) In situ detection, isolation, and physiological properties of a thin filamentous microorganism abundant in methanogenic granular sludge: a novel isolate affiliated with a clone cluster, the green non-sulfur bacteria, subdivision I. Appl Environ Microbiol 67(12):5740–5749. doi:10.1128/AEM.67.12.5740-5749.2001

Sliekers AO, Third k, Abma W, Kuenen JG, Jetten MSM (2003) Canon and anammox in a gas-lift reactor. FEMS Microbiol Lett 218:339–344. doi:10.1016/S0378-1097(02)01177-1

Strous M, van Gerven E, Kuenen JG, Jetten M (1997) Ammonium removal from concentrated wate steams with the anaerobic ammonium oxidation (Anammox) process in different reactor configurations. Water Res 31:1955–1962. doi:10.1016/S0043-1354(97)00055-9

Strous M, Fuerst JA, Kramer EHM, Logemann S, Muyzer G, van de Passchoonen KT et al (1999a) Missing lithotroph identified as new planctomycete. Nature 400:446–449. doi:10.1038/22749

Strous M, Kuenen JG, Jetten MSM (1999b) Key physiological parameters of anaerobic ammonium oxidation. Appl Microbiol Biotechnol 65:3248–3250

Van de Graaf AA, de Bruijn P, Robertson LA, Jetten MSM, Kuenen JG (1996) Autotrophic growth of anaerobic ammonium-oxidezing microofganisms in a fluidized bed reactor. Appl Environ Microbiol 142:2187–2196

Wingender J, Neu TR, Flemming H-C (1999) What are bacterial extracellular polymeric substances? In: Wingender J, Neu TR, Flemming H-C (eds) Microbial extracellular polymeric substances: characterization, structure and function. Springer, Berlin

Yamada T, Sekiguchi Y, Imachi H, Kamagata Y, Ohashi A, Harada H (2005) Diversity, localizaiont, and physiological properties of filamentous microbes belonging to Chloroflexi subphylum I in mesophilic and thermophilic methanogenic sludge granules. Appl Environ Microbiol 71(11):7493–7503. doi:10.1128/AEM.71.11.7493-7503.2005