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Characterization of Sludge from Single-Stage Nitrogen Removal Using Anammox and Partial Nitritation (SNAP)

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Abstract

Single-stage Nitrogen removal using Anammox and Partial nitritation (SNAP) was demonstrated under continuous-treatment conditions to be reliable and stable. An average ammonium conversion of 89.2 ± 6.2% and a nitrogen removal of 76.3 ± 7.2% were obtained for 60 days of continuous operation under operational conditions of 35℃, pH 7.5~7.8, and 0.06~0.10 vvm aeration rate at a loading of 0.6 kg-N/m^3/d using a simulated landfill leachate as influent. The observed sludge yield of the SNAP process was calculated to be as low as 0.045 mg-VSS/mg-N removed. The SNAP sludge had a particle size distribution of 420~550 μm and showed a tendency to form aggregates inside void spaces of the net-type acryl-resin biomass carrier. Specific ammonium oxidizing activities of the sludge ranged from 430 to 610 mg-N/g-VSS/d and 32 to 51 mg-N/g-VSS/d under total aerobic and anaerobic conditions, respectively. Levels of ammonium oxidizers and nitrite oxidizers were determined to be 10^7~10^9 MPN/g-VSS and 10^6 MPN/g-VSS, respectively. DNA analysis of the SNAP biomass revealed the existence of three dominant groups of bacteria including ammonium oxidizers closely related to *Nitrosomonas europaea*, nitrite oxidizers closely related to *Nitrospira* sp.; and anammox bacteria closely related to strains KU2 and KSU-1. These bacteria were suggested to exist in a dynamic equilibrium.

Key words: bacterial composition, landfill leachate, nitrogen removal, sludge activity, SNAP.

INTRODUCTION

Development of novel biological processes for nitrogen removal has gained much attention in recent years. In particular, the wholly autotrophic conversion of ammonium to nitrogen gas by a two-step biological process has been of much interest. The two reactions involved in this process consist of partial nitritation and anammox (equations 1 and 2, respectively) and can be understood as a combined reaction (equation 3). Accordingly, these reactions can be expressed in two separate reactors or combined as a single-stage unit process.

$$2NH_4^+ + 1.5O_2 \rightarrow NH_4^+ + NO_2^- + H_2O + 2H^+ \quad (1)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (2)$$

$$2NH_4^+ + 1.5O_2 \rightarrow N_2 + 3H_2O + 2H^+ \quad (3)$$

Some single-stage autotrophic processes including CANON[1)] and OLAND[2)] are already widely known. In addition, we recently reported the SNAP (single-stage nitrogen removal using anammox and partial nitritation) process[3)]. This process involves the co-attachment of aerobic and anaerobic

ammonium oxidizing bacteria on a common biomass carrier consisting of an acryl-resin fiber net. In continuous treatment studies of the SNAP process using a simulated landfill leachate, ammonium conversions as high as 88% and total nitrogen removals (as nitrogen gas) up to 78% have been demonstrated at a loading rate of 0.6 kg-N/m^3/d.

A single-stage autotrophic process requires that the operational conditions be controlled in such a manner that both aerobic and anaerobic ammonium oxidation can occur in the same unit reactor. Thus, both aerobic ammonium oxidizing bacteria (AOB) and anaerobic ammonium oxidizing (anammox) bacteria must be able to obtain dominance in the same general environment. Biomass communities of the CANON and OLAND processes have been shown to contain three basic types of bacteria – predominately including AOB and anammox bacteria and, in smaller numbers, nitrite-oxidizing bacteria (NOB)[4, 5]. In our previous study, the bacterial composition in biomass of a reactor with the firstly discovered SNAP process was shown to contain AOB (99% similarities to a *Nitrosomonas* sp.) and anammox strains (88~100% similarities to strain KSU-1) accounting for 8.7% and 15%, respectively, of the total clones[6].

For an oxygen-limited process, knowing the composition of the bacterial community and the activities of its members under total aerobic and anaerobic conditions would yield valuable information toward understanding and controlling a single-stage autotrophic process. It is thought that these characteristics are specific to different process conditions depending on bacterial composition and mode of operation. In this paper we introduce information pertinent to the operation of the SNAP process and define characteristics of ammonium oxidizing activities and community composition in the biomass.

MATERIALS AND METHODS

Reactor and operation

The SNAP process was operated continuously using two identical reactors, SN-2 and SN-3 with liquid volumes of 4.65 *l* as described previously[3]. For research reported here, operation was extended beyond that of our previous work to testing periods 14, 15 and 16 as shown in Table 1. The composition of the influent was selected to simulate treated landfill leachate and was the same as that used in the previous study.

Representative samples of sludge were drawn from reactors SN-2 and SN-3 for batch tests, MPN tests and bacterial analyses. For reactor SN-2, three samples were taken on operational days 335, 344 and 442. Two samples were taken from reactor SN-3 on the same days 344 and 442 with reactor SN-2.

Batch tests for activity of SNAP sludge

In the previous study[3], during anaerobic batch tests simultaneous removals of ammonium and nitrite by SNAP sludge were demonstrated. However, those tests were carried out using loosely attached sludge. In this study, we conducted the batch tests using a mix of well-attached and loosely attached sludge. Two sets of tests were conducted with sludge drawn on days 344 and 442 from the SN-2 reactor.

Aerobic activity test

Batch tests were conducted to evaluate the nitrifying activity of the SNAP sludge under aerobic conditions. The inorganic test medium consisted of 381.7 mg/*l* NH_4Cl, 88 mg/*l* KH_2PO_4, 630 mg/*l* $NaHCO_3$, 750 mg/*l* $KHCO_3$, 20 mg/*l* $CaCl_2 \cdot 2H_2O$ and 50 mg/*l* $MgSO_4 \cdot 7H_2O$ plus 1 m*l* of a trace element solution per liter as reported by Lipponen *et al.*[7]. A specific amount of sludge was used in order

Table 1 Experimental periods for reactor SN-2

Period (term)	Operational conditions			
	HRT (h)	Temp. (℃)	pH	Aeration rate (vvm)
1 (0~17)	6	35	7.5	0.10
2 (18~31)	6	35	7.5	0.06
.....				
13 (243~298)[a]	6	35	7.5	0.10
14 (299~322)	10	35	7.5	0.10
15 (323~344)[b]	10	35	7.8	0.10
16 (345~464)	10	35	7.5~7.8	0.06~0.10

[a] *Periods from 1 to 13 were described in previous paper*[3]
[b] *Operation was stopped and then restarted within the day 344.*

to get final biomass concentrations of 0.8 to 1.2 g-VSS/*l*. The sludge was washed three times by vortex mixing, centrifuging and decanting and then suspended in the medium by using strong aeration in a 1-*l* Erlenmeyer flask in a water bath at 35℃. Samples were taken initially and then once every hour thereafter (at least 5 times) to analyze NH_4-N, NO_2-N, NO_3-N and alkalinity. From this data, the maximum specific ammonium oxidation rate was determined using a Lineweaver-Burk analysis.

Anaerobic activity test

Batch tests were conducted for the evaluation of the anammox activity of the SNAP sludge under anaerobic conditions. The inorganic test medium consisted of 117.9 mg/*l* $(NH_4)_2SO_4$, 197 mg/*l* $NaNO_2$, 27.2 mg/*l* KH_2PO_4, 500 mg/*l* $KHCO_3$, 20 mg/*l* $CaCl_2 \cdot 2H_2O$, 50 mg/*l* $MgSO_4 \cdot 7H_2O$ and 5 mg/*l* $FeSO_4 \cdot 7H_2O$ plus 1 m*l* of a trace element solution per liter as reported by Imajo *et al.*[8]. Sludge samples were prepared in the same manner as for the aerobic test (above) and used at concentrations of 0.9 to 1.1 g-VSS/*l*. Washed sludge and medium were mixed in a 0.5-*l* Erlenmeyer flask with a rubber stopper and gas-tight syringe and flushed with argon gas before incubating on a shaker at 100 rpm at 35℃. There were some modifications to the anaerobic test compared to the previous study[3] as follows. The initial ammonium concentration was lower than the nitrite level and argon gas was used instead of nitrogen gas for purging oxygen. The former modification allowed for better application of Monod kinetics and the latter allowed for overcoming reverse effects of N_2 gas on the anammox reaction (in which N_2 gas is the principal product).

MPN test

The MPN test used for enumeration of AOB and NOB in the SNAP sludge consisted of 10-fold serial dilutions in five 5-m*l* culture tubes. The 5-m*l* test tubes were used to reduce the required volume of culture medium. All samples were treated identically as follows. A 50-m*l* sludge sample was mixed well, then 25 m*l* was used for determining SS and VSS and the remaining 25 m*l* was used for the MPN test. The latter portion was washed with distilled water four or five times by vortex mixing, centrifuging and decanting to remove background concentrations of nitrogenous compounds. Finally, a suspension was obtained by gentle blending using an ultrasonic homogenizer (US-150T, Nippon Seiki Co., Japan) at a low output (5/10 dial) with a very short time (30 seconds) and low temperature (by dipping in ice water).

The media used in this study were adopted from Lipponen *et al.*[7]. Serial 10-fold dilutions were made by adding 0.5 m*l* of the suspension to 4.5 m*l* sterile medium. Each successive dilution was vortex-mixed for 30 seconds before a 0.5 m*l* portion was taken. For both AOB and NOB, 10^{-3} to 10^{-7} dilutions were applied and 5 replicate tubes for each dilution were used.

Test tubes were incubated at 28.0 ± 0.5℃ for 30 days. After incubation, the activity of AOB was determined by the disappearance of the blue color of bromothymol blue and the activity of NOB was detected by a colorless signal when Griess-Ilosvay was added. From positive and negative records, the MPN values were determined by using the MPN Calculator program (VB6 Version, at http://www.i2workout.com/mcuriale/mpn/index.html). Finally, the results were calculated as MPN per g-VSS (based on the data from the VSS determination). Two sets of tests were carried out for each sample and the results were averaged.

Analytical methods

NH_4-N, NO_2-N, NO_3-N and alkalinity were analyzed according to standard methods[3]. Particle size distribution analysis of sludge samples was conducted using a Laser Scattering Particle Size Distribution Analyzer LA-920 (HORIBA, Japan).

Bacterial community analyses

The existence of nitrifying and anammox bacteria in the SNAP sludge was examined by 16S rDNA analysis. Three procedures, as summarized in Table 2, were carried out for amplifying partial 16S rDNA sequences. Primers 357F and 543R were specific for the 16S rDNA regions in the different bacterial species which correspond to positions 341 to

Table 2 Summary of DNA analysis methods

	Procedure #1	Procedure #2	Procedure #3
Analysis steps	DNA extraction, 1st PCR amplification, 2nd PCR amplification, DGGE, 3rd PCR amplification of excised bands, DNA sequencing	DNA extraction, PCR amplification, directly DNA sequencing	DNA extraction, 1st PCR amplification, DGGE, 2nd PCR amplification of excised bands, thermal cycling for sequencing, DNA sequencing
DNA extraction	Use ISOPLANT Kit (Nippon Gene)	Use ISOPLANT Kit (Nippon Gene)	Use Soil DNA Isolation Kit (MOBIO)
Primers for PCR	- For 1st and 3rd PCR: 357F and 534R - For 2nd PCR: 357F-GC clamp and 534R	Ana-5' and Ana-3'	- For 1st PCR: GM5F-GC clamp and 907R - For 2nd PCR: GM5F and 907R
DGGE conditions	8% polyacrylamide gel, 30%-65% denaturant gradient. At 60℃, 100 V, for 16 hours Stained by SYBR Gold	Not applied	8% polyacrylamide gel, 20%-80% and 30%-60% denaturant gradient. At 60℃, 200 V, for 6 hours Stained by SYBR Green

Sequences of primers
357F: 5'-CCTACGGGAGGCTGCAG-3'; **534R**: 5'- ATTACCGCGGCTGCTGG-3'
Ana-5': 5'-TAGAGGGGTTTTGATTAT-3'; **Ana-3'**: 5'-GGACTGGATACCGATCGT-3'
GM5F: 5'- CCTACGGGAGGCAGCAG-3'; **907R**: 5'-CCCCGTCAATTCCTTTGAGTTT-3'

534 in *E.coli*[9]. Primers Ana-5' and Ana-3', whose nucleotide sequences correspond respectively to positions 811 to 828 and 1004 to 1022 in the 16S rDNA of anammox KSU-1 strain, were designed for anammox bacteria specificity. Pair of bacterial primer GMF5 and universal primer 907R was used for amplifying the 16S rDNA of members belonging to the domain *Bacteria*[10]. Procedures #1 and #2 were applied to all 5 samples, while procedure #3 was applied to only 2 samples on day 442. The retrieved sequences were evaluated for homologies using BLAST at NCBI databases (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS AND DISCUSSION

Stability of SNAP performance

Figure 1 shows the results of continuous operation of the SNAP process in reactor SN-2. These results include three periods having the same operational conditions with the previous study (periods 13, 14 and 15 compared to periods 1, 9 and 5, respectively) and one period of long-term operation (period 16) with flexible control of pH and aeration rate. Average treatment results of these repeated periods, as shown in Table 3, indicated repeatability in the performance of the SNAP process.

During period 16, random changes in pH and aeration rate within small ranges resulted in only insignificant changes in performance. The pH range in the reactor was 7.5~7.8 and bulk DO levels were from 1.00 to 2.75 mg/*l*. In only two cases when pH dropped below 7.0 due to a problem with the $NaHCO_3$ pump (days 370~375) and the aeration rate increased to over 0.16 vvm (bulk DO about 3.00 mg/*l*) due to a problem with the air flow meter (days 438~446), did the effluent nitrate levels increase significantly. The high effluent nitrate levels can be explained by the sudden increase in NOB activity under favorable conditions such as low pH and high DO (2.75~3.30 mg/*l*).

Together with previously obtained experimental results[3], this suggests a dynamic co-existence between bacterial groups (AOB, NOB and anammox) in the SNAP sludge. The average ammonium conversion and N-removal for the last 60 days of operation were 89.2 ± 6.2% and 76.3 ± 7.2%, respectively.

General characteristics of SNAP sludge

Sludge production rate

Reactor SN-2 had been re-started using 13 g-SS of nitritation sludge 15 days before the SNAP process occurred as mentioned in the

previous study[3]. Operation was stopped on day 191, re-started on day 210 with 16 g-SS of SNAP sludge and stopped again on day 344. When operation was stopped, total sludge quantity and VSS content were determined. Six grams (as SS) of loosely attached sludge was removed on day 128. From these data, shown in Table 4, the average sludge production rate was estimated to be 148 mg-SS/d or 102 mg-VSS/d. The average VSS content of the SNAP sludge was 69% of SS.

Considering a typical nitrogen removal rate of 0.45 kg-N/m^3/d[3] and the reactor volume of 5.0 *l*, the observed biomass yield of the SNAP process was calculated to be 0.045 mg-VSS/mg-N. The biomass yield for SNAP process was much lower than the theoretical value of 0.13 g-VSS/g-N for both AOB and anammox bacteria[11]. This is understandable if taking into account that the SNAP is an attached-immobilized growth process, thus operated with a very long sludge retention time (SRT). Theoretically, the lower observed biomass yield is obtained for the longer SRT. As a low sludge production process, the handling of excess sludge would thus be minimized. Corroboratively, only a small

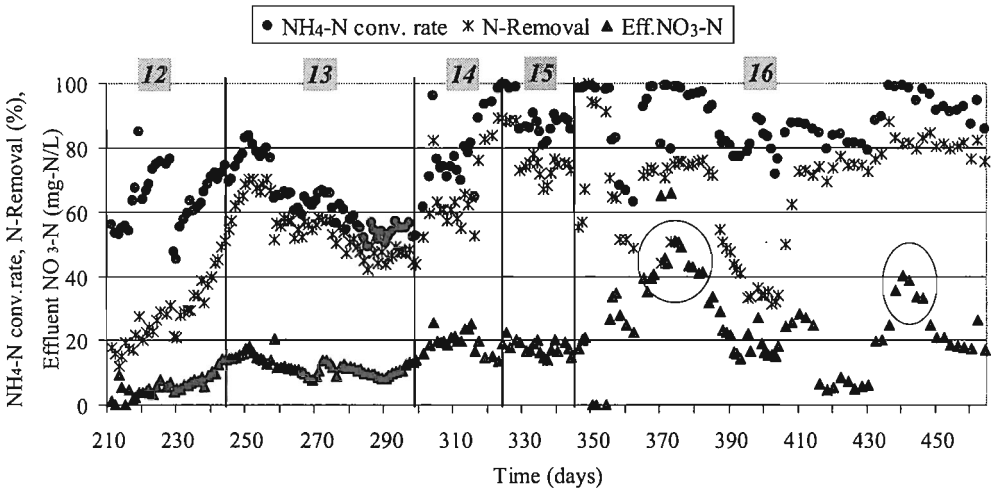

Fig. 1 Operation of the SNAP process in extended phases (reactor SN-2)

Table 3 Repeatability in performance of the SNAP process

Period[a]	1	13	9	14	5	15
NH_4-N conversion, %	52.2 ± 2.4	58.9 ± 5.2	77.0 ± 2.4	76.4 ± 11.2	88.1 ± 3.1	89.8 ± 6.2
N-removal, %	44.9 ± 2.8	51.4 ± 4.8	60.9 ± 1.7	63.4 ± 10.8	78.5 ± 2.8	77.4 ± 7.3

[a]*Periods 1, 5 and 9 are of the previous study*[3]

Table 4 Sludge production data during SNAP operation

Time duration (days)	Sludge quantity, g-SS				Daily production	
	Initial	Final	Removed	Increased	mg-SS/d	mg-VSS/d
206	13	34	6	27	131	90
134	16	38	0	22	164	113
Average					*148*	*102*

amount of excess sludge was removed from reactor SN-2 after about 200 days of operation.

Particle size distribution

Sludge samples taken from SN-2 reactor on day 344 were analyzed for particle size distribution. Sample 1 was mixed sludge and sample 2 was loosely attached sludge. As shown in Fig. 2, there was not much difference in the distribution patterns of two sludge samples. The loosely attached sludge contained a greater portion of large size particles, reflecting a trend to aggregate. The SNAP sludge particles were mostly in the size range of 420~550 μm. High concentrations of inorganic components such as Ca, Mg, and Fe may have facilitated the formation of aggregates in spaces inside the biomass carrier. These aggregates might play an important role in creating anoxic conditions for anammox bacteria.

Activities of SNAP sludge

Aerobic activity

Averaged data of aerobic activity tests for the SNAP sludge from reactor SN-2 are shown in Fig. 3. From these data, the maximum specific ammonium oxidation rates were calculated to be 430 mg-N/g-VSS/d (or 297 mg-N/g-SS/d) and 610 mg-N/g-VSS/d (or 421 mg-N/g-SS/d) for the tests on days 344 and 442, respectively.

Anaerobic activity

Figure 4 shows data of anaerobic activity tests for the sludge from reactor SN-2 on days 344 and 442. Maximum specific ammonium consumption rates were calculated to be 32 mg-N/g-VSS/d (or 22 mg-N/g-SS/d) and 51 mg-N/g-VSS/d (or 35 mg-N/g-SS/d) for these two cases, respectively. Averaged ratios of nitrite-to-ammonium consumption were 1.26 and 1.39, which are closed to the value 1.32 for the anammox reaction. The specific ammonium consumption rate also exhibited an increase with time.

The increase in aerobic and anaerobic activities of sludge samples from day 344 to day 442 might be related to the increase in bacterial level, or overall activity of the SNAP sludge. This can be supported by the increases in ammonium conversion and nitrogen removal rates from 87.9 to 92.4%, and from

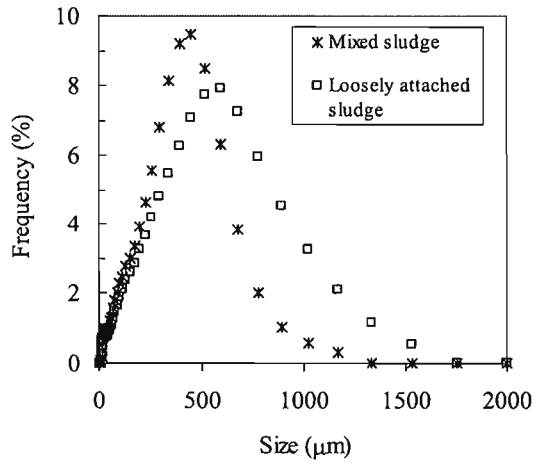

Fig. 2 Particle size distribution of the SNAP sludge

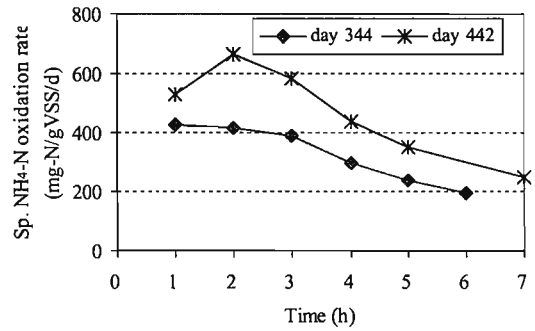

Fig. 3 Data profile of aerobic activity of the SNAP sludge from reactor SN-2

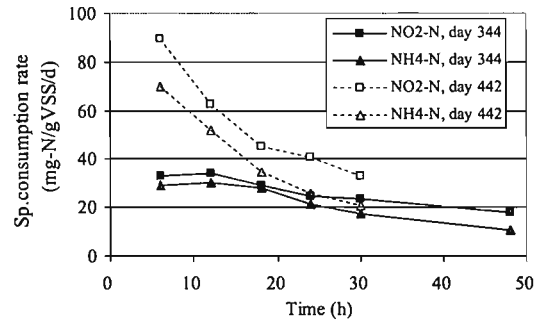

Fig. 4 Data profile of anaerobic activity of the SNAP sludge from reactor SN-2

74.4 to 80.6%, respectively, just before taking sludge samples.

Aerobic-to-anaerobic activity ratios of the SNAP sludge decreased from 13.4 to 12 from day 344 to day 442. This decrease indicates that the relative amount of anammox bacteria in the biomass increased with time. Thus, it appears this ratio could serve as an indicator of the ratio of active AOB to active anammox bacteria in the SNAP sludge. In this case, the SNAP sludge contained 12 to 13 times more active AOB than anammox bacteria. Combinations of this index and data from MPN test (next section) could give information about bacterial composition when there is no data from biomolecular analyses. In addition, molecular analyses that target the ribosomal RNA gene, give indications only about the presence of microorganisms, but not about their activity[12].

Maximum aerobic and anaerobic activities of CANON sludge under ammonium limitation were reported as 33 nmoles/mg protein/min and 7 nmoles/mg protein/min, respectively[4]. In that study, protein concentration was calculated as half of SS by which aerobic and anaerobic activities were 333 mg-N/g-SS/d and 70.5 mg-N/g-SS/d, respectively. Compared to the CANON process, the SNAP sludge had the same aerobic activity but only about half the anaerobic activity. This difference was quite clear by the fact that the CANON sludge had 45±15% AOB and 40±15% anammox bacteria[1], while the active AOB level was 12-13 times higher than that of the anammox bacteria in the SNAP sludge.

For the OLAND process, batch tests showed an aerobic activity of 147.8±7.6 mg-N/g-VSS/d and an anoxic activity of 76.5 ± 6.4 mg-N/g-VSS/d[5].

Enumeration of nitrifiers by MPN

Results of MPN tests for seeding sludge and SNAP sludge samples are shown in Table 5. Data ranges of SNAP sludge were obtained from the samples taken on operational days 298, 344 and 442 for reactor SN-2. Table 5 also gives a comparison of MPN data of the SNAP process with those of the OLAND process. The MPN data showed that the SNAP sludge still contained high numbers of NOB together with AOB. However, under conditions of low DO, high temperature and high pH in the reactor, the NOB activity was inhibited with effluent nitrate levels being only about 10% of influent ammonium concentration (as nitrogen). This inhibition was also discussed by Yun and Kim[13]. In that study, the MPNs of AOB and NOB in nitrifying biofilm were found to be 4.7×10^5 and 3.8×10^4 per m*l*, respectively, while the nitrite content was accumulated up to 95% in the effluent. These results suggest that MPN data reflect the presence of bacteria, but not their activity in an attached-growth system. Compared to the seed sludge, the levels of AOB were higher while the levels of NOB were lower.

A sludge sample from SN-3, taken on day 344, had AOB and NOB of 9.10×10^6 and 2.55×10^6 MPN/g-VSS, respectively. Higher ammonium and nitrite concentrations in reactor SN-3 may have exhibited more inhibition to AOB, resulting in the smaller difference between the numbers of AOB and NOB. This observation is supported by the fact that the average ammonium conversion rate of reactor SN-3 was 66.5 ± 12.9%, which was lower than that of reactor SN-2.

From Table 5, the number of AOB was six orders of magnitude higher than that of NOB in the OLAND sludge, while increases of only one to three orders of magnitude were observed in the SNAP sludge. Firstly, this can be understood considering the differences in the seed sludges of the two processes. While the seed sludge in the OLAND process

Table 5 Distribution of nitrifiers in the SNAP and OLAND sludges

Bacteria	SNAP process		OLAND process[2]	
	Seed sludge	SN-2 sludge	Seed sludge	OLAND sludge
AOB, MPN/g-VSS	1.43×10^7	$2.23 \times 10^7 \sim 2.71 \times 10^9$	3.6×10^{11}	2.5×10^{10}
NOB, MPN/g-VSS	6.65×10^6	$1.40 \times 10^6 \sim 9.66 \times 10^6$	1.4×10^{11}	4.5×10^4

was inoculated with an ammonium-containing medium used to enrich nitrifiers before seeding[2], the seed sludge in the SNAP process was cultivated with a medium composed mainly of peptone and meat extract. The second reason might be in the difference between suspended-growth and attached-growth systems. The OLAND system used a suspension culture while the SNAP was an attached-immobilization system. Wash-out effects are usually more significant in suspension systems than attached-growth systems. Thus, the wash-out effects explain the relative higher proportion of NOB in the attached-growth reactor compared with the OLAND system even these microorganisms were subject to continued inhibition by free ammonia and oxygen-limited condition. This explanation was adopted from Yun and Kim[13] when they rationalized their similar results mentioned above.

16S rDNA analysis of SNAP sludge

Figure 5 shows the DDGE profile of bacterial DNA of the SNAP sludge amplified by PCR using primers 357F and 543R. The picture indicates clearly the presence of band A in all lanes and of band B in three of five lanes corresponding to various SNAP sludge samples. These common bands were cut out; DNA was extracted from gel and amplified. Then, amplified DNA was sequenced directly. Results of a homology search for the sequences of bands A and B in the NCBI database are shown in Table 6.

Initially, the existence of anammox bacteria appeared evident from the specific bands of markers. To confirm their existence, the 16S rDNA of anammox bacteria in the SNAP sludge was amplified by PCR using anammox-specific primers Ana-3' and Ana-5'. Then, the amplified DNA was directly sequenced. A homology search using NCBI BLAST for five of the sequences obtained here is presented in Table 7.

Figure 6 shows the DGGE bands of DNA fragments amplified by PCR using primer sets GM5F-907R with different DGGE denaturant gradients (20~80% and 30~60%). Results of BLAST searches in the NCBI database for sequences obtained from excised bands are summarized in Table 8. Because of failures in PCR amplification in the cycle sequencing step, result for band 3 is not displayed in Table 8.

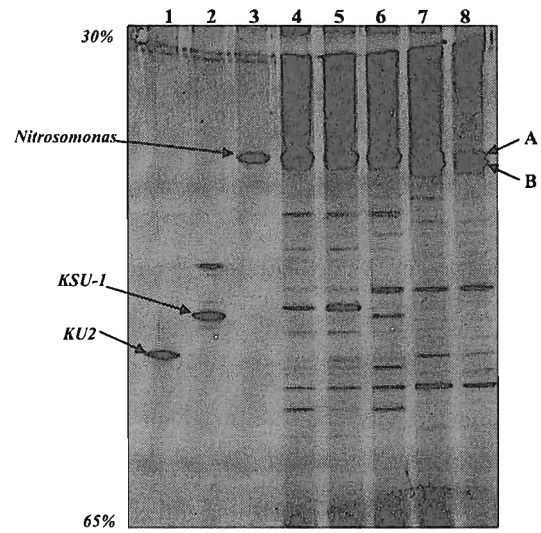

Fig. 5 DGGE profile of partial 16S rDNA amplified by PCR with primers 357F and 534R
1: KU2 Marker, 2: KSU-1 Marker,
3: Nitrosomonas Marker, 4: SN-2 (day 335),
5: SN-2 (day 344),6: SN-2 (day 442),
7: SN-3 (day 344), 8: SN-3 (day 442).

Table 6 Homology search results for sequences of bands A and B

Band	Highest homology (Accession No.)	Identity (%)	Notes from database
A	- Uncultured beta proteobacterium clone 7.18B (AF266837)	100/105 (95.2%)	Ammonia-oxidizing bacteria from a hypersaline lake
	- *Nitrosomonas europaea* (AJ245759)	99/104 (95.2%)	
B	- Uncultured bacterium clone AD-4 (AY609342)	140/145 (96.6%)	Autotrophic nitrogen removal with anaerobic granular sludge
	- Uncultured bacterium clone 1-2 (AY548931)	140/145 (96.6%)	Microbial communities of ANAMMOX sludge

Table 7 Homology search results for DNA sequences amplified with anammox-specific primers

Sample	Highest homology (Accession No.)	Identity (%)
SN-2 (day 335)	Uncultured anoxic sludge bacterium KU2 (AB054007)	104/105 (99%)
SN-2 (day 344)	Uncultured anoxic sludge bacterium KU2 (AB054007)	116/116 (100%)
SN-2 (day 442)	Planctomycete KSU-1 (AB057453)	116/116 (100%)
SN-3 (day 344)	Uncultured anoxic sludge bacterium KU2 (AB054007)	131/131 (100%)
SN-3 (day 442)	Uncultured anoxic sludge bacterium KU2 (AB054007)	105/106 (99%)

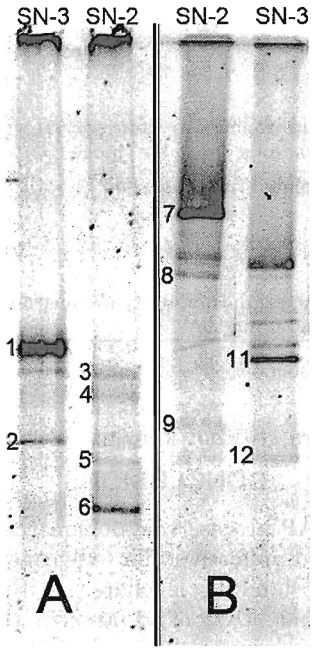

Fig. 6 DGGE profile of partial 16S rDNA amplified by PCR with primer set GM5F-907R.
A; 20-80% denaturant gradient, B; 30-60% denaturant gradient

The existence of *Nitrospira* in two samples SN-2 (day 442) and SN-3 (day 335) thereafter was confirmed by DGGE analysis following the procedure #1 with some modifications. The modifications included the uses of 30~70% denaturant gradient (instead of 30~65%) and 10% polyacrylamide gel (instead of 8%). These modifications allowed overcoming the runoff and elution of amplified DNA fragments of *Nitrospira* from the gel due to their high GC content.

All of the above results of bacterial analyses confirmed the co-existence of anammox bacteria, AOB and NOB in the SNAP sludge as suggested by the activity and MPN tests. *Nitrosomonas europaea* and two clones of *Nitrospira* sp. were identified as the bacteria responsible for oxidizing ammonium and nitrite, respectively, in the SNAP process. Different combinations of AOB/NOB have been observed in various nitrogen removal processes[10]. From results of the MPN tests, concentrations of *Nitrosomonas europaea* were always much higher than that of *Nitrospira* sp. in the SNAP sludge and the complete inhibition of *Nitrospira* sp., as desired, was not achieved.

Anammox bacteria similar to the KU2 strain were detected in all sludge samples from reactors SN-2 and SN-3 and a bacterium identified as a KSU-1 strain was detected in one SN-2 sludge sample. These anammox strains were previously detected in column reactors packed with a non-woven biomass carrier in our laboratory[8, 14]. The source of the bacteria was thought to be the tap water, originated from groundwater, used for preparation of influent. The detection of the KU2 strain as the dominant anammox bacteria in this study differed from the results of our previous study on SNAP process[6]. In this study, the SNAP process was operated for a longer time period and with higher concentration of influent ammonium and inorganic salts. These differences might have contributed to the shift in anammox populations. It was found from BLAST searches that all sequences homologizing with KU2 bacterium also homologize at same identities with planctomycete KOLL2a strain. The KOLL2a strain is an anammox bacterium detected in a rotating biological contactor treating ammonium-rich leachate[15].

Table 8 Homology search results for sequences of DNA bands in Fig 7

Band	Sample	Highest homology (Accession No.)	Identity (%)	Notes from data source
1	SN-3 (day 442)	Uncultured bacterium clone 1-2 (AY548931)	540/541 (99.8%)	Microbial communities of ANAMMOX sludge
2	SN-3 (day 442)	Uncultured bacterium DGGE gel band (AF540052)	517/549 (94.2%)	Bacterial community of a bioreactor fed with lake water
4	SN-2 (day 442)	*Nitrosomonas europaea* (BX321856)	515/531 (97.0%)	
5	SN-2 (day 442)	*Nitrospira* sp. *clone b2* (AJ224038)	447/464 (96.3%)	
6	SN-2 (day 442)	*Nitrospira* sp. *clone b30* (AJ224041)	524/533 (98.3%)	
7	SN-2 (day 442)	Uncultured bacterium clone 1-2 (AY548931)	533/541 (98.5%)	
8	SN-2 (day 442)	Uncultured bacterium clone AD- 8 (AY609346)	377/393 (95.9%)	Autotrophic nitrogen removal with anaerobic granular sludge
9	SN-2 (day 442)	Uncultured Chloroflexi bacterium (AY921747)	503/538 (93.5%)	Isolated from farm soil
11	SN-3 (day 442)	*Nitrospira* sp. *clone b30* (AJ224041)	528/537 (98.3%)	
12	SN-3 (day 442)	Uncultured bacterium (AB195873)	484/542 (89.3%)	Bacterial community of anaerobic reactor degrading VFA.

In the CANON process, neither *Nitrobacter* nor *Nitrospira* were firstly detected in sludge samples with *Nitrosomonas*-like and planctomycetes-like bacteria[1]. However, later their presence was detected and interaction and competition effects between these groups of bacteria in the CANON process were suggested[4]. In the OLAND process, the existence of small numbers of nitrite oxidizers was also assumed and then demonstrated with the presence of predominant AOB (*Nitrosomonas*-like) and anammox bacteria (close relations to *Kuenenia stuttgartiensis*)[5].

Other interesting results were obtained in the bacterial analyses of the SNAP sludge. Sequences similar to the uncultured bacterium clone 1-2 (AY548931) (band B of Fig. 5; bands 1 and 7 of Fig. 6) were repeatedly found in the SNAP sludge from both reactors. This bacterium was found in the microbial community of anammox sludge. In addition, sequences were also found having high similarities to the uncultured eubacterium clone 19 (AJ412669) that was found in the microbial community of a denitrifying reactor treating landfill leachate[16]. However, the functions of these bacteria are still not known.

CONCLUSIONS

The SNAP process was demonstrated to be stable and effective for treatment of a simulated landfill leachate. With pH of 7.5~7.8 and DO of 1.00~2.75 mg/*l*, an ammonium conversion rate of 89.2 ± 6.2% and a nitrogen removal rate of 76.3 ± 7.2% were achieved over 60 days of operation at a loading rate of 0.6 kg-N/m^3/d. With a sludge production rate of 102 mg-VSS/d or sludge yield of 0.045 mg-VSS/mg-N removed, this process would minimize the need for excess sludge disposal. In addition, use of the net-type acryl-resin fiber carrier made it possible to maintain sufficient biomass inside the reactor. The retained sludge was shown to be partially in aggregate form.

Results of aerobic and anaerobic activity tests, MPN enumeration, and DNA analyses agreed well with each other and suggested that the SNAP sludge contained three dominate kinds of bacteria: AOB (close relatives of *Nitrosomonas europaea*), NOB (close relatives of *Nitrospira* sp.) and anammox bacteria (close relatives of KU2

and KSU-1 strains). MPN results showed AOB numbers were one to three orders of magnitude higher than NOB, and activity tests indicated AOB was about one order of magnitude higher than anammox bacteria. Although most NOB might be inhibited under operational conditions used here, a reduction in numbers of NOB in the bacterial community would be desirable for improving the performance of the SNAP process.

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