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Anammox Treatment Potential in an Up-Flow Column Reactor using a Novel Acrylic Fiber Biomass Carrier

SEN QIAO[1], YINGJUN CHENG[1], ZHIJUN LIU[2], YASUNORI KAWAGOSHI[1], AYA FUJIMOTO[1], TOHICHIROH KOYAMA[3], and KENJI FURUKAWA[1]

[1] Graduate School of Science and Technology, Kumamoto University /2-39-1 Kurokami, Kumamoto, 860-8555, Japan
[2] Department of Chemical Equipment and Control Engineering, School of Chemical Engineering, Dalian University of Technology/Dalian, 116000, China
[3] NET Co. Ltd./Kohyohdai 3-6-216, Kawanishi, 666-0115, Japan

Abstract

In this study, a novel acryl fiber biomass carrier (ABC) was applied for anammox research in an up-flow column reactor. This material could retain anammox microorganism efficiently and the average weight of attached sludge in the bottom part of the reactor was calculated to be 0.14 g-TSS/cm^3 ABC. High removal efficiencies of ammonium, nitrite and TN were obtained at a high volumetric loading rate of 2.0 kg-TN/m^3/d. Furthermore, anammox activity did not decrease significantly with decreasing temperature, e.g., when the temperature decreased from 33.0℃ to 26.2℃, nitrite removal efficiencies decreased only about 5% (from 94% to 89%). Scanning electronic microphotographs revealed that the microorganisms were in a compacted state when attached on the support material. Based on the analysis of 16S rDNA, two close matches of anammox bacteria, KSU-1 and KOLL2a, were detected. This material showed potential for treatment of wastewater with low concentrations of nitrogen using a short hydraulic retention time.

Key words: Anammox, nitrogen removal, temperature, volume loading rate

INTRODUCTION

Recently, a newly discovered biologically mediated segment of the nitrogen cycle consisting of anaerobic ammonium oxidation[1,2,3] has been of increasing interest as a potentially useful means of nitrogen abatement. In this reaction, ammonium serves as the electron donor using equal molar amounts of nitrite as the electron acceptor directly resulting in production of dinitrogen gas. The potential existence of microorganisms capable of this energetically favorable reaction had been predicted three decades ago by Broda[4]. Only several years ago, an organism capable of the process was identified for the first time as a deeply branching *planctomycete*[5] and the stoichiometry of the combined catabolic and anabolic reactions was determined by Strous et al.[6] to be:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

As shown in Eq. (1), a relatively small amount of nitrate is produced, evidently from the oxidation of nitrite, by which electron equivalents are generated for the reduction of inorganic carbon into biomass[7].

However, the growth rate of anammox bacteria is extremely slow with a doubling time of about two weeks, thus the anammox

process needs a reactor system with highly efficient biomass retention. It is therefore of importance for anammox process development to find a suitable biomass support material to retain the bacteria as efficiently as possible.

The purpose of this study is to investigate the suitability of a novel acrylic fiber biomass carrier (ABC) for anammox study as a packing material in an up-flow column reactor.

MATERIAL AND METHODS

Experimental setup The reactor used in this research was constructed of polymethyl methacrylate having an inner diameter of 140 cm and height to the effluent port of 985 cm, shown in Fig. 1. The liquid volume was 17.3 *l* and the reaction zone, including influent distribution and biomass retention sections, was 15.2 *l*. Experiments were conducted at 35℃, controlled thermostatically using a hot water jacket and darkness was maintained with a black-vinyl sheet enclosure. The feeding solution was introduced from the bottom in an up-flow mode by a peristaltic pump (Eyela Co., Ltd., Tokyo). Dinitrogen gas purging was used to remove dissolved oxygen (DO) from the influent. The DO concentrations in the influent were consistently maintained below 1.0 mg/*l*. Acrylic fiber biomass carriers (ABC: NET Co., Ltd., Kawanishi, Japan) shown in Fig. 2 were used as biomass carrier, and 700 pieces of ABC were placed in the reaction zone. One ABC was approximately 1.07 g and the average volume of one ABC was about 6.0 cm^3 with a specific surface area was 146.5 m^2/m^3.

Synthetic wastewater The reactor was fed with synthetic wastewater, whose composition was shown in Table 1. In this research, $(NH_4)_2SO_4$ and $NaNO_2$ were used as the ammonium and nitrite sources, in equal molar amounts. The other influent components are shown in Table 2. All chemicals were analytical grade.

Operational conditions For starting up of the reactor, seed sludge was taken from another upflow column reactor using nonwoven as biomass carriers[7]. 6.1 g anammox sludge, which was equivalent to about 400 mg/*l* of MLSS in the reactor, was seeded.

In the first phase (A), volumetric loading rate (VLR) was increased stepwisely from 0.04 kg-TN/m^3/d to 0.27 kg-TN/m^3/d by increasing the influent concentrations as shown in Table 3. During the second phase (B), experimental strategies of decreasing the hydraulic retention time (HRT) and increasing influent concentrations were employed to

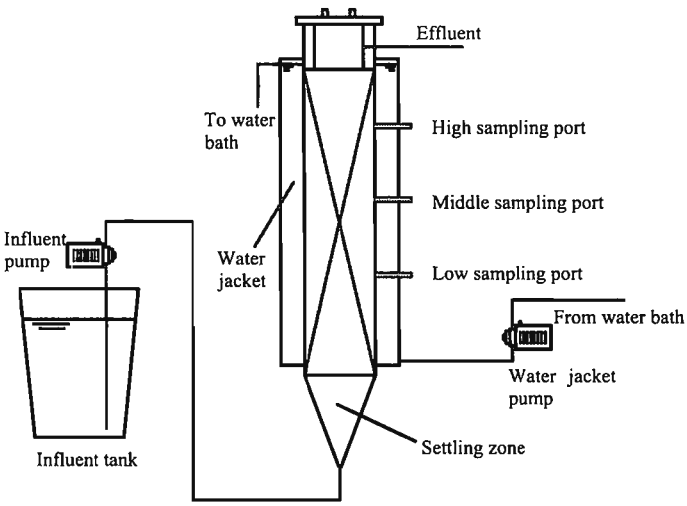


Fig. 1 Schematic diagram of up-flow anammox column reactor used in this research

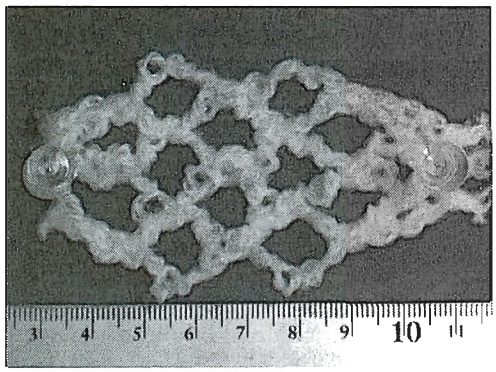


Fig. 2 Photo of acrylic fiber biomass carrier (ABC)

Table 1 Composition of synthetic medium

Compound	Concentration (mg/*l*)
$(NH_4)_2SO_4$	20-200 (as N)
$NaNO_2$	20-200 (as N)
$KHCO_3$	125
KH_2PO_4	54
$FeSO_4 \cdot 7H_2O$	9
EDTA·2Na	5
Trace element solution#	1.0 m*l*/*l*
Mine salt solution#	2.0 m*l*/*l*

Table 2 Trace element and mine salt solution compositions

Trace element solution compositions		Mine salt solution compositions	
Compound	Concentration (mg/*l*)	Compound	Concentration (mg/*l*)
$CoCl_2 \cdot 6H_2O$	0.24	$CaCl_2$	700
$CuSO_4 \cdot 5H_2O$	0.25	KCl	700
H_3BO_3	0.014	MgSO4	500
$MnCl_2 \cdot 4H_2O$	0.99	NaCl	500
$Na_2MoO_4 \cdot 2H_2O$	0.22		
Na_2SeO_4	0.11		
$NiCl_2 \cdot 6H_2O$	0.19		
$ZnSO_4 \cdot 7H_2O$	0.43		

Table 3 Operational conditions

	A-I	A-II	A-III	A-IV	A-V	B-I	B-II	B-III	B-IV	B-V
Days	1-7	8-16	17-23	24-53	54-109	110-128	129-141	142-150	151-198	199-261
Flow rates (*l*/h)	0.64	0.64	0.64	0.64	0.64	1.00	1.00	1.25	1.25	1.92
HRT (h)	23.4	23.4	23.4	23.4	23.4	15.0	15.0	12.0	12.0	7.81
TN (mg/*l*)	40.0	100	140	180	240	240	240	300	400	400
VLR (kg-TN/m^3/d)	0.04	0.10	0.16	0.22	0.27	0.41	0.48	0.60	0.80	1.20

Table 3 (continued)

	C-I	C-II	C-III	C-IV	C-V	C-VI
Days	262-269	270-277	278-308	309-347	348-364	365-392
Flow rates (*l*/h)	1.92	1.92	1.92	2.55	3.40	4.25
HRT (h)	7.81	7.81	7.81	5.88	4.41	3.53
TN (mg/*l*)	300	200	300	300	300	300
VLR (kg-TN/m^3/d)	0.90	0.60	0.90	1.20	1.60	2.00

increases in VLR for investigation of the treatment potential of ABC in an anammox application. In phase B, the VLR was increased to 1.2 kg-TN/m^3/d, while influent ammonium and nitrite concentrations finally reached to 200 mg/*l*. In the phase C, the VLR was decreased to 0.6 kg-TN/m^3/d via 0.9 kg-TN/m^3/d due to inferior performance at the end of phase B. After adaptation, VLR was once again increased in order to evaluate the tolerance and recovery capacity to VLR shock loading.

Analytical methods Ammonium was quantified based on the indophenol reaction with o-pheniphenole[8]. Nitrite was quantified by the colorimetric method[9] and nitrate by the ultraviolet spectrophotometric screening method[9]. DO was measured using a DO meter (HORIBA, pH/DO meter D-55).

For scanning electron microscopy (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 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).

DGGE using 16S rDNA was performed for bacterial community analysis. An amount of sample taken from one support material was centrifuged at 12,000×g for 5 min and this harvested cells were used for the extraction of DNA. DNA extraction and purification were performed by UltraClean Soil DNA Isolation Kit (Mo Bio, West Carlsbad, CA, USA). PCR reaction mixture was prepared with 2 µl of the extracted DNA, 10 µl of Taq-& Go ready mix (Qbiogene, Irvine, CA, USA), 1 µl of each 25µM forward and reverse primer solution. The forward primers were GM-5F[10] with GC-clamp (5'-CGCCCGCCGC GCGCGGCGGGCGGGGCGGGGGCACGGGG GGCCTACGGGAGGCTGCAG-3') and Akuf1 (5'-CCTACGGGAGGCTGCAG-3') (made by our laboratory); the reverse primers were DS907R[10] (5'-CCCCGTCAATTCCTTTGAGTT T-3') and Akur1 (5'-CCCCGTCAATTCTTTT GAGTTT-3') (made by our laboratory). PCR was performed according to hot-start[11] and touch-down[12] methods. The PCR tubes were set in PC708 thermal cycler (ASTEC, Fukuoka) and kept at 94℃ for 2 min, then the temperature was maintained at 80℃ and 2 µl of the above extracted DNA was added as a template. The reaction was continued as follows: annealing (65℃ for 30 sec.), elongation (72℃ for 1.5 min) and denaturation (94℃ for 20 sec.); the PCR cycle was repeated with annealing temperature being lowered to 55℃ in 1.0℃ increments (total of 19 cycles) and 10 cycles were continued at 55℃ for annealing. Amplified DNA was verified by an agarose gel electrophoresis using Mupid S electrophoresis system (Advance, Tokyo). DGGE gel was made by SJ-1060GF gradient gel maker (ATTO, Tokyo) with 40–50% formamide concentration gradient. Electrophoresis was performed using AE-6290E system (ATTO) at 200 V for 6 h according to the manufacturer's protocol. After electrophoresis, the gel was stained with SYBR GREEN I for 30 min and DNA bands were verified using EM-20E UV-transilluminator (UVP, Upland, CA, USA). Distinct DNA bands were cut out from the gel using sterilized plastic pipette tips. Each gel section was placed into a PCR tube and the DNA fragment was reamplified. The cycle sequence was performed with the same forward primers and DTCS Quick Start Kit (Beckman coulter, USA), and temperature conditions were as follows: 94℃(20 sec.) –57℃(30 sec.) –72℃ (1.5 min)×35 cycles. The products were purified by Ultra Clean PCR Clean-up Kit (Mo Bio). DNA sequencing was performed using DTCS Quick Start Kit and CEQ8000 (Beckman Coulter, Fullerton, CA, USA) in accordance with manufacturer's protocol. The sequences of DNA bands were compared with BLAST DNA database. The sequence alignment and phylogenetic analysis were performed using the ClustalW software[13].

RESULTS AND DISCUSSIONS

Phase A: Start up In the first period of this phase, operation was focused on successful startup. The influent flow rate was set at a low level of 0.64 *l*/h (HRT of 23.4 h) and the initial influent concentrations of ammonium and nitrite were both 20 mg/*l*. During the first 23 days, the influent nitrite levels were increased stepwisely to 70 mg/*l* and the TN removal efficiencies of more than 80% were constantly observed. The results of phase A are summarized in Fig. 3. Before the influent nitrite concentration increased to 120 mg/*l*, the nitrite removal efficiencies were stable and almost over 90%. In this stage, the average reaction ratio of NH_4-N:NO_2-N was 1:1.11, which was a little lower than the stoichiometric ratio of 1.32 determined by others[6]. The influent nitrite was then increased to 120 mg/*l* for a VLR of 0.27 kg-TN/m^3/d near the end of phase A, and the ammonium, nitrite and TN removal efficiencies were 73%, 87% and 68%, respectively.

Phase B: VLR effects After successfully establishing of the anammox process, experiments were conducted to evaluate tolerance capacity to shock loading. In this phase, the VLR was increased by adjusting influent concentrations and HRT, alternatively. During the first 40 days of this period, the VLR had increased to 0.6 kg-TN/m^3/d with a HRT of 12 h and influent TN of 300 mg/*l*. When the influent TN concentration was increased to 400 mg-N/*l* for a VLR of 0.8 kg-TN/m^3/d, it took another 40 days for the adaptation. Once the process adapted to high VLR, the ammonium, nitrite and TN removal efficiencies reached 83%, 95% and 81%, respectively. However, the effluent nitrogen

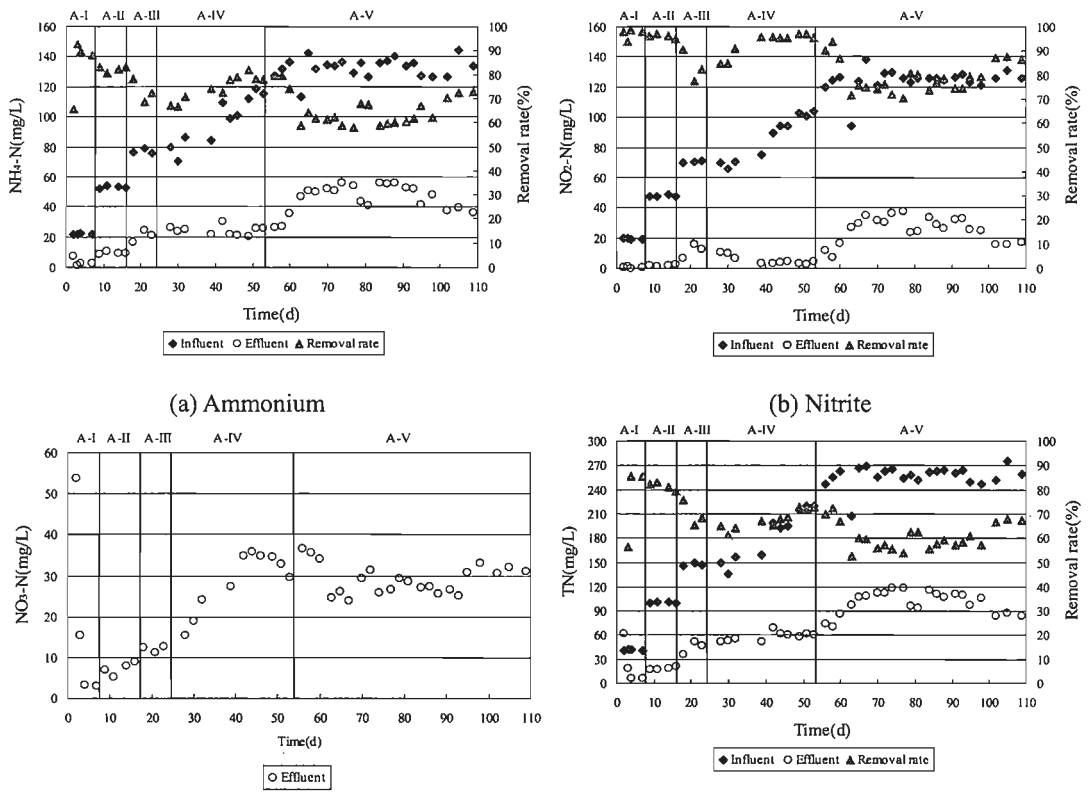


Fig. 3 Time courses of nitrogen concentrations during phase A
◆ Influent, ○ Effluent, ▲ Removal rates

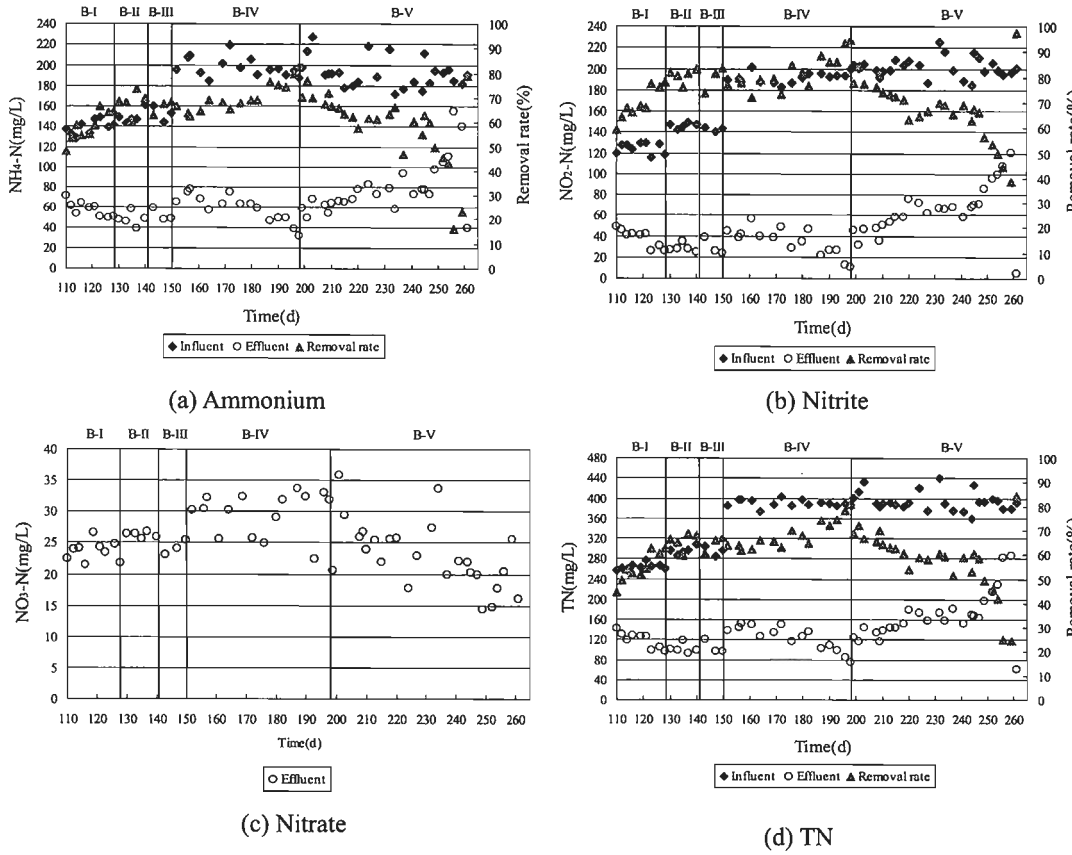


Fig. 4 Time courses of nitrogen concentrations during phase B
◆ Influent, ○ Effluent, ▲ Removal rates

concentration fluctuated during the following two months after decrease in HRT to 8.0 h (VLR of 1.2 kg-TN/m³/d). The anammox activity gradually became inhibited due to high nitrite concentrations in the reactor with the removal efficiencies of ammonium, nitrite and TN decreasing to 23%, 39% and 24%, respectively. But the removal efficiencies recovered to 79%, 97% and 84%, respectively, after one day stopping of influent. These results suggest that this kind of inhibition is reversible and anammox activity can recover given enough time for nutrients degradation.

Concentration profiles of nitrogenous compounds in the reactor at a VLR of 0.8 kg-TN/m³/d are shown in Fig. 5. From these results, it was found that most nitrogen was removed at the inlet part of the reactor. The ammonium, nitrite and TN removal

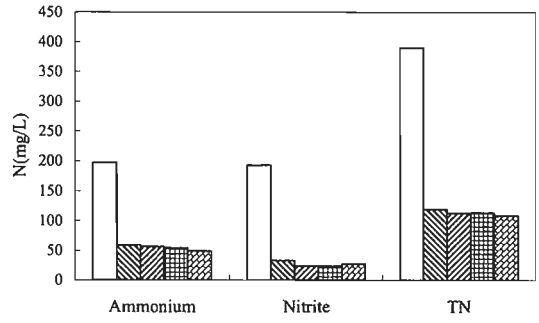


Fig. 5 Changes in nitrogen concentrations at different height level (0.8 kg-TN/m³/d)
□ Influent, ▨ Low port, ▨ Middle port, ▦ High port, ▨ Effluent

efficiencies at the inlet part of the reactor were 70%, 83% and 70%, respectively, while the removal rates in the effluent were 75%, 86% and 72%, respectively. Consequently, it is apparent that most of the anammox activity was concentrated in the inlet part, which could be attributed to the high filling density and good absorption capacity of the support material.

Phase C: Recovery capacity Due to inferior performance in the previous phase, the VLR was reduced to 0.9 kg-TN/m^3/d at the beginning of phase C by decreasing influent ammonium and nitrite concentrations from 200 mg/*l* to 150 mg/*l*. And the VLR decreased to 0.9 kg-TN/m^3/d from 1.2 kg-TN/m^3/d, TN removal efficiency increased to 34%, only 10% higher than that of 1.2 kg-TN/m^3/d, not as expected. So the VLR was decreased to 0.6 kg-TN/m^3/d by further reducing the influent ammonium and nitrite to 100 mg/*l* each. After another seven days of operation, the anammox activity began to improve greatly. The VLR was restored to 0.9 kg-TN/m^3/d, and the removal efficiencies of ammonium, nitrite and TN increased to 78%, 90% and 71%, respectively. Subsequently, the influent concentrations were kept consistent and VLRs were increased by decreasing HRT. On the basis of accumulation of sufficient anammox biomass, the VLRs increased again to 1.20 kg-TN/m^3/d and then to 1.6 kg-TN/m^3/d in a short time, with maintaining nitrite removal efficiencies as high as 95% and 83%, respectively. In addition, the nitrogen removal efficiencies were not fluctuated after the VLR was increased to 2.0 kg-TN/m^3/d. During the following 28 days of operation for this VLR,

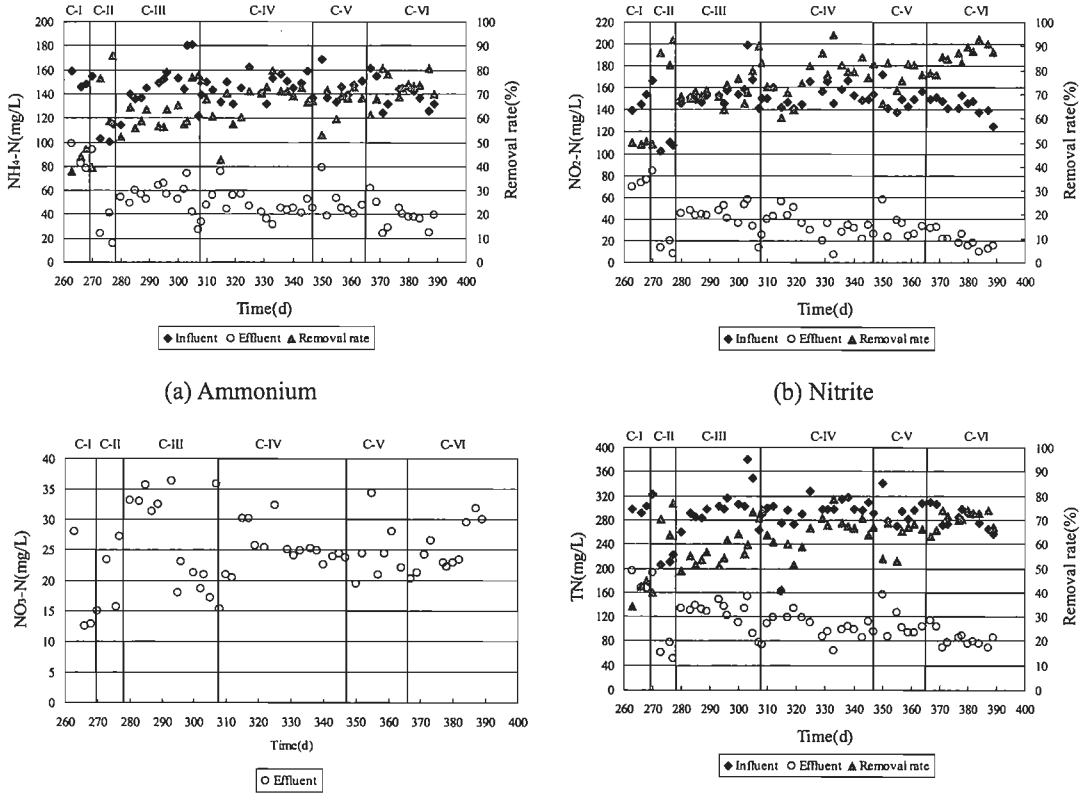


Fig. 6 Time courses of nitrogen concentrations during phase C
◆ Influent, ○ Effluent, ▲ Removal rates

the ammonium, nitrite and TN removal efficiencies as high as 74%, 93% and 73%, were obtained, respectively.

Temperature effect on anammox performances After achieving stable T-N removal efficiencies at a high VLR of 2.0 kg-TN/m³/d, the effects of temperature were evaluated by varying reactor temperatures from 15.3℃ to 33.0℃. Figure 7 shows the changes in nitrite removal rates at different height levels under various operational temperatures. After wastewater passed the low and middle parts of the reactor, almost 50% and 70% of the nitrite were removed, respectively, and the nitrite removal efficiencies were kept fairly stable, especially in the low part of the reactor. This could be attributed to the thick layer of anammox biomass accumulated in the bottom and middle parts of the reactor. It was suggested that high enough anammox biomass concentration would reduce the effect of temperature and benefit the stable nitrogen removals. With decreasing temperature, the anammox activity was not greatly affected with a high nitrite removal efficiency of 89% at 26.2℃. This nitrite removal efficiency was only 5% lower than that obtained at 33.0℃. When reactor was operated without temperature control, the nitrite removal efficiency decreased to 60% at ambient temperature of 15.3℃. However, the nitrite removal efficiency recovered to 80% within one day after increasing reactor temperature to 23℃. This result indicated that anammox activity can quickly recover from low operational temperature conditions. Furthermore, similar results were observed for the removal profiles of ammonium and TN.

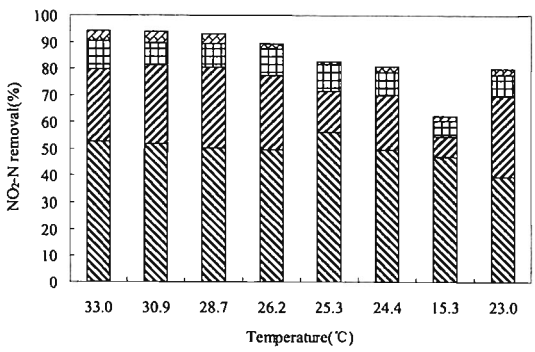
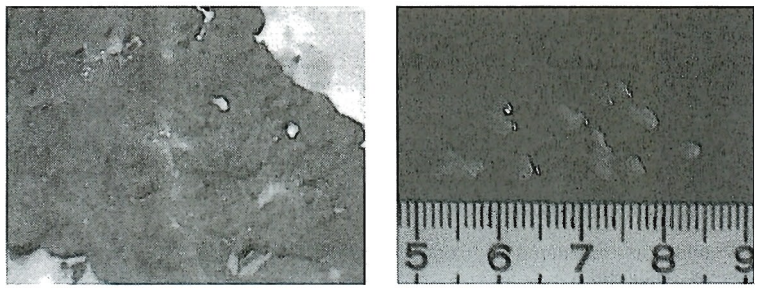


Fig. 7 Effect of temperature on nitrite removal efficiencies in different height levels under VLR of 2.0 kg-TN/m³/d
▨ Low port, ▨ Middle port,
▦ High port, ▨ Effluent

For Fig. 5 (on day 190), the slow growth rate of anammox biomass would make most anammox microorganism concentrating at the bottom part since seeding and long HRT of 12 h will also benefit anammox bacteria of bottom part for degrading nutrients. While results in Fig. 7 were determined on day 390. It was possible for anammox biomass to expand inside the reactor, but the short HRT of 3.53 h made it difficult to degrade most nitrogen only by bottom part. Therefore, the extended anammox biomass in middle part worked and caused the difference between Fig. 5 and Fig. 7.

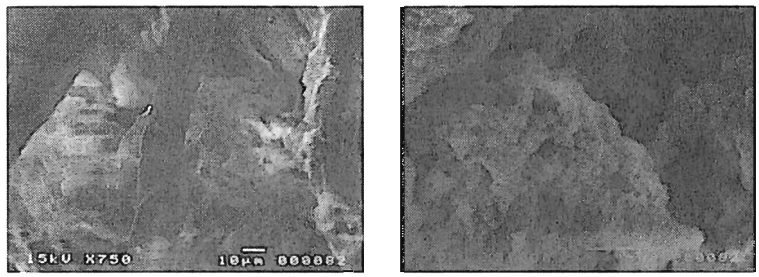
Anammox sludge Figure 8 shows the attached anammox sludge on ABC. Compared with the original appearance of ABC, a large amount of anammox bacteria was attached on the support material, on both the inside and the outside of ABC. Granular sludge formed in the inside of ABC was shown in Fig. 8 (b). The extraordinary merit of this kind material for stable retaining of anammox sludge was clearly shown. Figure 9 shows the scanning electron micrographs (SEM) of anammox sludge on ABC. From these SEM photographs, it was found the microorganisms attached on the support materials in a compacted state. On day 284, all the carriers was taken out of the reactor and five pieces of ABC were chose randomly for determining the weight of biomass. The average weight of attached biomass was determined to be 0.035 g-TSS/cm³ ABC. On day 390, with the same means, other five pieces of ABC from the bottom part samples were used for measuring the biomass weight on the carriers. The average weight was calculated as 0.14 g-TSS/cm³ ABC.

DNA Analysis At first GM5F-DS907R[10] were tried for anammox bacteria identification, but failed. Later we designed the primer set of Akuf1-Akur1 for specific detection of anmmox bacteria consulting other



(a) (b)

Fig. 8 Anammox sludge attach-immobilized on the acrylic biomass carrier



(a) (b)

Fig. 9 SEM photograph of anammox sludge attached on ABC

references[5,14]. So both primers were applied for anammox bacteria identifying in that time. On the support material, two close matches to anammox bacteria were detected by the phylogenetical analysis of 16S rDNA sequences. One was KOLL2a (AJ250882) and the other was KSU-1(AB057453). KOLL2a was first isolated from a rotating disk contactor in Switzerland and is quite similar to *Candidatus B. anammoxidans*, though it exhibits higher tolerances to phosphate (up to 20 mM) and to nitrite (up to 13 mM) and was active at lower cell densities. Its optimum pH was reported to be 8.0 and optimum temperature was 37℃[14]. KSU-1 was discovered from a laboratory-scaled reactor using a polyester nonwoven biomass carrier in Japan; by fluorensence imaging, KSU-1 has been shown to form spherical clusters wrapped in a thin layer of *Zoogloea* sp.[15]. Furthermore, the KSU-1 strain was found dominated in the sludge from Single-stage Nitrogen removal using Anammox and Partial nitritation (SNAP) process[16]. Table 4 showed all the strains in this reactor.

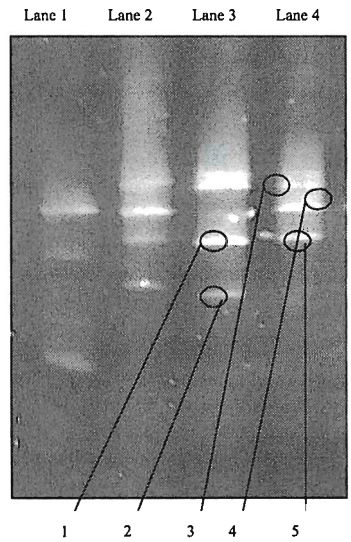


Fig. 10 DGGE profile of partial 16S rDNA amplified by PCR

Note: Lane 1 and Lane 3 were samples from the sludge in the settling zone and support material in the bottom area of reactor, respectively, using the same primer of Akuf1-Akur1; Lane 2 and Lane 4 were samples from the sludge in the settling zone and support material in the bottom area of reactor, respectively, using the same primer of GM5F-DS907R.

Table 4 Results of PCR analysis of bacterial community

Band	Homology (Accession No.)	Identidy (%)
1	Anaerobic ammonium-oxidizing palnctomycete KOLL2a partial 16S rRNA gene (AJ250882)	325/331(98%)
	Candidatus Kuenenia Stuttgartiensis 16S ribosomal RNA gene (AF375995)	322/351(97%)
2	Planctomycete KSU-1 gene for 16S rRNA (AB057453)	367/370(99%)
	Candidatus Kuenenia Stuttgartiensis 16S ribosomal RNA gene (AF375995)	364/370(98%)
3	Beta proteobacterium Rufe9b 16S rRNA gene (AY235688.1)	485/518(93%)
	Beta proteobacterium Rufe9 16S ribosomal RNA gene (AY235687.1)	485/518(93%)
4	Anaerobic filamentous bacterium GOMl-1 gene for 16S rRNA (AB243672)	448/503(89%)
	Anaerobic filamentous bacterium KOME-1 gene for 16S rRNA (AB243673)	433/489(88%)
5	Dehalococcoides sp. BHI80-15 16S rRNA gene (AJ431246)	363/425(85%)
	Caldilinea aerophila gene for 16S rRNA (AB067647)	359/425(84%)

CONCLUSIONS

The novel acrylic fiber biomass carrier (ABC) demonstrated a high anammox sludge retention capacity in an up-flow column-type reactor. At a high VLR of 2.0 kg-TN/m^3/d, TN removal efficiency as high as 73% was obtained. With a decrease in reactor temperature, nitrogen removal efficiencies did not decrease sharply. And even without temperature control, ammonium, nitrite and TN removal efficiencies of 48%, 60% and 48%, respectively, were obtained at ambient temperature of 15.3℃. In addition, the removal efficiencies of ammonium, nitrite and TN increased to 64%, 80% and 64%, respectively, within one day later after increasing operational temperature to 23℃. Through the SEM observation, it was found that the anammox sludge had a quite compact state when attached on the support material and the average weight of attached biomass in the bottom part of the reactor was revealed to be 0.14 g-TSS/cm^3 ABC. DNA analysis revealed the existence of two close matches of anammox bacteria identified as KSU-1 and Koll2a. This new acrylic resin fiber biomass carrier also has a high potential for nitrogen removal of wastewaters with relatively low levels of nitrogen under a high loading rate.

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