Korean J. Chem. Eng., **25**(6), 1448-1455 (2008)

Nitrifying bacterial communities and its activities in aerobic biofilm reactors under different temperature conditions

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(*Received 18 February 2008 • accepted 13 April 2008*)

Abstract−Tests were performed to investigate nitrifying bacterial communities and activities in aerobic biofilm reactors with different temperature conditions, denaturing gradient gel electrophoresis (DGGE) based on polymerase chain reaction targeting 16S rRNA and *amoA* gene, fluorescence *in situ* hybridization (FISH) and dehydrogenase activity (DHA). T1, T2 and T3 reactors operated at different temperatures (5, 10 and 30 °C, respectively) were set up in the thermostat and acclimated. Nitrification was considerably limited in T1 and T2 reactors. DGGE revealed specific genera of ammonium-oxidizing bacteria (AOB) and some *Nitrosomonas* genera endured at the low temperatures. FISH revealed a decreased distribution ratio between AOB and nitrate-oxidizing bacteria at 5 °C, and showed that the decrease of AOB also affected the nitrification failure in the aerobic biofilm reactor. The mean attached biomass of the T1, T2 and T3 reactors was 69.6, 80.6 and 112.9 mg/L, respectively, and the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride dehydrogenase activity of the respective reactors was 73.6, 87.4 and 134.2 mgO_2^*/g VSS/day. The results demonstrate that a low temperature condition in an aerobic biofilm reactor decreases the attached biomass, distribution ratio and activity of nitrifying bacteria, and produces a change in the composition of the AOB species, which results in the failure of nitrification.

Key words: Biofilm, Nitrification, Temperature, Denaturing Gradient Gel Electrophoresis, Fluorescence *In Situ* Hybridization, INT-Dehydrogenase Activity

INTRODUCTION

A suspended growth reactor system consists of three basic components: a reaction tank to keep biomass in suspension, a clarifier to separate biomass from the treated wastewater, and a recycle system for returning solids from the clarifier back to the reaction tank [1,2]. Compared with the suspended growth reactor, the attached growth reactor does not need the clarifier and recycle system, making it convenient to operate since the bulking problem in the clarifier is avoided. The suspended growth reactor is problematic for the nitrification process because of the lower growth rate of nitrifying bacteria, which consist of ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), compared to heterotrophic bacteria [3,4]. The most common and effective method to overcome this problem is to use the attached growth reactor, whose relevant microorganisms can be maintained independently of hydraulic retention times (HRT) [3,5,6].

The influence of temperature on the nitrification process is important for the design and operation of the attached growth reactor. The van't Hoff-Arrhenius equation has been used to estimate the impact of temperature change on the nitrification rate in the suspended growth reactor [7,8]. For the attached growth reactor, however, it is difficult to address the effects of temperature on nitrification kinetics because the process is also influenced by other temperature-dependent phenomena and parameters [9]. Zhu and Chen [4] reported that the impact of temperature on the attached growth reactor is not as significant as that predicted by the van't Hoff-Arrhenius equation, and diffusion mass transport plays an important role in the nitrification process in the attached growth reactor. The impact of temperature change on nitrification in this reactor remains poorly understood.

Temperature affects nitrification in two opposite ways. First, nitrification rates decrease as the temperature drops below the optimal temperature. Above the optimal temperature, nitrification rates decrease as enzymatic proteins denature [10]. Secondly, as temperature drops, nitrification rates increase because dissolved oxygen (DO) also increases. Ødegaard and Rusten [11] reported that under oxygen-limiting conditions the dependence of nitrification does not significantly affect removal rates at different temperatures. This is due to the fact that at lower temperatures the nitrification rate is reduced, but at the same time the DO concentration increases. Lazarova et al. [12] and Hur et al. [13] reported that the maximum nitrification rate measured for completely developed biofilms and the transition value of the bulk DO concentration was 5.2 mg/L and 5.0 mg/L, respectively. Therefore, to investigate the temperature effects only on the aerobic biofilm reactors, different temperatures should be used above 5.0 mg DO/L.

Only active biomass is responsible for nitrification and organic material removal, rather than the total biomass present in biofilm [14-16]. Therefore, it is important to understand the nitrifying bacterial community depending on the different temperature conditions of the aerobic biofilm reactors. Moreover, AOB play an important role in wastewater treatment [17], as they are responsible for the first and frequently rate-limiting step in the process of nitrification.

Most microorganisms cannot be cultured by conventional spread-plate or most-probable-number count approaches of bacteriological enumeration [18,19]. Indeed, only 0.5%-10% of prokaryote biodi-

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versity has actually been identified [20]. The advent of culture-independent, molecular biological techniques such as polymerase chain reaction (PCR)-based approaches has changed our historical perception of microbial diversity. One approach that has been successfully used is denaturing gradient gel electrophoresis (DGGE) of PCR-amplified genes, which can be used to evaluate the diversity of complex microbial systems [21,22].

DGGE studies of AOB diversity can be classified into two approaches. One is based on 16S rDNA. The major problem with this approach is the limited specificity of commonly used primers [23, 24]. The other approach is to use a translated gene as a molecular marker [25-27]. Rotthauwe et al. (1997) developed a primer pair (AmoA-1F and AmoA-2R), which amplify a 491 bp fragment of the ammonia monooxygenase subunit A gene (*amoA*). This gene is present in all autotrophic AOB and is believed to contain enough information to make phylogenetic inferences based on its sequence [23,28].

Understanding the microbial distribution of AOB and NOB in sludge flocs or biofilms is important in interpreting the sequential oxidations of NH_4^+-N and NO_2^--N to NO_3^--N with coupled oxygen mass transfer to the microorganisms [29]. The fluorescence *in situ* hybridization (FISH) technique using rRNA-targeted oligonucleotide probes, a non-PCR-based method, has been utilized and proven to be an effective method for the analysis of microbial distribution [30, 31].

Like other biochemical reactions, microbial nitrification activity is affected by temperature. Biodegradation of an organic compound proceeds through a series of oxidation reactions involving loss of electrons or removal of hydrogen atoms from organic compounds. The process of removal of hydrogen atoms from an organic compound is called dehydrogenation. Enzymes that catalyze dehydrogenation reactions are called dehydrogenases. If the number of dehydrogenases in the biodegradation can be measured, microorganism activity can be determined [32]. Bitton and Koopman [33] developed a tetrazolium reduction assay to determine the physiological activity of filamentous microorganisms in activated sludge, which involves the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan (INTF) by the active bacterial electron transport system; the procedure is named the INT-dehydrogenase activity (DHA) test. This method of measuring DHA is quite easy to perform and is very sensitive. A close correlation has been reported between INT-DHA and oxygen consumption [34-36]. In this study, DHA concentration was used to indicate microorganism activity.

Because of these concerns, we operated the aerobic biofilm reactors at three different temperature conditions (5, 10 and 30 °C). DGGE based on the 16S rRNA and *amoA* approaches was performed to investigate the community composition of AOB. For the quantitative analysis of nitrifying bacteria, FISH was also utilized. INT-DHA analysis would be expected to provide the activity of microorganisms influenced by temperature.

EXPERIMENTAL

1. Reactor Operation

The experimental apparatus consists of three aerobic biofilm reactors with a fixed-bed (Fig. 1). Each reactor was filled with ceramic media whose packing ratio was 0.15. The effective volume of each reactor was 5.3 L. All reactors were set up in the each thermostat to maintain a constant temperature. The reactors were inoculated with activated sludge obtained from a municipal sewage treatment plant and were acclimated with raw wastewater, whose characteristics are shown in Table 1. The hydraulic retention time of all reactors was 6 h.

Nitrification is very limited when wastewater temperature is below 10 °C [37]. Therefore, to investigate the effect of temperature, reactors designated T1, T2 and T3 were maintained with a temperature of 5, 10 and 30 °C, respectively. Initially, the reactors were acclimated at 5.0 mg O_2/L and 20 °C for 60 days, during which time they acquired nitrification efficiencies above 80%. Thereafter, the reac-

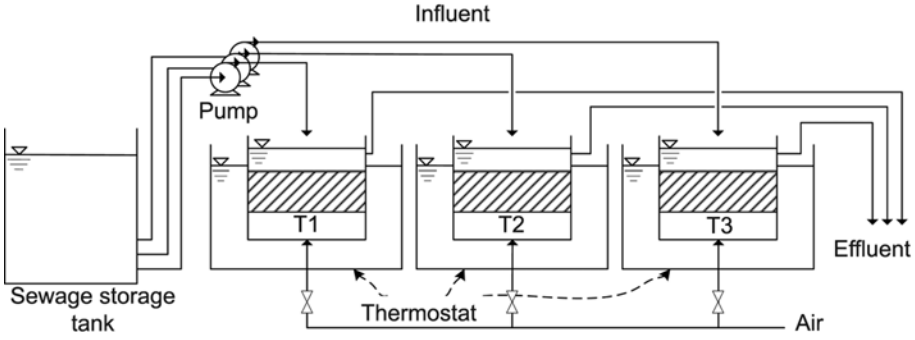

Fig. 1. Schematic diagram of the aerobic biofilm reactors with different temperatures.

Table 1. Characteristics of the raw wastewater

pH	$TCOD_{Cr}$ (mg/L)	BOD_5 (mg/L)	$SCOD_{Cr}$ (mg/L)	SS (mg/L)	NH_4^+-N (mg/L)	PO_4^{3-}-P (mg/L)	Alkalinity ($mgCaCO_3$/L)
7.09-7.32 (7.28)[a]	104.5-175.5 (135.0)	52.5-70.4 (69.2)	65.9-136.6 (97.1)	43.6-58.9 (51.7)	26.3-39.9 (33.7)	2.4-3.2 (2.8)	210-340 (279)

[a]( ) is mean value during the operating days

tors were operated with the particular temperature for 100 days.

2. DNA Extraction and Amplification

DNA samples were extracted from the aerobic biofilm reactors. Samples were centrifuged at 10,000×g for 1 min and the supernatant was removed and resuspended in 1 mL of distilled water. PowerSoil™ DNA kit (Mo Bio Labs, Carlsbad, CA) was used to extract DNA according to the manufacturer's instructions. The electrophoretic profile of the DNA products was determined by using a 1% agarose gel. The extracted DNA was amplified by PCR using a Mastercycler gradient automated thermal cycler (Eppendorf, Hamburg, Germany). Tables 2 and 3 summarize the primers used in this study and PCR conditions, respectively. The reactions were performed in a 25 μL volume containing 1 μL of template DNA, 0.25 μL of the forward and reverse primers (10 pmol), 2.5 μL of 10×Taq buffer, 10 μL of 10 mM dNTP, and 0.125 μL of DNA polymerase (Solgent, Daejeon, Korea). PCR products were checked electrophoretically as described above, and were purified by using a PCR purification kit (Bioneer, Alameda, CA).

3. DGGE and Sequencing

DGGE was performed by using 16s rDNA gene fragments and *amoA* to characterize the microbial communities of AOB in the reactors. PCR products were separated with a DCode system (Bio-Rad, Hercules, CA). Samples were loaded onto a 6% polyacrylamide gel in 0.5×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3). A denaturant gradient ranging from 20-50% denaturant (100% denaturant constituted 7 M urea and 40% v/v formamide) was used. Gels were run at 60 °C for 8 h at a constant voltage of 200 V and then were stained for 30 min in ethidium bromide (Bio-Rad). The band profile was identified with an ultraviolet transilluminator (Uvitec, Cambridge, UK). DGGE bands were excised with a sterile pipette tip and transferred to 30 μL TE buffer. To resuspend DNA, each sample was heated at 60 °C and frozen at −20 °C three times for 15 min. The resuspended DNA was reamplified by using the same primers. After purification, PCR products were sequenced with an ABI 3730XL capillary DNA sequencer (Applied Biosystems, Franklin Lakes, NJ) and an ABI Prism™ Bigdye™ terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystems).

4. Oligonucleotide Probes

The following rRNA-targeted oligonucleotides were used: EUB 338, Nso190, Nsv 443, Nsm 156, Ntspa662 and Nit3. Oligonucle-

Table 2. PCR primers used in this study

Primer	Sequence(5'-3')	Target	Reference
CTO 189F	GGA GRA AAG CAG GGG ATC G	*β*-AOB	[8]
CTO 654R	CTA GCY TTG TAG TTT CAA ACG C		
CTO 189F-GC Clamp[a]	GC Clamp - GGA GRA AAG CAG GGG ATC G		
amoA 1F	GGG GTT TCT ACT GGT GGT	AOB	[38]
amoA 2R-TC	CCC CTC TGC AAA GCC TTC TTC		
amoA 1F-GC Clamp[a]	GC Clamp - GGG GTT TCT ACT GGT GGT		
M13F	AGT CAC GAC GTT GTA	pUC vector	[39]
M13R	CAG GAA ACA GCT ATG AC		

[a]The GC Clamp added for DGGE-PCR: 5'-CGC CGC GCG GCG GGC GGG GCG GGG GC-3'

Table 3. PCR conditions used in this study

Primer	PCR Conditions
CTO 189F(GC Clamp)/654R	1 min 93 °C, followed by 39 cycles of 1 min at 92 °C 1 min at 57 °C, 45 s at 68 °C followed by a 5 min final extension at 68 °C
amoA 1F(GC Clamp)/2R-TC	5 min 94 °C, followed by 42 cycles of 1 min at 94 °C 90 s at 60 °C, 90 s at 72 °C followed by a 10 min final extension at 72 °C
M13 F/R	9 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 55 °C, and 2 min at 72 °C, followed by 10 min final extension at 72 °C

Table 4. Oligonucleotide probes

Probe	Specificity	Sequence(5'-3')	Target site[a]	% FA[b]	[NaCl] (mM)[c]	Reference
EUB338(II)	Eubacteria	GCAGCCACCCGTAGGTGGT	338-355	20	215	[40]
Nso190	Ammonia-oxidizing *β-proteobactria*	CGATCCCCTGCTTTTCTCC	190-208	20	215	[41]
Nsv443	*Nitrosospira* spp.	CCGTGACCGTTTCGTTCCG	444-462	30	102	[41]
Nsm156	*Nitrosomonas* spp.	TATTAGCACATCTTTCGAT	156-174	5	630	[41]
Ntspa662	*Nitrospira* spp.	GGAATTCCGCGCTCCTCT	662-679	20	215	[42]
Nit3	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	1035-1048	40	46	[43]

[a]16S rRNA position according to *Escherichia coli* numbering

[b]Formamide concentration in the hybridization buffer

[c]Sodium chloride concentration in the washing buffer

otides were synthesized and fluorescently labelled with fluorescein isothiocyanate (FITC), a hydrophilic sulfoindocyanine dye (CY3) and rhodamine red at the 5' end by CoreBioSystem (Seoul, Korea). All probe sequences, the hybridization conditions and references are given in Table 4.

5. FISH

The biofilms of each reactor were vortexed strongly with 0.1% sodium pyrophosphate solution and fixed by immersing in freshly prepared paraformaldehyde solution (4% in phosphate buffered saline) overnight at 4 °C. Thereafter, the biofilms were rinsed with phosphate buffered saline. Each sample was immobilized on a gelatin coated slide glass. The sample was finally dehydrated by successive passage through ethanol solution, and air-dried. The fixed samples were hybridized by spiking sequentially 8 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.2, 0.01% sodium dodecyl sulphate) and 2 μL of fluorescent probes, and then quickly transferring them to pre-warmed (46 °C) moisture chamber. Finally, the slide was dipped into the washing solution at 48 °C. After the hybridization, digital images of the aggregates were taken with a Zeiss Axioskop 2plus fluorescence microscope (Carl Zeiss, Hamburg, Germany) and visualized through Zeiss Axiovision digital imaging software. Analyses were performed with the Carl Zeiss Imaging Solution system.

6. INT-DHA test

The general method of Koopman et al. [34] was used. A 0.2% (w/v) solution of INT (Sigma-Aldrich, St. Louis, MO) was prepared by using deionized, filter-sterilized water. Triplicate 5 mL samples were amended with 1.0 mL of INT, incubated in the dark at room temperature (20±2 °C) for 30 min and fixed with 1.0 mL of 37% formalin. Treated samples were centrifuged at 2,500 rpm for 10 min and excess water was removed. Pellets were extracted with 5 mL 2+3 acetone/dimethyl sulfoxide for 20 min in the dark, after which the extracts were centrifuged for 10 min and optical density of the supernatants determined at 465 nm. INT-DHA activity was calculated in equivalent oxygen (O_2^*) units by the following equation:

$$DHA = 905 D_{465}\, \nu / VCtF \quad (1)$$

Where DHA=INT-DHA activity (mgO_2^*/g VSS/day), D_{465}=optical density through a path length of 1.0 cm, ν=final solvent volume (mL), V=volume of INT-treated sample used in the extraction procedure (mL), C=initial biomass concentration of the sample (g VSS/L), t=incubation time (min) and F=factor to adjust for dilution caused by adding INT and formalin.

Specific inhibitors of nitrification, ATU (allyl-thiourea) and $NaOCl_3$, were used to distinguish the activities of *Nitrosomonas* spp., *Nitrobactor* spp., and heterotrophic bacteria, respectively. $NaClO_3$ inhibited total nitrifying activity, whereas ATU inhibited only *Nitrosomonas* spp. Accordingly, the fractional DHA of nitrifying bacteria and heterotrophic bacteria can be calculated as follows [44,45]:

$$H = (B-D)/(A-D) \quad (2)$$

$$NS = [(A-D)-(C-D)]/(A-D) \quad (3)$$

$$NB = [(C-D)-(B-D)]/(A-D) \quad (4)$$

Where H, NS and NB=the factional DHA of heterotrophic bacteria, *Nitrosomonas* spp. and *Nitrobacter* spp., respectively, A=absorbance of unamended sample at 480 nm, B=absorbance of samples incorporating ATU and $NaClO_3$ at 480 nm, C=absorbance of sample with added ATU at 480 nm and D=absorbance of sample amended with 37% formalin at 480 nm.

7. Analytical Methods

Influent and effluent samples for each reactor were tested within three days of sampling. The pH and DO concentrations were measured by using a model 230A pH meter (Orion Research, Pittsburgh, PA) and a model 58 DO meter (YSI, Yellow Springs, OH), respectively. The soluble chemical oxygen demand (SCOD), NH_4^+-N, NO_3^--N and NO_2^--N concentrations were measured after filtering the sample through a 0.45 μm membrane filter with an AA3 auto analyzer (Bran+Luebbe, Norderstedt, Germany). Total chemical oxygen demand (TCOD), alkalinity and suspended solids (SS) were measured by Standard Methods (APHA, 2005). The stored samples were kept refrigerated at 4 °C until tested.

RESULTS AND DISCUSSION

1. COD removal and Nitrification Efficiencies

The SCOD removal and nitrification efficiencies of each reactor are given in Figs. 2(a) and 2(b), respectively. Shown in Fig. 2(a), the low temperature conditions of 5 °C and 10 °C led to a decrease in SCOD removal. The mean effluent SCOD concentrations of the T1, T2 and T3 reactors were 54.8, 48.6 and 36.4 mg/L, respectively,

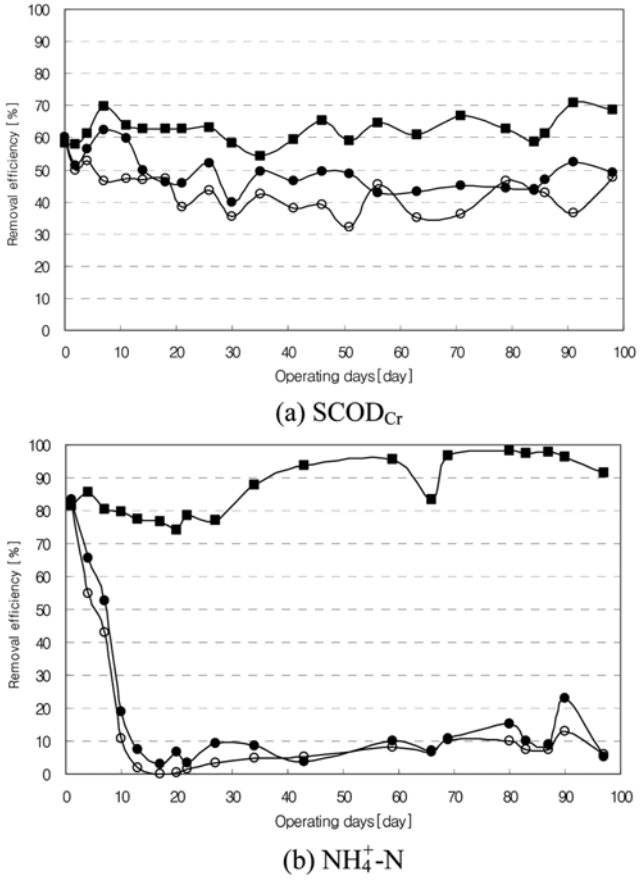

Fig. 2. Removal efficiencies of the each reactor with different temperature conditions. T1 (○), T2 (●) and T3 (■).

and the mean SCOD removal efficiencies were 43.3%, 49.3% and 62.4%, respectively. Concerning the relationship between BOD_5 and COD (Table 1), residual SCOD matters were assumed to hardly biodegradable matters. The non-biodegraded SCOD of 18.4 mg/L and 12.2 mg/L of the T1 and T2 reactors, respectively, were not removed by the low temperature. Therefore, it assumed that heterotrophic bacteria were influenced by low temperature.

Fig. 2(b) displays the nitrification efficiencies of each reactor. The mean nitrification efficiencies of T1, T2 and T3 reactors were 14.8%, 18.5% and 86.8%, respectively, and the mean effluent concentrations of NH_4^+-N were 29.2, 27.9 and 4.2 mg/L, respectively. Nitrification was considerably limited when wastewater temperature was below 10 °C. It is clear that nitrifying bacteria are more sensitive than heterotrophic bacteria.

2. DGGE

The studies on 16S rDNA explained that physiological and ecological differences exist between the *β*-proteobacteria AOB genera and clusters. The major problem with the 16S rDNA approach is the limited detection of AOB belonging to *β*-proteobacteria. However, the enzyme-encoding *amoA* is present in all AOB. Thus, the *amoA* approach is feasible to detect AOB.

DGGE was run on both 16S rDNA and *amoA* amplicons from each sample to reveal the differences in band pattern (Fig. 3). Samples were selected from the seeded sludge on operation days one, 20 and 45. The band patterns for *amoA* amplicons are generally more complex than the band patterns obtained by the 16S rDNA amplicons [46]. Presently, we acquired more clear and complex DGGE profiles using *amoA* amplicons than 16S rDNA amplicons (Fig. 3).

As apparent in the DGGE profiles using CTO primers, the TCD band detected in seeded sludge disappeared after acclimation and, over time, TCB and TCC bands appeared. It is assumed that the AOB in the TCB and TCC bands are the microorganisms related to the attached growth reactor, whereas the AOB in the TCD band represent microorganisms related to the suspended growth reactor. The TCC band was *Nitrosomonas* sp. NM 59 (Table 5) belonging to the *Nitrosomonas oligotropha* cluster, which are found in low ammonium aquatic environments. The TCB band was similar to uncultured *Nitrosomonas* genus. This *Nitrosomonas* genus was considered as the AOB that endured at low temperature, because it was detected in both the T1 and T2 reactors.

Fig. 3(b) displays the DGGE profiles based on *amoA* primers. AOB species in the biofilms changed little with differing temperatures. However, *Nitrosospira multiformis* (TAE band) was detected.

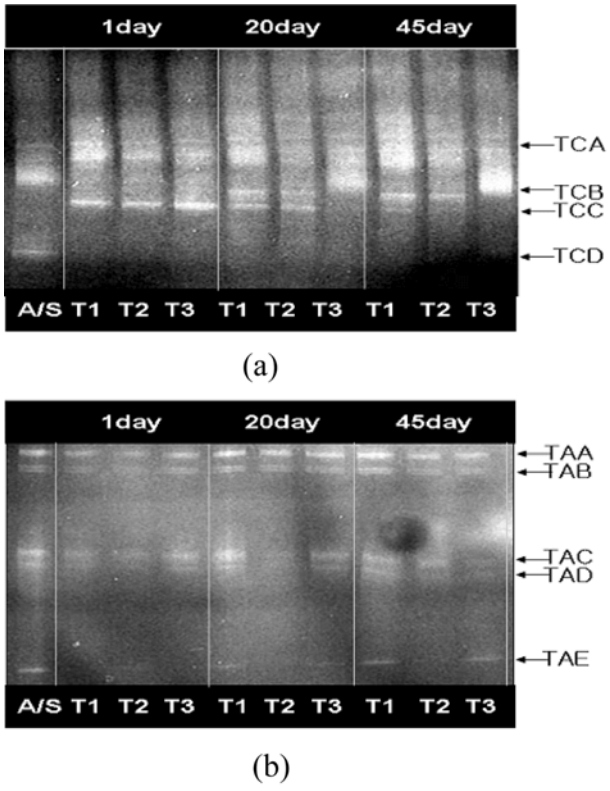

Fig. 3. DGGE profiles. The profiles are based on (a) 16S rDNA with CTO primers and (b) *amoA* gene with *amoA* primers; A/S: seeded sludge, T1, T2 and T3: each reactor.

Typically, scanty amounts of *Nitrosospira* genus are present in wastewater treatment systems [47]. This is consistent with the idea that *Nitrosospira* genus is not an influential AOB in the nitrification process. However, this cannot be concluded rigorously, since *Nitrosospira* could be detected only through the *amoA* approach, not 16S rDNA approach.

3. Distribution of Microorganisms Using FISH

To investigate the distribution ratios of microorganisms, FISH was performed by the combination of two probes. The distribution ratios of AOB (probe Nso190), *Nitrobactor* spp. (probe Nit3) and *Nitrospira* genus (probe Ntspa662) were calculated by the relative distribution of eubacteria (EUB338(II)). Also, those of *Nitrosomonas* spp. (probe Nsm156) and *Nitrosospira* spp. (probe Nsv443) were

Table 5. Sequences of extracted DGGE bands

DGGE band[a]	Accession No.	Closest species in GenBank	Similarity (%)
TCA	AY543074	Uncultured *Nitrosomonas*	97
TCB	AF527015	Uncultured *Nitrosomonas*	99
TCC	AY123811	*Nitrosomonas* sp. Nm59	96
TCD	AF495429	Uncultured bacterium	98
TAA	DQ911633	Uncultured AOB	95
TAB	AF293069	Uncultured AOB	98
TAC	DQ911630	Uncultured AOB	98
TAD	DQ911630	Uncultured AOB	95
TAE	U91603	*Nitrosospira multiformis* ATCC 25196	98

[a]Name of DGGE band in Fig. 3.

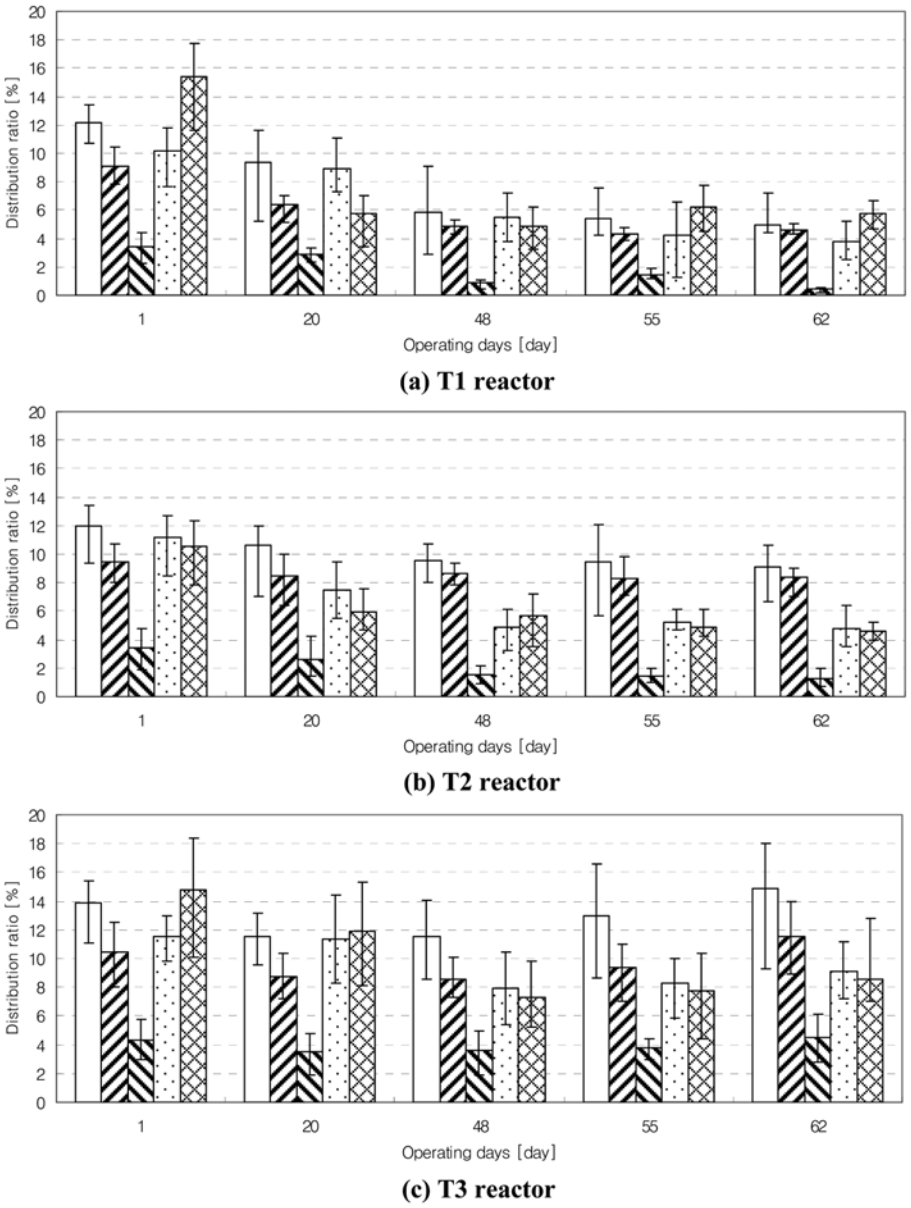

Fig. 4. Distribution ratios in each reactor. AOB (□), *Nitrosomonas* spp. (▨), *Nitrosospira* spp. (▧), *Nitrobacter* spp. (▦) and *Nitrospira* spp. (▩).

calculated by the relative distribution of AOB (probe Nso190). Fig. 4 shows the bacterial distribution ratios of each reactor operating at the different temperatures. Initially, all the target bacterial communities had similar distribution ratios.

AOB oxidize ammonia to nitrite and are important microorganisms in the control of nitrification [48] The distribution ratios of AOB in the T1, T2 and T3 reactors were 12.2%, 12.0% and 13.9%, respectively, on day 1 of operation. By day 62, the distribution ratios of T1 and T2 reactors had decreased to 5.0 and 9.1%, respectively. However, the distribution ratios of T3 at day 62 increased to 14.8%. The mean ratios of AOB in all three reactors from day 20 were 6.4%, 9.7% and 12.7%, respectively. These results are consistent with the idea that a temperature under 10 °C facilitated the decrease in the AOB distribution ratios, and further that the decrease of AOB also affects nitrification failure in aerobic biofilm reactors.

The composition of AOB communities consisting of *Nitrosomonas* spp. and *Nitrosospira* spp. was also investigated. The distribution ratio of *Nitrosomonas* spp. was higher than that of *Nitrosospira* spp. in all three reactors. At day 62 of reactor operation, the distribution ratios of *Nitrosomonas* spp. in AOB of the T1, T2 and T3 reactors were 92.0%, 92.1% and 77.6%, respectively. Initially, we assumed that *Nitrosospira* spp. are more sensitive than *Nitrosomonas* spp. at lower temperatures. Comparison of these results with those obtained using DGGE revealed that *Nitrosomonas* spp. is more important than *Nitrosospira* spp. in the nitrifying biofilm reactor.

In the case of NOB, the mean distribution ratio of *Nitrobacter* spp. in the T1, T2 and T3 reactors on day 20 of operation was 5.6%, 5.6% and 9.2%, respectively, and that of *Nitrospira* spp. was 5.7%, 5.3% and 8.9%, respectively. In this study, the higher temperature led to a higher distribution ratio. However, the higher temperature did

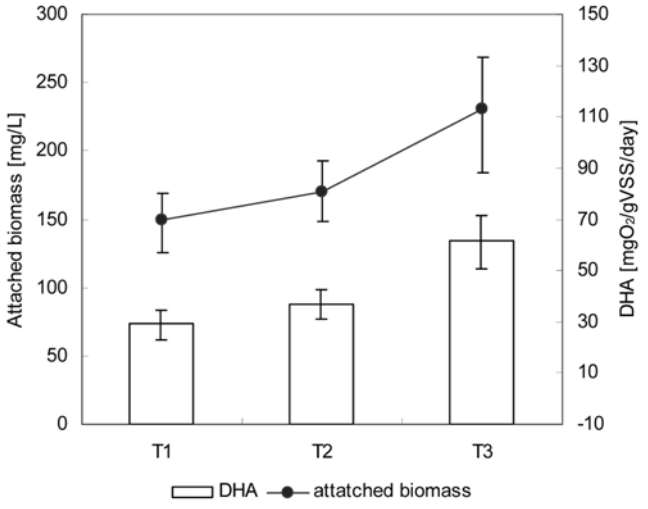

Fig. 5. Relationship between DHA and attached biomass in each reactor.

not lead to a shift in dominance of *Nitrobacter* spp. and *Nitrospira* spp. because their distribution ratios were similar for all three reactors.

4. INT-DHA

Fig. 5 shows the relationship between the INT-DHA activity and the attached biomass in each reactor. Attached biomass [mg/L] was calculated from the biomass [g] vortexed with 0.1% sodium pyrophosphate solution and detached from some ceramic media, divided by effective volume of the reactor [L]. In considering the relationship between attached biomass and nitrification efficiency, only active biomass influences nitrification efficiency [12-14,16]. In this study, the mean attached biomass of the T1, T2 and T3 reactors was 69.6, 80.6 and 112.9 mg/L, respectively, and INT-DHA of each reactor was 73.6, 87.4 and 134.2 mg O_2^*/g VSS/day, respectively. Hur et al. [13] reported that a relatively lower DO condition leads to higher INT-DHA activity, because filamentous microorganisms affect the INT-DHA activity [34-36]. However, in this study, no filamentous microorganisms were detected according to the temperature variation. Indeed, as the reactor was operated at lower temperatures, attached biomass and DHA simultaneously decreased (Fig. 5). These decreases fueled the failure of nitrification.

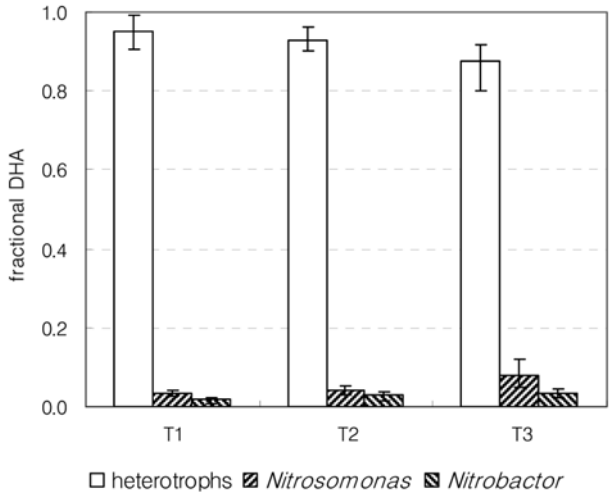

Fig. 6. Fractional DHA in each reactor.

Fig. 6 depicts the fractional DHA of heterotrophic microorganisms and nitrifying bacteria calculated by Eqs. (2)-(4). The fractional DHA of heterotrophs in the T1, T2 and T3 reactors was 0.950, 0.930 and 0.877, respectively. That of *Nitrosomonas* spp. was 0.033, 0.041 and 0.079, respectively, and that of *Nitrobacter* spp. was 0.017, 0.029 and 0.034, respectively. In the reactors operating at lower temperature, the fractional DHA of heterotrophic bacteria increased, whereas that of nitrifying bacteria decreased.

CONCLUSIONS

For the biological nitrogen removal process, the attached growth reactor system is valuable because the constituent nitrifying bacteria, which grow more slowly than heterotrophic bacteria, can be maintained independently of the hydraulic retention time. The influence of temperature, especially the microbial diversity and activity according to the temperature change, in the attached growth reactor has remained poorly understood. To investigate the nitrifying bacterial community and activity in aerobic biofilm reactor with different temperature conditions, DGGE, FISH and INT-DHA methods were performed.

We demonstrate that nitrification is considerably limited when the wastewater temperature is below 10 ºC. DGGE reveals that specific AOB genera are available in the aerobic biofilm reactors compared with seeded sludge, and some *Nitrosomonas* genera endure at low temperature. In this study, *Nitrosospira* could be detected only using the *amoA* approach.

FISH analyses show that a reactor temperature under 10 ºC decreases the distribution ratios of AOB and NOB, and that the AOB decrease affects the nitrification failure. *Nitrosospira* spp. are more sensitive than *Nitrosomonas* spp. at a lower temperature and their distribution ratio is markedly lower than that of *Nitrosomonas* spp. Therefore, *Nitrosomonas* spp. is likely more important than *Nitrosospira* spp. in aerobic biofilm reactors.

As the reactor is operated at a lower temperature, attached biomass, DHA and the fractional DHA of nitrifying bacteria simultaneously decrease. Considering all of the results, we conclude that a low temperature in the aerobic biofilm reactor causes decreases in attached biomass, distribution ratio and activity of nitrifying bacteria, and facilitates an altered AOB species composition. These phenomena cause the failure of nitrification in aerobic biofilm reactors.

ACKNOWLEDGMENT

This work was supported by grant No. R01-2003-000-11640-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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