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Laboratory assessment of biofilm process and its microbial characteristics for treating nonpoint source pollution

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Abstract−A biofilm process with the attached bacterial growth onto ceramic media was applied to remove carbonaceous and nitrogenous pollutants from nonpoint water source. The packing ratios of ceramic media were 0.05 and 0.15 (v/v). Thereafter, the reactors were operated intermittently in sequencing batch mode with different cycle periods: 0, 5, 10 and 15 d. The COD and NH_4^+-N removal efficiencies were investigated under different operating conditions, such as media packing ratio, temperature and interevent period. Additionally, polymerase chain reaction (PCR)-denaturing gel gradient electrophoresis (DGGE) and INT-dehydrogenase activity (DHA) tests were conducted to observe the microbial community and activity in the biofilm. Consequently, the removal efficiency of the organic matter after 8 h remained stable, even with longer interevent periods, regardless of the packing ratio. The interevent period and packing ratio seemed to have no significant influence on the COD removal efficiency. However, stable nitrification efficiency, with longer interevent period, was only achieved with a packing ratio of 0.15. Therefore, a packing ratio above 0.15 was required to simultaneously achieve stable COD removal and nitrification efficiency. The DGGE profiles revealed that the prevalent microorganism species were changed from that of the seeded activated sludge into those detected in the sediments. Due to the prevalence of microorganisms related to the sediment, their activities did not decrease, even after a 15 d interevent period.

Key words: Biofilm, INT-dehydrogenase Activity, Microbial Community, Nonpoint Source, PCR-DGGE

INTRODUCTION

In terms of the discharge characteristics into the watershed, water pollution can be divided into two types: point source (PS) pollution and nonpoint source (NPS) pollution. Compared with PS, NPS is more hydrological, intermittent and complicated, is also difficult to control, due to its large variety of sources and prevalence, with the possibility of occurrence at any time from any type of land use [1,2]. For these reasons, NPS is associated with more legislative and infrastructural issues at a national level prior to investment activities. Recently, regulations and investigations relating to NPS pollution have become an important issue in Korea, as NPS is responsible for about 22-37% of BOD river pollution [3].

There are a variety of best management practices (BMPs) currently used to reduce NPS pollution. However, there are currently no methods being used that combine conventional wastewater treatment technologies and pollution prevention methods for the management of NPS pollution.

Typical technologies for wastewater treatment include suspended and attached growth systems. Under a suspended growth system, the microorganisms are freely mobile in the liquid, which provides direct contact between the bacteria cells and bulk water. In an attached growth system, however, the microorganisms are grown in a viscoelastic layer of biofilm attached to the surface of the carrier. Thus, this process is also called a biofilm process, where the individual bacteria are immobilized [4]. The suspended growth system consists of three basic components: a reaction tank to maintain a suspension, a clarifier to separate the biomass from the treated wastewater, and a recycle system for returning solids from the clarifier back to the reaction tank [5,6]. Compared with the suspended growth system, the attached growth system does not need the clarifier and recycle system, making it possible for treating NPS pollutants as they do not occur continuously but intermittently. Thus, in this study, a biofilm process was introduced for treating NPS pollutants for the purpose of combining conventional wastewater treatment technologies and NPS pollution managements.

NPS pollution occurs when it rains, with the problem being that the biofilm process has to be maintained in an empty state when it does not rain. Therefore, it is important that the microorganisms attached to the biofilm carrier in an empty vessel remain active.

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 hydrogen atom removal 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, the microorganism activity can be determined [7]. Bitton and Koopman [8] 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

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(DHA) test. This method of measuring DHA is quite easy and very sensitive. A close correlation has been reported between INT-DHA and oxygen consumption [9-11]. In this study, the DHA concentration was used to indicate the microorganism activity.

Most microorganisms cannot be cultured by conventional spread-plate or most-probable-number count approaches for bacteriological enumeration [12,13]. Indeed, only 0.5-10% of prokaryote biodiversity has actually been identified [14]. 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 [15,16]. Thus, PCR-DGGE based on 16S rRNA was performed to investigate the microbial characteristics in an intermittently operated biofilm process.

Because of these concerns, the biofilm process was operated for different interevent periods (0, 5, 10, 15 d), with INT-DHA and PCR-DGGE analysis then performed.

MATERIALS AND METHODS

1. Reactor Operation

Two reactors, with effective volumes of 5.3 L, were set up with a thermostat to maintain a constant temperature of 25 °C and were filled with ceramic media with packing ratios of 0.05 and 0.15 (v/v), respectively. Fig. 1 describes the operation of the reactors through four phases: injection, aeration, discharge and idle. The reactors were inoculated with activated sludge obtained from a municipal sewage treatment plant, and acclimatized continuously for 20 d. Table 1 shows the concentrations of the synthetic wastewater, which was selected to reflect the characteristics of NPS pollutant [17,18]. Table 2 presents the operating conditions used in this study. As shown, the reactors were operated intermittently: 5, 10 and 15 d. To investigate the COD removal and nitrification efficiencies with different packing ratios, the reactor was operated under conditions A and B. After conducting experiments of two conditions, we operated the reactor under condition C to evaluate the effect of temperature.

Table 2. Operating conditions in this study

Condition	Packing ratio (%)	Temperature (°C)	Interevent period (d)	DO (mg/L)
A	5	25		
B	15	25	0, 5, 10, 15	5.0±0.2
C	15	10		

Table 3. PCR primers used in this study

Primer	Sequence (5′-3′)	Target
*EUB*10-F	AGAGTTTGATCMTGGCTCAG	16s rDNA
*EUB*1400-R	ACGGGCGGTGTGTACAAG	
*EUB*341-F	CCTACGGGAGGCAGCAG	16s rDNA
*EUB*518-R	ATTACCGCGGCTGCTGG	

GC Clamp, CGC CGC GCG GCG GGC GGG GCG GGG GC was attached to *EUB*341-F for DGGE

Lazarova et al. [19] and Hur et al. [20] reported that the maximum nitrification rate measured for a completely developed biofilm and the transition value of the bulk DO concentration were 5.2 and 5.0 mg/L, respectively. Therefore, to exclude the effect of the DO concentration, all tests were performed at 5 mg DO/L.

2. DNA Extraction and Amplification

DNA samples were extracted from aerobic biofilm reactors, and centrifuged at 10,000 ×g for 1 min, with the supernatant removed and the pellet then resuspended in 1 mL of distilled water. A PowerSoil™ DNA kit (Mo Bio Labs. Inc., CA) was used to extract DNA, according to the manufacturer's instructions. DNA products were checked by their electrophoresis profile using 1% agarose gel. The extracted DNA was amplified by PCR using an automated thermal cycler (Mastercycler gradient, Eppendorf, Germany). Tables 3 and 4 summarize the *EUB* primers used in this study, and the PCR conditions, respectively. The use of *EUB* primers enables the detection of predominant bacteria species. The reactions were carried out in a volume of 25 μL containing 1 μL of template DNA, 0.25 μL of the forward and reverse primers (10 pmol), 2.5 μL of 10×Taq

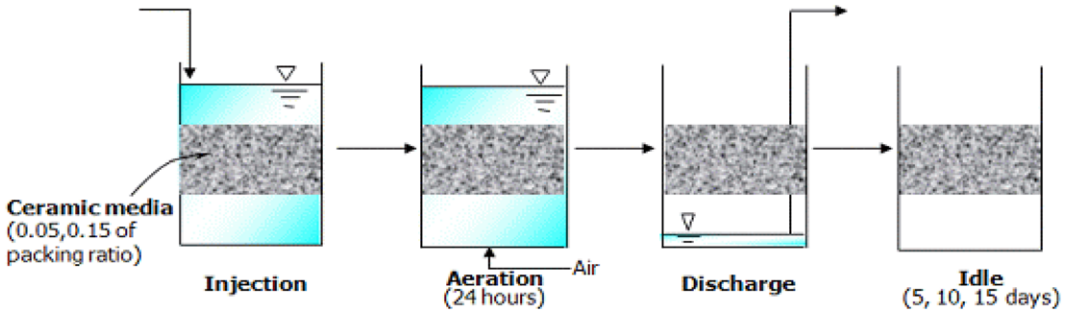

Fig. 1. Operating procedure of the biofilm reactor for treating non-point source pollutant.

Table 1. Characteristics of the synthetic wastewater used in this study

Item	pH	COD_{Cr} (mg/L)	NH_4^+-N (mg/L)	Alkalinity (mg $CaCO_3$/L)
Concentration	7.9-7.7 (7.8)[a]	56.9-63.4 (61.2)	7.1-10.1 (9.4)	97.8-105.4 (102.3)

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

Table 4. PCR conditions used in this study

Primer	PCR conditions
*EUB*10-F/*EUB*1400-R	9 min 95 °C, followed by 35 cycles of 1 min at 95 °C 1 min at 53 °C, 2 min at 72 °C followed by a 10 min final extension at 72 °C
*EUB*341-F/*EUB*518-R	9 min 95 °C, followed by 30 cycles of 1 min at 95 °C 1 min at 53 °C, 2 min at 72 °C followed by a 10 min final extension at 72 °C

buffer, 10 μL of 10 mM dNTP and 0.125 μL of Taq DNA polymerase (Solgent Co., Korea). PCR products were checked by their electrophoresis profile using 1% agarose gel. PCR products were purified with a PCR purification kit (Bioneer Co., Korea).

3. DGGE and Sequencing

DGGE was performed using 16s rRNA gene fragments to characterize the microbial communities in the reactors. PCR products were separated by using 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% to 50% denaturant (100% denaturant is 7 M urea plus 40% v/v formamide) was used. Gels were run at 60 °C for 8 h at a constant 200 V, stained for 30 min in ethidium bromide (Bio-Rad, Hercules, CA), and the band profile identified with a UV transilluminator (Uvitec gel documentation system, UK). DGGE bands were excised with a sterile pipette tip and transferred to 30 μL of TE buffer. To resuspend the DNA, it was heated at 60 °C and frozen at –20 °C three times for 15 min. The resuspended DNA was re-amplified using the same primers. After purification, the PCR products were sequenced with an ABI 3730XL capillary DNA sequencer (Applied Biosystems Co., USA) and ABI prism™ Bigdye™ Terminator cycle sequencing Ready reaction kit (Ver. 3.1, USA).

4. INT-dehydrogenase Activity Test

The general method of Koopman et al. [8] was used for the INT-dehydrogenase activity test. A 0.2% (w/v) solution of INT (Sigma-Aldrich Co., USA) was prepared using deionized, filter-sterilized water. 5 mL triplicate 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 removed. Pallets 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, with the optical density of the supernatants determined at 465 nm.

5. Analytical Methods

The influent and effluent samples for each reactor were tested within 3 d of sampling. The pH and dissolved oxygen concentration were measured with an Orion Research pH meter (Model230A, USA) and a YSI DO meter (Model58, USA), respectively. The soluble chemical oxygen demand (SCOD), NH_4^+-N, NO_3^--N and NO_2^--N concentrations were measured, after the sample was filtered through a 0.45 μm membrane filter with an auto-analyzer (AA3, Bran+Luebbe, Germany). Alkalinity and suspended solids (SS) were measured using Standard Methods [21]. The stored samples were kept refrigerated at 4 °C until tested.

RESULTS AND DISCUSSION

1. COD Removal

Fig. 2 shows the COD concentration according to the reaction time for different interevent periods. After 6 h, the mean COD removal efficiencies under conditions A and B were 76 and 73%, respectively. COD removal rate is higher in condition A because the acclimation period is different between conditions A and B. However, COD concentrations after 8 h with interevent periods of 0, 5, 10 and 15 d were 9.3, 13.5, 12.0 and 11.8 mg/L under condition A, respectively, and 13.5, 12.6, 9.6 and 11.9 mg/L under condition B, respectively. After 8 h, no significant COD removal was observed, and the COD concentrations after 12 h had similar values, regardless of the interevent period. It was shown that the removal efficiencies of organic matter remained stable, despite longer interevent periods. These results suggest the COD removal is not strongly related to the packing ratio or interevent period. Therefore, the most cost-effective packing ratio for COD removal in this removal would be

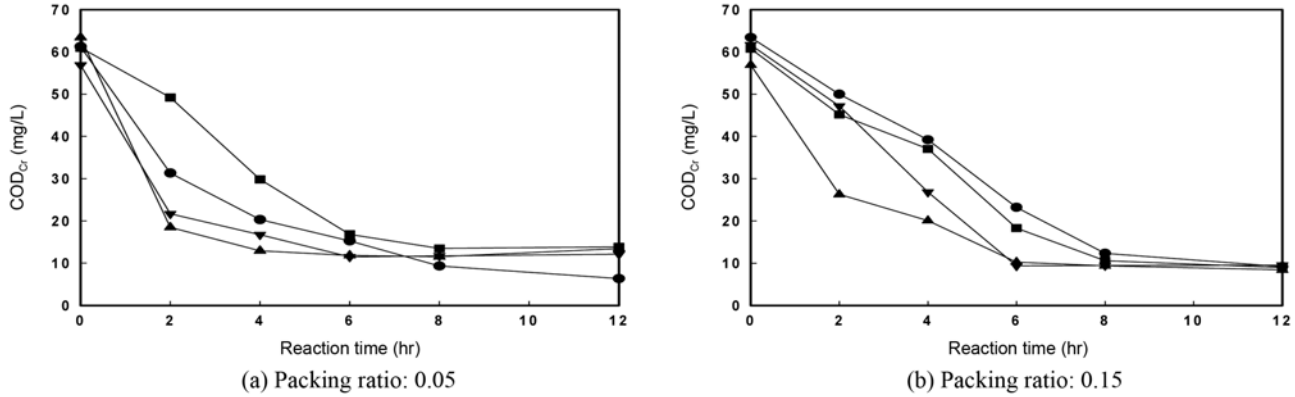

Fig. 2. Variation in the COD_{Cr} concentration according to the reaction time for different interevent periods; 0 d (●), 5 d (▲), 10 d (■) and 15 d (▼).

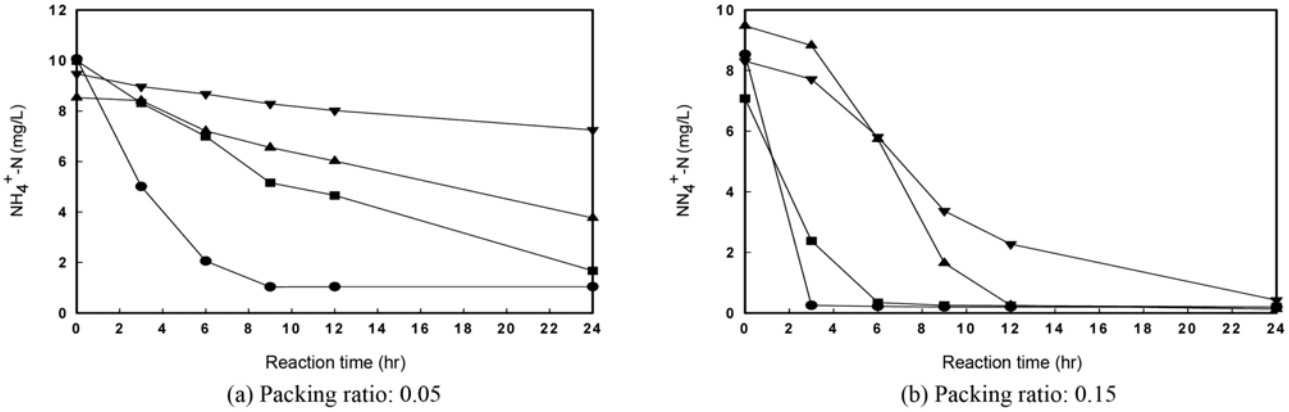

Fig. 3. Variation in the NH_4^+-N concentration according to the reaction time for different interevent periods; 0 d (●), 5 d (■), 10 d (▲) and 15 d (▼).

0.05.

2. Nitrification

Fig. 3 shows the nitrification efficiency with reaction time under conditions A and B. The NH_4^+-N concentrations after 24 h with interevent periods of 0, 5, 10, 15 d were 1.0, 1.7, 3.8 and 7.3 mg/L under condition A, respectively, and 0.2, 0.2, 0.3 and 0.9 mg/L under condition B, respectively. These results indicate that the interevent period and packing ratio are very important factors for achieving stable nitrification efficiency. Under condition A, the nitrification efficiency decreased the longer the interevent period. However, under condition B, while the nitrification rate decreased the longer the interevent period, the NH_4^+-N concentrations after 24 h were similar, regardless of the interevent period. Therefore, a packing ratio above 0.15 is required to achieve simultaneous stable COD removal and nitrification efficiency.

3. Effect of Temperature

The influence of temperature on the nitrification process is important in the design and operation of an attached growth reactor. The van't Hoff-Arrhenius equation has been employed to estimate the impact of temperature change on the nitrification rate in a suspended growth reactor [22,23]. For an attached growth reactor, however, it is difficult to address the effects of temperature on the nitrification kinetics because the process is also influenced by other temperature-dependent phenomena and parameters [24]. Zhu et al. [25] reported that the impact of temperature on the attached growth reactor was not as significant as that predicted by the van't Hoff-Arrhenius equation, but diffusion mass transport does play an important role in an attached growth reactor process.

Fig. 4 shows the variations in the COD and NH_4^+-N concentrations under condition C performed to evaluate the effect of temperature on the pollutant removal efficiency. After a reaction time of 24 h, the COD concentrations for interevent periods of 0, 5, 10 and 15 d were 12.7, 23.6, 18.8 and 35.9 mg/L, respectively, and NH_4^+-N concentrations were 0.4, 0.3, 4.8 and 11.0 mg/L, respectively. As shown in Fig. 4(a), a reaction time of at least 15 h was required to accomplish stable COD removal at 10 °C. The nitrification efficiencies after 24 hs for interevent periods of 0, 5, 10 and 15 d were 96.1, 96.9, 56.3 and 4.8%, respectively. These results indicate that a low temperature has a greater influence on nitrification than on COD removal; nitrification is considerably limited with interevent periods above 10 d. Park et al. [26] reported that a low temperature in an aerobic biofilm reactor causes decreases in attached biomass, distribution ratio and activity of nitrifying bacteria, as well as facilitating an altered ammonia-oxidizing bacteria (AOB) species composition. In this study, it also became clear that the nitrifying bacteria were more sensitive to temperature than were heterotrophic bacteria.

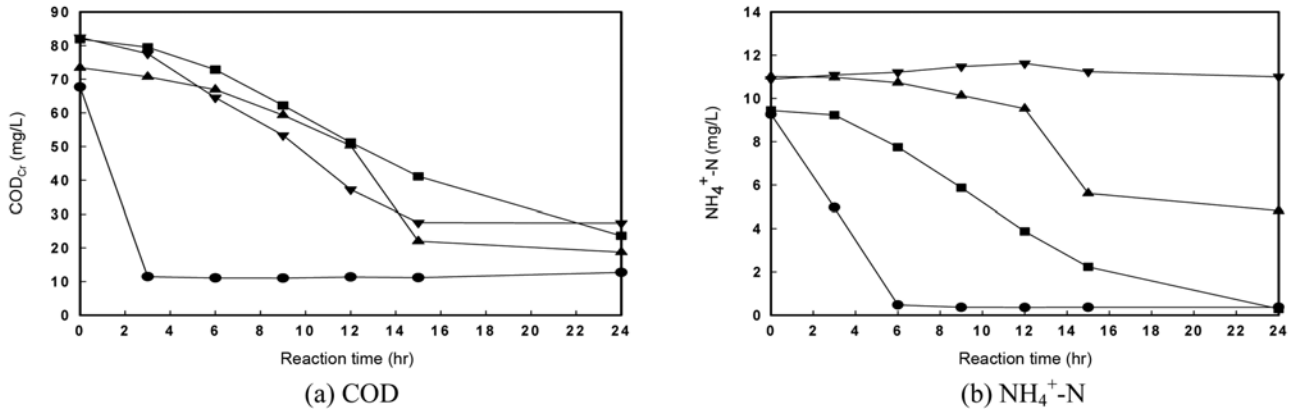

Fig. 4. Variation in the (a) COD_{Cr} and (b) NH_4^+-N concentrations according to the reaction time for different interevent periods under condition C; 0 d (●), 5 d (■), 10 d (▲) and 15 d (▼).

4. DGGE

Fig. 5 shows the ceramic media used in this study, which was used for an interevent period of 15 d under condition B. As shown in Fig. 5(b), the attached biomass of the media surface was dry when the interevent period of 15 d had passed. Fig. 6 presents the DGGE profiles obtained using 16S rDNA with *EUB* primers. Seven bands consisted of the main bacterial population, showing a more diverse community in initial DGGE profile. As the interevent period was longer, a significant shift in population structure was denoted by appearance of new bands. As shown in Fig. 6, bands N-1 and N-7 were primary microorganism in the first operation. With a longer interevent period, however, these bands decreased. Thereafter, bands N-4 and N-5 strengthened in intensity. According to the 16S rDNA sequence analysis shown in Table 5, band N-1 was most closely related to the *Pseudomonas fluorescens*, which is found in cultivated soils and sewage sludge [27]. Many studies have also indicated its presence within the marine environment [28,29]. Band N-3 was *Bacillus funiculus* strain MXC 3-4-2, which is found in soil [30]. Band N-7 was primarily detected in activated sludge, which was injected as a seeded activated sludge. Thus, in the initial condition of reactor operation, microorganisms detected in soil and activated sludge existed simultaneously. However, as the interevent period increased, diversity of microbial community decreased. Bands N-1, N-2, N-3 and N-7 disappeared with longer interevent periods and bands N-4 and N-5 became prevalent in the reactor with longer interevent periods, and were *bacterium* DR2A-7G21 and uncultured bacterium, respectively, which are primarily found in sediment [31]. These results indicate that as the interevent period increases, the bacteria related to sediment become prevalent, whereas bacteria related to activated sludge and soil decreased. It is apparent that the interevent period changed the indigenous microbial community greatly.

(a) (b)

Fig. 5. Photographs of (a) ceramic media and (b) ceramic media with attached biomass after a 15 d interevent period.

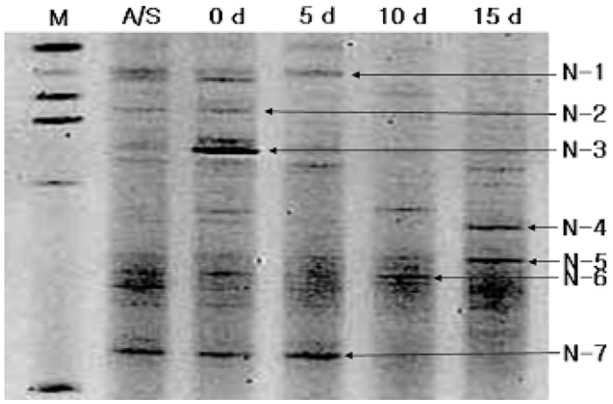

Fig. 6. DGGE profiles of 16S rDNA gene fragments; A/S: seeding sludge, M: DNA ladder, 0 d, 5 d, 10 d and 15 d: resting period.

5. INT-DHA

The most important consideration for a biofilm process is the microorganism activity because the rate of biochemical transformation is directly dependent on the metabolic activity of the microorganism rather than on the total biomass concentration in the reactor [32]. Fig. 7 shows the INT-DHA of two reactors. The initial INT-DHA under conditions A and B was 25.5 and 30.3 mg O_2/g VSS/day, respectively. After interevent periods of 15 d, the INT-DHA was 25.4 and 22.4 mg O_2/g VSS/day, respectively. The INT-DHA of the microorganisms was constant despite the different interevent periods. From the DGGE results, the microorganisms related to the

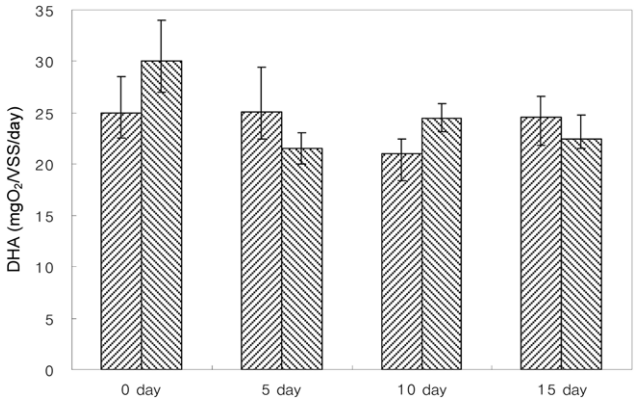

Fig. 7. DHA under conditions A and B with the different interevent periods; condition A (▨), condition B (▧).

Table 5. Characteristics of 16S rDNA fragments obtained from DGGE gel

DGGE band[a]	Access No.	Closest species in GenBank	Sources	Identity (%)
N-1	AF094732	*Pseudomonas fluorescens*	-	91
N-2	DQ419742	Uncultured bacterium	-	97
N-3	DQ792752	*Bacillus funiculus* strain MXC 3-4-2	-	97
N-4	AB127860	Bacterium DR2A-7G21	Freshwater sediment	100
N-5	AY711255	Uncultured bacterium	Sediment	100
N-6	DQ838095	Uncultured bacterium	-	97
N-7	DQ105621	*Uncultured proteobacterium*	Activated sludge	91

[a]Name of DGGE bands in Fig. 6

Table 6. COD removal and nitrification efficiencies at reaction times of 6 and 12 h under each condition

Interevent period (d)	Condition A				Condition B				Condition C			
	6 h		12 h		6 h		12 h		6 h		12 h	
	CE[a]	NE[b]	CE	NE	CE	NE	CE	NE	CE	NE	CE	NE
5	72	30	77	53	36	81	79	97	11	18	71	59
10	80	15	82	29	43	24	83	71	9	3	74	14
15	79	9	79	15	64	27	81	62	9	4	56	5

[a]COD removal efficiency (%)
[b]Nitrification efficiency (%)

sediment increased the longer the interevent period. These microorganisms were assumed to cause a constant INT-DHA activity. These results show why COD removal and nitrification were achieved, even after a 15 d interevent period. Thus, the biofilm process was concluded to be applicable for treating NPS pollutants.

6. Design Criteria of the Biofilm Process for NPS Pollution

Table 6 summarizes the COD removal and nitrification efficiencies under each condition after reaction times of 6 and 12 h. As shown in Table 6, stable COD removal was achieved under conditions of a packing ratio and reaction time of 0.05 and 6 h, respectively. However, to achieve high nitrification efficiency, a packing ratio and reaction time of 0.15 and 12 h, respectively, were required. It is generally known that the growth rate of nitrifying bacteria, which consist of AOB and nitrite-oxidizing bacteria (NOB), is slower than of heterotrophic bacteria [25,33,34]. Due to the difference in the growth rate, more heterotrophic bacteria were concentrated on the ceramic media surface. Therefore, the required reaction time for nitrification was longer than that for COD removal. In addition, considering that microbial death and decay, which causes loss of nitrifying bacteria, occurs only during the interevent period, maintaining the biomass of nitrifying bacteria during the interevent period with the a packing ratio is difficult. Thus, maintenance of biomass by increasing the packing ratio is important in achieving high nitrification efficiency.

Under condition C, operated at 10 °C, it was difficult to achieve stable nitrification efficiency, despite the packing ratio and reaction time of 0.15 and 12 h, respectively, that was required to achieve stable COD removal. Therefore, it was assumed that COD removal and nitrification efficiency were influenced by low temperature. These results can be used for the design and operation of a biofilm process for treating NPS pollutants.

CONCLUSIONS

This study was performed to treat NPS pollutants with a biofilm process. The ceramic media provided for biofilm growth in the reactors had packing ratios of 0.05 and 0.15. Thereafter, the reactors were operated intermittently for different interevent periods: 0, 5, 10 and 15 d.

The removal efficiency of the organic matter after 8 h remained stable, even with a longer interevent period, regardless of the packing ratio. The interevent period and packing ratio seemed to have no significant influence on the COD removal efficiency. However, stable nitrification efficiency, with longer interevent period, was achieved only under the condition of a packing ratio of 0.15. These results indicate that a packing ratio above 0.15 is needed to achieve simultaneous stable COD removal and nitrification efficiency.

As the reactors were operated at 10 °C, to achieve stable COD removal, a reaction time of at least 15 h is required, whereas nitrification efficiency sharply decreased with a longer interevent period, even with a packing ratio below 0.15. Thus, a low temperature has a greater influence on nitrification than on COD removal and nitrification.

The DGGE profiles showed that the tendency of the bands changed with differing interevent periods. The microorganism found in the seeded sludge decreased the longer the interevent periods, while the microorganisms related to the sediment remained prevalent. Because of the prevalence of these microorganisms with a longer interevent period, the INT-DHA of the microorganisms was constant, despite the different interevent periods. These results show that the biofilm process can be applied to treating NPS.

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