Journal of the Environmental Sciences
21(12); 1455~1462; December 2012

pISSN: 1225-4517 eISSN: 2287-3503
http://dx.doi.org/10.5322/JES.2012.21.12.1455

ORIGINAL ARTICLE

Correlationship of Vertical Distribution for Ammonia Ion, Nitrate Ion and Nitrifying Bacteria in a Fixed Bed Nitrifying Biofilm

Gi-Chung Choi, Im-Gyu Byun[1)*]

Suengineering Co. Ltd., Busan, Gyeongsangnam-do 626-800, Korea
[1)]Institute for Environmental Technology and Industry, Pusan National University, Busan 609-735, Korea

Abstract

The vertical distributions of nitrifying bacteria in aerobic fixed biofilm were investigated to evaluate the relationship between nitrification performance and microbial community at different HRT. Fluorescent *in situ* hybridization (FISH) and portable ion selective microelectrode system were adopted to analyze microbial communities and ions profiles according to the biofilm depth. Cilia media packed MLE (Modified Ludzack-Ettinger) like reactor composed of anoxic, aerobic Ⅰ/Ⅱ was operated with synthetic wastewater having COD 200 mg/L and NH_4^+-N 40 mg/L at HRT of 6 hrs and 4 hrs. Total biofilm thickness of aerobic Ⅰ, Ⅱ reactor at 4 hrs condition was over two times than that of 6 hrs condition due to the sufficient substrate supply at 4 hrs condition (6 hrs; aerobic Ⅰ 380 ㎛ and Ⅱ 400 ㎛, 4 hrs; aerobic Ⅰ 830 ㎛ and Ⅱ 1040 ㎛). As deepen the biofilm detection point, the ratio of ammonia oxidizing bacteria (AOB) was decreased while the ratio of nitrite oxidizing bacteria (NOB) was maintained similar distribution at both HRT condition. The ratio of AOB was higher at 4 hrs than 6 hrs condition and NH_4^+-N removal efficiency was also higher at 4 hrs with 89.2% than 65.4% of 6 hrs. However, the ratio of NOB was decreased when HRT was reduced from 6 hrs to 4 hrs and NO_2^--N accumulation was observed at 4 hrs condition. Therefore, it is considered that insufficient HRT condition could supply sufficient substrate and enrichment of AOB in all depth of fixed biofilm but cause decrease of NOB and nitrite accumulation.

Key words : Biofilm, Microelectrode, Fluorescent *in situ* hybridization (FISH), Ammonia oxidizing bacteria, Nitrite oxidizing bacteria, Nitrite accumulation

1. Introduction

It is well known that total nitrogen can be removed by nitrification and denitrification in a biological treatment plant. Biofilm reactors are more efficient for nitrification than the activated sludge process due to compactness of process, long SRT and stable process operation for the environmental disturbance period (Park et al., 1998). Nitrifying bacteria in biofilm is more sensitive to a number of environmental factors such as, pH, temperature, and inhibitory chemicals, and the growth rate is very slow. Therefore, it is important to characterize nitrifying bacteria community in biofilm to optimize nitrification process.

To characterize nitrifying bacterial communities, the traditional cultivation techniques, such as spread-

Received 15 October, 2012; **Revised** 26 October, 2012;
Accepted 26 December, 2012
***Corresponding author** : Im-Gyu Byun, Institute for Environmental Technology and Industry, Pusan National University, Busan 609-735, Korea
Phone: +82-51-510-7381
E-mail: big815@pusan.ac.kr

plate and most-probable-number count, has been studied. However, these methods are not recommended because it takes several weeks to incubate before enumerating. Also these techniques can often detect only a few species of microbial community in biofilm and may cause statistical uncertainty of the numeration. Recently, development of fluorescence *in situ* hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes has made it possible to analyze complex microbial community structures (Jang et al., 2002). Especially, as ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria detecting FISH probes are proved and commercially provided, many researcher has been trying to analyze the nitrifying bacteria group in various kinds of biological nutrient removal (BNR) processes (Hisashi et al., 2003, 2006; Okabe et al., 2004).

Microelectrodes have been used to investigate the spatial distribution of various microbial activities in biofilms (De Beer et al., 1997; Okabe et al., 1999). The combination of these methods apparently provides reliable and direct information for the fate and the occurrence of specific microorganisms.

The objectives of this study were to investigate vertical distribution of AOB, NOB, ammonia ion and nitrate ion at different HRTs in a fixed bed nitrifying biofilm. The correlationship of each parameter was also evaluated. These experiments were implemented by combining FISH and Liquid ion-exchanging membrane (LIX) microelectrode measurements of NH_4^+ and NO_3^-.

2. Materials and Methods

2.1. Experimental apparatus

Experimental apparatus was MLE (Modified Ludzack-Ettinger) like process and composed of anoxic and aerobic Ⅰ, Ⅱ reactors (Fig. 1). Each aerobic reactor was packed with cilia media and packing ratio was 1.0 V/V%. The effective volume of each reactor was 6.0 L. During the experiments, the biofilm was cultured with the synthetic nutrient medium containing on 200 COD mg/L, 40 NH_4^+-N mg/L. The reactor was operated with 6h (anoxic 2 hrs; aerobic Ⅰ 2 hrs; aerobic Ⅱ 2 hrs) and 4 hrs (anoxic 1 hr 20 min; aerobic Ⅰ 1 hr 20 min; aerobic Ⅱ 1 hr 20 min) hydraulic retention time.

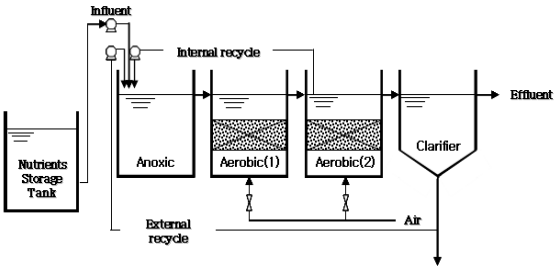


Fig. 1. Schematics of cilia media packed MLE reactor system.

2.2. Microelectrode measurements of ammonia ion and nitrate ion

Liquid ion-exchanging membrane (LIX) microelectrodes for NH_4^+ and NO_3^- were prepared as described by Miller(1995) and De Beer et al.(1997). Ammonium microelectrodes were calibrated in 0.14-1,400 mg N/L NH_4Cl solutions, and nitrate microelectrodes were calibrated in 0.14-1,400 mg N/L $NaNO_3$ solutions. The ion-selective microelectrode readings tended to shift over time, so it was necessary to calibrate the ion-selective microelectrodes before and after experiments, and any necessary corrections were made to the calibration curves (Li et al., 2002).

2.3. Biofilm fixation and cryosectioning

After measuring ion profiles with the micro-electrode, the biofilm samples were fixed freshly prepared 4% paraformaldehyde solution for 8h at 4℃ immediately. Then, the biofilms were embedded in Tissue-Tek OCT compound (Sakura Finetek, USA) and frozen to -20℃ in cryomicrotome to facilitate cryosectioning. The frozen samples were mounted and horizontality cut with substratum to a thickness

of 15 ㎛. The sections were placed in hybridization wells on a gelatin-coated microscopic slide and immobilized by air dryer. Finally, the sections were dehydrated in an ethanol dilution series (50%, 80% and 90%) and stored at room temperature.

2.4. Oligonucleotide probes

The following rRNA-targeted oligonucleotides were used: EUB338, Nso190, Ntspa662, and Nit3. Oligonucleotides were synthesized and fluorescently labeled with fluorescein isothiocyanate (FITC) or hydrophilic sulphoindocyanine (CY3). All of the probe sequences, the specificity and the hybridization conditions of the probes are given in Table 1.

2.5. Fluorescence *in situ* hybridization

All *in situ* hybridizations were performed according to the procedure described by Manz et al. (1992) and Amann (1995) in hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl; pH 7.2, 0.01% sodium dodecyl sulfate (SDS) and formamide concentration] at 46℃ for 3 hrs. The final probe concentration was 5 ng/μL. Finally, the slide was dipped into the washing solution at 48℃ for 20 min. After the hybridization, digital images of the aggregates were taken by a fluorescence microscope (Zeiss Axioskop 2plus, Germany) and visualized using Zeiss Axiovision digital imaging software. Analysis was performed with the standard software package using Carl Zeiss Imaging Solution system (Zeiss, Germany).

3. Results and Discussion

3.1. Removal of SCOD and ammonia ion in cilia media packed MLE reactor system

Initially, biofilm reactor was acclimated at HRT 8 hrs and has almost 99% nitrification efficiency. Fig. 2 shows SCOD and NH_4^+-N removal of each HRT. Fig. 2(a) shows the effluent SCOD concentrations slightly increased as the HRT decrease from 6 hrs to 4 hrs. The SCOD removal efficiencies at the two different HRT were 95.0% and 92.3%, respectively. Fig. 2(b) shows that the removal efficiencies of NH_4^+-N at HRT of 6 hrs and 4 hrs were 65.4% and 89.2%, respectively.

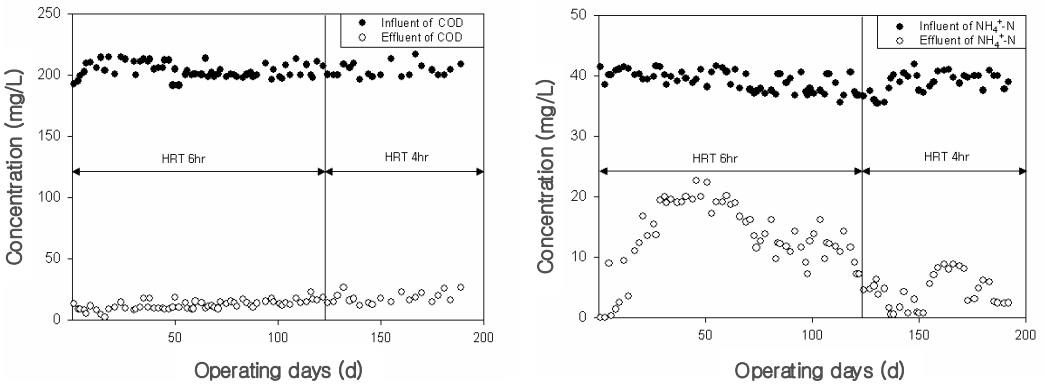
Table 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence(5'-3') of probe	Target site[a]	FA[b](%)	[NaCl] (mM)[c]	Reference
EUB338 (Ⅲ)	Eubacteria	GCTGCCACCCGTAGGTGGT	338-355	20	215	
Nso190	Ammonia-oxidizing β-Proteobacteria	CGATCCCCTGCTTTTCTCC	190-208	20	215	Mobarry et al., 1996
Ntspa662	Nitrospira genus	GGAATTCCGCGCTCCTCT	662-679	20	215	Daims et al., 2000
Nit3	Nitrobacter spp.	CCTGTGCTCCATGCTCCG	1035-1048	40	46	Wagner et al., 1996

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

[b] Formamide concentration in the hybridization buffer

[c] Sodium chloride concentration in the washing buffer



(a) SCOD (b) NH_4^+-N

Fig. 2. SCOD and NH_4^+-N profiles of cilia media packed MLE process during operation period.

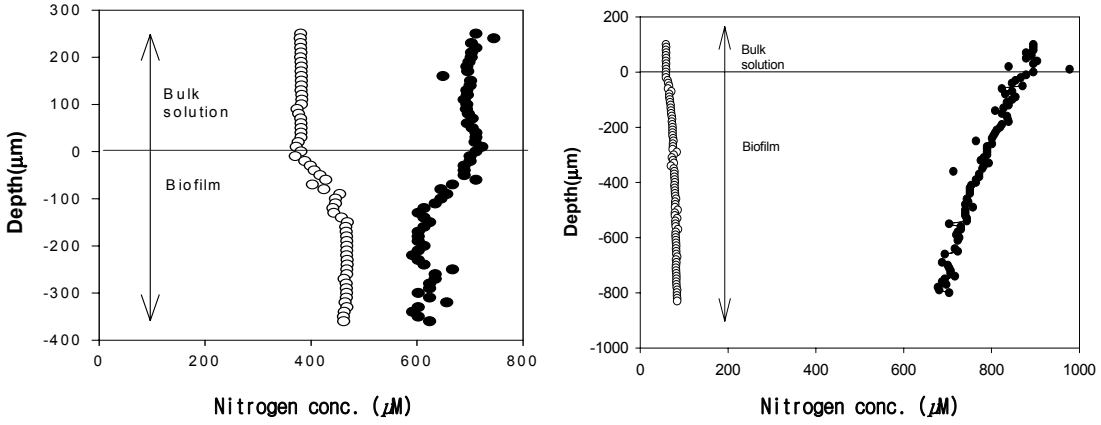
3.2. Vertical ion profiles in a fixed bed nitrifying biofilm by portable ISME system

Ion concentration profiles of NH_4^+-N and NO_3^--N in the biofilms were determined with Liquid ion-exchanging membrane(LIX) microelectrodes. The concentration profile between bulk solution and biofilm changed greatly as the HRT decreased 6 hrs to 4 hrs. The increases of the nitrogen removal efficiencies according to the decrease of HRT were related to the change of ion profiles and biofilm thickness in both HRT condition. After reaching the steady state, biofilm thicknesses were 380 ㎛ (6 hrs, aerobic Ⅰ), 400 ㎛ (6 hrs, aerobic Ⅱ), 830 ㎛ (4 hrs, aerobic Ⅰ), 1040 ㎛ (4 hrs, aerobic Ⅱ).

NH_4^+-N and NO_3^--N profiles of aerobic reactor Ⅰ, Ⅱ on HRT 6 hrs showed that abrupt decrease of NH_4^+-N concentration at the boundary layer between bulk solution and biofilm. As deepen the biofilm thickness, NH_4^+-N concentration was reduced almost linearly until 130 ㎛. This removed ammonia nitrogen was completely oxidized to nitrate and nitrate concentration was increased until 130 ㎛ depth of the biofilm (Fig. 3(a)). As no more decrease of NH_4^+-N concentration was observed below 130 ㎛, it is considered that oxygen transfer was limited to the biofilm depth of 130 ㎛.

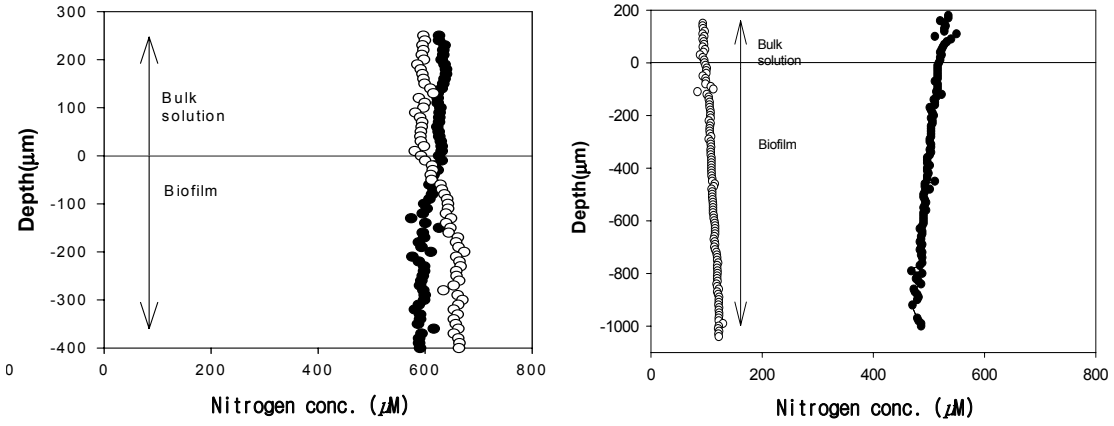
At 4 hrs HRT, decrease of NH_4^+-N concentration was almost linearly occurred from boundary layer to bottom of the biofilm. This is because there are much pore and channel and the oxygen was transferred to the bottom of the biofilm due to the low density (Fig. 3(b) and Fig. 4(b)). However, remarkable increase of NO_3^--N concentration was not observed according to deepen the biofilm depth. Therefore it is considered that complete nitrification was not occurred and nitrite was accumulated. This result was corresponded to the increase of nitrite concentration in the final effluent. Nitrite concentration was observed by ion chromatography but the data was not presented.

In previous studies, researchers reported that one of the most important factor which affects nitrification in the mixed biofilm (the condition which heterotrophs and nitrifying bacteria) is DO diffusion limitation (Jang et al., 1998). And the ion profiles changed greatly due to the higher DO and this result is corresponding to other studies (Jang et al., 1998).



(a) Ion profiles of HRT 6 hrs (b) Ion profiles of HRT 4 hrs

Fig. 3. The vertical ions profiles in bulk solution and fixed biofilm for aerobic Ⅰ ; NH_4^+-N (●), NO_3^--N (○).



(a) Ion profiles of HRT 6 hrs (b) Ion profiles of HRT 4 hrs

Fig. 4. The vertical ions profiles in bulk solution and fixed biofilm for aerobic Ⅱ ; NH_4^+-N (●), NO_3^--N (○).

3.3. Vertical distribution of nitrifying bacteria in a fixed bed nitrifying biofilm

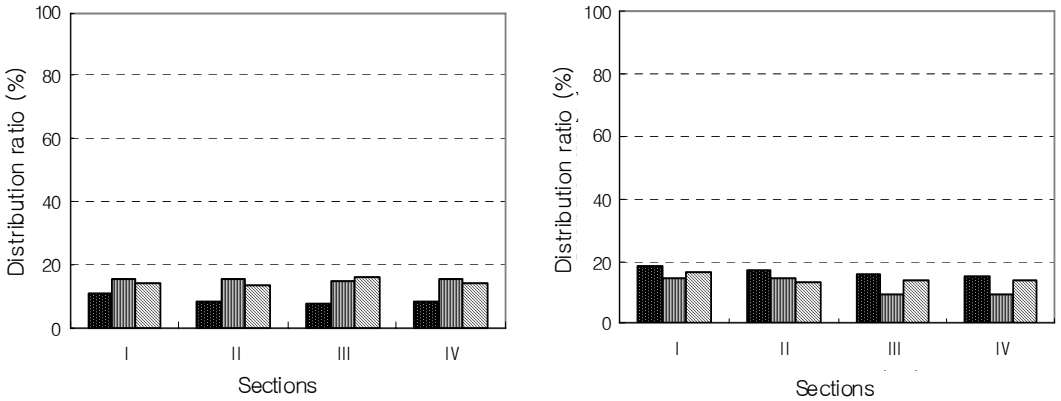
To observe vertical distribution of nitrifying bacteria depending on the biofilm depth, biofilms were sliced with 15 ㎛ thickness horizontally and were analyzed by FISH. Biofilms in each condition were quadrisected according to the depth and the details is presented in Table 2. The spatial distribution of nitrifying bacteria in aerobic Ⅰ and aerobic Ⅱ are shown in Fig. 5 and Fig. 6, respectively.

At the HRT of 6 hrs, the ratio of AOB was decreased as the depth of biofilm was increased in aerobic Ⅰ. The ratios of AOB in the biofilm of aerobic Ⅰ were 10.8%, 8.1%, 7.9% and 8.4% at each depth of 50 ㎛ (Section Ⅰ), 150 ㎛ (Section Ⅱ), 250 ㎛ (Section Ⅲ) and 350 ㎛ (Section Ⅳ) given in Table 2. However, *Nitrobacter spp.* did not change in different sections; the ratio was 15±0.5%. *Nitrospira genus* was 16.0% in Ⅲ section and 14.0% in other sections (Fig. 5(a)).

Table 2. The total thickness of biofilms and four depth section for each biofilms

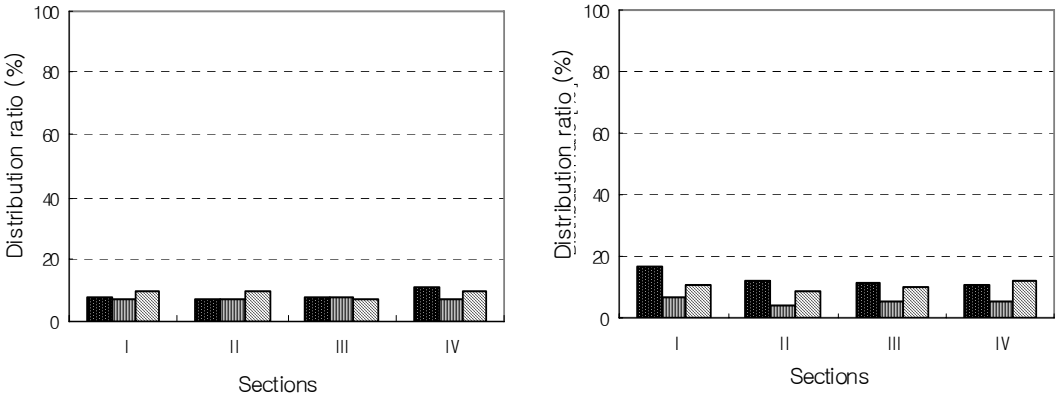
Condition		Biofilm thickness (㎛)	Section Ⅰ	Ⅱ	Ⅲ	Ⅳ
HRT 6 hrs	Aerobic Ⅰ	380	0-100	100-200	200-300	300-380
	Aerobic Ⅱ	400	0-100	100-200	200-300	300-400
HRT 4 hrs	Aerobic Ⅰ	830	0-210	210-420	420-630	630-830
	Aerobic Ⅱ	1040	0-260	260-520	520-780	780-1040

In aerobic Ⅱ, the ratios were almost same for all depth. The ratios of AOB in aerobic Ⅰ were 18.1%, 17.0%, 15.6% and 15.2%, respectively, in each section. The ratios were decreased depending on the depth and relatively high compared with the HRT 6 hrs. In Fig. 5(b), *Nitrobacter spp.* and *Nitrospira genus* decreased as the depth was deepen.



(a) HRT 6 hrs (b) HRT 4 hrs

Fig. 5. The distribution of AOB (■), *Nitrobacter spp.* (▥), *Nitrospira genus* (▨) in the aerobic Ⅰ biofilms according to biofilm depth.



(a) HRT 6 hrs (b) HRT 4 hrs

Fig. 6. The distribution of AOB (■), *Nitrobacter spp.* (▥), *Nitrospira genus* (▨) in the aerobic Ⅱ biofilms according to biofilm depth.

As *Nitrobacter spp*. and *Nitrospira genus* are nitrite oxidizing bacteria (oxidizing of NO_2^--N to NO_3^--N), it was considered that the decrease of *Nitrobacter spp*. and *Nitrospira genus* in aerobic biofilm as deepen the biofilm depth induce nitrite accumulation. Therefore, the combination of the FISH analysis and the portable ISME system for the monitoring of a fixed biofilm nutrient removal process could be a appropriate method which has good correlationship between microbial communities and ion profiles in the nitrifying biofilms.

4. Conclusions

The relationship between nitrification performance and microbial community was evaluated through the investigation of vertical distributions of nitrifying bacteria and ions profiles in aerobic fixed biofilm at different HRTs.

When the HRT was 6 hrs and 4 hrs, the COD removal efficiencies were 95.0% and 92.3%, respectively.

From FISH analysis, the ratio of AOB was increased while the ratio of *Nitrospira genus* and *Nitrobacter spp.* was decreased as the HRT was reduced from 6 hrs to 4 hrs. The analysis of FISH and NH_4^+-N removal efficiencies were corresponded to each others. And the ISME analysis showed that the ISME analysis showed that the ratio of AOB increase enhances the removal efficiency of NH_4^+-N but NOB decrease result in nitrite accumulation. The thickness of the biofilm increased as the HRT was decreased and the thicknesses of the biofilm in aerobic reactor Ⅰ and aerobic reactor Ⅱ were nearly same. Therefore, it is considered that the FISH analysis and the portable ISME system could be appropriate methods to monitor the performance and microbial community of a fixed biofilm nutrient removal process.

References

Amann, R. I., Ludwig, W., Schleifer, K. H., 1995, Phylogenetic identification and in situ detection of individual microbial cells without cultivation, Microbiol. Rev., 59(1), 143-169.

Daims, H., Nielsen, P. H., Nielsen, J. L., Juretschko, S., Wagner, M., 2000, Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and in situ physiology, Wat. Sci. Technol., 41(4/5), 85-90.

De Beer, D., Schramm, A., Santegoted, C. M., Kuhl, M., 1997, A nitrite microsensor for profiling environmental biofilms, Appl. Environ. Microbiol., 63(3), 973-977.

Hisashi, S., Okabe, S., Yamaguchi, Y., Watanabe, Y., 2003, Evaluation of the impact of bioaugmentation and biostimulation by in situ hybridization and microelectrode, Wat. Res., 37, 2206-2216.

Jang, A., Bishop, P. L., Okabe, S., Lee, S. G., Kim, I. S., 2002, Effect of dissolved oxygen concentration on the biofilm and in situ analysis by fluorescence in situ hybridization (FISH) and microelectrodes, Wat. Sci. Technol., 47(1), 49-57.

Li, J., Bishop, P. L., 2002, In situ identification of azo dye inhibition effects on nitrifying biofilms using microelectrodes, Wat. Sci. Technol., 46(1/2), 207-215.

Manz, W., Amann, R. I., Ludwig, W., Wagner, M., Schleifer, K., H., 1992, Phylogenetic oligodeoxynucleotide probe for the major subclasses of proteo bacteria: problems and solutions system, Appl. Microbiol., 15, 593-600.

Miller, A. J., 1995, Ion-selective microelectrodes for measurement of intracellular ion concentration, Method. Cell Biol., 49, 275-291.

Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, E., Stahl, D. A., 1996, Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria, Appl. Environ. Microbiol., 62, 2156-2162.

Okabe, S., Hisashi, S., Watanabe, Y., 1999, In situ analysis of nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes, Appl. Environ. Microbiol., 65, 3182-3191.

Okabe, S., Tomonori, K., Tsukasa, I., Hisashi, S., 2004, Analysis of size distribution and areal cell density of ammonia-oxidizing bacterial microcolonies in

relation to substrate microprofiles in biofilms, Biotechnol. Bioeng., 85(1), 86-95.

Park, T. J., Lee, K. H., Lee, J. H., 1998, Simultaneous organic and nutrient removal from municipal wastewater in BSACNR process, Kor. J. Chem. Eng., 15(1), 9-16.

Satoh, H., Yamakawa, T., Kindaichi, T., Ito, T., Okabe, S., 2006, Community structures and activities of nitrifying and denitrifying bacteria in industrial wastewater-treating biofilms, Biotechnol. Bioeng., 94(4), 763-772.

Wagner, M., Rath, G., Koops, H. -P., Flood, J., Amann, R., 1996, In situ analysis of nitrifying bacteria in sewage treatment plants, Wat. Sci. Technol., 34(1/2), 237-244.

Wijeyekoon, S., Mino, T., Satoh, H., Matsuo, T., 2004, Effects of substrate loading rate on biofilm structure, Wat. Res., 38, 2479-2488.