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|보 문|

Comparison of Biofilm Formed on Stainless Steel and Copper Pipe Through the Each Process of Water Treatment Plant

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정수처리 공정 단계별 스테인리스관과 동관에 형성된 생물막 비교

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Biofilm formed on stainless and copper in water treatment plant was investigated for sixteen weeks. Biofilm reactor was specially designed for this study. It was similar to that of a real distribution pipe. Raw water, coagulated, settled, filtered and treated water were used in this study. The average number of heterotrophic bacteria counts was 1.6×10^4 CFU/ml, 5.8×10^3 CFU/ml, 1.8×10^3 CFU/ml, 1.3×10^2 CFU/ml, 1 CFU/ml, respectively. Density of biofilm bacteria formed on stainless and copper pipes in raw, coagulated and settled water increased above 2.9×10^3 CFU/cm^2 within second weeks while more biofilm bacteria counts were found on the stainless pipe than on the copper pipe. In case of filtered water (free residue chlorine 0.44 mg/L), there was no significant difference in the number of biofilm bacteria on both pipes and biofilm bacteria below 18 CFU/cm^2 were detected on both pipe materials after fifth weeks. Biofilm bacteria were not detected on both pipe materials in treated water (free residue chlorine 0.88 mg/L). According to the results of DGGE analysis, Sphingomonadacae was a dominant species of biofilm bacteria formed on the stainless pipe while the copper pipe had Bradyrhizobiaceae and Sphingomonadaceae as dominant bands. In case of filtered water, a few bands (similar to *Propionibacterium* sp., *Sphingomonas* sp., *Escherichia* sp., and etc.) that have 16S rRNA sequences were detected in biofilm bacteria formed on both pipes after fifth weeks. Stainless pipe had higher species richness and diversity than the copper pipe.

***Keywords*:** biofilm, copper pipe, DGGE, distribution pipe, drinking water, stainless pipe

It was reported in Korea and other countries that microbes undetected in water treatment plant were found in tap water at the end of distribution network. (LeChervallier *et al.*, 1987; Charackils, 1988; Park *et al.*, 1993; Yoon *et al.*, 2002). This situation is not because of the microbials that survived in disinfection and grew in tap water but because of the detachment of microbes that attached themselves to inner surface of the distribution pipe (LeChervallier *et al.*, 1987; Charackils, 1988; Van der wende *et al.*, 1989). Biofilm could cause various problems such as chlorine demand, coliform regrowth, pipe corrosion, offensive odor, and etc. (Hu *et al.*, 2005). Tap water in distribution system contains biodegradable organic material, inorganic nutrient and microbes that survived the treatment process and microorganisms that entered the distribution system through the pipe network (DiGiano *et al.*,

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2000; EPA, 2002). Also, most of these microbes can attach to the pipe wall and become part of a biofilm (EPA, 2002). Biofilm in water distribution system is a complex mixture of microbes, organic and inorganic materials, which is attached to the inner surface of the distribution system, being surrounded by EPS (Extracellular polymeric substance) (EPA, 2002). Biofilm formation is physically and chemically affected by various factors such as nutrient, water temperature, water velocity, free residual chlorine, pipe material, and etc. (Park *et al.*, 1993; Manuel *et al.*, 2007).

Park *et al.* (1994) reported that climax biofilm would be formed in the pipes of distribution system about three months after installation and that although no microbes from water treatment are not introduced into distribution system, high density of microorganisms could be detected from distribution pipe due to desorption or detachment of biofilms. Researchers have investigated difference in biofilm formation by pipe materials because various pipe types are used for distribution pipes and found that density of heterotrophic bacteria is different by pipe types even under same condition (Zacheus *et al.*, 1999; Hallam *et al.*, 2001; Lee, 2004; Lee *et al.*, 2004; Momba and Makala, 2004; Kim *et al.*, 2007).

Most of past studies isolated bacteria and confirmed density of heterotrophic bacteria using R2A medium (LeChevallier *et al.*, 1987; Park *et al.*, 1993, 1994; Lehtola *et al.*, 2001). However, metabolically active cells in biofilms account for 17–35% of the total cells and 6–18% of them are able to form colony units in R2A medium (Manuel *et al.*, 2007). As analysis of microbial community using traditional culture method has some limits, microbial community is recently analyzed by molecular techniques such as DGGE (Denaturing gradient gel electrophoresis), Pyrosequencing, RFLP (Restriction fragment length polymorphism) and etc., instead of traditional culture method (Saiki *et al.*, 1985; Muyzer *et al.*, 1993; Ronaghi, 2001). In this study, biofilm reactor was designed using connected pipe materials instead of annular biofilm reactor and we investigated difference and change in microbial community of biofilm formed on two pipe types using DGGE of molecular techniques.

Materials and Methods

Region and period for investigation

We investigated biofilm formation by pipe materials in each processed water samples (raw, coagulated, settled, filtered and treated water) of K water treatment plant, which is located in In-cheon city, for four months.

Biofilm formation reactors and operation condition

The reactors for biofilm formation were designed with distribution pipe (inner diameter 20 mm). Stainless pipe (KSD 3595) and Copper pipe (KSD 5301) were cut in fixed length (6 cm) and then twenty pipe fragments were connected using EZ-Joint and Ring Union respectively (Supplementary data Fig. S1). Inner surface of each fragment was 37.68 cm^2. Flow velocity for biofilm formation was adjusted at 0.3 m/sec.

Heterotropic plate counts

Heterotrophic bacteria of collected biofilm and each process waters were enumerated by pour plate method. Samples and their ten-fold dilutions were cultivated on R2A medium (Sigma, USA) at 21℃ for 7 days (LeChevallier *et al.*, 1990; Chang *et al.*, 2002). When sample contained residual chlorine, 10% sodium thio-sulfate solution was added to adjust the final concentration of the sample to 0.03%.

Biofilm collection

Fragments collected each week were washed by 100 ml sterilize distilled water for removal of reversibly adhered microbes. After one side of fragment was sealed with parafilm, fragments were filled with 10 ml sterilized distilled water and then fragments were sonicated at 40 W and 20 Hz for 2 min by Sonic Dismembrator 550 (Fisher scientific, USA) (Clark and Sivaganesan, 1999). Another side was sealed and then 20ml of biofilm sample was obtained using same methods (Park *et al.*, 2006).

DNA extraction and Nested-PCR

Collected biofilm samples were concentrated by centrifuge at 10,000×g for 2 min. DNA extraction was performed using Microbial DNA Isolation kit (Mo-Bio, USA) under the manufacture's protocol. DNA extract was amplified with two primer sets for V3 region of 16S rRNA gene. Primer sets for first PCR were universal primer 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) and primer sets for Semi-Nested PCR were GC clamp-341F (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG) and 518R (ATT ACC GCG GCT GCT GG). The condition for first PCR program was as follows: initial denatruration at 94℃ for 5 min; 30 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 1 min 30 sec; and final extension at 72℃ for 5 min. Semi nested PCR was performed using the first PCR products as template DNA. PCR program was used by touchdown (Muyzer *et al.*, 1993); initial denaturation at 94℃ for 5 min; cycling step of denaturation 94℃ for 30 sec, annealing at 65 to 55℃ for 30 sec, extension 72℃ for 30 sec; and final extension at 72℃ for 7 min. Annealing temperature of cycling step was decreased by

Table 1. Turbidity, PH, free residual chlorine and water temperature in each of treatment processes during experimentation period

	Turbidity (NTU)	pH	Free Residual Chlorine (mg/L)	Water temperature (℃)
Raw water	4.00-390.00 (23.99)	6.70-8.30 (7.50)	0.03-0.18 (0.08)	18.0-26.0 (19.9)
Coagulated water	> 99.99	6.82-7.28 (7.07)	0.07-0.10 (0.08)	11.7-28.0 (24.0)
Settled water	0.25-3.60 (0.74)	6.80-7.70 (7.20)	0.01-0.12 (0.04)	11.3-28.0 (24.0)
Filtered water	0.05-0.07 (0.06)	6.80-7.70 (7.26)	0.13-0.80 (0.45)	11.3-27.1 (23.9)
Treated water	0.05-0.07 (0.05)	6.70-7.90 (7.20)	0.11-0.99 (0.88)	10.0-26.1 (22.5)

1℃ every second cycle until touchdown at 55℃. Amplified products were analyzed by electrophoresis in 1.5% (w/v) of agarose gel.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed with D-Code system (Bio-Rad, USA). PCR product with 2× loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol) was loaded onto 8% (w/v) of polyacrylamide gel in 1× TAE (40 mM Tris, 20 mM acetic acid, 50 mM EDTA, pH 8.0) using denaturing gradient ranges of 45% to 60%, in which 100% denaturant contained 7 M urea and 40% (v/v) of deionized formaide. The electrophoresis was run at 60℃ and 50 V for 820 min. The gel was stained for 10 min with EtBr and then the stained gel was immediately photographed under UV light.

DGGE bands sequencing and analysis

Representative bands were excised from gel using sterilize razor blades, placed in 50 µl sterile distilled water, and then overnight at 4℃ after freezing-thawing step was repeated three times (Cho and Cho, 2008). Extracted products such as DNA template were amplified by 341F and 518R primer. The following PCR program was used; initial denaturation at 94℃ 5 min; 30 cycles of denaturation 94℃ for 30 sec, annealing at 55℃ for 30 sec, extension 72℃ for 30 sec; and final extension at 72℃ for 7 min. Sequencing of amplified products was performed by Macrogen, Inc. (Korea). Species similarity of 16S rRNA sequences were confirmed by BLAST tool in NCBI (National center for Biotechnology Information) website. The Number and intensity of bands were measured by Quantity One software (Bio-Rad).

Results

Water quality in each of processes

Turbidity, pH, residual chlorine and water temperature were measured (Table 1). Water temperature in all processed water samples decreased from 28℃ to 10℃. PH of each processed water samples was maintained neutral, ranging from 7.07 to 7.50. Average concentration of free residual chlorine in raw to settled water was 0.04 to 0.08 mg/L, filtered water was 0.45 mg/L and treated water was 0.88 mg/L. Turbidity in raw water was 23.99 NTU on average and turbidity rapidly went up after a heavy rain. Coagulated water was measured out of range because of added coagulase, settled water was 0.74 NTU, filtered water was 0.06 NTU and treated water was 0.05 NTU.

Turbidity in raw and settled water remained stable during experiment period, regardless of turbidity level in source, coagulated and settled water.

Heterotrophic plate counts of processing waters and biofilms

Average density of heterotrophic bacteria in each processed water samples was 1.6×10^4 CFU/ml (1.0×10^4 CFU/ml – 3.1 $\times 10^4$ CFU/ml) in raw water, 5.8×10^3 CFU/ml (1.7×10^3 CFU/ml – 1.5×10^4 CFU/ml) in coagulated water, 1.8×10^3 CFU/ml (5.3×10^3 CFU/ml – 2.5×10^3 CFU/ml) in settled water and 1.3×10^2 CFU/ml (10 CFU/ml – 3.4×10^2 CFU/ml) in filtered water, respectively. Heterotrophic bacteria were mostly undetected in treated water but some below 4 CFU/ml were found during some weeks (Fig. 1).

Average density of biofilm bacteria on stainless and copper pipes in raw water ranged from 4.8×10^2 CFU/cm^2 to 6.0×10^5 CFU/cm^2 and from 3.2×10^2 CFU/cm^2 to 1.4×10^4 CFU/cm^2, respectively (Fig. 2A). In coagulated water, both pipes showed 53 CFU/cm^2 on first week. After second week, stainless pipes had average density of biofilm bacteria from 1.2×10^3 CFU/cm^2 to 1.4×10^5 CFU/cm^2, while copper pipes recorded

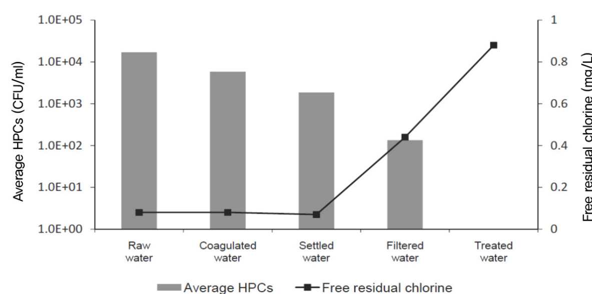

Fig. 1. Average density of heterotrophic bacteria and concentration of free residual chlorine from raw, coagulated, settled, filtered, and treated water. Each sample was cultivated on R2A at 21℃ for 7 days.

from 6.3 × 10^2 CFU/cm^2 to 5.3 × 10^3 CFU/cm^2 (Fig. 2). In settled water, both pipes recorded 23 CFU/cm^2 on the first week. After second week, stainless pipe had a range from 2.9 × 10^3 CFU/cm^2 to 4.5 × 10^4 CFU/cm^2 and copper pipe showed a range from 5.8 × 10^2 CFU/cm^2 to 1.2 × 10^4 CFU/cm^2 (Fig. 2C). Density of biofilm bacteria on stainless pipe was higher than on copper pipe in raw, coagulated and settled water. In filtered water, biofilm bacteria on stainless pipe ranged from 1 CFU/cm^2 to 12 CFU/cm^2 from seventh to sixteenth weeks and biofilm bacteria on copper pipe ranged from 1 CFU/cm^2 to 18 CFU/cm^2 from fifth to twelfth weeks (Fig. 2D). Biofilm bacteria were not detected on both pipes in treated water.

Analysis of DGGE profile

The number of bands on DGGE profile from DGGE electrophoresis declined from raw to filtered water (Figs. 3 and 4). In case of treat water, we didn't perform electrophoresis because 16S rRNA gene was not amplified from biofilm on both pipes during experimental period. Phylogenetic tree was analyzed by MEGA 4.0 software based on sequencing data from each band.

Dominant band on stainless pipe was Sphingomonadaceae (Supplementary data Fig. S2). Bradyrhizobiaceae and Spingomonadaceae were dominant in biofilm on copper pipe (Supplementary data Fig. S3). Composition change of biofilm bacteria was confirmed by light intensity of each band and sequencing data (Fig. 5). In raw water (Fig. 5A and 5B), relative abundance of Uncultured bacterium in biofilm on both pipes was high for initial two weeks. Rate of Spingomonadaceae in biofilm on stainless pipe drastically increased after second weeks. Rates of Brdayrhizobiaceae and others increased in twelfth week and similar rate of Spingomonadaceae and Bradyrhizobiaceae were found in sixteenth week. Rate of Bradyrhizobiaceae and others in biofilm on copper pipe continued to remain high but rate of uncultured bacterium was high in sixteenth weeks. In coagulated water (Fig. 5C and 5D), Sphingomonadaceae was dominant in biofilm on stainless pipe throughout the experiment period. Sphingomonadaceae was dominant and Bradyrhizobiaceae was subdominant in biofilm on copper pipe. Relative abundance of Bradyrhizobiaceae was

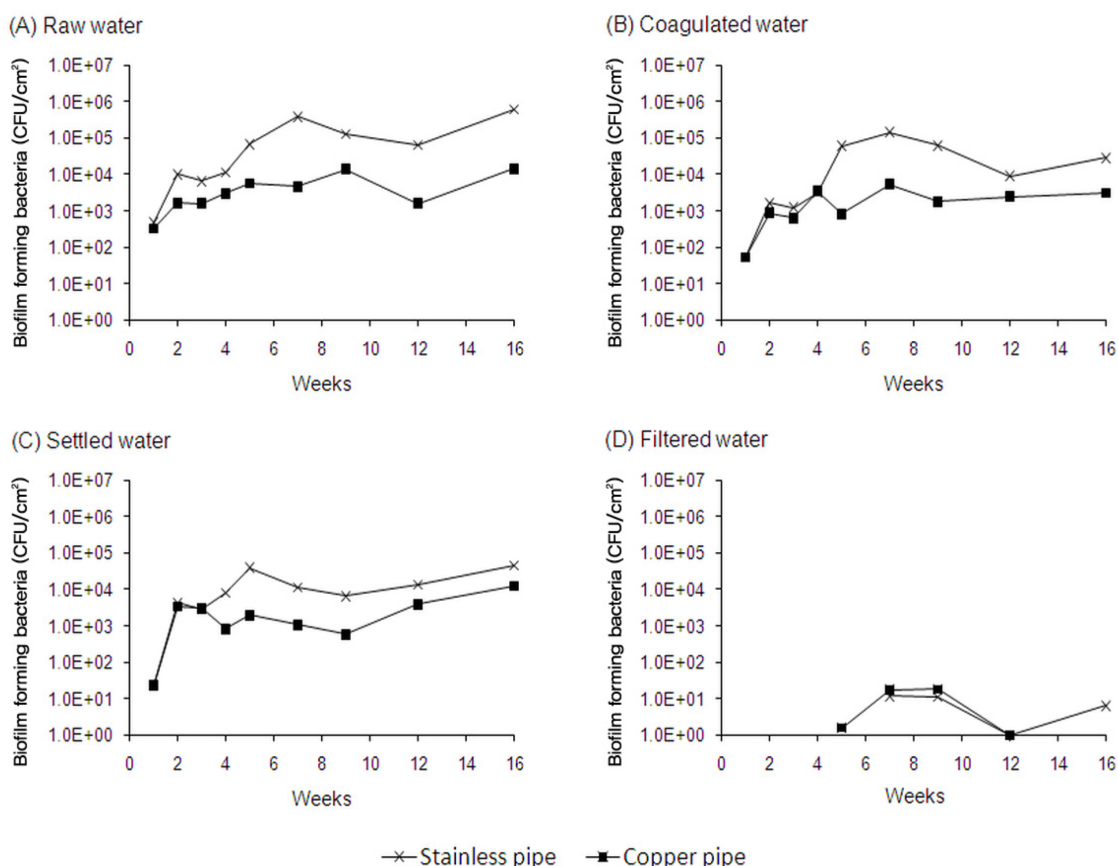

Fig. 2. Density of biofilm forming bacteria formed on stainless and copper pipes for sixteen weeks. Biofilm formed on both pipes in treated water was not detected.

higher than Spingomonadaceae in some weeks on cooper pipe. In settled water (Fig. 5E and 5F), Spingomonadaeae was dominant and Uncultured bacterium was sub dominant in biofilm on stainless and copper pipe. Bradyrhziboiaceae and Uncultured bacterium were especially dominant in biofilm on stainless and copper pipe, respectively, in ninth week. In filtered water (Fig. 5G and 5H), bands similar with 16S rRNA gene and *E. coli* (JQ266006.1) and *Propionibacterium* sp. (HM489920.1) were detected in biofilm on stainless pipe after seventh week. Bands with 16SrRNA gene, which are similar with *Sphingomonas* sp. (HM484311.1), *E. coli* (JQ26006.1), and *Propionibacterium* sp. (HM489920.1), were found from biofilm on copper pipe after fifth week. 16S rRNA gene wasn't amplified from biofilm on both pipes in treated water.

Richness and Shannon-Weaver index were calculated from the number and intensity of the bands detected for sixteen weeks (Fig. 6). These indexes declined from raw to settled water. Results of species richness (Fig. 6A) were 4.06 and 2.45 on stainless and copper pipe, respectively, in raw water. While, the results were 1.94 and 1.78 in coagulated water and 1.06 and 1.03 in settled water, respectively. According to the results of Shannon-Weaver index (Fig. 6B), biofilm on stainless and copper pipe was 2.67 and 2.14 in raw water, 1.89 and 1.81 in coagulated water, 1.22 and 1.18 in settled water, respectively.

Discussion

Average density of heterotrophic bacteria in each processed water samples declined through treatment process. However, density of heterotrophic bacteria was not sensitive to decline in temperature from seasonal change (Yoon *et al*., 2002). Average concentration of free residue chlorine from raw to settled water ranged from 0.04 to 0.08 mg/L because of pre-chlorination for removal of organic and inorganic compounds. In filtered and treated water, the figures were relatively high with 0.44 mg/L and 0.88 mg/L, respectively, which was due to the middle- and post- chlorination for disinfection. Although filtered and treated water included high concentration of free residue chlorine, heterotrophic bacteria was detected in low density. It was due to re-growth of bacteria damaged by disinfectant as chlorine (LeChevallier *et al*., 1987). Regrowth of damaged bacteria was closely related to concentration of free chlorine residue and its concentration declined as it got further away from water treatment plant, but density and diversity of heterotrophic bacteria increased (Jegatheesan *et al*., 2000; Lehtola *et al*., 2004; Yoon and Lee, 2004).

Density of biofilm bacteria on both pipes in raw, coagulated and settled water increased above 2.9×10^3 CFU/cm^2 within second weeks. After second weeks, biofilm bacteria counts

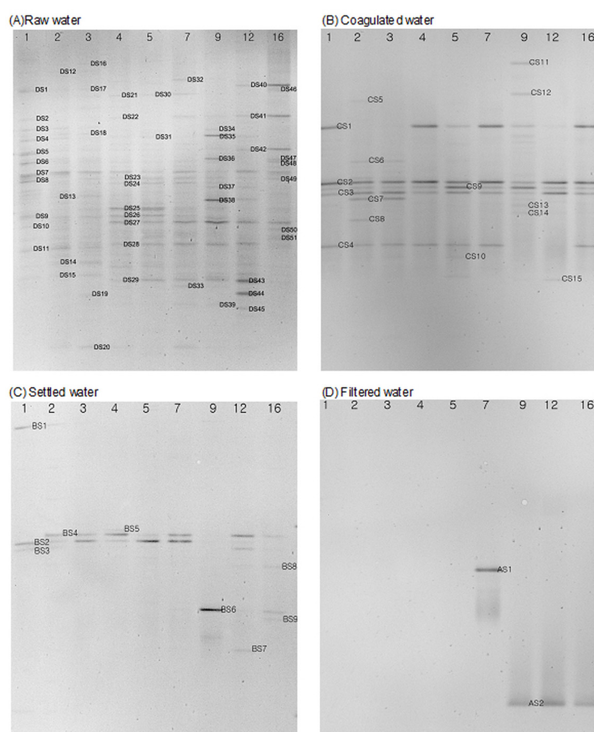

Fig. 3. DGGE profile of V3 domain fragments of bacterial 16S rRNA genes amplified from biofilms formed on stainless pipe in each processed water samples for sixteen weeks.

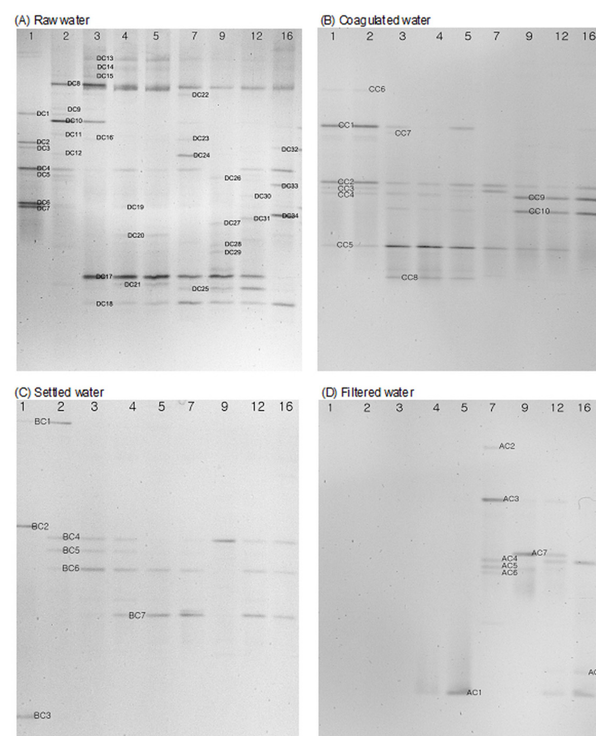

Fig. 4. DGGE profile of V3 domain fragments of bacterial 16S rRNA genes amplified from biofilms formed on copper pipe in each processed water samples for sixteen weeks.

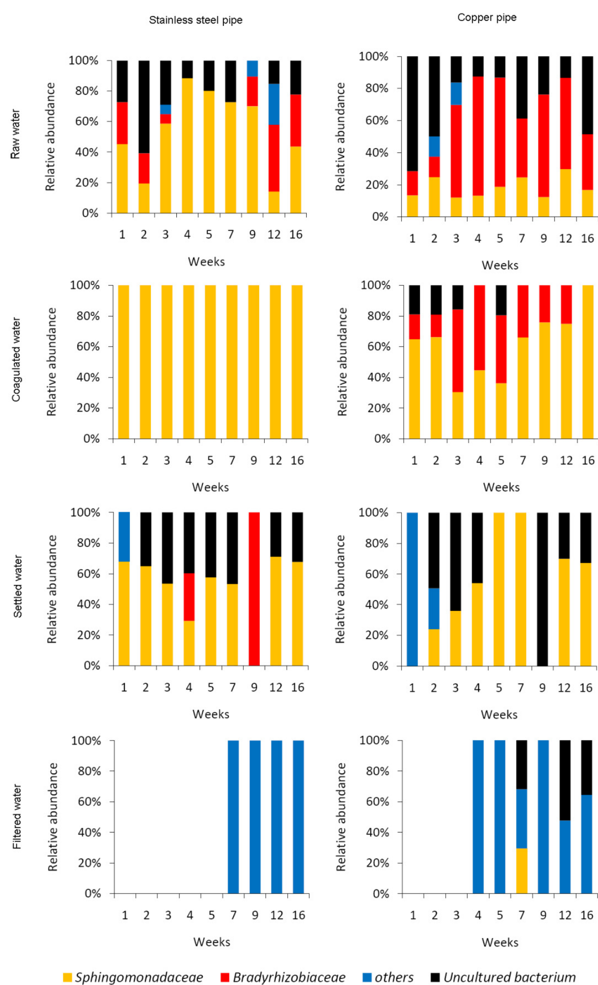

Fig. 5. Composition of biofilm formed on stainless and copper pipe in raw, coagulated and settled water. Each of relative abundances was calculated from the number and intensity of bands from DGGE Profile.

were detected higher on stainless pipe than on copper pipe. Stainless and PCV pipe had higher density of biofilm bacteria than carbon steel and galvanized iron pipes (Kim *et al.*, 2006). In filtered and treated water, low concentration of or no biofilm bacteria counts were detected on both pipes as a result of disinfection effect of free residual chlorine. Zhou *et al.* (2008) reported that density of biofilm bacteria on copper pipe was lower than on stainless pipe because of synergistic effects between copper and residual chloramines. We didn't confirm definite difference of biofilm bacteria between stainless and copper pipe when free residual chlorine was present as disinfectant. Injection of free residual chlorine was useful to control floating bacteria but it was difficult to control biofilm formed in pipes because of its resistance to disinfectant and antibiotics (LeChevallier *et al.*, 1990; Van der Wende and Charackilis, 1990; Kim *et al.*, 2006; Folkesson *et al.*, 2008). Therefore, control of biofilm formation would be possible when a certain level of free residual chlorine is continuously maintained.

According to the result of DGGE analysis, dominant species of biofilm formed on stainless pipe mainly belong to Sphingomonadacae, but biofilm formed on copper pipe mainly belong to Sphingomonadaceae and Bradyrhizobiaceae. This family was dominant species in biofilm formed in real distribution network (Schmeisser *et al.*, 2003; Hong *et al.*, 2010). It was reported that Sphngomonadaceae produces extracellular polymeric substance for biofilm formation (Hidetoshi *et al.*, 2003; Bereschenko *et al.*, 2010; Ivone *et al.*, 2011). However, we didn't find what role bacteria related to Bradyrhizobiaceae play in biofilm formation. This is, in large part, because that the earlier studies on biofilm formation have mainly focused on pathogenic bacteria and single species (O'Toole *et al.*, 2000). Also, it was not clearly identified that what factor of bacteria affect biofilm formation (Kim *et al.*, 2005). Because bacterial composition of biofilm changes continuously, we didn't find clear pattern in results of richness and diversity. Higher richness and diversity of biofilm were formed on stainless pipe

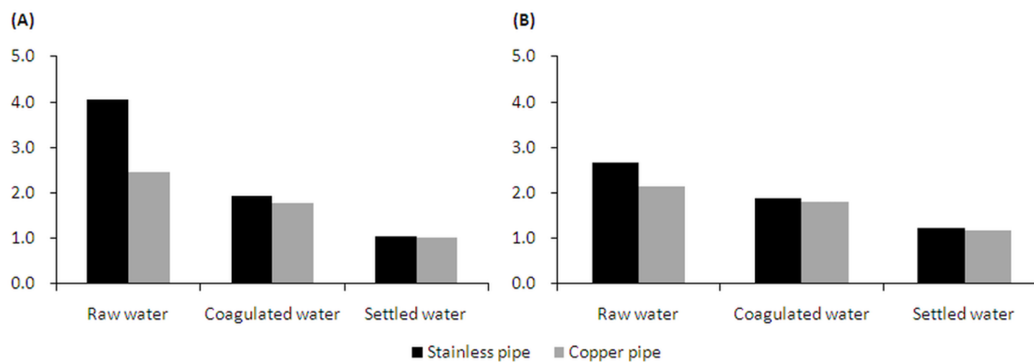

Fig. 6. Average of richness (A) and Shannon-Weaver index (B) of biofilm formed on stainless and copper pipe in raw, coagulated and settled water for sixteen weeks.

than on copper pipe, in general. In filtered water, bands with 16S rRNA gene that are similar with *Spingomonas* sp. (HM484311.1), *E. coli* (JQ26006.1) and *Propionibacterium* sp. (HM489920.1) were identified in both pipes after fifth weeks. Any bands weren't detected from biofilm on both pipes in treated water. These results were similar with the density of biofilm bacteria described in the earlier section.

적 요

정수처리 시설에서 급·배수관으로 많이 사용되는 스테인리스관과 동관에 형성되는 생물막의 특성에 대해 16주 동안 조사하였다. 생물막 반응기는 실제 배급수관의 구조와 유사하게 설계하였으며, 정수처리장으로 유입되는 상수원수와 약품혼화 응집수, 침전수, 여과수, 처리수를 사용하였다. 평균 종속영양세균수는 1.6×10^4 CFU/ml, 5.8×10^3 CFU/ml, 1.8×10^3 CFU/ml, 1.3×10^2 CFU/ml, 1 CFU/ml로 각 처리 과정을 거치면서 감소하였다.

스테인리스관과 동관에 형성된 생물막 세균수는 원수, 응집수, 침전수에서 2주만에 2.9×10^3 CFU/cm^2 이상으로 증가하였고, 동관보다 스테인리스관에서 생물막 세균수가 높게 검출되었다. 여과수(평균 잔류염소 0.44 mg/L)에서는 두 관 재질에 따른 생물막 세균수의 명확한 차이는 없었으며, 5주 이후부터 두 관 재질 모두 18 CFU/cm^2 이하의 생물막 세균이 검출되었다. 정수(평균 잔류염소 0.88 mg/L)에서는 두 관 재질 모두 생물막 세균이 검출되지 않았다. DGGE 분석결과, 원수, 응집수, 침전수에서 스테인리스관은 Sphingomonadaceae가 우점이였고, 동관에서는 Bradyrhizobiaceae와 함께 Sphingomonadaceae도 우점이였다. 여과수의경우, 5주차 이후 스테인리스관과 동관에 형성된 생물막에서 *Propionibacterium* sp., *Sphingomonas* sp., *Escherichia* sp. 등과 유사한 16S rRNA 유전자 서열을 가지는 밴드들이 검출되었다. 종 풍부도 및 다양성은 동관에 비해 스테인리스관이 더 높게 나타났다.

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