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Effects of Phosphate Addition on Biofilm Bacterial Communities and Water Quality in Annular Reactors Equipped with Stainless Steel and Ductile Cast Iron Pipes

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The impact of orthophosphate addition on biofilm formation and water quality was studied in corrosion-resistant stainless steel (STS) pipe and corrosion-susceptible ductile cast iron (DCI) pipe using cultivation and culture-independent approaches. Sample coupons of DCI pipe and STS pipe were installed in annular reactors, which were operated for 9 months under hydraulic conditions similar to a domestic plumbing system. Addition of 5 mg/L of phosphate to the plumbing systems, under low residual chlorine conditions, promoted a more significant growth of biofilm and led to a greater rate reduction of disinfection by-products in DCI pipe than in STS pipe. While the level of THMs (trihalomethanes) increased under conditions of low biofilm concentration, the levels of HAAs (halo acetic acids) and CH (chloral hydrate) decreased in all cases in proportion to the amount of biofilm. It was also observed that chloroform, the main species of THM, was not readily decomposed biologically and decomposition was not proportional to the biofilm concentration; however, it was easily biodegraded after the addition of phosphate. Analysis of the 16S rDNA sequences of 102 biofilm isolates revealed that *Proteobacteria* (50%) was the most frequently detected phylum, followed by *Firmicutes* (10%) and *Actinobacteria* (2%), with 37% of the bacteria unclassified. *Bradyrhizobium* was the dominant genus on corroded DCI pipe, while *Sphingomonas* was predominant on non-corroded STS pipe. *Methylobacterium* and *Afipia* were detected only in the reactor without added phosphate. PCR-DGGE analysis showed that the diversity of species in biofilm tended to increase when phosphate was added regardless of the pipe material, indicating that phosphate addition upset the biological stability in the plumbing systems.

Keywords: biofilm, phosphate, disinfection by-products, drinking water distribution system, stainless steel pipe, ductile cast iron pipe

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Introduction

Systematic management of drinking water distribution systems (DWDS) is very important for improving the final quality of tap water in households, as the quality of drinking water produced at the water treatment plant (WTP) can be adulterated by cross connection, backflow, corrosion, DBPs (disinfection by-products) and biofilm. Biofilms are composed of bacterial cells attached to a pipe surface, which are embedded in a matrix of secreted extracellular polymeric substance (EPS) (Percival *et al.*, 2000). Biofilms can affect the final quality of tap water in a number of ways, including bacterial regrowth, increased disinfection demand, and taste-odor problems in DWDS. Factors affecting microbial growth of biofilms in DWDS include temperature, available nutrients, disinfection method, pipe material, corrosion, and hydraulic effects (LeChevallier, 1990; Percival and Walker, 1999).

Corrosion products of metallic pipes are known to: provide bacteria with hiding places by increasing the surface area available for bacterial adsorption, provide iron oxide particles as potential electron acceptors that may increase bacterial activity, absorb organic matter that may subsequently be biodegraded by microorganisms, and consume residual disinfectant (Camper *et al.*, 1996; Appenzeller *et al.*, 2002). The high affinity of phosphate anions for corroded surface metals leads to the formation of a stable phosphate-metal complex, which limits further corrosion (Persson *et al.*, 1996). Although carbon is usually considered the limiting nutrient for microbial growth in DWDS, phosphorus has been suggested as another limiting nutrient for microbial growth in certain types of water (Miettinen *et al.*, 1997; Sathasivan and Ohgaki, 1999). Therefore, the use of phosphorus-based corrosion inhibitors may be of concern to water utilities, because phosphate addition can influence the growth of bacteria and microbiologically-related water quality problems such as taste and odor issues and violation of the TCR (Total Coliform Rule) (Abernathy and Camper, 1998; McNeill and Edwards, 2002). In Korea, drinking water standards were revised in 2007 to lower the concentration of corrosion inhibitors from 10 mg/L (as P_2O_5) to 5 mg/L in order to avoid adverse effects. Previous studies have shown that very low amounts of phosphates led to increased microbial growth of biofilms in DWDS (Miettien *et al.*, 1997; Lehtola *et al.*, 2002; Chu *et al.*, 2004). Conversely, other studies have yielded contradictory results. Appenzellar *et al.* (2001) reported that the addition of phosphate (2.0 mg/L) had no impact on the bacterial growth of biofilm on gray cast iron

pipe while causing only a moderate increase of biofilm cell count on slightly corroded stainless steel pipe in Propella reactors. Batté *et al.* (2003) reported that the addition of 0.5 mg/L of phosphate in an annular reactor system fed with dechlorinated water had no impact on biofilm growth. Gouider *et al.* (2009) also reported that the addition of 1 mg PO_4^{3-}/L did not affect the bacterial density in a biofilm reactor containing glass beads. These differences were ascribed in part to the nutrient limiting conditions of the original water (C and N ratio), but also may be attributable to the presence of disinfectants and corrosion products of the plumbing material. It was also reported that phosphate treatment could affect the metabolic potential and microbial composition of biofilms (Keinänen *et al.*, 2002; Batté *et al.*, 2003).

The control of bacterial regrowth in DWDS is usually achieved by removing organic carbon or by chlorination. The residual chlorine level for management of drinking water quality in Seoul was reduced from 0.2 mg/L to 0.1 mg/L as an increased residual chlorine content in DWDS resulted in increases of DBPs as well as taste-odor problems (van der Kooij *et al.*, 1999, 2005). The concentration of THMs (trihalomethanes) is known to increase in drinking water supply systems with increased hydraulic residence time and to peak at the highest residual chlorine level (Annick *et al.*, 2009). It has also been reported that halo acetic acids (HAAs) could be decomposed by biofilm where residual chlorine was completely consumed, while THMs increased with distance from the WTP to the end point of the DWDS (Chen and Weisel, 1998; Baribeau *et al.*, 2005). Rossman *et al.* (2001) pointed out that the rate of DBP production in a distribution system would not necessarily be reduced by increased chlorine consumption due to non-DBP producing reactions with deposits on the pipe wall. Therefore, the change of activity in the biofilm due to the injection of phosphate could have an impact on the degradation of disinfection by-products in environments such as indoor plumbing systems where the disinfectant concentration is reduced with an increase of residence time.

In this study, using cultivation and culture-independent approaches, we investigated the effects of phosphorus-based corrosion inhibitors on biofilm formation, species diversity, and changes in the biofilm bacterial community structures with different pipe materials such as corrosion-resistant STS and corrsion-prone DCI under hydraulic conditions close to actual domestic plumbing systems. We also studied the effect of corrosion inhibitors on the biodegradability of DBPs.

Materials and Methods

Experimental devices

Four annular reactors (BioSurface Technologies, USA) were used to study the effect of phosphate addition on pipe corrosion and biofilm control. Each device was operated to simulate the hydraulic conditions of drinking water supply systems. Stainless steel 304 and ductile cast iron were selected as sample materials because they are the pipe materials most frequently used for distribution and service lines in Seoul. The chemical composition of pipe materials used in annular reactors are shown in Table 1. Sample pipe coupons of STS and DCI were installed in the annular reactors, i.e., 20 coupons in a reactor. The influent was tap water treated at the nearby Guui water treatment plant, which produces tap water through conventional water treatment technology (coagulation-precipitation-filtration-chlorination process) using the Han river as the water source. The Guui plant produces approximately 10^6 m^3/day. Characteristics of the drinking water used in the biofilm reactors are given in Table 2. The concentration of the phosphorus-based corrosion inhibitor (Na_2HPO_4) used in this study was 5 mg/L, which is by regulation the maximum allowable level. The reactors were operated under similar hydraulic conditions to domestic plumbing systems, i.e., at a speed of 50 rpm, which translates to a shear stress of 0.25 N/m^2 (Camper, 1996), corresponding to a flow rate of approximately 0.3 m/sec in a 100 mm diameter smooth pipe. The hydraulic residence time was determined by the injection rate. Reactors were operated with an injection rate of 170 ml/min (i.e., 40 L/day) for 9 months.

Table 1. Composition (wt%) of the investigated pipe materials (Korean Standards)

Metal composition	DCI (D 4311)	STS 304 (D 3698)
C	>2.5	<0.08
Si	<2.7	<1
Fe	*	*
S	<0.02	<0.03
Ni	-	8–10.5
Cr	-	18–20.00
P	<0.08	<0.045
Mn	<0.4	<2
Mg	<0.09	-

* Remainder of the ions present

Water quality

The residual chlorine concentration was measured using the *N,N*-diethyl-*p*-phenylenediamine (DPD) method with a portable colorimeter (HACH Pocket Colorimeter). Turbidity was analyzed with a 2100P turbidimeter (Hach Co., USA).

Table 2. The characteristics of the drinking water used in the annular reactors

Parameters (Unit)	Mean (S.D.)	Max.	Min.
Water Temp. (°C)	22 (±2.76)	26.5	11.6
Turbidity (NTU)	0.15 (±0.06)	0.43	0.07
Chlorine residual (mg/L)	0.4 (±0.09)	0.67	0.20
pH (U)	7.16 (±0.1)	7.02	7.38
TOC (mg/L)	1.2 (±0.1)	1.5	0.9
Fe (mg/L)	0.004 (±0.0029)	0.01	0.00
Al (mg/L)	0.29 (±0.34)	1.18	0.06
NO_3-N (mg/L)	1.7 (±0.3)	2	1.2
Hardness (mg/L as $CaCO_3$)	65.4 (±9.6)	80.4	53
Alkalinity(mg/L as $CaCO_3$)	46.2 (±6.3)	58.1	23.6
Cl^- (mg/L)	16.1 (±3.3)	22	11
SO_4^{2-} (mg/L)	15.4 (±3.2)	21.0	10.0
$KMnO_4$ (mg/L)	1.4 (±0.3)	1.9	1
Total solids (mg/L)	114 (±16.1)	139.3	93.9

Phosphate was analyzed by ionic chromatography (Dionex, USA). pH and total organic carbon (TOC) were analyzed with a pH meter (Metrohm 780) and TOC analyzer (Ionic, Sivers 820), respectively. Iron, copper, zinc, and manganese were measured with an ICP (Horiba Jobin Yvon/Activa M) according to standard procedures (APHA *et al.*, 1998). THMs were analyzed by GC/MSD (Agilent 6890N/Agilent 5979) after purge and trap pre-treatment (Tekmar 3000 concentrator) according to the US EPA method 524.2. THM content was determined by the summation of three compounds, chloroform, bromodichloromethane (BDCM), and dibromochloromethane (DBCM). HAAs were analyzed by GC/ECD (Agilent 6890N) after liquid-liquid extraction according to the US EPA method 552.3. HAA content was determined by the summation of dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromochloroacetic acid (BCAA), dibromoacetic acid (DBAA), monochloroacetic acid (MCAA), and monobromoacetic acid (MBAA). Haloacetonitriles (HANs), haloketones (HKs), and chloral hydrate (CH) were analyzed by GC/ECD (Varian CP-3800) after liquid-liquid extraction according to the US EPA method 551.1. HAN content was determined by totaling the 4 compounds that included trichloroacetonitrile (TCAN), dichloroacetonitrile (DCAN), bromochloroacetonitrile (BCAN), and dibromoacetonitrile (DBAN), while HK content was the total of 1,1-dichloroacetone and 1,1,1-trichloroacetone.

Sampling and bacterial counts

The number of bacteria in biofilm was analyzed by the protocol for biofilm analysis of the Center for Biofilm Engineering (Heersink, 2002). The coupon (1.5 × 15 cm) was aseptically removed from the reactor and placed into a 50 ml sterilized conical tube containing 20 ml of sterile phosphate buffer solution and 25 L of sterile sodium thiosulfate (10% w/v) prior to scraping. The coupon was held at one end with a flame-sterilized hemostat, and the slide was firmly scraped in a downward direction at least 5 times with a sterile cell lifter (Corning Inc.). The scraper was stirred periodically into the sterile dilution buffer contained in the 50 ml tube. After sufficient scraping, the slide was rinsed by pipetting the dilution buffer in the conical tube down the slide at least 3 times (Gagnon and Slawson, 1999). The sample specimen was treated twice with ultrasound for 30 sec to completely disperse the material in the liquid. The treated sample was diluted to the appropriate concentration by the 10-fold serial dilution method and then spread onto replicate plates of R2A agar. The inoculated plates were incubated at 28°C for 7 days, and plates with 30–300 colonies were selected. The number of bacteria per ml were averaged and converted into the number of bacteria per unit area (i.e., CFU/cm^2).

DNA extraction and PCR for the 16S rRNA gene

In order to identify bacterial species, representative colonies formed on the plates were selected based on colony morphology and were cultivated in R2A medium at 28°C for 3–4 days. Total genomic DNA was extracted from the isolates with a Qiagen DNeasy Tissue Kit (QIAGEN, Germany). PCR was carried out using the primers 27mf (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The primers 27f and 1492r anneal to positions 8–27 and 1,492–1,513 of 16S rRNA genes (*E. coli* 16S rRNA gene sequence numbering) (Lane, 1991), respectively. PCR amplification of nearly full-length 16S rRNA genes was performed in 50 µl reaction mixtures containing 10× PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris/HCl; pH 8.8, 25 mM $MgCl_2$, 0.1% Tween 20], 1 µl of template DNA, 25 pmol of each primer, 200 mM of each dNTP (GeneCraft, Germany), and 2.5 U of *Taq* polymerase (GeneCraft). The PCR cycle consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. After PCR amplification, 2 µl samples of the PCR products were checked by electrophoresis on 1.5% agarose gels. PCR products were purified using a QIAquick PCR Purification kit (QIAGEN).

Sequencing of the PCR-amplified 16S rRNA gene

Sequencing was performed using an ABI Prism BigDye Terminator Cycle Sequencing Ready kit (Applied Biosystems, USA) with the sequencing primers 27f and 1492r according to the manufacturer's instructions (Hugenholtz *et al.*, 1998). Sequences of approximately 400 unambiguous nucleotide bases were used for comparison with GenBank data using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

Denaturing gradient gel electrophoresis (DGGE) analysis of the biofilm bacterial community

Biofilm bacterial community DNA (April to July and October to November) was extracted using a FastDNA Spin kit (Qbiogene, USA). PCR amplification of 16S rDNA genes was performed as described previously with the primers 352TA (5′-ACTCCTACGGGAGGC-3′) and 515r (5′-ACCGCGGCTGCTGGCAC-3′) that target the highly variable V3 region (Ahn *et al.*, 2009). The PCR product contained a GC clamp of 40 bases added to the 515r reverse primer. Real-time PCR amplification of 16S rDNA genes was performed in 50 µl reaction mixtures containing 1× PCR buffer composed of 1.5 mM $MgCl_2$ (TaKaRa, Japan), 1 µl of template DNA, 0.5 µM of each of the forward and reverse primers, 0.2 mM of each dNTP, 2.5 U of *Taq* polymerase, 1 mg/ml of bovine serum albumin (Q-BIO Gene, USA), and 0.5× SYBR Green 1 (Cambrex Bio Science, USA). The real-time PCR reaction was performed using a Roche LightCycler 480 instrument (Roche, Germany). The PCR cycle consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 30 min. PCR products were subjected to DGGE analysis with the Dcode Universal Mutation Detection System (Bio-Rad, USA) as described previously (Kim *et al.*, 2005). After electrophoresis, gels were stained with SYBR Green I for 15 min, rinsed for 25 min, and photographed by UV transillumination (302 nm).

Microscopy of biofilms

Biofilms were observed by field emission scanning electron microscopy (SUPRA 55VP, Carl Zeiss) using 3,000-fold

magnification.

Statistical analysis

Statistical analysis of experimental data utilized the paired t-test. Each experimental value was compared with a corresponding control value. Statistical significance was deemed acceptable when the probability of the result, assuming the null hypothesis (p), was less than 0.05.

Results and Discussion

Effects of orthophosphate on water quality

The influent water quality of the reactors met the Korean drinking water standards, in particular for parameters (pH, Al, Cl^-, SO_4^{2-}, TS, Alkalinity, Hardness) that could affect the corrosion condition of pipe samples (Table 2). The calculated corrosion index (Langelier saturation index) of the tap water was evaluated to be -1.4 (-1.8–1.0), which correlates to being slightly corrosive (Tang *et al.*, 2011). The average water temperature was 22°C (11.6–26.5°C). The temperature of the influent increased to over 20°C from May to October with a peak over 24°C in the summer season (July to September), a favorable condition for microbial growth and corrosion. Iron, turbidity, residual chlorine, pH, and TOC before and after orthophosphate addition were investigated. The addition of 5 mg/L of phosphate reduced the iron concentration in DCI pipe approximately 7 fold. Thus, after 3 months of operation, the iron concentration met the drinking water quality standards (0.3 mg/L), demonstrating that phosphate effectively inhibited corrosion. Inhibition of corrosion resulted in an approximately 78% reduction of turbidity on average (from 5.98 NTU to 1.31 NTU) in DCI pipe and continuously decreased to a stabilized level of 0.5–1.0 NTU after 3 months of operation (Fig. 1). By contrast, during 9 months of operation, the addition of phosphate caused the average turbidity of STS pipe to increase from 0.17 NTU to 0.27 NTU ($p<0.001$), which still complies with standards (<0.5 NTU). Previous studies have shown that phosphate addition causes a reversal of surface charge and a more negative zeta potential (Appenzeller *et al.*, 2002;

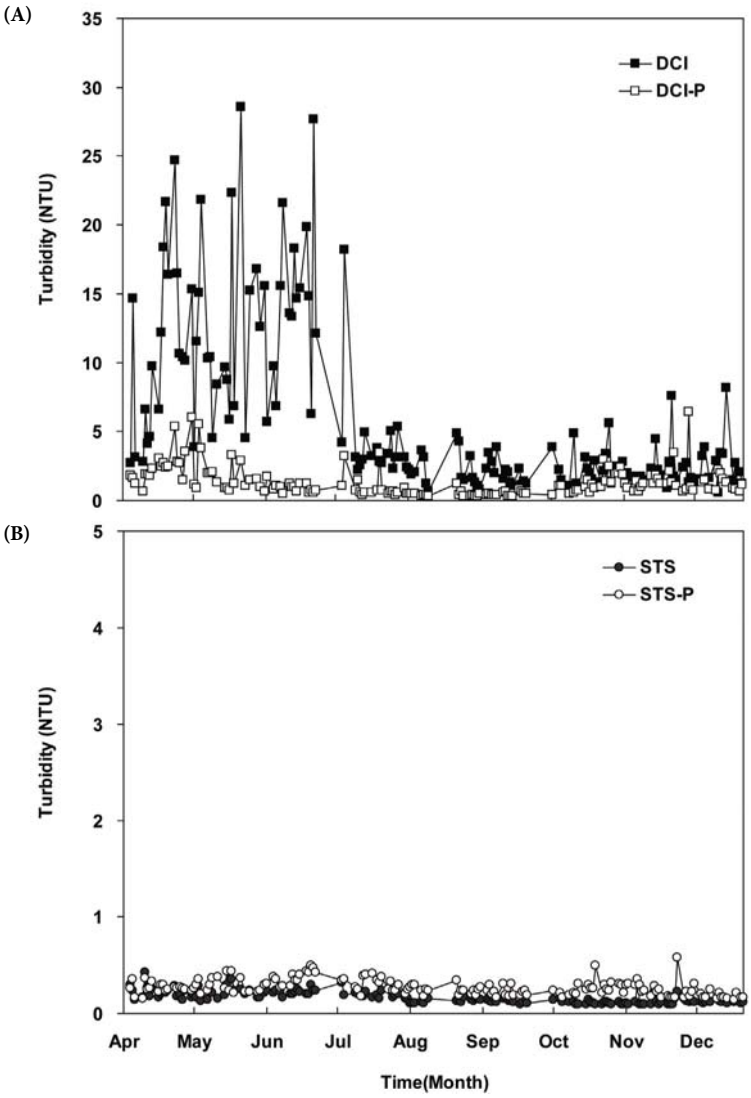

Fig. 1. Changes in turbidities in the reactors of DCI and STS pipes with and without phosphate addition. The sample size obtained from each reactor was 156.

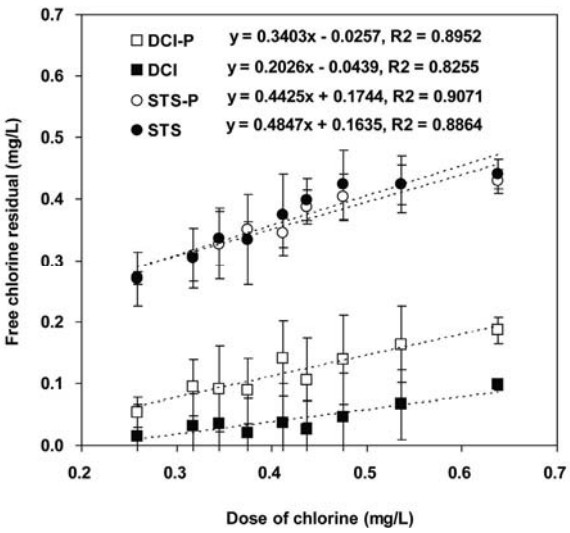

Fig. 2. Changes of chlorine residual in the reactors of DCI and STS pipes with and without phosphate addition according to the chlorine residual of influent water. Data are the averages of at least 18 independent experiments and presented as the Mean±SD.

Lytle and Snoeyink, 2002). Stainless steel is a moderately hydrophobic material, as indicated by the water contact angle (95.0±2.6°) and negative charge with a zeta potential value at pH 7.4 of approximately -25 mV (Nguyen *et al.*, 2011). The bacteria were negatively charged in the pH range between pH 1.2 and 10 (Appenzeller *et al.*, 2001). It has been reported that bacteria can attach to both hydrophilic and hydrophobic surfaces but the strength of bacterial attachment to hydrophobic surfaces is not as strong as one to hydrophilic surfaces (Boks *et al.*, 2008). The negative electrostatic force on the STS pipe surface may hinder the stability of biofilm community structure while attracting more cationic material. Furthermore, phosphate addition was observed to increase the metabolic potential and cultivability of biofilm cells (Juhna *et al.*, 2007; Fang *et al.*, 2009), which could be responsible for an increase in the detachment rate of daughter cells accompanied by a high growth rate. The concentration of phosphate in the DCI pipe decreased by 11% during the experiment, while no change was observed with the STS pipe. Appenzeller *et al.* (2001) reported that the respective phosphate concentrations decreased by 88% and 77% in cast iron pipe with 1 mg/L and 5 mg/L of added phosphate, while it decreased by 12% in slightly corroded STS pipe with 1 mg/L of added phosphate. This distinction may be attributable to differences in the test environmental factors such as the degree of pipe corrosion, presence of disinfectant, and hydraulic conditions. In our study, none of the STS 304 pipes were corroded, and consequently, phosphate anion was not used for the formation of stable phosphate-metal complexes.

The concentration of residual chlorine in the influent was approximately 0.4 mg/L (0.2–0.7 mg/L). Residual chlorine was consumed very rapidly by corrosion products in DCI pipe to a level of 0.04 mg/L (82.4–98.4% reduction of residual chlorine from the influent), while it was reduced to 0.11 mg/L (59.8–89.4%) in DCI pipe with added phosphate as a corrosion inhibitor (Fig. 2). Reduction rates of residual chlorine in DCI pipes were 0.050 mg/L·min and 0.062 mg/L·min with and without added phosphate, respectively. This result suggests that the concentration of residual chlorine is closely related to corrosion in the metal pipe and phosphate addition functions to reduce chlorine consumption by corrosion control. Unlike DCI pipes, there was no significant difference ($p>0.05$) in the residual chlorine levels between phosphate-treated and control STS pipes (0–24.6% of the chlorine was consumed).

The TOC concentration was reduced more substantially in DCI pipe (0.9 mg/L) than in STS pipe (1.2 mg/L) regardless of phosphate addition ($p<0.001$). Organic carbon is an essential nutrient for bacterial growth. Adsorption of organic carbon by iron oxide-containing corrosion products can promote biofilm growth (Butterfield *et al.*, 2002). Hence organic carbon might be consumed more rapidly in DCI pipe containing substantial amounts of corrosion products and biofilm than in STS pipe. By contrast, the addition of phosphate caused no significant change in TOC concentration in STS pipes. In DCI pipe, the TOC concentration was lower ($p<0.001$) without the addition of phosphate (avg. 0.87 mg/L) than with the addition of phosphate (avg. 0.91 mg/L). This distinction may be attributable to the differences in nutrient availability and EPS (exopolysaccharide) production conditions from the corroded surface of the pipe. The production of EPS in biofilm requires the uptake and conversion of large amounts of organic carbon and other nutrients. Fang *et al.* (2009) suggested that phosphate addition can promote biofilm cell growth while decreasing EPS production. This may be due to an alteration of the nutrient conditions from phosphorus limiting to carbon limiting. As the carbon source becomes limiting, cells utilize carbon to synthesize cellular components and to produce energy instead of producing extracellular polymers to protect cells enclosed in the biofilm.

Effects of orthophosphate on disinfection by-products (DBPs)

The concentration of DBPs in the distribution system can be affected by several factors, such as residual chlorine, TOC, bromide concentration, pH, water temperature, residence time, and microbial activity (Krasner *et al.*, 1989; Summer *et al.*, 1996; Singer *et al.*, 2002). In this study, to

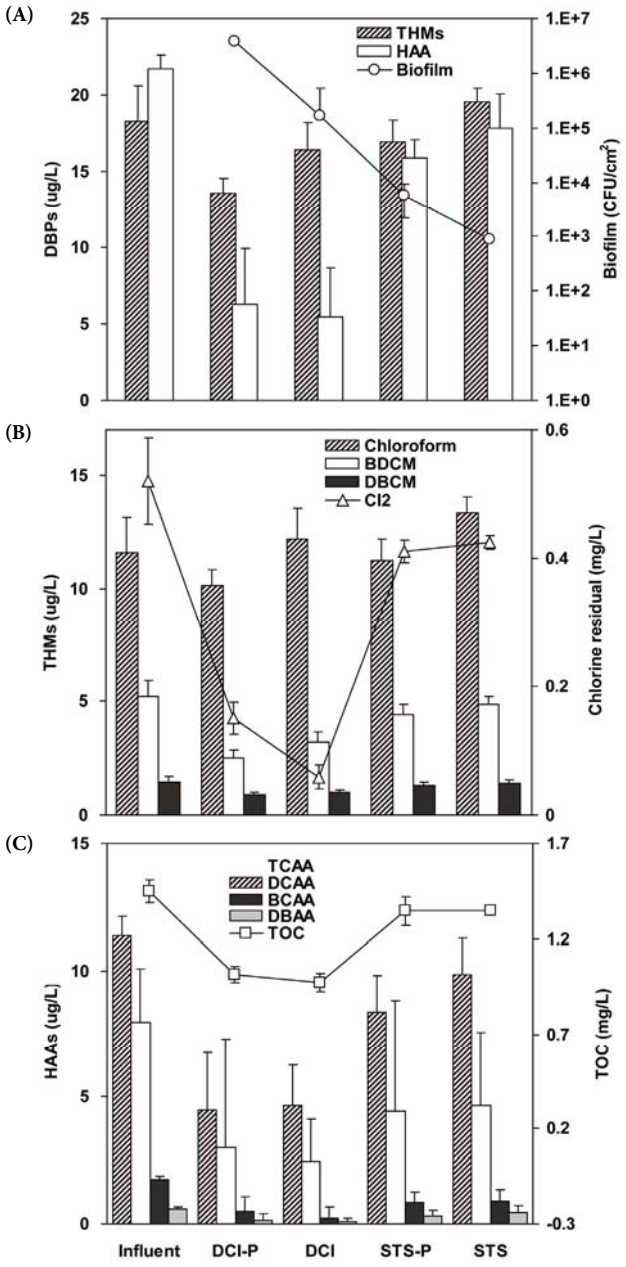

Fig. 3. Comparison of concentrations of disinfection-by-products in the annular reactors. (A) DBPs decreased inversely proportional to the concentration of bacteria in biofilm, (B) THMs increased proportional to the concentration of the chlorine residual, and (C) HAAs increased proportional to the concentration of the TOC.

determine whether phosphate addition could affect the biodegradability of DBPs, the concentrations of DBPs were analyzed after 6 months of operation when the biofilm was stable and mature. The concentration of bacteria in the biofilm was initially maintained at approximately 10^3 CFU/cm^2 and 10^5 CFU/cm^2 for STS pipe and DCI pipe, respectively. Addition of phosphate increased the respective biofilm cell count in STS and DCI pipes by approximately 1 log unit (Fig. 4). The concentration of chlorinated DBPs decreased proportionally to the bacterial cell count in the biofilm (Fig. 3). HAAs and CH decreased in all of the pipe samples while THMs increased depending on the pipe material (presence of corrosion product) and environmental conditions (phosphate addition). Although the concentration of THMs decreased (16–38%) for the first month, it increased in November and December when the water temperature was lower and the residual chlorine content was higher. The THM concentration decreased by 22.9% in DCI pipe with the addition of phosphate, while it increased by 7% in STS pipe without the addition of phosphate. The large reduction of THMs in DCI pipe might be caused by the consumption of residual chlorine by corrosion products in the early stage of the reaction and by decomposition of previously-formed THMs by higher biofilm content under conditions of low residual chlorine. Chloroform, which was the dominant THM component (63.4%), decreased by 9.6% only in the DCI pipe treated with phosphate, while it increased by 8.6% and 20.2% in the DCI pipe and STS pipe without phosphate treatment, respectively. Unlike other DBPs, chloroform was relatively refractory to degradation

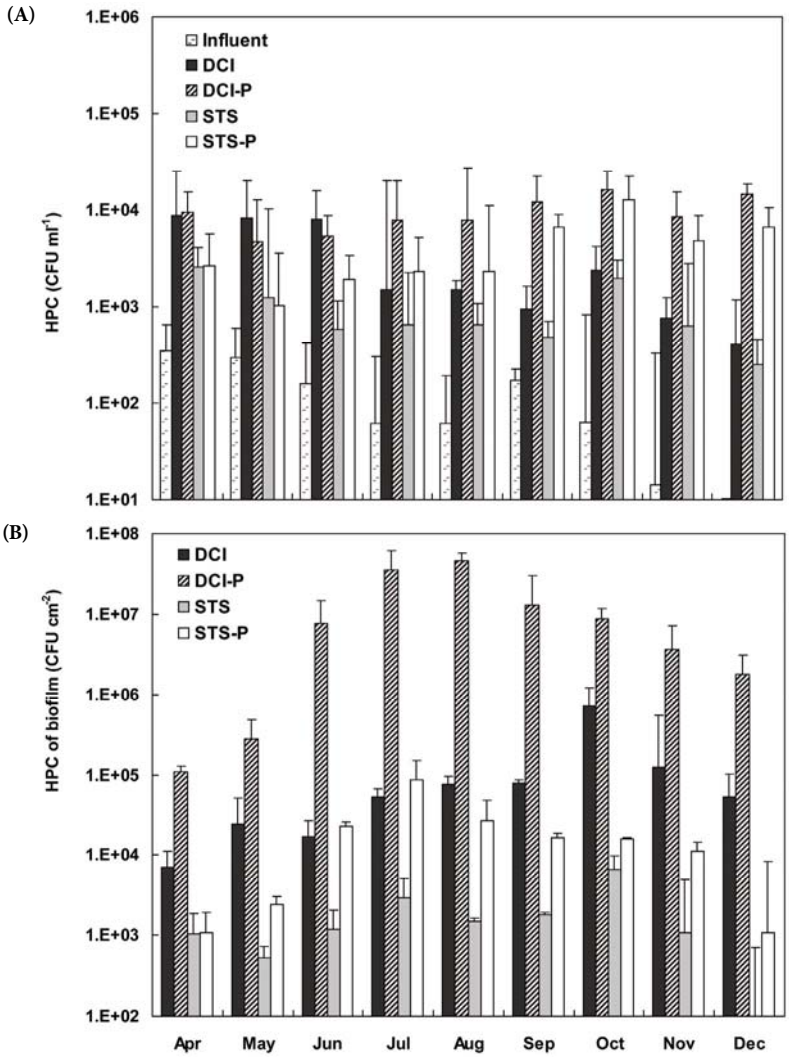

Fig. 4. Monthly changes of bacterial cell counts in the effluent (A) and biofilm (B) on the DCI and STS pipes with and without phosphate addition. Bacterial cells were grown on R2A agar at 28°C for 7 days. Data are the averages of at least 2 (A) to 12 (B) independent experiments and presented as the Mean±SD

by the biofilm community, but was more susceptible to biodegradation under phosphate-treatment conditions. BDCM and DBCM decreased up to 50% and 33.4%, respectively, relative to the concentration of bacteria in the biofilm (Fig. 3). This observation is in agreement with a report that the increase in substrate utilization potential of biofilm communities in response to the addition of biodegradable organic carbon was more prominent in combination with the addition of phosphorus compounds (Park and Hu, 2010). Wahman *et al.* (2005) reported that THMs could be decomposed by co-metabolism with nitrifiers such as *Nitrosomonas,* and the THM decomposition rate constant (K_{1THM}) increased with increased bromide substitution in the THM (i.e., TBM>DBCM>BDCM>TCM).

Table 3. Concentrations of disinfection-by-products (DBPs) of influent water and average reduction rate (%) of DBPs in the annular reactors

DBPs	Influent Average (μg/L)	Average reduction rate (%)			
		DCI-P	DCI	STS-P	STS
THMs	18.3 (10.1–25.8)	22.9	6.7	3.7	-12.3
Chloroform	11.6 (5.5–15.3)	9.6	-8.6	-1.0	-20.2
BDCM	5.2 (3.4–8.0)	50.0	36.1	14.1	3.5
DBCM	1.5 (1.0–2.5)	33.4	26.8	9.3	2.3
HAAs	21.7 (16.8–25.7)	66.6	63.5	34.9	25.4
TCAA	11.4 (8.0–15.3)	60.2	61.1	26.6	13.7
DCAA	8.0 (6.7–9.6)	70.1	63.5	44.0	40.8
BCAA	1.7 (1.5–2.0)	87.1	71.8	50.5	47.8
DBAA	0.6 (0.5–0.8)	85.8	75.0	42.5	20.5
CH	6.3 (4.6–11.9)	89.6	93.2	32.5	20.2

The HAA concentration showed a decreasing tendency in all sample pipes. The rate of HAA content reduction was more than 2 fold higher (1.8 fold with phosphate, 2.6 fold without phosphate) in DCI pipe than in STS pipe (Table 3), and these rates for HAAs were 2.8–10.0 fold higher than those for THMs. This observation is in agreement with a report that HAA concentrations decreased in environments of low residual chlorine and high bioactivity due to a higher level of biological decomposition (Chen and Weisel, 1998; Tung and Xie, 2009). This result was interpreted as lower molecular weight compounds requiring less cell energy for their uptake and utilization. Chen and Weisel (1998) reported that HAA, HAN, HK, and chloropicrin (CP) concentrations decreased in the summer when the water temperature was higher, while the THM concentration increased with residence time. Among the HAA species, TCAA and DCAA were 48.5% and 32.5%, respectively, and their reduction rates were more than 60% in DCI pipe regardless of phosphate addition. Baribeau *et al.* (2005) reported that, unlike DCAA, higher halogen substituted HAA species such as TCAA were not easily biodegraded. In this study, unlike DCAA, TCAA was substantially reduced in STS pipes when phosphate was added. HAA biodegradation occurred when the residual chlorine concentration was lower than 0.3 mg/L and HPC (Heterotrophic Plate Count) concentrations were high (>10,000 CFU/ml). Even with a higher HPC concentration, no HAA reduction was observed when the residual chlorine concentration was higher than 0.7 mg/L (Tung and Xie, 2009). However, in this study, despite a high concentration of chlorine, HAAs were highly degraded by the biofilm under stagnant conditions commonly found in plumbing systems. On the other hand, chloral hydrate (CH), haloacetonitriles (HANs), and haloketones (HKs) were detected in limited amounts and followed the same trend as HAAs.

Effects of orthophosphate on the bacterial concentration of biofilms

The concentration of suspended bacteria was 3.5 fold higher in DCI pipes than in STS pipes (Fig. 4A). The concentration of bacteria increased up to 13 fold and 23 fold in DCI and STS pipes, respectively, after July when the water temperature had increased and the red color of the water (due to corrosion) had dissipated. The concentration of biofilm cells was approximately 1 log unit higher in DCI pipe than in STS pipe (Fig. 4B). Niquette *et al.* (2000) reported that the concentration of bacteria was 10–45 fold higher in the biofilm formed in cast iron pipe (1–1.2 μg C/cm^2) than in PE and PVC pipe (less than 0.1 μg C/cm^2) when the concentration of residual chlorine was low (i.e., 0.0–0.03 mg/L). Corrosion products formed on the surface of cast iron pipe increases the surface area, moderating hydraulic mixing and increasing the precipitation of organic materials that supply nutrients to bacteria in the biofilm (Camper *et al.*, 2003). Chlorine consumption by iron oxides could also modulate the effect of disinfectants on bacteria (LeChevaillier *et al.*, 1993, 1996).

The addition of phosphate caused approximately 2 log and 1 log unit increases in the biofilm cell counts in DCI pipe and STS pipe, respectively (Fig. 4B). This observation is in agreement with previous reports that the addition of phosphate could significantly increase the microbial growth in DWDS even at very low levels, i.e., 0.1 mg PO_4-P/L (Critchley and Fallowfield, 2001; Lehtola *et al.*, 2002; Chu *et al.*, 2005; Torvinen *et al.*, 2007; Fang *et al.*, 2009; Vrouwenvelder *et al.*, 2010). Juhna *et al.* (2007) reported that an increased concentration of phosphate could extend the life

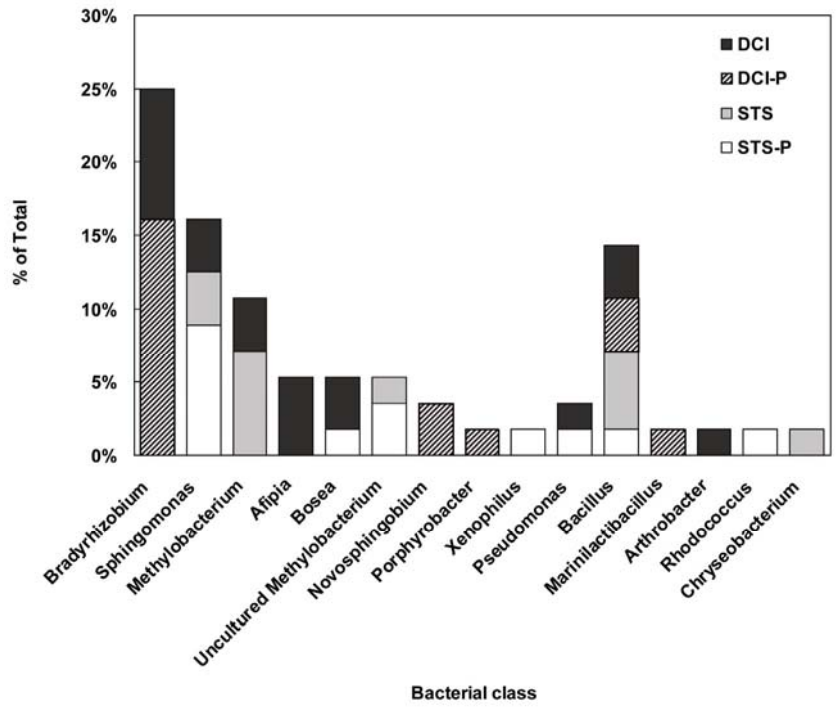

Fig. 5. **Taxon distribution of bacteria isolated from biofilms of the DCI and STS pipes with and without phosphate addition.**

of *E. coli* in the biofilm of DWDS. In contrast, Appenzeller *et al.* (2001) reported that the addition of phosphate at appropriate levels (i.e., 0.1–2 mg/L) was effective in controlling bacteria in gray cast iron pipe, while the number of bacteria increased to a small extent in slightly corroded STS pipe. Batté *et al.* (2003) reported that the addition of 0.5 mg/L of phosphate in an annular reactor system fed with dechlorinated water had no impact on biofilm growth. Gouider *et al.* (2009) also reported that 1 mg PO_4^{3-}/L addition did not affect the bacterial density in a biofilm reactor containing glass beads. These apparently contradictory results can be interpreted as being a consequence of differences in the nutrient limiting conditions of the original influent. The molar ratio for carbon, nitrogen and phosphorus of microbial biomass is ~100:20:1.7, showing that, compared to carbon, lower phosphorus levels are needed for microbial growth. The drinking water used in this study was observed to contain low levels of phosphorus as compared with AOC, especially during the dry season (Choi and Choi, 2010).

Many water utilities are apprehensive of using phosphorus-based corrosion inhibitors because the addition of phosphorus may result in an increase in microbiologically-related water quality problems such as taste and odor issues and violation of the TCR (Abernathy and Camper, 1998). Phosphorus-based corrosion inhibitors have been used to control corrosion and scale in pipes for more than 60 years. Since the US EPA announced the LCR (Lead and Copper Rule) in 1991, many water utilities have added phosphorus-based corrosion inhibitors to drinking water to meet the revised lead and copper level regulation, although a variety of negative effects, such as microbial regrowth, increased treatment cost, and scale formation in hot water, have been reported (McNeill and Edwards, 2002). Corrosion control by phosphorus-based corrosion inhibitors of microbial growth is not only affected by the type and concentration of the corrosion inhibitor, but also by the pipe material and type of disinfectant. LeChevaillier *et al.* (1996) reported that detection of coliform decreased with phosphorus-based corrosion inhibitors used to control corrosion in drinking water supply systems. Volk *et al.* (2000) reported that the corrosion rate of gray cast iron pipe could be controlled by less than 3 mpy (milli-inch per year) of added zinc phosphate (1.5–2.0 mg PO_4-P/L) and there was no microbial regrowth with an average chloramine concentration of 3.4 mg/L (2.8–4.3 mg/L). Abernathy and Camper (1998) reported that polyphosphate was not very effective for biofilm control while zinc orthophosphate could reduce biofilm by 1.5 log units when treated with chloramines. Unlike corrosion resistant materials, corrosion-susceptible material like gray cast iron causes a high chlorine demand, and hence, is less effective for disinfection of biofilm bacteria. Thus, microbial regrowth due to phosphate addition can be compensated for by the increased disinfection capability from corrosion inhibition. Therefore, to use a phosphate-based corrosion inhibitor in a plumbing system, it is necessary to determine the type and optimum concentration of the inhibitor to avoid affecting microbial growth.

Effects on bacterial community structure of biofilm

16S rRNA gene sequences of 102 bacterial colonies cultivated in R2A agar medium were analyzed to identify the species of bacteria in the biofilm, which were then categorized into 6 bacterial phyla. The dominant phylum was *Proteobacteria* (50%) followed by *Firmicutes* (10%), *Actinobacteria* (2%), *Bacteroidetes* (1%), and unknown (37%). *Proteobacteria*, composed of α-subclasses except for *Xenophilus*

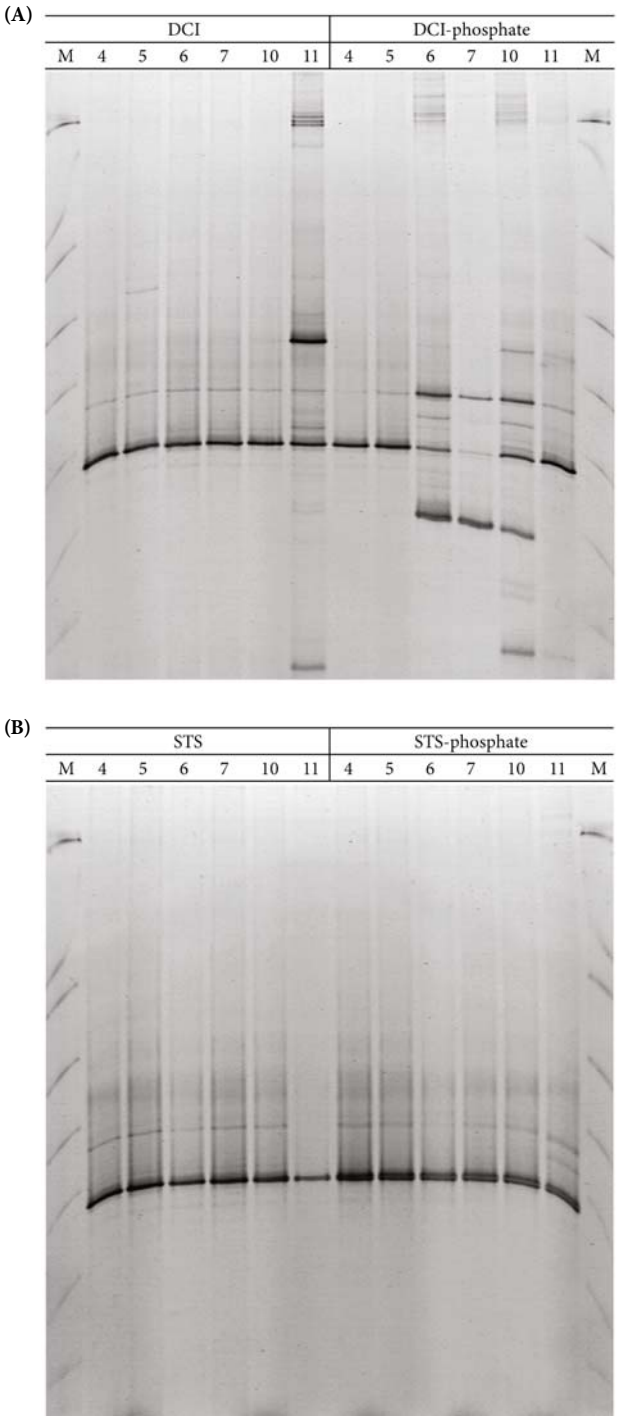

Fig. 6. DGGE profiles of the bacterial community in biofilm of the DCI and STS reactors with (P) and without phosphate. Lanes: 4-11, April-November; M, 100-bp DNA size marker.

(β-*Proteobacteria*) and *Pseudomonas* (γ-*Proteobacteria*), were classified into 15 bacterial genera (Fig. 5). The dominant genus from DCI pipe, regardless of the addition of phosphate was *Bradyrhizobium*, with *Bacillus* the next most common genus. *Badyrhizobium* is known to produce many soluble EPS composed of acid hetero-polysaccharides (80–90%) (Krieg and Holt, 1984; Kuykendall *et al*., 1988), and they can survive even under conditions of scarce H_2 and CO_2 by chemolithotrophic growth. In addition, *Bradyrhizobium japonicum* was reported to precipitate metal ions such as Fe^{3+} and Al^{3+} by acidic EPS (glucose, mannose, galactose, and galacturonic acid) formation (Dudman, 1976; Corzo *et al*., 1994). Capsular poly-γ-glutamate, which is a polymer formed by *Bacillus licheniformis*, is also known to react with Fe^{3+} to develop rust-colored ferrihydrite (McLean *et al*., 1990). McRae *et al*. (2004) reported that TCAA and MCAA are decomposed by *Xanthobacter*, *Sphingomonas*, *Rhizobium*, *Bradyrhizobium elkanii*, *Comamonas testosterone*, and *Bdellovibrio bacterioborus*. *Bacillus* is also known to be responsible for dehalogenation (Olaniran, 2001). Hence there is a possibility that *Bradyrhizobium* and *Bacillus* could grow well under corrosive conditions and contribute to the decomposition of DBPs. *Methylobacterium* and *Afipia* were detectable only in the reactor without added phosphate. *Sphingomonas*, the dominant genus in STS pipe, is known to appear at an early stage of biofilm formation. Kelly *et al*. (2004) reported that *Sphingomonas* and *Methylobacterium* could use various carbon sources as nutrients. In addition to the amount of biofilm found, pipe materials also could affect the composition as well as metabolic activity of microbes present in biofilm (Schwartz *et al*., 1998; Jang *et al*., 2011). Norton and LeChevallier (2000) observed significant differences in the growth and composition of biofilms formed in iron pipes as well as their ability to withstand disinfectants compared with biofilms formed in PVC pipes.

16S rRNA gene PCR-DGGE was carried out to investigate the bacterial community structure of biofilm. The biofilm DGGE profiles of STS pipes were relatively simple showing two major bands over the course of the experiment, which did not depend on phosphate addition (Fig. 6B). Similar DGGE DNA band patterns were observed from the biofilm of DCI pipes without phosphate addition from April to October (Fig. 6A, left panel, lanes 4–10). However, with the addition of phosphate, the number of bands increased from 2 to 8, demonstrating an increase in bacterial diversity in DCI pipes (Fig. 6A, right panel, lanes 6–10). These results suggest that bacterial diversity is greatly increased in the biofilm of DCI pipes with added phosphate, which is in agreement with the results from the above cultivation experiments as well as other previous research (Keinänen *et al*., 2002; Batte *et al*., 2003).

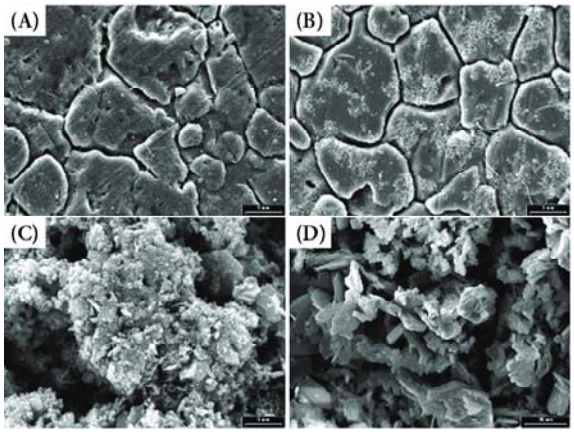

Fig. 7. Field emission scanning electron micrograph (FESEM) of biofilm. (A) STS pipe, (B) STS pipe with phosphate, (C) DCI pipe, and (D) DCI pipe with phosphate. Scale bar=5 μm.

Field emission scanning electron microscopy (FESEM)

After the biofilm became stable, on day 60 of operating the annular reactor, sample coupons were analyzed by FESEM (Fig. 7). For STS pipe, more biofilm and sediment materials formed on the surface of the sample coupons when phosphate had been added. The surface of the STS was marked with numerous channels and crevices. Attachment appeared to be random relative to the position of the polishing grooves. This indicates that the biofilm microorganisms had sufficient preformed polysaccharides to adhere firmly to all portions of the surface. In DCI pipe, phosphate addition could have transformed the pipe surface characteristics thereby affecting the release of iron oxide particles.

In conclusion, in this study, the addition of 5 mg/L of phosphate to plumbing systems significantly promoted the growth of biofilm regardless of the level of corrosion on the plumbing materials. Phosphate treatment also enhanced the biodegradability of DBPs. Species diversity in biofilm from DCI pipe significantly increased with the addition of phosphate, which suggests that phosphate treatment impaired the biological stability in the plumbing systems. As phosphorus is a limiting factor for microbial growth, when selecting the phosphate-based corrosion inhibitor for use in a plumbing system, it is necessary to determine the optimum concentration to avoid affecting microbial growth.

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