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Influence of Pipe Materials and VBNC Cells on Culturable Bacteria in a Chlorinated Drinking Water Model System

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Abstract To elucidate the influence of pipe materials on the VBNC (viable but nonculturable) state and bacterial numbers in drinking water, biofilm and effluent from stainless steel, galvanized iron, and polyvinyl chloride pipe wafers were analyzed. Although no HPC (heterotrophic plate count) was detected in the chlorinated influent of the model system, a DVC (direct viable count) still existed in the range between 3- and 4-log cells/ml. Significantly high numbers of HPC and DVC were found both in biofilm and in the effluent of the model system. The pipe material, exposure time, and the season were all relevant to the concentrations of VBNC and HPC bacteria detected. These findings indicate the importance of determining the number of VBNC cells and the type of pipe materials to estimate the HPC concentration in water distribution systems and thus the need of determining a DVC in evaluating disinfection efficiency.

Keywords: Viable but nonculturable (VBNC), direct viable count (DVC), drinking water, biofilm, pipe material

Bacteria in a VBNC state have metabolic activity, even though they are unable to be cultivated by the use of conventional procedures. However, VBNC bacteria can recover culturability under appropriate conditions [3, 25] and may even maintain their pathogenicity in a VBNC state [3]. Biofilms are communities of microbes that are attached and grow on surfaces, and the occurrence of biofilms can cause technical and hygiene problems with tap water [9, 14, 20, 21]. When the application of diverse disinfectants to control biofilms in a water distribution system was studied [8, 20], an increase in bacterial concentration was reported to occur at the household distribution system or the household tap, rather than from source waters or from the distribution system [26]. Water distribution systems consist of reservoirs and pipes, made from a diversity of materials, such as iron, stainless steel, polyvinyl chloride, *etc.* These materials are also common in household and building distribution systems. VBNC cells in drinking water [6, 21] can recover their culturability; pipe composition might affect the recovery of culturability. Although there have been reports on the effect of pipe composition, these focused on bacterial concentrations and not on the level or recovery of the culturability of VBNC bacteria [10, 16]. This study aimed to elucidate whether the VBNC bacteria could recover their culturability in drinking water by use of a model system and examined the

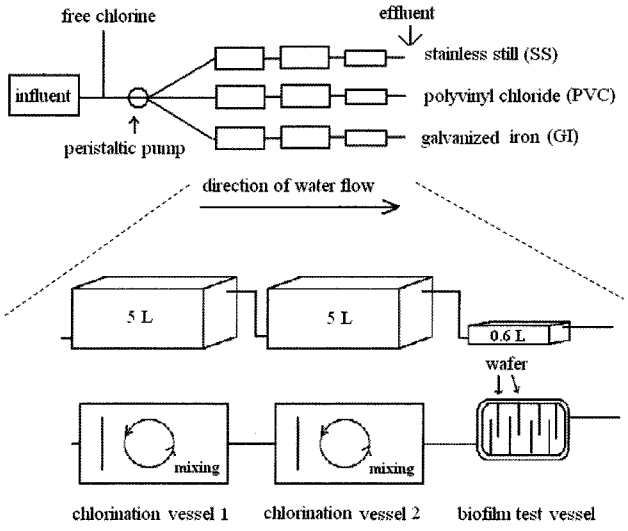

Fig. 1. Schematic diagram of the experimental model system. Vessels were made of stainless steel.
The effective volume and contact time with each vessel was 4.5 l and 150 min, respectively. Target concentration was 1.0 mg/l free chlorine residual and the retention time in the disinfection vessel was 300 min. The flow rate was 30 ml/min. Wafers were inserted in every other groove on both sides of the biofilm test vessels.

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Table 1. Water quality parameters of source water and tap water before chlorination.

Source water		Tap water	
HPC in R2A media (CFU/ml)	10^2–10^5	HPC in R2A media (CFU/ml)	10^1–10^3
pH	7.8–8.5	pH	6.5–7.2
Water temperature (°C)	19, 25, 5	Chloride residual (mg/l)	0.2–0.6
Dissolved oxygen (mg/l)	5.0–8.9	NH_4^+-N (mg/l)	N.D.–0.23
Biochemical oxygen demand (mg/l)	1.2–2.0	NO_3^--N (mg/l)	1.0–2.6
Chemical oxygen demand (mg/l)	2.8–4.2	Hardness (mg/l)	50–68
Suspended solids (mg/l)	3.0–6.0	$KMnO_4$ (mg/l)	0.7–2.1
Total nitrogen (mg/l)	1.99–2.64	Suspended solid (mg/l)	0.88–1.24
Total phosphorus (mg/l)	0.04–0.12	Turbidity (NTU)	0.05–0.3

HPC, pH, and chloride residual were measured in this study and the other parameters were obtained from the homepage of the Ministry of Environment, Korea (http://www.me.go.kr/). The average water temperatures of the source water were 19, 25, and 5°C for May, August, and December, respectively.

influence of pipe materials on the VBNC state and bacterial concentrations in drinking water.

Chlorination and biofilm test vessels (Fig. 1) contained separated wafers of different pipe materials. The effective volume and contact time with free chlorine residuals was 4.5 l and 150 min, respectively, for each chlorination vessel. The wafers were made from polyvinyl chloride, stainless steel, and galvanized iron. The biofilm vessels were arranged in three rows by wafer materials as shown in Fig. 1. The wafers (25×75×1.0 mm) were inserted in every other groove on both inside walls of the biofilm test vessels (Fig. 1) after confirming that the wafers were free from HPC after being immersed in 70% (vol/vol) ethanol for 24 h. A multichannel peristaltic pump (Masterflex, Cole Parmer Instrument Co., U.S.A.) was used for each vessel. Chlorination vessels and biofilm test vessels were tightly closed with covers.

A sand filtrate of source water was added to tap water and was chlorinated [17]. The source water was sampled from the catchment area in May (spring), August (summer), and December (winter) and was then passed through an autoclaved sand filter (effective diameter: 6.5 cm; effective height: 30 cm) at the laboratory within 4 h of sampling. The influent, a mixture of tap water and filtrate (200:1), was made everyday and retained in a one-hundred liter plastic container. Free chlorine was continuously supplied to the mixture with a target dose of 1.0 mg/l at the effluent (Fig. 1) after confirming the complete reduction of HPC by pre-tests before the experiment proceeded. This experimental system was operated for one week with a flow rate of 30 ml/min, and a 0.05 min^{-1} dilution rate at the biofilm test vessel. The experimental system was operated at 6–13.3°C (May), 18.4–23.6°C (August), and 1.4–6.7°C (December).

Reversibly bound bacteria were washed out and biofilm bacteria were detached by the Lee *et al.* method [13, 14]. The pour plating method was performed by the use of minimal R2A agar (Merck) in triplicate and 0.05% (wt/vol) sodium pyruvate was added [1]. Colony-forming units (CFU) were counted after culturing at 20°C for 1 week. Chlorinated water and influent was sampled with autoclaved polypropylene tubes at chlorination vessel 2 (Fig. 1) and the plastic container, respectively. DVC and TBN (total bacterial number) were enumerated by the use of fluorescent microscopy (Zeiss, Göttingen, Germany) according to the Standard Methods [2].

All statistical analyses were performed with the SAS (Statistical Analysis System) software package for Windows (ver. 8.1) (SAS Institute, Cary, NC, U.S.A.) based on 95% confidence levels. After normalization, analyses were performed using paired *t*-tests and analysis of variance (ANOVA).

Water quality parameters of source and tap waters are shown in Table 1. Consumption of MnO_4 was below 2.1 mg/l, indicating that organic pollution was low in the tap water. The influence of chemical factors of the source water to the influent of the system would be negligible as the volume of filtrate to tap water was about 5%.

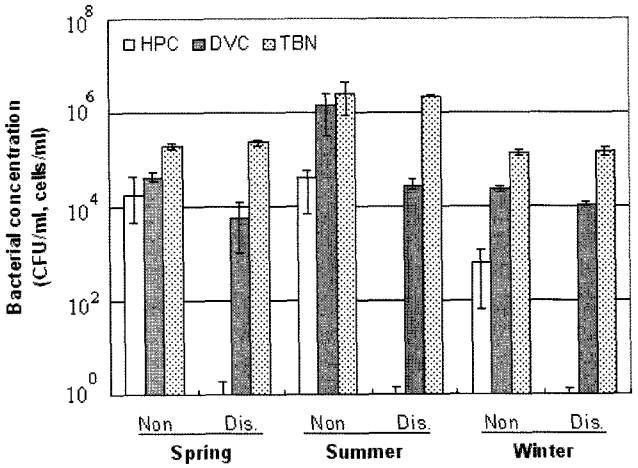

Fig. 2. Changes in the heterotrophic plate count (HPC, CFU/ml), direct viable count (DVC, cells/ml) and total bacterial number (TBN, cells/ml) in the influent (mixture of tap water and source water) before chlorination (Non) and after chlorination (Dis).

Fig. 2 shows the effect of chlorination on the HPC, DVC, and TBN of the influent. Residual chlorine at the effluent was measured periodically, more than 3 times (Advantec, Japan) in a day, and the concentration was always about 1.0 mg/l. Chlorination eliminated almost all HPC and the disinfection efficiency was 4-log (99.99%) or more (Fig. 2), as has been reported in previously published study [12]. The DVC showed about 10^4–10^6 cells/ml, and 2-log (99%) or less reduction was observed in the spring and summer, respectively (Fig. 2). Less than 1-log reduction was observed in the winter. The TBN was in the range of 10^5–10^6 cells/ml before and after chlorination, which indicates that chlorination caused only a slight decrease in the TBN in this study. The survived DVC and TBN bacteria after chlorination would be VBNC cells. The TBN, the acridine orange direct count, has the limitations that it does not discriminate between viable and dead cells. This might result in a slight decrease of the TBN by chlorination (Fig. 2). Therefore, we concluded that the survived DVC rather than the TBN cells in the disinfected influent where no HPC bacteria were detected are in a viable but nonculturable state. This study suggests that VBNC bacteria would attach, regain their culturability, and result in biofilm formation, and may shed many bacteria into the water column. We confirmed complete reduction of HPC by pre-tests before proceeding with the experiment (data not shown) and almost all media contained no HPC; however, sometimes there were one or two colonies in one of the triplicated media. C×t values (disinfectant concentration (C) multiplied by contact time (t) between the disinfectant and microorganisms) were lower in this study compared with the guidelines of the U.S. E.P.A. [22]. However, Gauthier and colleagues [12] have reported the survival of bacteria in high C×t values, and Baudart and colleagues [5] reported that VBNC cells were present in drinking water. Hence, we thought that our model system conditions were close to those seen in actual water distribution systems.

Fig. 3 represents the distribution of bacterial concentrations on biofilm at each substratum. Culturable growth of bacteria on pipe materials was observed from day 1 and reached 10^1–10^4 CFU/cm^2 according to the substratum and the season (Fig. 3A). Almost all wafers showed a 3-log DVC level at the day-1 samples and increased 1-log during 7 days in spring and winter (Fig. 3B). All wafers harbored a 5-log (cells/cm^2) level DVC from day-1 samples in the summer (Fig. 3B). The TBN showed ca. 1-log higher values than DVC, although they exhibited similar patterns (Fig. 3C). Galvanized iron (GI) showed a higher level of culturable bacteria than stainless steel (SS) and polyvinyl chloride (PVC). Others [9, 17] have reported that PVC had a lower biomass inventory than GI, as seen in this study. This trend was similar for both pristine and aged materials [9]. There were no differences in the HPC level between PVC and SS systems [27] as also seen from our results. HPCs

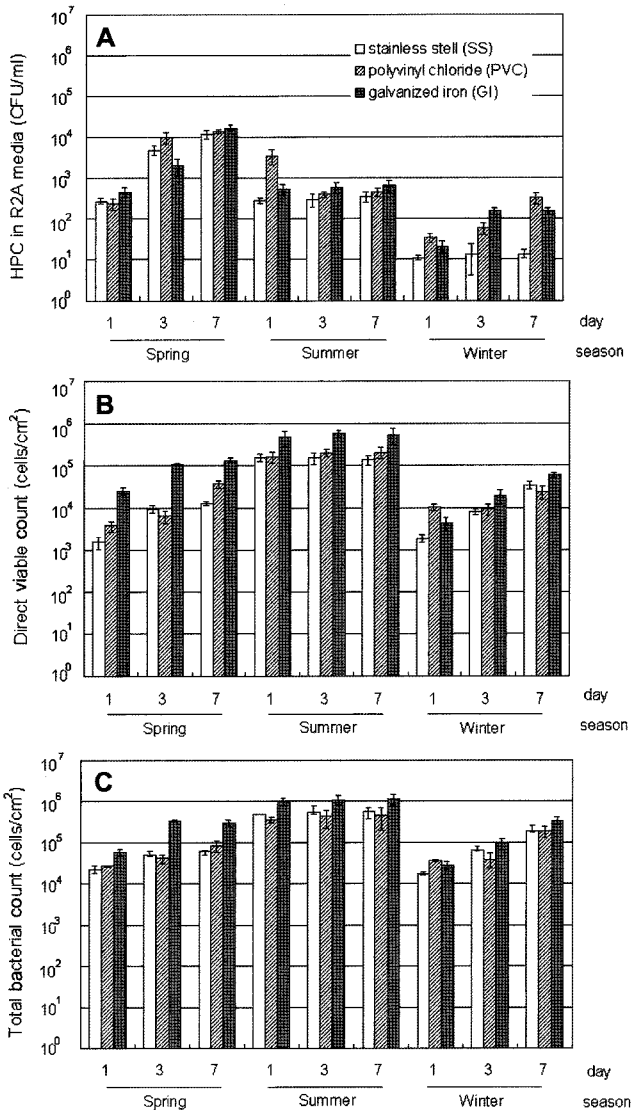

Fig. 3. Patterns of HPC (CFU/cm^2), DVC (cells/cm^2), and TBN (cells/cm^2) in biofilm on stainless steel (□), polyvinyl chloride (▨), and galvanized iron (■) wafers at days 1, 3, and 7.

were significantly different with relation to the substratum (P=0.01), exposure time (P<0.001), and season (P<0.001) as determined by ANOVA. There can be a different view in that pipe materials may not have a significant relationship with the concentration of bacteria in the biofilm as the detachment efficiency might be different among the three substrata. However, our results were based on the same detaching method that was applied to all substrata as others have employed [9, 17, 27], indicating that the pipe materials had a significant relationship with the bacterial concentration in the effluent. Although all of the HPCs in the influent were attached to the substratum, the biofilm showed a 0.1–29.8 (mean 6.6)-fold higher HPC than the disinfected influent at day 1. The doubling time of the HPC was 1–12 days [11, 19] and even more than 20 days [6] in the biofilm

of water distribution networks, which means our finding results from the recovery of culturability of VBNC bacteria. However, there is another possibility that bacterial species grown in each season have different growth rates, or that a small population of culturable cells is responsible, rather than requiring a change in the growth properties of temporarily nonculturable cells [4, 24]. These results illustrate that a large portion of VBNC bacteria could be in the biofilm of drinking water and can recover their culturability. Health-threatening bacteria and indicators were detected on biofilms in drinking water in previous studies [13, 15, 18, 20, 21]. Wong and colleagues [25] have shown, in addition to bacterial pathogens maintaining their pathogenicity in the VBNC state, that they were able to recover culturability under appropriate conditions. This might imply that more strict and safe disinfection is required and disinfection efficiency should be evaluated not only by HPC but also by DVC.

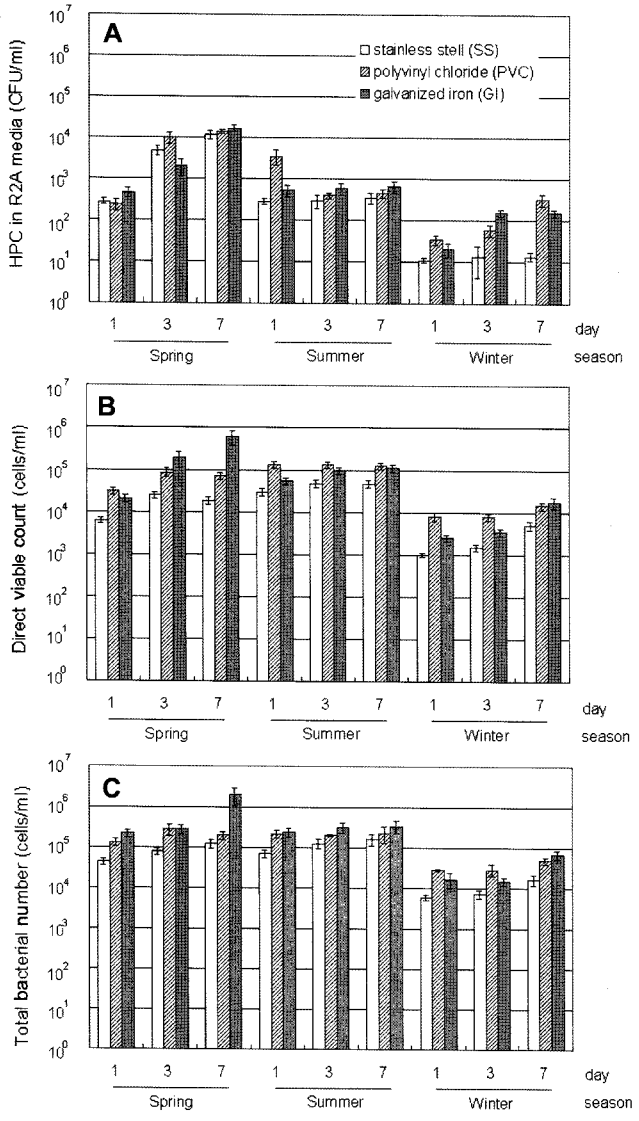

Fig. 4. Patterns of HPC (CFU/ml), DVC (cells/ml), and TBN (cells/ml) in the effluent of stainless steel (□), polyvinyl chloride (▨), and galvanized iron (■) wafers at days 1, 3, and 7.

Fig. 4 illustrates the distribution of bacterial concentrations in the effluent at different substratums. Effluents of three pipe materials showed HPC from day-1 samples. As there were almost no culturable bacteria in the disinfected influent (Fig. 2), and it is impossible for bacteria to grow in the planktonic state at the dilution rate (0.05/min) of this study, the bacteria in the effluent (Fig. 4A) would have originated directly from the biofilm. SS showed the least HPC, DVC, and TBN by the paired *t*-test ($P \leq 0.02$), whereas there were no differences between PVC and GI. Differences in the cell concentration of the effluent may be due to differences in the strength of binding to the different surfaces. Wäsche and colleagues [23] found that hydrodynamic conditions had a strong influence on the growth of biofilms and the detachment of biomass from biofilms. However, our results are rather controversial to those of Wäsche and colleagues [23], as all the hydrodynamic conditions were equal in all of the tested seasons in this study. Boe-Hansen and colleagues [7] emphasized the relationship of the net growth rate to the detachment rate in the quasi-stationary phase biofilm. These investigators have suggested that there was another factor besides the strength of binding to the different surfaces for differences of bacterial densities in biofilm. Considering the relation between bacteria in the effluent and the biofilms, the HPC/DVC (%) varied from 0.03 to 16.4 (mean 2.34) in the biofilm and from 0.28–63.9 (mean 5.38) in the effluent. The biofilm and effluent showed significantly different ratios (P=0.008, *t*-test). This result might also support the recovery of the culturability of VBNC bacteria in biofilms and suggest that biofilms are detached when the HPC/DVC ratio is increased.

In conclusion, our results indicate the importance of VBNC bacteria on biofilm development in chlorinated water, pipe materials on the microbiological water quality of drinking water, and the need of DVC determination in evaluating disinfection efficiency.

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