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Effects of Pipe Materials on Chlorine-resistant Biofilm Formation Under Long-term High Chlorine Level

Zebing Zhu · Chenguang Wu · Dan Zhong · Yixing Yuan · Lili Shan · Jie Zhang

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Abstract Drinking water distribution systems are composed of various pipe materials and may harbor biofilms even in the continuous presence of disinfectants. Biofilms formation on five pipe materials (copper (Cu), polyethylene (PE), stainless steel (STS), cast iron (CI), and concrete-coated polycarbonate (CP)) within drinking water containing 1.20 mg/L free chlorine, was investigated by flow cytometry, heterotrophic plate counts, and denaturing gradient gel electrophoresis analysis. Results showed that the biofilms formation varied in pipe materials. The biofilm formed on CP initially emerged the highest biomass in 12 days, but CI presented the significantly highest biomass after 28 days, and Cu showed the lowest bacterial numbers before 120 days, while STS expressed the lowest bacterial numbers after 159 days. In the biofilm community structure, *Moraxella osloensis* and *Sphingomonas* sp. were observed in all the pipe materials while *Bacillus* sp. was detected except in the CP pipe and *Stenotrophomonas maltophila* was found from three pipe materials (Cu, PE, and STS). Other bacteria were only found from one or two pipe materials. It is noteworthy that there are 11 opportunistic pathogens in the 17 classified bacterial strains. This research has afforded crucial information regarding the influence of pipe materials on chlorine-resistant biofilm formation.

Keywords Drinking water distribution systems · Biofilm · Pipe material · Chlorine-resistant bacteria · Pathogenic bacteria

Introduction

Many problems in drinking water distribution systems (DWDS) are microbial in nature, including biofilm growth, nitrification, microbially mediated corrosion, and the persistence of pathogens [1,2]. In DWDS, owing to the large surface to volume ratio, at least 95 % of the total biomass has been reported to be found adhered to the surface of pipelines [3]. There is

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Z. Zhu · C. Wu · D. Zhong (✉) · Y. Yuan · L. Shan · J. Zhang
State Key Laboratory of Urban Water Resource and Environment,
School of Municipal and Environmental Engineering, Harbin Institute of Technology, 150090 Harbin, China
e-mail: zhongdan2001@163.com

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hardly any pipe material that does not allow biofilm formation [1]. The ability of pipe materials to support drinking water biofilm varies dramatically from plastic to metal pipes as they exhibit different degrees of surface roughness and chemical activity [4]. Moreover, the type of material can also affect the disinfectant efficiency of biofilms [1,5]. Biofilms grown on copper, polyethylene (PE), PVC, and cement-lined ductile iron were inactivation with a much lower amount of free chlorine or monochloramine than those grown on unlined iron surfaces, probably because free chlorine is known to preferentially react with ferrous iron to produce the insoluble ferric hydroxide [1,6]. However, there were some controversial results reporting that plastic materials, such as PVC and polyethylene (PE), were also found to promote more biofilm growth than iron alloys and cements [7]. Some reports even suggested that the choice of most approved pipe material had only little impact on the ultimate biofilm density in the long-term studies [7]. The discrepancies may be a result of differences not only in analytical methods and sampling protocols but also in water chemistry, flow regimen, disinfectant efficiency, pipe service ages, and temperature [5,7].

Most of the previous studies have provided sufficient information regarding the effects of pipe materials on biofilm biomass and microbial diversity at low chlorine levels (<1.0 mg/L free chlorine) [3,6–11]. However, municipal water supplies are usually disinfected with 0.5~ 3.0 mg/L chlorine to control bacterial growth [12]. The average free chlorine concentrations are usually more than 1.0 mg/L free chlorine at the outlet of the treatment plant [7,12–14]. Consequently, there is a pressing need to continually improve the understanding of the effect of pipe materials on chlorine-resistant biofilm formation (biomass and microbial diversity) near the treatment plant pipelines (>1.0 mg/L free chlorine). Moreover, the potential of pipe material to promote growth of pathogenic bacteria [1,9] (e.g., *Mycobacterium* spp., *Klebsiella* spp., *Aeromonas* spp., *Salmonella* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Legionella* spp., and *Vibrio* spp.) is an issue of great concern that deserves special attention. Therefore, more data are needed to evaluate the long-term effects of these pipe materials on pathogens at high disinfectant levels (>1.0 mg/L free chlorine).

To detailedly investigate the effects of pipe materials on chlorine-resistant biofilms formation and pathogenic bacteria under long-term high disinfectant level, five kinds of commonly used pipe materials were fed with long-term high free chlorine drinking water from the same pilot distribution system and were sampled after different trial periods. Bacterial concentrations were determined by flow cytometry and heterotrophic plate count (HPC) measurements, and microbial diversity was analyzed with denaturing gradient gel electrophoresis (DGGE).

Material and Methods

Model Distribution System

In order to study the effects of different pipe materials on microbial biofilm formation, five commonly used pipe materials (i.e., copper (Cu), polyethylene (PE), stainless steel 316 (STS), cast iron (CI), and concrete-coated polycarbonate (CP)) were selected. Cu, STS, CI, and CP coupons were purchased from BioSurface Technologies Corp. (Bozeman, MT, USA), and PE coupons were purchased from a tube manufacture firm based on the Chinese mandatory National Standard (GB/T13663-2000). A biofilm annular reactor (BAR) (Model 1320LJ, BioSurface Technologies Co., USA) was used to simulate drinking water distribution systems. The BAR consists of two concentric glass cylinders and a rotating inner drum that houses 20 flush-mounted removable coupons. Each coupon has an exposed surface area of 15.9~ 18.9 cm^2 for biofilm growth. The BAR and new pipe coupons were cleaned with soap and

water, then washed with 70 % (*v/v*) of ethanol and distilled water three times, and dried for removal of bacteria and impurities on these surfaces. Shear stress was applied to the slide surfaces by rotating the inner cylinder at 100 rpm, which produces shear on the coupon surface similar to that produced by flow of 0.3 m/s (1 ft/s) in a 152 mm (6 in.) diameter pipe [15].

The BAR was supplied with running drinking water from the City of Harbin DWDS (Harbin, China), which was fed to the reactor at a rate of 120 mL/h (Fig. 1). Clorox bleach was diluted to a range of 60~80 mg/L in amber Schott bottle (0.5 L) prepared by adding Clorox bleach (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) to autoclaved deionized water and added to BAR bulk phase at 3 mL/h, which maintained free chlorine residual at 1.20 ±0.23 mg/L (n=39). The hydraulic retention time (HRT) is 8 h. General inlet water quality parameters are listed in Table 1. It has been reported that bacteria could regrow when assimilable organic carbon (AOC) were more than 100 μg/L in chlorinated drinking water [9]. In the experiments described here, the concentration of AOC in the inlet water was 184.38 ±26.16 μg/L, which would result in bacterial regrowth according to previous research.

Sampling of Biofilms and Water

Water was sampled at the inlet of the BAR weekly, collected in 1-L autoclaved glass carboys containing 20-mg sodium thiosulfate to neutralize the chlorine reaction and was stored at 4ºC for less than 12 h before physico-chemical and bacterial analysis.

Biofilm samples were obtained from different pipes removed from the reactor after 12, 28, 49, 70, 91, 120, 159, 196 days and were placed in sterile tubes containing 30 mL of bacteria-free distilled water. Then, the new coupons were re-inserted into the sampled sites in case more samples were needed. Biofilms were released by sonication in an ice water bath (1 min, 40 W) and vortexed vigorously for 1 min to disrupt the biofilms structure with this procedure repeated two times. Samples of biofilms suspensions were diluted in sterile distilled water. Subsequently, the mixture was filtered through 0.22-μm micropore membranes (Millipore, Billerica, MA, USA) and the filtered materials were kept in the dark and at −20ºC for subsequent DNA

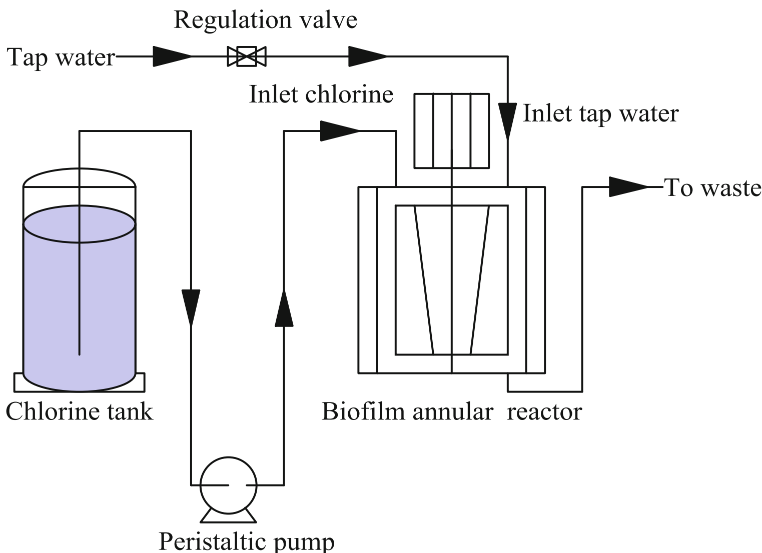

Fig. 1 Schematic diagram of the annular reactor

Table 1 Main parameters of water samples used in the experiments

Parameter (unit)	Mean	SD	Min.	Max.	Number
Water temp. (°C)	15.1	4.7	7.8	23.5	39
Turbidity (NTU)	0.52	0.28	0.25	1.45	39
pH (U)	6.75	0.12	6.52	7.02	39
Chlorine residual (mg/L)	1.20	0.23	0.85	1.64	39
NH_4^+ (mg/L)	0.059	0.016	0.042	0.098	11
Cl^- (mg/L)	13.08	1.87	10.32	16.38	11
SO_4^{2-} (mg/L)	13.30	1.33	10.74	14.94	11
NO_3^-(mg/L)	2.17	0.86	0.84	3.26	11
TOC (mg/L)	2.36	0.36	1.57	2.93	11
AOC (μg/L)	182.11	24.23	134.66	215.76	11
TP (mg/L)	<0.02				11
Fe (mg/L)	0.17	0.11	0.09	0.43	11
TDS (mg/L)	117.31	16.88	99.92	151.43	11
TN (mg/L)	3.49	0.57	2.64	4.43	11
HPC (R2A, CFU/mL)	5802	–*	587	10,467	11

*Value of SD is much bigger than the mean value

extraction. The biofilms samples obtained at 12, 28, 49, 91, 159 days were used for bacteria counting while the biofilms samples obtained at 70, 120, 196 days were used for bacteria counting and DNA extraction.

Chemical and Physical Water Analysis

Water quality parameters including water temperature, chloride (Cl^-), sulfate (SO_4^{2-}), nitrate (NO_3^-), ammonium (NH_4^+), total nitrogen (TN), total phosphorus (TP), and total dissolved solids (TDS) were measured according to standard methods [16]. Turbidity and free chlorine were measured using a Hach 2100P Turbidimeter and Hach Pocket Colorimeter™ II Cl_2, respectively (Hach Co., Loveland, CO, USA). Inductively coupled plasma mass spectrometers (ICP/MS) (Plasma Quad 3, VG, England) were used to analyze the total iron (Fe). TOC was analyzed by a high-temperature combustion method with a Shimadzu TOC-VCPH analyzer (Kyoto, Japan). Assimilable organic carbon (AOC) was analyzed by a modification of Van der Kooij et al.'s method, the modification included addition of inorganic nutrients [17]. Growth of *Pseudomonas fluorescens* P17 and *Spirillum* sp. strain NOX in water samples were calculated to correspond to acetate equivalents. Water temperature, turbidity, pH, and chlorine residual were analyzed weekly while TOC, AOC, TN, TP, TDS, Fe, and other inorganic ion were analyzed semi-monthly.

Bacteria Enumeration

Total heterotrophic cultivable bacteria were enumerated on R2A medium, a non-selective medium recommended for the examination of total heterotrophic bacteria in DWDS [17]. The treated sample was diluted to an appropriate concentration by 10-fold dilution and spread on a plate of R2A agar. All measurements were undertaken in triplicate. The inoculated R2A agar plates were incubated for 7 days at 22ºC before colony counting [17] and the plates on which

20~300 colonies formed were selected. The numbers of bacteria per milliliter were averaged and converted to the number of bacteria per unit area (i.e., CFU/cm^2, CFU/mL).

Total cell concentration (TCC) was measured by an Accuri™ C6 flow cytometry (Becton Dickinson, San Jose, California, USA) equipped with 488 solid-state lasers. Staining and flow cytometric analysis was performed as described previously [18]. In short, for a working solution, SYBR® Green I (SG) (Invitrogen AG, Basel, Switzerland) was diluted 100× in anhydrous dimethyl sulfoxide (DMSO). This working solution was stored at −20°C until use. Biofilm suspensions were diluted 1/10 just before measurement using 0.22 μm filtered commercially available bottled water (Evian, France) so that the concentration measured with the flow cytometer (FCM) was always less than 2×10^5 cells/mL. From every diluted biofilm suspensions, 0.5 mL was stained with 5 μL of SG in order to measure TCC.

DNA Extraction and PCR-DGGE

Total genomic DNA was extracted with the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA). The DNA concentration and purity were measured by micro-spectrophotometry (NanoDrop® ND-2000, NanoDrop Technologies, Wilmington, DE, USA). The DNA samples were stored at −20°C before the next analyses.

Total genomic DNA was used as template for PCR amplification from V1 to V3 variable regions of 16S rRNA gene with the primer 8 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and primer 518R with a GC clamp (5′-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGC AC GGGGGGATTACCGCGGCTGCTGG-3′). PCR reactions were performed in a total reaction volume of 50 μL containing 200 mM of each deoxyribonucleotide triphosphate (dNTP), 0.5 mM of each primer, 1× PCR buffer containing 1.5-mM $MgCl_2$, 4 U of Ex Taq DNA polymerase (Takara Biotechnology Co. Ltd., Japan) and 10~25 ng of template DNA. A negative control reaction, with ddH_2O instead of template, was carried out simultaneously. DNA amplification was carried out on a Bio-Rad S1000™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) as follows: initial denaturation at 94°C for 8 min, followed by 35 cycles of 50 s at 94°C, 50 s at 55°C, and 50 s at 72°C with a final extension step at 72°C for 10 min. The product sizes were verified on 1 % (*w/v*) agarose gel electrophoresis.

DGGE analysis was conducted using a DCode™ universal mutation detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Briefly, approximately 500 ng of PCR products were loaded onto an 8 % (*w/v*) polyacrylamide gel cast in 1×TAE (20-mM Tris, 10-mM acetate, and 0.5-mM EDTA, pH 8.2). To separate the DNA fragments polyacrylamide gels with a gradient from 40 to 60 % (100 % denaturant contained 7-M urea and 40 % formamide) were used. Electrophoresis was run at 60°C and 90 V for 12 h, after which the gels were stained with silver as described previously and photographed [19].

The major DGGE bands were excised using a sterile blade. Acrylamide slices were crushed and dissolved in 30 μL 1×TE at 40°C for 4 h, and then centrifuged at 10,000 rpm for 10 min. The 3-μL supernatant was used as the template and PCR amplification conducted under the conditions as described above using the same primers without GC clamp. The PCR products were recovered by agarose gel electrophoresis and purified using a E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) then ligated into vector pMD 18-T (Takara Biotechnology Co. Ltd., Japan), and cloned into *Escherichia coli* DH5α. Five white clones from each sample were randomly selected for PCR detection, and three positive clones were selected for sequencing by ABI3730. The sequences were aligned and compared in the GenBank database using BLAST programs of the National Center for Biotechnology Information (NCBI). All sequences included in this paper were deposited to GenBank under accession numbers KF704655 to KF704676.

Statistical Analysis

Data for total cell concentration and heterotrophic bacteria counts over different sampling dates or/and materials were compared using analysis of variance (ANOVA), post-hoc Tukey's test. The relationship between total and cultivable counts in different types of water was assessed based on a Pearson correlation analysis. These analyses were supported by SPSS software 11.0 for Windows. Biofilm cultivability (cfu) was calculated by the following expression: biofilm cultivability (%)=[HPC/TCC]×100; inactivation efficiency=$\log_{10}$ (HPC_{inlet}/HPC_{outlet}).

Results and Discussion

Bacterial Levels in Water

It is widely accepted that bacterial regrowth in DWDS can occur even in the presence of disinfectant in bulk water [20]. The HPC values at the inlet and outlet water of the BAR are shown in Fig. 2. During the initial 100 days, the HPC of the BAR outlet water (HPC_{outlet}) exhibited only a slight, non-significant increase (from 0.9 to 169 CFU/mL, $p>0.05$). Chlorine was initially strikingly efficient in reducing bacterial growth and eliminated 2–4 log units of the HPC of the BAR inlet water (HPC_{inlet}). However, from 100 to 166 days, rapid, significant ($p<0.01$) bacterial growth occurred with the HPC of the BAR effluents ranging from 362 to 1,387 CFU/mL demonstrating that, at these later times, chlorine had weak inactivation efficiency (<1.0 log units of HPC). After 166 days, the HPC_{outlet} did not change and was typically about 1,300 CFU/mL. Many researchers have confirmed that the increase of bulk water free bacteria with time in DWDS is mainly due to biofilm bacterial growth and detachment, not the growth of free bacteria [21,22]. It is well known that bacterial growth in water is almost negligible in drinking water systems, and only attached cells are capable of proliferating [23]. The hydraulic retention time of the BAR system tested in the study was

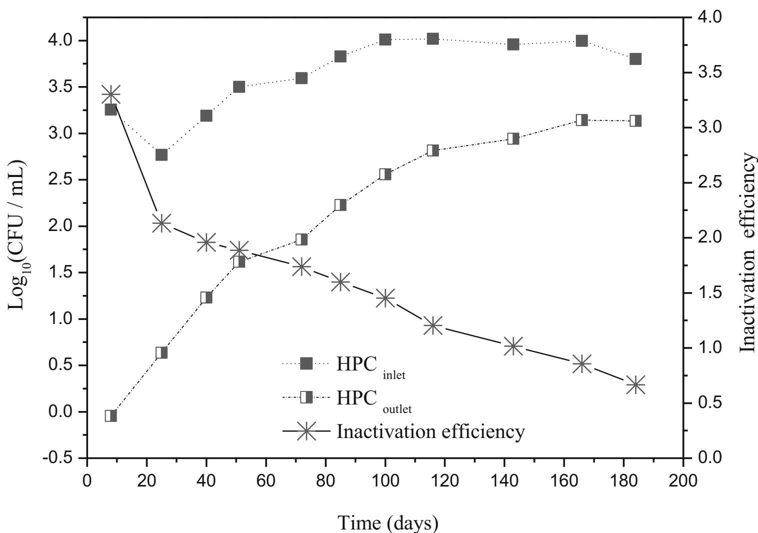

Fig. 2 Variation of suspended bacterial concentration during the experiment

about 8 h, which was far lower than the bacteria generation time of 1~17 days [23,24]. The increase or stability in the number of bacteria in bulk water derives from the multiplication of bacteria attached to the walls of pipes, and from their continuous detachment [23]. Based on the above, it is rationally inferred that the biofilm bacterial density should be at steady state after 166 days as the bulk bacterial number reached steady state from then on.

Biofilm Growth on Different Pipe Materials

Total cell counts (TCC) were enumerated based on flow cytometer (FCM) enumeration for each biofilm sample on the coupons (Fig. 3). TCC varied significantly ($p<0.01$) over the eight sampling dates (12, 28, 49, 70, 91, 120, 159, and 196 days) in the biofilm formed on the coupons (Fig. 3). The biofilm formed on cast iron presented the highest total cell counts ranged from 5.1×10^4 to 1.7×10^7 and was 3- to 431-fold higher ($p<0.01$) than those formed contemporaneously on other pipe materials throughout the experiment except at the first 12 days on the concrete-coated polycarbonate biofilm (Fig. 3). The biofilm formed on concrete-coated polycarbonate exhibited the second highest TCC with 7.0×10^4~3.6×10^6 cells/mL after 28 days but only became significantly ($p<0.05$) higher than the TCC on the other three pipe materials (copper, stainless steel, polyethylene) after 159 days of the later stage. For the TCC in the biofilms formed on pipes during the first 12 days of the early trial stage (Fig. 3), the five experimental pipe materials were ranked in the order: concrete-coated polycarbonate (CP) > cast iron (CI) > polyethylene (PE) > stainless steel (STS) > copper (Cu). But at the end of trial stage (196 days), the TCC in the biofilms formed on the five pipe materials were ranked in the order: CI > CP > Cu> PE >TS. Although the biofilms on Cu pipe surfaces showed the lowest concentration of bacteria during the first 120 days of the early stage, the extent of biofilm formation on Cu pipe increased with corrosion, and presented the third highest bacterial concentration at the end of the experiment. Jang et al. also observed the

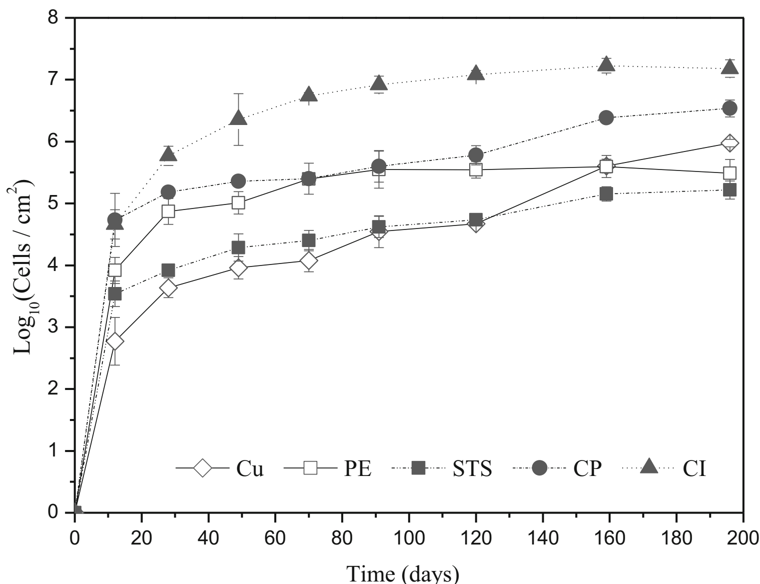

Fig. 3 Total cell counts of biofilms on the inner surfaces of the reactor coupons over different dates

same phenomenon in copper pipe [8]. Apart from Cu pipes, the total number of bacteria attached to pipe materials reached steady state at different stages. In PE pipes, microbial concentrations reached steady state within the 91 sampling days and in CI pipes, microbial densities reached steady state within the 120 sampling days. In STS and CP pipes, microbial densities reached steady state within the third 159 sampling days of the later stage, but in copper pipe, microbial densities continued to increase for more than 196 days (Fig. 3). These results are similar to those reported by others. For example, Lehtola et al. observed that the steady state was reached on PE pipes within the first 37 days, but in copper pipes, microbial densities continued to increase for more than 200 days [17]. Holden et al. found that biofilm on cast iron reached steady state within 100 and 130 days [25]. This would suggest that pipe materials can dramatically influence the time taken to build up the biofilm to a steady state.

For the number of total heterotrophic cultivable bacteria colonized on the pipe surfaces (Fig. 4), similar trends to those of total cell counts were observed. The HPC attached to pipe surfaces exhibited significant ($p<0.01$) variations throughout the experiment. The HPC in the biofilm on CI pipe also emerged to be the highest and was 25~504-fold higher than those on other pipe materials (Fig. 4). The biofilm on PE pipe colonized higher HPC than the other three pipe materials (Cu, STS, CP) before 120 days, but after 159 days, CP replaced PE, and colonized higher HPC than Cu, STS, and PE pipe materials. During the first 12 days of the early stage (Fig. 4), the order of HPC (from more to less) was CI, CP, PE, STS, and Cu.

The entire microbial community in a biofilm sample can be visualized with fluorescent dyes (SYBR® Green I) coupled with flow cytometer (FCM) enumeration. This allows assessment of all microorganisms, including so-called unculturable heterotrophic bacteria, autotrophic bacteria, and so-called viable-but-not-culturable (VBNC) cells [18]. The number of total cells was 1 to 2 log units higher than the number of cultivable cells as adjudged by HPC [18]. In this study, we observed that the culturability in the biofilms was significantly ($p<0.001$) different for different pipe materials but no significant difference ($p=0.982$) was observed between the

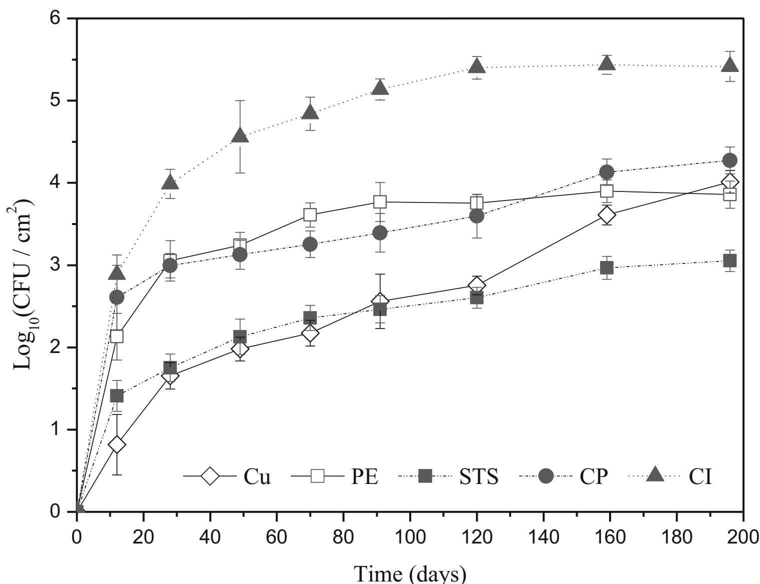

Fig. 4 Heterotrophic plate counts of biofilms on the inner surfaces of the reactor coupons over different dates

consecutive sampling times (Fig. 5). The biofilms on the PE pipe showed the highest values of cultivability in the range of 1.52~2.37 % while biofilms on CI pipe displayed the second highest values of cultivability in the range of 1.33~2.12 %. The biofilms on Cu pipe exhibited intermediate values of cultivability in the range of 1.04~1.27 %, while biofilms on CP and STS presented low values of cultivability in the range of 0.58~0.77 % and 0.65~0.92 %, respectively. Apart from PE pipes, the bacterial cultivabilities attached to pipe materials keep constant in slight fluctuation within time. Vaz-Moreira et al. concluded that chlorination can promote a reduction on bacterial diversity and cultivability [26], but it is not suitable for all pipe materials. The difference of biofilm cultivabilities on different materials were most probably due to different culturable chlorine-resistant bacteria species from various pipe materials.

Based on the results presented above, it is clear that pipe materials have a strong influence on chlorine-resistant microbial densities and culturabilities in biofilm formation. The lowest microbial numbers in Cu pipes during the early stage is attributed to the release of copper ions from Cu pipe with these metal ions known to be growth-inhibiting to bacteria [7]. Subsequently, bacteria in the biofilm formed on the surface of Cu pipes were encased by EPS which significantly increased the biofilm tolerance to antibiotics and disinfectants such as copper and chlorine [27]. At the same time, corrosion of copper was promoted by the bacteria attached to the surface of the Cu pipe, and the bacterial levels in the biofilm were higher than those on the STS pipe. This is presumably because copper reacted with the disinfectant resulting in greater chlorine consumption, and the bacterial population had adapted or been selected to live on the copper surface [17].

Stainless steel pipe showed the lowest bacterial number after 159 days. Jang also reported that STS pipe showed the lowest bacterial number at the end of the operation [8]. Stainless steel is a moderately hydrophobic material, with a slightly negative surface charge at the pH conditions used in the experiment described here. The negative electrostatic force on the surface hindered bacterial adhesion, as the surface of bacteria was also slightly negatively

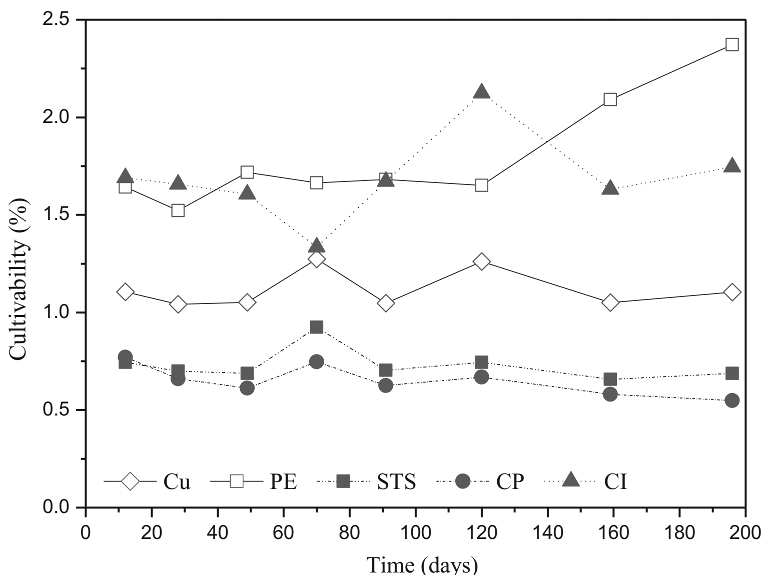

Fig. 5 Culturability of biofilms on the inner surfaces of the reactor coupons over different dates

charged [8]. Also, stainless steel is a corrosion resistance metal material, depending on the almost instantaneous formation of a thin, durable chromium-oxide film on exposure to air and water [28]. Therefore, the bacteria are not easy to attach to the surface of stainless steels. In the five new commonly used pipe materials, bacterial levels in the biofilm formed on the surface of PE pipe exhibited the highest values of cultivability, and increased slightly with time. Also, the biofilm biomass were 1~2 logs higher than those on STS and Cu pipe during the first 70 days of the early stage. It has been reported that significant amounts of dissolved organic carbon (DOC) and phosphorus may be released from PE pipe material to water [17]. Phosphorus was found to increase the biofilm cell number and enhance the culturability of bacteria in the biofilm, but decrease the exopolysaccharides (EPS) production. As such, during the early stage, bacterial growth could be promoted in the reactor with PE pipe, but when the disinfectant continued, less exopolysaccharides (EPS) production would influence bacterial regrowth.

The biofilm formed on cast iron presented the highest biomass and the second highest culturability throughout the experiment. Disinfectants could substantially affect cast iron corrosion [7] and Appenzeller et al. demonstrated that iron in drinking water may promote both growth and cultivability of *E. coli* [29]. Iron rust not only increases pipe surface porosity and roughness for microbial attachment, but also can serve as a nutrient [4]. Also, iron pipes are more reactive with disinfectants and quench their antimicrobial effects [1]. Therefore, bacteria colonized on cast iron were more protected from chlorine residual than those colonized on other materials. When comparing total bacterial counts attached to non-corroding materials (PE and concrete) incubated in the same conditions, total bacterial counts attached to cement were proportional (ratio cement/PE of 5.79) to those measured on PE. Furthermore, the highest total bacterial counts initially adhered to CP inner surface in 12 days. Concrete is substantially more porous than CI, Cu, STS, or PE material [30]. It is possible that the more porous the structure of concrete, the more internal surface area it will provide for biofilm to grow. As a result, it provides bacterial contaminants with more protection from disinfectants initially [30]. But compared to cast iron pipe, high chlorine doses can accelerate chemically induced corrosion in cast iron pipes [7]. Iron rust not only increases pipe surface porosity and roughness for microbial attachment but also can serve as a nutrient, and the rust scavenges free residual chlorine, resulting in its removal before it can reach and react with the biofilm microorganisms [1,4]. Therefore, after 28 days, the relatively lower biomass of CP pipe seems to come from its surface porosity and roughness, not from its release of chemical compounds and low reactivity with chlorine. Thus, the characteristics of pipe materials should be considered as important parameters influencing bacterial regrowth in a distribution system.

Microbial Community in Biofilm

Due to the thin biofilms grown on Cu and STS coupons in 70 and 120 days, the DNA extraction resulted in low yields (<5 ng/μL, A_{260}/A_{280} ratios <1.30) were insufficient for amplification of the 16S rRNA genes. Consequently, only 196 days biofilm samples were performed by PCR-DGGE to investigate the phylogenetic diversity of the biofilm microbial communities in the reactor (Fig. 6). To obtain further insight into the taxa prevailing in each sample, 22 distinct and non-overlapping bands out of the 41 PCR-DGGE bands classes detected were excised, cloned, and sequenced. In all samples, the closest neighbors of the majority of the bands corresponded to organisms of the phylum Proteobacteria, Alpha- and Gamma-proteobacteria were the dominant bacterial groups in all pipe samples under high free residual chlorine concentrations (1.20 mg/L), while Betaproteobacteria was a lesser extent, and only found abundant in cast iron and concrete pipes (Fig. 7, Table 1S). Douterelo et al., McCoy

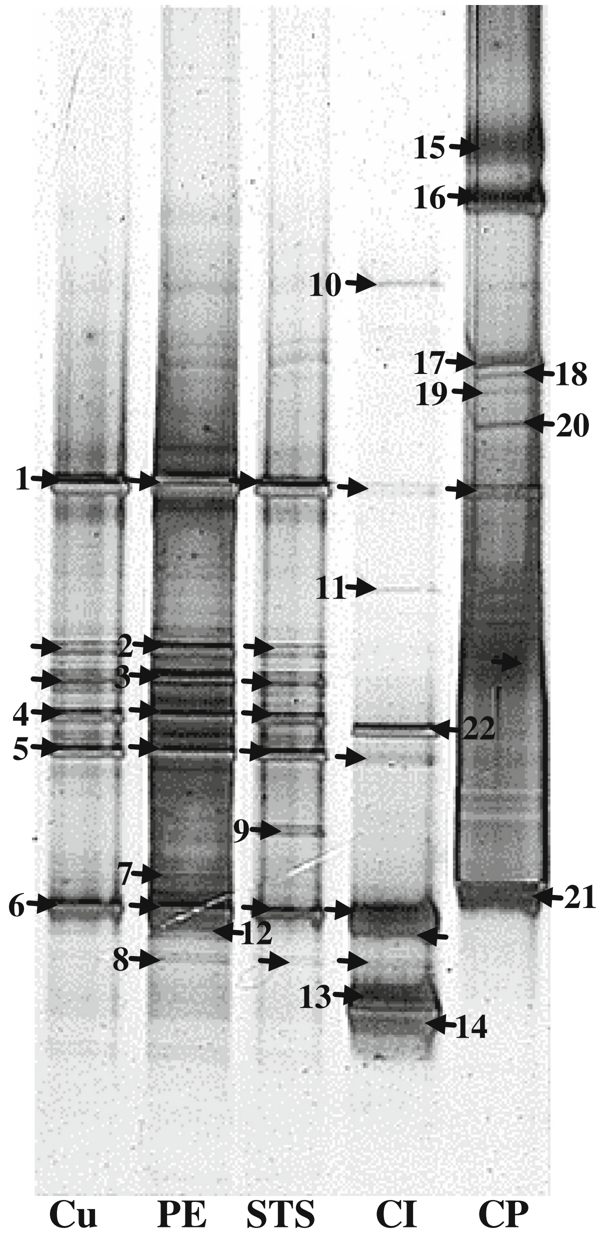

Fig. 6 DGGE gel image for material samples

and VanBriesen,.and Poitelon et al. reported that Betaproteobacteria are sensitive to higher disinfectant residuals, where as the Alpha- and Gamma-proteobacteria population may be enriched in the presence of higher chlorine concentrations [31–33]. The Betaproteobacteria population within the biofilms of cast iron and concrete pipes may be due to the pipe surface porosity and roughness. The *Alpha* subclass was an oligotrophic population which could survive oligotrophic conditions where disinfectant residuals are commonly maintained [34]. The Gamma subclass, described as "pioneers" during the formation of drinking water biofilm,

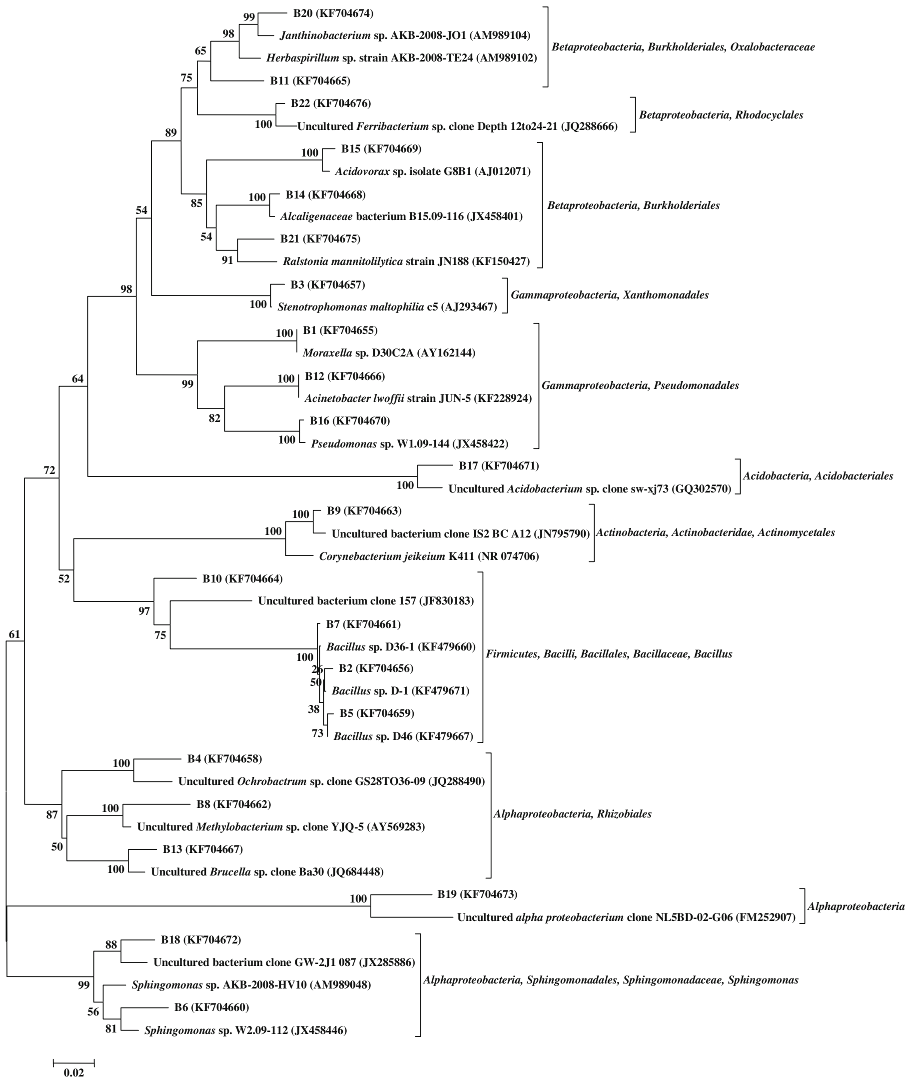

Fig. 7 Dendrogram constructed on basis of partial 16S rDNA sequences of the respective DGGE bands marked in Fig. 6. Bootstrap analysis was based on 1,000 replicates. The dendrogram was generated using the neighbor-joining method based on the model of Kimura 2-parameter. Bootstrap values, generated from 1000 resamplings, at or above 50 % are indicated at the branch points. The scale bar indicates 0.02 nucleotide substitution per site

could be located deeper within the biofilm and thus be less susceptible to chlorine with diffusion of this disinfectant limited by reaction–diffusion interactions within the biofilm matrix [35]. Additionally, Firmicutes were also frequently detected except in the concrete pipe.

In this study, the biofilms on pipes were developed under identical laboratory conditions with the same high chlorinated water, incubations at the same time produced biofilms that differed in diversity (Fig. 7, Table 1S). Pseudomonadales (mainly *Moraxella osloensis*) and Sphingomonadales (only *Sphingomonas* sp.) were found abundant in all pipes, while

Bacillales (only *Bacillus* sp.) and Rhizobiales (Brucellaceae and Methylobacteriaceae) were found abundant in Cu, PE, STS, and CI pipes, and Burkholderiales (four different families) was only found abundant in CI or CP pipes. *Moraxella osloensis* is one of aerobic chlorine-resistant bacteria, which has often been isolated from environments within high free chlorine such as biofilms on drinking water clearwall, chlorinated dental unit waterlines [12,36]. *Sphingomonas* sp. was likely to be responsible for the formation of biofilms in chlorinated drinking water, maybe because it could rapidly colonize the pipe surfaces and cover them with their EPS, and it could use various carbon sources as nutrients [12]. *Bacillus* sp. possesses an impressive physiological diversity due to its endospore formation. The species is highly resistant to unfavorable conditions and is associated with iron corrosion [37]. For example, spores could not be completely removed from stainless steel, cast iron, copper, and PVC slides [6,38], despite using free chlorine concentrations as high as 75 mg/L [38]. However, in this study, *Bacillus* spores disappeared from the concrete-lined coupons at the end of experimentation. Shane et al. also found the interesting results on the concrete-coated pipe [38]. *Bacillus* spores have a hydrophobic surface due to their outer coat proteins and exosporium [39], and they have peptidoglycan on their cell surface which may weaken the attachment capability on surfaces with porous and hydrophilic structure such as concrete-coated material. Like the spores, PE is hydrophobic, and this could have promoted adhesion, while the rust on the metal pipe surfaces could be positively charged, which could enhance the negatively charged spore attachment. Many reports found that Rhizobiales (especially Methylobacteriaceae) were dominant in PVC, stainless steel, copper, and cast iron [3,8], probably because they could use various carbon resources as their nutrients [8]. However, in this study and in the research of Luo et al. [40], Rhizobiales were not detected in cement biofilm. It is unclear why the number of attached Rhizobiales was so low, maybe because the viability of the attached Rhizobiales was inhibited by cement. Additionally, in this study, *Stenotrophomonas maltophila* (belonging to Gammaproteobacteria) was also frequently detected, mainly in copper, stainless steel, and PE pipe samples. This species has been previously isolated from copper, stainless steel, PE, and PVC pipes biofilms, and is associated with copper corrosion [11,41].

Other bacteria were only found on one or two pipe material (Fig. 7, Table 1S). For example, *Acinetobacter* sp. were found in PE and cast iron pipe, while *Herbaspirillum* sp., *Ferribacterium* sp., and Alcaligenaceae were only found in cast iron pipe. These species have been related to corrosion in iron water distribution systems [10,37,42,43]. *Janthinobacterium* sp., *Acidovorax* sp., *Ralstonia* sp., and *Acidobacterium* sp. were only found in cement pipe. These species have also been found in biofilms of cement-lined steel pipes [40]. *Mycobacterium* sp. is known to grow in drinking water systems and is very resistant to chlorine [4]. In this study, it was of surprise that *Mycobacterium* sp. were absent in all of the five pilot biofilms. However, this is not a special case. Even though pyrosequencing method was used, in some Asian countries, Zhang et al., Kwon et al., and Hong et al. also did not report the presence of this genus under high chlorine residual (0.6~3 mg/L) [12–14]. It is unlikely that the absences were due to the problem of the viability of *Mycobacterium* or the experimental error. The discrepancies may be a result of differences not only in analytical methods and sampling protocols but also in water chemistry, flow regimen, disinfectant efficiency, pipe services ages, and temperature [5,7].

Potential Pathogens

The persistence and growth of pathogens is a central concern in DWDSs [2]. In our study, bacteria such as *Sphingomonas* sp., *Brucella* sp., *Herbaspirillum* sp., Alcaligenaceae, *Ralstonia mannitolilytica*, *Stenotrophomonas maltophila*, *Moraxella osloensis*, *Acinetobacter*

sp., *Pseudomonas* sp., *Corynebacterium jeikeium*, and *Bacillus* sp. could cause an infection risk in an immunocompromised host [9,44–47]. The presence of high concentrations of disinfectants was not sufficient to eliminate pathogens in biofilms, and they could be released to the bulk water used for human consumption.

It is worth noting that there are 11 opportunistic pathogens in our 17 classified bacterial species. These opportunistic pathogens are all relatively resistant to chlorination. These pipe surfaces were all colonized by 4~7 species of pathogens. The pathogens adhering to surfaces greatly differ between pipe materials (Fig. 7, Table 1S): cast iron pipe surface was colonized by species of higher pathogenicity, while copper and cement pipe surfaces were colonized by species of the lower pathogenicity. Perhaps it is inevitable that chlorine-resistant microorganisms may contain opportunistic pathogenic bacteria in DWDS. Therefore, particular attention should be given to the long-term effects of pipe materials on chlorine-resistant biofilm formation.

Conclusion

Pipe material has strong effects on speed, biomass, and community of chlorine-resistant biofilm stabilization in high chlorinated water distribution system biofilms. The biomass in stabilized biofilms formed on the pipes ranked in the order: CI > CP > Cu > PE > STS. Molecular analysis clearly showed that Alpha- and Gamma-proteobacteria dominated in all pipe biofilms, and Bacilli were frequently detected in biofilms of Cu, PE, STS, and CI pipes, while Betaproteobacteria was only found in CI and CP pipes. Moreover, two thirds of the identified bacteria were closely associated with human health effects, and CI pipe harbors more pathogens.

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