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Pyrosequencing analysis of bacterial communities in biofilms from different pipe materials in a city drinking water distribution system of East China

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Abstract Biofilms in drinking water distribution systems (DWDSs) could cause several types of problems, such as the deterioration of water quality, corrosion of pipe walls, and potential proliferation of opportunistic pathogens. In this study, ten biofilm samples from different pipe materials, including ductile cast iron pipe (DCIP), gray cast iron pipe (GCIP), galvanized steel pipe (GSP), stainless steel clad pipe (SSCP), and polyvinyl chloride (PVC), were collected from an actual DWDS to investigate the effect of pipe material on bacterial community. Real-time quantitative polymerase chain reaction (qPCR) and culture-based method were used to quantify bacteria. 454 pyrosequencing was used for bacterial community analysis. The results showed that the numbers of total bacteria and culturable heterotrophic bacteria from iron pipes were higher than that in PVC, while the numbers of *Shigella* and vibrios were low in biofilms from iron pipes. Bacterial community analysis showed that *Hyphomicrobium* or *Desulfovibrio* were the predominant microorganism in iron pipes, whereas *Sphingomonas* or *Pseudomonas* were dominant in other types of pipe. This study revealed differences in bacterial communities in biofilms among different pipe materials, and the results were useful for pipeline material selection in DWDSs.

Keywords Drinking water distribution system · Biofilms · Pipe materials · Bacterial community

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Introduction

Drinking water distribution systems (DWDSs) function to supply treated water safe for human consumption. However, the available scientific literature indicates that a variety of microbes could survive and grow in DWDSs (Marciano-Cabral et al. 2010; Wu et al. 2014), and they tend to attach to pipe surface to form biofilms (Douterelo et al. 2014a, b). There is a common notion that the number and diversity of bacteria in biofilms is greater than that in the bulk water (Camper et al. 1999; Zacheus et al. 2001; Liu et al. 2013; Liu et al. 2014). Biofilms formation could induce several unpleasant problems in DWDSs, including the deterioration of water quality (Martiny et al. 2005), corrosion of pipe walls (Nawrocki et al. 2010), and potential proliferation of opportunistic pathogens (Buse et al. 2012).

The formation and growth of pipe wall biofilms might be affected by many factors, such as the pipe material (Allion et al. 2011), water source (Zeng et al. 2013), nutrient availability (Park and Hu 2010), type and concentration of disinfectant (Xue and Seo 2013), and liquid shear stress conditions (Douterelo et al. 2013). Pipe material was found to be a major factor governing the drinking water microbiome in simulated

DWDSs (Wang et al. 2014). Distinct bacterial communities were observed on steel, copper, stainless steel, and polyvinyl chloride coupons exposed to the same drinking water in annular reactors (Jang et al. 2011). In addition, the density of bacteria varied in different pipe materials (Niquette et al. 2000). However, these previous studies generally employed reactors and simulated pipelines (Revetta et al. 2013) because biofilm samples from actual DWDSs were difficult to obtain; the simulated conditions might not precisely represent the characteristics of bacterial communities in natural DWDSs that have been in operation for long periods of time and in complicated environments (Douterelo et al. 2013). Although many conditions such as the pipe diameter and daily regime are not constant in actual DWDSs, pipe material is still considered a key factor affecting bacterial composition (Douterelo et al. 2014b).

In this study, ten biofilm samples were obtained from different pipe materials, including ductile cast iron pipe (DCIP), gray cast iron pipe (GCIP), galvanized steel pipe (GSP), stainless steel clad pipe (SSCP), and polyvinyl chloride (PVC), in operation for approximately 11 years in a city DWDS in East China. This study aimed to investigate the bacterial community diversity in biofilms among different pipe materials in an actual DWDS and to study the effect of the pipe material on the biofilm bacterial community.

Materials and methods

The drinking water distribution system and biofilm sampling

This study performed in a city DWDS supplied by reservoir water in Shaoxing, East China. The drinking water treatment plant employs the process of flocculation followed by sand filtration and chlorination before discharging to the DWDS. The produced water quality meets the national drinking water standards. The water treatment plant, with drinking water yield of 500,000 m^3/day, supplies 2,000,000 people.

Five locations (L1, L2, L3, L4, and L5) located at the end of the DWDS were chosen. The distance of the location to the treatment plant was 18–22 km. The flow rates in these locations were lower than 0.1 m s^{-1}. The pipes in these locations are made of DCIP, GCIP, GSP, SSCP, and PVC, respectively. The service time of these pipes was nearly 11 years. Two pipe sections were cut at each location, and ten biofilm samples were sampled during Sept 2013 to Oct 2013, as shown in Table 1. The sampling process was as follow: water supply of the location was cut off and water in pipes was drained off slowly at a flow rate of less than 0.03 m/s to prevent biofilm drop off before cutting the pipe. Then, soil around the buried pipe was excavated and the surface of the pipe was physically decontaminated with tap water. Two pipe sections were cut off at each location, sealed with a sterile membrane, and transported to the laboratory in an ice box within 6 h. The biofilm was collected by swabbing the inner pipe wall with a sterile brush while continuously washing the pipe wall with sterile water.

Water quality analysis

The pH, turbidity, oxygen consumption (OC), iron, manganese, and free chlorine of the bulk water at each location were measured weekly during the 9 months prior to sampling. pH was monitored using a portable pH 10 series meter (Oakton Instruments, Vernon Hills, IL, USA). Turbidity was measured using a 2100P portable turbidimeter based on manufacturer's instruction (HACH, Loveland, CO, USA). Oxygen consumption measurement was performed using $KMnO_4$ as oxidants according to GB/T-5750-2006 (China). The concentration of iron and manganese was determined using acid digestion with nitric acid before analysis by inductively coupled plasma-atomic emission spectroscopy as previously reported (Douterelo et al. 2014b). Free chlorine was measured using a HACH DR 890 colorimeter based on manufacturer's instruction (HACH, Loveland, CO).

Biofilm processing

Collected biofilms were shaken in sterile glass bottles with sterile glass beads (size of 4–5 mm) on a shaker for 20 min to break up microbial clusters and filtered using sterile mesh (80 meshes) to remove impurities such as metal particles and sand sediments. Then the biofilm slurries were centrifuged with 50 mL sterile centrifuge tubes at a speed of 4500 rpm for 15 min. The biofilm biomass (sediment) was measured, and the supernatant was remained. Collected sediment was split into two parts: one (biomass measured) was stored at −80 °C for DNA extraction and the other was resuspended with remained supernatant and stored at 4 °C for measurements of physicochemical parameters and incubation experiment. All the processing was finished within 12 h after the pipe was cut.

Properties of biofilm analysis

The contents of total solids (TS), volatile solids (VS), iron, manganese of the biofilms were determined (APHA 2012). The TS content of the biofilm was determined as the residue after drying a slurry sample at 105 °C to constant weight. Further heating at 550 °C (to constant weight) drives off the VS. The concentration of iron and manganese was determined using acid digestion with nitric acid before analysis by Inductively Coupled Plasma-Atomic Emission Spectroscopy.

Table 1 Detailed information on the sampled pipes

Locations	Pipe material	Pipe age (year)	Inner diameter (mm)	Sampling date
L1	DCIP	11	150	25 Sept 2013
L2	GCIP	11	150	22 Oct 2013
L3	GSP	12	80	29 Sept 2013
L4	SSCP	10	80	10 Oct 2013
L5	PVC	13	100	17 Sept 2013

Enumeration of bacteria by culturing

Resuspended biofilm slurry (volume of 20 mL) was shaken in a sterile glass bottle (volume of 50 mL) again with sterile glass beads (4-5 mm) for 15mins on a shaker before culturing. An equal volume of sterile water was used for control. The number of heterotrophic bacteria was determined using the heterotrophic plate count (HPC) method. HPC analysis was performed using the spread plate method with R2A agar and a 7-day incubation period at 25 °C (Reasoner and Geldreich 1985; Thayanukul et al. 2013). The number of pathogens was determined using plate-counting method with different medium. Total coliforms was cultured with eosin methylene blue (EMB) medium for 48 h at 37 °C as previously described (An et al. 2002). *Salmonella* and *Shigella* were cultured with *Salmonella* chromogenic medium and *Shigella* chromogenic medium (Tian Tan Biologic Technology Company, China), overnight at 37 °C, respectively (Zhang et al. 2014). Vibrios were cultured with thiosulfate-citrate-bile salt-sucrose agar (TCBS) overnight at 30 °C (Williams et al. 2013). *Legionella* was cultured with buffer charcoal yeast extract agar for 9 days at 37 °C and further identified by inoculating on buffer charcoal yeast extract agar without L-cysteine (BCYE-cys) for 2 days at 37 °C (Bartie et al. 2003). The number of colonies was counted on plates with between 30 and 300 colonies.

DNA extraction and quantitative PCR

Total DNA was extracted from the biofilm (0.25 g) using a Power Soil DNA Kit (Mo Bio Laboratories, Carlsbad, CA, USA), as described by the manufacturer. The extracted total DNA was examined on 1.0 % agarose gels via electrophoresis and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, USA) (Hu et al. 2011). The DNA was stored at −20 °C for future ploymerase chain reaction (PCR) amplification. Quantitative PCR (qPCR) was used to quantify total bacteria in biofilm samples. The qPCR was performed using an iCycler iQ5 thermocycler (Bio-Rad, CA). Forward primer 338F:5′-ACTCCTA CGGGAGGCAGCAG-3′ (Lane et al. 1991) and reverse primer 518R:5′-ATTACCG CGGCTGCTGG-3′ (Muyzer et al. 1993) were chosen; the reaction systems and conditions were previously reported (Hu et al. 2012). The construction of the standard curve was conducted from a series of 10-fold dilutions of plasmid DNA inserted by 16S ribosomal RNA (rRNA) genes of biofilm bacteria in the DWDS amplified through qPCR. Three replicates of each sample and plasmid DNA were conducted. The copy numbers of the samples were determined through a calculation based on comparison with the threshold cycle values of the standard curve.

454 pyrosequencing

Two DNA samples of each location, representing one type of pipe material, were mixed based on the same DNA amount. The mixture was used for bacterial 16S rRNA gene pyrosequencing to analyze bacterial community composition. The primer pairs 357F (5′-CCTACGGGAGGCAGCAG-3′) and 926R (5′-CCGTCAATTCMTT TRAGT-3′) were used to amplify the V3–V5 region of the bacterial 16S rRNA genes. A barcode was permuted for each sample to allow for the identification of individual samples in a mixture within a single pyrosequencing run. Each sample was amplified in triplicate using a 25-μL reaction system (sterile water, 16.375 μL; buffer (10×), 2.50 μL; dNTPs (2.5 mM), 2.00 μL; DNA template (20 ng/μL), 2.00 μL; forward primer (10 μM), 1.00 μL; reverse primer (10 μM), 1.00 μL; Takara polymerase (5 U/μL), 0.125 μL) using the following protocol: 94 °C for 5 min, 26 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Three replicate PCR products from each sample were combined and purified using an AxyPrep DNA purification kit (AXYGEN). All of the samples were quantified by TBS-380 and mixed at an equimolar ratio in a single tube to be run on a Roche FLX+ 454 pyrosequencing system (Roche Diagnostics Corporation, Branford, CT, USA).

Sequences generated from the pyrosequencing analysis were subsequently processed using the Mothur software package (http://www.mothur.org). After denoising and chimera inspection, the high-quality sequences (quality score >25, exact match to barcode and primer, 200–600 bp in length, and containing no chimeras) were generated. Operational taxonomic units (OTUs) were generated using a 97 % sequence-identity threshold (Schloss and Handelsman 2005). Representative OTUs were selected based on the most

abundant sequence, and taxonomic assignment was conducted using the Ribosomal Database Project (RDP) classifier with data sets from the RDP pyrosequencing pipeline (Cole et al. 2009).

Data analysis

The Chao1 and Shannon community diversity indices were analyzed. The coverage of the amplicon libraries was calculated as $(1-(n1/N))\times 100$, where $n1$ is the number of unique OTUs and N is the total number of OTUs in a library. The ecological distribution of the biofilm communities and their associations with environmental factors were determined using principal components analysis (PCA) and redundancy analysis (RDA), respectively, using CANOCO software based on normalized values of the abundance of each OTU in bifilm samples. A Pearson correlation analysis (significance level $p < 0.05$) was used to test the correlations between biofilm parameters including physicochemical and biotic factors. One-way ANOVA was used to determine whether the biofilm samples had significant difference (Hu et al. 2014).

Results

Physicochemical parameters of bulk water

The physicochemical properties of bulk water at five locations are summarized in Table 2 (The detailed data are supplied in Tables S2, S3, S4, S5, and S6). The pH of the bulk water approximately remained at 7.2. The turbidity ranged from 0.09 to 0.12 NTU, and the OC was between 0.9 and 1.0 mg/L. However, the free chlorine and total iron contents varied from 0.23 to 0.44 mg/L and 0.008 to 0.015 mg/L, respectively. The highest and lowest concentrations of free chlorine were detected in samples from SSCP and PVC, whereas the highest total iron concentration was measured in the bulk from PVC followed by the sample from GSP. In the bulk water from DCIP, GCIP, and SSCP, the total iron content was less than 0.009 mg/L.

Physicochemical parameters of biofilm

The biofilms from different pipes were diverse in form (Fig. 1). The biofilm in DCIP was evenly distributed on the pipe wall. However, abundant entire tubercles without a layered structure were found on the surface of GCIP. Compared with DCIP and GCIP, GSP was more resistant to corrosion, and the corresponding biofilm was irregularly distributed on pipe walls with tubercles. In SSCP and PVC, the biofilms were relatively thin. The mass of biofilms accumulated on pipe walls ranged from 1.96 to 140.79 mg·cm^{-2} with divergence among different pipes (Table 3). The maximum biofilm biomass was detected in GCIP, and the minimum was found on PVC. The concentrations of TS and VS ranged from 0.19 to 59.97 mg cm^{-2} and from 0.02 to 3.19 mg cm^{-2}, which were consistent with biofilm mass. The VS accounted for 5–13 % of the TS in different biofilms. The VS/TS ratios were higher in biofilms from SSCP and PVC than that from iron pipes. The content of Fe and Mn was highest in biofilm from GCIP with 9.0 and 0.2 mg cm^{-2}, while Fe and Mn in biofilm from PVC was below the detection limit. Moreover, correlation analysis, according to Pearson test, showed there were significantly positive correlation between biofilm mass, TS, VS, and Fe (Table 4, sample numbers = 10). The biofilm mass, TS, VS, and Fe had a significant difference in biofilms from various material pipes, with $p < 0.001$ (Table 5, sample numbers = 10).

Bacterial quantification

Quantitative PCR and HPC results are shown in Fig. 2. The total bacterial 16S rRNA gene copies in the biofilms ranged from 6.68×10^5 to 4.29×10^7 gene copies cm^{-2}. GCIP had the highest level of 16S rRNA gene copy numbers at 10^7 gene copies cm^{-2}, compared with other pipes. The biofilms of DCIP, GSP, and SSCP had the copy numbers of 16S rRNA gene varying from 2×10^6 to 7×10^6 gene copies cm^{-2}, while in biofilm of PVC, there was just 10^5 gene copies cm^{-2}, which was the lowest level in the all sampled biofilms. HPCs, which indicate the number of culturable heterotrophic microorganisms, ranged from 10^4 to 10^6 CFU cm^{-2}. The highest HPC was observed in DCIP, which had 10^6 CFU cm^{-2}, followed by GCIP, SSCP, and GSP with 10^4–10^5 CFU cm^{-2}. The HPC of PVC was 10^3–10^4 CFU cm^{-2}, which was the lowest value among all samples. Correlation analysis, according to Pearson test, showed that qPCR was significantly positively correlated with mass, TS, VS, and Fe, while HPC was not (Table 4, sample numbers = 10). The qPCR and HPC results had a difference or significant difference in biofilms from various material pipes, with p values of 0.096 and 0.038, respectively (Table 5, sample numbers = 10).

Total coliforms, *Salmonella*, *Shigella*, vibrios were detected in biofilms (Table 6). The highest number of total coliforms was detected in GSP with 10^3 CFU cm^{-2}, while the lowest was in PVC with tens of CFU cm^{-2}. The highest numbers of *Shigella* and vibrios were detected in one GSP with 10^5 CFU cm^{-2} (however, *Shigella* and vibrios were not detected in the other GSP), while vibrios were not found in GCIP. *Salmonella* were only detected in one DCIP with 2.1×10^2 CFU cm^{-2}.

The composition of the bacterial communities

A total of 125,131 sequences were obtained from five biofilm samples, with an average yield of more than 20,000 sequences

Table 2 Physicochemical properties of bulk water during the 9 months prior to sampling

Locations	pH	Turbidity (NTU)	Oxygen consumption (mg/L)	Iron (mg/L)	Free chlorine (mg/L)
L1	7.2 ± 0.1	0.12 ± 0.05	0.9 ± 0.1	0.009 ± 0.007	0.40 ± 0.07
L2	7.2 ± 0.1	0.10 ± 0.04	1.0 ± 0.1	0.008 ± 0.006	0.31 ± 0.07
L3	7.2 ± 0.1	0.09 ± 0.05	1.0 ± 0.1	0.011 ± 0.009	0.29 ± 0.06
L4	7.2 ± 0.1	0.10 ± 0.06	1.0 ± 0.1	0.008 ± 0.006	0.44 ± 0.05
L5	7.2 ± 0.1	0.12 ± 0.04	0.9 ± 0.1	0.015 ± 0.008	0.23 ± 0.07

DCIP ductile cast iron pipe, *GCIP* gray cast iron pipe, *GSP* galvanized steel pipe, *SSCP* stainless steel clad pipe, *PVC* polyvinyl chloride pipe

for each sample. Sample coverage reached to 99 %, suggesting that the medium-depth pyrosequencing captured the majority of unique bacterial OTUs. Raw sequences are available from the NCBI Sequence Read Archive (SRA accession number: SRR1734621; BioProject ID: PRJNA270958). Sequences were assigned to 22 phyla, of which *Proteobacteria* were the most abundant, accounting for 92.24 % of total sequences.

In biofilm from DCIP, sequences were assigned to 22 phyla (Fig. 3). *Proteobacteria*, *Cyanobacteria*, and *Bacteroidetes* represented 71.34, 6.79, and 6.36 % of the total sequences, respectively. An additional 19 phyla accounted for 22 % of the total sequences. Among the subclasses of *Proteobacteria*, the biofilm community from the DCIP was dominated by *Alphaproteobacteria* (68.74 %), *Betaproteobacteria* (15.62 %), and *Gammaproteobacteria* (12.08 %). The dominant bacterial genera in biofilm from the DCIP, in descending order of relative abundance, were *Hyphomicrobium* (27.69 %), *Rhizobium* (9.05 %), *Craurococcus* (1.54 %), *Methylocaldus* (1.44 %), and *Paludibacter* (1.34 %) (Fig. S1).

In the biofilm from GCIP, sequences were assigned to 14 phyla (Fig. 3). *Proteobacteria* represented 92.79 % of the total sequences, followed by *Actinobacteria* (3.14 %) and *Acidobacteria* (2.31 %). The remaining 11 phyla contributed to 1.77 % of the total sequences. Among the subclasses of *Proteobacteria*, the bacteria from GCIP were dominated by *Betaproteobacteria* (40.58 %), *Alphaproteobacteria* (32.58 %), *Deltaproteobacteria* (18.88 %), and *Gammaproteobacteria* (7.92 %). By contrast to the DCIP samples, the dominant bacterial genus in GCIP was *Desulfovibrio* (16.90 %), followed by *Afipia* (8.06 %), *Rhodanobacter* (5.72 %), *Bradyrhizobium* (2.32 %), *Geothrix* (2.17 %), and *Sphingomonas* (1.38 %); *Hyphomicrobium* only accounted for 1.06 % of the total sequences.

In the GSP biofilm, sequences were assigned to only ten phyla (Fig. 3). *Proteobacteria* represented 98.98 % of the total sequences, and an additional nine phyla accounted for 1.12 %. Among the subclasses of *Proteobacteria*, the bacterial community from the GSP was dominated by *Gammaproteobacteria* (45.41 %), *Alphaproteobacteria* (37.80 %), and *Betaproteobacteria* (16.73 %). The dominant bacterial genera were *Pseudomonas* (43.33 %) and *Sphingomonas* (30.22 %), followed by *Limnobacter* (8.05 %), *Delftia* (6.18 %), and *Acidovorax* (1.89 %). This distribution among genera was different from that of the biofilms in iron pipes.

Sequences from the SSCP biofilm were assigned to 11 phyla (Fig. 3). *Proteobacteria* represented 97.72 % of the total sequences, and an additional ten phyla accounted for 2.28 %

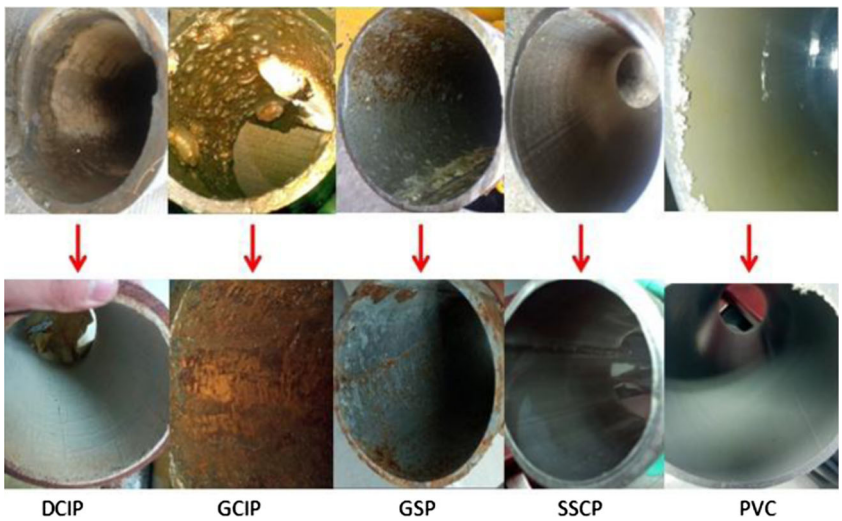

Fig. 1 Morphology of biofilms on inner pipe walls before sampling and morphology of pipe walls after sampling

Table 3 Physicochemical properties of biofilm and the contents of iron (Fe), zinc (Zn), and manganese (Mn) in biofilm per unit pipe area

Pipe material	Samples	Biofilm mass (mg/cm^2)	TS (mg/cm^2)	VS (mg/cm^2)	Fe (mg/cm^2)	Zn (mg/cm^2)	Mn (mg/cm^2)
DCIP	1	5.77	3.08	0.26	0.294	0.023	0.156
	2	5.28	2.46	0.12	0.134	0.007	0.116
GCIP	1	116.32	51.71	2.92	8.175	0.025	0.417
	2	140.79	59.97	3.19	10.825	0.982	0.069
GSP	1	18.74	5.24	0.58	0.298	0.148	0.025
	2	11.29	7.30	0.19	0.082	0	0
SSCP	1	5.02	0.56	0.07	0.026	0.008	0.003
	2	5.08	1.15	0.13	0.010	0	0.007
PVC	1	2.40	0.21	0.02	0[b]	0	0
	2	1.96	0.19	0.02	0	0	0

[a] The contents of COD, TS, VS, Fe, Zn, and Mn in volume of 10 mL biofim suspension were examined with three replications, and the total masses of these items were obtained according to the total biofilm suspension volume. Then, the contents of COD, TS, VS, Fe, Zn, and Mn in biofilms per unit of pipe wall area were calculated according to the pipe wall surface area, and the unit—milligrams per square centimeter refers to the content of component in biofilm per unit of pipe area

[b] The value was too small to be detected using flame atomic absorption spectrometry

of the total sequences. Among the subclasses of *Proteobacteria*, the bacterial community from SSCP was dominated by *Alphaproteobacteria* (82.88 %), followed by *Gammaproteobacteria* (14.43 %). Similar to the results from the GSP biofilm, *Sphingomonas* (72.48 %) and *Pseudomonas* (14.43 %) were the dominant genera in the SSCP biofilm (Fig. S1).

In contrast to the other pipes, sequences from PVC were assigned to only seven phyla (Fig. 3). *Proteobacteria* represented 98.86 % of the total sequences, and other six phyla accounted for 1.14 %. Among the subclasses of *Proteobacteria*, the bacterial community from SSCP was strongly dominated by *Gammaproteobacteria* (72.38 %) followed by *Alphaproteobacteria* (25.31 %). *Betaproteobacteria* and *Deltaproteobacteria* together composed 2.33 % of the *Proteobacteria*. The most abundant genus in PVC was *Pseudomonas*, which accounted for 63.08 % of the total sequences. The remaining genera, in descending order of relative abundance, were *Brevundimonas* (22.42 %), *Aeromonas* (4.95 %), *Stenotrophomonas* (2.19 %), and *Herbaspirillum* (1.38 %) (Fig. S1). In all biofilm samples, sequences assigned to the family *Enterobacteriaceae*, including total coliform, *Salmonella*, and *Shigella*, were accounted for 0.07–0.42 % of the sequences, while sequences assigned to the family *Vibrionaceae* or *Legionellales* were undetected.

Apparent differences in bacterial community diversity were observed among the five biofilms. The highest bacterial diversity was found in the DCIP, having a Chao1 index of 701.64 and a Shannon index of 4.22 (Table 7). The bacterial community in the biofilm from the SSCP had the lowest diversity, with a Chao1 index of 249.43 and a Shannon index of 1.44.

A RDA was performed to test the relationship between the environmental factors and bacterial community composition (Fig. 4) with p value in Table S1 in the supplementary material. The RDA results indicated that the total iron content of

Table 4 Pearson correlation analyses of physicochemical parameters and biotic factors in biofilm samples collected from different material pipes

Parameters	The Pearson correlation coefficient (sample numbers = 10)						
	Biofilm mass	TS	VS	Fe	Mn	qPCR	HPC
Biofilm mass	1	0.998**	0.997**	0.996**	0.747*	0.792**	0.149
TS	0.998**	1	0.994**	0.994**	0.725*	0.813**	0.188
VS	0.997**	0.994**	1	0.989**	0.710*	0.821**	0.160
Fe	0.996**	0.994**	0.989**	1	0.770**	0.764*	0.148
Mn	0.747*	0.725*	0.710*	0.770**	1	0.195	0.146
qPCR	0.792**	0.813**	0.821**	0.764*	0.195	1	0.182
HPC	0.149	0.188	0.160	0.148	0.146	0.182	1

*$p < 0.05$; **$p < 0.01$—as determined by SPSS version 20.0 (SPSS program, Chicago, IL, USA)

Table 5 Effects of pipe materials on biofilm physicochemical characteristics and bacterial communities using one-way ANOVA

Factor	*p* value (pipe samples = 10)						
	Biofilm mass	TS	VS	Fe	Mn	qPCR	HPC
Pipe material	0.001	0.001	0.001	0.001	0.485	0.096	0.032

bulk water was the most significant factor influencing the bacterial community distribution of biofilm ($p < 0.05$, 1000 Monte Carlo permutations, sample numbers = 5). In addition, the free chlorine content was correlated with the bacterial community composition, although this effect did not reach a significant level.

Discussion

Biofilms are always inhabited in pipes in all DWDS and accumulated easily in iron pipes due to their high surface roughness compared with plastic pipes (Berry et al. 2006). However, the density of biofilms accumulated in pipe walls in actual DWDSs was rarely reported. In this study, the density of biofilms on surface of pipes in operation for 11 years ranged from 1.96 to 140.79 mg cm^{-2}. The amount of biofilms in iron pipes was greater than that in SSCP and PVC as expected, while the higher VS/TS ratios were found in SSCP and PVC. The corrosion scale of iron pipes accounted a great proportion of TS (Nawrocki et al. 2010), which made a low VS/TS ratio in iron pipes.

The total bacterial 16S rRNA gene abundance in biofilms from different pipe materials ranged from 10^5 to 10^7 gene copies cm^{-2}, and the numbers of culturable heterotrophic bacteria ranged from 10^4 to 10^6 CFU cm^{-2}. Both of qPCR and HPC values are lower than that previously reported (Batté et al. 2003; Lehtola et al. 2006). The growth of biofilms could be affected by organic matter content in DWDSs (Yadav et al. 2014). Low concentration of total organic carbon limited biofilm growth (Van Nevel et al. 2013). The DWDS in this study was supplied with reservoir water having a low concentration of total organic carbon (the concentration ranged from 1.0 to 1.5 mg L^{-1}), which might have led to the reduced bacterial concentrations observed in this study. The numbers of total bacteria and culturable heterotrophic bacteria from iron pipes were detected 5- to 30-fold and 36- to 93-fold higher than that in the PVC biofilm, respectively. Higher numbers of bacteria were detected in cast iron pipes than in PVC in a model DWDS (Neden et al. 1992), and it has been reported that iron pipes supported 10- to 45-fold more growth than plastic (Jang et al. 2011). The results in this study provided further evidence that the biofilm might be more easily inhabited in iron pipe walls than in others in actual DWDS. Bacterial concentration in biofilms was affected by various variables, such as pipe materials, pipe ages, flow rate, and chlorine residual (Liu et al. 2013). They interact with each other to create distinct ecological niches with different physiochemical conditions, in which various microbes can be selected and enriched in drinking water systems (Wang et al. 2014). Pipes sampled in this study had similar pipe age and flow rate. The concentration of chlorine residual of bulk water in the distribution system was below 0.5 mg L^{-1}. It was reported disinfection with chlorine dioxide and chlorite could reduce the concentration of planktonic bacteria but had little effect on the concentration of biofilm bacteria (Gagnon et al. 2005). Therefore, pipe material might be the main factor influencing the biofilm bacterial concentration in the study. There have been some explanations

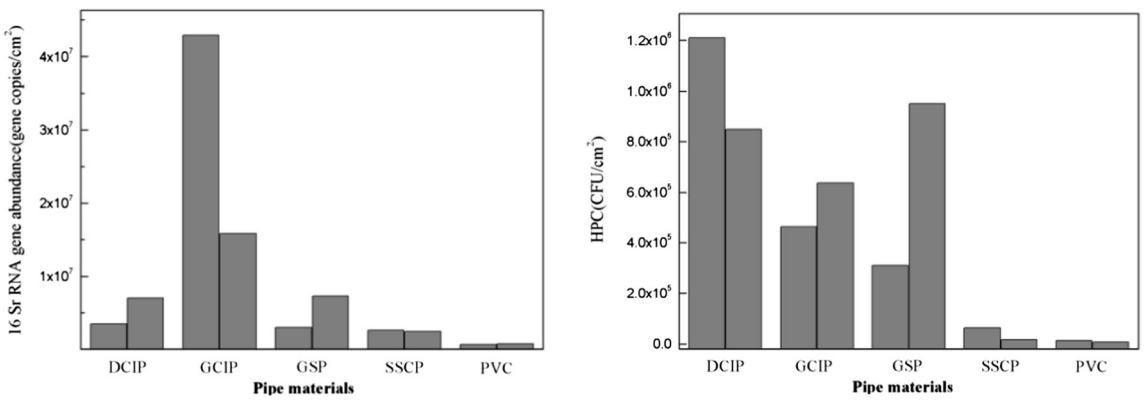

Fig. 2 The numbers of total bacterial 16S rRNA gene copies and culturable heterotrophic microorganisms in biofilms from different pipe materials as determined by qPCR and HPC

Table 6 The numbers of total coliforms, *Salmonella*, *Shigella*, vibrios, and *Legionella* in biofilms from different pipe materials as determined by culturing

Pipe material	Samples	Total coliforms (CFU/cm^2)	*Salmonella* (CFU/cm^2)	*Shigella* (CFU/cm^2)	Vibrios (CFU/cm^2)	*Legionella* (CFU/cm^2)
DCIP	1	5.3×10^2	2.1×10^2	0	2.9×10^2	0
	2	1.4×10^1	0	0	0	0
GCIP	1	2.1×10^2	0	0	0	0
	2	1.0×10^2	0	0	0	0
GSP	1	6.6×10^3	0	1.2×10^5	2.1×10^5	0
	2	5.8×10^3	0	0	0	0
SSCP	1	2.5×10^2	0	1.2×10^2	5.0×10^2	0
	2	8.7×10^2	0	2.2×10^4	0	0
PVC	1	6.5×10^1	0	1.3×10^4	3.5×10^4	0
	2	3.2×10^1	0	1.3×10^3	2.7×10^2	0

for this evidence: iron pipes have more surface area than PVC due to their high surface roughness, which is favorable for biofilm establishment (Jang et al. 2011). In addition to a high surface roughness, the iron corrosion observed in chlorinated and chloraminated pipes is a complex process involving chemical (e.g., iron reacts with disinfectants) and biological (e.g., microbial corrosion) reactions, which can result in lower levels of disinfectant residuals and a lower efficacy against biofilms (Zhu et al. 2014). Furthermore, iron is an essential element for bacterial proliferation and promotes biofilm growth when released from iron pipes (Wang et al. 2014). Though the number of culturable heterotrophic bacteria might be high in some biofilms with high total bacterial 16S rRNA gene number, there was no obvious positive correlation between HPC results and qPCR results (Table 4). HPC yielded only information about a limited fraction (less than 10 %) of the whole microbial community. qPCR was used to detect and quantify bacteria in biofilms based on quantifying the number of 16S rRNA gene copies present in samples. It was possible that quite a few dead bacteria might have been identified as part of the community. Furthermore, extracellular DNA associated with biofilm extracellular polymeric substances (EPS) could also account for some of the DNA identified (Gomez-Alvarez et al. 2012).

Although all conditions such as pipe sites and daily regime were not consistent in a DWDS, the key factor affecting the bacterial composition of the samples was the type of pipe material (Douterelo et al. 2014b). It is

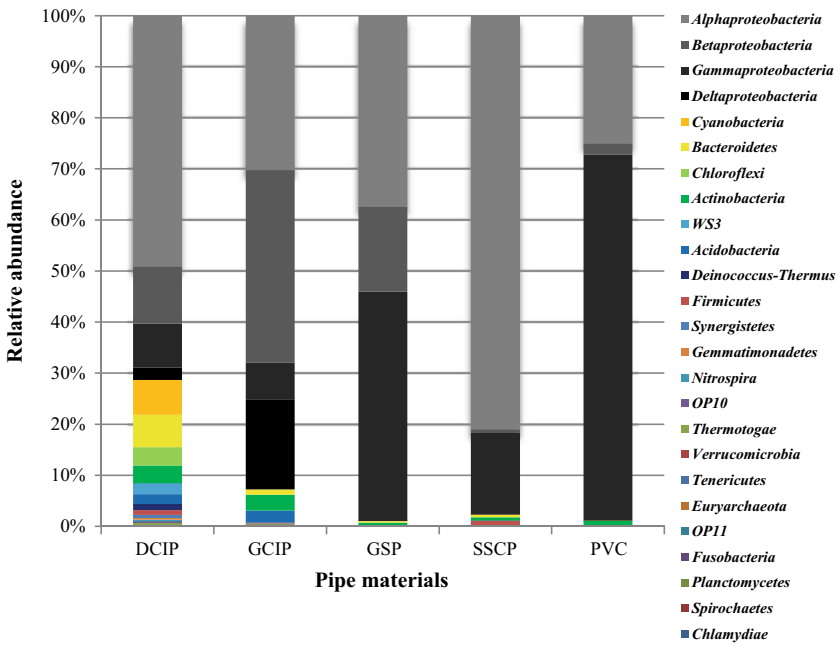

Fig. 3 Relative abundance of phyla and subclasses of *Proteobacteria* in biofilms from different pipe materials. The phylum *Proteobacteria* is divided into the subclasses *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* (shown in *black* and *white* in the *upper part of the column*)

Table 7 The Chao1 richness index, Shannon diversity index, and coverage of bacteria in biofilms at 97 % of sequence similarity

Pipe material	Chao1 index	Shannon index	Coverage (%)
DCIP	701.64	4.22	99
GCIP	354.17	3.43	99
GSP	361.18	2.01	99
SSCP	249.43	1.44	99
PVC	350.71	2.06	99

reported that daily hydraulic regime influenced the physical characteristics of biofilms such as the strength of attachment to the pipes, but they did not seem to obviously affect their bacterial composition (Douterelo et al. 2013). Differences in bacterial communities from various pipe materials were observed in this study, as shown in Fig. 3. In the sample obtained from DCIP, a high abundance of stalked bacteria, such as *Hyphomicrobium* (27.69 %), was particularly noted. It has been observed using microscopy-based methods that stalks recovered from such bacteria inhabiting water distributions systems were coated with insoluble ferric salt deposits (Ridgway and Olson 1981). Bacteria belonging to the genus *Hyphomicrobium*, which is naturally present in iron-rich environments and can oxidize iron and manganese, are generally involved in iron and manganese deposition (Cerrato et al. 2010).

Compared with the DCIP biofilm, a lower abundance of *Hyphomicrobium* (1 %) but higher abundance of *Desulfovibrio* (16.90 %) was detected in the biofilm from GCIP. The genus *Desulfovibrio* as one type of sulfate-reducing bacteria (SRB) is an important bacterial community with regard to iron corrosion in anaerobic environments. These bacteria can use sulfate as an electron acceptor (Rainha and Fonseca 1997). However, the presence of SRB in DWDSs has rarely been reported except in corrosion scales from iron pipes (Yang et al. 2014). This discrepancy may be explained by the fact that all previous investigations were performed using simulated pipe loops, and the biofilms were too thin to form anaerobic layers necessary for SRB growth. The high contents of iron and manganese detected in the biofilms from GCIP (Table 3) formed environmental niches favorable for iron corrosion-related bacteria, including *Hyphomicrobium* and *Desulfovibrio*. Moreover, the biofilm in GCIP was hundreds of micrometers thick, which could allow for the formation of an anaerobic layer in which the bacteria *Desulfovibrio* could survive (Liu et al. 2013). Iron ions reacted with hydrogen sulfide, a metabolite produced by *Desulfovibrio*, to form rust tubercles, creating more severe corrosion in GCIP. *Cyanobacteria* were found in this system with chlorine disinfection. Metagenomic analysis previously demonstrated the presence of *Cyanobacteria* in chlorinated water but absent in chloramined system (Gomez-Alvarez et al. 2012). This case provided further evidence that these species might be present in DWDSs. *Cyanobacteria* typically depend on light for survival. They might enter the distribution system from the reservoir water, although how they attached to biofilm in the dark was not clear. It was possible that some of the *Cyanobacteria* populations could temporarily survive in darkness (Richardson and Castenholz 1987), and it was also

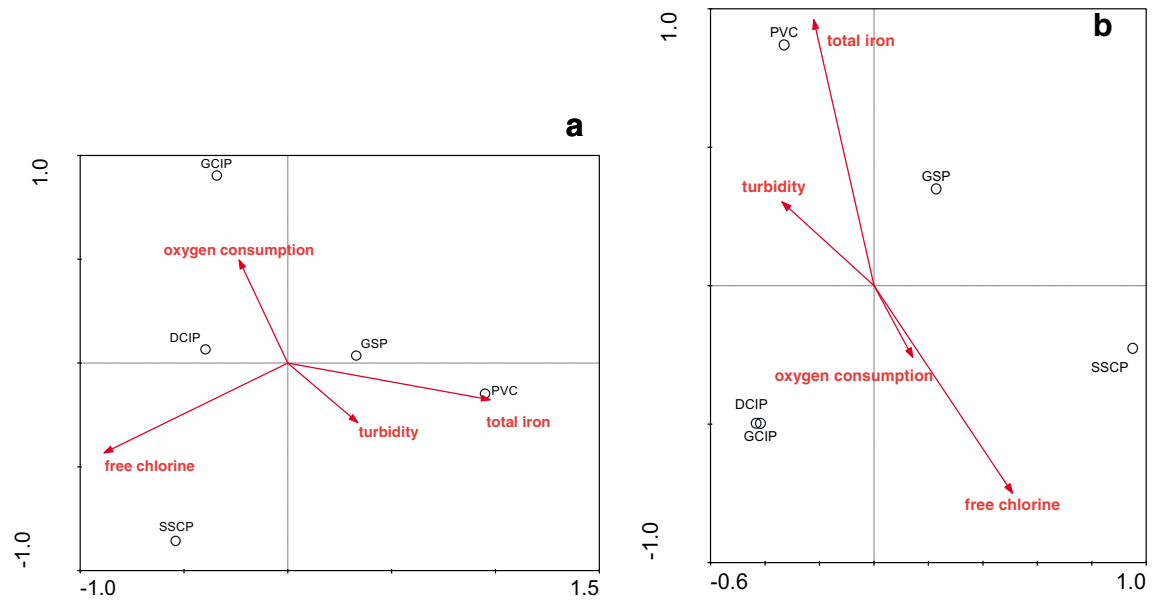

Fig. 4 RDA ordination plots for the first dimensions showing the relationship between the biofilm bacterial community structure and physicochemical properties in bulk water: **a** the level of class and **b** the level of genus

possible that they were only trapped in the biofilm and were not metabolically active (Douterelo et al. 2014b). It was surprising that *Cyanobacteria* only existed in DCIP in this DWDS, but the reason why they exist in the DCIP needs to be check with more detections and tests.

Members of the genera *Sphingomonas* (30.22 %) and *Pseudomonas* (43.33 %) were predominant in the biofilm from GSP. *Sphingomonas* and *Pseudomonas* have been reported to be the predominant bacterial genera detected in pipe wall biofilms (Niquette et al. 2000; Berry et al. 2006; Liu et al. 2014). *Sphingomonas*, which use oxygen as the terminal electron acceptor, are chemoheterotrophic, aerobic, and metabolically versatile bacteria that have high-affinity uptake systems under low nutrient conditions (White et al. 1996; Bereschenko et al. 2010), and they are commonly found in oligotrophic conditions such as DWDSs (Simoes et al. 2010). *Pseudomonas* has been reported to be the most abundant bacterial organisms in DWDSs because of their ability to form biofilms (Wang et al. 2013). Most *Pseudomonas* have aerobic metabolism and use oxygen as the terminal electron acceptor; however, in some cases, nitrate can be used as an alternate electron acceptor, allowing for anaerobic growth (GM et al. 2005). It was reported that the oxygen diffusion distance was around 200 μm by using suspended carrier mathematical model (Mašić et al. 2010) and about 270 μm by using microelectrodes (Ning et al. 2014). The biofilm in GSP was thin, and oxygen in the bulk water could easily diffuse into the biofilm, thereby allowing the growth of *Sphingomonas* and *Pseudomonas*.

Sphingomonas were also found in the sample from SSCP although at a different relative abundance (72.48 %) than in the GSP sample (30.22 %). *Sphingomonas* are known to act as pioneers in the initial stages of biofilm formation in drinking water systems (Navarro-Noya et al. 2013), and several strains have been observed to have strong resistance to disinfectants such as chlorine and chloramine (Sun et al. 2013). The concentration of free chlorine was the highest in the bulk water in SSCP at 0.44 mg L^{-1}; this situation may have facilitated the predominance of *Sphingomonas* in the biofilm. In contrast to the other pipes, the predominant bacteria detected in the sample from PVC were classified as *Pseudomonas* and *Brevundimonas*, which belong to the family *Sphingomonadaceae*.

The existence and growth of pathogens is found in DWDSs. The presence of total coliform, *Salmonella*, *Shigella*, and *vibrios* in the biofilms determined by culture-based method implies these pathogens could grow in the DWDS and biofilm sloughing might lead to human exposure to pathogens through water (Buse et al. 2012). *Legionella* are typical pathogenic species, and they were usually determined in US DWDSs (Marciano-Cabral et al. 2010). *Legionella* were undetected in all biofilms in this study. The number of pathogens varied in biofilms from different types of pipes. The GCIP contained the minimum number of pathogens, while the *Shigella* and vibrios were detected more in GSP and PVC. However, in this study, only culture-based methods were performed to detect the pathogens, which had certain limitations. None culture-based methods, such as PCR-based approach were required for better evaluation of the pathogens in the biofilms.

Diverse distributions of bacterial communities were observed in different pipe materials and may have been influenced by the physiochemical properties of bulk water. RDA analysis indicated that the total iron content had a significant correlation with the distribution of bacterial communities in the examined biofilm samples. In this study, the concentration of total iron of bulk water detected in DCIP was lower than that in the PVC pipe. It was reported biofilms can accumulate trace amounts of materials from DWDSs, such as heavy metals (Ashbolt et al. 2010). In this study, we also detected high contents of iron and manganese in biofilms from iron pipes. High content of iron and manganese in biofilms from iron pipes was due to the pipeline corrosion which caused the iron and manganese release from the pipe into the biofilms. And at the same time, the heavy metal also can be absorbed into the biofilms from the bulk water, which may be the main reason why the concentration of total iron of bulk water detected in DCIP was lower than that in the PVC.

In this study, a significant diversity of the bacterial abundance and community distribution in biofilms from different pipe materials in actual DWDSs was found. The biomass in DWDSs tended to accumulate in iron pipes, such as GCIP and DCIP. A high abundance of iron bacteria, including *Hyphomicrobium* and *Desulfovibrio*, was detected in samples from iron pipes, and the diversity of bacterial communities was also highest in iron pipes. The members of the genera *Sphingomonas* and *Pseudomonas* were found in biofilm samples from SCP, SSCP, and PVC pipe, although the relative abundance of the bacteria differed among these biofilms.

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