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Effect of flushing on the detachment of biofilms attached to the walls of metal pipes in water distribution systems*

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Abstract: Biofilm detachment caused by flushing can result in secondary contamination in drinking water distribution systems (DWDSs). To evaluate the impact of flushing on biofilm detachment, actual water supply pipes including ductile cast iron pipes (DCIPs), gray cast iron pipes (GCIPs), and stainless steel compound pipes (SSCPs) were used in this study. Real-time quantitative polymerase chain reaction and 454 pyrosequencing were used to quantify bacteria and analyse microbial community composition, respectively. The results showed that the pipe material greatly influences the resistance of a biofilm to flushing. Biofilms attached to DCIPs were able to resist quite strong flushing, while those attached to GCIPs and SSCPs were sensitive to flushing. Both flush-resistant and flush-sensitive bacteria were present in all the biofilms, but their frequency differed among the different metal pipes. Thus, the resistance to flushing of bacteria is related not only to the nature of the bacteria, but also to the pipe material. Although flushing can remove some of the biofilm and may be a good way to clean the DWDS, the shear stress needed to remove the biofilm differs among different pipe types. The results of this study provide technical support for the management and operation of DWDS.

Key words: Drinking water distribution system (DWDS); Biofilms; Flushing; Metal pipe
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1 Introduction

Drinking water distribution systems (DWDSs) are the connection between water treatment plants and consumers. Their function is to deliver treated water to consumers safely. Previous studies indicate that although DWDSs are an extreme environment with oligotrophic conditions where residual disinfectant commonly persists, microorganisms are able to survive, in particular by attaching to the internal surfaces of pipes forming biofilms (Simões *et al.*, 2007). These biofilms can be associated with several unpleasant problems in DWDS, such as deterioration of water quality (Martiny *et al.*, 2005), corrosion of pipe walls (Nawrocki *et al.*, 2010), adsorption and trapping of substances from the bulk water, and hosting opportunistic pathogens (Szewzyk *et al.*, 2000; Beech and Sunner, 2004). When biofilms accumulate, various substances and undesirable microorganisms may be hosted leading to discolouration

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and deterioration of the microbiological drinking water quality (LeChevallier, 1999). Many factors affect the detachment of pipe wall biofilms, such as flushing (Labib and Lai, 2000), scratches of protozoa (Bryers, 1988), aging of the biofilm, and nutrient starvation (Sanin *et al.*, 2003; Hunt *et al.*, 2004). Since flushing often occurs in DWDS, it should be considered the major factor inducing biofilm detachment. Therefore, it is reasonable to pay more attention to exploring the effect of flushing on the detachment of biofilms.

It has been reported that new drinking water biofilms are complex microbial systems composed mainly of patchy and non-uniformly distributed aggregates (Abe *et al.*, 2012). After a long period of growth and development, mature biofilms are intrinsically stratified with respect to cohesion, density, and porosity (Möhle *et al.*, 2007; Derlon *et al.*, 2008; Paul *et al.*, 2012). A cohesive basal layer and a soft top layer have been shown to be always present in these mature biofilms. When flushing occurs, the top layer of a biofilm is easily detached, while the basal layer has high resistance to detachment (Paul *et al.*, 2012). When higher hydraulic shear stress occurs, more extensive detachment of the biofilm generally occurs (Rittman, 1982; Paul *et al.*, 2012; Douterelo *et al.*, 2014). Increasing hydraulic shear stress, however, cannot make the biofilm detach completely (Douterelo *et al.*, 2013). The detachment of biofilms leads to the release of accumulated materials into bulk water, causing deterioration of water quality (Lehtola *et al.*, 2007). In addition, flushing affects not only the biomass of biofilms, but also the composition of their bacterial communities, which suggests variation in sensitivity to hydraulic shear stress among different bacteria (Douterelo *et al.*, 2013; 2014). However, because biofilm samples from DWDS are difficult to obtain, previous studies generally employed idealized conditions such as bench-top reactors (Choi and Morgenroth, 2003; Abe *et al.*, 2012; Paul *et al.*, 2012) and scaled pipelines (Horn *et al.*, 2003) or semi-idealized conditions such as a full-scale circular pipeline facility with a trickle feed set to give a system residence time of 24 h (Douterelo *et al.*, 2013). Therefore, previous studies did not ideally reveal the effect of flushing on the detachment of biofilms in actual DWDS.

Metal pipes, including ductile cast iron pipe (DCIP), gray cast iron pipe (GCIP), and stainless steel clad pipe (SSCP), are commonly used in actual DWDS and the microbial risks of these metal pipes are prominent and obvious (Douterelo *et al.*, 2014; Wang *et al.*, 2015). Previous research on the detachment of biofilms was often carried out using plastic substrates such as polyvinyl chloride (PVC) (Manuel *et al.*, 2009), organic glass (Mathieu *et al.*, 2014) or high-density polyethylene (HDPE) (Douterelo *et al.*, 2013). The lack of studies using metal pipes has greatly affected the systematic understanding of the detachment of biofilms when flushing occurs in DWDS. To overcome these limitations, our study was carried out in a unique full-scale pipeline facility. This facility can realistically generate two hydraulic shear stress loads (low and high) to flush biofilms attached to the walls of metal pipe including DCIPs, GCIPs, and SSCPs. Flushing at low shear stress was used to simulate abnormal flow change, while flushing at high shear stress was used to simulate water cleaning by supply companies. Actual metal pipe samples were obtained from a municipal DWDS in Eastern China. In this study, we aimed to investigate the effect of flushing on the detachment of biofilms and the flushing resistance of biofilms developing in different metal pipes. It is important to understand the properties of biofilms within DWDS and any associations with risks to potable water quality, so that systems can be better operated and managed in the future.

2 Materials and methods

2.1 Pipe segments

In this study, all metal pipe segments were obtained from an operational municipal DWDS of a medium-sized city in Eastern China. A water reservoir serves as the water source for the city. Raw water is treated by conventional water treatment processes (coagulation-sedimentation-filtration-disinfection) with lime added to adjust the pH. The treated drinking water (about 500 000 m^3/d) is supplied for two million people within a 2942 km^2 area. Based on their similarity in terms of water quality, flow velocity, water age, and pipe age (Table 1), three typical locations in the DWDS (Fig. 1) were chosen for sampling. Flow velocity and water age were obtained by calculating and analysing a hydraulic model, while water quality

data were obtained by site monitoring during the previous 12-month period. At these locations, different metal pipe segments were collected for the flushing experiment. The collecting process was as follows: the water supply at the location was cut off and water in the pipes was drained off slowly at a flow velocity of less than 0.03 m/s to prevent biofilm loss before cutting the pipe. Then, the soil around the buried pipe was excavated and the surface of the pipe was physically decontaminated with tap water. Six pipe segments were cut out at locations A and B, but only three at location C because of location-specific constraints. The pipe segments were sealed with sterile plastic wrap, and transported to the laboratory in an ice box within 6 h. *In-situ* water samples, sufficient for the downstream flushing experiment, were collected and taken to the laboratory within 6 h.

2.2 Experimental facility and operating conditions

The experimental facility consisted of two recirculating pipe loops (loops A and B) fed by a common pump from a reservoir (Fig. 2). Loops A and B shared a section of the pipeline. When the 150 mm (inner diameter, ID) pipe segments needed flushing, two valves of loop B had to be closed and loop A had to be changed into a closed-loop. In contrast, when 80 mm (ID) pipe segments needed flushing, two valves of loop A had to be closed and loop B had to be changed into a closed-loop. Experimental connection sections, a flow meter, motor-control valve, circulating pump, butterfly valve, pressure meter, exhaust port, and sample tap were mounted in the pipeline. Pipe segments collected from the DWDS were mounted in the connection section for flushing experiments. A circulating pump with an 11 m design head and a 120 m^3/h design flow rate provided the required hydrodynamic conditions to flush the pipe segments. A motor-control valve controlled by computer was used to change the flow velocity (hydraulic shear stress) with closing time set. Flow velocity and pressure were measured by the flow meter and pressure meter, respectively. Butterfly valves were used to isolate loops A and B. An exhaust port was used to release trapped air in the pipelines and a water sample tap for sampling.

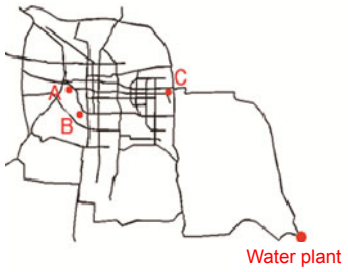

Fig. 1 Locations of sampling sites (locations A, B, and C were chosen by considering their similarity in terms of water quality, flow velocity, water age, and pipe age)

To avoid the impact of water quality changes on detachment of the biofilm, the water used to flush the pipe segments was that collected from the sampled locations in the actual DWDS. Before flushing, the free chlorine of the bulk water was adjusted to be similar to that of the sampled locations, to compensate for free chlorine decay during transportation.

Before starting the experiment, the facility was disinfected (free chlorine was present at 20 mg/L in bulk water) for 24 h with sodium hypochlorite solution. The system was then flushed repeatedly at maximum flow velocity (about 3 m/s) with fresh water until the levels of free chlorine and turbidity were similar to those of the water in the DWDS system. After disinfecting, pipe segments were mounted in the connection section. The facility was then filled with water collected from the sampling locations within the DWDS until the water pressure reached 0.1 MPa. Then the circulation pump and motor-control valve

Table 1 Detailed information on the sampled pipes and the physicochemical properties of bulk water during the 12 months prior to sampling

Location	Pipe material	Pipe age (year)	Pipe length (mm)	Pipe inner diameter (mm)	Free chlorine (mg/L)	pH	TOC (mg/L)	AOC (μg/L)	Flow velocity (m/s)	Water age[b] (h)
A	DCIP	11	500	150	0.39±0.07	7.2±0.1	1.1±0.10	103±13	<0.1	<8.5
B	GCIP	11	500	150	0.31±0.06	7.1±0.1	1.3±0.17	98±9	<0.2	<9.0
C	SSCP	10	500	80[a]	0.35±0.10	7.2±0.1	1.2±0.07	108±22	<0.1	<8.7

[a] The maximum inner diameter of SSCPs in this city was 80 mm; [b] Water age is the residence time of water from the point of entry to the distribution system to the consumer's tap. TOC: total organic carbon; AOC: assimilable organic carbon

were started to flush the pipe segments. Two different flushing regimes were applied by controlling the degree and duration of opening of the motor-control valves. Shear stress was calculated by Eq. (1), with results shown in Table 2. The two regimes were: a low shear stress flushing regime, operating at a flow velocity of 0.1 m/s (similar to the flow velocity at the sampled locations) for 2 min, then flushing at a shear stress of 2.0 N/m^2 for the next 10 min; a high shear stress flushing regime, operating at a flow velocity of 0.1 m/s for 2 min, then flushing at a shear stress of 9.0 N/m^2 for the next 10 min. These two flushing regimes were based on daily operation data in the actual DWDS. Flow statistics showed that the average flow velocity in the DN150/DN80 pipes in the actual DWDS was about 0.1 m/s, while in some circumstances, such as rezoning, changing seasonal demand or pipe bursts, the shear stress could rise to about 2.0 N/m^2, or even to 9.0 N/m^2 during flushing.

$$\tau_0=\frac{\lambda}{8}\rho v^2, \tag{1}$$

where τ_0 is the shear stress (N/m^2), λ is the frictional resistant coefficient, ρ is the fluid density (kg/m^3), and v is the flow velocity (m/s).

To determine the effect of flushing on the detachment of biofilm attached to the walls of the different metal pipes, two pipe segments of DCIP or GCIP were sampled without flushing, two after low shear stress flushing, and another two after high shear stress flushing. While only one pipe segment of SSCP was sampled without flushing, one was sampled after low shear stress flushing, and another after high shear stress flushing. The samples were then stored in refrigerators at 4 °C for downstream detection.

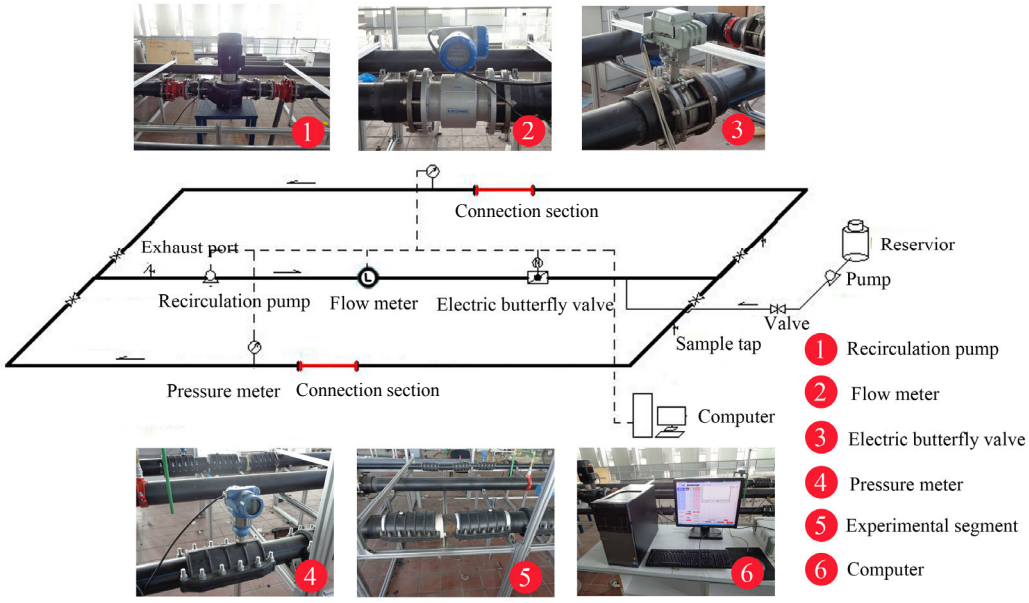

Fig. 2 Experimental facility of full-scale laboratory pipe loop used to flush pipe segments

Table 2 Details of the flushing regimes

Pipe material	Flushing regime	d (mm)	Re	Δ (mm)	Δ/d	λ	τ_0 (N/m^2)	v (m/s)
DCIP	Low shear stress (Flush1.1)	150	129 259	0.15	0.0010	0.022	2.0	0.86
	High shear stress (Flush1.2)	150	278 056		0.0010	0.021	9.0	1.85
GCIP	Low shear stress (Flush2.1)	150	100 701	1.2	0.0080	0.036	2.0	0.67
	High shear stress (Flush2.2)	150	213 427		0.0080	0.036	9.0	1.41
SSCP	Low shear stress (Flush3.1)	80	68 938	0.025	0.0003	0.022	2.0	0.86
	High shear stress (Flush3.2)	80	152 305		0.0003	0.020	9.0	1.90

d: pipe inner diameter; Re: Reynolds number; Δ: equivalent roughness

2.3 Sampling and biofilm preprocessing

The biofilm attached to the walls of the metal pipes was sampled by swabbing the inner pipe wall with a sterile brush while continuously washing the pipe wall with sterile water. When the turbidity of water flowing down the pipe wall after washing was less than 0.5 NTU, biofilm collection was considered to be complete. The biofilm slurry was collected into sterile glass bottles and stored in refrigerators at 4 °C.

The collected biofilms were shaken in sterile glass bottles with sterile glass beads of 4–5 mm on a shaker for 20 min to break up microbial clusters and then filtered using sterile mesh (80 meshes) to remove impurities such as metal particles and sand sediments. The treated biofilm slurries were split into two parts: one part was stored at 4 °C for an incubation experiment and the other was centrifuged in 50 ml sterile centrifuge tubes at a speed of 4500 r/min for 15 min. The biofilm biomass (sediment) was retained and the supernatant was discarded. Collected sediment was stored at −80 °C for DNA extraction.

2.4 Water quality analysis

The temperature, turbidity, pH, free chlorine, and total organic carbon (TOC) of the bulk water were measured before and after flushing. Turbidity was measured using a portable turbidity meter HACH 2100Q (HACH, USA) following the manufacturer's instructions. Temperature and pH were both measured using a HACH HQ30d multimeter (HACH, USA). Free chlorine was measured using a portable free chlorine meter HACH DR 890 (HACH, USA) following the manufacturer's instructions. TOC was analyzed by a high temperature combustion method with a Shimadzu 5000 TOC analyzer (Shimadzu Corporation, Japan).

2.5 Heterotrophic plate count

Resuspended biofilm slurry (20 ml) was shaken in a sterile glass bottle (50 ml) again with sterile glass beads (4–5 mm) for 15 min on a shaker before culturing. An equal volume of sterile water was used as a 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-d incubation period at 25 °C (Reasoner and Geldreich, 1985; Thayanukul *et al.*, 2013).

2.6 DNA extraction and real-time polymerase chain reaction (PCR)

Quantitative real-time PCR (qPCR) was used to quantify the total bacteria in the biofilm samples (Edwards, 2013; Shen *et al.*, 2014). Total DNA was extracted from the biofilm (0.25 g) using a Power Soil DNA Kit (Mo BioLaboratories, USA), as described by the manufacturer. The extracted genomic DNA was examined on 1.0% (0.01 g/ml) agarose gels via electrophoresis and quantified using a NanoDrop ND-1000 spectrophotometer (Hu *et al.*, 2011). The DNA was stored at −20 °C for future PCR amplification.

The SYBR® GREEN qPCR assay for bacteria targets the 16S rRNA gene, which exists as a single copy within bacteria. Forward primer 338F (5'-ACT CCTACGGGAGGCAGC-3') and reverse primer 518R (5'-ATTACCGCGGCTGCTGG-3') amplify a 180 bp region within the 16S rRNA gene (Muyzer *et al.*, 1993). Each real-time PCR reaction contained 12.5 μl of SYBR® GREEN (Bio-Rad), 1 μl of each primer, 9.5 μl of double-distilled water (ddH_2O), and 1 μl of DNA template for a total volume of 25 μl. Reactions were carried out with a Bio-Rad CFX96 real time system. The thermal cycle profile consisted of initial incubation for 3 min at 96 °C, 40 cycles of 30 s at 96 °C (denaturing), 30 s at 55 °C (annealing), and 30 s at 72 °C (extension) (Hu *et al.*, 2012). Negative DNA controls (template DNA replaced by sterile Nanopure water) and 10-fold serial dilutions of known amounts of positive control DNA were included in triplicate in each qPCR run. Each sample was also tested in triplicate and the mean value was used for statistical analysis. A representative calibration curve, spanning six orders of magnitude, was obtained, with a standard error estimate (R^2) of 0.9995.

2.7 Statistical analysis and 454 pyrosequencing

The extracted genomic DNA was used for bacterial 16S rRNA gene tag-encoded pyrosequencing to analyze bacterial community composition, and the primer pairs 357F (5'-CCTACGGGAGGCAGCAG-3') and 926R (5'-CCGTCAATTCMTTTRAGT-3') were used to amplify the V3–V5 region of the bacterial 16S rRNA gene copy numbers. 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 mmol/L): 2.00 μl; DNA template (20 ng/μl): 2.00 μl; forward primer (10 μmol/L): 1.00 μl; reverse primer (10 μmol/L): 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 and 72 °C for 1 min, and a final extension at 72 °C for 7 min. Three replicated PCR products from each sample were combined and purified using an Axy-Prep DNA purification kit (Axygen, USA). 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 machine (Roche Diagnostics Corporation, USA). A total of 125 131 sequences were generated from the pyrosequencing analysis and subsequently processed using the Mothur software package (http://www.mothur.org). After denoising and chimera inspection, the high-quality reads were used to generate a distance matrix and calculate the clustering of operational taxonomic units (OTUs) at 95% (class level) and 97% (genus level) confidence levels (Liu *et al.*, 2013). 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 (Revetta *et al.*, 2013; Liu *et al.*, 2014).

2.8 Control of the experimental time

To finish the experiment successfully, experimental time control was necessary. Details of the pipe cut-out/water sample collection and lab trials were as follows:

1. The pipe segments were sealed with sterile plastic wrap after cut-out, and transported to the laboratory in an ice box within 6 h. *In-situ* water (samples) sufficient for the downstream flushing experiment was collected within 6 h.

2. Sampling and biofilm preprocessing were finished within 12 h after the pipes were cut.

3. TOC and other field tests of water quality were finished within 24 h.

4. HPC incubation started within 24 h after biofilm preprocessing.

5. DNA extraction and real-time PCR were finished within 48 h after biofilm preprocessing.

6. The 454 pyrosequencing started within 48 h after DNA extraction.

3 Results

3.1 Morphology and physicochemical parameters of biofilm

Biofilms attached to the different metal pipe walls showed diverse forms (Fig. 3). In DCIPs, there was no obvious corrosion, and the biofilms were evenly distributed on the pipe wall. However, severe corrosion was found in GCIPs with lots of tubercles formed all over the pipe wall. Compared with DCIPs and GCIPs, the inner walls of SSCPs were smooth with no corrosion and the biofilms were relatively thin.

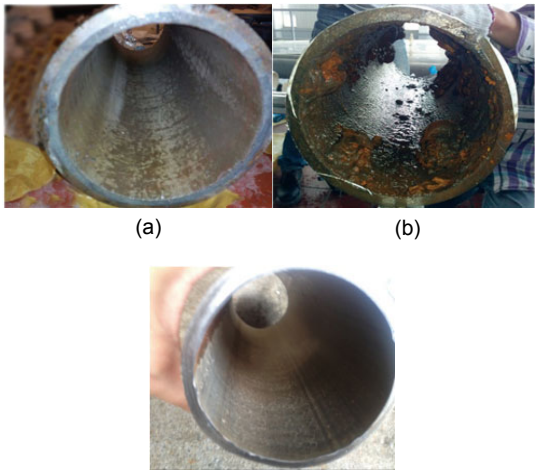

Fig. 3 Morphology of biofilms on inner pipe walls before sampling: (a) DCIP; (b) GCIP; (c) SSCP

The mass of biofilms accumulated on the pipe walls ranged from 5.02 to 128.56 mg/cm^2 with divergence among the different pipes (Table 3). The maximum biofilm biomass was detected in GCIPs, and the minimum in SSCPs. The concentrations of total solid (TS) and volatile solid (VS) ranged from 0.56 to 55.75 mg/cm^2 and from 0.07 to 3.06 mg/cm^2, respectively, which were consistent with the biofilm mass. The VS accounted for 5%–13% of the TS in the different biofilms. The VS/TS ratios were higher in biofilms from SSCPs than in those from iron pipes. The contents of Fe and Mn were the highest in

biofilms from GCIPs (9.50 and 0.24 mg/cm^2, respectively) and the lowest in biofilms from SSCPs (0.026 and 0.003 mg/cm^2, respectively).

3.2 Physicochemical analysis

The physicochemical properties of bulk water before and after flushing, and of the biofilm mass on the pipe walls after flushing, are summarized in Table 4. A significant increase in turbidity, TOC, and pH was found after flushing, and these physicochemical properties increased as hydraulic shear stress increased (Table 4). Flushing GCIPs caused a much greater change in turbidity than did flushing DCIPs and SSCPs. However, no significant differences were found in TOC after flushing. Compared with DCIPs and GCIPs, the concentration of TOC after flushing SSCPs was relatively low. The pH of bulk water increased after flushing, but there were no significant differences among DCIPs, GCIPs, and SSCPs. Compared with Table 3, biofilm mass showed significant reductions after flushing, especially in GCIPs. However, there was no significant difference between the results of low and high shear stress flushing.

Big changes were found in the physicochemical properties of bulk water after flushing, regardless of the shear stress. There were only slight differences between the results for low and high shear stress flushing, except in GCIP. In GCIP, increasing shear stress made turbidity increase significantly, but there was only a slight increase in TOC.

3.3 Bacterial quantification

The total bacterial 16S rRNA gene copy numbers in the biofilms decreased significantly after flushing, and showed an inverse ratio with the shear stress (Fig. 4). Before flushing, GCIPs had the highest levels of 16S rRNA gene copy numbers at 3.82×10^7 copies/cm^2, compared with DCIPs and SSCPs which had 2.87×10^6 and 2.61×10^6 copies/cm^2, respectively. In DCIPs, 16S rRNA gene copy numbers had only a slightly lower ratio (2.11×10^6 copies/cm^2) after flushing at low shear stress, but a significantly lower ratio (1.47×10^5 copies/cm^2) after flushing at high shear stress. In GCIPs, 16S rRNA gene copy numbers were significantly lower (4.70×10^6 copies/cm^2) after flushing at low shear stress, but showed only a slight further decrease (to 4.02×10^6 copies/cm^2) after flushing

Table 3 Physicochemical properties and the contents of Fe and Mn in biofilms per unit pipe area

Pipe	Biofilm mass (mg/cm^2)	Content per unit pipe area (mg/cm^2)			
		TS	VS	Fe	Mn
DCIP	5.53±0.35	2.77±0.44	0.19±0.10	0.21±0.11	0.14±0.03
GCIP	128.56±17.30	55.75±5.71	3.06±0.19	9.50±1.87	0.24±0.25
SSCP	5.02	0.56	0.07	0.026	0.003

The contents of TS, VS, Fe, and Mn in a volume of 10 ml of biofilm suspension were examined with three replications, and the total masses of these components were obtained according to the total biofilm suspension volume. Then, the contents of TS, VS, Fe, and Mn in biofilms per unit of pipe wall area were calculated according to the pipe wall surface area

Table 4 Physicochemical properties of bulk water and biofilm mass on pipe walls before and after flushing

Pipe	Flushing regime	T (°C)	Turbidity (NTU)	TOC (mg/L)	pH	Biofilm mass[b] (mg/cm^2)
DCIP	Inlet[a]	21.5±0.1	0.13±0.06	1.10±0.10	7.10±0.05	–
	Flush1.1	21.7±0.1	1.75±0.12	2.39±0.13	7.28±0.05	2.24±0.18
	Flush1.2	21.8±0.2	1.78±0.15	2.24±0.15	7.30±0.06	1.93±0.16
GCIP	Inlet	20.5±0.2	0.14±0.04	1.30±0.17	7.10±0.10	–
	Flush2.1	20.8±0.2	89.60±2.33	2.19±0.17	7.33±0.03	45.90±5.05
	Flush2.2	20.8±0.1	147.00±2.83	2.67±0.23	7.43±0.04	45.70±6.10
SSCP	Inlet	21.2±0.1	0.12±0.02	1.20±0.08	7.20±0.06	–
	Flush3.1	21.3±0.2	1.39±0.09	1.21±0.11	7.45±0.05	1.77
	Flush3.2	21.5±0.1	1.54±0.10	1.23±0.09	7.47±0.03	2.14

[a] Inlet water was *in-situ* water (samples) collected and moved to the laboratory for the downstream flushing experiment; [b] The biofilm masses before flushing are given in Table 3 and after flushing in Table 4

at high shear stress. Results for SSCPs were similar to those for GCIPs: 16S rRNA gene copy numbers were significantly lower (7.87×10^4 copies/cm^2) after flushing at low shear stress, but showed only a slight further decrease (to 4.41×10^4 copies/cm^2) after flushing at high shear stress. Compared with DCIPs and SSCPs, the total bacterial 16S rRNA gene copy numbers in the biofilms of GCIPs after flushing were still the highest.

The HPCs in the biofilms decreased significantly after flushing, and were lower at high shear stress (Fig. 5). Before flushing, GCIPs had the highest HPCs at 3.74×10^5 CFU/cm^2, compared with 7.80×10^4 CFU/cm^2 for DCIPs and 6.37×10^4 CFU/cm^2 for SSCPs. In DCIPs, HPCs were significantly lower (6.06×10^3 CFU/cm^2) after flushing at low shear stress, and decreased further (to 8.24×10^2 CFU/cm^2) after flushing at high shear stress. In GCIPs, HPCs showed a slight decrease (to 2.31×10^5 CFU/cm^2) after flushing at low shear stress, but a sharp decrease (to 2.31×10^4 CFU/cm^2) after flushing at high shear stress. Unlike DCIPs and GCIPs, SSCPs showed a sharp decrease in HPCs (to 3.22×10^3 CFU/cm^2) after flushing at low shear stress, but only a slight further decrease (to 2.74×10^3 CFU/cm^2) after flushing at high shear stress.

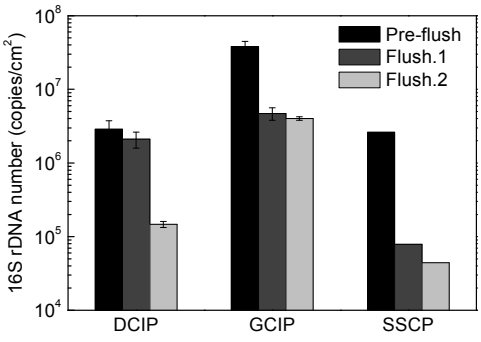

Fig. 4 Numbers of total bacterial 16S rRNA gene copies in biofilms before and after flushing, as determined by qPCR. Flush.1 refers to flushing at low shear stress ($\tau_0\approx$ 2.0 N/m^2); Flush.2 refers to flushing at high shear stress ($\tau_0\approx$9.0 N/m^2). Error bars indicate standard errors

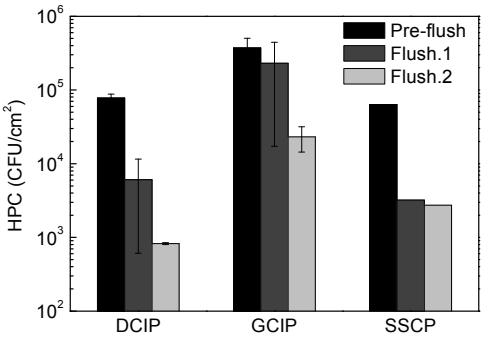

Fig. 5 Numbers of culturable heterotrophic microorganisms in biofilms before and after flushing, as determined by HPC

3.4 Composition of the bacterial communities

Shifts in the bacterial community structure at the class level were found by comparing the composition of biofilm samples before and after flushing (Fig. 6). In DCIP, the relative abundance of some bacterial classes increased significantly after flushing at low shear stress, but showed little change after flushing at high shear stress. These classes included *Betaproteobacteria* (10.3% for Pre.Flush1, 18.86% for Flush1.1, and 7.49% for Flush1.2) and *Gammaproteobacteria* (7.90% for Pre.Flush1, 23.7% for Flush1.1, and 9.02% for Flush1.2). This suggests that these bacterial classes are unlikely to detach under low shear stress, but detach under high shear stress. Some bacterial classes decreased significantly after flushing regardless of whether the shear stress was low or high. These classes included *Alphaproteobacteria* (45.6% for Pre.Flush1, 21.7% for Flush1.1, and 20.1% for Flush1.2) and *Anaerolineae* (2.50% for Pre.Flush1, 0% for Flush1.1, and 0% for Flush1.2). This suggests that these bacterial classes had largely detached under low shear stress, and little further detachment occurred when shear stress increased. However, other bacterial classes increased significantly after flushing occurred, regardless of whether the shear stress was low or high. These classes included *Clostridia* (0.20% for Pre.Flush1, 1.3% for Flush1.1, and 3.1% for Flush1.2) and *Methanomicrobia* (0.10% for Pre.Flush1, 3.2% for Flush1.1, and 11.0% for Flush1.2). This suggests that these bacterial classes had stronger resistance to flushing than other bacteria.

A similar phenomenon was found in GCIP. Classes that increased significantly after flushing at low shear stress, but decreased after flushing at high shear stress included *Betaproteobacteria* (36.97% for Pre.Flush2, 54.56% for Flush2.1, and 18.61% for Flush2.2). Classes that increased significantly after

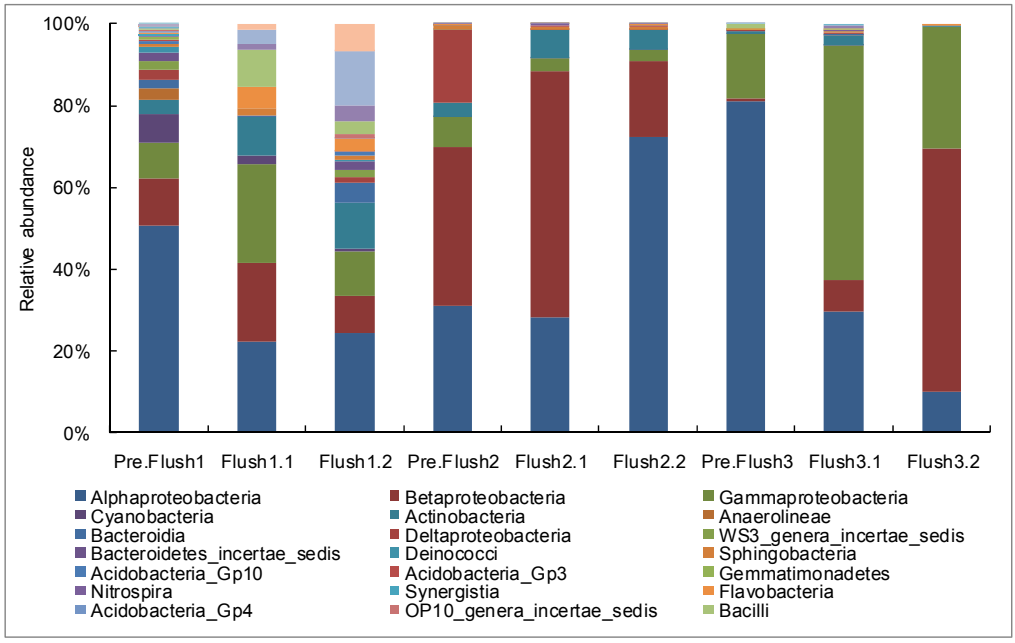

Fig. 6 Comparison of the relative abundance of the major phylotypes found in biofilms before and after flushing the internal pipe surfaces

Pre.Flush1, Pre.Flush2, and Pre.Flush3 refer to DCIP, GCIP, and SSCP before flushing the internal pipe surfaces

flushing at high shear stress, but changed little after flushing at low shear stress included *Alphaproteobacteria* (29.62% for Pre.Flush2, 25.28% for Flush2.1, and 71.18% for Flush2.2). Classes that decreased significantly after flushing, regardless of whether shear stress was low or high, included *Gammaproteobacteria* (7.22% for Pre.Flush2, 2.80% for Flush2.1, and 2.63% for Flush2.2) and *Deltaproteobacteria* (17.20% for Pre.Flush2, 0.02% for Flush2.1, and 0% for Flush2.2). Classes that increased after flushing, regardless of whether shear stress was low or high, included *Actinobacteria* (3.10% for Pre.Flush2, 6.37% for Flush2.1, and 4.77% for Flush2.2) and *Clostridia* (0.01% for Pre.Flush2, 0.03% for Flush2.1, and 0.05% for Flush2.2).

Similar results were obtained in SSCP. Classes that increased significantly after flushing at low shear stress, but changed little after flushing at high shear stress included *Gammaproteobacteria* (15.9% for Pre.Flush3, 55.9% for Flush3.1, and 29.4% for Flush3.2) and *Actinobacteria* (0.65% for Pre.Flush3, 2.3% for Flush3.1, and 0.4% for Flush3.2). Classes that decreased after flushing, regardless of whether shear stress was low or high, included *Alphaproteobacteria* (80.98% for Pre.Flush3, 28.7% for Flush3.1, and 9.8% for Flush3.2) and *Bacilli* (1.02% for Pre.Flush3, 0.4% for Flush3.1, and 0% for Flush3.2). Classes that increased after flushing occurred, regardless of whether shear stress was low or high included *Betaproteobacteria* (0.6% for Pre.Flush3, 7.8% for Flush3.1, and 59.2% for Flush3.2).

At the genus level, compositional shifts in the bacterial community structure were also observed by comparing the composition of biofilm samples from different metal pipes before and after flushing (Fig. 7). The community composition of biofilm showed different degrees of change due to the flushing regimes. In DCIP, the relative abundance of some bacterial genera increased significantly after flushing at low shear stress, but showed only a slight change after flushing at high shear stress. These genera included *Staphylococcus* (<0.01% for Pre.Flush1, 7.6% for Flush1.1, and <0.01% for Flush1.2), *Luteococcus* (<0.01% for Pre.Flush1, 6.6% for Flush1.1, and <0.01% for Flush1.2), and *Clostridium* (<0.01% for Pre.Flush1, 6.2% for Flush1.1, and 0.02% for Flush1.2). Some bacterial genera decreased significantly after flushing, regardless of whether shear stress was low or high. These genera included *Hyphomicrobium* (28.0% for

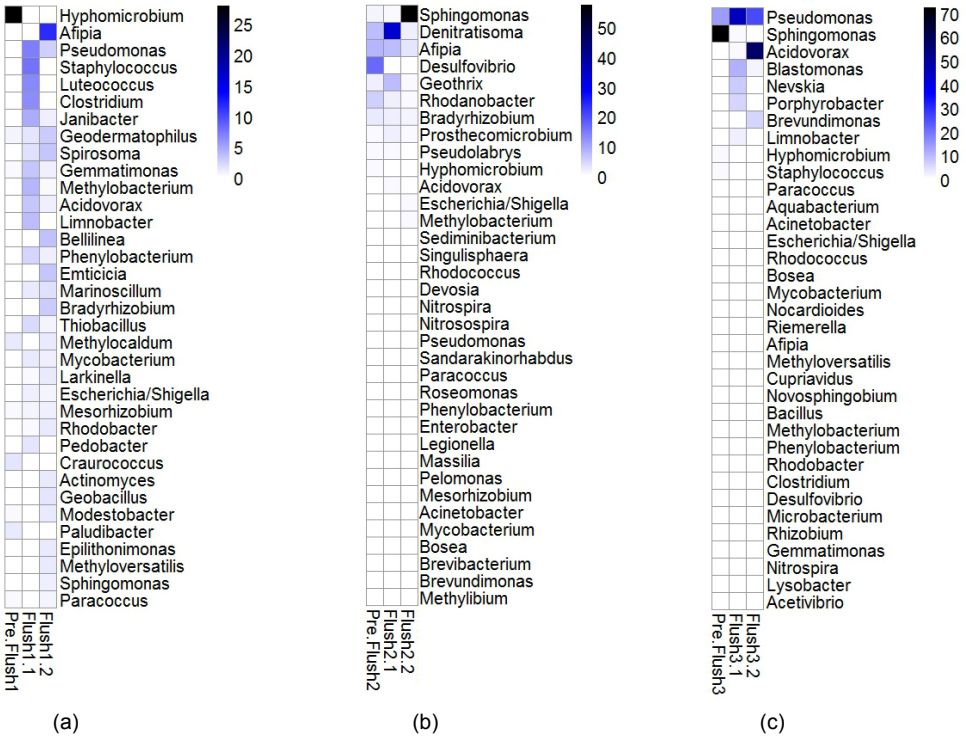

Fig. 7 Heatmaps showing the percentages (unit: %) of the most abundant species at the genus level in biofilms before and after flushing: (a) DCIP; (b) GCIP; (c) SSCP

Pre.Flush1, 0.1% for Flush1.1, and 0.07% for Flush1.2), *Craurococcus* (1.6% for Pre.Flush1, <0.01% for Flush1.1, and <0.01% for Flush1.2), and *Paludibacter* (1.3% for Pre.Flush1, <0.01% for Flush1.1, and <0.01% for Flush1.2). However, other bacterial genera increased after flushing regardless of whether shear stress was low or high. These included *Geodermatophilus* (0.8% for Pre.Flush1, 1.6% for Flush1.1, and 2.8% for Flush1.2) and *Spirosoma* (<0.01% for Pre.Flush1, 1.8% for Flush1.1, and 3.2% for Flush1.2).

In GCIP, genera that increased significantly after flushing at low shear stress, but showed only a slight decrease after flushing with high shear stress included *Denitratisoma* (7.6% for Pre.Flush2, 34.3% for Flush2.1, and 1.9% for Flush2.2) and *Geothrix* (2.10% for Pre.Flush2, 8.0% for Flush2.1, and 0.7% for Flush2.2). Genera that decreased significantly after flushing regardless of whether shear stress was low or high included *Rhodanobacter* (5.7% for Pre.Flush2, 2.1% for Flush2.1, and 0.6% for Flush2.2) and *Desulfovibrio* (17.0% for Pre.Flush2, <0.01% for Flush2.1, and <0.01% for Flush2.2). *Sphingomonas* increased sharply from 1.4% to 57.5% after flushing at high shear stress, which suggests that these bacteria are associated with the more strongly adhering material.

In SSCP, genera that increased significantly after flushing at low shear stress, but showed only a slight change after flushing at high shear stress included *Blastomonas* (0.1% for Pre.Flush3, 11.2% for Flush3.1, and 2.1% for Flush3.2), *Nevskia* (<0.01% for Pre.Flush3, 7.3% for Flush3.1, and 0.20% for Flush3.2), and *Porphyrobacter* (<0.01% for Pre.Flush3, 6.50% for Flush3.1, and 0.20% for Flush3.2). Genera that showed a slight change after flushing at low shear stress, but a considerable increase after flushing at high shear stress included *Acidovorax* (0.1% for Pre.Flush3, 1.1% for Flush3.1, and 57.80% for Flush3.2) and *Brevundimonas* (0.1% for Pre.Flush3, 0.1% for Flush3.1, and 6.1% for Flush3.2). *Sphingomonas* decreased after flushing regardless of whether shear stress was low or high (72.5% for Pre.Flush3, 1.3% for Flush3.1, and 0.2% for Flush3.2), suggesting that *Sphingomonas* is very sensitive to flow change.

4 Discussion

4.1 Effect of flushing on water physicochemical characteristics in different metal pipes

Significant differences were found among DCIPs, GCIPs, and SSCPs in the sensitivity of biofilms against flushing. Results of physicochemical analysis indicated that all the DCIP, GCIP, and SSCP segments accumulated material that was mobilized by increasing of the flows and corresponding shear stress on the pipe walls. These results also suggested that higher quantities of material develop on GCIPs over time and that the material accumulated in this type of pipe appears to be more sensitive to flow changes than the material in other pipes (Tables 2 and 3). Similar results were found by Husband and Boxall (2011) and Douterelo *et al.* (2013) using HDPE pipe. Our results add weight to their suggestion that in different types of pipes there is ubiquitous material accumulation resulting from the background concentrations of particulate and soluble materials within the bulk water (Douterelo *et al.*, 2013), and that in GCIPs there are additional accumulation processes associated with corrosion. The results of this study provide further evidence that GCIPs are not appropriate for use in actual DWDS. The morphology and physicochemical parameters of biofilm attached to the inner pipe walls reinforce this conclusion (Fig. 3 and Table 2).

In addition, big changes in the physicochemical index were observed due to flushing, but there were no significant differences among the different metal pipes in relation to low or high shear stress, except in GCIPs. This result suggests that material accumulated in the surface of the biofilms in DCIPs and SSCPs tends to detach and is sensitive to flow change, while inner material has a strong resistance to flushing. A probable explanation is that biofilms generally consist of a porous surface layer and a compact basal layer. Low shear stress can remove a large amount of biomass and deposits from the surface layer. However, the biomass of the basal layer adheres strongly to the pipe walls and is difficult to remove, even in the case of increasing shear stress. This finding supports the results of Paul *et al.* (2012), adding weight to their suggestion that a mature biofilm is intrinsically stratified with respect to its cohesion. A strong basal layer is always present and is more cohesive and dense than the outer layers. In GCIPs, however, a different result was observed. There were significant differences in the turbidity index in response to low and high shear stress flushing, but the change in the TOC index was not significant. This discrepancy may be explained by the fact that the biofilm in a GCIP probably has a loose inner structure containing a large amount of inorganic substances (Douterelo *et al.*, 2014). This result may be related to corrosion processes acting on the pipe wall.

4.2 Effect of flushing on the bacterial communities of biofilms

The biofilms detached significantly from the inner pipe walls after flushing, and further detachment could be observed as shear stress increased (Fig. 4). Moreover, biofilms attached to different metal pipes showed differences in their resistance to flushing. The variation in total bacteria (16S rRNA gene copies, Fig. 4) during flushing indicated that the biofilms attached to DCIPs had relatively strong cohesion, providing resistance against shear stress as high as 2.0 N/m^2, while the biofilms attached to GCIPs and SSCPs were sensitive to flushing. The weak resistance of GCIP biofilms to flushing could be explained by at least two assumptions. The first is that corrosion makes the surface of GCIPs form abundant tubercles which are comparatively loose and porous (Sarin *et al.*, 2004). The high degree of porosity may reduce the number of contact points within the extracellular polymeric substances (EPS) of the biofilm network structure. In this context, Mathieu *et al.* (2014) have demonstrated that reducing the number of contact points within the polymeric matrix can weaken the cohesion of biofilms. The second assumption is that the GCIP biofilm was not only eroded by hydraulic shear but also flaked by hydraulic shock caused by the extremely uneven surface. The weak resistance of the SSCP and strong resistance of DCIP biofilms could be explained by the roughness of the bare surface of the pipe walls. Oh *et al.* (2009) suggested that cells are aggregated with extra-cellular matrix (ECM) and that this matrix on the biofilm reinforces cohesion. In this study, we observed that decreasing the surface roughness resulted in less ECM, indicating that the cohesion of the biofilm was

weak. However, as the surface of SSCPs is smooth (with a surface roughness of new pipe of ≤0.6 μm), there is not enough ECM to resist a change in shear stress. The surfaces of DCIPs are rough (with a surface roughness of new pipe of ≥30 μm) and hence, enough ECM is present. Another explanation is related to electrostatic interactions. Chen and Stewart (2002) demonstrated that high-valence cations (e.g., Fe^{3+}, Al^{3+}) are potent cross linkers of the biofilm matrix, which could increase biofilm cohesion significantly. The ferric and aluminum ion contents of DCIP biofilms are higher than those of SSCPs, so it is plausible that biofilms of DCIPs are more cohesive than those of SSCPs. The data show that increasing shear stress can lead to further detachment of the biofilm, but some is retained and can resist high shear stress. This conclusion is identical to observations made by Douterelo *et al.* (2013), who claimed that the mechanical removal of biofilms by flushing did not completely remove bacteria from the pipe walls. The results also prove that a mature biofilm is intrinsically stratified with respect to its cohesion, including a loose surface layer and a strong basal layer.

It is understandable that a biofilm detaches from the inner pipe wall when the external shear stress is greater than the cohesive strength of the biofilm (Peyton and Characklis, 1993; Stewart, 1993). However, HPCs and 16S rRNA gene copy numbers may show different trends during flushing. This discrepancy suggests that the total bacteria (16S rRNA gene copy numbers) and culturable heterotrophic bacteria (HPCs) may have different sensitivities when exposed to flushing. In this study, we found that the total bacteria and culturable heterotrophic bacteria showed different responses to GCIP flushing (R^2= 0.696, possibility P=0.124). In GCIP, the total bacteria could mostly detach under low shear stress, and little effect was observed when shear stress increased. However, culturable heterotrophic bacteria detached only slightly under low shear stress, but detachment was clearly apparent under high shear stress. This may be because the biofilm in the GCIP was hundreds of micrometers thick, which could allow for the formation of an anaerobic layer in which the bacteria *Desulfovibrio*, *Denitratisoma*, and *Rhodanobacter* could survive. Moreover, *Desulfovibrio* (17.1%), *Denitratisoma* (7.6%), and *Rhodanobacter* (5.7%) were the major genera in GCIP before flushing (Fig. 7), and these genera are all anaerobic bacteria which cannot be measured by the incubation method (HPC-R2A). Therefore, HPCs yielded information about only a tiny fraction of the whole microbial community in the GCIP, suggesting that HPCs cannot accurately reflect the total bacteria. The other assumption is that the biofilm of GCIPs is loose and polyporous, containing many holes in which culturable heterotrophic bacteria may live (Wang *et al.*, 2015). Hence, both the surface and interior may be suitable for the survival of culturable heterotrophic bacteria. Low shear stress makes culturable heterotrophic bacteria living in the surface layer detach into the bulk water, while those living in the interior layer need higher shear stress to detach. Therefore, further detachment of HPCs could be observed as shear stress increased.

In SSCP, the total bacteria and HPCs showed similar variation during flushing (R^2=1.000, P= 0.003). They both had mostly detached under low shear stress, and little effect was observed when shear stress increased. This may be because *Sphingomonas* (72.5%), an aerobic bacterium which can be cultured by the incubation method (HPC-R2A), was the major genus in the GCIP before flushing (Fig. 7). Therefore, HPCs yielded information about a huge fraction of the whole microbial community in SSCP, suggesting that HPCs can effectively reflect total bacteria in this case. The relative abundance of *Sphingomonas* dropped significantly to 1.3% after low shear stress flushing, while high shear stress flushing led to no further significant drop. This is consistent with the variation in total bacteria and HPCs and supports the observations by Manuel *et al.* (2009) using HDPE.

In DCIP, the variation in total bacteria and HPCs showed different trends during flushing (R^2=0.728, P=0.101). Detachment is unlikely to affect the total bacteria count under low shear stress, but appears to have an effect under high shear stress. However, HPCs detach as long as flushing occurs. This may reflect the living conditions of aerobic heterotrophic bacteria. A biofilm attached to DCIP has an intrinsically stratified structure comprising a compact basal layer and a loose surface layer. Thus, the living conditions for bacteria in the surface and interior layers are very different. The surface layer is full of oxygen

and has easy access to nutrition, while the basal layer lacks enough oxygen and nutrition for heterotrophic bacteria. So most of the culturable heterotrophic bacteria live in the surface layer of the biofilm and are easy to detach by flushing (Manuel *et al.*, 2009). Note that *Hyphomicrobium*, which is an aerobic bacterium that can be measured by the incubation method (HPC-R2A), accounted for only 28% of all bacteria in the DCIP before flushing (Fig. 7), and the relative abundance of other aerobic bacteria was insignificant. HPCs yielded information about a limited fraction of the whole microbial community in SSCP, which suggests that HPCs cannot adequately reflect the total bacteria.

4.3 Influence of flushing on the bacterial community structure in pipes of different material

When samples were analyzed, taking into account the type of pipe material and independent of the flushing steps, clear differences in the structure of the bacterial communities at different taxonomic levels were found between samples of polyethylene and cast iron pipes (Douterelo *et al.*, 2013). Manuel *et al.* (2009) found that in the same source water distribution system, there were obvious differences in bacterial community structure in biofilms attached to pipes made of different metal materials. Similar differences can be seen in Figs. 6 and 7.

Furthermore, the resistance to flushing of different bacteria was significantly different at the levels of both class and genus (Figs. 7 and 8). In DCIPs, *Geodermatophilus* and *Spirosoma* were able to resist quite strong flushing, showing that these bacteria are shear resistant communities, while *Hyphomicrobium*, *Craurococcus*, and *Paludibacter* had little resistance to flushing, showing that these bacteria are sensitive to flushing and easily detached into bulk water. In addition, *Staphylococcus*, *Luteococcus*, and *Clostridium* resisted flushing at low shear stress but detached during flushing at high shear stress, which means these bacteria have limited shear resistance. Similar results were observed in GCIP and SSCP flushing experiments. In GCIPs, *Sphingomonas* was able to resist quite strong flushing, while *Rhodanobacter* and *Desulfovibrio* were sensitive to flushing and easily detached into bulk water. In addition, *Denitratisoma* and *Geothrix* resisted flushing at low shear stress but not at high shear stress, which suggests these bacteria have limited shear resistance. In SSCPs, *Acidovorax* and *Brevundimonas* were able to resist quite strong flushing, while *Sphingomonas* was sensitive to flushing and easily detached into bulk water. In addition, *Blastomonas*, *Nevskia*, and *Porphyrobacter* resisted flushing at low shear stress but not at high shear stress, which means these bacteria have limited shear resistance. Douterelo *et al.* (2014) monitored changes in the bacterial community structure of bulk water during the flushing process. Their results were in accordance with those of this study. In DCIP systems mentioned in the literature, *Hyphomicrobium* rises to become the most abundant in bulk water (from 2.0% to 8.0%), which means its relative abundance decreases in the biofilm. Accordingly, *Hyphomicrobium* was the most abundant genus before flushing in DCIP biofilms in this study and decreased from 28% for Pre.Flush1 to 0.12% for Flush1.1 and 0.07% for Flush1.2. In this study, results also revealed that a bacteria's resistance to flushing may differ markedly in pipes made of different metal materials. In DCIP, the relative abundance of *Alphaproteobacteria* (45.6% for Pre.Flush1, 21.7% for Flush1.1, and 20.1% for Flush1.2) was similar after low or high shear stress flushing. In GCIP, the relative abundance of *Alphaproteobacteria* (29.6% for Pre.Flush2, 25.2% for Flush2.1, and 71.2% for Flush2.2) became the highest after high shear stress flushing, at 71.2%. In SSCP, the relative abundance of *Alphaproteobacteria* (80.98% for Pre.Flush3, 28.70% for Flush3.1, and 9.80% for Flush3.2) decreased continuously after low and high shear stress flushing. Eventually, the percentage dropped to only 3.2%. For *Sphingomonas* in DCIP pipes, the percentage was too small and can be disregarded (0.10% for Pre.Flush1, 0.14% for Flush1.1, and 0.96% for Flush1.2). No obvious change was observed when flow velocity varied. In GCIP pipes, *Sphingomonas* increased sharply from 1.40% to 57.50% after flushing at high shear stress, which suggests that these bacteria are associated with the more strongly adhered material. In SSCP pipes, *Sphingomonas* decreased significantly as long as flushing occurred, regardless of whether the shear stress was high or low (72.50% for Pre.Flush3, 1.30% for Flush3.1, and 0.20% for Flush3.2).

The results above show that all pipes contained both flush-resistant and flush-sensitive bacteria. However, flush resistance varied among the different pipes. The sensitivity to flushing of different bacteria showed significant variation which was related to the nature of the bacteria and the pipe material.

5 Conclusions

The main conclusions from this study are: (1) Biofilms attached to the walls of different metal pipes have different resistance to flushing. The sensitivity of biofilms to flushing was significantly different among DCIPs, GCIPs, and SSCPs. Biofilms attached to DCIPs had relatively strong cohesion which could resist a shear stress as high as 2.0 N/m^2, while biofilms attached to GCIPs or SSCPs were more sensitive. (2) The resistance to flushing of different bacteria in metal pipes showed significant variation at both the class and genus levels. The resistance of bacteria to flushing was related not only to the nature of the bacteria, but also to the pipe material.

These findings clearly show the importance of the selection of pipe material for delivering safe drinking water in DWDS. The investigation of the cohesive strength of biofilms also provided a basis for improving methods for cleaning pipes. It is important to understand and characterize the detachment processes when biofilms undergo flushing, so that the associated risk level can be evaluated.

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中文概要

题　目： 供水系统中冲刷对金属管道内壁生物膜脱落的影响作用

目　的： 在市政供水管网系统中，由冲刷引起的管道内壁生物膜脱落可能造成饮用水二次污染。本文旨在通过研究冲刷前后生物膜理化特性和微生物种群结构的变化，探讨不同金属管材管道和不同冲刷流速对生物膜脱落的影响，从而为管道生物安全的风险评估提供科学依据。

创新点： 不同菌属的细菌对水力冲刷的敏感程度不同；各菌属对水力变化的敏感程度既与菌属本身的性质有关，也与其附着生长的管材有关。

方　法： 1. 采用 R2A 培养基平板计数、16S rDNA 检测、荧光定量聚合酶链式反应及 454 焦磷酸测序技术。2. 通过理化指标检测与测序相结合的方式进行分析，并以丰度图和热度图的形式呈现结果。

结　论： 1. 球墨铸铁管内壁生物膜的抗水力剪切能力较强，能够抵抗低剪切冲刷，在高剪切力冲刷后才会明显脱落，而灰口铸铁管和不锈钢复合管内壁生物膜的抗水力剪切能力较弱，低剪切力冲刷工况便会导致生物膜的明显脱落。2. 生物膜对冲刷的抵抗作用在其群落的属水平和纲水平上有显著变化，这与群落本身和管材特性均有关联。

关键词： 供水管网系统；生物膜；冲刷；金属管道