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Biofilm microbial diversity under conditions of different pipe materials and chlorine residual levels

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ABSTRACT

Microbial diversity and community structure of biofilm in water distribution network (WDN) was investigated through experiments in laboratory in order to secure water quality in WDN. The results suggested that the 47 identified bacteria strains belong to 18 genera from 13 families, among which *Sphingobium* and *Micro-bacterium* were dominant. 14 bacteria strains were isolated from cast iron pipes, whose microbial diversity was the highest, followed by stainless steel, 7 bacterial strains. Only three bacteria strains were isolated from polyethylene pipe. *Sphingomonas* existed in all the three types of pipe materials. Effect of chlorine residual on biofilm microbial diversity varies with pipe materials. Results of this research could support theoretical basis for the control of chlorine-resistant bacteria in WDN, enhancing water quality security in WDN.

Keywords: Water distribution network (WDN); Water quality; Biofilm; Chlorine-resistant bacteria; Microbial diversity

1. Introduction

In the presence of organic matter, microorganisms will attach to pipe wall, and biofilms will form. Finished water could meet the drinking water standard after water treatment processes. While during water conveyance from water treatment plant to consumers, water directly contacts with biofilms in pipe wall for a long period of time [1–3], causing "secondary pollution" [4,5]. When organic matter levels are high, traditional chlorination couldn't control microorganism growth effectively, and disinfection by-products may increase. Therefore, it is important to investigate construction, growth, and microbial diversity of biofilm. In recent years, it has been the focus of the urban water supply industry to investigate measures for controlling "secondary pollution" and to develop techniques for maintenance of water quality security in order to ensure that water provided to consumers meets the appropriate drinking water standard.

Berry et al. [6] suggested that chlorine resistance of biofilm in drinking water was closely related to microbial community structure. Therefore, in order to control chlorine-resistant bacteria, it is important to investigate biofilm microbial diversity under different chlorine levels.

In recent years, there are limited research results about biofilm microbial diversity and community structure in water distribution network (WDN) through molecular microbiology method. In addition, in those researches, biofilm samples were taken from actual network and were under the condition of certain kind of water [7]. There are very few results about the effects of chlorine residual levels and pipe materials on biofilm microbial diversity and community structure in WDN.

In this study, biofilm community structure on pipe wall in WDN was investigated under different chlorine residual levels and pipe materials. Furthermore, we compared the

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biofilm microbial diversity under different conditions. The results of this paper contribute to control pathogenic microorganism and chlorine-resistant bacteria in biofilms and are beneficial for water quality security in WDN. What's more, based on the results, the water suppliers could decrease chlorine dosage, saving the running cost of water treatment plants.

2. Material and methods

2.1. Material

Schematic diagram of drinking water distribution system simulator is shown in Fig. 1. Water used in the experiment is from water supply network. And water quality of experimental water is shown in Table 1.

2.2. Method

Eight sets of devices were used in this research (as shown in Table 3). The devices were running from early August to early October 2014. Eight coupons (as shown in Table 3 for details) were put in the devices. After running for days, coupons were taken out, respectively, with the number of No. 1, No. 2, No. 4, No. 6, No. 8, No. 10, No. 12, and No. 14. Before put in the devices, coupons were soaked in 10.0 mg/L sodium hypochlorite solution for 24 h. Biomass on the coupons was measured by heterotrophic plate counts.

In October, the biofilms on pipe wall of biofilm annulus reactor (BAR) were collected by ultrasonic oscillation. The procedures were as follows: coupons in BAR were taken from the devices and washed with sterile deionized water; after that, the coupons were put into a jar with sterile distilled water in the jar; the jar was put in ultrasonic oscillator and oscillated for 2 min with 80 W, and then, after an interval of 2 min, the jar was oscillated again. The procedures above were repeated five times. It was reported that 80%–85% permanent adhesion bacteria on coupons could be released into water and become suspended [8].

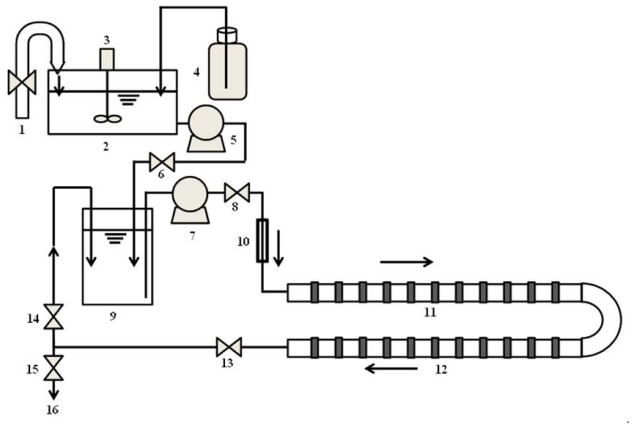

Fig. 1. Schematic diagram of drinking water distribution system simulator. (1) Tap water; (2) head water tank; (3) stirred tank; (4) device throwing disinfectant; (5, 7) pump; (6, 8, 13–15) valve; (9) low water tank; (10) flowmeter; (11, 12) biofilm annulus reactors (BARs); (16) outfall.

Bacteria attached to biofilms were isolated with streak plate method. Then, bacteria were identified by amplification of the 16S rDNA gene-based method. The sequence were aligned and compared by BLAST programs. Phylogenetic trees were constructed by MEGA 5.1 software package [9] and Neighbor-Joining method. Other conventional water quality parameters were analyzed according to the Chinese mandatory National Standards [10].

2.2.1. DNA extraction

In this study, DNA of pure cultures was extracted with the CTAB-proteinase K method. The detailed procedures were as follows:

2.2.1.1. Bacterial enrichment culture 40 mL inoculated R2A liquid medium was cultivated in shaking incubators (125 rpm) for 3–5 d at 25°C. 3–5 d later, the turbidity was about 0.6 NTU, and the concentration of bacteria was about 108–109 CFU/mL, changing from logarithmic phase to stable phase. 2 mL culture solution was transferred to 2 mL centrifuge tube, and was then centrifuged at 13,000 rpm for 10 min. The supernatant was abandoned. The procedures above were repeated five times.

2.2.1.2. Cell wall rupture by lysozyme 1 mL buffer solution I and 250 μL lysozyme (20 mg/mL) were added into the 2 mL centrifuge tube in Section 2.2.1.1. The tube was laid in water bath at 37°C for 2 h, and was shaken every 10–15 min. Then, the tube was laid in refrigerator at −20°C for 10 min, followed by 100°C water bath for 10 min. The tube was frozen and thawed for three times. After that, the tube was centrifuged at 12,000 rpm for 20 min, and the supernatant was abandoned.

2.2.1.3. Dissolution of DNA 1 mL DNA extracting solution II and 10 μL proteinase K (20 mg/mL) were added into the precipitate obtained in Section 2.2.1.2. The mixture was blended and shaken at 225 rpm for 30 min at 37°C.

After that, 200 μL 10% sodium dodecyl sulfate (SDS) solution was added into the centrifuge tube. The mixture was shaken and then laid in water bath at 65°C for 90 min, shaken

Table 1
Water quality of experimental water

Index of water quality	Maximum	Minimum	Average
Turbidity (NTU)	0.48	0.34	0.41
pH	7.20	6.80	7.02
Alkalinity (mg/L)	38.60	21.50	26.07
Chloride (mg/L)	5.06	4.54	5.02
Nitrogen nitrate (mg/L)	4.02	3.25	3.52
Sulfate (mg/L)	8.76	7.42	8.06
TOC (mg/L)	2.59	1.42	2.10
Total iron (mg/L)	0.48	0.35	0.41
Phosphate radical (μg/L)	4.20	2.94	3.65
Heterotrophic counts (CFU)	40	10	30

every 15–20 min. After centrifugation at 9,000 rpm for 10 min, the supernatant was pipetted into a 2 mL centrifuge tube.

Then, 0.5 mL DNA extracting solution II and 50 μL SDS were added into the precipitation. The mixture was blended for 30 s, and then 5 μL proteinase K was added. The tube was laid in water bath at 65°C for 10 min. After centrifugation at 9,000 rpm for 10 min, the supernatant was collected. The procedures above were repeated for three times and the supernatant was collected in a 2 mL centrifuge tube.

2.2.1.4. Purification of DNA The supernatant obtained in Section 2.2.1.3 was added with prepared mixture of phenol/chloroform/isoamylol by the ratio of 1:1. After 10 min standing, the mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred into a new 2 mL centrifuge tube with a pipette. The procedures were repeated twice.

After that, isopropanol was added to the supernatant by the ratio of supernatant:isopropanol = 1:0.6. After 30 min standing, the blended mixture was centrifuged at 12,000 rpm for 10 min, and then the supernatant was abandoned.

1 mL prepared 4°C 70% alcohol was added to the precipitate. The mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was abandoned, and let the precipitate dry naturally.

2.2.1.5. Redissolution and detection of DNA 30 μL–100 μL TE buffer solution was added to the precipitate. The mixture was blended by pipette. The precipitate was separately stored in refrigerator at −20°C.

Additionally, 5 μL DNA was used for agarose gel electrophoresis.

2.2.2. 16S rDNA full length PCR amplification method

The volume of constituents in polymerase chain reaction (PCR) is shown in Table 2. EUB27F and EUB1492R were used as primers. Thermal cycler was as follows: initial denaturation at 94°C for 600 s, followed by 32 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s, extension at 72°C for 60 s, and a final extension at 72°C for 600 s. After that, PCR products were handled with agarose gel electrophoresis at 75 V for 40 min, before gelation cutting and gel recovery. Finally, 16S rDNA genes of each strain were sequenced by Shanghai Biology Engineering Company (Shanghai) and the length of genes was between 1,369 and 1,440 bp. Sequences were compared with the National Center for Biotechnology Information (NCBI) 16S rDNA database using BLAST sequences. Operational taxonomic units were defined by a 99% similarity. Combined with observation of previous morphology, species of strains could be basically determined.

Table 2
The volume of constituents in PCR

Constituents	Volume (μL)
10 × PCR buffer	5
dNTP	4
Primer 1	1
Primer 2	1
rtap	1
Template	1.2
ddH_2O	Up to 50 μL

3. Results and discussion

3.1. Biomass and ecological characteristics of strain

Biofilm growth curve under the condition of 1 mg/L chlorine residual is shown in Fig. 2.

As can be seen in Fig. 2, the three curves show a similar trend. Biofilm biomass on pipe walls of the three pipe materials increased rapidly during the first 4 d, and then became stable. Biomass of stainless steel became stable from 4 to 6 d and that of polyethylene (PE) and cast iron became stable from 6 to 8 d. Wu et al. [11] investigated the growth laws of biofilm on cast iron without chlorine residual in BAR. Their results also indicated that biofilm biomass could become stable in 1 week. Guan et al. [12] investigated the growth laws of biofilm on PE pipe walls under two kinds of hydraulic conditions in BAR. Their results illustrated that biomass could become stable in 7 d, remaining about $10^5/cm^2$.

It also showed that cultured bacterial colonies of biofilm samples on R2A medium were mostly white and yellow on 1, 2, and 4 d. 4 d later, part of bacterial colonies were crimson or brick red. Based on the results, it can be concluded that although biofilm biomass became stable after 1 week, the microbial community of biofilm still changed. Their research also indicated that biofilm sampling of this experiment was reasonable and representative. The results were in agreement with those previously reported by Martiny et al. [13].

Pipe material, chlorine dosage, and classified bacterial strains in each equipment are shown in Table 3. As can be seen in Table 3, 59 bacterial strains were isolated according to different colonial morphologies on R2A solid medium. The bacterial colonies were all opaque and circular in surface. The size of diameters was not more than 5 mm. And the colors were yellow, white, red, and green.

Gram stain revealed that most of the 59 bacteria were gram-negative (Table 3). Cell walls of gram-positive bacteria were thick and solid, with abundant peptidoglycan, which could effectively reduce bacteria inactivation by chlorine. Therefore, the proportion of gram-positive bacteria should be higher in WDN. However, results of experiment were quite

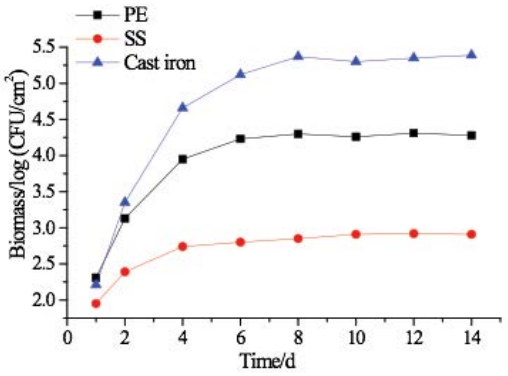

Fig. 2. Biofilm growth curve.

contrary to the analysis, and this agrees with previous studies [14–16]. It may be because the adhesion ability of gram-positive bacteria is poor, so it is not easy for them to adhere to pipe walls.

Scanning electron microscope observation (40,000×) revealed that the isolated bacteria included *Coccus, Brevibacterium*, and *Long bacilli*. 40% of the isolated bacteria were *Coccus*, and 60% were *Brevibacterium* and *Long bacilli*. Most of the bacteria were tiny. The length of most *Bacilli* was less than 1 μm, and the diameter of *Coccus* was even no more than 0.5 μm. The smaller the bacteria is, the bigger the specific surface area is, so that it is easier to absorb nutrients. That is the consequence of bacteria's long-term surviving in oligotrophic evolvement in WDN. Bacteria with the diameter less than 0.3 μm are called *Ultramicrobacteria* (UMB). UMB grows slowly on surface of nutritious agar, while it predominates in oligotrophic environment [17,18]. Thus, most of the microorganisms in biofilm on pipe walls are UMB in WDN.

3.2. Microorganism identification

3.2.1. Biofilm community structure on PE pipes

Four, three, and six bacterial strains were isolated from No. 2, No. 3, and No. 4 devices, respectively. Nine bacterial strains were identified successfully, and the results are shown in Table 4. The 16S rDNA is shown in Fig. 3.

It is clear that the three bacteria isolated from No. 2 device belong to two families and two genera. Bacteria 2-1 and 2-2 belong to *Sphingobium,* bacteria 2-3 belongs to *Cupriavidus*. Two bacterial strains were isolated from *Sphingobium* accounting for 66.7%. Only one bacterial strain was isolated from *Cupriavidus*, accounting for 33.3%. The bacteria isolated from No. 3 device belong to *Sphingobium*. Five bacterial strains isolated from No. 4 device belong to one family and two genera. Bacteria 4-1 and 4-5 belong to *Sphingomonas,* bacteria 4-2, 4-3, and 4-4 belong to *Sphingobium*. Three bacterial strains belong to *Sphingobium,* accounting for 60% and *Sphingomonas* accounts for 40%.

3.2.2. Biofilm community structure on stainless steel pipes

Nine bacterial strains were isolated from No. 6 and 7 devices. Fifteen bacterial strains were identified successfully and their 16S rDNA were obtained.

The results indicated that eight bacterial strains isolated from No. 6 device belong to three families and three genera, including *Cupriavidus, Sphingomonas*, and *Microbacterium*. Four bacterial strains belong to *Microbacterium* which

Table 3
Pipe material, chlorine dosage, and classified bacterial strains in each equipment

Number of device	Chlorine (mg/L)	Pipe material	Isolated bacteria/strain	Gram-negative bacteria/strain	Proportion of gram-negative bacteria (%)	Gram-positive bacteria/strain	Proportion of gram-positive bacteria (%)
2#	1.00	PE	4	4	100	0	0
3#	3.00	PE	3	3	100	0	0
4#	4.00	PE	6	5	83	1	17
6#	1.00	SS	9	6	67	3	33
7#	3.00	SS	9	9	100	0	0
10#	1.00	Cast iron	9	9	100	0	0
11#	3.00	Cast iron	9	8	89	1	11
12#	4.00	Cast iron	10	7	70	3	30
Total			59	51	86	8	14

Table 4
Isolated bacteria and identification results in PE
Note: a-b: a – device number, b – number of bacteria isolated from the device.

Number	Length of sequences (bp)	Name of bacteria	Bacterial number	Relevancy (%)
2-1	1,377	*Sphingobium* sp.	JQ433940	99
2-2	1,374	*Sphingobium yanoikuyae*	EU307932	99
2-3	1,429	*Cupriavidus* sp.	JX233516	99
		Cupriavidus sp.	AB542387	99
3-1	1,378	*Sphingobium yanoikuyae*	JN700070	99
		Sphingobium sp.	JQ433940	99
4-1	1,372	*Sphingomonas aerolata*	FR691420	99
4-2	1,382	*Sphingobium yanoikuyae*	JF681288	99
4-3	1,374	*Sphingobium yanoikuyae*	EU307932	99
4-4	1,382	*Sphingobium yanoikuyae*	JN700070	99
4-5	1,407	*Sphingomonas* sp.	DQ840049	99

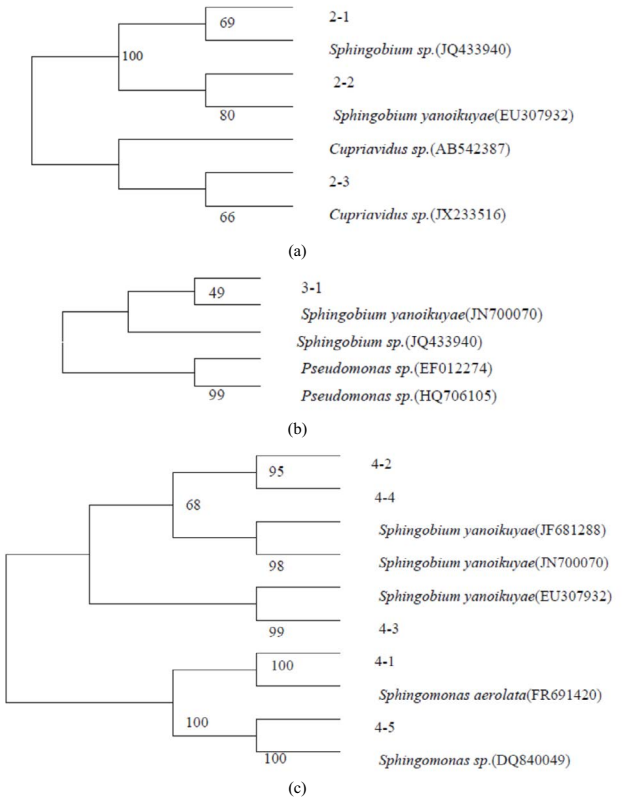

Fig. 3. Phylogenetic tree of isolated bacteria in PE. (a) Phylogenetic tree of three isolated bacteria from No. 2 device, (b) phylogenetic tree of one isolated bacteria from No. 3 device, and (c) phylogenetic tree of one isolated bacteria from No. 4 device.

is the most, accounting for 50%. Three bacteria belong to *Cupriavidus* and only one bacterial strain belongs to *Sphingomonas*. Seven bacteria identified in No. 7 device belong to four families and four genera, including *Acinetobacter*, *Acidovorax*, *Arcicella*, and *Sphingobium*. Four strains belong to *Acinetobacter*, accounting for 57% and one strain belongs to the other three genera.

3.2.3. Biofilm community structure on cast iron pipes

Nine, nine, and ten bacterial strains were isolated from No. 10, No. 11, and No. 12 devices, respectively. Twenty-three bacterial strains were identified successfully and the 16S rDNA were obtained.

Seven bacteria isolated from No. 10 device belong to six families and seven genera, including *Porphyrobacter*, *Sphingomonas*, *Acidovorax*, *Roseateles*, *Blastomonas*, *Bosea*, and *Dechloromonas*. *Sphingomonas* and *Blastomonas* belong to *Sphingomonadaceae*. Only one strain belongs to the other genera.

Eight bacterial strains isolated from No. 11 device belong to five families and six genera, including *Sphingomonas*, *Novosphingobium*, *Microbacterium*, *Dechloromonas*, *Sediminibacterium*, and *Methylobacterium*. Both *Sphingomonas* and *Novosphingobium* were from *Sphingomonadaceae*. Two bacterial strains were isolated from *Dechloromonas* and *Sediminibacterium*, respectively. One bacterial strain was isolated from other genera.

Eight bacterial strains isolated from No. 12 device belong to six families and six genera, including *Sphingomonas*, *Rhodocyclus*, *Acidovorax*, *Undibacterium*, and *Sphingomonadaceae*. Two bacterial strains belong to *Rhodocyclus* and *Microbacterium*, respectively. One bacterial strain was isolated from other genera.

As can be seen from the results, microbial diversity of biofilm on the three pipe materials was significantly different. Only *Sphingomonas* was found in all the three pipe materials. *Arcicella* existed only in stainless steel pipe. *Blastomonas* and other 11 genera existed only in cast iron pipe.

3.3. Comparison of microbial diversity

In this research, biofilms grew under eight kinds of conditions (different pipe materials and different chlorine residual levels) were investigated. Biofilm biomass and microbial diversity were compared through the same culturing time, isolation, and identification methods.

All the bacteria belong to 13 families and 18 genera. Biofilm biomass and microbial diversity of biofilms under different conditions were quite different. Isolated bacteria in each equipment are shown in Fig. 4. As can be seen from Fig. 4, bacteria isolated from each device were different both in species and in proportions. Forty-seven bacteria belong to eighteen genera, including *Sphingobium*, *Cupriavidus*, *Sphingomonas*, *Microbacterium*, *Acinetobacter*, *Acidovorax*, *Arcicella*, *Blastomonas*, *Porphyrobacter*, *Bosea*, *Roseateles*, *Novosphingobium*, *Methylobacterium*, *Dechloromonas*, *Sediminibacterium*, *Rhodocyclus*, *Undibacterium*, and *Bacillus*. This agrees with previous studies [13,14].

Hong et al. [19] investigated biofilm from water meters in actual pipe network. The results showed that bacterial strains isolated from the biofilm were very similar to the strains isolated in our experiment. Hwang et al. [20] also demonstrated that *Methylobacteriaceae* and *Sphingomonadaceae* microbes were abundant in chlorinated water in WDN.

The proportion of *Sphingobium* was highest in No. 2, No. 3, and No. 4 devices. The proportion of *Cupriavidus* was highest in No. 6 device. And the proportion of *Acinetobacter* was highest in No. 7 device. The proportions of each strain were almost the same in No. 10, No. 11, and No. 12 devices.

Bacterial strains isolated from each bacterial genus are shown in Fig. 5. Seven bacterial strains belong to *Sphingobium* isolated from four types of biofilm. Seven bacterial strains belong to *Microbacterium* isolated from three types of biofilm. Six bacterial strains belong to *Sphingomonas* isolated from five types of biofilm. Four bacterial strains were isolated from *Cupriavidus* and *Acinetobacter*. Three bacterial strains were isolated from *Acidovorax* and *Dechloromonas*. Two bacterial strains were isolated from *Sediminibacterium* and *Rhodocyclus*, respectively. And one bacterial strain was isolated from the other genera. It has been verified that *Sphingomonas* has strong chlorine resistance. It could survive for 4 h under 4 mg/L chlorine residual, and the inactivation ratio was only 5% [21].

In terms of pathogenicity, *Acinetobacter* is opportunistic pathogenic bacteria, and *Bacillus anthracis* is a pathogen. There are no reports about pathogenicity for the other strains.

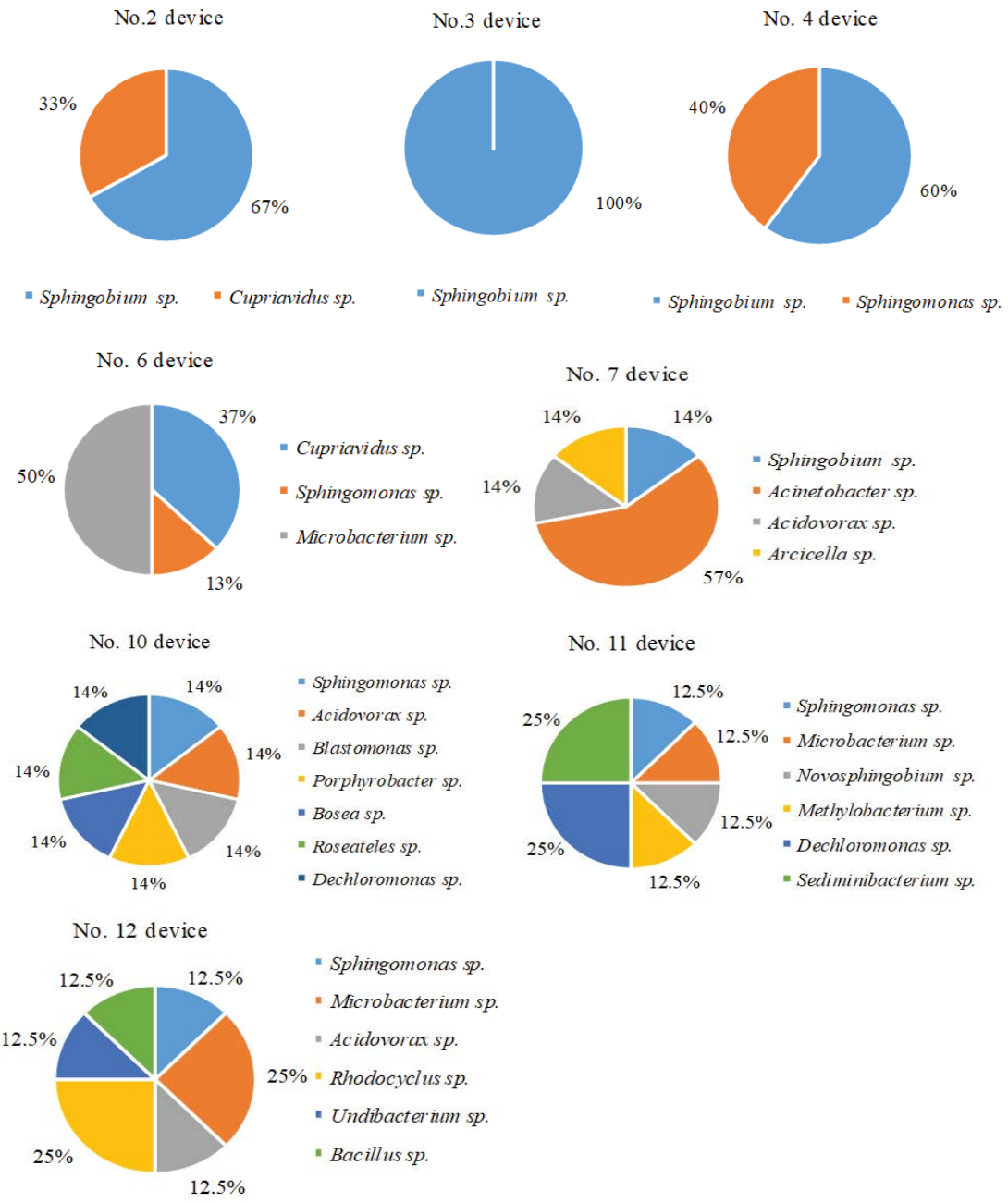

Fig. 4. Isolated bacteria in each equipment.

3.3.1. Comparison of microbial diversity in different pipe materials

Microbial diversity in different pipe materials were investigated by analyzing bacterial strains isolated from each pipe material. Biofilm community structure in different pipe materials is shown in Fig. 6.

Nine bacterial strains isolated from PE pipe belonged to *Sphingobium*, *Cupriavidus*, and *Sphingomonas*. The proportion of *Sphingobium* was much higher than the other two strains. Fifteen bacterial strains isolated from stainless steel pipe belonged to seven genera, the majority of which were *Microbacterium* and *Acinetobacter*. Twenty three bacterial strains isolated from cast iron pipe belonged to fourteen genera. There was no big difference between the proportion of each strain. The proportions of *Sphingomonas*, *Microbacterium*, and *Dechloromonas* were relatively higher.

Based on these results, it could be concluded that the microbial diversity in different pipe materials were in the order of: cast iron > stainless steel > PE. The biofilm community structure in cast iron pipe was the most complex, and

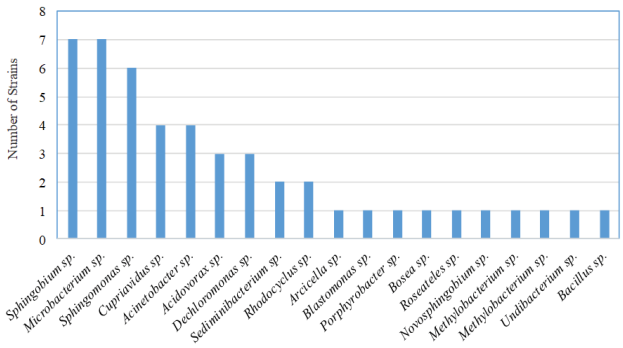

Fig. 5. Bacterial strains isolated from each bacterial genus.

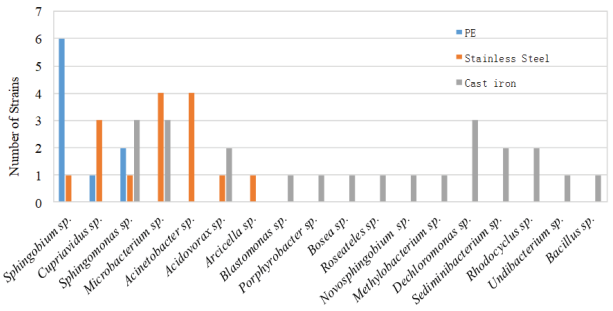

Fig. 6. Comparison of biofilm community structure in different pipe materials.

its microbial diversity was the highest. Twenty three bacterial strains belonged to fourteen genera were isolated from cast iron pipe. In contrast the biofilm community structure in PE pipe was extremely simple, and its microbial diversity was low. Only nine bacterial strains belonged to three genera were isolated from PE pipe. Compared with PE pipe, microbial diversity in stainless steel pipe was higher. Therefore, there was big difference between the bacterial species in different pipe materials, and only *Sphingomonas* existed in all of the three kinds of pipes.

3.3.2. Comparison of biofilm microbial diversity under different chlorine concentrations

As can be seen from the results, the relationship between biofilm community structure and chlorine concentrations in PE pipe was not evident. Compared with PE pipe, the situation in stainless steel pipe was different. Under the condition of 1 mg/L chlorine concentration, the proportion of *Acinetobacter* was the highest, accounting for 57%; under the condition of 3 mg/L chlorine concentration, the proportion of *Microbacterium* was the highest, accounting for 50%, followed by *Cupriavidus*, accounting for 37%; there was no same bacterial strain under the two conditions. As can be seen from the results, chlorine concentration has a great impact on microbial diversity in stainless steel pipe. While in cast iron pipe, microbial diversity was abundant and biofilm community structure was complex regardless of what chlorine concentration it is. The proportion of each genus was similar, so there was no dominant bacterium. Microbial diversity of biofilms cultivated under different chlorine concentrations was very different, and only *Sphingomonas* existed in all the three chlorine concentrations.

4. Conclusions

This study investigated the impacts of pipe material and chlorine concentration on biofilm microbial diversity. The results indicated that most of the 59 isolated bacteria were Gram-negative bacteria and *Bacillus*. The 47 identified bacterial strains belong to 18 genera from 13 families, most of which were *Sphingobium* and *Microbacterium*. The order of biofilm microbial diversity in different pipe materials was: cast iron pipe > stainless steel pipe > PE pipe. The impact of chlorine concentration on community structure and microbial diversity was significant in stainless steel pipe, while it was less influenced by chlorine concentration in PE pipe. In cast iron pipe, biofilm microbial diversity was very high for all the investigated conditions, but community structure was different. Only *Sphingomonas* was found in all the investigated chlorine concentrations and pipe materials. The results of this paper contribute to control pathogenic microorganism and chlorine-resistant bacteria in biofilms and are beneficial for water quality security in WDN. The chlorine dosage could also be decreased and the disinfectant could be evenly distributed in WDN, saving the running cost of water treatment plants.

Acknowledgment

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