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Impact of disinfectant on bacterial antibiotic resistance transfer between biofilm and tap water in a simulated distribution network☆

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ABSTRACT

Bacterial antibiotic resistance (BAR) is profoundly important to human health, but the environmental reservoirs of resistance determinants are poorly understood. BAR of biofilm and tap water were analyzed by using a water distribution simulator where different doses of chlorine and chloramine were used in this study. The results revealed that the disinfectants (≥2 mg/L) suppressed antibiotic resistant bacteria (ARB) in tap water and biofilms, while disinfected water and biofilms had a high relative abundance of ARB. The difference of ARB concentration and ARB percentage between the samples obtained from a disinfected pipeline and a non-disinfected pipeline became smaller over time. Because the water supply system is a unidirectional process, it is unclear how planktonic bacteria in water transfer BAR over time, although biofilm is suspected to play a role in this process. Compared with the biofilm samples without disinfectant, the disinfected biofilm had lower ICC and HPC/ICC percentage, lower AOC and AOC/TOC percentage, indicating that the disinfectant inhibited the bacteria growth in biofilm, and the disinfected biofilm had high proportion of non-culturable bacteria and low biodegradability, which affected BAR in biofilms. High throughput sequencing showed that in biofilms, the relative abundance of genera (*uncultured_f_Rhodocyclaceae*, *Brevundimonas*, and *Brevibacillus* in chlorinated systems, and *Brevundimonas*, *Brevibacillus* in chloraminated systems) with multiple antibiotic resistance and high abundance (up to 78.5%), were positively associated with disinfectant concentration and ARB percentage. The major prevalent genera in biofilms were also detected in tap water, suggesting that biofilm growth or biofilm detachment caused by external environmental factors will allow the movement of biofilm clusters with higher ARB concentration and percentage into bulk water, thereby increasing the antibiotic resistance of bacteria in tap water.

1. Introduction

The overuse and misuse of antibiotics during the last century have stimulated the emergence and enrichment of bacterial antibiotic resistance (BAR) mainly detected as antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment, which have become a public concern (Hall and Mah, 2017; Hu et al., 2016; Li et al., 2015). ARGs can be transferred in aquatic environments such as waste water, surface water and drinking water which can threaten human health (Li et al., 2015; Su et al., 2018). Thus, their abundance and transfer mechanisms in drinking water distribution systems (DWDSs) needs to be understood.

Modern water supply systems protect the public against microbial contamination by the use of treatment process (filtration and disinfection) to limit bacterial regrowth in DWDSs (Li et al., 2018; Proctor and Hammes, 2015). However, within DWDS, microorganisms can form multi-species biofilms resident on internal pipe surfaces (Liu et al., 2016a). In these biofilms, bacteria can grow in an environment with high density of bacteria concentration and close distance between bacteria, compared to suspended bacteria in aquatic environment (Flemming et al., 2016). Biofilms in DWDSs can facilitate bacterial antibiotic resistance and transmission by

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harboring ARB and supplying nutrients and protecting bacteria from disinfection, resulting in an increased likelihood of ARB occurrence (Zhang et al., 2018a). Biofilm growth confers many advantages to bacteria, improving survival and adaptation to diverse environmental stresses (Fish et al., 2016; Flemming et al., 2016). Bacteria in biofilms had higher antibiotic resistance than planktonic cells (Van Acker et al., 2014). A lot of bacteria in biofilm had higher resistance and tolerance to disinfectant and antibiotics than planktonic cells (Hall and Mah, 2017). This is likely due to the production of extracellular polymeric substances (EPS) and its protection, meanwhile the presence of stressful microenvironments leading to phenotypic differences in cells is also likely to contribute to this phenotypic heterogeneity within biofilms (Dao et al., 2011; Fish et al., 2016). Detachment is a common process in biofilm formation and the detachment rate was constant in a steady environment in DWDSs (Mathieu et al., 2014). When the biofilm adhesive strength was overcome by the shear force or disinfection the bacteria in biofilm will detach into tap water which may affect water quality (Fish et al., 2017; Fish et al., 2016). Lee indicated that chlorine can promote biofilm sloughing and increased bacteria concentration of drinking water (Lee et al., 2018). Obviously, changes in bacterial levels can affect the quality of water supplied to customers. Due to the direct contact of tap water with human, understanding the factors which affect the antibiotic resistance in DWDSs is critical to develop effective management strategies.

Disinfectant residuals are commonly applied to lower the numbers of microorganisms in DWDS. In China, disinfectant residuals are maintained in DWDSs to limit bacterial regrowth. However, even at a high dosage, disinfectant cannot eliminate microbial regrowth and biofilm formation. Moreover, stressful environments such as extreme pH, high salinity, nutrient deprivation, oxidation, or chlorine exposure promote growth of populations with greater resistance both in biofilm and bulk water (Khan et al., 2016). Biofilm bacteria experience a gradient of nutrients and antimicrobials, while bacteria are exposed to uniform environmental conditions at a section of drinking water distribution pipeline. Biofilm can cause the concentration of antimicrobials to diffuse to a low level, which may promote selection for an antimicrobial resistance population and increase the resistance of biofilm (Liu et al., 2016b). Understanding the mechanisms that contribute to biofilm-specific antibiotic resistance and tolerance will help guide the development of water treatments to limit the prevalence of antibiotic resistance in DWDSs.

In this study, we investigated the impact of disinfectant residual on the BAR transfer between biofilm and tap water. A simulated drinking water system was designed to evaluate the variation of BAR in a water distribution system treated with different dosages and types of disinfectant. High throughput sequencing was used to study the community shift from inlet water to outlet water at different disinfectant levels. Specifically, we aimed to address the following questions: (i) What is the impact of residual disinfectant on ARB concentration and ARB percentage in tap water and biofilm? (ii) What factors explain the different ARB concentration and ARB percentage of tap water and biofilm? (iii) What mechanism caused the changes of the ARB concentration and ARB percentage in tap water by biofilm detachment?

2. Materials and methods

2.1. Simulated drinking water distribution system operation and monitoring

A water distribution simulator (WDS) was designed to simulate the conditions of continuous water flow that drinking water might experience in a typical DWDS (such as daily use) (Fig. 1). The WDS consists of four parallel branch pipes in the chlorinated system and three parallel branch pipes in the chloraminated system, resulting in different residual concentrations of disinfectant (0 mg/L, 2 mg/L, 3 mg/L, and 4 mg/L in the chlorinated system, and 0 mg/L, 2 mg/L, and 3 mg/L in the chloraminated system). Firstly, the different parallel branch pipes (length 50 cm, diameter 20 mm) were connected in the WDS, and eight cast iron coupons ($H \times W \times L = 15.0\ cm \times 1.0\ cm \times 1.0\ cm$) were installed in the middle of each pipeline to form the biofilm. The WDS was flushed continuously using tap water without disinfectant to form biofilms until reaching the stable stage (almost one month, Figure S1). The inlet water of WDS was prepared by adding diluted sodium hypochlorite solution (active chlorine > 5%, Sinopharm Chemical Reagent Co., Ltd, China) a final chlorine concentration of 2 mg/L, 3 mg/L, and 4 mg/L. The chloramine disinfection was prepared by adding ammonium chloride and sodium hypochlorite solution to reach a final chloramine concentration of 2 mg/L and 3 mg/L. The flow rate was 20 ml/min. The retention time of each pipeline was 10 h. The WDS was operated under disinfected condition for eight weeks, and was maintained at normal temperature (18–23 °C) by using a heat rod. The chlorinated water samples were designated as CW0, CW2, CW3, and CW4, and the biofilm samples were designated as CB0, CB2, CB3, and CB4. The chloraminated water samples were named W0, W2, and W3, with B0, B2, and B3 as the biofilm samples. The water quality of the inlet water (IW) and outlet water (OW) was measured every week, as shown in Table S1 and Table S2.

2.2. Biological and chemical indexes detection

Glass bottles were sterile for samples collection. After sampling, the disinfectant residual was immediately neutralized. Turbidity, DOC, UV_{254}, pH, ammonia, total particle count, assimilable organic carbon (AOC) concentration, intact cell concentration (ICC), and total cell concentration (TCC) were detected.

ICC and TCC was detected by using flow cytometry (Prest et al., 2013). SYBR green was added to samples (Life Technologies Ltd., USA) for TCC detection. Both SYBR green and propidium iodide were added (Life Technologies Ltd., USA) for ICC detection. The ICC and TCC were counted by FACSCalibur flow cytometer (BD). AOC analysis was conduct by using an assay determined with flow cytometry previously (Li et al., 2018). The other chemical indexes investigated in this experiment were shown in Table S3.

2.3. BAR detection method

Four kinds of antibiotics (tetracycline, sulfamethoxazole, clindamycin, norfloxacin) frequently detected in surface water of south China (Jiang et al., 2013; Zhang et al., 2018b) were chosen to investigate antibiotic resistant bacteria, as shown in Table 1.

Heterotrophic plate count (HPC) and ARB were measured as below. Water samples were diluted and plated on R2A agar. After incubated at 22 °C for 7 days, the number of total colonies was regarded as HPC_{total} (Figure S2). Selective agar was prepared by adding the antibiotics at the maximum value of the minimum inhibitory concentrations (MICs) for *Pseudomonas aeruginosa*, *Enterococcus spp.*, and *Staphylococcus spp.*, bacteria that were previously identified in tap water (Cockerill, 2011; Guo et al., 2013). The medium was heat sterilized at 121 °C for 15 min and cooled to 55 °C. Antibiotics were then added to the medium, and thoroughly mixed before pouring. After plates were incubated at 22 °C for 7 days, the number of colonies were counted as $HPC_{22°C\ 7days}$ (Figure S3-S6). Sterilized phosphate buffer was used as negative control, the number of colonies were counted as HPC_{blank}. All samples were measured three times. The results were calculated as follows (Bai et al., 2015):

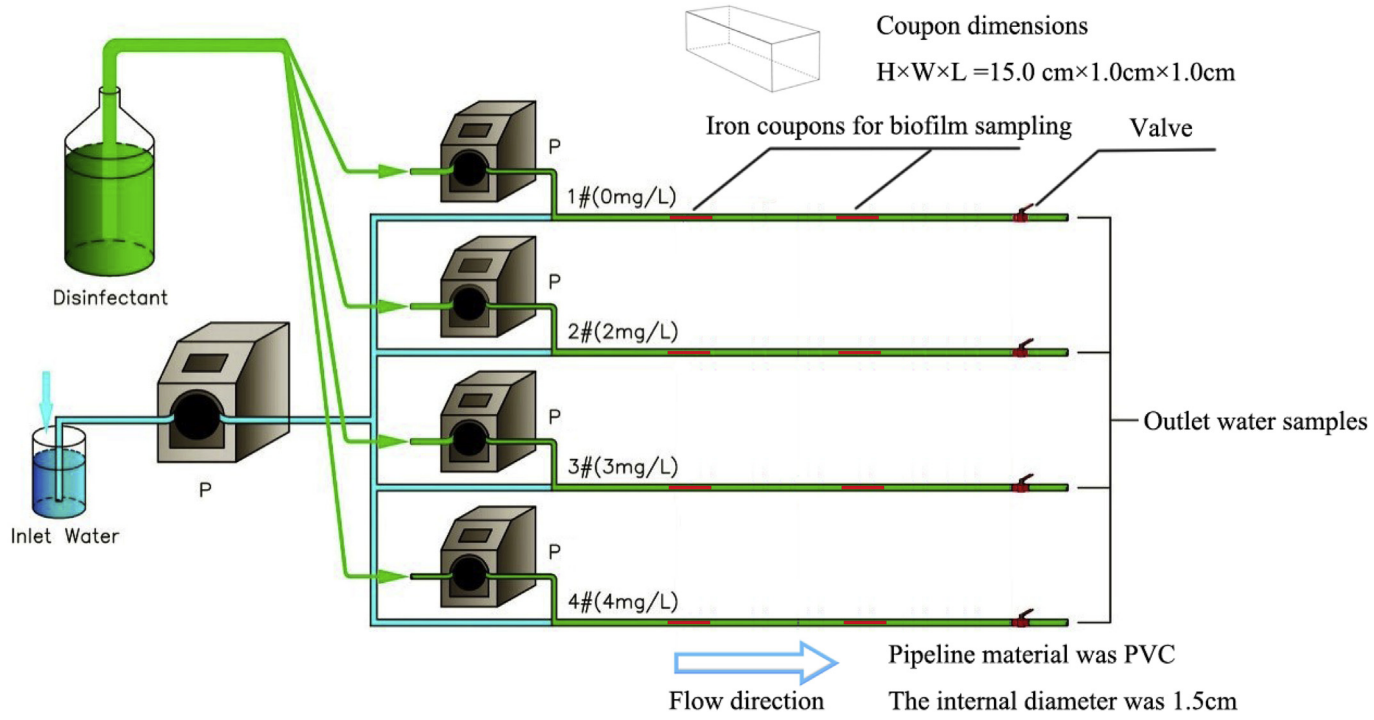

Fig. 1. Sketch map of the water distribution simulator (WDS) which was treated with different dosages of disinfectant (P is short for pumps). The simulator was single pass and the retention time was 10 h.

Table 1
Antibiotics chosen in this study.

Antibiotics	Tetracycline	Sulfamethoxazole	Clindamycin	Norfloxacin
Category	Tetracyclines	Sulfonamides	Macrolides	Quinolones
Resistance mechanism	Efflux pump	Dihydromyric acid synthesis changes	Efflux pump/erythromycin inactivation	Changes of the DNA gyrase protein
MIC mg/L	16 (Cockerill, 2011)	50.4 (Cockerill, 2011)	1 (Cockerill, 2011)	8 (Cockerill, 2011)
Percentage of ARB determined previously	0.5–34.6%	0–20%	/	/

$$\text{ARB concentration} = HPC_{22^{\circ}C\ 7days} - HPC_{blank}$$

$$\text{ARB percentage} = \text{ARB concentration}/\ HPC_{total}$$

The proportion of uncultivable cells was calculated as follows:

$$\text{Proportion of uncultivable cells} = (1\text{- HPC/ICC}) *100\%$$

2.4. Water and biofilm sampling

The inlet water (IW) and outlet water (OW) (5 L each) was sampled at the 2nd, 4th, 6th and 8th week. The water samples were collected in sterile glass bottle and then the BAR was detected. At the eighth week, 4L water was filtered through a polycarbonate membrane (0.22 μm, Millipore, USA) to extract DNA. Biofilm samples were collected at the eighth week by swabbing the coupon surface with sterile cotton, which was then put into a glass bottle filled with 100 ml sterile phosphate buffer. The bottle was placed into an ultrasonic vibration chamber (SB-800D, Ningbo Scientz Biotechnology Co., LTD, China) for 5 min of ultrasound treatment in ice water to detach the biomass from the cotton to the phosphate buffer (Proctor et al., 2016). Subsequently, the phosphate buffer was filtered through a 0.22 μm membrane for further DNA extraction.

2.5. DNA extraction and sequencing

FastDNA SPIN Kit (MP Biomedicals, CA) was used for DNA extraction. The DNA concentration and purity were detected by Nano DropND-2000 (NanoDrop Technologies, Willmington, DE). Then, PCR amplification was conducted to test the integrity of the DNA. The 16S rRNA genes were amplified from all DNA extracts using barcoded primers 515F/907R (515F 5′-barcode-GTGCCAGCMGCCGCGG)-3′ and 907R 5′-CCGTCAATTCMTTTRAGTTT-3′), the reaction system was as follows: 95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30s and a final extension at 72 °C for 10 min. PCR products were separated on 2% agarose gels. The extraction and purification was performed using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.). After preliminary assessment by electrophoresis, the PCR products were accurately quantified with the QuantiFluor -ST blue fluorescent quantitative system (Promega company), and then the samples were prepared at the corresponding proportions according to the sequencing requirements of each sample. One end of the DNA fragment is complementary to the primer base and is fixed on the chip. Finally, purified amplicons

were sequenced on an Illumina MiSeq platform.

2.6. Data analysis

After sequencing, QIIME (version 1.9.1) was used to process the sequencing data. The 300 bp reads were truncated at any position where the average quality score was less than 20, and the truncated reads were shorter than 50bp. Only sequences with overlap longer than 10 bp were retained and assembled according to their overlapping sequence. After removing the chimeric sequences, the sequences with a similarity greater than 97% using UPARSE (version 7.1 http://drive5.com/uparse/) were classified as belonging to the same OTU, and taxonomic analysis of the representative OTU sequences was performed. Based on the results of OTU cluster analysis, OTU can be analyzed for multiple diversity indices and detection of sequencing depth. Based on taxonomic information, the detection data was analyzed using R software and Origin 9.1. P value below 0.05 was regarded as significant for all tests.

3. Results and discussion

3.1. Effect of disinfectant types and concentration on BAR of water samples in the simulated system

As illustrated in Fig. 2(a), biofilm samples were removed every two weeks from the WDS with different disinfectant levels and four kinds of ARB were quantified. The average concentration of four kinds of ARB (tetracycline resistance, sulfamethoxazole resistance, clindamycin resistance, and norfloxacin resistance) with different chlorine levels ranged from 2 to 197 CFU/mL, 2–243 CFU/mL, 25–245 CFU/mL, and from 3 to 286 CFU/mL, respectively. As shown in Fig. 2(b), the average concentration of the four kinds of ARB (tetracycline resistance, sulfamethoxazole resistance, clindamycin resistance, and norfloxacin resistance) in chloraminated system levels were 36.5 ± 29.5, 72.5 ± 60.5, 136.5 ± 95.5, and 69.5 ± 61.5 CFU/mL, respectively. For eight weeks of operation, the ARB concentrations of IW and OW without residual chlorine or chloramine were statistically higher than the levels in OW samples with high chlorine or chloramine concentration ($P < 0.05$), which suggested that the disinfectants suppressed ARB in tap water. Anti-clindamycin resistant bacteria were present at a higher level than the other kinds of ARB in both chlorinated and chloraminated systems. The ARB concentrations were higher in the inlet water than in the samples with chlorine disinfection at the second week and fourth week. However, at the sixth week, the ARB concentrations of inlet water were only significantly higher than levels in samples with 3 mg/L chlorine and 4 mg/L chlorine. At the eighth week, only samples from the 4 mg/L chlorine system showed ARB concentrations that were significantly lower than those of inlet water, suggesting that the differences of ARB concentrations between IW and OW decreased with operation time. Because the inlet water and chlorine dosages were kept constant, this change likely reflects adaptation of bacteria in tap water. The same phenomenon was observed in the chloraminated system. The ARB concentrations were higher in inlet water than in all samples with chloramine when measured at the second week and fourth week. However, at the sixth week and eighth week, the ARB concentrations of inlet water were only significantly higher than the concentrations in samples with 3 mg/L chloramine. The analysis of variance between samples with different operation time indicated lower ARB and ARB percentage difference between the disinfected pipeline and non-disinfected pipeline over time. The ARB concentrations were

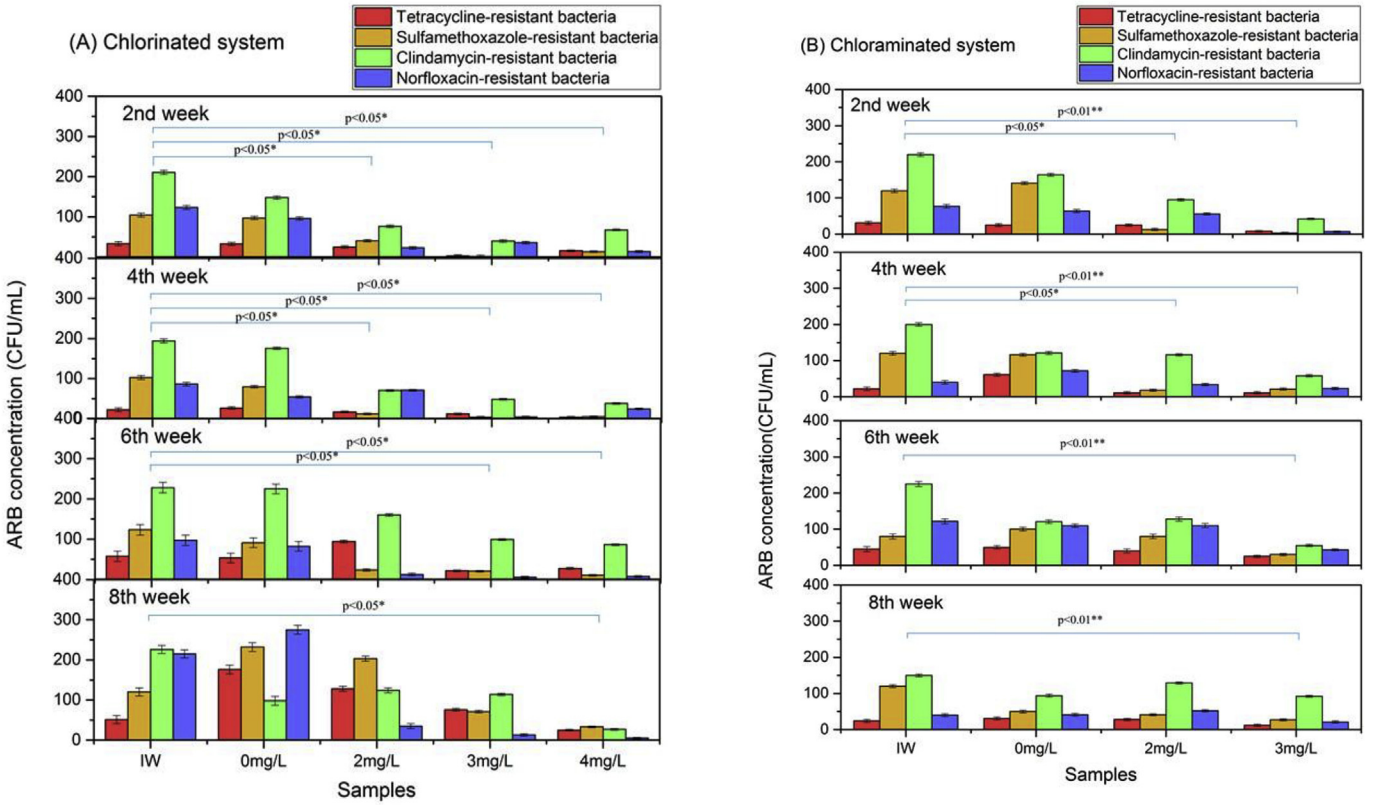

Fig. 2. Variation of ARB concentrations in IW and OW of pipelines with different disinfectant concentration (A) chlorinated and (B) chloraminated systems. The p value showed the difference between ARB concentration/percentage of water samples from pipelines with different dosages of disinfectant.

influenced by both disinfectant and treatment time.

As illustrated in Figure S7, the average ARB percentages of the four kinds of ARB (tetracycline resistance, sulfamethoxazole resistance, clindamycin resistance, and norfloxacin resistance) in water samples with chlorine disinfected ranged from 0.65% to 17.13%, 3.48%–22.62%, 4.85%–23.75%, and from 0.84% to 21.40%, respectively. The average percentages of these four kinds of ARB with different chloramine level ranged from 0.36% to 8.16%, 0.98%–13.08%, 2.28%–21.48%, and 0.60%–16.88%, respectively. The ARB percentage was higher in water samples with disinfectant than in the inlet water. Statistically higher ARB percentages were obtained in 2 mg/L chlorinated and chloraminated water samples ($P < 0.05$) than in other disinfected samples, and more than 2 mg/L of disinfectant lowered the amount of ARB, which suggested that chlorine reduced the ARB concentration in tap water. Lin targeted antibiotic-resistance genes and mobile genetic elements in secondary effluents from a municipal wastewater treatment plant after chlorination and indicated that chlorination was effective in reducing ARGs and MGEs (Lin et al., 2016). Clindamycin resistant bacteria were present at a higher level than the other kinds of ARB in the system. At the second week and fourth week of operation, the ARB concentrations were higher in 2 mg/L chlorinated water samples than in other samples with higher amounts of chlorine. However, at the sixth week and eighth week, the ARB concentrations in 2 mg/L chlorinated water samples were not significantly higher than those of other samples. In the chloraminated system, the ARB percentage was low at the second week in chloraminated samples, and the ARB percentage was higher in the 3 mg/L chloraminated water samples compared to those in the inlet water. At the sixth week, the ARB percentages of the 2 mg/L and 3 mg/L chloraminated water samples were significantly higher than the percentage in inlet water. At the eighth week, the ARB percentage in the 2 mg/L chloraminated water samples was significantly higher than that in inlet water. An interesting outlier to this trend was that the ARB percentage was higher in the outlet water of chlorinated water samples than in the inlet water at the second week, but this was observed later in the chloraminated system. This different behavior was attributed to the different oxidation properties of the two disinfectants. Compared with chlorinated system, the chloraminated system had lower ARB concentrations and lower ARB percentages. After more than two weeks operation time, the ARB percentage decreased in the chlorinated system, but this decrease was delayed in the chloraminated system, probably due to its low oxidation ability.

3.2. *Effect of disinfectant types and concentration on BAR of biofilm samples in the simulated system*

As illustrated in Fig. 3, the BAR of biofilm were detected at the eighth week. In the two systems, the ARB percentage of IW, OW, and biofilm ranged from 0.81% to 9.85%, 1.08%–25.89%, and 7.31%–27.81%, respectively. The biofilm without chlorine treatment had the highest ARB concentration, and the ARB percentage was the highest in the biofilm treated by 4.0 mg/L chlorine. The results indicated that the disinfectant suppressed ARB concentration but increased the ARB percentage in biofilm. In the chloraminated system, biofilm showed the highest ARB concentration with 2 mg/L chloramine, and the ARB percentage was highest with 3 mg/L chloramine. The relative abundances of ARB in the biofilm and OW had higher level than that in IW. Due to the high concentration of disinfectant added in IW and the disinfectant residual detected in OW, the bacteria regrowth was suppressed in bulk water, how did the OW have higher ARB concentration and percentage than IW? The biofilm detachment might have great effect on OW and resulted in high BAR.

As illustrated in Fig. 4(a) and (b), compared with biofilm samples without disinfectant added, the disinfected biofilms had lower ICC, HPC concentration, and HPC/ICC percentage, which suggested suppression of bacteria in biofilm. The low HPC/ICC percentage in the disinfected biofilm indicated a high proportion of uncultivable bacteria. As shown in Fig. 4(c) and (d), compared with the biofilm samples without disinfection, the disinfected biofilms had higher TOC level, lower AOC concentration, and lower AOC/TOC percentage, indicating the organic carbon in biofilm was centralized and disinfected biofilm had low biodegradability. These results were consistent with the previous finding that the mean stiffness of monochloramine- or free-chlorine-treated biofilms was 4–9 times higher than that of untreated samples (Shen et al., 2016). However, the AOC concentration available for direct use by bacteria decreased in biofilm after disinfection. Thus, there may be limited organic carbon in the disinfected biofilm. Nutrient limitation can induce specific stress responses which lead to antibiotic tolerance (Poole, 2012). The AOC concentration was negatively correlated to the ARB percentage in the biofilm ($P < 0.05$), indicating that nutrient conditions have a significant impact on antibiotic tolerance and adaptive resistance. The low HPC/ICC percentage in disinfected biofilm suggested that disinfection resulted in a high proportion of uncultivable bacteria. Chen indicated that after treatment with chlorine and chloramine more than 1 mg/L, the remaining viable *E. coli* cells may enter a viable but non culturable (VBNC) state (Chen et al., 2018), which may be the main reason for the observed HPC/ICC increase in biofilms after chlorine and chloramine disinfection. Biofilms create a microenvironment with less oxygen and nutrients, promoting the formation of both antibiotic-tolerant bacteria and VBNC cells. During biofilm growth, the oxidation stress which affect the cellular pathways can render BAR (Ayrapetyan et al., 2015). The HPC/ICC percentage was correlated to the ARB percentage in the biofilm ($P < 0.05$), which revealed that uncultivable cell production increased the bacterial antibiotic resistance and enhanced antioxidant capacity (Khakimova et al., 2013; Khan et al., 2016). L. Mathieu pointed out that the biofilm can release large biofilm clusters under high disinfectant condition (Mathieu et al., 2014). These findings suggest how bacteria can transfer the adaptability of the disinfectant to increase the bacterial antibiotic resistance in unidirectional water supply process. Microorganisms in the water supply system can prevent inactivation by high-concentration disinfectants in water by forming biofilms, producing resistant integrons and plasmids in the biofilm by adapting to the external environment, and are capable of horizontal gene transfer between bacteria. Biofilm growth or biofilm detachment caused by external environmental factors will allow biofilm clusters with higher ARB concentration and percentage to enter the bulk water, thereby increasing the antibiotic resistance and anti-disinfectant activity of bacteria in tap water. The biofilm can continue to form additional biofilms that connect the pipe wall to other areas of the pipe network, which ultimately affects the water quality of the terminal tap water, resulting in an increase in bacterial antibiotic resistance.

The disinfectants ($\geq$2 mg/L) was found to suppress ARB concentration in tap water while led to a high relative abundance of ARB in this study. Meanwhile, the use of disinfectant suppressed ARB concentration but increased the ARB percentage in the biofilm, because low nutrient conditions and uncultivable cells increased bacterial antibiotic resistance. Compared with chloraminated condition, the relative abundance of ARB in chlorinated condition was higher, which indicated that chlorine had higher ability to select antibiotic resistance. Thus, using chloramine and higher dose than 2 mg/l can reduce the risk of antibiotic resistance in biofilm and water samples.

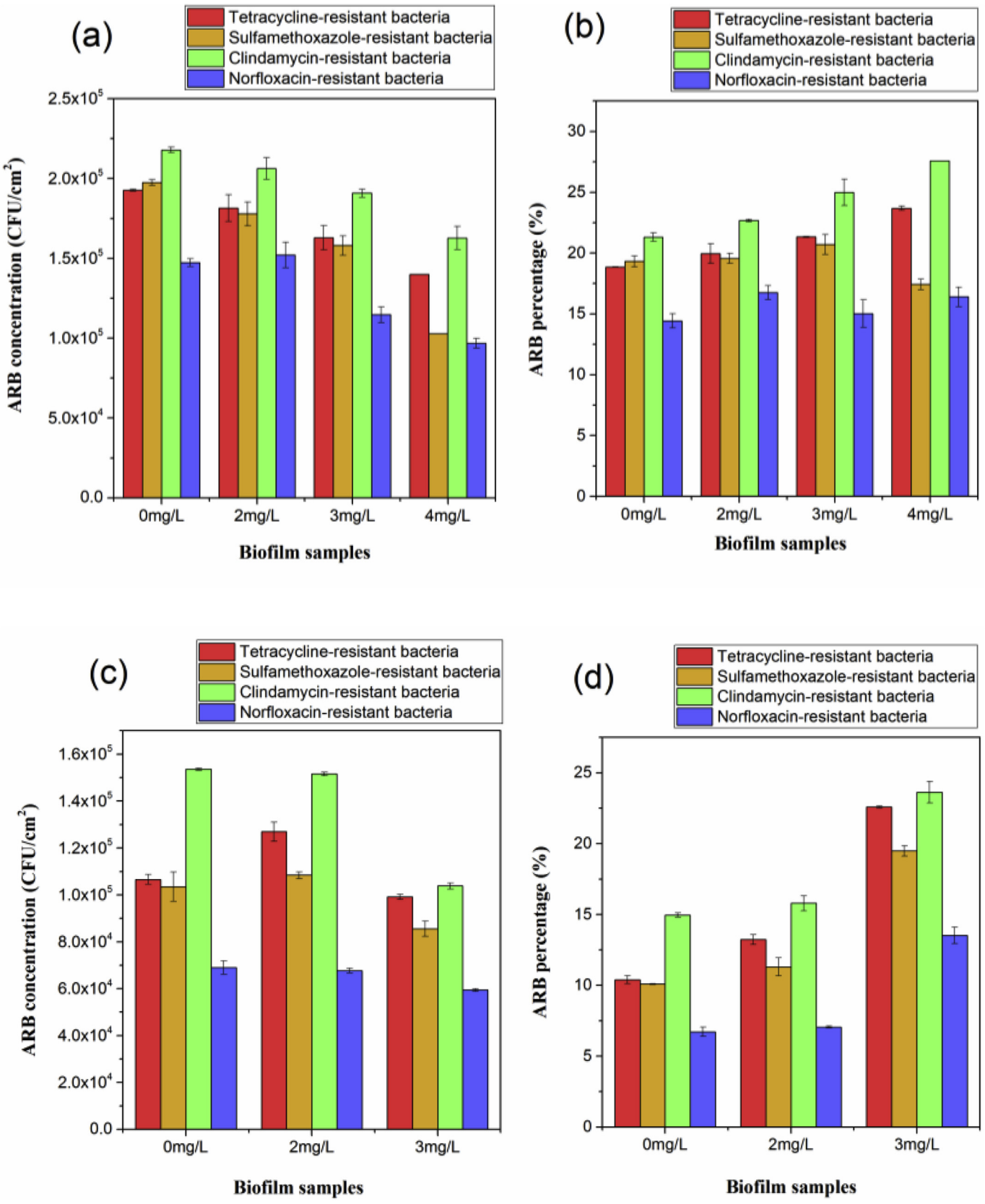

Fig. 3. Variation of ARB concentration and ARB percentage in biofilm samples in chlorinated and chloraminated systems (a and b were chlorinated system, c and d were chloraminated system).

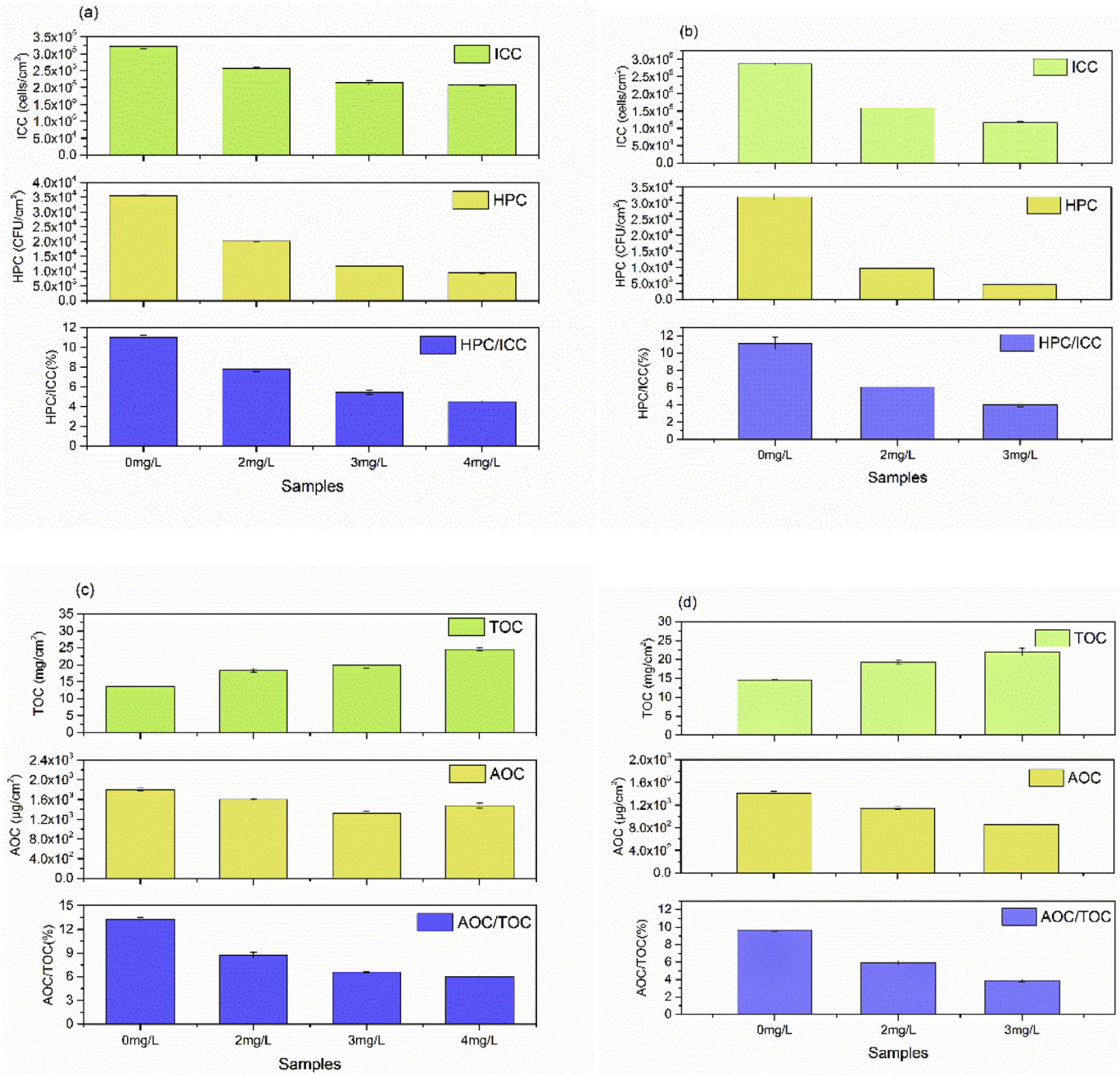

Fig. 4. HPC concentration, ICC, HPC/ICC percentage, TOC concentration, AOC concentration and AOC/TOC percentage in biofilm samples (a and c were chlorinated system, b and d were chloraminated system).

3.3. 16S rRNA gene profiling revealed major microbial community shifts for the different disinfectants and different concentrations in tap water and biofilm samples

High throughput sequencing revealed that the Chao 1 and Simpson indices of biofilm samples was lower than those of water samples, suggesting biofilm had low microbial diversity. As shown in Figure S8, in the chlorinated system, 10 known phyla were found in all water sampling sites: *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Nitrospirae*, *Chloroflexi*, *Planctomycetes*, and *Chlamydiae*. *Proteobacteria* was predominant in water and biofilm samples, with four main classes (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria*). Classes *Alphaproteobacteria*, *Gammaproteobacteria*, and *Cyanobacteria* were present at higher relative abundance in the water samples than in the biofilm samples. In contrast, the relative abundance of members of the classes *Betaproteobacteria*, *Bacilli*, *Sphingobacteriia* and *Acidobacteria* were higher in biofilm samples than in water samples. At the family level, the relative abundances of *Rhodocyclaceae*, *Caulobacteraceae*, and *Comamonadaceae* were 56.15%, 42.73% and 12.44% in biofilm samples, higher than those in tap water (Figure S9). The family of *Rhodocyclaceae* contains mainly of aerobic or denitrifying rod-shaped bacteria that have diverse metabolic capabilities, produce extracellular polymers (EPS) (Adav et al., 2009), and prefer oligotrophic conditions (Carlson et al., 2017). Their presence was

consistent with the oligotrophic condition in disinfected biofilm with low AOC concentration, as shown in Fig. 5. *Caulobacteraceae* are frequently studied for their swarm cell growth phase and attachment and continuous dispersal within biofilms (Gullberg et al., 2011). *Comamonadaceae* are dominant groups in biofilm and suspended communities in other fast-flowing, low-temperature, and oligotrophic aquatic ecosystems (Ling et al., 2016). Additionally, these groups have been associated with biological instability (Proctor et al., 2016), and their high levels may reflect detachment from biofilms.

In the chloraminated system, 10 known phyla were found in all water sampling sites. *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Actinobacteria*, *Acidobacteria*, *Nitrospirae*, *Bacteroidetes*, *Planctomycetes*, *Parcubateria* and *Chlamydiae* were detected in all water and biofilm samples (Figure S10). *Proteobacteria* predominated in water and biofilm samples. Classes *Alphaproteobacteria*, *Deltaproteobacteria*, *Nitrospira*, *Acdiobcateria,* and *Cyanobacteria* presented higher relative abundances in the water samples than in the biofilm samples. In contrast, the relative abundance of members of the classes *Betaproteobacteria*, *Bacilli,* and *Actinobacteria* were higher in biofilm samples than in water samples. At the family level (Figure S11), *Caulobacteraceae, Rhodocyclaceae*, *Paenibacillus,* and *Comamonadaceae* made up 76.65%, 35.15%, 18.86%, and 14.31% of biofilm samples, had were present at higher levels in biofilm than in tap water, so should be considered important contributors to biofilm formation in drinking water. The major families in the chlorimanated system were similar with those in the chlorinated system, supporting the idea that biofilm detachment affected the bacterial antibiotic resistance in tap water.

Biofilms are encased in an extracellular polymeric matrix composed of polysaccharides, proteins, and DNA, but bacteria with degrading, chelating, or detergent-like functions such as *Sphingomonas spp*., *Pseudomonas aeruginosa,* and *Acinetobacter spp* can easily detach from the biofilm (An et al., 2010). As shown in Figure S12, the results identified several genera in biofilms and outlet water (*Sphingomanadales*, *Brecundimonas, and Brevibacillus* in the chlorinated system, and *Brevundimonas*, *Brevibacillus*, *Sphingomanadales and Mycobacterium* in the chloraminated system) which prefer an oligotrophic environment and have multiple antibiotic resistance (Yang et al., 2016a; Yang et al., 2016b). In the chlorinated system, the relative abundance of *uncultured_f_Rhodocyclaceae*, *Brevundimonas*, and *Brevibacillus* made up 39.0% of the biofilm and was positively associated with chlorine concentration ($P < 0.05$) and ARB percentage ($P < 0.05$) and negatively correlated with AOC concentration ($P < 0.05$). Thus, the oligotrophic condition in biofilm selected these bacteria and the increase in these genera increased the ARB percentage of biofilm. In the chloraminated system, *Brevundimonas* and *Brevibacillus* with antibiotic resistance (Khan et al., 2016) constituted more than 78.5% of the biofilm and showed a positive correlation with chloramine concentration ($P < 0.05$) and ARB percentage ($P < 0.05$), indicating that the disinfectant selected these bacteria in the biofilm and these genera increased the proportion of ARB in biofilm. In tap water, the relative abundance of genera (*Sphingomanadales, Brecundimonas* and *Brevibacillus* in the chlorinated system, and *Brevundimonas, Brevibacillus, Sphingomanadales*, and *Mycobacterium in the chloraminated system*) was correlated to the ARB percentage ($P < 0.05$), suggesting these genera increased the ARB percentage in tap water. In particular, these bacteria contained bacitracin resistance genes, contributing to the increase of ARB percentage. Because of disinfectant can promote biofilm detachment, the higher level of these bacteria in biofilm and OW than in IW, indicating that disinfectant

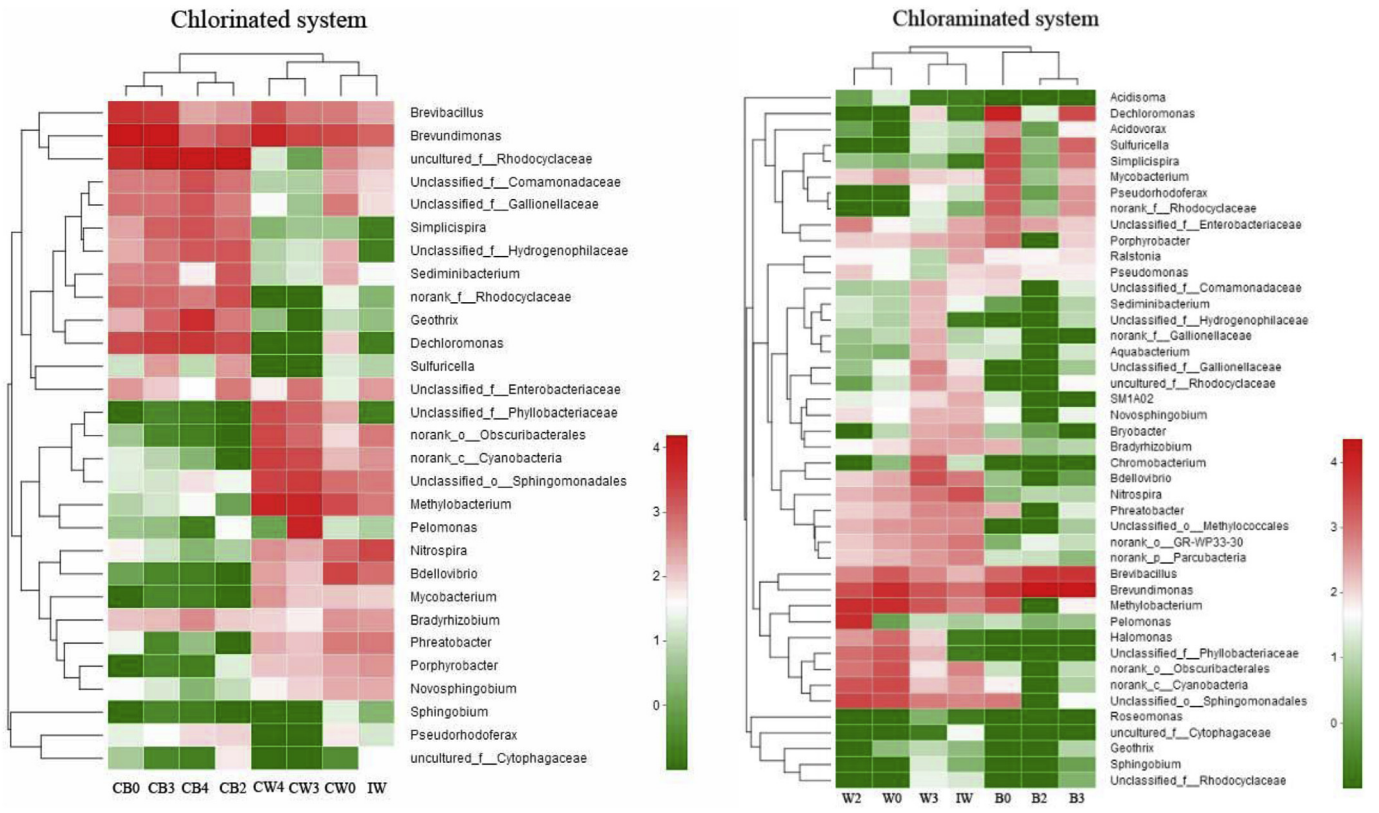

Fig. 5. Heat map showing the most abundant genera in water and biofilm samples and the community similarity analysis of biofilm and water samples in chlorinated and chloraminated systems at the eighth week.

could promote the ARB proportion in biofilm and the interaction between biofilm and water. Thus, the biofilm present in DWDS select multiple ARB due to the oligotrophic conditions (low AOC concentration and high disinfectant concentration) and the detachment of ARB from the biofilm increased the ARB percentage in tap water.

4. Conclusions

This study reveals how novel resistance mechanisms of biofilm affect tap water with pressure from different disinfectants. The results revealed that the ARB concentrations were influenced by both disinfectant and reaction time. A statistically higher fraction of ARB was obtained in 2 mg/L chlorinated and chloraminated water samples ($P < 0.05$) than in other samples, and when disinfectant was present above 2 mg/L concentration, there was a lower ARB percentage in the water. There was a higher ARB percentage in the biofilm samples than that in tap water. The ARB percentage was negatively correlated to the AOC concentration ($P < 0.05$) in the biofilm, suggesting that low nutrient conditions had a significant impact on antibiotic tolerance and adaptive resistance. The percentage of non-culturable bacteria was also correlated to the ARB percentage ($P < 0.05$), which revealed that the increase of non-culturable bacteria led to the high relative abundance of ARB in biofilms. High throughput sequencing of biofilm samples identified microbes (*uncultured_f_Rhodocyclaceae*, *Brevundimonas*, and *Brevibacillus* in chlorinated systems, and *Brevundimonas* and *Brevibacillus* in chloraminated systems) which had multiple antibiotic resistance and had high abundance (up to 78.5%). Biofilm growth or biofilm detachment caused by environmental factors will cause release of high ARB-containing biofilm clusters into the bulk water, thereby increasing the antibiotic resistance in tap water. The biofilm can repeat the process by re-adhering to the pipe wall and connect to other areas of the pipe network to form another biofilm, which ultimately affects the water quality of the terminal tap water.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.11.077.

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