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Chlorination contributes to multi-antibiotic resistance in a pilot-scale water distribution system

Jinmei Li^a, Shuting Zhang^b, Lizheng Guo^c, Lihua Chen^d and Zhisheng Yu^{a,*}

^a College of Resources and Environment, University of Chinese Academy of Sciences, 19 A Yuquan Road, Shijingshan District, Beijing 100049, China

^b School of Environment and Civil Engineering, DongGuan University of Technology, No.1, Daxue Rd., Songshan Lake, Dongguan, Guangdong Province, China

^c Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China

^d Delft University of Technology, Mekelweg 4, 2628 CD Delft, The Netherlands

*Corresponding author. E-mail: yuzs@ucas.ac.cn

ABSTRACT

The generation and dissemination of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment has become a critical risk to human health. This study is based on a pilot-scale simulated water distribution system to understand the effects of chlorine disinfection treatment (without free chlorine) on ARB and ARGs in biofilms. The hydraulic parameters and pipe materials of the system were simulated based on a drinking water system. The results of the colony counts showed that bacterial multi-antibiotic resistance could be enhanced 13-fold in the biofilms of the pipeline. The use of high-throughput qPCR (HT-qPCR) indicated that the total relative abundance of ARGs in biofilm samples increased significantly ($p < 0.05$), while the diversity of bacteria was shown to be reduced via taxonomic analysis of the V3–V4 region of 16S rRNA. The prominent types of ARGs were conferred resistance by aminoglycoside and β -lactam after the chlorine disinfection treatment, and antibiotic deactivation was the main mechanism. Phyla *Proteobacteria* had the highest abundance in both treatment and control groups but decreased from 70.81% (initial biofilm sample) to 26.09% (the sixth-month biofilm sample) in the treatment groups. The results show that the chlorine disinfection plays a role in the risk of development of bacterial antibiotic resistance in pipe networks owing to bacteria in biofilms. This study was the first to investigate the contribution of chlorination without free chlorine to the bacterial community shift and resistome alteration in biofilms at a pilot-test level.

Key words: antibiotic resistance, ARG, biofilm, DBPs, multi-drug resistance

HIGHLIGHTS

- Chlorine disinfection can enhance bacterial multi-antibiotic resistance.
- The total relative abundance of ARGs in biofilm samples increased significantly.
- The diversity of the treated biofilm bacteria increased.
- Antibiotic deactivation was the main mechanism after chlorine disinfection.

INTRODUCTION

The use of antibiotics has induced a number of environmental issues. The generation and dissemination of ARB and ARGs represent a global risk to public health (Pruden *et al.* 2006). ARGs are brought into sharp focus as a concern for researchers as new emerging contaminants (Pruden *et al.* 2006). Antibiotic resistance is encoded by ARGs (Allen *et al.* 2010), which are genes that can be readily shared among bacteria, and even spread from bacteria, human and animal sources to drinking water systems (Armstrong *et al.* 1981; Faria *et al.* 2009; Zhong *et al.* 2010). A wide number of ARB and ARGs in drinking water systems have been detected during the last several decades (Pavlov *et al.* 2004; Zhang *et al.* 2009; Jia *et al.* 2015). In tap water, studies have shown that the quantities of both ARB and ARGs were larger compared with the drinking water achieved after treatment (Xi *et al.* 2009; Lv *et al.* 2014).

Biofilms make up an extraordinary section of the microorganisms in water distribution systems (WDS) (Liu *et al.* 2014a). They contain diverse microbial species at high abundance that are ubiquitous in pipe network systems (Shih & Huang 2002; Kivanç *et al.* 2016; Qi *et al.* 2016), and the ubiquitous presence of bacteria in biofilms could spread antibiotic resistance through horizontal gene transfer (HGT) in both water treatment processes and pipelines (Madsen *et al.* 2012). In general,

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biofilm is produced in the pipe walls of water distribution network systems and is mainly composed of microbial and extracellular polymeric substances (EPS). EPS are largely composed of polysaccharides, proteins and extracellular DNA (Flemming & Wingender 2001). The formation of biofilms will not only accelerate the corrosion rate of water distribution pipelines (Quignon *et al.* 1997), but also provide numerous advantages to the embedded bacteria, including increases in bacterial against various environmental stresses, sharing of nutrients and metabolic products among bacteria, and especially facilitate the horizontal transfer of ARGs between bacteria (Liu *et al.* 2014b; Mi *et al.* 2015; Shi *et al.* 2015). The increase in biofilm-associated antibiotic resistance infections is boosting the demand for new advanced and more effective treatment. In this sense, nanotechnology offers a ground-breaking platform to address this challenge (Moshtaghi *et al.* 2016; Zinatloo-Ajabshir *et al.* 2020; Zinatloo-Ajabshir *et al.* 2021a, 2021b, 2021c, 2021d). One of the most promising environmental utilizations of nanotechnology has been in water treatment and remediation where various nanomaterials can purify the water by adsorbing dyes, heavy metals, and other pollutants, inactivating and removing pathogens, and converting harmful materials into less harmful compounds (Saleem & Zaidi 2020). Additionally, the nanomaterials have been added to membranes (Vallet-Regi *et al.* 2019).

Chlorination is an extensively used method for disinfecting water because of its low cost and ability to effectively remove microorganisms from water to prevent serious waterborne diseases in water treatment. Notably, previous reports have shown that chlorination can contribute to the enrichment of ARB and ARGs (Armstrong *et al.* 1982). Chlorination could also play a role in increasing the total relative abundance of ARGs in opportunistic bacteria (Chao *et al.* 2015; Jia *et al.* 2015). Cross- or co-resistance of disinfectants could be the underlying mechanisms responsible for the promotion of ARB and ARGs (Nakajima *et al.* 1995). Additionally, it is widely known that the chlorination processes can produce disinfection by-products (DBPs), which are regarded as water contaminants (Pilotto 1995; Costet *et al.* 2011; Hrudey *et al.* 2015), and result in extensive contact with humans and other organisms (Rahman *et al.* 2010). For example, trihalomethanes (THMs) are widely known DBPs (Golfinopoulos *et al.* 2003). There is growing evidence that suggests that DBPs could cause an increase in antibiotic resistance (Lv *et al.* 2015; Li *et al.* 2016) and the continuous detection of ARB and ARGs in tap water after disinfection treatment (Lv *et al.* 2014; Li *et al.* 2016). However, whether it is DBPs or residual chlorine that causes antibiotic resistance remains unclear, as far as we know, the contribution of chlorination without free chlorine to the resistome alteration in WDS has not been evaluated.

Here, we hypothesize that chlorination treatment (without free chlorine) in water distribution pipe network systems plays a role in enhancing bacterial resistance during biofilm formation. To test this hypothesis, the main research included the following: first, bacterial community composition was investigated during biofilm formation by Illumina sequencing and bioinformatics analysis in the distribution system; secondly, HT-qPCR was used to detect genetic changes and mechanisms related to antibiotic resistance. In addition, HPC and q-PCR methods were applied to assess the abundance of antibiotic bacteria and total bacterial counts. This study can help to understand the contributions of chlorination treatment on ARB or ARGs and the promotion mechanisms of antibiotic resistance during biofilm formation after chlorine disinfection in WDS.

MATERIALS AND METHODS

Setup and materials

New PPR (Polypropylene-Random) material pipes were operated to simulate the WDS. Four identical pilot drinking water distribution pipes were established, and three of them were used for chlorination. Free chlorine was then removed by acid-based neutralization after chlorination and dechlorination treatment. The concentration of THMs was used to evaluate the chlorination treatment. Four 200 L plastic containers were used to store raw water, which was manipulated as the influent water distributed to the four pilot pipes by four submersible pumps, replacing the water every five days as a cycle. The flow rate of the system was approximately 15 L/min (Figure 1).

In addition, this research experiment was designed for two stages. In stage 1, the feed water of the four pipe systems without any treatment were used for influent, and stage 1 continued for 30 days, so that the initial biofilm could form on the four pipe systems. In stage 2, three of the pipe systems were regarded as an exposure group that used chlorination and dechlorination water (without free chlorine) as influent to continue culturing the biofilms. The control group system was established based on inlet water without chlorination.

THM standard solutions (chromatographic pure) including trichloromethane (CHCl_3), chlorodibromomethane (CHClBr_2), bromodichloromethane (CHCl_2Br) and tribromomethane (CHBr_3) (Sigma-Aldrich, USA) were used to test the formation of

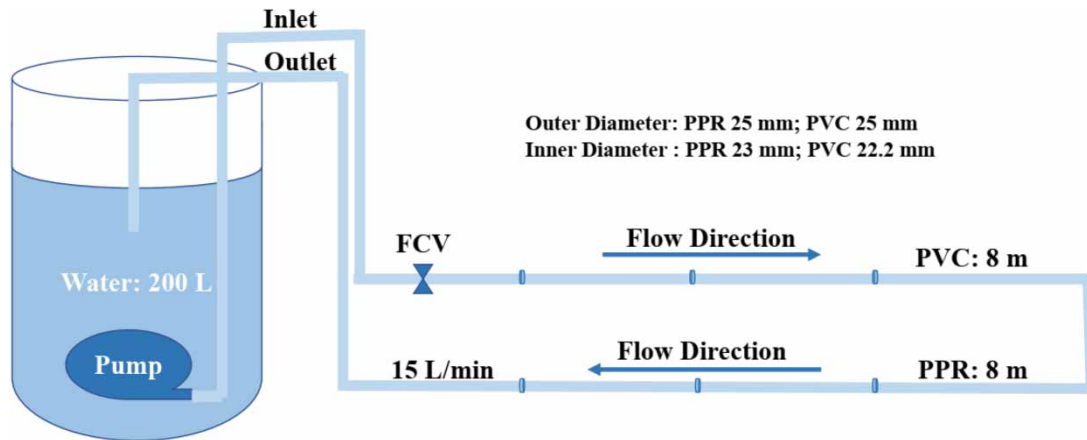


Figure 1 | Schematic diagram of the pipe network. The pipe network was a closed loop, and the inlet water was replaced every 5 days. Four identical water distribution pipe systems were established. Three of them were used as an exposure group in parallel. The water was used as inlet water after chlorination and the process of removal of free chlorine. The control group system was established based on inlet water without chlorination. FCV: flow control valve; PVC: polyvinyl chloride; PPR: pentatrico peptide repeats. The mean flow rate of water in the pipe network was 15 L/min. The inlet water quality during the culture time in the present study is shown in [Table 1](#).

by-products (please refer to the Supplementary Material, Text S1); ampicillin (Amp), vancomycin (Van), kanamycin (Kan), erythromycin (Ery), sulfamethoxazole (Sul), clarithromycin (Cla), rifampin (Rif) and gentamicin (Gen) were obtained from Sigma-Aldrich to investigate the antibiotic resistance bacteria, and the section ‘ARB determination’ describes the detection method of the ARB in detail; R₂A nutrient agar was obtained from Qingdao Hope Bio-Technology (China), and it was used to investigate the concentration of ARB with corresponding antibiotics (Lv *et al.* 2014). Sodium hypochlorite (NaClO) was purchased from Sinopharm Group Chemical Reagent (China), and was applied to chlorination in this research. Sodium thiosulfate (Na₂S₂O₃) was purchased from Sinopharm Group Chemical Reagent (China) for dechlorination of disinfected water.

Determination of physicochemical water properties

Raw water was pumped from the manmade stream in the Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China (Zeng *et al.* 2020). The same feed water was used for the simulated system biofilm culture after chlorination treatment, and the control group without chlorination. The pH, temperature, dissolved oxygen (DO), and turbidity (NTU) were measured on site with a portable multi-parameter water quality meter (Muti 3420, WTW, Germany). The physicochemical properties of the influent water included the following: total nitrogen content (TN), total organic carbon content (TOC), chemical oxygen demand (COD_{Mn}), total phosphorus (TP), the residual chlorine, UVA₂₅₄, and ammonia nitrogen (NH₄⁺-N), nitrate (NO₃⁻-N) and nitrite (NO₂⁻-N) concentrations were measured according to China’s national standards (Chen *et al.* 2017; Wan *et al.* 2019). The detailed data are listed in [Table 1](#). A portable residual chlorine detector (Hach, USA) was used to measure the free residual chlorine.

Chlorine-based disinfectants can produce DBPs, such as THMs, which are considered as conditionally carcinogenic compounds (Valdivia-Garcia *et al.* 2016), and include CHCl₃, CHBrCl₂, CHClBr₂ and CHBr₃. THMs were investigated as representatives of chlorinated DBPs in this study, the analytical method details are described in the Supplementary Material (Text S1) and results are shown in [Figure S1](#). The concentration of THMs were maintained at 1,000–1,500 micrograms per litre.

Biofilm sampling

Biofilm samples were collected from the pilot system at the first, third, and sixth months. The PPR pipes were cut into 10-cm-long sections, and the external surface of the pipes was wiped with alcohol cotton balls before placement in a 1,000 mL sterilized beaker with 300 mL aseptic deionized water, and the inside of the pipes was scraped with aseptic cotton buds after the application of ultrasonic device treatment (1 min interval at 40 kHz, repeated five times) (Lin *et al.* 2016). A volume of 2 mL of the biofilm bacteria suspension was transferred to a sterile centrifuge tube and homogenized.

Table 1 | The feed water quality during the culture time in the present study ($n = 50$)

Parameter (units)	Mean	SD	Max	Min
Temperature (°C)	19.9	10	23.8	13.5
Turbidity (NTU)	2.7	1.6	5.14	1.83
Dissolved oxygen (mg/L)	8.87	1.8	12.01	7.81
Residual chlorine (mg/L)	0.04	0.03	0.09	0.00
Total organic carbon (mg/L)	4.46	0.87	6.79	2.88
pH	8.3	0.31	8.69	7.92
UVA ₂₅₄ (cm ⁻¹)	0.2	0.05	0.23	0.17
Chemical oxygen demand (mg/L)	49.16	0.92	52.67	43.64
NH ₄ ⁺ -N (mg/L)	0.39	0.41	0.9	0.17
NO ₂ ⁻ -N (mg/L)	0.06	0.06	0.2	0.02
NO ₃ ⁻ -N (mg/L)	1.37	0.9	2.39	0

SD, standard deviation; NTU, nephelometric turbidity units.

ARB determination

To identify the ARB in the exposure and control groups, the biofilm obtained was used to coat R₂A agar plates with eight different antibiotics or containing two types of a random combination of antibiotics. The biofilm bacterial solution obtained was diluted ten times with a 0.9% NaCl solution and coated on R₂A nutrient agar with Amp (32 µg/mL), Van (32 µg/mL), Gen (8 µg/mL), Kan (32 µg/mL), Ery (8 µg/mL), Cla (8 µg/mL), Rif (8 µg/mL) and Sul (350 µg/mL) to investigate the effect of different antibiotics on bacteria in the pipeline biofilm samples. The biofilm bacteria obtained that exhibited multiple resistance to Gen + Cla, Van + Gen, Cla + Amp, Rif + Van, Van + Cla, Ery + Cla, Sul + Cla and Rif + Gen were tested similarly, and the total number of biofilm bacteria were measured. The biofilm bacterial solution was sequentially diluted with a 0.9% NaCl solution and cultured on R₂A nutrient agar. A total of 100 µL of diluted samples were coated on R₂A nutrient agar and cultured for seven days at 28 °C. Finally, the colonies that were on triplicate plates that had between 30 and 300 colonies of bacteria were counted (Lv *et al.* 2014).

DNA extraction

The remaining biofilm bacterial suspension samples were filtered with a 0.22 µm filter membrane (GPWP04700; Millipore, Ireland), which was used for DNA extraction, and some of the biofilm bacteria were treated with PMA before the biofilm bacterial DNA extraction protocol, which was based on the Fast DNA SPIN Kit for Soil (MP Biomedicals, USA), using microspectrophotometry (Nano-100, China) to detect the concentration and purity of DNA. All samples were stored at -80 °C before DNA extraction and chemical analysis.

HT-qPCR determination

A total of 299 validated primer sets were applied, and the primers contained a 16S rRNA gene, 286 ARG primer sets, nine transposase gene primer sets and three integrase gene primer sets (Zhu *et al.* 2013; Liu *et al.* 2014b; Wan *et al.* 2019). Thus, this experiment used primers that contained almost all the resistant gene classes that have been studied so far.

All of the DNA samples were diluted to 50 ng/µL before manipulation in the HT-qPCR. The melting curves of each reaction were automatically generated by WaferGen software (Lin *et al.* 2016). WaferGen software is included in the WaferGen SmartChip Realtime PCR system (WaferGen Biosystems, USA). This system played an important role in HT-qPCR, and all the manipulations followed methods that had been previously described (Zhu *et al.* 2013; Wang *et al.* 2014a). The qPCR process was utilized as described by Lin *et al.* (2016). The thermal cycle consisted of 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s (Zhu *et al.* 2013; Wang *et al.* 2014a). All the manipulation of quantitative PCRs was performed in technical triplicates.

SmartChip qPCR v 2.7.0.1 software (WaferGen Biosystems) was applied to manipulate data from the HT-qPCR outcome. Only data multiple melting peak and amplification efficiencies no more than 1.8–2.2 were manipulated with the software. All the HT-qPCR manipulations were performed in triplicate, and a threshold cycle of 28 was used as the detection limit (Lin

et al. 2016). The number of ARGs discovered was used to evaluate the richness of ARGs, and the relative abundance of ARG was regarded as normalized ARG copies to the 16S rRNA copies (Lin *et al.* 2016; Wan *et al.* 2019).

Bacterial community structure analysis

The extracted DNA samples were used for Illumina high-throughput sequencing (HTS) by Shanghai Majorbio Bio-pharm Technology Co., Ltd (China). The V3–V4 region of 16S rRNA was selected for amplification to investigate the bacterial community structure in the biofilm samples. The forward primer was 338F: ACTCCTACGGGAGGCAGCAG, and the reverse primer was 806R: GGACTACHVGGGTWTCTAAT. The polymerase chain reaction conditions were as follows: 95 °C for three minutes, followed by 35 cycles of (95 °C for 30s, 56 °C for 30s and 72 °C for 45s), and finally, an extension of ten minutes at 72 °C. The amplification products were purified and used for Illumina HTS. The open-reference operational taxonomic unit (OTU) was carried out in accordance with the following principles (Wan *et al.* 2019): (1) the non-repeated sequence was extracted for the optimized sequence, so that the redundant calculation amount of the middle process of the analysis was reduced without duplication; (2) OTU clustering of non-repetitive sequences (excluding single sequences) was carried out according to 97% similarity. The chimerism was removed in the clustering process, and the representative sequence of OTU was obtained; (3) all the optimized sequences mapped to OTU representative sequences, and the sequences with 97% similarity to representative sequences were selected to generate OTU tables; (4) the RDP classifier Bayesian algorithm was applied to analyze 97% of the OTU representative sequences with similar level, and analyzed the taxonomy at domain, kingdom, phylum, class, order, family, and genus level, respectively, to calculate the community composition of each sample (<https://www.arb-silva.de/>). All Illumina high-throughput sequences have been submitted to the National Center for Biotechnology Information Sequence Read Archive under the accession number PRJNA541806.

Statistical analysis

Microsoft Excel 2016 (Microsoft Inc., USA) was used to manipulate the mean, standard deviation and fold change of the data. The bar graph was drawn using Origin 8.0 software (<http://origin.en.softonic.com/>). An independent-sample *t*-test was applied to statistically analyze the differences of antibiotic resistance frequency between the control and treatment by the chlorination process in each biofilm sample. The tests were conducted using SPSS 19.0 (IBM, USA). Three parallels were used for each comparison, and $p < 0.05$ was considered significant. Heatmap graphs were produced using RStudio with the gplots package (<http://www.r-project.org/>).

RESULTS

Antibiotic resistance detection

The antibiotic resistance investigation was regarded as proof to recognize the phenotype of resistance to antibiotics. The resistances to random combinations of eight pairs of antibiotics were determined after the system ran for six months. Before the detection of antibiotic resistance, a total of culturable bacteria in each sample was investigated by the heterotrophic plate count (HPC) method, coated on R₂A agar plates without any antibiotic, and the colonies were counted. Three parallels were taken for coating plates in every replicate. The colony count results of multi-antibiotic resistance are shown in Figure 2. The bacteria that grew in antibiotic-containing R₂A agar nutrient plates were regarded as having resistance to the corresponding antibiotics (Lv *et al.* 2014). The percentage of resistance was used to quantify the rate of ARB, which was determined 'by dividing the number of colonies on the antibiotic plate by the total bacteria count' (Lv *et al.* 2014).

The results show that multi-antibiotic resistance could be increased by the chlorination process on bacterial antibiotic resistance in biofilms of the pipeline. The resistances to Sul + Cla and Ery + Cla antibiotics were enhanced up to 13- and nine-fold, respectively. The resistance of all the pairs of the randomly combined antibiotics was significantly increased with the chlorination treatment process of the exposure groups compared with controls except the Gen + Cla antibiotics.

Richness and diversity of ARGs

An HT-qPCR-based method was used to detect the ARGs of the biofilm samples; 70 distinct ARGs were discovered, and the richness of these ARGs presented a different trend during the formation of biofilm (Figure 3(a)). A total of 21, 22 and 27 ARGs were discovered at the first, third and sixth months in the exposure group, respectively (Figure S2). This result indicated that the richness of the resistance genes increased during the formation of biofilm with chlorinated and dechlorinated influent, and the mobile genetic elements (MGEs) of the exposure group changed and increased during exposure of the cultures

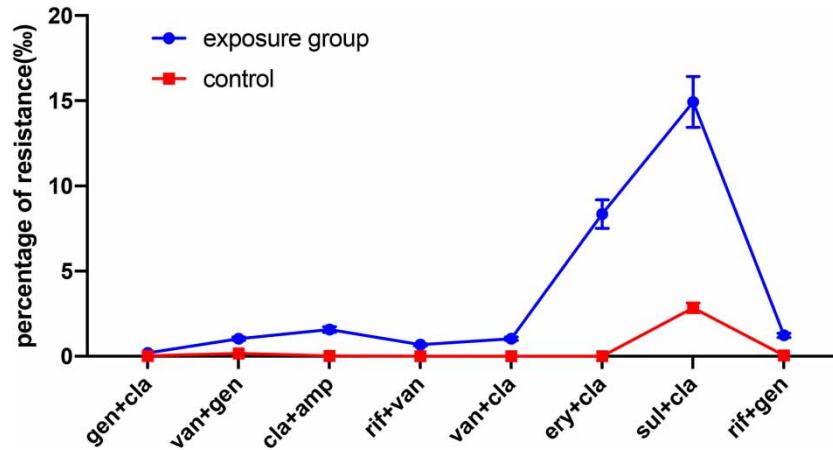


Figure 2 | The abundance of antibiotic resistant bacteria for multi-antibiotic resistance. All manipulations were performed in triplicate, and the error bars represent \pm SD.

(Table S2). However, the ARGs of control groups decreased gradually; a total of 45, 37 and 22 ARGs were detected in the controls at the first, third and sixth month, respectively (Figure S2, Table S2). These results show that chlorine disinfection treatment (without free chlorine) can evidently enhance the extent of resistance genes in the biofilms of a water distribution system during biofilm formation.

The ARGs that were detected in the exposure group included the three main resistance mechanisms (efflux pumps, antibiotic deactivation and cellular protection) (Figure 3(b), Table S2). Four ARGs of vancomycin, tetracycline and FCA were detected, respectively (Table S3). Only two ARGs (*sul2* and *dfrA12*) of sulfonamide, seven ARGs of aminoglycoside, nine of β -lactamase, ten of MLSB, and eight other/efflux ARGs were detected (Table S2). In addition, the diversity of aminoglycoside and β -lactamase of ARGs decreased in the first month but increased significantly in the third and sixth months ($p < 0.05$). However, the diversity of the controls decreased except for aminoglycoside of the ARGs (Table S2). The diversity of resistance genes detected in exposure biofilm samples that were classified based on the mechanism of resistance showed that only the deactivation of ARGs increased, and all the mechanism of resistance genes of the controls decreased during biofilm formation. Additionally, the diversity of ARGs of aminoglycoside, β -lactamase and transposons increased more significantly ($p < 0.05$) in the exposure groups, and only *int1* 1, *qacEdelta1-01* and *sul 2* were detected in every sample of the exposure groups. The ARGs of tetracycline were not detected in the initial sample but were detected in all pipeline bacterial biofilm samples after the chlorination and dechlorination treatment processes (Table S2).

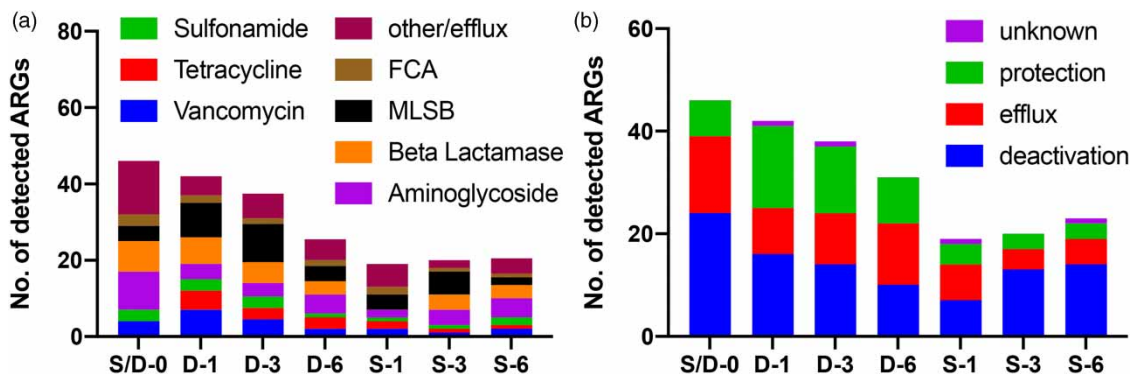


Figure 3 | Number of distinct resistance genes discovered in each sample. (a) antibiotic resistance gene detection statistics based on the antibiotic to which they conferred resistance; (b) the resistance genes discovered in each sample classified taking into account the mechanism of resistance. Biofilm bacterial sampling was conducted in triplicate. S is the exposure group sample; D is the control group sample; -1, -3 and -6 were obtained biofilm samples obtained from stage 2 of the pilot system at the first, third, and sixth months after chlorination treatment, respectively. S/D-0 was the sample from the stage 1 pipe biofilms without any treatment in the exposure and control groups.

Relative abundance of ARGs

Based on the results of HT-qPCR, the $2^{-\Delta\Delta CT}$ method was used to determine the relative abundance of ARGs (Lin *et al.* 2016). ARGs were enriched much more in the exposure group biofilm samples than in the controls (Figure 4). The sum of the enrichment of all unique genes that were detected in a sample was regarded as the total enrichment in the biofilm samples (Zhu *et al.* 2013). The enrichment of ARGs occurred in the exposure samples, demonstrating that the chlorination treatment can increase the concentration of ARGs. Moreover, *sul 2*, *qacEdelta1-01*, and *intl 1* increased significantly ($p < 0.01$), *intl 1* was the most enriched integron gene with an enrichment up to 5,401.6-fold compared with the initial control (Table S4 provides the enrichment details for all the genes detected). However, the abundance of ARGs in the control group changed dynamically. For example, the relative abundance of *tnpA-2* increased with culture time, *ere* (36) decreased, and *sul 2* remained almost the same.

Bacterial community structure during biofilm formation

Taxonomic analysis, which was employed to investigate the bacterial community composition, and the high-throughput sequencing (HTS) method were used to study the 16S rRNA gene of bacteria. The results indicated that *Proteobacteria* had the highest abundance on both of the exposure groups and control groups at the phylum level (Figure 5), but the abundance of *Proteobacteria* decreased from 70.81% (initial biofilm sample) to 26.09% (the sixth-month biofilm sample) in the exposure groups. The control groups were 25.64%, 40.29% and 30.97% at the first, third, and sixth month biofilm samples, respectively. This result shows that DBPs can shift the abundance of the phylum *Proteobacteria* during biofilm formation in the system, whereas other phyla, such as *Chloroflexi*, *Actinobacteria* and *Bacteroidetes*, had a relatively higher abundance in the exposure groups. However, the relative abundance of *Actinobacteria* and *Planctomycetes* increased considerably during

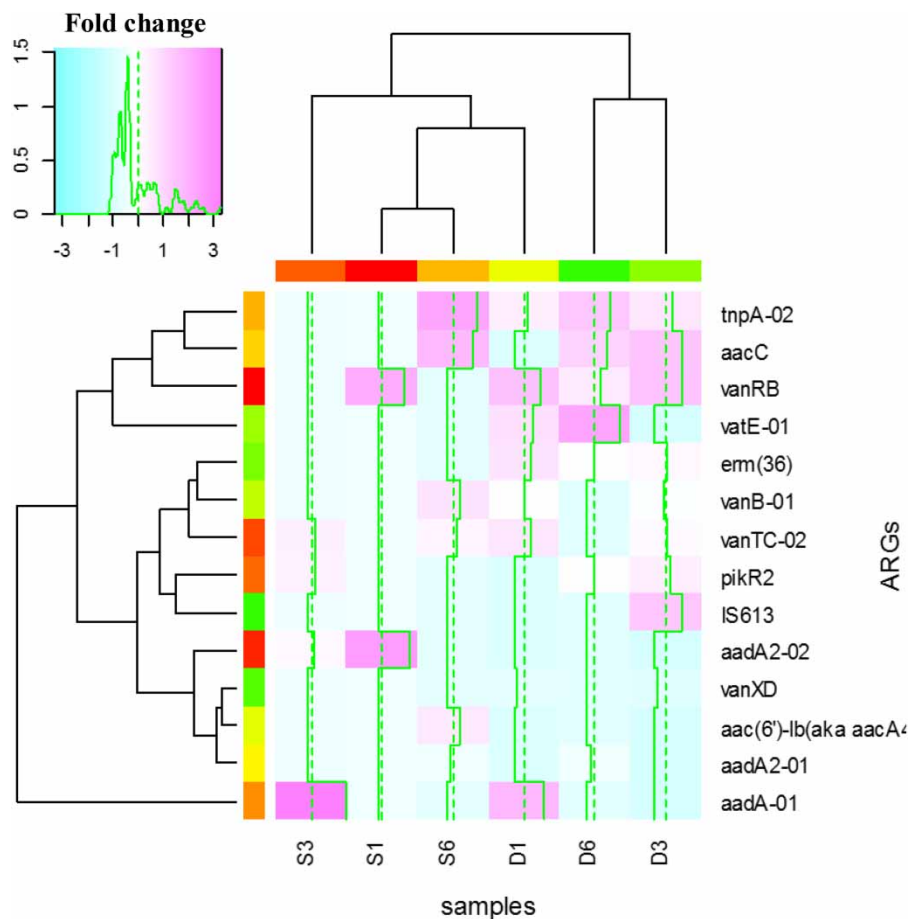


Figure 4 | Relative abundance of the antibiotic resistance genes in each sample. S is the exposure group sample; D is the control group sample; -1, -3 and -6 were the biofilm samples obtained from the pilot system at the first, third, sixth months after chlorination treatment, respectively.

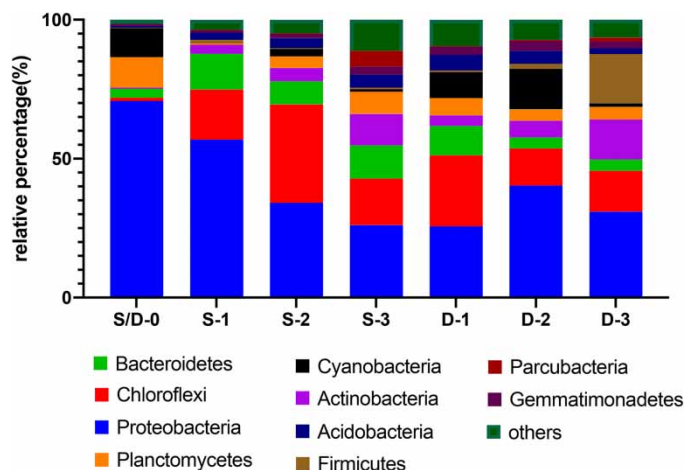


Figure 5 | Bacterial community composition as classified by RDP Classifier based on the average percentage of the top ten largest bacterial abundance at the phylum level. S is the exposure group sample; D is the control group sample; -1, -2 and -3 were biofilm samples obtained from the pilot system at the first, third, and sixth months after the chlorination treatment, respectively. S/D-0 was the sample from the phase 1 pipe biofilms without any treatment in exposure and control groups.

the culture time in the exposure group (Figure 5). Evaluating the diversity (Table 2) of the biofilm samples indicated that the DBPs might accelerate the generation of a greater variety of bacterial community composition in WDS, compared with the controls. The *Betaproteobacteria* and *Alphaproteobacteria* classes had a relatively higher abundance in all the biofilm samples, followed by the *Betaproteobacteria*, *Gammaproteobacteria*, *Anaerolineae* and *Actinobacteria*. However, the *Betaproteobacteria* and *Alphaproteobacteria* were the most abundant at class level in the exposure and control groups, respectively (Figure S3).

The bacterial diversities of the exposure group increased during the culture time (Table 2), and the taxonomic analysis showed that the composition of the bacterial community changed during biofilm formation and development. For example, the relative abundance of *Actinobacteria*, *Planctomycetes*, *Acidobacteria*, *Gemmatimonadetes*, *Parcubacteria* and *Chlamydiae* phyla (Figure 5), and the *Actinobacteria*, *Caldilineae*, *Deltaproteobacteria*, *OM190* and *Flavobacteriia* classes (Figure S3), increased considerably during the culture time in the exposure group. However, only the *Actinobacteria* and *Firmicutes* phyla and the *Actinobacteria* and *Bacilli* classes increased significantly in the controls, while in the exposure group, we found that the trends of the *Proteobacteria* phylum and the *Betaproteobacteria* class decreased markedly.

SEM images of the pipe network biofilm

The process details of the SEM analysis operation are described in the Supplementary Material (Text S2). The SEM profiles clearly showed the density of the biofilm in the simulated drinking water distribution system. Images were captured from

Table 2 | Diversity analysis of biofilm samples using the RDP pipeline

Sample	Shannon	Simpson	Chao 1	Shannoneven
S/D-0	3.88	0.059	663.85	0.61
S1	4.43	0.036	653.3	0.70
S3	4.81	0.026	848.39	0.73
S6	5.54	0.009	1,070.61	0.81
D1	5.34	0.013	1,013.19	0.79
D3	5.04	0.028	1,040.76	0.74
D6	4.95	0.033	1,080.36	0.71

S is the exposure group sample; D is the control group sample; -1, -3 and -6 were biofilm samples obtained from the pilot system at the first, third, and sixth months after chlorination treatment, respectively. S/D-0 was the sample from the phase 1 pipe biofilms without any treatment in the exposure and control groups.

various positions of each membrane surface, and a representative one was chosen. The membrane surfaces of the exposure group were much denser than those of the controls, and we found the bacterial morphology on the biofilm of the exposure group also differed from the control. The results indicated that the bacteria on the surface of biofilm in the experimental group were stunted, while those in the control group were smooth and slender (Figure 6).

The present study found that the concentration of bacteria can increase up to 10^7 CFU/cm² in the exposure treatment biofilms after chlorination without residual chlorine; however, the concentration of bacteria in the control group fluctuated highly (Figure S4). Biofilms are mainly composed of microorganisms and EPS, and EPS is mainly composed of polysaccharides, proteins and extracellular DNA (Flemming & Wingender 2001). Previous studies indicated that biofilm could grow on various pipe materials, and bacterial concentration reached 10^7 CFU/cm² in 30 days in chlorinated drinking water systems. Thus, our study results are consistent with those previously reported.

DISCUSSION

The resistance of all the pairs of the randomly combined antibiotics was significantly increased with the chlorination treatment process, the resistances to Sul + Cla and Ery + Cla antibiotics was enhanced up to 13- and nine-fold, respectively. And a total of 27 ARGs were discovered at the sixth month, and four ARGs of vancomycin were detected in this study. The diversity of resistance genes that have been discovered and proven could possibly confer resistance to the main classes of antibiotics, including vancomycin, aminoglycosides, β -lactam and macrolides. These antibiotics play a key role for human medical treatment (Zhu *et al.* 2013). Furthermore, vancomycin is considered as the final line of antibiotics in clinical life-saving procedures (Ziglam & Finch 2001).

Notably, previous studies indicated that chlorination can contribute to the enrichment of multiple antibiotic resistance and ARGs (Armstrong *et al.* 1982; Shi *et al.* 2013). Residual chlorine was regarded as an important contributing factor driving

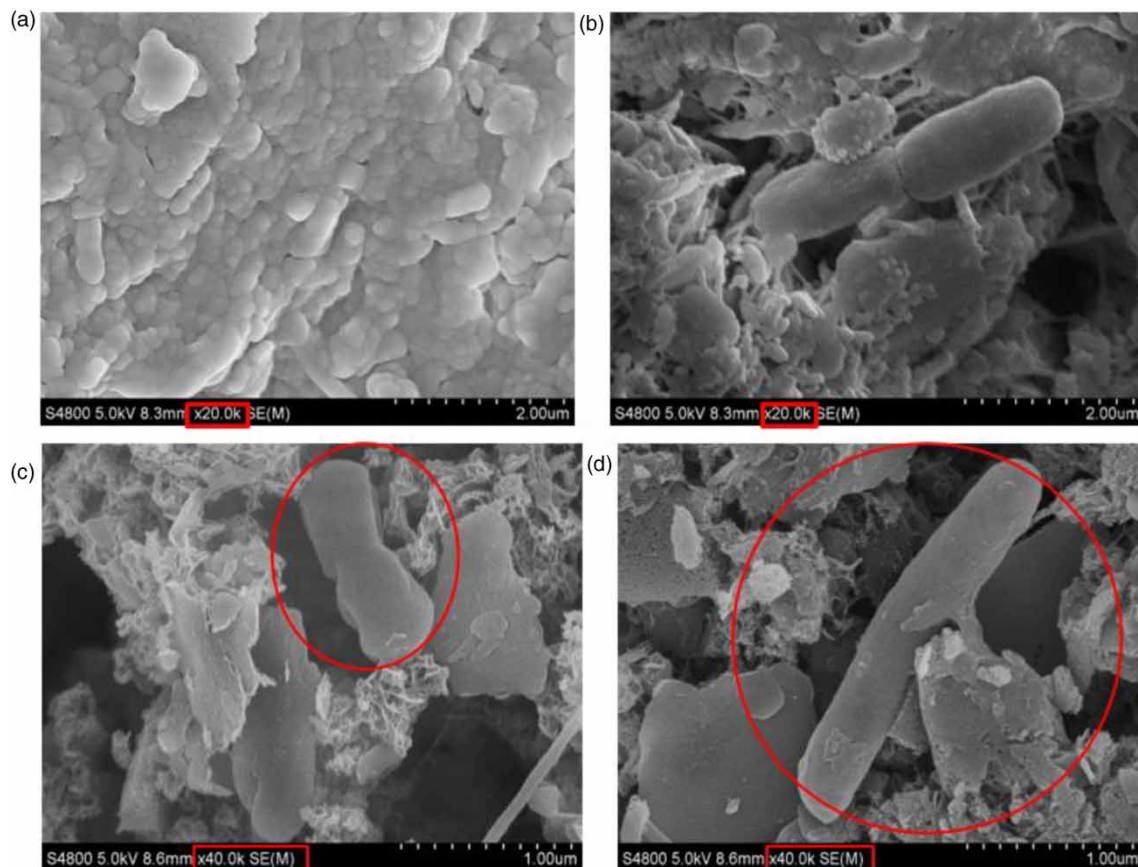


Figure 6 | SEM images of biofilm in the system: (a) and (c) are exposure group samples amplified at $\times 20.0$ k, $\times 40.0$ k, respectively; (b) and (d) are control group samples amplified at $\times 20.0$ k, $\times 40.0$ k, respectively.

multidrug resistance genes and affecting the antibiotic resistome (Jia *et al.* 2015). Chlorination could concentrate various ARGs and integrons involved in horizontal gene transfer (Shi *et al.* 2013); cross- or co-resistance of disinfectants may be the underlying mechanisms (Nakajima *et al.* 1995; Dukan & Touati 1996). In this study, biofilms of the experimental group emerge as much denser than those of the control group, which may help to bring useful assistance to the implanted biofilm bacteria and may enhance the ability of bacteria to overcome various stresses in WDS, such as DBPs, and other various environmental stresses (Kievit 2009), therefore, these conditions contributed to enhancing the richness of resistance genes in the biofilms of WDS during the formation of biofilms. In addition, the development of biofilms can facilitate the utilization of all available resources by the embedded bacteria, including nutrients and metabolic products, and even contribute to HGT within the complex biofilm composed of bacteria (Kievit 2009). Horizontal gene transfer through MGEs is also identified as the underlying mechanism contributing to the resistome formation (Caro-Quintero *et al.* 2011; Smillie *et al.* 2011). For example, *intI 1* was the most enriched integron gene with an enrichment up to 5,401.6-fold compared with the initial control in this study. Hence, the number of ARGs and the increase of the total relative abundance of ARGs can be explained.

Previous studies have found that MGEs may have relevance to human health (Eikmeyer *et al.* 2012), when considering the influence of chlorinated treatment on bacterial antibiotic resistance in pipeline biofilms. In addition, when the abundances of ARGs in biofilm bacterial samples are estimated, an abundance of MGEs is inevitably detected. Once there are MGEs that harbor ARGs in biofilm bacteria, with their high mobility and versatility they may contribute to the spread of ARGs among many biofilm bacteria and increase the abundances of ARGs. This study shows that *intI 1*, *qacEdelta1-01* and *sul 2* were detected in every sample of the experimental group, and *intI 1* was the most enriched integron gene with an enrichment up to 5,401.6-fold compared with the initial control. Previous studies have used the same detection methods indicating that *qacEdelta1-01* and *sul 2* were also the most enriched genes in Chinese swine farms (Zhu *et al.* 2013). Research by Guerin *et al.* (2009) indicated that exposure to antibiotics could enhance the over-expression of SOS-triggered *intI 1* integrase (Guerin *et al.* 2009). Thus, chlorination processes may contribute to the enrichment of integrons (Beaber *et al.* 2004), and further affect resistance in WDS.

Furthermore, chlorination evidently improves the relative abundance while reducing the diversity of bacteria (Jia *et al.* 2015). However, our results indicate an increase in the diversity of the bacterial community. Residual chlorine was regarded as having the most significant role in affecting bacterial communities (Jia *et al.* 2015). But the residual chlorine in this study has been removed. A previous study indicated that biofilms in WDS can cause pipe corrosion (Zhu *et al.* 2014), the draining of residual chlorine (Liu *et al.* 2012) and product taste and odor problems (Codony *et al.* 2005). Thus, the pressures of DBPs and biofilm conditions played the most important role in increasing bacterial diversity. The chlorination process not only changes genetics and may contribute to the development of biofilms, it also increases the stress resistance of bacteria (Laganenka & Sourjik 2017). At the phylum level, *Proteobacteria* and *Chloroflexi* represented the two most abundant genera. However, the relative abundance of *Proteobacteria* decreased considerably in the exposure treatment group with the time of culture. A previous report showed that *Proteobacteria* was also dominant in the WDS biofilms (Chao *et al.* 2015). In this study, several bacteria, such as *Actinobacteria* and *Planctomycetes*, also increased during biofilm cultivation. Although most of these bacteria have been determined in previous studies of drinking water distribution biofilms (Wang *et al.* 2014b; Chao *et al.* 2015), this study reported on the dynamics of these bacteria after chlorination without free chlorine during the formation of biofilm, which has not been previously reported to date.

However, the results cannot be taken as evidence that chlorinated DBPs caused the changes in antibiotic resistance and bacterial community composition. After the chlorination treatment, not only are DBPs generated, but also the feed water quality/composition varied. Nevertheless, results indicate that chlorination is likely to lead to an increased risk of antibiotic resistance of biofilm bacteria in the pipe networks.

CONCLUSIONS

This study investigated the effects of the chlorine disinfection process on bacterial antibiotic resistance in a water distribution system at a pilot-test level. The main findings of the study included the following: (1) chlorine disinfection can enhance bacterial multi-antibiotic resistance; (2) the total relative abundance of ARGs in biofilm samples increased significantly; (3) the diversity of the treated biofilm bacteria increased; (4) antibiotic deactivation was the main mechanism after chlorine disinfection. This experiment indicated that the chlorination process in a distribution system, such as the production of disinfection

by-products, may contribute to the increase in relative abundance of ARGs and MGEs in biofilm bacteria and contribute to the multiple resistance of biofilm bacteria in pipe networks. Overall, these findings are important for understanding the current state of antibiotic resistance and its relationship to the prevalence and diversity of ARGs in water distribution systems, and specifically, the mechanism of persistent ARB and ARGs. To our knowledge, this is the first study to verify that chlorine disinfection without free residual chlorine plays an important role in the prevalence of antibiotic resistance in water distribution systems.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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