The drinking water treatment process as a potential source of affecting the bacterial antibiotic resistance

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HIGHLIGHTS

- Bacterial antibiotic resistance rate increased as the water treatment progressed.
- Carbon filtration plays a key role in enhancing bacterial antibiotic resistance rate.
- Multidrug resistant bacteria were isolated and identified in processed water.
- Ozone, BAC and disinfection can greatly affect the community abundance.

GRAPHICAL ABSTRACT

ABSTRACT

Two waterworks, with source water derived from the Huangpu or Yangtze River in Shanghai, were investigated, and the effluents were plate-screened for antibiotic-resistant bacteria (ARB) using five antibiotics: ampicillin (AMP), kanamycin (KAN), rifampicin (RFP), chloramphenicol (CM) and streptomycin (STR). The influence of water treatment procedures on the bacterial antibiotic resistance rate and the changes that bacteria underwent when exposed to the five antibiotics at concentration levels ranging from 1 to 100 μg/mL were studied. Multidrug resistance was also analyzed using drug sensitivity tests. The results indicated that bacteria derived from water treatment plant effluent that used the Huangpu River rather than the Yangtze River as source water exhibited higher antibiotic resistance rates against AMP, STR, RFP and CM but lower antibiotic resistance rates against KAN. When the antibiotic concentration levels ranged from 1 to 10 μg/mL, the antibiotic resistance rates of the bacteria in the water increased as water treatment progressed. Biological activated carbon (BAC) filtration played a key role in increasing the antibiotic resistance rate of bacteria. Chloramine disinfection can enhance antibiotic resistance. Among the isolated ARB, 75% were resistant to multiple antibiotics. Ozone oxidation, BAC filtration and chloramine disinfection can greatly affect the relative abundance of bacteria in the community.

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1. Introduction

The emergence of bacterial antibiotic resistance is common in areas where antibiotics are used (Julian and Dorothy, 2010). The widespread use of antibiotics in medicine, intensive animal husbandry, industrial

settings and their release in wastewater treatment plants are main sources of the selective pressure exerted on bacteria (Marti et al., 2013, 2014; Schwartz et al., 2003; Sheetal et al., 2013). Because antibiotic selection pressures are so prevalent (Kemper, 2008; Kümmerer, 2003), the antibiotic resistance of pathogenic bacteria represents a global health problem that requires a better understanding of the fate of antibiotic-resistant bacteria (ARB) in water environments and their spread in the water supply system (Baquero et al., 2008; Huerta et al., 2013; Jiang et al., 2013; Ramanan et al., 2013; Timothy et al., 2011). The wide and excessive use of antibiotics has resulted in the pollution of several of the world's surface and ground water sources by antibiotics and antibiotic-resistance genes (ARGs) (Chigor et al., 2010; Machado and Bordalo, 2014; Martinez, 2009; Olusegun et al., 2009). The concentrations of tetracyclines (TCs), sulfa antibiotics, and chloromycetin (CAP) antibiotics in the Huangpu River, which supplies the city of Shanghai, China with some of its drinking wat-8()-411(Cw)4y1lia (2012tg11-395(4A0n)-15.mme
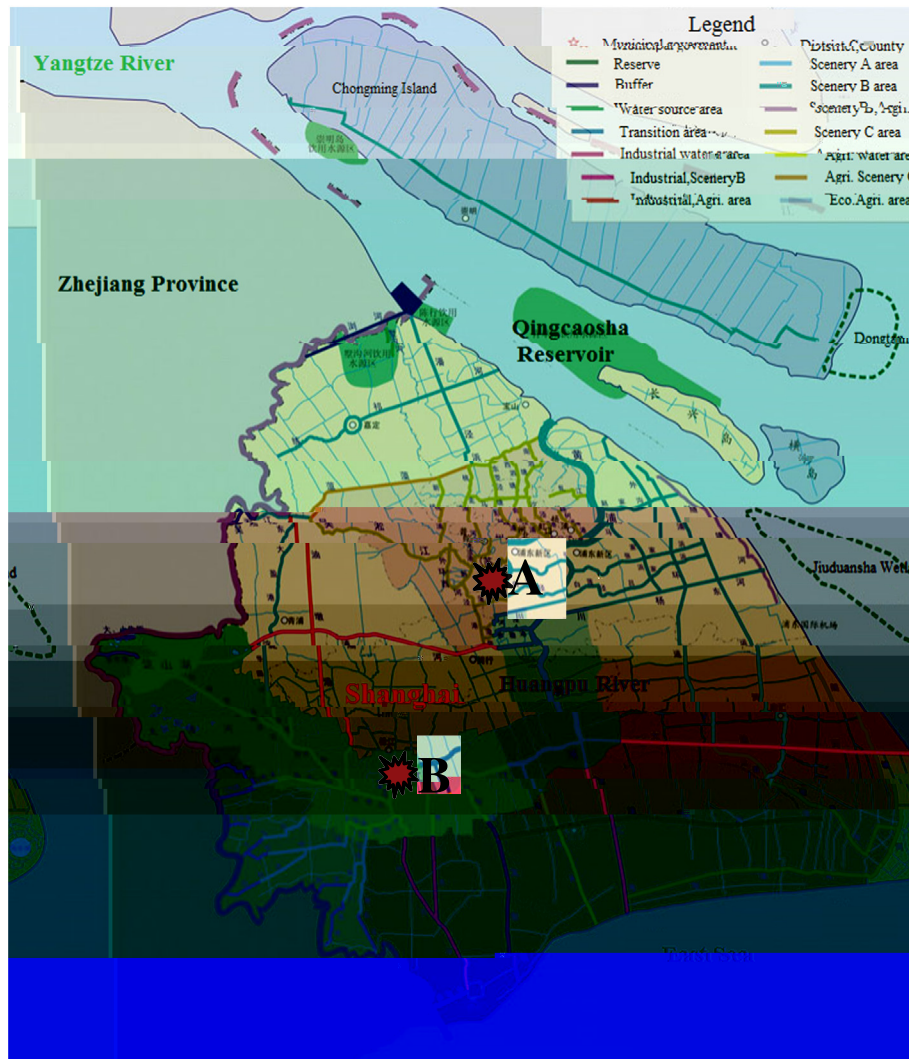
Fig. 1. Water sources and target waterworks distribution in Shanghai, China.

resistance rate was calculated as the ratio of the average number of incubated ARB to the average heterotrophic plate count.

The antibiotic resistance rate was calculated for medium that contained AMP, KAN, CM, RFP or STR at a concentration of 1 μg/mL, 2 μg/mL, 5 μg/mL, 10 μg/mL, 50 μg/mL or 100 μg/mL. Thus, the highest degree of bacterial antibiotic resistance and the influence of the water treatment process on bacterial antibiotic resistance were investigated using this method. Measurement of antibiotic sensitivity by the disk method was used to study the multi-drug resistance of the isolated

bacteria according to the 2013 CLSI M100-S23 performance standards for antimicrobial susceptibility testing.

2.4. Scanning electron microscopy (SEM) observation and biomass measurement

The activated carbon samples were washed 3 times using sterile water and air-dried on a UV-sterilized super-clean bench. The samples were then soaked in 2.5% precooled glutaraldehyde for 4 h at 4 °C and
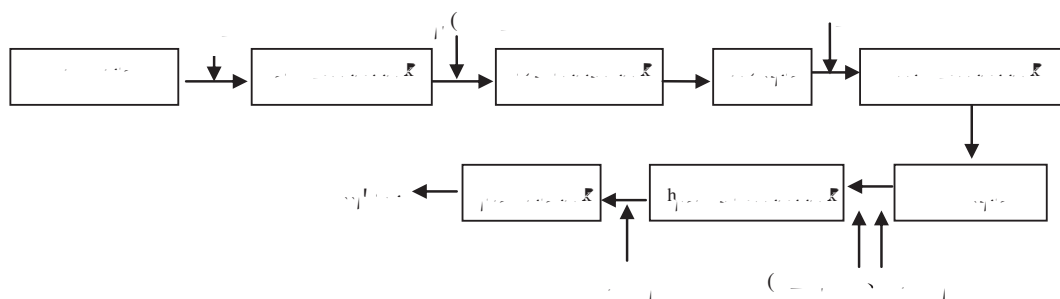

Fig. 2. Water treatment process in one of the target waterworks in Shanghai.

Table 1
Characteristics of raw water taken from Huangpu River and Qingcaosha Reservoir (mg/L).

Source	COD$_{Mn}$	COD$_{Cr}$	BOD$_5$	TP	TN	NH$_3$-N	Nitrate	Sulfate	Chloride	Fluoride
Yangtze River	1.93	8.83	0.93	0.05	1.75	0.07	1.51	38.25	34.67	0.29
Huangpu River	5.23	19.25	2.53	0.14	3.73	1.03	2.16	95.67	76.42	0.81

were sequentially immersed in 50%, 70%, 80%, and 90% ethanol for 30 min, and in 100% ethanol for 1 h. The samples were subsequently dried on a super-clean bench. After sputter coating, the samples were observed by SEM (FEI SIRION 200, USA).

The biomass on the surface of the activated carbon was examined by measuring the phospholipids in the biological membranes of the cells (Findlay et al., 1989).

2.5. DNA extraction and high-through sequencing of the environmental metagenome

Because there is low biomass present in processed drinking water, a 0.22-μm microporous membrane (Millipore, USA) was used to concentrate bacterial cells from 4-L water samples and mixed these cells with purified water (30 mL). The membrane attached bacterial cells were separated using ultrasound oscillation for 30 min at 53 kHz, and the filtered materials were then used for total genomic DNA extraction with a Water DNA Kit (OMEGA, Bio-Tek, Doraville, GA, USA). The DNA concentration and purity were measured by microspectrophotometry (NanoDrop_ND-2000, NanoDrop Technologies, Wilmington, DE).

Next generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, Inc. (Beijing, China). DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and the quality of the DNA was assessed on a 0.8% agarose gel. Sequencing libraries were constructed using the MetaVx™ Library Preparation Kit (GENEWIZ, Inc., South Plainfield, NJ, USA). Briefly, 5 to 50 ng of DNA was used to generate amplicons that covered the V3, V4, and V5 hypervariable regions of bacterial and Achaea16S rDNA. Indexed adapters were added to the ends of the 16S rDNA amplicons using limited-cycle PCR. The DNA libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified using Qubit and real time PCR (Applied Biosystems, Carlsbad, CA, USA). The DNA libraries were then multiplexed and loaded on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Sequencing was performed using a 2 × 250 paired-end (PE) configuration; image analysis and base calling were conducted using the MiSeq Control Software (MCS) on the MiSeq instrument. Initial taxonomy analysis was carried out on an Illumina BaseSpace cloud computing platform.

The isolated multidrug resistance bacteria in the effluent of waterworks were identified based on the analysis of the 16S rDNA sequencing, using primers 27F and 1492R. The nucleotide sequences with about 1400 bp were used for BLAST DNA homology searches with the DNA database from the internet address: http://www.ncbi.nlm.nih.gov.

2.6. Data analysis

The QIIME data analysis package was used for 16S rDNA data analysis. The forward and reverse reads were joined using 'make.contig' from the Mothur software package. Quality filtering was performed on the joined sequences, and sequences that did not fulfill the following criteria were discarded.

After quality filtering, a total of 662,746 sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program UCLUST against the Greengenes 13_8 database pre-clustered at 97% sequence identity. The Ribosomal Database Project (RDP) classifier was used to assign a taxonomic category to all OTUs at a confidence threshold of 0.8. The RDP classifier uses the 16SrRNA RDP database, which has taxonomic categories predicted to the genus level.

3. Results and discussion

3.1. The influence of raw water quality on bacterial antibiotic resistance

When the raw water was polluted by antibiotics, antibiotics can exist in all processed water and form a selective pressure for ARB (Guo et al., 2014; Xi et al., 2009). Table 1 showed the characteristics of raw water taken from the Huangpu River and the Qingcaosha Reservoir. It can be found that water quality in the Qingcaosha Reservoir is better than that in the Huangpu River. Some investigations (Davison, 2013; Shen et al., 2012) showed that various antibiotics were existed in the Huangpu River. Fig. 3 showed the antibiotic resistance rates of bacteria from the waterworks effluent from the Huangpu River or the Qingcaosha Estuary Reservoir against the five types of antibiotics studied. The five types of antibiotics and six concentration levels were used for plate incubation and counting. The results indicated that different raw water sources had different antibiotic contamination characteristics and exhibited different effects on the antibiotic resistance of bacteria from the waterworks effluent. Compared with the bacteria from the waterworks effluent from the Qingcaosha Estuary Reservoir, bacteria from the waterworks effluent from the Huangpu River exhibited a higher antibiotic resistance rate to AMP, STR, RFP, and CM but lower antibiotic resistance rate to KAN. This result suggested that the Huangpu River was polluted by antibiotics more severe than the Qingcaosha Estuary Reservoir and KAN was present in the Huangpu River basin at relatively low levels. The ARB isolated from the effluents also showed their great adaptability to such selective pressure as chloramine disinfection in water treatment process. In this investigation, although absolute values of antibiotic resistance rates were low and the ARB identified might not directly pose a health threat, the potential risk of human damage did exist.

3.2. The influence of water treatment process on bacterial antibiotic resistance rate

When antibiotic concentrations were maintained within a range of 1 to 10 μg/mL, the bacterial antibiotic resistance rates increased in drinking water as the water treatment process progressed (Fig. 4). There was
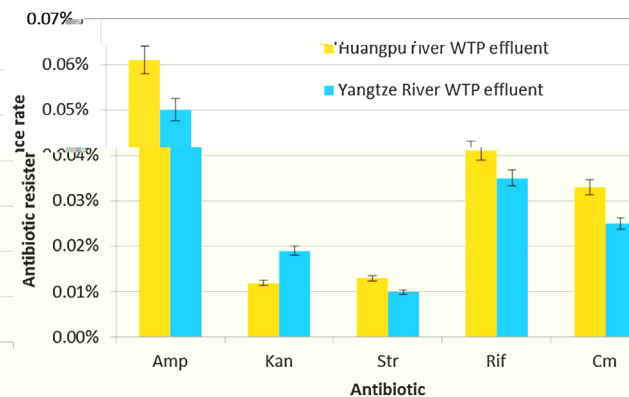
Fig. 3. Average antibiotic resistance rates of bacteria under all six concentration levels in effluents from Huangpu River and Qingcaosha Reservoir waterworks.
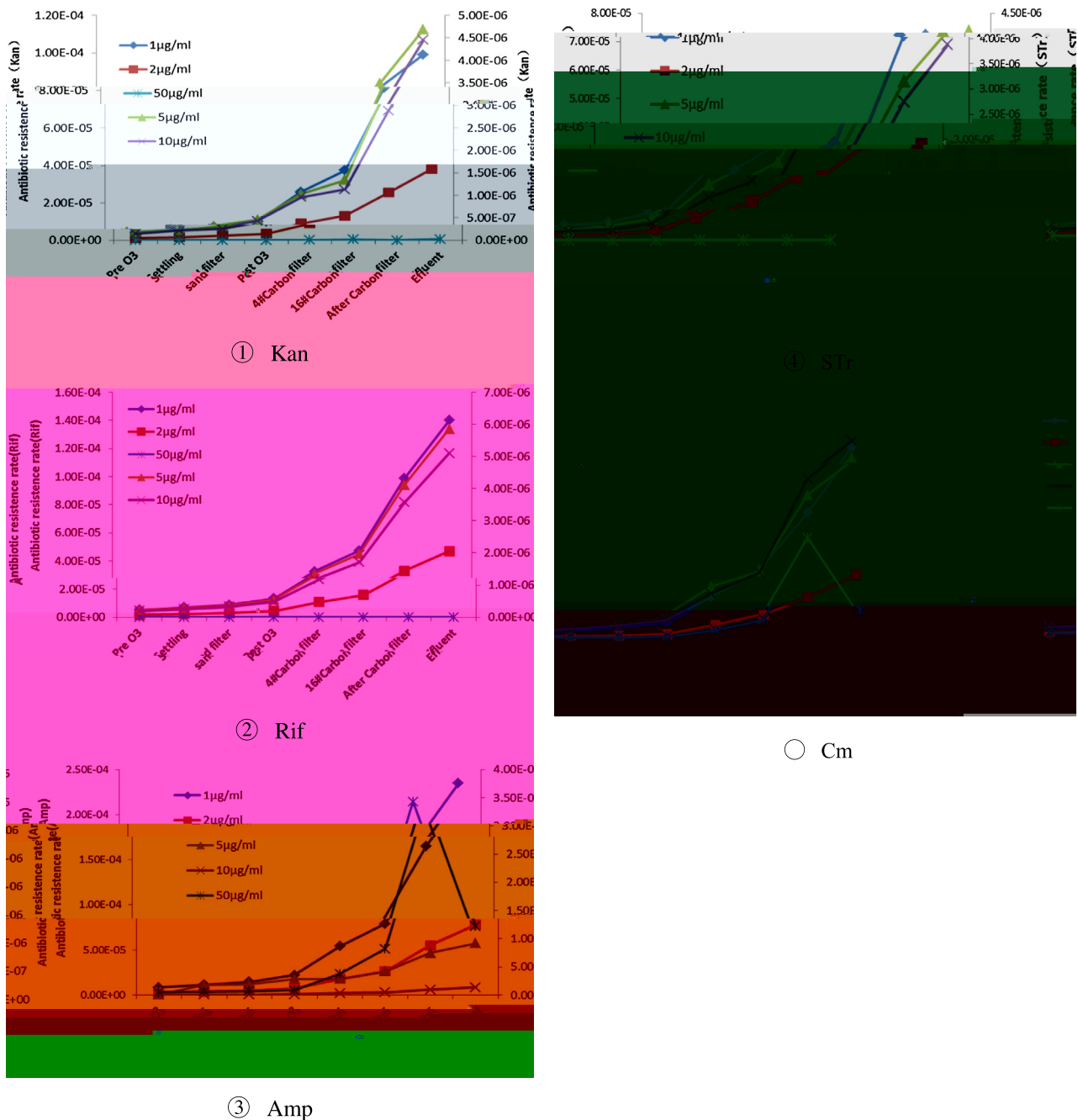
Fig. 4. The influence of water treatment procedures on the antibiotic resistance rates of bacteria at various antibiotic concentrations for different antibiotics.

a particularly significant increase after BAC filtration procedure, during which a large amount of microbes aggregate. Broken carbon particle filtration contributed to the antibiotic resistance rate to a great extent than broken carbon column filtration. The chloramine disinfection procedure also had a large effect on bacterial antibiotic resistance. In contrast, the antibiotic resistance rate did not significantly increase after physical or chemical treatment procedures such as coagulation, sedimentation or sand filtration. As the antibiotic concentration increased, and particularly when the concentration exceeded 10 μg/mL, nearly all of the bacteria were inhibited or killed and were not able to reproduce on the R2A medium.

The occurrence of antibiotic and disinfectant resistance and its spread to bacteria in drinking water have great significance for public health. The data in Fig. 4 showed that BAC filtration played a key role on enhancing bacterial antibiotic resistance and bacterial antioxidant capacity during chloramine disinfection in the drinking water treatment process.

In this study, a large number of bacteria attached to the surface of the activated carbon and formed a biofilm (Fig. 5) and the biomass on the surface of broken carbon particles is larger than that on broken carbon column (Fig. 6). A corresponding phenomenon that broken carbon particle filtration contributed more to the antibiotic resistance rate than broken carbon columns filtration was also observed as shown in Fig. 4. In the water supply system, the biofilm on activated carbon was an ideal environment for possible dynamic sharing of the genetic elements responsible for antimicrobial resistance between different bacterial species (Juhas et al., 2009). After the BAC treatment procedure, a higher number of bacteria may acquire antibiotic resistance, which would presumably occur through the horizontal transfer of ARGs (Schwartz et al., 2003; Xi et al., 2009; Zhang et al., 2009).
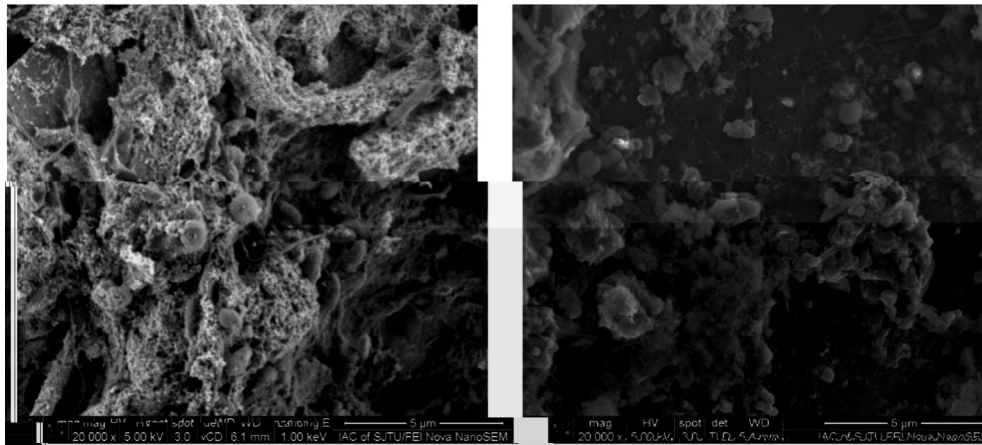
Fig. 5. Bacteria attached on the activated carbon. Particle broken carbon (16#, left); column broken carbon (4#, right).
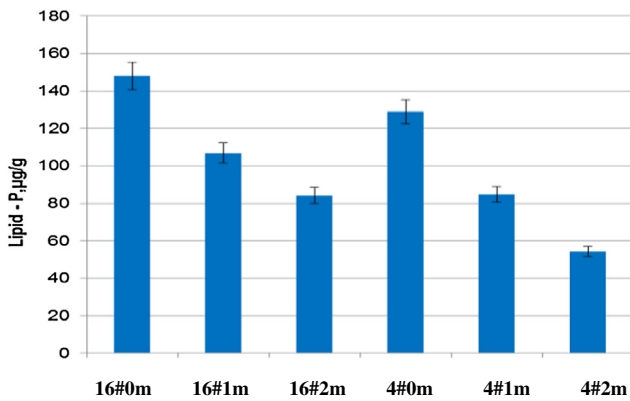

Fig. 6. Biomass attached on the surface of activated carbon. Particle broken carbon (16#); column broken carbon (4#).

There were many selective pressures in this process, including pre-ozone oxidation, post-ozone oxidation and chloramine disinfection, which may enhance bacterial antibiotic resistance. Molecular biology techniques have been adopted to investigate the influence of free chlorine disinfection in waterworks on the antibiotic resistance of bacteria in water (Huang et al., 2013; Shi et al., 2013; Shrivastava et al., 2004). Previous results indicated that the numbers of ARB, ARGs

and the relative abundance of mobile genetic elements increased through chlorine disinfection. Their results were well matched with our studies as shown in Fig. 4. Because of antibiotic-resistant and possible dnd antioxidant genes (Xie et al., 2012), ARB can have a complete structure and cannot be easily destroyed by antibiotics, oxidants and disinfectant compared with general bacteria. Therefore, ARB can survive under several selective pressures throughout the water treatment process.

3.3. Multi-drug resistance analysis

Assessment of antibiotic sensitivity using the disk method was adopted for multidrug-resistance analysis. Test papers that were soaked in a 5 μg/mL solution of each of the five antibiotics were patched onto a plate of ARB that had been screened and incubated. Each multi-drug resistance analysis for a bacterial was repeated three times. The resistance of the bacteria to the various antibiotics was determined according to the 2013 CLSI standards for antimicrobial susceptibility testing.

As shown in Fig. 7, 16 types of ARB were isolated and identified by 16S rDNA sequencing in the effluent of waterworks. They mainly belonged to the phylum of Proteobacteria and Firmicutes. The numbers of ARB that were resistant to one, two, three, four, or five types of antibiotics were 4, 6, 3, 2, and 1, respectively. Furthermore, 75% of the ARB was multi-drug resistant. A strain of Bradyrhizobiaceae sp. was even
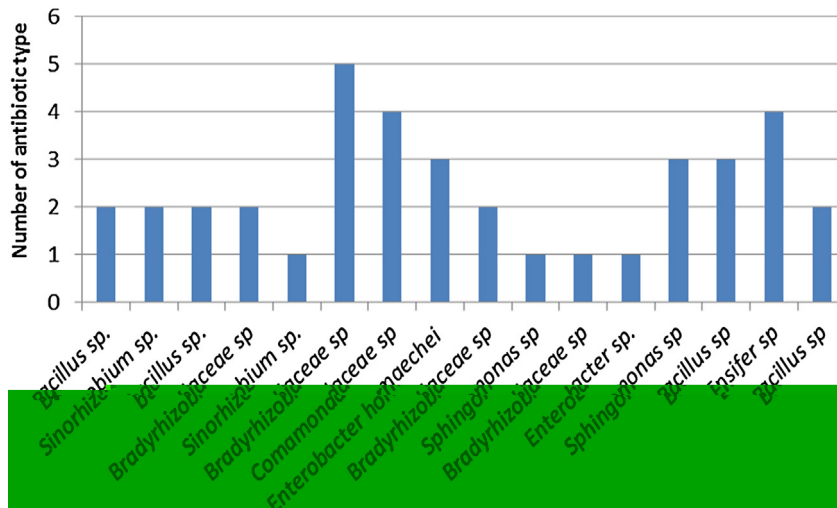

Fig. 7. Isolated antibiotic resistant bacteria and their multidrug resistance counts.

resistant to all five types of antibiotics. Although the ARB was only resistant to antibiotics at a concentration of 10 μg/mL or less, multi-drug resistance bacteria were present in Shanghai's drinking water. These phenomena have been observed in drinking water produced in karstic hydrosystems (Ribeiro et al., 2014) or treated by chlorination (Shrivastava et al., 2004).

3.4. Effec of a e ea men oce on he bac e ia me agenome

As Fig. 4 showed, BAC filtration and chloramine disinfection had a significant effect on the bacterial antibiotic resistance rates in processed water. To get more about the viable but non-culturable bacterial information with related to antibiotic resistance in drinking water before and after BAC filtration and chloramine disinfection, metagenomic analysis was conducted via 16S rRNA high-throughput sequencing. As Fig. 8 showed, in all four samples sourced from the Huangpu River, before and after BAC filtration and after chloramine disinfection, the dominant bacteria in the community at the phylum level were *P o eobac e ia*, *Ac inobac e ia*, *Bac e oide e* , *Ve comic obia*, *Planc om ce e* , and *Acidobac e ia*. *P o eobac e ia* and *Ac inobac e ia* accounted for the majority of the community. According to Lang's research, there were widespread gene transfer agent genes in α-proteobacteria (Lang and Beatty, 2007). Ozone oxidation, BAC filtration and chloramine disinfection greatly affected the relative abundance of bacteria in the community but did not greatly affect taxonomic composition, as shown in Table 2. This is different from Pinto's research in which the authors thought that bacterial community structure in the drinking water microbiome is governed by filtration processes (Pinto et al., 2012). *P o eobac e ia* and *Ac inobac e ia* were both sensitive to ozone oxidation and chloramine disinfection, after which the abundance of *P o eobac e ia* and *Ac inobac e ia* greatly increased and decreased respectively. Thus, *P o eobac e ia* showed a stronger antioxidant capacity than did *Ac inobac e ia*. In contrast, *Bac e oide e* was not sensitive to ozone oxidation and chloramine disinfection. The BAC filter could have greatly increased the abundance of *Ac inobac e ia*. *Ac inobac e ia* can always produce certain antibiotic products. Many

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