Science of the Total Environment 533 (2015) 24–31

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

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

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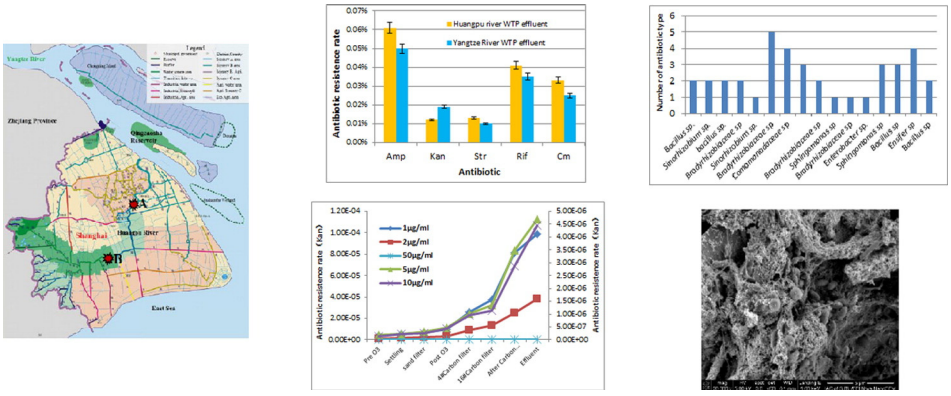
HIGHLIGHTS

- 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



ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 21 June 2015
Accepted 21 June 2015
Available online xxxx

Editor: A. Covaci

Keywords:
Drinking water
Water treatment process
Antibiotic resistance bacteria (ARB)
Biological activated carbon
Multidrug resistance

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

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http://dx.doi.org/10.1016/j.scitotenv.2015.06.082
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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 water, are in the range of 0.44 to 2.69 $\mu g \cdot L^{-1}$, 0.97 to 1.96 $\mu g \cdot L^{-1}$ and 0.03 to 0.26 $\mu g \cdot L^{-1}$ respectively (Shen et al., 2012). In early March 2013, over 16,000 dead pigs were found in this river; the pigs had been dumped by farmers from neighboring Jiaxing, Zhejiang Province, a major pig-farming area upstream of Huangpu River where antibiotics were used very popular for livestock (Davison, 2013). Thus, the aquatic ecosystem has been considered to be a critical source of ARB and ARGs (Tao et al., 2010; Zhang et al., 2009). The current water treatment process cannot entirely eliminate the existing antibiotics in the drinking water (Figueira et al., 2011; Guo et al., 2014; Laroche et al., 2010; Xi et al., 2009). Moreover, horizontal transmission of ARGs among microbes in the water supply system could facilitate the emergence and dissemination of bacterial antibiotic resistance in drinking water and influence human health (Ribeiro et al., 2014). However, there have been no systematic investigations examining the influence of different water treatment processes on bacterial antibiotic resistance, particularly in high-microbe-density environments, including biological activated carbon (BAC) and during oxidizing processes such as ozone oxidation and chloramine disinfection. These processes are crucial for the control of microbiological risk in drinking water.

Shanghai is the largest international metropolis in China, and Huangpu River is the primary source of drinking water in Shanghai. However, because of water pollution in the Huangpu River, the Qingcaosha Reservoir, which is the largest estuary and river reservoir in the world and which draws its water from the Yangtze River, has replaced a large portion of Shanghai's water source. This reservoir is located at the estuary, where various pollutants including certain antibiotics, may accumulate from the upstream cities' discharge. These two water sources are both facing antibiotic and ARG pollution and deserve more attention as a public health concern.

The current study compared the antibiotic resistance rates of bacteria in effluents from Shanghai waterworks using two main water sources and investigated the influence of water treatment procedures on bacterial antibiotic resistance using the following five antibiotics: ampicillin (AMP), kanamycin (KAN), rifampicin (RFP), chloramphenicol (CM), and streptomycin (STR). The multidrug resistance of isolated ARB was tested and the influence of the water treatment process on the environmental water metagenome was also analyzed using 16S rDNA high-throughput sequencing.

2. Materials and methods

2.1. Water sampling

As Fig. 1 showed, all water samples were taken from the effluent of each treatment process unit in two waterworks (A and B) that respectively used the Qingcaosha Estuary Reservoir or the Huangpu River for source water in 2013 spring and summer (March to August). All the samplings were under the help of the waterworks' engineers. The samples were taken once a month. All the sampling procedures met the standard examination methods for drinking water-collection and preservation of water samples (GB/T 5750.2-2006). 10 L water was collected at each site for antibiotic resistance bacteria isolation and DNA extraction. All water samples were stored in 2.5 L pre-cleaned and sterilized glass bottles, maintained at ice boxes and transported to the laboratory at once by a car. The water treatment process involved pre-ozonization, coagulation and sedimentation, sand filtration, post-ozonization, BAC filtration and chloramine disinfection, as shown in Fig. 2. In the BAC unit, two different types of activated carbon were applied: broken activated carbon particles used in 16# carbon filter and broken activated carbon columns in 4# carbon filter. We set 8 sampling points along the process as introduced above.

2.2. Antibiotics selection

Five popular used antibiotics in human health treatment and animal farms (AMP, KAN, RFP, CM and STR) were selected for this study. AMP is broad-spectrum semisynthetic penicillin that has low toxicity. The antibacterial spectrum of AMP is similar to that of penicillin. *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Proteus*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* are sensitive to KAN, whereas *Pseudomonas aeruginosa*, Gram-positive bacteria (except *S. aureus*), anaerobes, atypical mycobacteria, *Rickettsia*, fungi and viruses are resistant to KAN. RFP can effectively kill *M. tuberculosis* and several of the nontuberculous mycobacteria inside and outside host cells. CM can inhibit the growth of Gram-positive and Gram-negative bacteria, and the inhibitory effect on Gram-negative bacteria is comparatively substantial. STR exerts a strong antibacterial effect on *M. tuberculosis*; conversely, nontuberculous mycobacteria are resistant to STR. There are many animal farms in the upstream watershed of Huangpu River, and the antibiotics like CM and STR were popular to be used for animal breeding. The bacteria affected by these five antibiotics include the primary bacteria that exist in drinking water.

2.3. ARB isolation and calculation of the antibiotic resistance rate

The steps for screening for ARB were as follows, using AMP as an example:

(a) An antibiotic solution was prepared by dissolving 50 mg of AMP in 10 mL of water; the concentration of the solution was thus 5 mg/mL. The colorless, transparent solution was then distributed into 10 tubes (1 mL per tube).
(b) A 1-L volume of R2A agar medium was prepared. The medium was heat sterilized under 121 °C and 15 min and cooled to a moderately warm temperature. AMP was then added to the medium, and thoroughly mixed. The medium was poured into plates and stored at room temperature overnight.
(c) A water sample extracted from the water treatment plant was filtered using a 0.22-μm filter membrane, which was then cut into pieces and placed in a 2-mL microcentrifuge tube. Then 2 mL of a phosphate buffer solution was added to the microcentrifuge tube, which was subsequently vortexed for 5 min at high speed.
(d) A general R2A medium-coated plate without antibiotics was employed for comparison with the medium-coated plate containing 5 μg/mL antibiotics. The later plate was inoculated and spread with the vortexed water sample, followed by incubation for 7 days at 28 °C. Each sample for ARB isolation at each antibiotic concentration level was repeated three times.
(e) The antibiotic resistance rate was calculated as follows.

Based on the aforementioned steps, ARB were counted and isolated on the medium-coated plates containing antibiotics. The antibiotic

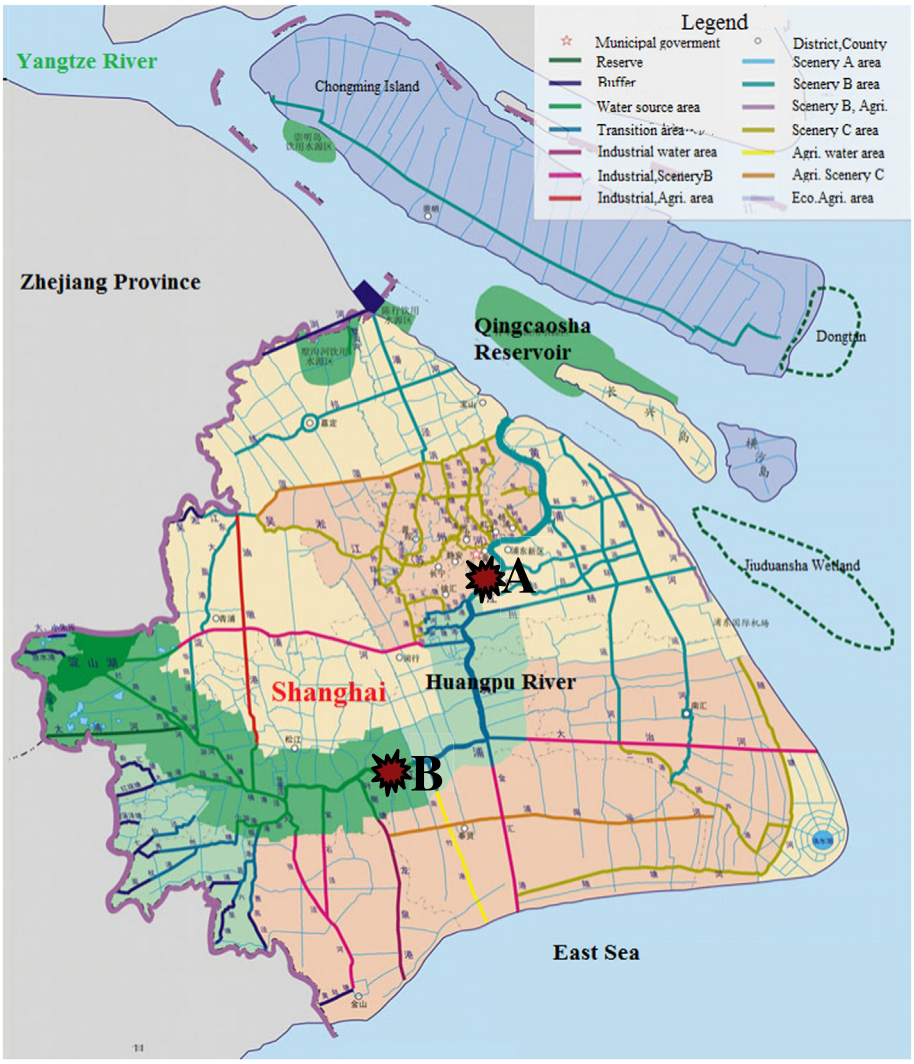

Fig. 1. Water sources and target waterworks distribution in Shanghai, China.

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

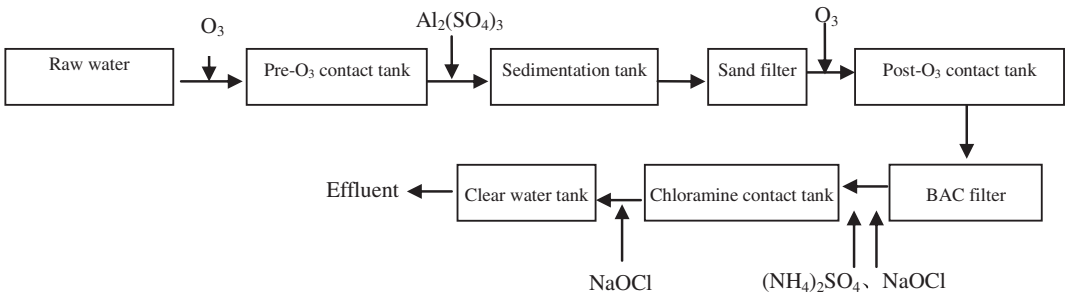

Fig. 2. Water treatment process in one of the target waterworks in Shanghai.

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-throughput 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 *Archaea*16S 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.contigs*' 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 procedures on bacterial antibiotic resistance rates

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

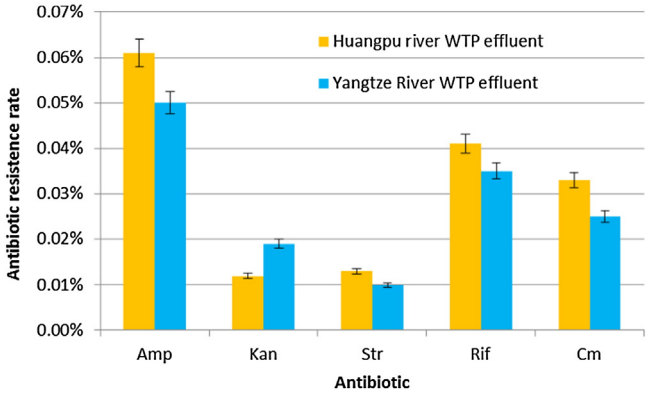

Fig. 3. Average antibiotic resistance rates of bacteria under all six concentration levels in effluents from Huangpu River and Qingcaosha Reservoir waterworks.

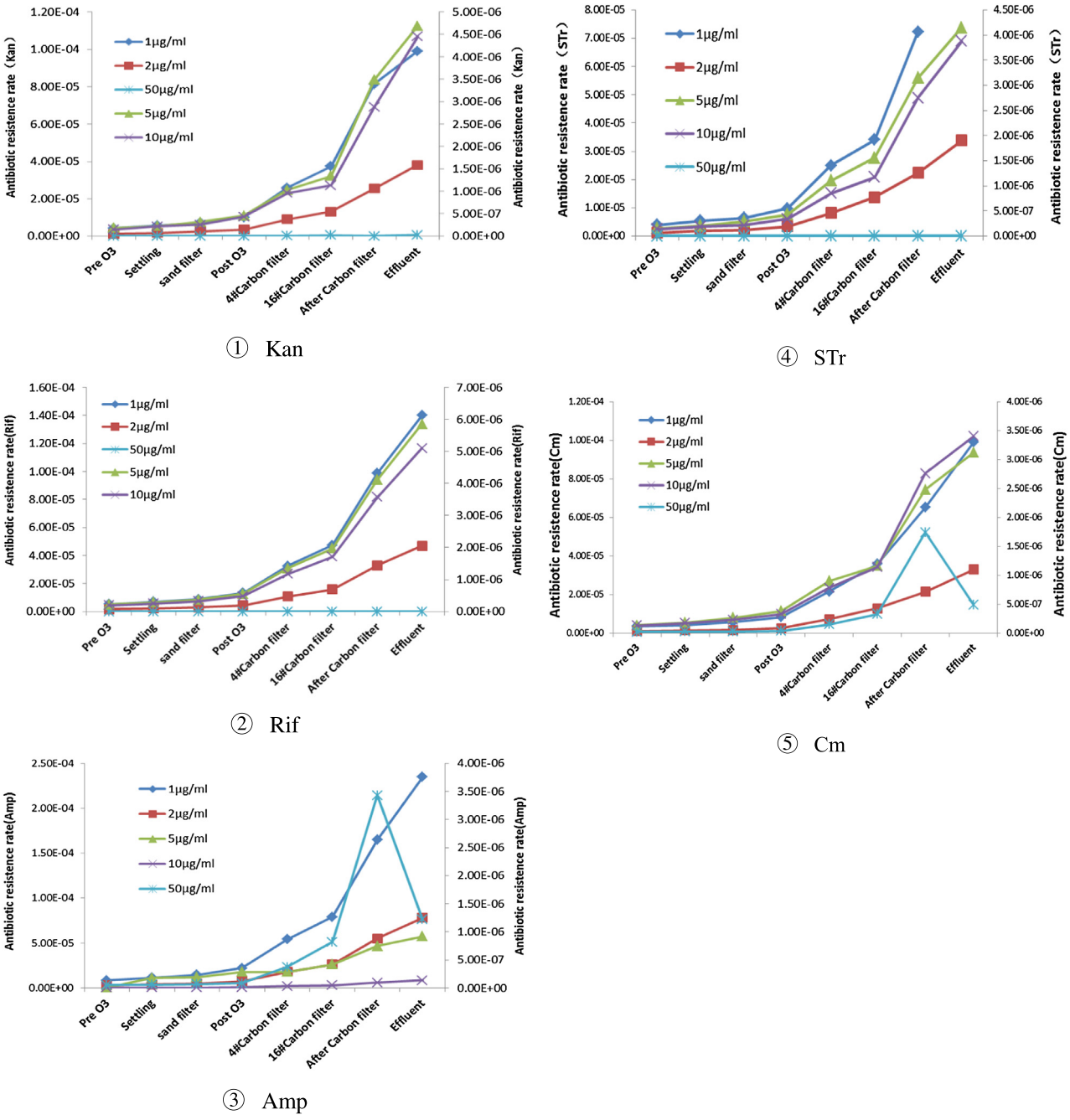

Fig. 4. The influence of water treatment procedures on the antibiotic resistance rates of bacteria at various antibiotic concentrations for different antibiotics.

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).

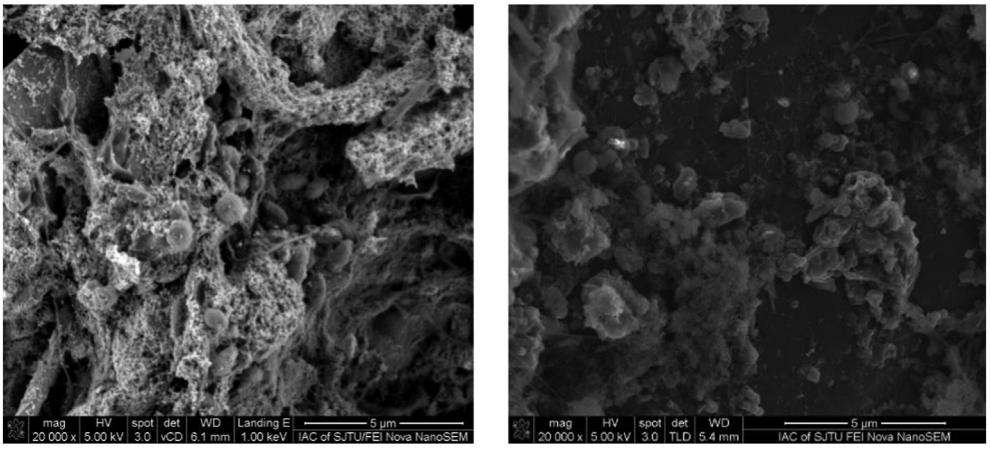

Fig. 5. Bacteria attached on the activated carbon. Particle broken carbon (16#, left); column broken carbon (4#, right).

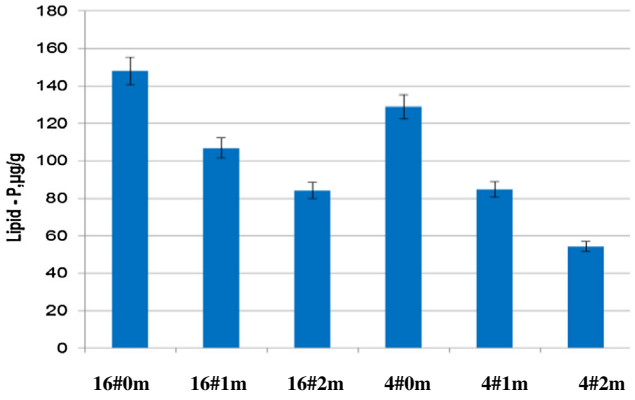

Fig. 6. Biomass attached on the surface of activated carbon. Particle broken carbon (16#); column broken carbon (4#).

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

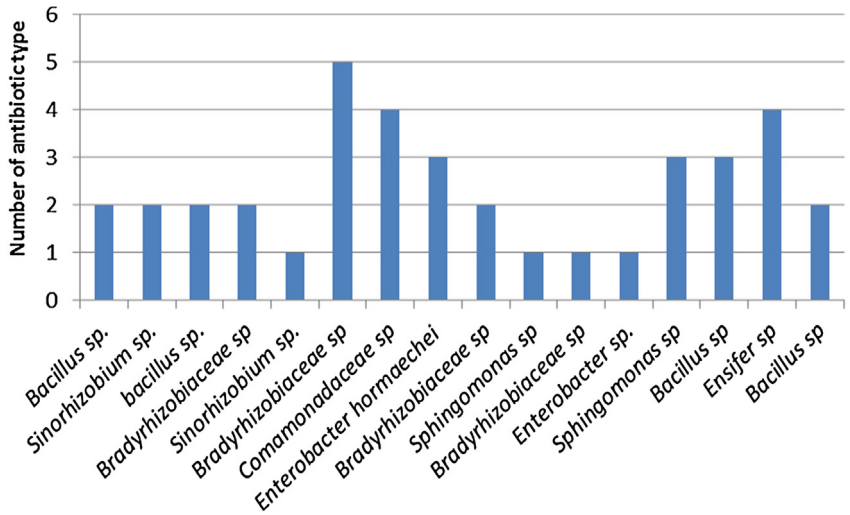

Fig. 7. Isolated antibiotic resistant bacteria and their multidrug resistance counts.

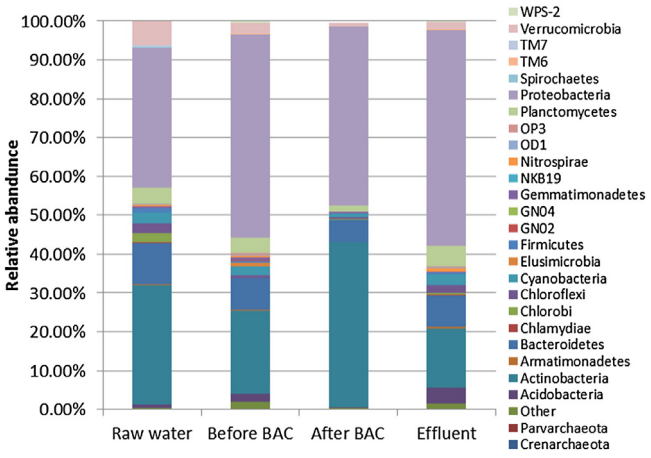

Fig. 8. Variation of bacterial community composition along the water treatment process.

resistant to all five types of antibiotics. Although the ARB was only resistant to antibiotics at a concentration of 10 μg/mL or less, multidrug 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. Effects of water treatment process on the bacteria metagenome

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 *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes*, and *Acidobacteria*. *Proteobacteria* and *Actinobacteria* 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). *Proteobacteria* and *Actinobacteria* were both sensitive to ozone oxidation and chloramine disinfection, after which the abundance of *Proteobacteria* and *Actinobacteria* greatly increased and decreased respectively. Thus, *Proteobacteria* showed a stronger antioxidant capacity than did *Actinobacteria*. In contrast, *Bacteroidetes* was not sensitive to ozone oxidation and chloramine disinfection. The BAC filter could have greatly increased the abundance of *Actinobacteria*. *Actinobacteria* can always produce certain antibiotic products. Many other types of bacteria that were chloramine- and antibiotic-resistant existed in the effluent from the waterworks with Huangpu River source water.

Because of surface water pollution in China, the number of drinking water treatment plants that employ ozone-BAC has increased greatly. In the near future, all Shanghai waterworks will upgrade their traditional water purification processes by adding ozone and BAC for better drinking water quality. However, the surface on BAC and the biofilms in water distribution systems are ideal environments for large amounts of microbe to aggregate and transfer antibiotic-resistant genes that could result in multidrug resistant bacteria. Although more supporting evidences and data are required for these potential impacts of BAC on antibiotic resistance in drinking water, process designers and water authority managers should start to pay attention to the balance of positive and negative functions of biological activated carbon filtration and should conduct further investigations to deepen their understanding to the function of BAC.

Table 2
The changes of taxonomic composition in Minhang water treatment process.

Samples	Phylum	Class	Order	Family	Genus
Raw water from Huangpu River	26	58	108	186	358
Water before BAC filter	22	51	95	165	320
Water after BAC filter	24	49	92	153	300
Effluent	24	50	95	161	307

4. Conclusions

Compared with the bacteria from effluent from the Qingcaosha Reservoir waterworks, the bacteria from the Huangpu River waterworks exhibited stronger antibiotic resistance against AMP, STR, RFP and CM but weaker antibiotic resistance against KAN. When the concentration of antibiotics was in the range of 1 to 10 μg/mL, the antibiotic resistance rates of bacteria in the water increased as the water treatment process progressed. In the drinking water treatment process, BAC filtration plays a key role in affecting bacterial antibiotic resistance. Multi-drug-resistant bacteria were isolated and identified from the processed drinking water. Ozone oxidation, BAC filtration and chloramine disinfection can greatly affect the relative abundance of bacteria in the community.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (J 1210047), the Key Project of the Shanghai Science and Technology Commission (12231201703, 13231202904) and Shanghai Water Authority(2013-03).

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