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Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water

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ABSTRACT

This study aimed to investigate the chlorination effects on microbial antibiotic resistance in a drinking water treatment plant. Biochemical identification, 16S rRNA gene cloning and metagenomic analysis consistently indicated that *Proteobacteria* were the main antibiotic resistant bacteria (ARB) dominating in the drinking water and chlorine disinfection greatly affected microbial community structure. After chlorination, higher proportion of the surviving bacteria was resistant to chloramphenicol, trimethoprim and cephalothin. Quantitative real-time PCRs revealed that *sulI* had the highest abundance among the antibiotic resistance genes (ARGs) detected in the drinking water, followed by *tetA* and *tetG*. Chlorination caused enrichment of *ampC*, *aphA2*, bla_{TEM-1}, *tetA*, *tetG*, *ermA* and *ermB*, but *sulI* was considerably removed ($p < 0.05$). Metagenomic analysis confirmed that drinking water chlorination could concentrate various ARGs, as well as of plasmids, insertion sequences and integrons involved in horizontal transfer of the ARGs. Water pipeline transportation tended to reduce the abundance of most ARGs, but various ARB and ARGs were still present in the tap water, which deserves more public health concerns. The results highlighted prevalence of ARB and ARGs in chlorinated drinking water and this study might be technologically useful for detecting the ARGs in water environments.

1. Introduction

The prevalence of antibiotic resistant bacteria (ARB) and resistance genes (ARGs) has conferred enormous and complicated impacts on environmental safety (Taylor et al., 2011) and human health (Walsh et al., 2011). Growing evidence suggests that drinking water is a reservoir for ARB and ARGs (Armstrong et al., 1981; Schwartz et al., 2003), thus the induced public health problems may receive great concerns (Ram et al., 2008). Notably, previous studies have indicated that chlorination, a commonly used disinfection technology in drinking water or wastewater treatment, can contribute to the enrichment of ARB (Armstrong et al., 1982) and spread of ARGs (Xi et al., 2009). Cross- or co-resistance of disinfectants and ARGs might be the underlying mechanisms responsible for the antibiotic resistance promotion (Dukan and Touati, 1996; Greenberg et al., 1990; Nakajima et al., 1995). Mobile genetic elements (MGEs) including plasmids, integrons and insertion sequences act as facilitators in the prevalence of ARB and ARGs in environment (Wright et al., 2008; Ciric et al., 2011), since bacteria can capture different ARGs housed on MGEs through horizontal transfer (Martinez, 2008). Furthermore, the mobile resistome can easily spread among species including human pathogens, which deserves more public health concerns (Canton, 2009; Nikaido, 2009).

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Currently, efforts are focused on the isolation of ARB from drinking water, while few studies have been conducted based on the metagenome DNA extraction due to the extremely low biomass and high microbial diversity (Lebkowska, 2009). However, comprehensive characterization of ARB and ARGs in natural environment or artificial compartments using culture-dependent methods remains challenging since most of environmental bacteria are unculturable (Schmieder and Edwards, 2012). Metagenomic analysis combined with high-throughput sequencing is considered as a promising tool for analysis of ARGs diversity (Zhang et al., 2011) and abundance (Uyaguari et al., 2011), as well as discoveries of novel genes (Torres-Cortes et al., 2011), since this method has been applied to detect ARGs in various environmental compartments including soils (Monier et al., 2011), activated sludge (Parsley et al., 2010), sediments (Kristiansson et al., 2011) and feces (Durso et al., 2011).

In this study, culture-independent methods including high-throughput sequencing and quantitative real-time PCR (q-PCR) were used for comprehensive assessment of chlorination effects on microbial resistance patterns in drinking water. This is the first study investigating the abundance and diversity of ARGs and MGEs in drinking water using metagenomic methods based on Illumina high-throughput sequencing. PCRs and q-PCRs were also performed to determine the effects of chlorination on occurrence and abundance of various ARGs. Meanwhile, we also characterized the antibiotic resistance phenotypes and genotypes of the bacterial isolates from drinking water after microbial community structure analysis. The results of this study may help to extend our knowledge about the antibiotic resistance in drinking water treatment system which might be practically useful for detecting the ARGs in water environments.

2. Materials and methods

2.1. Water sampling and DNA extraction

As illustrated in Figure S1, filtered water (FW) and chlorine-disinfected water (DW) were simultaneously sampled from chlorination tank and clear water tank of Beihekou Tap Water Plant (Nanjing, China), and tap water (TW) was also sampled at Environmental Biotechnology Laboratory of Nanjing University (Nanjing, China) at the same time. To avoid the temporal variation, the water sampling was repeated for four times separately in August, September, October and November of 2011. The information about water quality of FW, DW and TW was generously provided by the water treatment plant (Table S1). The chlorine disinfection lasted for 2–4 h in the drinking water plant and the finished drinking water was transported to laboratory through pipelines within 7–8 h. Considering the low biomass in drinking water, we used water purifiers (Toray Industries Inc., Japan) to concentrate bacterial cells by filtering about 2000 L water for 48 h, and the multilayer hollow fiber membrane was removed from filter cartridge and mixed with purified water (200 mL). The bacterial cells attached on the membrane were separated out by ultrasound oscillation for 30 min at 53 kHZ (KuDos HK3310HP, China). Subsequently, the mixture was filtered through 0.45-μm micropore membrane and the filtered materials were used for total genomic DNA extraction using the FastDNA Soil Kit (MP Biomedicals, CA, USA). The DNA concentration and purity were measured by microspectrophotometry (NanoDrop®ND-2000, NanoDrop Technologies, Wilmington, DE) (Table S2), and equal mass of each DNA sample extracted in the four months was pooled together to minimize the temporal variation.

2.2. Bacterial isolation, biochemical identification and antibiotic susceptibility analysis

About 5 L of FW, DW and TW were filtered through 0.45-μm micropore membrane and the membranes were then spread on the MacConKey nutrient agar overnight at 37 °C. Intestinal bacteria, an important group of human pathogen, were cultured and further isolated after streaking plate. A total of 100 bacterial clones (25 clones in each monthly sample) were isolated from each of FW, DW and TW, among which 54 each were randomly selected and subject to species identification using ID 32 identification test strips (BioMérieux, Italia). The parameters were read and interpreted by ATB 1525 EXPRESSION (BioMérieux, Italia). Bacterial susceptibility of the selected 54 bacterial clones from each sampling location was assessed for ampicillin, cephalothin, tetracycline, chloramphenicol, trimethoprim and gentamycin using Kirby–Bauer disc diffusion method according to the recommended protocol of Clinical and Laboratory Standards Institute (M100-S20 Vol. 30, No. 1). The relative abundance (%) of the ARB was calculated by normalizing against the total tested bacterial clones.

2.3. Phylogenetic analysis

After DNA extraction from the water samples, 16S rRNA gene was amplified by PCR with 27F/1492R primers. PCR products were further purified by DNA Fragment Purification Kit (TaKaRa, Japan) and then cloned into JM109 competent cells using pMDTM19-T Vector (TaKaRa, Japan). After positive recombinants were identified with PCR using M13F/M13R primer set, recombinant plasmids were extracted from 60 colonies in each sample and purified using MiniBest Plasmid DNA Extraction Kit (TaKaRa, Japan). The sequencing of these plasmids was performed by using M13F and M13R primers on ABI 3730xl capillary sequencers (PE Applied Biosystems, Foster City, USA). Mothur software (Schloss et al., 2009) was applied to identify operational taxonomic units (OTUs) to generate a representative sequence for each OTU. The nucleotide sequences were compared against the NCBI nucleotide database using BLASTn algorithm (Altschul et al., 1990) to retrieve the reference sequences from the NCBI GenBank. Phylogenetic trees were constructed with MEGA 5.0 software (Tamura et al., 2011) using the neighbor-joining method at bootstrap value 1000. The sequences of 16S rRNA genes obtained in this study have been deposited in GenBank under accession numbers JQ905978–JQ906083.

2.4. Detection of ARGs in isolated clones and environmental DNA

PCRs were conducted to detect various ARGs in the isolated clones and the environmental DNA samples of FW, DW and

TW. The ARGs include the genes encoding resistance to β-Lactam (bla_{TEM-1} and *ampC*), tetracycline (*tetA*, *tetB*, *tetC* and *tetG*), aminoglycoside (*aphA2*), sulfonamide (*sulI*), erythromycin (*ermA* and *ermB*) and trimethoprim (*drfA17*), and detailed information of the ARGs and their primers is shown in Table S3. The PCR protocols were as follows: (1) For *ampC*, bla_{TEM-1}, *ermA*, *ermB*, *sulI*, *tetA*, *tetC* and *tetG*, denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, annealing at 58 °C (*ampC* and bla_{TEM-1}) or 55 °C (*ermA*, *ermB*, *sulI*, *tetA*, *tetC* and *tetG*) for 30 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min; (2) For *tetB*, *aphA2* and *drfA17*, denaturation at 94 °C for 5 min, followed by 35 cycles of 60 s at 94 °C, annealing at 55 °C for 60 s and extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR system (30 μL) contained 1 × PCR buffer, 100 μM dNTP, 2 pmol of each primer, 150 ng of template DNA and 1 U of ^{EX}Taq polymerase (TaKaRa, Japan). PCR products were analyzed by gel electrophoresis using 1% (w/v) agarose in 1 × TAE buffer. To ensure reproducibility, duplicate reactions were performed for each permutation of sample and primer set, and sterile water was used as negative control. The PCR products of the ARGs were further confirmed by DNA sequencing, and the nucleotide sequences have been deposited in GenBank under accession numbers JQ937275–JQ937280.

2.5. Quantitative real-time PCR

Eight genes (bla_{TEM-1}, *ampC*, *tetA*, *tetG*, *aphA2*, *sulI*, *ermA* and *ermB*) were selected for q-PCR assay. The plasmids containing target genes were obtained by molecular cloning. q-PCRs were conducted in a final volume of 20 μL containing SYBR Premix EX Taq Super Mix (TaKaRa, Japan) (10 μL), 10 μM primer (0.2 μL each) (Table S3), DNA templates (40 ng) and ddH_2O (Zhang et al., 2009). Thermal cycling and fluorescence detection were performed in Corbett Real-Time PCR Machine with the Rotor-Gene 6000 Series Software 1.7 (QIAGEN, the Netherlands) using the following protocol: 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at the given temperatures (Table S3) for 20 s and extension at 72 °C for 40 s. Each reaction was conducted in triplicate.

Five to seven-point calibration curves (C_t values versus log of initial target gene copy) were generated using 10-fold serial dilution of the plasmid containing target gene. PCR efficiency of each gene ranged from 93.19% to 106.20% with R^2 value more than 0.992 for the calibration curves. In order to minimize the variation of extraction efficiency, the eubacterial 16S rRNA gene was quantified simultaneously using the method recommended by López-Gutiérrez et al. (2004), and the relative abundance of target genes was normalized to each total bacterial community.

2.6. High-throughput sequencing of environmental metagenome

High-throughput sequencing of the drinking water metagenome was performed in Beijing Genome Institute (Shenzhen, China) by using Illumina Hiseq 2000. The sequencing strategy of Index 101 PE (Paired-End sequencing, 101-bp reads and 8-bp index sequence) was applied to generate nearly equal amount of clean reads for each sample. The raw reads containing three or more "N" or contaminated by adapter (>15 bp overlap) were removed, and the filtered clean reads (about 1.6 Gb per each sample) were used for metagenomic analyses (Table S4). The metagenomic data have been deposited in NCBI Sequence Read Archive under accession number SRA050945.

2.7. Bioinformatic analysis on high-throughput sequencing

All the clean reads were assembled *de novo* by using SOAPdenovo (BGI, Shenzhen, China) with optimal Kmer at 43 (FW), 45 (DW) and 47 (TW) to generate contigs. The detailed information about the sequencing reads and assembling results was described in Tables S4 and S5. Both the nucleotide and protein database of ARGs were established by downloading the sequences in the MvirDB which collected all publicly available organized sequences (5642 sequences of both nucleotide and protein) of ARGs (Zhou et al., 2007). A read was annotated as ARG according to its best BLAST hit (BLASTn or BLASTx with the *E*-value cut-off at 10^{-5}) if (1) the similarity was above 90% and (2) the alignments had at least 50 bp (for nucleotide database) or 25 amino acids (for protein database) (Kristiansson et al., 2011).

The nucleotide sequences of MGEs including integrons, insertion sequences and plasmids were downloaded from INTEGRALL (1447 integrase genes and 8053 gene cassettes) (Moura et al., 2009), ISfinder (2578 sequences, 22 families of insertion sequences) (Siguier et al., 2006) and NCBI RefSeq database (2408 plasmid genome sequences), respectively. A read was identified as integron or insertion sequence if the BLAST hit (BLASTn with the *E*-value cut-off at 10^{-5}) had a sequence identity of more than 90% over an alignment of at least 50 bp (Kristiansson et al., 2011). The threshold of identified plasmids was determined as the BLAST hits (BLASTn with the *E*-value cut-off at 10^{-5}) with a nucleotide sequence identity of above 95% over an alignment of at least 90 bp (Kristiansson et al., 2011).

Based on the assembled contigs (Table S5), open reading frames (ORFs) were predicted by using MetaGeneMark, and amino acid sequences of the putative ORFs were compared against the NCBI non-redundant protein database to generate BLAST results (format 0). MEGAN 4 software (Huson et al., 2011) was used to assign BLAST results to NCBI taxonomies with the Lowest Common Ancestor algorithm using default parameters (absolute cutoff: BLAST bitscore 35; relative cutoff: 10% of the top hits). The relative abundance of each classification was calculated by normalizing the assigned sequences to the total number of ORFs.

3. Results

3.1. Chlorination effect on antibiotic resistance pattern of intestinal bacteria in drinking water

Phylogenic analysis on the bacterial clones showed that *Escherichia* dominated in FW (63.0%), followed by *Enterobacter* (18.5%), *Klebsiella* (16.7%) and *Acinetobacter* (1.9%) (Figure S2).

After chlorine disinfection in the water treatment plant, *Pseudomonas* was enriched in DW (27.8%). Drinking water pipeline transportation further enhanced the relative abundance of *Pseudomonas* (63.0%) and *Citrobacter* (33.3%) in TW. Antibiotic susceptibility analyses demonstrated that FW, DW and TW contained the resistant bacteria for all the tested antibiotics except for gentamicin. After chlorination, the relative abundance of chloramphenicol, trimethoprim and cephalothin resistant bacteria was enhanced by 4.1 fold, 1.5 fold and 3.0 fold, respectively. The potentiation effect continued during the water transportation. Compared with FW, TW had the increased relative abundance of ampicillin (by 1.5 fold), chloramphenicol (by 2.8 fold), tetracycline (by 4.7 fold), trimethoprim (by 3.3 fold) and cephalothin (by 4.8 fold) resistance bacteria (Figure S3).

Due to the prevalence of ARB in drinking water, we also investigated the occurrence of the ARGs in the bacterial clones. Among the eight ARGs detected in this study, seven (except *drfA17* encoding resistance to trimethoprim) were present in the isolated clones (Figure S4). In general, *ampC* and *tetA* in the isolated bacteria had relative higher detection frequencies of 79.8% and 31.7%, respectively. However, PCRs showed that chlorination and transportation caused no obvious increase in detection frequency of some ARGs (*ampC*, *aphA2*, $bla_{\text{TEM-1}}$, *tetB* and *tetC*) in the bacterial clones. The various genetic determinants could be detected after water treatment, but the changing tendency was not completely consistent with the resistance phenotypes in the bacterial clones isolated from drinking water (Figure S4).

3.2. Chlorination effect on microbial structure in drinking water

After phylogenic analysis of the 16S rRNA sequences, a total of 29, 20 and 29 OTUs were identified for FW, DW and TW, respectively, among which only 15 (FW), 11 (DW) and 18 (TW) OTUs could be assigned to known groups. Most of the clones were grouped into the phylum *Proteobacteria* (Figures S5–S7). Chlorination seemed to decrease the microbial diversity in drinking water since *Bacteroidetes*, *Actinobacteria*, β-*Proteobacteria* disappeared after chlorination, but some of them occurred again in TW. In DW, 98.4% of the clones were affiliated to *Proteobacteria* (Figure S6), suggesting that this phylum is mainly responsible for the microbial resistance to chlorination in drinking water. Interestingly, a total of 34 OTUs in the three water samples could not be successfully assigned to any known bacteria in NCBI database, so we collected these unassigned OTUs in the three samples to establish a new phylogenetic tree (Figure S8). The results showed that these unassigned OTUs from the different samples were closely clustered in the phylogenetic tree.

Metagenomic analysis on community structure showed that the relative abundance of *Proteobacteria* gradually increased from 29.2% to 47.2% after chlorination and to 90.9% after transportation. However, the abundance of *Actinobacteria* demonstrated a decreasing trend, taking up 36.7% in FW, 11.1% in DW and 0.25% in TW (Figure S9). Water chlorination and transportation caused obvious microbial community shift at genus level since *Polynucleobacter*, *Candidatus Pelagibacter* and *Acidimicrobium* were the dominant genera in FW, while *Agrobacterium* and *Pseudomonas* were prevalent in DW. The predominant community members in TW were *Hyphomicrobium*, *Erythrobacter*, and *Sphingomonas*, occupying 4.5%, 3.3% and 2.0% of *Proteobacteria*, respectively (Figure S10).

3.3. Chlorination effect on occurrence and abundance of ARGs in drinking water

PCRs of the ARGs in environmental DNA showed that $bla_{\text{TEM-1}}$, *tetA*, *tetG*, *sulI*, *ermA* and *ermB* were present in FW, DW and TW (Figure S11). *AmpC* was not detectable in FW but was found to be present in DW and TW, while *aphA2* occurred in FW and DW but disappeared in TW. Three genes (*tetB*, *tetC* and *drfA17*) were absent in each water sample. q-PCR results showed that *sulI* had the highest abundance among the tested ARGs, followed by *tetA* and *tetG* (Fig. 1). Chlorination significantly caused enrichment of seven ARGs (*ampC*, *aphA2*, $bla_{\text{TEM-1}}$, *tetA*, *tetG*, *ermA* and *ermB*) ($p < 0.05$), but the water transportation decreased the levels of all the eight ARGs in drinking water ($p < 0.05$) (Fig. 1). The water treatment posed different effects on the abundance of the ARGs. The relative abundance increased by 1.4 fold for *ermA* and 1.6 fold for *ermB* in the drinking water after disinfection and transportation ($p < 0.05$), while *tetA*, *tetG* and *sulI* levels decreased by 7.9 fold, 65.8 fold and 52.6 fold, respectively ($p < 0.05$). $bla_{\text{TEM-1}}$ abundance showed no significant difference between FW and TW ($p > 0.05$).

3.4. Metagenomic analysis of chlorination effect on abundance and diversity of ARGs in drinking water

BLAST against the MvirDB protein database showed that a total of 62 reads (0.0052‰) for FW, 842 reads (0.0705‰) for DW and 44 reads (0.0037‰) for TW were assigned to 18 (Table S6), 63 (Table S7) and 20 (Table S8) types of the known ARGs, respectively (Fig. 2A). Obviously, both the diversity and abundance of the detectable ARGs increased significantly after chlorination, but a huge decrease occurred in water transportation. The water chlorination induced an increase in the abundance of *mexF*, bla_{TEM}, *aph33ib* and *cml_e3* by 106.0 fold, 10.3 fold, 10.3 fold and 8.5 fold, respectively (Fig. 2B). The abundance of *mexF*, bla_{TEM}, *qacEΔ1* and bla_{KLUA} was reduced by 34.7 fold, 2.5 fold, 17.0 fold and 3.0 fold after transportation, respectively. The change of bla_{TEM} levels was consistent with the q-PCR results (Fig. 1). Furthermore, we summarized the types of the ARGs detected in the drinking water (Fig. 3). In general, the ARGs present in DW could encode more types of antibiotic resistant protein (11 types) than those in FW (9 types) and TW (5 types). β-Lactam resistance genes were prevalent in drinking water since most of the identified sequencing reads were found to be involved in β-Lactam resistance in FW (39.2%), DW (34.9%) and TW (76.5%), respectively. In addition, chlorination promoted multidrug and aminoglycoside resistances in drinking water, and water transportation seemed to reduce the diversity of ARGs in the drinking water.

Alignments against MvirDB nucleotide database and protein database showed similar results for the changes in diversity and abundance of ARGs in drinking water. Search in the nucleotide database showed that a number of sequencing

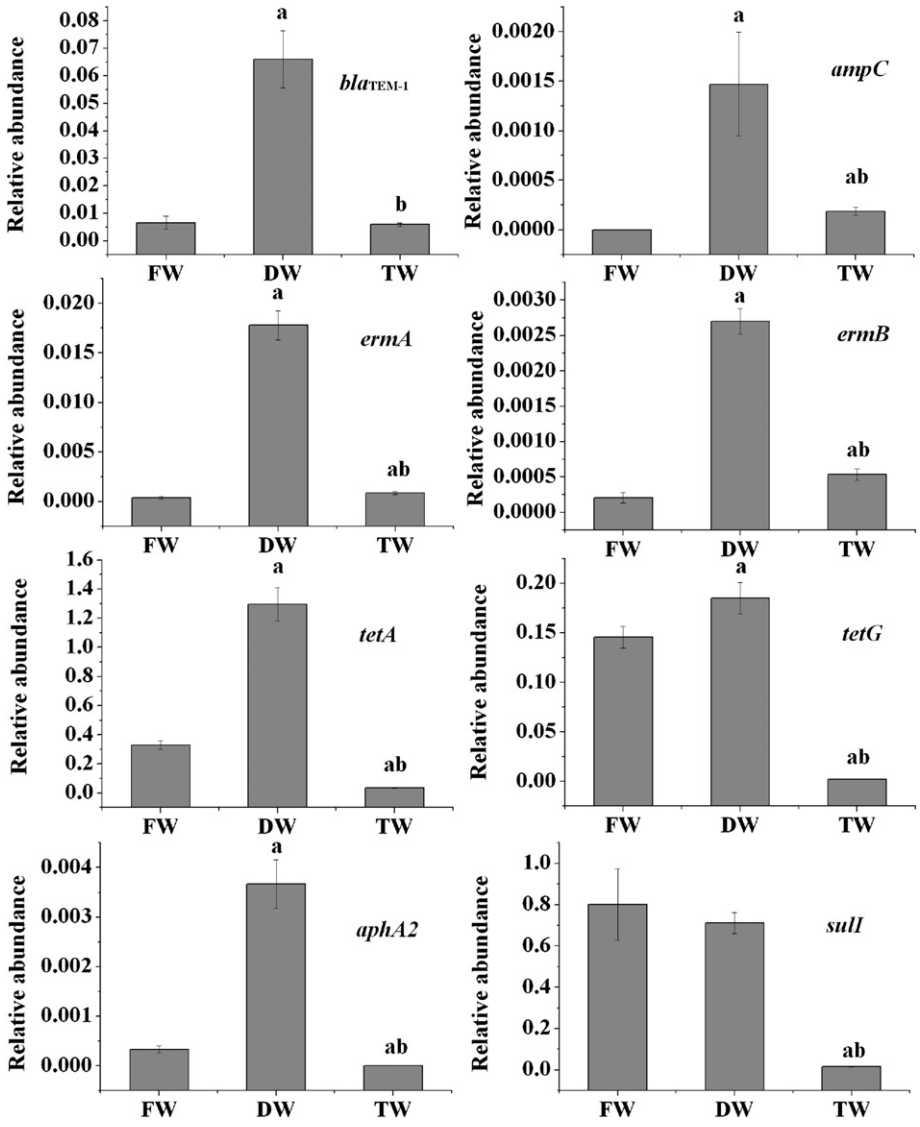

Fig. 1 – Relative abundance of antibiotic resistance genes (ARGs) in filtered water (FW), disinfected water (DW) and tap water (TW). q-PCR was used to determine the relative abundance normalized to the total copy number of 16S rRNA genes in corresponding samples. a: $p < 0.05$, comparing FW with DW and TW; b: $p < 0.05$, comparing DW with TW.

reads from the three water samples of FW (63 reads, 0.00529‰), DW (846 reads, 0.0709‰) and TW (46 reads, 0.00387‰) were identified as different ARGs, which belonged to 21 (Table S9), 51 (Table S10) and 6 types (Table S11), respectively.

3.5. *Chlorination effect on abundance and diversity of mobile genetic elements in drinking water*

Alignment against the INTEGRALL database revealed that a total of 102 reads (0.00856‰) for FW (Table S12), 1000 reads (0.0838‰) for DW (Table S13) and 196 reads (0.0165‰) for TW (Table S14) could match known integrons, and class 1 integronase gene *intI1* was predominant in each water sample. On the whole, the relative abundance of integrons increased by 8.8 fold after chlorination and then decreased in TW (Fig. 4A). More types of integrases genes were detected in DW including *intI1* (85.0%), *intI2* (1.2%), *intI3* (0.2%), *intI9* (0.1%), *intI* (0.6%), *intIPac* (0.3%) and unknown integrases genes (12.6%). Gene cassettes carried on the integrons contained miscellaneous ARGs encoding resistance to β-Lactam, aminoglycoside, sulphonamides, trimethoprim, tetracycline, streptomycin, chloramphenicol and quaternary ammonium compounds in the drinking water.

Search in ISfinder database showed that a total of 108 reads (0.00906‰) for FW (Table S15), 2206 reads (0.185‰) for DW (Table S16) and 92 reads (0.00773‰) for TW (Table S17) could match the known transposons. Chlorination accumulated insertion sequences in DW by up to 19.4 fold (Fig. 4B). A total of 20 types were detected in FW and IS*Ppu12* had the highest abundance (26.9%), followed by IS*Aba2* (13.0%), IS*Pps1* (10.2%) and IS*Pst2* (9.3%). After chlorination, more types of transposons (82 types) were detectable in DW. Different from the transposons present in FW, the predominant ones in DW were IS*Aba125* (11.5%), IS*Aba5* (8.1%) and IS*Ppu9* (7.4%).

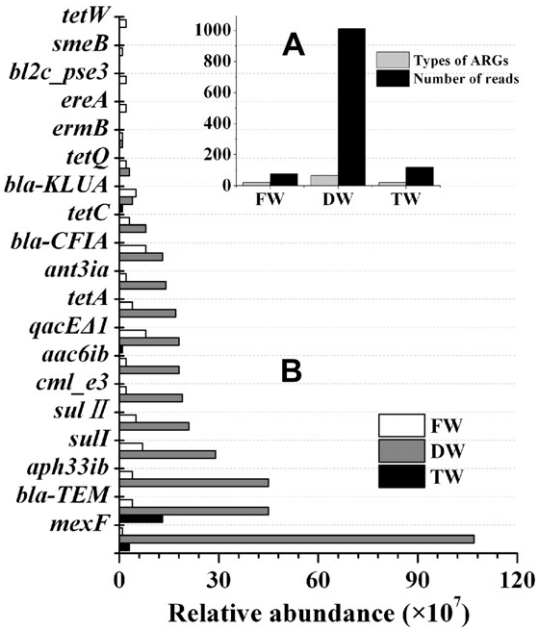

Fig. 2 – Assigned types (A) and relative abundance (B) of antibiotic resistance genes (ARGs) in filtered water (FW), disinfected water (DW) and tap water (TW). After search in MvirDB database, the relative abundance was obtained with the matched Illumina sequencing reads normalized to the total reads of each water sample.

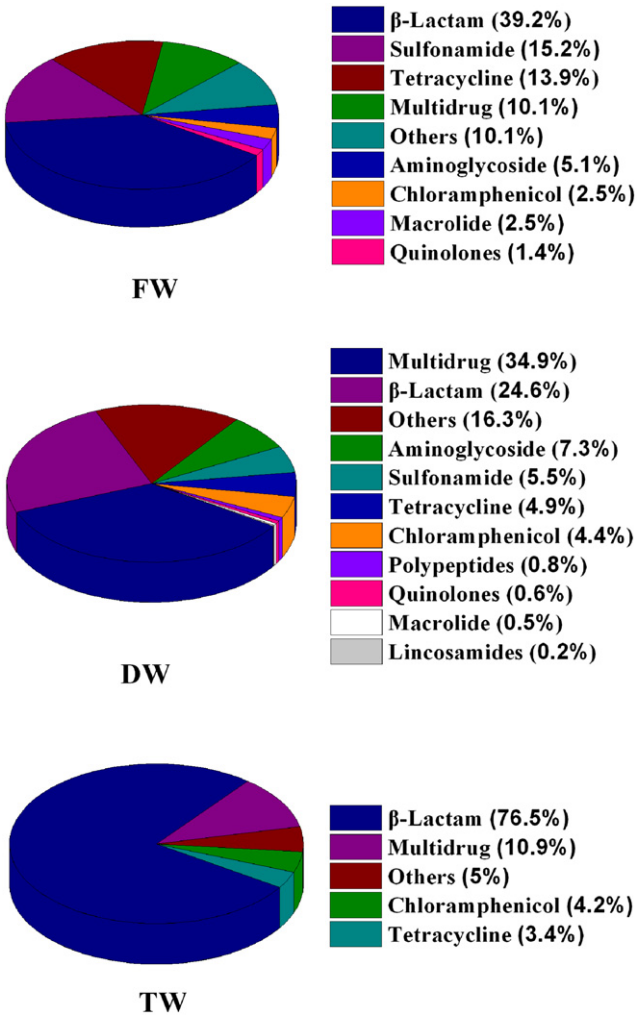

Fig. 3 – Antibiotic resistance patterns in filtered water (FW), disinfected water (DW) and tap water (TW). The resistance genes detected in drinking water were grouped according to antibiotic types after alignment of the high-throughput sequencing reads against MvirDB database.

Mapping all the reads to NCBI Plasmid Genome Database revealed that the number of matched reads was 4908 (Table S18) for FW (0.412‰), 23659 (Table S19) for DW (1.982‰) and 9160 (Table S20) for TW (0.770‰), and these plasmids were further classified into 133, 326 and 180 types, respectively. DW had the most copies of the total plasmids, which were 4.8 fold and 2.6 fold of FW and TW, respectively (Fig. 4C). The abundance of pGMI1000MP was the highest both in FW (1111 reads) and DW (2673 reads), occupying 22.6% and 11.3% of corresponding total matched reads, but it decreased to 350 reads in TW. Similarly, chlorine disinfection also increased the abundance of p3ABAYE, pMRAD01, pA81 and megaplasmid in drinking water, but the abundance of these plasmids in TW was sharply reduced after transportation (Figure S12).

4. Discussion

In this study, intestinal bacteria were isolated from FW, DW and TW to preliminarily investigate the antibiotic resistance patterns in drinking water. Antibiotic susceptibility assay suggested elevated resistance to several tested antibiotics after chlorine disinfection of drinking water. These results are not completely consistent with the PCRs with environmental DNA as template due to unfeasibility for culture of most environmental bacteria, the diversiform genetic determinants for antibiotic resistance, and resistance phenotype–genotype discrepancies of some characterized bacteria in given environments (Davis et al., 2011). Similarly, a previous study showed that chlorination of sewage influent could result in an increase in the proportion of lactose-fermenting bacteria resistant to ampicillin and cephalothin (Murray et al., 1984). In this study, most of the bacterial clones were found to be resistant to ampicillin, cephalothin and tetracycline, and *ampC* and *tetA* had relatively higher detection frequencies than other ARGs in the bacteria. It was previously indicated that resistance to cephalothin, amoxicillin and sulphamethoxazole in *Enterobacteriaceae* was more prevalent in drinking water than in wastewater and surface water (Figueira et al., 2012).

The microbial diversity of the drinking water samples was also examined to characterize the influence of chlorination on bacterial community shift. Biochemical identification, 16S rRNA gene cloning and metagenomic analysis consistently indicated that chlorine disinfection and pipeline transportation altered the microbial community structure, but *Proteobacteria* persisted along the water treatment and distribution system. This result was supported by Poitelon et al. (2009)

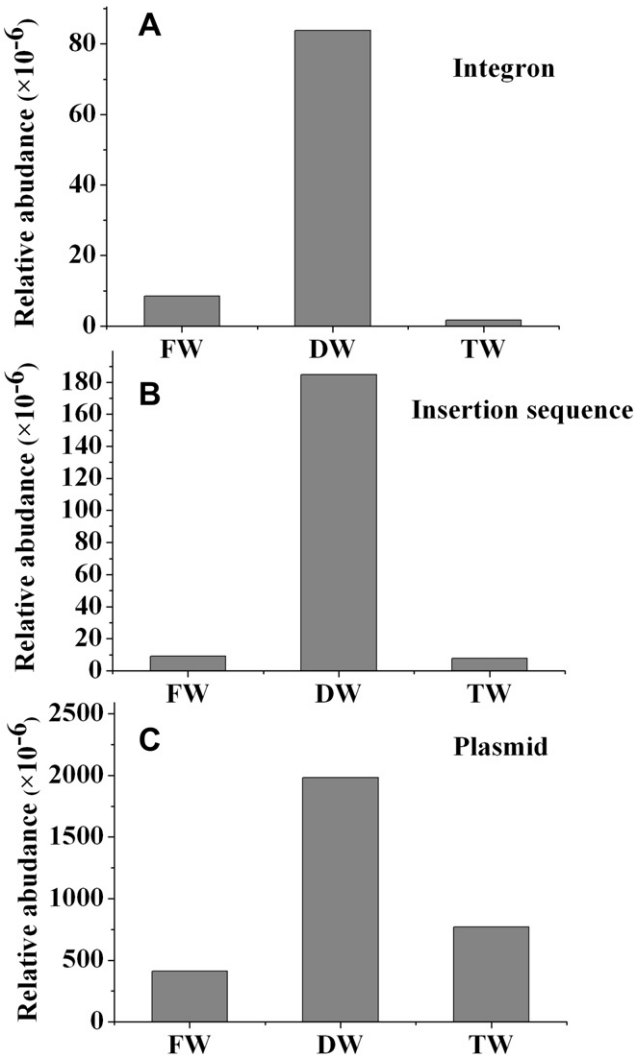

Fig. 4 – Relative abundance of integrons (A), insertion sequences (B) and plasmids (C) in filtered water (FW), disinfected water (DW) and tap water (TW). After BLAST against INTEGRALL, ISfinder and NCBI Plasmid Genome Database, the relative abundance was obtained with the matched Illumina sequencing reads normalized to the total reads of each water sample.

indicating that *Proteobacteria* (57.2–77.4%) dominated in chlorinated drinking water. Compared with 16S rRNA gene cloning, metagenomic analysis provided more detailed information about the microbial membership and community shift in the drinking water. A metagenomic survey of biofilm in drinking water also showed that the majority of the microbes were closely related to the *Proteobacteria* with members of *Rhizobium*, *Pseudomonas* and *Escherichia* (Schmeisser et al., 2003). Furthermore, a group of unassigned bacteria representing novel bacterial clades persisted in the drinking water. Similarly, Revetta et al. (2010) also found that a significant fraction of the bacteria in drinking water were difficult to isolate and classify due to the unique oligotrophic environment.

In this study, some ARGs responsible for the observed antibiotic resistance were selected for analyzing occurrence and abundance by PCR and q-PCR. Comparatively, the genes *sulI*, *tetA* and *tetG* had significantly higher abundance, which may result from long-term exposure and huge residual amount of tetracycline and sulphonamide in drinking water source of China (Luo et al., 2011; Zhou et al., 2011). This study also showed that bla_{TEM-1}, *ampC*, *ermA* and *ermB* were prevalent in the drinking water. Plasmid-mediated bla_{TEM-1} and *ampC* are the most common genes coding β-lactamases and have been frequently detected in drinking water (Schwartz et al., 2003; Xi et al., 2009). Previous studies have revealed the occurrence of erythromycin resistant bacteria in drinking water (Faria et al., 2009), and our results further indicated that the prevalence of resistance genes *ermA* and *ermB* in the finished water of the plant. Additionally, this study showed that *aphA2* conferring aminoglycoside resistance was abundant in intestinal bacteria and environmental DNA of the drinking water, which is supported by a previous study demonstrating that *aphA2* was present in *Escherichia coli* isolated from drinking water (Cernat et al., 2007). Few studies have been conducted to quantitatively assess the effects of chlorination on ARGs in drinking water. In this study, q-PCR results showed that short-term chlorination (2–4 h) could promote the antibiotic resistance in drinking water, but *sulI* gene was effectively removed by chlorination. This result was supported by Xi et al. (2009) demonstrating that chlorination could reduce the abundance of *sulI*, but increase the abundance of *cat* and bla_{SHV} genes in treated drinking water.

To further characterize the comprehensive effects of chlorination on antibiotic resistance in drinking water, we used high-throughput sequencing and metagenomic analysis to investigate the abundance and diversity of ARGs and MGEs. The results demonstrated that chlorination could evidently concentrate various ARGs in drinking water normalized to the total microbial community, which is consistent with q-PCR results. Some ARGs like bla_{TEM-1}, *sulI*, *tetA* and *tetC* present in the bacterial isolates were also identified by metagenomic analysis, but the changing tendency was not completely consistent due to the bias of bacterial culture. Similarly, more MGEs including integrons, insertion sequences and plasmids could be detected with higher abundance after the chlorine disinfection. It is well known that antibiotic resistance can be achieved by acquisition of MGEs through horizontal gene transfer and the mobility of ARGs is closely related to integrons, transposons and plasmids (Boucher et al., 2007; Boerlin and Reid-Smith, 2008). In this study, chlorination was found to evidently enhance the abundance of class 1 integrons and the diversity of gene cassettes containing various ARGs, which may accelerate the mobility of the ARGs in the microbial community (Fluit and Schmitz, 1999). IS*Aba125* was dominant in drinking water after chlorination and the insertion sequence often carry *aphA6* encoding aminoglycoside resistance (Nigro et al., 2011) and can act as facilitator for duplication of β-lactamase gene in *Acinetobacter* (Evans et al., 2010). Plasmid pGMI1000MP with the highest abundance in FW and DW contained ARGs encoding antibiotic efflux pump involved in fosmidomycin resistance (Salanoubat et al., 2002).

Several studies have been conducted to investigate the underlying molecular mechanisms of chlorination effects on antibiotic resistance. Although most bacterial cells were damaged after chlorination, but the surviving bacteria may

grow during the disinfection. Similarly, Huang et al. (2011) have indicated that even 10-min chlorination can contribute to selection of antibiotic resistance bacteria and alteration of microbial community in sewage. Previous insight into the microbial ecology of drinking water also revealed that resistance to chlorination was affected by bacterial community structure and interspecies relationships (Berry et al., 2006), so the microbial community shift might subsequently drive the alteration of antibiotic resistance patterns in drinking water. Moreover, extracellular stress can promote replication of plasmids in bacterial cells (Wegrzyn and Wegrzyn, 2002), so chlorination might increase the copy number of plasmids in the cells of surviving bacteria, resulting in the higher relative abundance of ARGs in disinfected water. Metagenomic analysis used in this study revealed that the relative abundance of plasmids was significantly enhanced after chlorine disinfection. Additionally, co-resistance may be involved in the antibiotic resistance promotion, since chlorination can decrease bacterial membrane permeability and inhibit molecular transport to result in antibiotic resistance (Krige, 2009). Previous studies also suggested that lagoon bacteria displayed elevated resistance to some selected antibiotics after chlorine disinfection (Macauley et al., 2006), and trimethoprim-resistant *E. coli* were observed more resistant to chlorine than the antibiotic-sensitive isolates (Templeton et al., 2009). Disinfectant-induced antibiotic resistance results from bacterial co-resistance between disinfectants (e.g. triclosan and benzalkonium chloride) and antibiotics, which may be mediated by multidrug efflux pumps (Chuanchuen et al., 2001). Addition of efflux pump inhibitor (carbonyl cyanide m-chlorophenylhydrazone) with exposure to chlorine can decrease enumeration levels of ARB in water and biofilm (Krige, 2009).

The increase of the total bacterial colonies in TW compared with DW indicated that reactivation of microbial community have been occurred in pipelines when microbial nutrition increased and residual chlorine decreased (Table S1). Because of the bacterial regrowth, both the metagenomic analysis and q-PCR results mirrored a dramatic decrease in the diversity and relative abundance of specific ARGs after drinking water transportation. Many factors can affect bacterial regrowth in pipelines including fluctuating temperatures, residence time, pipe materials and pipe diameters (Lautenschlager et al., 2010). The microbial community shift may contribute to the alteration of antibiotic resistance in the pipeline, so the relative abundance of ARGs will decrease if more nonresistant bacteria survive in the environment of low-level chlorine. However, a variety of ARB and ARGs were still present in TW, deserving great public health concerns. In future, more advanced water treatment technologies have to be developed to effectively remove the ARB and ARGs in drinking water so as to minimize the related public health risks. Some efforts also should be motivated to reduce the possibility of ARB and ARGs entering into and spread in drinking water source.

5. Conclusions

- Results of PCR, high-throughput sequencing, metagenomic analysis and culture-dependent methods reveal prevalence of a variety of ARB and ARGs in drinking water.
- Chlorine disinfection can greatly affect the microbial structure and cause enrichment of ARB, ARGs and MGEs in drinking water. Plasmid over-replication, microbial community shift and multidrug efflux pumps-mediated co-resistance might be responsible for the enrichment of ARGs under chlorine stress.
- Although water pipeline transportation can alleviate bacterial antibiotic resistance in the disinfected water, the tap water still contains various ARB and ARGs, which might induce health risks for the consumers.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.watres.2012.09.046.

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