Environmental Pollution 259 (2020) 113818

Contents lists available at ScienceDirect

Environmental Pollution

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

Behavior of last resort antibiotic resistance genes (*mcr-1* and bla_{NDM-1}) in a drinking water supply system and their possible acquisition by the mouse gut flora☆

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ARTICLE INFO

Article history:
Received 2 October 2019
Received in revised form
5 December 2019
Accepted 13 December 2019
Available online 16 December 2019

Keywords:
mcr-1
bla_{NDM-1}
Antibiotic resistance gene
Drinking water
Mouse gut
Horizontal gene transfer

ABSTRACT

Mcr-1 and bla_{NDM-1} antibiotic resistance genes (ARGs) confer resistance to colistins and carbapenems, which are often antibiotics used as a last resort in tertiary care hospitals. Dissemination of these two ARGs in drinking water supply systems and their effect on healthy gut bacteria are poorly studied. In this study, the dissemination of *mcr-1* and bla_{NDM-1} in a drinking water supply system, and their effect on the antibiotic resistance of mouse gut bacteria are explored.

Metagenome analysis revealed that source water (Taipu river and Jinze reservoir) was polluted with ARGs. *Mcr-1* and bla_{NDM-1} can be disseminated through the water distribution system. Even advanced water treatments (ozone and biological activated carbon (BAC)) could not effectively remove *mcr-1* and bla_{NDM-1}. Low concentrations of chloramine disinfectants in the water distribution system were not effective at limiting ARG abundance. Mobile genetic elements were also found to play a major role in the dissemination of ARGs via horizontal gene transfer (HGT) throughout the water supply system. Statistical analysis revealed that there was no effect of temperature on the abundance of *mcr-1* and bla_{NDM-1} throughout the water supply system.

A last resort ARG, *mcr-1* can disseminate from drinking water to the healthy mouse gut. The presence of *mcr-1* in a strain belonging to *Enterococcus hirae*, which is different from the strain belonging to the *Bacillus cereus* group isolated from drinking water, strongly supports the phenomena of HGT inside the gut.

This research provides novel insights into the role of drinking water in disseminating ARGs to the gut and strongly suggests that drinking water may also play a major role apart from other factors known to be involved in the prevalence of last resort ARGs in the gut.

1. Introduction

Drinking water (DW) quality is a major issue throughout the world, with more than a billion people who are unable to get access to safe water for drinking (Shannon et al., 2008). The presence of contaminants and increasing pollution in water poses a threat to the environment and human health (Hong et al., 2015; Richardson and Ternes, 2018). An important cause of water pollution is pharmaceutical contamination, and antibiotics play a major role in this category (Khetan and Collins, 2007). A total of 210,000 tons of antibiotics are produced annually in China, of which 46% are used for animal husbandry (Su et al., 2014b).

Antibiotics get released into the environment through direct discharge from factories, human and animal wastes and unused antibiotics (Berendonk et al., 2015; Luo et al., 2014; Martinez, 2008; Verlicchi et al., 2015). A large amount of antibiotics have been detected in water resources, which is a threat to human health because they can promote antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) in the surface water (Berendonk et al., 2015). ARGs have the ability to spread among bacteria, and therefore, they can move from humans to animals and to natural environments, including DW (Berendonk et al., 2015; Gillings et al., 2015; Martinez, 2008; Storteboom et al., 2010).

The removal of antibiotics from DW is difficult with existing

☆ This paper has been recommended for acceptance by Wen Chen.

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https://doi.org/10.1016/j.envpol.2019.113818
0269-7491/

water treatment processes (Figueira et al., 2011; Guo et al., 2014; Xi et al., 2009). In addition, the phenomena of ARG horizontal transmission among microbes expedites the emergence and dissemination of antibiotic resistance (AR) in DW (Flores Ribeiro et al., 2014).

Aquatic environments, including surface, ground and tap water, are known reservoirs of ARB and ARGs (Marti et al., 2014; Xi et al., 2009; Zhang et al., 2009). For instance, the detection of bla_{NDM-1} in DW in New Delhi in 2011 drew worldwide attention (Walsh et al., 2011).

Colistin has become the last line of defense against infections caused by carbapenem-resistant bacteria (Paterson and Harris, 2016). However, worldwide attention was again drawn to the identification of plasmid-mediated transferable colistin resistance encoded by the *mcr-1* gene from patients and animal sources in China in 2016 (Liu et al., 2016). The *mcr-1* gene was later identified in *E.coli* and *K. pneumoniae* isolates from animals, foodstuffs and humans all over the world (Olaitan et al., 2016; Tse and Yuen, 2016). The identification of *mcr-1* in a multidrug resistant plasmid (Malhotra-Kumar et al., 2016) and its co-occurrence with NDM-9 in a strain isolated from chicken meat sample are worrisome, because the transfer of these resistant strains to humans may lead to untreatable infections (Yao et al., 2016). Considering the fact that ARGs attained by human pathogens may have an environmental origin (Martinez, 2008), it was suspected that *mcr-1* and bla_{NDM-1} might exist in water environments.

Previous studies have reported the occurrence of *mcr-1* in the Haihe river of China (Yang et al., 2017), and its presence in the human microbiome has also been reported in China (Ruppe et al., 2016). However, until now, no attention has been given to the fact that DW could also serve as a path to facilitate the dissemination of last resort ARGs to the gut flora.

Qingcaosha and Jinze reservoirs obtain water from the Yangtze and Taipu rivers and provide 70% and 30% of Shanghai's DW, respectively. Due to discharge from upstream cities and its geographic location, different pollutants, including certain antibiotics, may contribute to the contamination of these reservoirs. Hence, these water sources are facing antibiotic and ARG pollution.

In this study, we hypothesized that DW could be one of the many reasons for the spread of ARGs to the gut flora. To our knowledge, detailed studies have not been conducted to examine the dissemination of *mcr-1* and bla_{NDM-1} in the DWSS, or their uptake by gut microbiota. Therefore, the aim of this study was to examine the relationship between the presence of these ARGs in the drinking water supply system (DWSS) and their dissemination to the healthy gut flora.

2. Materials and methods

2.1. Study site and sample collection

Water samples were collected from source water (SW) (SW1: start of Taipu river, SW2: middle of Taipu river, SW3: Jinze reservoir influent and SW4: Jinze reservoir effluent), the water treatment plant (the influent and effluent of the water treatment plant) and the distribution system (DS) (primary water supply system (PWSS) and secondary water supply system (SWSS)) (Fig. 1). The samples were collected over four months: May 2018 (25 °C), October 2018 (20 °C), December 2018 (10 °C) and March 2019 (13 °C). The water treatment process in the plant included coagulation, sedimentation, sand filtration, ozonization, biological activated carbon (BAC) filtration and chloramine disinfection. The schematic diagram of the samples and their basic characteristics are shown in detail in Fig. S1, Table S1 and Table S2 in the Supplementary material. Sampling procedures were carried out with the help of waterworks engineers, and the standard examination methods for DW collection and preservation of water samples were met (GB/T 5750.2-2006).

A total of 10 L water samples were collected from each site, kept in 5 L sterilized glass bottles, stored on ice and transported to the laboratory for further analysis. Total chlorine and turbidity were measured on site using the standard examination methods for drinking water (GB/T 5750.11-2006, GB/T 5750.4-2006).

2.2. DNA extraction and qPCR

The bacterial biomass is very low in treated DW, therefore a 0.22-μm microporous membrane (Millipore, USA) was used to concentrate bacterial cells from the collected samples. Total genomic DNA was extracted from membrane-attached bacterial cells using a Water DNA Kit (OMEGA, Bio-Tek, Doraville, GA, USA) in accordance with the manufacturer's protocol. The concentration and purity of DNA was measured by agarose gel electrophoresis and a OneDrop 2000 UV−vis spectrophotometer. Qualified DNA was adjusted to 50 ng/μl and stored at −80 °C for further analysis.

All qPCR reactions were performed using the Applied Biosystems 7500 system with Hieff™ qPCR SYBR® Green Master Mix (YEASEN). A total of 5 primer sets were used to quantify the gene targets in the present study, including two last resort ARGs (*mcr-1* and bla_{NDM-1}), two MGEs (*intI1* and *tnpA*) and the 16S rRNA gene. Primer sequences for 16S rRNA, *tnpA*, *intI1*, *mcr-1* and bla_{NDM-1} were the same as those published in previous studies (Zhu et al., 2013; Gillings et al., 2015; Ahammad et al., 2014; Walsh et al., 2011). qPCR reaction conditions and data processing were carried out as mentioned before (Chen et al., 2017a; Wang et al., 2014). Briefly, the gene copy number was calculated using the following equation: Gene Copy Number $= 10^{(31-CT)/(10/3)}$, where CT (cycle threshold) refers to the qPCR result with a detection limit set to 31 (Looft et al., 2012). The relative abundance of a gene was calculated by normalizing the gene copy number to the 16S rRNA copy number (Zheng et al., 2018). All qPCR reactions were carried out with three replicates and negative controls and any genes identified in only one of the three replicates were regarded as false positives and removed.

2.3. ARB isolation and identification of resistant strains

Polymyxin B sulphate and meropenem, at 8 μg/ml and 4 μg/ml, respectively, were used for the selection of *mcr-1*- and bla_{NDM-1}-resistant bacterial strains, as previously described (Bai et al., 2015). For the isolation of strains from feces in the mouse model, both aerobic and anaerobic isolation methods were used.

Isolated bacterial strains were used for genomic DNA extraction using a boiling method (Gueimonde et al., 2004; Naas et al., 2007). Briefly, a few colonies of a bacterial isolate were resuspended in 100 μl of sterilized water, boiled at 100 °C for 20 min and then cooled for 10 min, followed by centrifugation at 12,000×*g* for 2 min. The recovered supernatant was then kept at −20 °C until further analysis.

The identification of bacterial isolates was carried out by 16S rDNA sequencing using the universal primers 27F and 1492R. The nucleotide sequences, approximately 1400 bp in length, were used for BLAST DNA homology searches (http://www.ncbi.nlm.nih.gov).

2.4. Metagenome sequencing, annotation and analysis of ARGs in source water

Metagenomic shotgun sequencing libraries were prepared and then sequenced using the HiSeq 2000 platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd., China). Raw reads were assembled

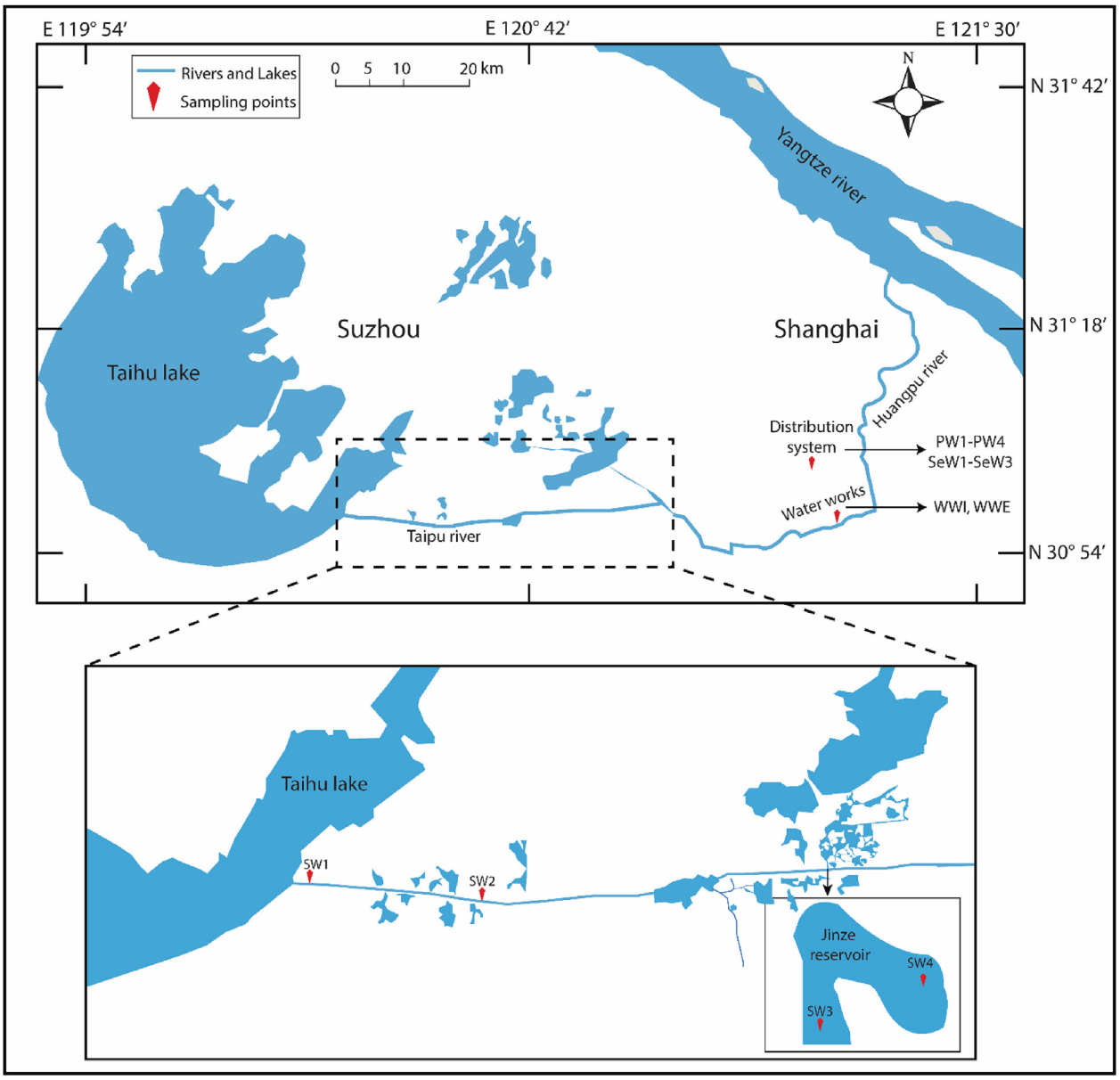

Fig. 1. Map showing the sampling sites in the water supply system; SW: Source water (SW3: Jinze reservoir influent, SW4: Jinze reservoir effluent), WW: waterworks (I: influent, E: effluent), PW: primary water supply, SeW: Secondary water supply.

using Seqprep, Sickle, BWA, and SOAPdenovo2 (Version 1.06), and contigs with lengths >300 bp were kept for bioinformatics analyses. ORFs were predicted using MetaGene, and a nonredundant gene catalog was made using CD-HIT. The SOAPaligner was used to compare high quality reads of each sample with the nonredundant gene set.

The predicted genes were assigned taxonomy using BLASTP alignment against the nonredundant database of the NCBI (Tatusov et al., 2003). BLASTP was used to search the protein sequences of the predicted genes in the evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database (Jensen et al., 2008). The nonredundant gene catalog was aligned against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2004) by BLAST (Version 2.2.28+) and was assigned KEGG functional annotation by KOBAS 2.0 according to previously described methods (Qin et al., 2010).

The nonredundant gene set was compared to the Antibiotic Resistance Genes Database (ARDB, http://ardb.cbcb.umd.edu) and the Comprehensive Antibiotic Resistance Database (CARD, http://arpcard.Mcmaster.ca, Version 1.1.3) using BLASTP (Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The expected value (e-value) of the alignment parameters was 1e-5 and the alignment parameters were set to "strict" alignment. The genes of the target species were then combined with the annotation information for drug resistance functions to obtain an annotated result. The abundance of the AR function was calculated using the sum of the gene abundance corresponding to the AR function.

2.5. Experimental design and sample preparation for animal models

BALB/c J Unib female mice of 18–20 days old were used for this study. They were kept in sterilized microisolators in aerated cages in a controlled environment (humidity, 60–80%; temperature, 22 ± 2 °C). A commercial autoclavable diet sterilized by steam was

given *ad libitum*. The diet was *mcr-1* and bla_{NDM-1} free as confirmed by PCR. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of SLAC Inc.

Four experimental groups (five animals per group) were constructed according to the type of ARG added to the water: C, M, N, and MN (Fig. 2). At the beginning of the experiment on day 0, feces was sampled from all animals and maintained at −20 °C. All groups received specified water for a total of 21 days and fecal samples were collected every 7 days.

Bacterial strains were stored at −20 °C, and those prepared for use in animal water were grown in fresh liquid R2A media overnight at 37 °C. Cultures were then centrifuged for 30 min at 4 °C at 3600×*g*. Cells were then washed twice with PBS (pH 7.2). The pellet was resuspended in 9 mL of PBS to a final concentration of 10^9 CFU/mL. A total of 0.01 mL of this suspension was then added to 1 L of ddH_2O to be given to mice to drink. The daily water intake of each mouse was approximately 20–25 mL.

Fecal DNA was extracted using the QIAamp ® Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol.

2.6. Data analysis

The data was organized in Microsoft Excel 2016, and graphs were plotted using GraphPad Prism 7.0 and Circos (Krzywinski et al., 2009). The differences in grouped data were analyzed using one-way Kruskal-Wallis ANOVA tests followed by Dunn's multiple comparison tests (Chen et al., 2017b). Pearson's and Spearman's correlations were performed using SPSS 21.0 software. All statistical tests were considered significant with a P-value < 0.05.

3. Results

In general, the total number of ARGs were detected in SW by metagenome sequencing. After sequencing, two last resort ARGs (*mcr-1* and bla_{NDM-1}) and two MGEs (*intI1* and *tnpA*) were selected to study their dissemination in WSS. Their abundance and diversity were detected from the source water to the DS over the course of four months with variable temperatures. Bacterial strains were isolated from the samples carrying *mcr-1* and bla_{NDM-1} and positive strains were used to determine the role of DW in carrying last resort ARGs to the healthy mouse gut.

3.1. Source water: A potential source of ARGs, including mcr-1 and bla_{NDM-1}, in the drinking water supply system

3.1.1. Metagenome analysis

Three samples from SW, SW1, SW3 and SW4, were used for shotgun metagenome analysis. The results detected a total of 334 ARGs belonging to 23 different classes specific to the type of antibiotic they are mainly resistant to (Fig. 3). Most of the ARGs in the SW samples belonged to multidrug resistant efflux pump classes, followed by other classes with resistance to certain antibiotics. There was not much difference observed in the relative abundance of the ARGs belonging to specific classes among the three SW samples. Interestingly, in all the three samples, ARGs belonging to classes resistant to beta-lactam and polymyxin antibiotics were identified. The relative abundance of ARGs belonging to the beta-lactam resistance gene family was 2.5%, 2.6% and 2.4% in SW1, SW3 and SW4, respectively. The relative abundance of ARGs belonging to the polymyxin resistance gene family was 3.1%, 3.9% and 2.9% in SW1, SW3 and SW4, respectively. Apart from all of the other ARGs, *mcr-1* and bla_{NDM-1} were detected by metagenome analysis in all the three SW samples.

An increase in the relative abundance could be seen in SW3 compared to SW1, suggesting that the pollution from other rivers coming into contact with the Taipu river contributes to the increased abundance of ARGs. Compared to the influent of the reservoir, the effluent showed less relative abundance of ARGs. The detection of last resort ARGs in SW drove us to study their dissemination throughout the WSS.

3.1.2. Absolute abundance of mcr-1 and bla_{NDM-1} by qPCR

The absolute abundance of *mcr-1* and bla_{NDM-1} was analyzed by qPCR in all four SW samples (SW1, SW2, SW3 and SW4) from different months. There were no significant differences in the absolute abundance of *mcr-1* and bla_{NDM-1} observed among the source water samples (KW test, P > 0.05; Fig. 4). Similarly, there were no significant differences in the occurrence of *mcr-1* and bla_{NDM-1} in the SW over the course of the four month sampling period (one-way ANOVA, F = 1.907 and F = 0.936, P > 0.05).

3.2. Advanced water treatment processes could not effectively remove mcr-1 and bla_{NDM-1}

To check the effects of the water treatment processes on the concentrations of *mcr-1* and bla_{NDM-1}, the influent (WWI) and

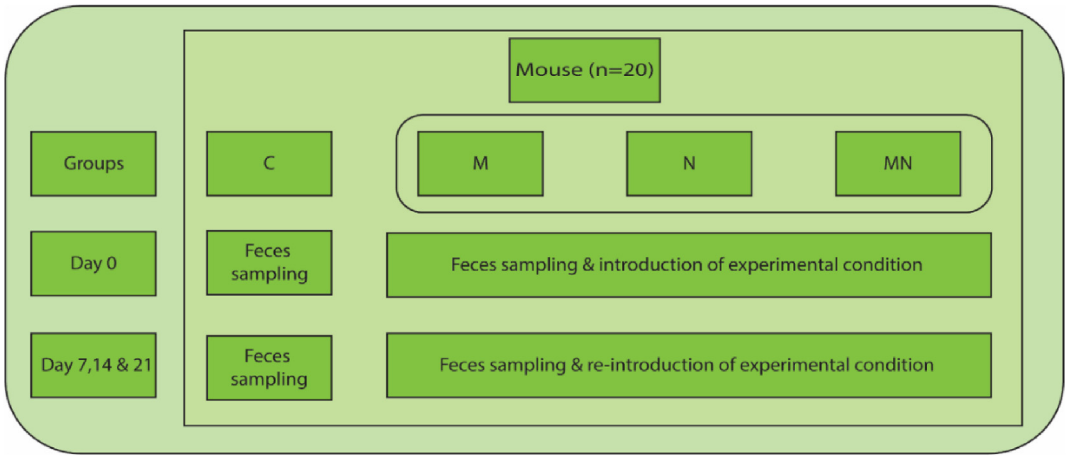

Fig. 2. Flow chart of the study design for testing the effect of ARGs in drinking water on the mouse gut. C: autoclaved water (control), M: mcr-1, N: bla_{NDM-1}, MN: mcr-1 and bla_{NDM-1}.

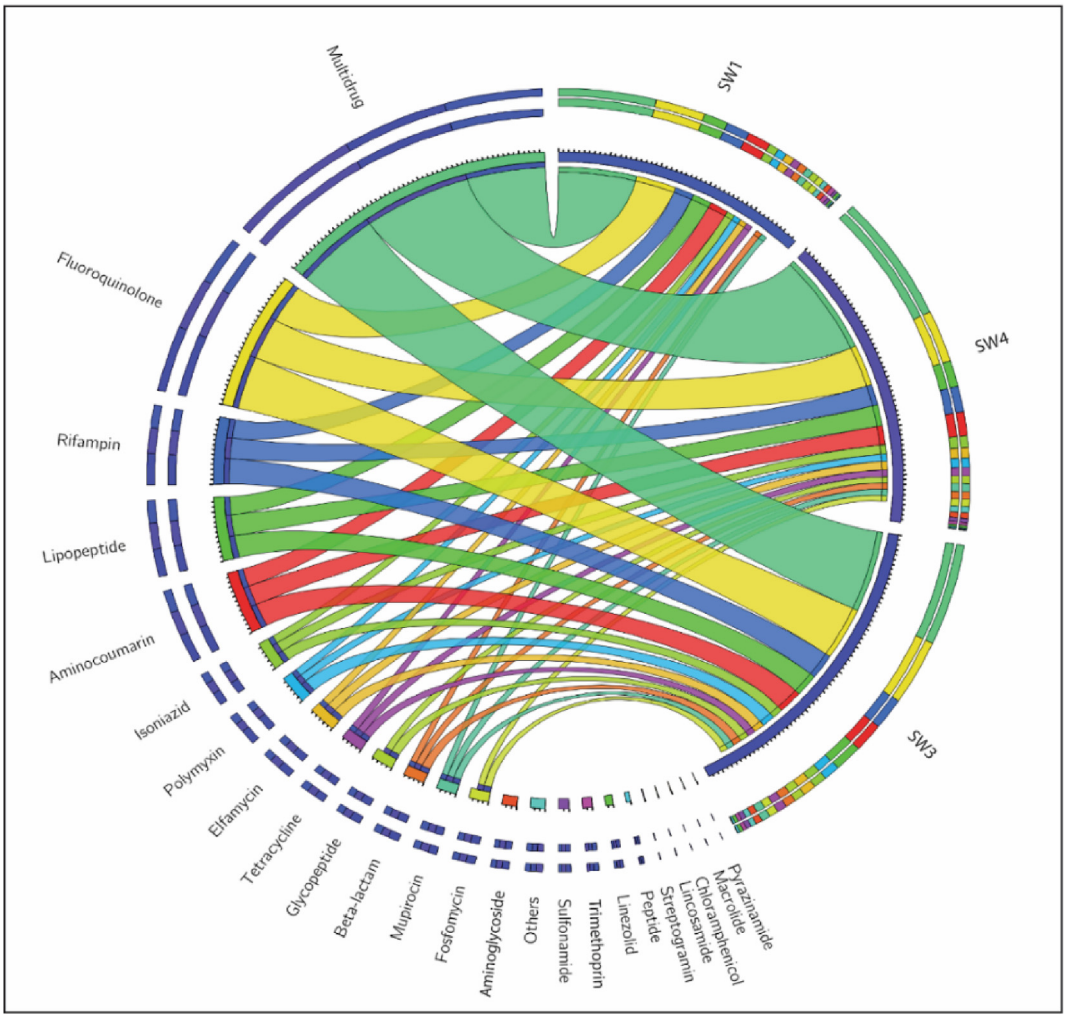

Fig. 3. Relative abundance of ARG classes in the three source water samples by metagenome analysis. SW1= Taipu River, SW3 = Jinze reservoir Influent, SW4 = Jinze reservoir Effluent.

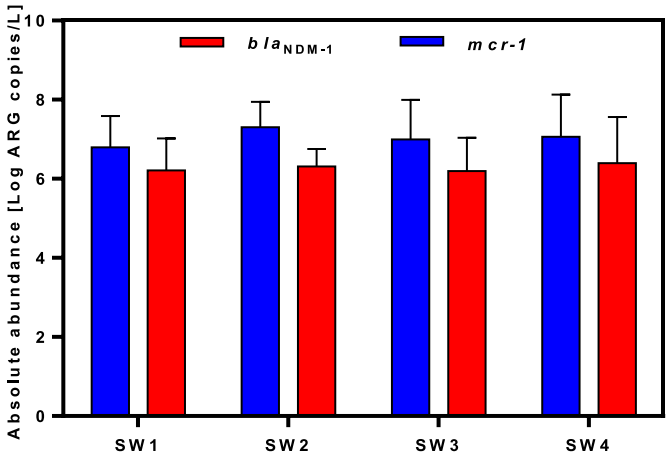

Fig. 4. Mean absolute abundance of ARGs in source water samples from four different months. SW=Source Water (1, 2, 3, 4 represents the different locations from where samples were taken).

effluent (WWE) of the waterworks were checked by qPCR. The results for the abundance of *mcr-1* and bla_{NDM-1} in the waterworks indicated that there was no significant difference between the WWI and the WWE (KW test, $P > 0.05$; Fig. 5). Similarly, there were no significant differences in the occurrence of *mcr-1* and bla_{NDM-1} within the water treatment plant over the four month sampling period (one-way ANOVA, $F = 8.344$ and $F = 0.786$, $P > 0.05$). The

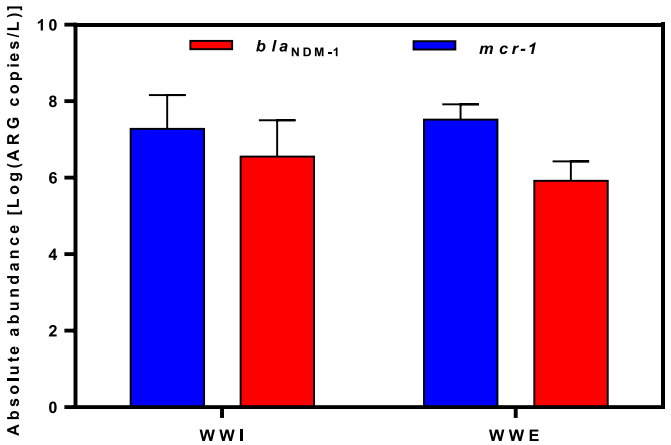

Fig. 5. Mean absolute abundance of ARGs in the waterworks influent and effluent from four different months. WW = waterworks, I = influent, E = effluent.

drinking water treatment process with multiple barriers does not have a significant effect on the removal of these two ARGs.

3.3. Prevalence of *mcr-1* and bla_{NDM-1} in the water distribution system (primary and secondary water supply system)

Four samples were used from the PWSS (i.e., PW1-PW4) and three samples were used from the SWSS (i.e., SeW1-SeW3) to detect *mcr-1* and bla_{NDM-1}.

qPCR results revealed that these two ARGs can be detected in the distribution system; the highest absolute abundance of *mcr-1* and bla_{NDM-1} was observed in PW1, which is the nearest pipeline sample to the water treatment plant (Fig. 6). There was no significant difference in the occurrence of *mcr-1* and bla_{NDM-1} from the DS during the four month sampling period (one-way ANOVA, $F = 0.9179$ and $F = 0.9432$, $P > 0.05$). However, significant differences in the absolute abundance of *mcr-1* and bla_{NDM-1} were observed in the DS over time, with the absolute abundance of *mcr-1* and bla_{NDM-1} being significantly higher in May than in December and March (KW test, $P < 0.05$; Fig. 7).

We found that the correlations between chloramine disinfection and *mcr-1* and bla_{NDM-1} were 0.19 and 0.12, respectively. No obvious correlations were observed.

3.4. The role of MGEs on the prevalence of *mcr-1* and bla_{NDM-1} in the water supply system

Two MGEs, *intI1* and *tnpA*, were used as a proxy for the detection of the absolute abundance of integrons and transposons, respectively, in all samples over the course of four months.

qPCR results revealed that the absolute abundance of *intI1* and *tnpA* increased from SW1 to SW2, then decreased in the reservoir influent but remained the same in the reservoir effluent. In the waterworks samples, we observed that the absolute abundance of both MGEs decreased in the effluent (WWE) compared to the influent (WWI), suggesting a role for the water treatment process in the removal of MGEs from WSS (Fig. 8). In addition to that, no regular pattern of MGE absolute abundance was observed in the PWSS and SWSS. The consistent presence of MGEs in the water supply system may suggest the presence of HGT in the water distribution system and even the gut.

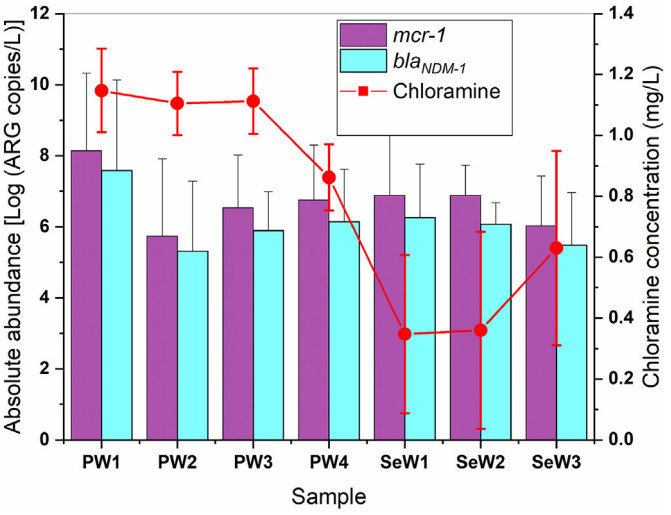

Fig. 6. Mean absolute abundance of ARGs in the primary and secondary distribution system from four different months. PW = primary water supply system, SeW = secondary water supply system.

3.5. Isolation of ARB from the water supply system

Polymyxin- and meropenem-resistant strains were isolated from all the samples in WSS and were used to identify the presence of *mcr-1* and bla_{NDM-1}. The isolated strains that were positive for *mcr-1* and bla_{NDM-1} are shown in Tables S3 and S4 (Supplementary material).

Among all the isolated strains from the WSS, strains carrying *mcr-1* from source to DS were found to be associated with one common genera (i.e., the *Bacillus cereus* group), hence the strains from this genus were used for further mouse experiments. For bla_{NDM-1}, we were only able to isolate strains carrying bla_{NDM-1} from the SW and waterworks, but no strains could be isolated from the DS.

3.6. Dissemination of *mcr-1* from drinking water to the healthy mouse gut

3.6.1. Absolute abundance of *mcr-1* in the mouse gut

Bacterial strains isolated from Shanghai and Beijing tap water belonging to the *Bacillus* and *Xanthobacter* genera were used to characterize the dissemination of *mcr-1* and bla_{NDM-1}, respectively, from DW to the healthy mouse gut.

As shown in Table S5 (Supplementary material), qPCR results suggested that *mcr-1* could disseminate from DW into the mouse gut. Before introducing the strain on Day 0, all samples (M1, MN1) were negative for *mcr-1*, but with the introduction of the strains positive for *mcr-1*, *mcr-1* presence was observed in the mouse feces of all the remaining samples (M2, MN2, M3, MN3, M4, MN4). For bla_{NDM-1}, results were negative throughout the experiment.

The highest increase in relative abundance and absolute gene copies of *mcr-1* was observed in M2, which was the first timepoint that a foreign strain was introduced into the mouse gut (Fig. 9). Later in M3, the relative abundance and the absolute gene copies decreased. Moreover, in M4 a slight increase in the absolute abundance of *mcr-1* was again observed.

3.6.2. Isolation of *mcr-1* positive strains from mouse feces

Polymyxin-resistant strains were isolated from fecal samples carrying *mcr-1* and to our surprise, a strain other than *Bacillus cereus* carrying *mcr-1* was identified in the gut from samples M1 and M4. This strain showed 97–98% similarity to *Enterococcus hirae* (Table S6 in Supplementary material).

To investigate further, some strains belonging to the *Enterococcus* genera were isolated on Day 0 and checked for the presence of *mcr-1*. All of these strains were negative for *mcr-1*, and one of these strains was identified and showed 97% similarity to *Enterococcus hirae* (Fig. S3 in Supplementary material). This suggested that the *Enterococcus* strain did not have *mcr-1* on Day 0, but *mcr-1* may have been transferred to *Enterococcus* by HGT after inoculation of the *Bacillus* strain carrying *mcr-1*, and this may have increased its abundance inside the gut.

3.7. Global spread of *mcr-1* and bla_{NDM-1} in the tap water

Tap water samples of 100 mL from different countries were used in this study to isolate polymyxin- and meropenem-resistant strains and to identify the presence of *mcr-1* and bla_{NDM-1}. We identified *mcr-1* in tap water from China (Shanghai, Beijing), USA (Philadelphia), Pakistan, Korea, Dubai and Germany. bla_{NDM-1} was detected in tap water samples from India and Pakistan. The isolated strains from different countries are shown in Tables S7 and S8 (Supplementary material).

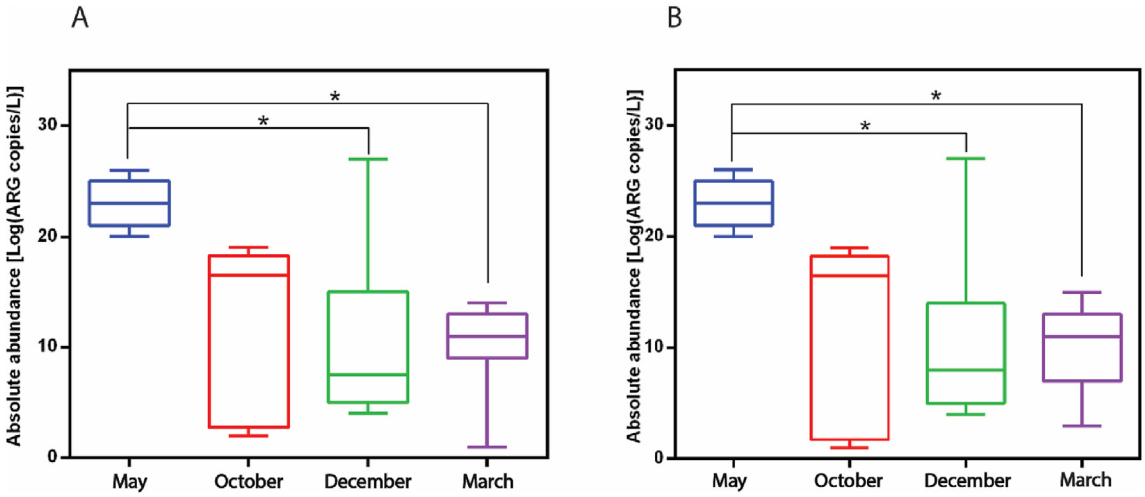

Fig. 7. Comparison of absolute abundance of mcr-1 and bla_{NDM-1} in a distribution system in four different months. Kruskal-Wallis tests were used to determine the significance of the effect on the variation (*: $P < 0.05$). A: mcr-1 and B: bla_{NDM-1}.

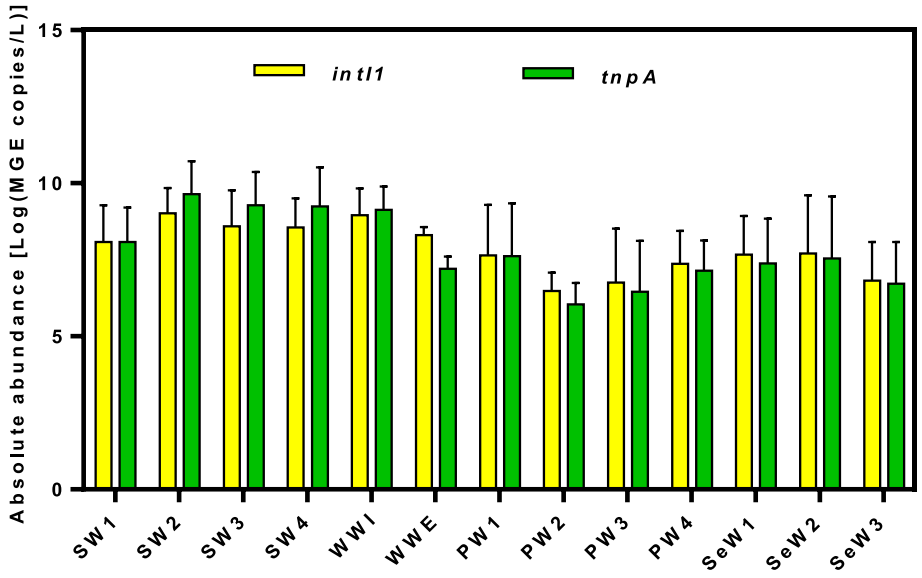

Fig. 8. Mean absolute abundance of MGEs in the water supply system from four different months. SW = source water, WW = waterworks (I=Influent, E = Effluent), PW = primary water supply, SeW = secondary water supply.

4. Discussion

4.1. Source water, a hotspot of ARGs

Due to high antibiotic intake by humans and animals, AR has become a severe risk for human health in China (Hvistendahl, 2012). Lakes along the Yangtze River, including lake Tai, have been polluted due to advancements in the rapid economic development in this region of China (Yang and Lu, 2014; Stange et al., 2019). Animal breeding and wastewater treatment plants may be the main cause of ARGs in these lakes. AR is widespread in the natural environment (Allen et al., 2010). Studies have shown that the Taihu basin is the third highest producer of antibiotics from anthropological, agricultural and aqua cultural activities in China (Zhang et al., 2015), with greater ARG and resistome risk compared to other global lakes, making the Taihu lake a hotspot for resistomes. Precautions are required to prevent the reentry of resistance genes from lakes to humans via DW (Chen et al., 2019).

Studies have shown that, compared to the reservoir system, river systems can be easily affected by anthropogenic activities (Liao et al., 2018; Su et al., 2014a). Therefore, it has been suggested that the river-reservoir system is a perfect system for the dissemination of ARGs by HGT among bacterial communities via MGEs (Lupo et al., 2012; Marti et al., 2014).

In the current study, metagenome analysis revealed the presence of ARGs in the SW (samples from the start of the Taipu river, the middle of the Taipu river, and the reservoir), with less variation found in sample abundance from the reservoir, indicating that lakes and rivers are the major source of ARG and MGE dissemination in the water supply system, which is in accordance with the published studies (Fig. 3).

A recent study reported the presence of bla_{NDM-1} in the Taihu lake but *mcr-1* was undetectable (Stange et al., 2019). By contrast, in this study, both ARGs were found in all SW samples including one sample from the start of the Taipu river (SW1), suggesting that the Taihu lake is also a contributor of *mcr-1* and bla_{NDM-1} in SW (Fig. 4).

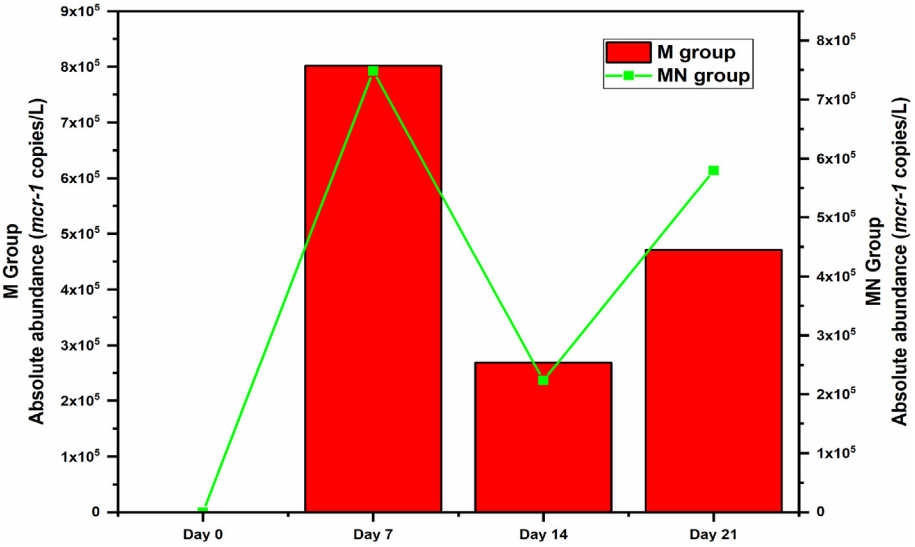

Fig. 9. Absolute abundance of mcr-1 in the mouse gut. M group = mouse group given a bacterial strain carrying mcr-1, MN group = mouse group given bacterial strain carrying mcr-1 and bla_{NDM-1} in drinking water.

4.2. Chloramine disinfection may not be effective at removing ARGs and plays a role in HGT

Guo et al. (2014) showed that the removal of ARGs varies depending on the overall treatment scheme. In the current study, a slight change in the copy number of *mcr-1* and bla_{NDM-1} was observed after the water treatment process, however, these genes could not be removed entirely (Fig. 5). An increase in the relative abundance of ARGs after treatment with BAC and after the final chlorination step has been reported previously (Xu et al., 2016). ARB can survive the selective pressures that occur during the water treatment process. More specifically, chlorine disinfection is known to play an important role in increasing ARB, ARGs and the relative abundance of MGEs during the water treatment process (Huang et al., 2013; Shi et al., 2013; Shrivastava et al., 2004; Xie et al., 2012). Chlorine disinfection prevents ARB survival but may not destroy ARGs resulting in the release of these surviving ARB and ARGs (especially within the deactivated bacterial cells) into aquatic environments (Furukawa et al., 2017). In this study, both *mcr-1* and bla_{NDM-1} were detected in the treated DW. A small increase in the absolute abundance of *mcr-1* was observed.

Water DS could serve as an incubator for ARB growth and as a reservoir for the spread of AR to opportunistic pathogens (Xi et al., 2009). Subinhibitory concentrations of disinfectants used in water systems increase the possibility of AR (Zhang et al., 2017). Correlation between *mcr-1* and/or bla_{NDM-1} was not observed with chloramine disinfectants, indicating that low concentrations of chloramine disinfectants in the DS may not be effective at limiting ARG abundance within the DWDS. Disinfectants and chemicals used in the water treatment process can influence the horizontal transfer of ARGs (Beaber et al., 2004; Prudhomme et al., 2006).

The total abundance of ARGs in the DS was higher than in the processed effluent, which is in agreement with the possibility that bacteria carrying these genes survive during the water treatment process (Su et al., 2018; Xi et al., 2009).

MGEs mediating HGT, such as integrons, transposons and plasmids, play an important role in the widespread dissemination of ARGs to the environment (Marti et al., 2014; Stalder et al., 2012). Various microorganisms acquire AR and ARGs via MGEs through HGT (Martinez et al., 2015). Therefore, in this study, two MGEs, one integron (*intI1*) and one transposon (*tnpA*), were quantified (Fig. 8). The class I integron *intI1* and tnpA were detected in all samples, indicating the influence of human activities because the *class 1* integron-integrase gene has been reported as a proxy for anthropogenic pollution (Gillings et al., 2015).

Certain bacteria are resistant to free chlorine, such as spore-forming bacteria (*Bacillus* or *Clostridium*), and can survive chlorine disinfection (Norton and LeChevallier, 2000). Therefore, strains of the *Bacillus* genera from treated water and DS were isolated and used for mouse experiments (Table S1 in Supplementary material).

Many factors, i.e., antibiotic pressure, high/low temperature, starvation, chlorination, changes in the pH, and oxygen stress, are responsible for stimulating the viable but nonculturable (VBNC) state of any bacteria (Pasquaroli et al., 2013; Patrone et al., 2013; Pawlowski et al., 2011). In this study, it was not possible to culture any strains carrying bla_{NDM-1} from the DS, indicating the possibility that bacteria stressed by chlorination might be in a VBNC state. VBNC bacteria can become reactivated in favorable conditions (Oliver, 2005), and therefore, their infection risk should not be overlooked in aquatic ecosystems (Furukawa et al., 2017).

4.3. Drinking water: A source for the dissemination of ARGs into the gut

The relationship between bacteria and the immune system is generally considered to be antagonistic, however, gut colonizing bacteria that are influenced by diet play an important role in early immune system development (Pannaraj et al., 2017). A previously published study suggested that DW could potentially affect the composition of the gut microbiota (Dias et al., 2018). In this study, we focused on the dissemination of ARGs to the gut through DW.

The innate immune system, which is the first line of defense in infants, is very weak during the early developmental stages compared to the later stages, making infants susceptible to invading pathogens (Simon et al., 2015). The increased levels of *mcr-1* observed after the first inoculation could be due to the weak immune system of the animal (Fig. 9). Further inoculations resulted in decreased *mcr-1* levels, possibly because of the immune system development in the mice. However, the increase in *mcr-1* on day 21 might be because of the colonization of foreign bacteria in the gut or the transfer of this gene into some other surviving strain/member of the gut microbiota.

Enterococcal strains isolated from mouse feces at timepoints pre- and post-inoculation might not belong to the same species, as 16S sequencing results are specific only up to the genus level (Fig. S3 in Supplementary material). The absence of *mcr-1* on day 0 (by qPCR and in the isolated strains) and then on day 21 and the presence of *mcr-1* in the strain separate from the *Bacillus cereus* inocula suggests that there might be some HGT taking place between the two genera inside the mouse gut. In addition, the identification of MGEs in the gut samples supports the fact that the presence of MGEs in the gut samples may facilitate HGT inside the gut (Fig. S2 in Supplementary material).

Dissemination of $bla_{\text{NDM-1}}$ in the mouse gut was not observed, and this may be because of the gut's natural barrier system, which prevents colonization of invading pathogens. Germ-free and/or antibiotic treated rodents might be helpful for further dissemination studies of $bla_{\text{NDM-1}}$ in the gut.

4.4. Temporal effect on ARGs in water supply system

Based on our study of the variations in temperature over four months, we found no change in the detected number of ARGs. In the source and treatment process samples, the absolute abundance of ARGs did not change with temporal variations, however, an increase in the absolute abundance was observed in the month of May (25 °C) in the DS samples (Fig. 7).

Bacterial biomass plays a significant role in influencing the absolute abundance of ARGs (Yang et al., 2018), therefore, a larger bacterial biomass in the DS has the chance to carry more ARGs, which is in accordance with the preference for microbial growth at higher temperatures (Li et al., 2019). Similarly, 16S rRNA sequencing and microbial growth studies have shown positive correlations with warmer temperatures (Li et al., 2018).

4.5. Global spread of mcr-1 and bla_{NDM-1}

The *mcr-1* and $bla_{\text{NDM-1}}$ genes have been emerging as last resort ARGs worldwide, especially in water environments. The countries where $bla_{\text{NDM-1}}$ and *mcr-1* have been detected are listed in Table S9 in the Supplementary material.

To add to the current knowledge, we detected $bla_{\text{NDM-1}}$ in tap water from Pakistan, in addition to India and China, which is in accordance with the previous studies (Tables S8 and S9 in Supplementary material). The *mcr-1* gene has been found to be more mobile compared to $bla_{\text{NDM-1}}$, especially in water environments. The detection of *mcr-1* in tap water from the USA, Pakistan, Korea, Dubai and Germany further supported this study in establishing that there is a vast spread of *mcr-1* globally in water environments, which certainly needs attention (Table S7 in Supplementary material).

Studies have shown that the bacterial community plays a vital part in building ARG profiles, with pathogenic bacteria being more susceptible to attaining and spreading ARGs compared to nonpathogenic bacteria (Forsberg et al., 2014; Wu et al., 2017; Zhou et al., 2017). In aquatic ecosystems most of the ARGs are carried by bacteria (Stokes and Gillings, 2011), therefore, in order to remove ARGs effectively from aquatic environments, ARB removal should be the first step. Consequently, measures should be taken to effectively remove ARB carrying last resort ARGs from the water treatment process to minimize their entrance into the gut. Biofilm growth in DWDS is another factor that needs to be considered to minimize the dissemination of ARGs from DW to the gut.

Hence, we can conclude that the abundant spread of *mcr-1* and $bla_{\text{NDM-1}}$ globally in water environments is posing a great threat to human health. The chance for the dissemination of last resort ARGs from water environments to the healthy gut is evident not only in China, but in other countries as well, which is a matter of great concern globally.

5. Conclusion

In this study, the dissemination of last resort *mcr-1* and $bla_{\text{NDM-1}}$ genes from DW sources to the healthy mouse gut was investigated. In particular, we identified that the DW treatment process could not remove the two ARGs studied and moreover, chloramine disinfection and MGEs play an important role in the dissemination of ARGs in DS.

The presence of *mcr-1* in DW and its dissemination to the healthy mouse gut has not yet been reported. We established that *mcr-1* could disseminate from DW into the healthy mouse gut. Many studies have used mouse models to study human diseases, and the mouse gut has many similarities to the human gut. The dissemination of *mcr-1* into mouse gut suggests that DW can also have an effect on the antibiotic resistance of the human gut microbiota. Therefore, based on this study, we also suggest that one of reason for prevalence of *mcr-1* in humans could be their DW, among other factors.

The microbiome of water not only affects the community structure in the gut when used for drinking purposes but also influences the antibiotic resistance of the gut microbiome through indirect uses, i.e., washing, bathing and cooking, which is a matter of great concern. Further studies are needed to identify effective measures to remove these last resort ARGs from the DW treatment process to make water safer for animal and human consumption.

CRediT authorship contribution statement

Hira Khan: Data curation, Formal analysis, Investigation, Writing - original draft. **Xiaocao Miao:** Data curation. **Mingkun Liu:** Data curation. **Shakeel Ahmad:** Visualization. **Xiaohui Bai:** Conceptualization, Methodology, Validation, Supervision, Writing - review & editing.

Acknowledgments

This research was supported by the key Special Program of the Science and Technology for the Pollution Control and Treatment of Water Bodies (2017ZX07207-003, 2017ZX07207-004, 2017ZX07207-005), the National Natural Science Foundation of China (51878406).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113818.

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