Distribution of antibiotic resistance in the effluents of ten municipal wastewater treatment plants in China and the effect of treatment processes

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HIGHLIGHTS

- The release of antibiotic resistance from WWTP effluents was quantified.
- Antibiotic resistance to sulfonamides was higher than that to tetracyclines.
- *int11* was closely involved in the dissemination of ARGs in WWTPs.
- Disinfection played an important role in eliminating antibiotic resistance.
- Ozone was less effective in reducing antibiotic resistance than UV and chlorine.

GRAPHICAL ABSTRACT

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ABSTRACT

Municipal wastewater treatment plant (WWTP) effluents represent an important contamination source of antibiotic resistance, threatening the ecological safety of receiving environments. In this study, the release of antibiotic resistance to sulfonamides and tetracyclines in the effluents of ten WWTPs in China was investigated. Results indicate that the concentrations of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) ranged from 1.1×10^1 to 8.9×10^3 CFU mL⁻¹ and 3.6×10^1 (*tetW*) to 5.4×10^6 (*tetX*) copies mL⁻¹, respectively. There were insignificant correlations of the concentrations of ARB and ARGs with those of corresponding antibiotics. Strong correlations were observed between the total concentrations of tetracycline resistance genes and sulfonamide resistance genes, and both of which were significantly correlated with *int11* concentrations. Statistical analysis of the effluent ARG concentrations in different WWTPs revealed an important role of disinfection in eliminating antibiotic resistance. The release rates of ARB and ARGs through the effluents of ten WWTPs ranged from 5.9×10^{12} to 4.8×10^{15} CFU d⁻¹ and 6.4×10^{12} (*tetW*) to 1.7×10^{18} (*sul1*) copies d⁻¹, respectively. This study helps the effective assessment and scientific management of ecological risks induced by antibiotic resistance discharged from WWTPs.

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1. Introduction

Nowadays, the emergence and spread of antibiotic resistance in

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the environment have raised increasing concerns. Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been frequently detected in a variety of environmental media, including soil (Burch et al., 2014), surface water (Stoll et al., 2012), and even drinking water (Bai et al., 2015). Owing to their emerging threat to public health, the World Health Organization (WHO) has classified the spread of antibiotic resistance as one of three most serious threats to public health in the 21st century (WHO, 2014).

Municipal wastewater treatment plants (WWTPs) serve as important reservoirs of antibiotic resistance. ARB and ARGs have been widely detected in WWTPs around the world, and the elimination of antibiotic resistance by wastewater treatment processes is generally inefficient (Du et al., 2014; Zhang and Zhang, 2011). Biological treatment facilities may provide a suitable environment for the spread of ARGs, where the high abundance and diversity of bacteria facilitate gene transfer (Zhang et al., 2009). Therefore, ARGs are not effectively eliminated but sometimes even increase inside the biological treatment facilities (Zanotto et al., 2016). Disinfection processes are important to improve the effluent biosecurity, but ARGs may still persist post disinfection with chlorine or UV light (Sharma et al., 2016). As a consequence, the discharge or reuse of WWTP effluents may lead to the dissemination of antibiotic resistance in receiving environments, such as rivers and wastewater-irrigated soils (Chen et al., 2014; Makowska et al., 2016). However, to date, the overall impact of the released antibiotic resistance from WWTPs has not yet been well evaluated. On the one hand, the information regarding the types and concentrations of antibiotic resistance is still limited; and on the other hand, factors which may significantly affect the dissemination of antibiotic resistance, such as antibiotics and horizontal transfer elements (e.g., integrons), need to be further investigated.

The effect of wastewater treatment processes on the transfer of antibiotic resistance has been examined based on field investigation of ARB and ARGs in WWTPs (Bouki et al., 2013; Makowska et al., 2016; Rizzo et al., 2013; Sharma et al., 2016). However, results of those studies were largely variable and sometimes even contradictory. Given the difference of target indicators of antibiotic resistance (e.g., bacterial groups and ARG types), the performance of different WWTPs in eliminating antibiotic resistance can be hardly compared with accuracy. Statistical analysis may help assess the effect of treatment processes on the elimination of antibiotic resistance.

Therefore, the present study was to: 1) quantify the release of antibiotic resistance from the effluents of ten WWTPs; 2) determine the correlation of antibiotic resistance with antibiotics and integrons in the effluents; 3) clarify the effect of treatment processes on the elimination of antibiotic resistance in the effluents. Sulfonamides (SAs) and tetracyclines (TCs) were selected as representative antibiotics due to their frequent detections in WWTPs in China (Yuan et al., 2015). The concentrations of heterotrophic bacteria encoding resistance to SAs and TCs in the effluents were determined. A total of seven *tet* genes (i.e., *tetA*, *tetC*, *tetG*, *tetO*, *tetW*, *tetM* and *tetX*), two *sul* genes (i.e., *sul1* and *sul2*), and one integrase gene (i.e., *int1*) were quantified by using the quantitative real-time polymerase chain reaction (q-PCR) method.

2. Materials and methods

2.1. Sample collection

The selected ten WWTPs were located in Beijing, Wuxi and Qingdao cities in China, with detailed information provided in Table 1. Their treatment processes were commonly adopted in other WWTPs of China. Domestic sewage was predominant in all influents of the selected WWTPs, with average flow rates ranging from 80,000 to 600,000 m³ d⁻¹. A proportion of 10–40% of the

effluents from WWTPs B1, B3, B4 and B5 were reused for lawn irrigation, landscaping and cooling water in thermal power plants, whilst all other effluents were discharged to nearby rivers. For each WWTP studied, two effluent samples were respectively collected in May (spring) and November (winter) in 2015, whose characteristics are listed in Table S1. During the sampling period, the WWTPs were confirmed to be under normal operations and no rainfall event was recorded. Flow-proportional (24 h) composite samples were collected using automatic samplers (SD 900, Hach, USA) and 10 L of composite sample were transferred into brown glass bottles, chilled in icebox, and then transported to the laboratory.

2.2. Analysis of antibiotics

The concentrations of seven SAs (i.e., sulfadiazine (SDZ), sulfathiazole (STZ), sulfamerazine (SMR), sulfamethazine (SMN), sulfamethizole (SML), sulfamethoxazole (SMX), and sulfadimethoxine (SDM)) and four TCs (i.e., tetracycline (TCN), oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DOX)) in the effluents were determined by the method developed in our previous study (Yuan et al., 2015). In brief, each effluent sample (500 mL) was extracted and purified by solid phase extraction with an Oasis HLB cartridge (500 mg/6 mL, Waters, USA), and the extract was analyzed by ultra-high performance liquid chromatography and tandem mass spectrometry (Agilent 6420, USA). The limits of quantification of tested SAs and TCs were in the range of 0.04–0.22 ng L⁻¹. The detection of antibiotics in each sample was performed in triplicate.

2.3. Quantification of ARB

Heterotrophic plate counts (HPCs) on antibiotic-selective media were used to determine the ARB concentrations in the effluents. Ten-fold serial dilutions (i.e., 10-, 100- and 1000-fold) were performed for the effluent samples with phosphate-buffered saline (pH 7.4) to obtain colony counts in the range of 30–300 per plate, and triplicate plate counts were conducted for each dilution. Diluted sample aliquots (1 mL each) were added into 10 mL of nutrient agar (peptone: 10 g L⁻¹, beef extract: 3 g L⁻¹, NaCl: 5 g L⁻¹, agar: 15 g L⁻¹, pH = 7.2) spiked with tested antibiotics in advance. The spiked concentrations of TCN and SMX were 16.0 and 50.4 mg L⁻¹, respectively, according to both the resistance break-points used for testing clinical ARB isolates (CDC, 2008) and the suggested concentrations in published literature (Gao et al., 2012). Plates were incubated at 37 °C for 2 d before counting the bacterial colonies. The total HPCs in each effluent sample were also determined using antibiotic-free nutrient agar.

2.4. Quantification of ARGs

Each effluent sample of 200 mL was filtered through 0.22-μm polyvinylidene fluoride membranes (Millipore, USA), and then the membranes were cut into pieces with pre-sterilized scissors and subjected to DNA extraction. All DNA extractions were performed with Fast DNA SPIN kit for soil (MP-bio, USA). The concentration and quality of the extracted DNA were determined by spectrophotometry (Genequant 1300, GE Healthcare, USA) and agarose gel electrophoresis, respectively. The extraction and analysis of DNA for each sample were performed in triplicate. The q-PCR method was applied to quantify the tested ARGs with the conditions provided in our former study (Wang et al., 2015). The amplification efficiencies and limits of detection for gene copy number are available in Table S2.

Table 1

Treatment processes and operation parameters of the ten selected WWTPs in China.

WWTP	Treatment processes	Flow rate ($\times 10^5 \text{ m}^3 \text{ d}^{-1}$)	HRT ^a (h)	SRT ^b (d)	Disinfection
B1	Anaerobic/anoxic/oxic → membrane bioreactor	6	9.3	15	O ₃
B2	Oxidation ditch → coagulation/sedimentation	2	16	17	UV
B3	Anaerobic/oxic → membrane bioreactor	1	21	18	UV
B4	Anaerobic/anoxic/oxic → ultra-filtration	5.5	16	20	O ₃
B5	Anaerobic/oxic → biofilter → ultra-filtration	5	10	21	UV
B6	Anaerobic/oxic	5	18	20	O ₃
W1	Oxidation ditch → rotary fiber disc filter	1.5	17	18	UV
W2	Anaerobic/anoxic/oxic → rotary fiber disc filter	2	20	21	UV
Q1	Anaerobic/anoxic/oxic → coagulation/sedimentation → rotary fiber disc filter	1.5	21	13	NaOCl
Q2	Anaerobic/anoxic/oxic → coagulation/sedimentation → rotary fiber disc filter	8	21	19	UV

^a HRT: hydraulic retention time.^b SRT: sludge retention time.

2.5. Statistical analysis

Correlations of the antibiotics, 16S rDNA, *intl2* and plant operation and effluent quality parameters with the target ARB and ARGs were evaluated by Pearson's bivariate correlation analysis (SPSS 19.0, IBM, USA). Paired samples *t*-test based on *p*-value was performed to evaluate the significance of difference among different samples. Principal component analysis (PCA) was used to cluster the effluent samples using Canoco 4.5 software for Windows.

3. Results and discussion

3.1. Antibiotics in effluents

The concentrations and detection frequencies of SAs and TCs in the effluents of ten WWTPs are presented in Fig. 1. All the target SAs and TCs were detected in the effluent samples with detection frequencies higher than 65% (except SMR with a low detection frequency of 20%), indicating an incomplete removal of antibiotics in the WWTPs. The mean concentration of total SAs (i.e., a sum of the concentrations of seven SAs) was 118.3 ng L^{-1} , which was significantly higher ($p < 0.01$) than that of total TCs (i.e., a sum of the concentrations of four TCs, 24.5 ng L^{-1}). The concentrations of individual antibiotics are listed in Table S3. SMX was the most

prevalent SA antibiotic in the effluents with a mean concentration of 82.8 ng L^{-1} , while OTC was detected with the highest mean concentration (i.e., 12.4 ng L^{-1}) among TCs. The relatively lower concentrations of TCs could be attributed to their reduced usage in China in recent years. Moreover, sludge adsorption was found to be the primary transfer pathway of TCs in WWTPs, which may also account for their low abundance in the effluents (Yuan et al., 2015).

3.2. ARB and ARGs in effluents

The concentrations of total HPCs, tetracycline-resistant bacteria (TRB) and sulfonamide-resistant bacteria (SRB) detected in the effluents of ten WWTPs are shown in Fig. 2. SRB and TRB were detected with concentrations ranged from 8.8×10^2 to $8.9 \times 10^3 \text{ CFU mL}^{-1}$ and 1.1×10^1 to $2.1 \times 10^3 \text{ CFU mL}^{-1}$, respectively. The mean concentrations of total HPCs, TRB and SRB were 3.3×10^4 , 2.7×10^3 and $4.5 \times 10^2 \text{ CFU mL}^{-1}$, respectively. The mean ratios of TRB and SRB to the total HPCs were 3% and 12%, and their highest ratios reached up to 12% and 36%, respectively, indicating a considerable fraction of resistance in bacteria in the effluents. In a WWTP located in Shanghai of China, SRB and TRB were found to occupy about 30% and 10% of heterotrophic bacteria in effluent samples, respectively (Yuan et al., 2014). Hence, it can be seen that SRB commonly exhibit higher abundances than TRB in WWTP effluents, which is most possibly related to the extensive application of SAs.

Fig. 1. Antibiotic concentrations in the effluents of ten WWTPs ($n = 20$). The detection frequency (%) of each antibiotic is indicated numerically at the top of the column. Total SAs: a sum of the concentrations of seven sulfonamides; total TCs: a sum of the concentrations of four tetracyclines.

Fig. 2. Antibiotic-resistant bacteria (ARB) concentrations and the ratios of ARB to total heterotrophic plate counts (HPCs) in the effluents of ten WWTPs ($n = 20$). TRB: tetracycline-resistant bacteria; SRB: sulfonamide-resistant bacteria.

The target TRGs in this work represent three main mechanisms of bacterial resistance to TCs, including genes encoding ribosomal protection (RPP), efflux pump (EFP), and enzymatic inactivation (EI) proteins. *Sul1* and *sul2* are mutated dihydropteroate synthase genes, representing the primary mechanism of bacterial resistance to SAs. These ARGs can be easily disseminated by means of bacterial reproduction and horizontal gene transfer among bacteria, which contributes to their frequent detection in the environment. The ARG concentrations in the effluents of ten WWTPs are shown in Fig. 3, with detailed concentration for each ARG listed in Table S4. Results indicate that the target ARGs were universal in all effluent samples, demonstrating a similar emergence pattern in the studied WWTPs.

Among the target ARGs, *sul1* and *sul2* were detected as the most abundant gene types with mean concentrations of 1.1×10^6 and 6.7×10^5 copies mL^{-1} , respectively. *TetX* was detected with distinctively high concentrations (i.e., 3.3×10^3 – 5.4×10^6 copies mL^{-1}) among the TRGs in all cases. *TetX* has drawn attention because it tends to elevate the minimum inhibitory concentrations of certain bacterial strains for tigecycline. Tigecycline belongs to a new generation of TCs developed in response to the increasing resistance to traditional TCs during recent decades (Bartha et al., 2011). Hence, the prevalence of *tetX* in the effluents may imply a high ecological risk of the bacteria encoding resistance to TCs. The EFP TRGs, including *tetA*, *tetC* and *tetG*, were detected with mean concentrations of 3.0×10^4 , 4.2×10^4 and 3.5×10^4 copies mL^{-1} , respectively. The RPP TRGs, including *tetM*, *tetO*, and *tetW*, showed relatively lower abundance, with mean concentrations of 1.9×10^4 , 7.7×10^4 and 1.0×10^4 copies mL^{-1} , respectively. EFP TRGs have a broad host range, which leads to their high prevalence in various environmental matrices. For example, Zhang and Zhang (2011) reported higher levels of *tetA* and *tetC* than other 12 TRGs in WWTPs. In a biological treatment system for oxytetracycline production wastewater, *tetA*, *tetC* and *tetG* were found to be the most abundant types among the tested TRGs (Liu et al., 2012). In contrast, the host bacteria of RPP TRGs are less adaptive to the environment (Wang et al., 2016), which may explain their relatively lower abundance in the effluents.

Fig. 3 also shows that the total concentrations of SRGs (2.0×10^4 – 6.4×10^6 copies mL^{-1}) were higher than those of TRGs (5.8×10^3 – 5.9×10^6 copies mL^{-1}), probably because SRGs are more recalcitrant to wastewater treatment processes than TRGs (Chen and Zhang, 2013; Munir et al., 2011).

Fig. 3. Antibiotic resistance gene (ARG) concentrations in the effluents of ten WWTPs ($n = 20$). Total TRGs: a sum of the concentrations of seven tetracycline resistance genes; total SRGs: a sum of the concentrations of two sulfonamide resistance genes.

3.3. Correlation analysis

The concentrations of total SAs and TCs showed weak correlations with the corresponding ARB and ARGs (either total gene copy numbers or a specific ARG) ($p > 0.05$) (Table S5). A possible explanation is that with low antibiotic concentrations, many other factors, such as co-selection and cross-selection, may affect the dissemination of antibiotic resistance in wastewater. Li et al. (2015) found insignificant correlations between the concentrations of SMX and TCN and their corresponding ARB ($p > 0.05$) in wastewater samples from a WWTP. Gao et al. (2012) also reported that TRGs (*tetO* and *tetW*) showed insignificant correlations with TC concentrations in the WWTP wastewater. It is seen that the abundance of antibiotic resistance does not directly correspond to the detected real-time concentrations of antibiotics; for the evolution of antibiotic resistance in bacteria, the time factor also counts. Therefore, it is not proper to use antibiotic concentrations as an indicator to forecast antibiotic resistance levels in WWTP effluents.

Integrins are greatly involved in the horizontal gene transfer of ARGs. As shown in Fig. 4a, both the total concentrations of TRGs and SRGs were significantly correlated with *int1*, showing Pearson $r = 0.778$ and 0.784 ($p < 0.01$), respectively. *Sul1* and *tetG* are directly associated with *int1* (Boyd et al., 2000; Mazel, 2006), whilst other ARGs (i.e., EFP TRGs and *sul2*) are likely associated with plasmids that can harbor integrins (Wang et al., 2015), which may explain these significant correlations. As the most common

Fig. 4. Pearson's bivariate correlation between ARGs and *int1* (a) and between total SRGs and total TRGs (b) in the effluents of ten WWTPs ($n = 20$).

integron type and an indicator of horizontal gene transfer potential, *intI1* has been identified to correlate with ARGs in various environmental matrices, such as soils (Wang et al., 2014) and surface water (Drudge et al., 2012). Hence, the positive correlation of tested ARGs with *intI1* in the effluents hints the risk of dissemination of ARGs through horizontal gene transfer when the effluents are discharged or reused.

The total concentrations of TRGs and SRGs showed a strong correlation with 16S rDNA, with Pearson $r = 0.690$ and 0.895 ($p < 0.01$), respectively (Table S5), implying that the abundance of bacteria in the effluents could play an important role in the horizontal transfer of both TRGs and SRGs. Fig. 4b shows a strong correlation between total SRGs and total TRGs (Pearson $r = 0.762$, $p < 0.01$), indicating the genetic linkage driven by co-occurrence and co-transfer of ARGs, which may result in multidrug resistance in bacteria. This result highlights the importance of co-transfer of ARGs contributing to the ARG dissemination in the environment.

3.4. Effect of treatment processes

Although the treatment processes were diverse in the investigated WWTPs (Table 1), no significant difference ($p > 0.05$) in the concentrations of ARB and ARGs was observed among various effluent samples. Moreover, no strong correlations were found between the concentrations of ARB and ARGs and the parameters of plant operation (i.e., flow rate, hydraulic retention time, and sludge retention time) and effluent quality (i.e., concentrations of suspended solids, chemical oxygen demand, and total nitrogen) (Table S6). PCA was performed using the ARG concentrations in the effluent samples. The resistance profiles of effluent samples showed two different clusters (i.e., (B1, B4, B6) vs. (B2, B5, Q1, Q2, W1, W2)) except B3 (Fig. 5). This classification pattern was probably ascribed to the different disinfection methods adopted in the selected WWTPs. As shown in Table 1, ozone was adopted in WWTPs B1, B4 and B6, and UV light or chlorine in all other WWTPs. According to published literature (Cengiz et al., 2010; Guo et al., 2015; Huang et al., 2013), disinfection processes using ozone, chlorine and UV light can all induce DNA damages and thus deactivate the ARG expression. However, their actual performances in reduction of antibiotic resistance depend on the operation conditions, such as wastewater characteristics, ozone dose, UV intensity,

and contact time with bacteria (Rizzo et al., 2013). In this work, the PCA result clearly differentiated ozone from other disinfection technologies. As shown in Table S4, the effluent ARG concentrations in the WWTPs with ozone disinfection were a little higher than those of other plants, indicating that ozone was relatively less effective in reducing ARGs than UV and chlorine. A few studies have recently reported that ozone dosages commonly used for disinfection could not significantly eliminate the antibiotic resistance in wastewater effluents (Czekalski et al., 2016; Pak et al., 2016). An important reason is that ozone tends to be primarily consumed by the abundant dissolved organic matter present in the effluents.

The PCA result highlights an important role of disinfection in controlling the release of antibiotic resistance from WWTPs. Hence, there is a need to explore the mechanisms for eliminating antibiotic resistance in WWTP effluents by various disinfection methods.

3.5. Release of ARB and ARGs through effluents

Recently, antibiotic resistance in aquatic environment has been frequently detected, which can often be traced to the antibiotic resistance in WWTP effluents (Iwane et al., 2001; Xu et al., 2015). The aquatic environment appears to allow the ARGs of sewage origin to persist and spread therein, which consequently increases the risk of gene transfer to human and animal pathogens via food chains or drinking water (Czekalski et al., 2015). Furthermore, the reuse of WWTP effluents as irrigation water may also promote the dissemination of ARGs into soils (Wang et al., 2014). These risks require special attention considering a continuously rising tendency to reuse the WWTP effluents worldwide.

The release rates of ARB and ARGs through the effluents of ten WWTPs were calculated by multiplying the concentrations of ARB and ARGs by the average flow rate, assuming a steady flow for all the treatment plants. As shown in Fig. 6, the release rates of SRB and TRB were in the ranges of 8.7×10^{13} to 4.8×10^{15} and 5.9×10^{12} to 1.1×10^{15} CFU d⁻¹, respectively. With respect to ARGs, their release rates ranged from 6.4×10^{12} (*tetW*) to 3.2×10^{18} (*tetX*) copies d⁻¹. For ARB and most of ARGs, higher release rates were observed in winter than in spring. Mezrioui and Baleux (1994) found that a high temperature favored the treatment of activated sludge, which resulted in a higher reduction of faecal coliforms in summer than in winter. In addition, higher antibiotic loading rates in WWTP influents in winter have been often reported (Castiglioni et al., 2006; Zhang et al., 2015), which also induces a higher selection pressure for ARB.

Fig. 5. Principal component analysis of ARG concentrations in the effluents of ten WWTPs. Circles indicate possible sample groups according to their origins.

Fig. 6. Release rates of ARGs and ARB through the effluents of ten WWTPs.

Munir et al. (2011) reported that in the effluents of five WWTPs in the United States (US), the release rates were around 10^{12} copies d^{-1} and 10^{11} CFU d^{-1} for ARGs (*tetW*, *tetO* and *sul1*) and ARB (TRB and SRB), respectively. Given similar wastewater treatment processes adopted in US and China, the considerably higher release rates observed in this work indicate a more serious pollution of antibiotic resistance in China's WWTPs. This result emphasizes the necessity of eliminating antibiotic resistance in WWTP effluents to reduce potential ecological risks in the receiving environments.

4. Conclusions

The results of this work clearly demonstrated the prevalence of antibiotic resistance in the effluents of WWTPs in China. The degree of antibiotic resistance to SAs was always higher than that to TCs in the effluents. The significant correlation of ARGs with *int11* indicated the risk of ARG dissemination in the environment through horizontal gene transfer along with the discharge or reuse of WWTP effluents. Moreover, the strong correlations between the total concentrations of TRGs and SRGs revealed the multidrug resistance in bacteria, which deserves more attention in the future. Disinfection played an important role in eliminating antibiotic resistance in the effluents, and further studies are needed to enhance the removal efficiency of ARB and ARGs through optimized disinfection processes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2017.01.041>.

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