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Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants

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ABSTRACT

The propagation of antibiotic resistance genes (ARGs) is an emerging health concern worldwide. Thus, it is important to understand and mitigate their occurrence in different systems. In this study, 30 ARGs that confer resistance to tetracyclines, sulfonamides, quinolones or macrolides were detected in two activated sludge wastewater treatment plants (WWTPs) in northern China. Bacteria harboring ARGs persisted through all treatment units, and survived disinfection by chlorination in greater percentages than total Bacteria (assessed by 16S rRNA genes). Although the absolute abundances of ARGs were reduced from the raw influent to the effluent by 89.0%–99.8%, considerable ARG levels [$(1.0 \pm 0.2) \times 10^3$ to $(9.5 \pm 1.8) \times 10^5$ copies/mL)] were found in WWTP effluent samples. ARGs were concentrated in the waste sludge (through settling of bacteria and sludge dewatering) at $(1.5 \pm 2.3) \times 10^9$ to $(2.2 \pm 2.8) \times 10^{11}$ copies/g dry weight. Twelve ARGs (*tet*A, *tet*B, *tet*E, *tet*G, *tet*H, *tet*S, *tet*T, *tet*X, *sul*1, *sul*2, *qnr*B, *erm*C) were discharged through the dewatered sludge and plant effluent at higher rates than influent values, indicating overall proliferation of resistant bacteria. Significant antibiotic concentrations (2%–50% of raw influent concentrations) remained throughout all treatment units. This apparently contributed selective pressure for ARG replication since the relative abundance of resistant bacteria (assessed by ARG/16S rRNA gene ratios) was significantly correlated to the corresponding effluent antibiotic concentrations. Similarly, the concentrations of various heavy metals (which induce a similar bacterial resistance mechanism as antibiotics – efflux pumps) were also correlated to the enrichment of some ARGs. Thus, curtailing the release of antibiotics and heavy metals to sewage systems (or enhancing their removal in pre-treatment units) may alleviate their selective pressure and mitigate ARG proliferation in WWTPs.

1. Introduction

A large number of studies have demonstrated that the widespread use of antibiotics to treat microbial infections in humans and to promote animal growth have led to the proliferation of antibiotic resistant bacteria (ARB) in the environment. Antibiotic resistance genes (ARGs) have been detected in surface water (Reinthaler et al., 2003), groundwater (Chee-Sanford et al., 2001), sediments (Pei et al., 2006; Storteboom et al., 2010) and wetlands (Cummings et al., 2010). High concentrations of multidrug-resistant bacteria have also been detected in domestic sewage, hospital wastewater, and drainage from livestock feeding operations (Pruden et al., 2006; Rysz and Alvarez, 2004).

Wastewater treatment plants (WWTPs) play a vital role in minimizing the discharge of many water pollutants, including antibiotics (Gulkowska et al., 2008; McArdell et al., 2003; Watkinson et al., 2007; Zhou et al., 2013) and pathogenic microorganisms (Frigon et al., 2013; Viau and Peccia, 2009) to the environment. However, WWTPs serve not only as collection points for resistant organisms and antimicrobials from a wide variety of sources (i.e., hospitals, industries, households), but are also potential breeding grounds and documented point sources for environmental

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dissemination of antibiotic resistance (Pruden et al., 2013). Paradoxically, biological treatment units in WWTPs promote bacterial growth and genetic exchange (Zhang et al., 2009), which in turn may lead to further ARG proliferation (Du et al., 2015, 2014; Luo et al., 2014; Szczepanowski et al., 2004; Zhang et al., 2009; Zhang and Zhang, 2011). Discharge of ARGs in the WWTP effluent to natural water systems, or in biosolids applied to agricultural soil may enhance the propagation of antibiotic resistance to indigenous bacteria (Auerbach et al., 2007; Fahrenfeld et al., 2013; Luo et al., 2014). For example, multi-drug resistant *Achromobacter* sp. isolated from a WWTP transferred the NDM-1 gene to a *Comamonas* sp. that was indigenous to the receiving waters of the Haihe River in China (Luo et al., 2014). Furthermore, extracellular DNA (eDNA) in river sediments (possibly stabilized by clay particles and organic matter that decrease susceptibility to degradation via nuclease attack) was shown to transform indigenous bacteria as a potential ARG propagation mechanism (Ghosh and LaPara, 2007; Mao et al., 2014; Nwosu, 2001; Riesenfeld et al., 2004).

Various types of antibiotics have been shown to exert selective pressure for the maintenance and propagation of ARGs in river sediments, irrigation ditches, dairy lagoons and WWTPs (Luo et al., 2010; Pruden et al., 2006). However, other studies have also shown ARG enrichment in the absence of antibiotics (Alonso et al., 2001; Baker-Austin et al., 2006; Chen et al., 2013; Wright et al., 2006). Antimicrobial drugs and other chemical stressors (e.g., heavy metals, biocides) are regularly found in waste streams that enter WWTPs. Along with antibiotic residues, these compounds may co-select for ARBs, enhance the reproduction of resistance vectors within ARBs, and possibly stimulate horizontal exchange of resistance genes. To date, the extent to which antibiotics entering WWTPs (and the associated treatment processes) influence ARGs dynamics (including propagation or removal) is poorly understood, and thus warrants further investigation. It is also important to understand the potential influence of other factors such as sewage-associated heavy metals that may induce similar bacterial defense mechanisms as antibiotics (e.g., efflux pumps) (Levy, 2002; Wright et al., 2006).

This study investigated the profiles of 30 ARG profiles in two WWTPs in northern China. Quantitative real-time PCR (qPCR) was used: 1) to determine the concentrations and flow of 23 of the most frequently detected ARGs through each WWTP treatment unit, treated WWTP effluent, and discharged biosolids; 2) to quantify how individual wastewater treatment processes affect the fluxes of antimicrobial drugs, antimicrobial resistant organisms, and antimicrobial resistance elements (e.g., their associated DNA). We analyzed 16S rRNA genes as a surrogate for estimating total Bacteria counts. The influence of antibiotic residues and heavy metals, which may contribute selective pressure on certain ARGs, was also investigated.

2. Methodology

2.1. Descriptions of wastewater treatment plants

Two full-scale activated sludge WWTPs in northern China were investigated. WWTP1 processes approximately 540,000 m^3 per day of mainly domestic sewage from a population of about 2.1 million. WWTP2 processes approximately 580,000 m^3 per day of both domestic and industrial wastewater for a population of about 2.2 million (Table S1). Both plants employ anaerobic and anoxic lagoons followed by a conventional activated sludge process, the contact time for chlorination of treated effluent is 30 min, with a chlorine (Cl_2) disinfection dose of 5 mg/L. A detailed description of the treatment processes is provided elsewhere (Luo et al., 2014). WWTPs operational parameters are presented in the SI section (Table S1).

2.2. Sample collection and DNA extraction

One-liter water samples were collected from the outlet of each treatment unit of each WWTP using a GRASP refrigerated automatic sampler (GRASP Science & Technology Co., LTD, Beijing, China). To avoid confounding effects associated with hydraulic loading fluctuations, composite samples were collected every 2 h for a 24 h period, and analyzed in triplicate. Dewatered sludge (DS) samples were collected in triplicate using a scraper. All samples were stored on ice, transported to the laboratory, and maintained at 4 °C until they were processed for analyses within 24 h of sample collection. Water samples were collected from the following locations (Fig. 1): (1) raw influent (RI), (2) primary clarifier tank (PCT), (3) anaerobic tank (AaT), (4) anoxic tank (AT), (5) aerated tank (AeT), (6) secondary clarifier (SCT) and (7) final effluent (FE). Sludge samples were obtained from units: (8) recycled active sludge, (9) combined primary and secondary waste sludge, and (10) dewatered sludge. The samples were collected in December 2011 and June 2012; final effluent samples were also collected in November and December 2011, and in June and July 2012 to account for potential seasonal variations in ARG flows and fate.

Water samples (0.5 L) were vacuum-filtered (0.22-μm filters) and the filters were placed in extraction tubes provided in the Ultraclean Water DNA Kit (MoBio Laboratories, Inc.). For sludge (0.5 L), DNA was extracted with the Soil DNA Isolation Kit (MoBio Laboratories, Inc.). The extracted DNA was further purified using the DNA pure-spin kit (Vigorousbio, Beijing, China) to minimize PCR inhibition. An internal standard (*Escherichia coli* DH5α cloned with the CESA9 gene) was used to determine DNA extraction efficiency as described previously (Luo et al., 2010).

2.3. Qualitative PCR

PCR assays targeting the 16S rRNA gene and 30 ARGs, including 20 tetracycline (*tet*), 4 sulfonamide (*sul*), 4 quinolone (*qnr*), and 2 macrolide (*erm*) resistance genes were conducted using a Biometra TGradient Thermal Cycler (Biometra Company, Germany). Table S2 summarizes primer sequences, amplicon sizes, and annealing temperatures. PCR amplification of template DNA was conducted with commercially available kits (TransgenR) according to manufacturer's protocols. Each 25 μL PCR reaction mixture contained PCR buffer, 400 μM each dNTP, 400 nM of each forward and reverse primer, 1.25 U Taq DNA polymerase, and 1 μL of template DNA. Duplicate PCR reactions were performed for each sample. Negative controls consisted of 1-μL sterile water in lieu of the DNA template, and positive controls were genome (for 16S rRNA) or plasmid DNA (cloned ARGs) extracted from *E. coli* DH5α. Both positive and negative controls were included in each PCR run.

Each PCR amplification for the target ARGs commenced with an initial 5-min DNA denaturation step at 95 °C, followed by 35 amplification cycles each consisting of: i) denaturing for 30 s at 95 °C; ii) annealing for 30 s at the temperatures specified in Table S2, and iii) extension for 1 min at 72 °C. A final 7-min extension at 72 °C was performed in all the analyses. PCR products were sequenced by BGI, Beijing China, and were confirmed by aligning to ARG sequences in Genbank database with BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Standard plasmid and quantitative real-time PCR (qPCR)

All qPCR assays were performed using the Bio-Rad IQ5 instrument (Bio-Rad Company, Hercules, CA, USA). Calibration standard curves for positive controls were generated as described previously

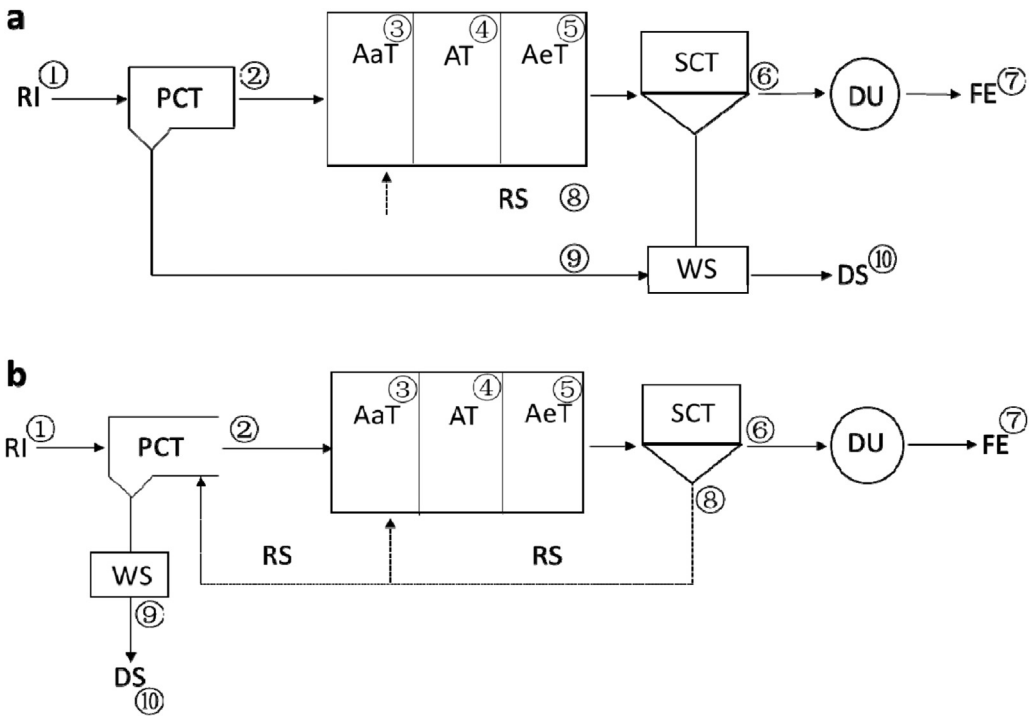

Fig. 1. Process flow diagrams and sampling points for two waste water treatment plants (WWTPs) in northern China: (a) WWTP1 and (b) WWTP2. Abbreviations: RI: raw influent, PCT: primary clarifier tank, AaT: anaerobic tank, AT: anoxic tank, AeT: aerated tank, SCT: secondary clarifier tank; DU: disinfection unit, FE: final effluent, RS: recycled sludge, WS: waste sludge, DS: dewatered sludge (also lime-treated for WWTP2).

(Pei et al., 2006). Negative and positive controls and qPCR reactions were also constructed as described previously (Luo et al., 2010). To assess the presence of qPCR inhibitors in the samples and determine the appropriate template DNA concentration, a series of dilutions of extracted DNA were analyzed using the relative standard curve method, per manufacturer's protocol. The qPCR amplification reactions were performed in 96-well plates with a final volume of 25 μL of reaction mixture (iQ SYBR Green Supermix, Bio-Rad), including 0.2 μM of each forward and reverse primer and 1 μL of template DNA. An initial 5 min denaturing step at 94 °C, followed by 40 amplification cycles of: (i) 30 s denaturation at 94 °C, (ii) annealing for 1 min at temperatures specified in Table S2, (iii) 30 s extension at 72 °C, followed by a final melt curve stage with temperature ramping from 55 °C to 95 °C. DNA samples were collected and analyzed in triplicate, and the specificity of qPCR products was corroborated with the melt curve. The amplification efficiencies of the standards for 16S rRNA and ARGs ranged between 92.6% and 108.6% The R^2 values of the standard curves for 16S rRNA and ARGs were in the range of 0.995–0.999. Internal standard genes (*CESA9*, coding for cellulose synthase A9 in *Arabidopsis thaliana* ecotype Columbia) were added to pretreated samples to determine DNA recovery and sample inhibition (Luo et al., 2010).

2.5. Plate counts

Viable plate counts were performed to assess whether ARB were generally more resistant to disinfection by chlorination (Cl_2). Wastewater samples were collected prior to entering and immediately upon exiting the disinfection units, and spread on agar plates to count colony forming units (CFUs) as stipulated by Standard Methods (Eaton et al., 2005). Total heterotrophs were cultivated on nutrient agar plates (Biolab). ARB were cultivated on agar plates, amended with sulfonamide (50 mg/L), tetracycline (10 mg/L), ciprofloxacin (10 mg/L) or erythromycin (50 mg/L). A dilution series was used to enumerate viable heterotrophs and ARBs. CFUs were counted for dilutions yielding number between 100 and 300 CFU/plate (Eaton et al., 2005). All agar plates were incubated at 37 °C for 48 h.

2.6. Antibiotics and heavy metal concentrations analyses

High-Performance Liquid Chromatography-Tandem Mass Spectrometry (LC–MS/MS) with internal standards was used to determine the concentrations of 16 antibiotics belonging to the tetracycline, sulfonamide, quinolone, and macrolide families in sewage and activated sludge samples. [Trimethyl-13C3] caffeine, sulfadiazine-13C6 (Cambridge Isotope Laboratories; Andover, MA), lomefloxacin and meclocycline (LOMX&MECL, Sigma–Aldrich, St. Louis, MO, USA) were added as internal standards to water and sludge samples to discern matrix effects during both water and sludge pretreatment. To enhance analytical precision, Simatone (Sigma–Aldrich, Germany) was added to the pre-treated water and sludge samples prior to LC–MS/MS analyses. Recoveries of (Trimethyl-13C3 caffeine, meclocycline, sulfadiazine-13C6 and lomefloxacin) for both water samples and sludge samples were 71%–90%. The limit of quantification (LOQ) for the 16 antibiotics under consideration ranged from 0.3 to 2.0 ng/l for water and from 0.5 to 3.2 ng/g for sludge samples. Details of sample preparation and chromatographic analyses are presented in the SI section.

The total concentrations of 7 heavy metals (i.e., As, Cd, Cr, Cu, Ni, Pb and Zn) in sewage and activated sludge samples were analyzed by ICP-MS (Perkin Elmer ELANDRC-e, USA). Detection limits were in the range of 0.006–0.030 μg/L, and recoveries were 97%–103%. Analytical precision was high, with a relative standard deviation smaller than 4%. ICP-MS analytical procedures are described in the SI section.

2.7. Statistical analyses

2.7.1. General analysis

Averages and standard errors were determined for all datasets. To quantify proliferation or removal of ARGs throughout different WWTP units, gene flow rates (gene copies/day) were calculated by multiplying average volumetric flow rates through a given unit by the corresponding influent and effluent concentrations of ARGs or 16S rRNA (as a surrogate for total Bacteria).

2.7.2. Cluster analysis

Clustering analysis was performed using R software (version 3.1.3, R Development Core Team). The $\log_{10}$ transformed flow ratios (daily output/input of each constituent of interest, including subtypes of genes) was used in the clustering analysis (LaPara et al., 2011).

2.7.3. Correlation and regression analysis

A Pearson correlation and regression analysis was performed to assess the relationship between ARG concentrations and other factors such as concentrations of antibiotics and heavy metals. Before performing this analysis, the Kolmogorov–Smirnov and Shapiro–Wilk tests were used to ensure that the datasets followed a normal (or log normal) distribution. Stepwise multivariate regression (SMR) analysis was performed between each antibiotic (or heavy metal) concentration and the relative abundance of each subtype of ARG (assessed as ARG/16S rRNA ratios) in the final effluent of both WWTPs. Partial correlation analysis was also used to further discern the significance of these correlations while eliminating potential effects of controlling variables. Specifically, partial correlations were conducted to assess the strength of the relationship between two variables (e.g., residual antibiotic or metal concentrations and the relative abundance of ARGs) while controlling the effects of other variables (e.g., physical/chemical/biological wastewater quality parameters, and sampling season). The Pearson partial correlation between the selected two variables, after controlling other variables in the Partial statement, is equivalent to the Pearson Correlation between the residuals of the two variables after regression of the controlling variables. All correlation and regression analyses were performed using SPSS for Windows Release 21 (SPSS Inc. USA).

3. Results and discussion

3.1. Prevalence of ARGs in WWTPs

Several studies have previously addressed the occurrence and proliferation of resistant bacteria in WWTPs (Auerbach et al., 2007; Christgen et al., 2015; Gao et al., 2012; Martins da Costa et al., 2006; Munir et al., 2011; Szczepanowski et al., 2009). However, these studies focused on characterizing influent versus effluent ARGs, and did not quantify their propagation or removal (and changes in their relative abundance) through different units in the wastewater treatment train. This information is important to understand ARB reservoirs and their fate in WWTPs, and to discern where propagation or inefficient removal occurs. Such knowledge could improve the design or operation of WWTP units.

All thirty ARGs considered (20 *tet*, 4 *sul*, 4 *qnr*, 2 *erm)* were detected in distinct water and sludge samples, illustrating the prevalence of ARB in WWTPs. Twenty three of these ARGs (15 *tet*, 3 *sul*, 3 *qnr*, 2 *erm)* were selected for further quantitative analysis because of their high detection frequencies in all WWTP units.

The average concentration of various ARG families was determined to characterize general trends in their proliferation and removal through different treatment units. Sulfonamide (*sul*) ARGs were generally detected at higher concentrations than macrolide (*erm*), tetracycline (*tet*) and quinolone (*qnr*) resistance genes in both sewage and sludge samples during the winter sampling event (Fig. 2). Sulfonamide resistance genes *sul*1 and *sul*2 were the most abundant in each treatment unit of both WWTPs, with average total *sul* concentrations of $(6.7 \pm 7.2) \times 10^5$ copies/mL in effluent samples, and $(2.2 \pm 2.8) \times 10^{11}$ copies/g dry weight in dewatered sludge. The prevalence of *sul*1 corroborates previous studies reporting it to be the dominant ARG in WWTP sludge (Calero-Cáceres et al., 2014; Christgen et al., 2015; Wang et al., 2013). The *erm* genes (*erm*B and *erm*C) were detected at an average of $(7.0 \pm 12) \times 10^4$ copies/mL in the effluent, and $(1.2 \pm 0.9) \times 10^{10}$ copies/g dry weight in dewatered sludge (DS). Average concentrations of *tet* genes were $(8.4 \pm 24) \times 10^4$ copies/mL in the effluent, and $(1.3 \pm 1.6) \times 10^{10}$ copies/g dry weight in DS. Quinolone resistance genes were found at lower concentrations: $(7.3 \pm 9.6) \times 10^3$ copies/mL in effluent, and $(1.5 \pm 2.3) \times 10^9$ copies/g dry weight in DS.

The concentrations of most of the screened ARGs in the WWTP water samples were reduced by approximately 0.95–2.67 logs (relative to the raw influent, RI) through the treatment units, with the bulk of the reduction attributable to bacteria settling in the secondary clarifier (Fig. 2). For comparison, a conventional activated sludge WWTP in Jeddah, Saudi Arabia, achieved 3.5 logs removal of heterotrophic bacteria and up to 3.5 logs removal of fecal coliforms (Al Jassim et al., 2015). Furthermore, 1 to 3 logs reductions in ARG concentrations were observed in four municipal WWTPs in China (Chen and Zhang, 2013); and 2.4 to 4.6 logs removal of ARGs and ARBs were achieved by two WWTPs in Michigan (Munir et al., 2011). Although a significant decrease in aqueous-phase ARG concentrations was achieved by both WWTPs in this study, relatively high concentrations were discharged in the treated effluents (up to 10^6 copies/mL) to receiving waters.

Significantly higher ARG discharges from the WWTPs occurred in the dewatered sludge (DS) with concentrations up to 10^{11} copies/g dry weight for *sul1* genes. The DS concentration factors, defined here as the ratio of average gene flow rates discharged through the DS normalized to the corresponding gene flow rates coming into the WWTP in the raw influent, were 99 ± 27% for *tet*, 128 ± 15% for *sul*, 115 ± 17% for *qnr*, and 98 ± 10% for *erm* genes in WWTP1. A similar trend was observed for WWTP2 (i.e., DS concentration factors were 107 ± 17% for *tet*, 127 ± 18% for *sul*, 133 ± 13% for *qnr*, and 124 ± 11% for *erm*) (Fig. 3). The high relative abundance of ARGs in DS (2.4×10^{-4}–8.6×10^{-2}, normalized to the corresponding 16S rRNA concentrations) may pose a risk of propagating antibiotic resistance to soil bacteria through land application of such ARG-rich biosolids.

3.2. Proliferation of ARGs through biological WWTP processes

The total load of ARGs (as well as 16S rRNA genes, as a surrogate for total Bacteria) entering the WWTPs and flowing through each unit was determined by multiplying the volumetric flow rates by the corresponding gene concentrations. Gene flow rates through various treatment units are summarized in Table 1. Significant proliferation of ARGs and 16S rRNA genes occurred in the aeration tank, where sewage biodegradation and microbial growth occurs. Consistent with the concentration of ARGs by bacteria settling, a relatively high total ARG discharge [$(1.0 \pm 0.2) \times 10^{17}$ to $(6.1 \pm 0.5) \times 10^{19}$ copies/day] occurred in dewatered sludge.

The enrichment of various ARG subtypes through the two WWTPs was determined by normalizing the total (effluent plus waste DS) gene discharge by the corresponding inflow rate (Fig. 3). Although several ARG subtypes were removed through the WWTPs, 12 ARGs were discharged at higher rates than influent

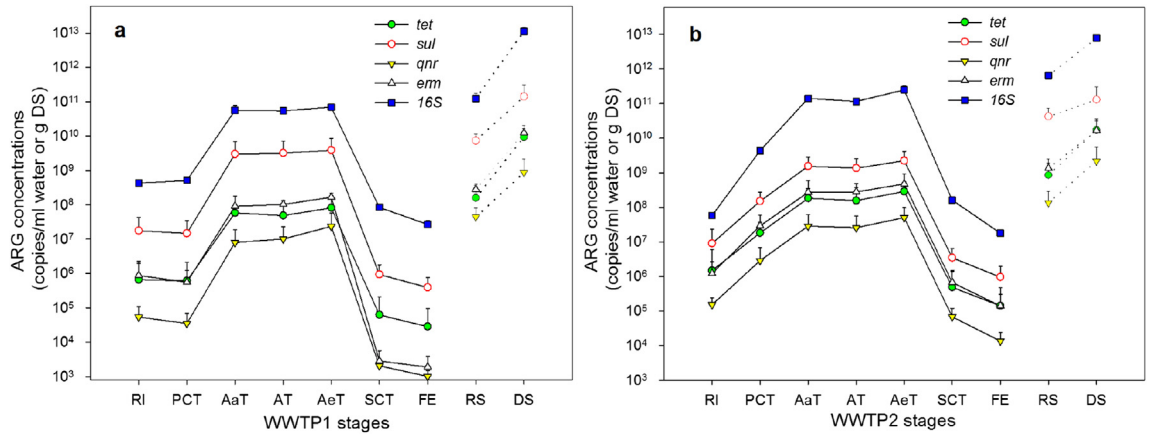

Fig. 2. Abundance of ARGs and 16S rRNA genes in two WWTPs (winter samples). The data represent the average concentrations of various ARG families, including *tet* (tetracycline resistance genes *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*H, *tet*M, *tet*L, *tet*O, *tet*Q, *tet*X, *tet*T, *tet*W, and *tet*S), *sul* (sulfonamide resistance genes *sul*1, *sul*2, and *sul*3), *qnr* (quinolone resistance genes *qnr*B, *qnr*D, and *qnr*S), and *erm* (macrolide resistance genes *erm*B and, *erm*C). Error bars depict ± one standard deviation. RI, PCT, AaT, AT, TeT, SCT, and FE are water samples, and RS (recycled sludge) and DS are sludge samples.

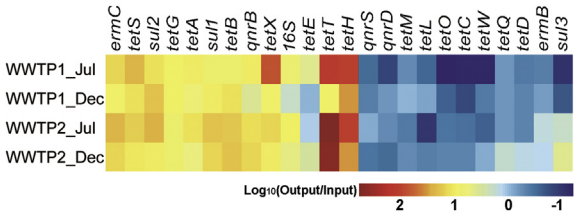

Fig. 3. Heat map of ARG enrichment through two WWTPs on different seasons, based on total (FE plus DS) gene outflow rates normalized to inflow rates (RI). The analysis includes the most frequently detected 23 ARGs and 16S rRNA (representative of total Bacteria). Columns show the influent-normalized gene outflow ratio. Yellow and red indicates an increase in gene flow through the WWTP, whereas blue indicates a decrease. No change corresponds to 0 in the color scale. For details of the enrichment of those 12 genes, please refer to Figure S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

values, indicating overall proliferation within the WWTPs (Figure S1). These ARGs (*tet*A, *tet*B, *tet*E, *tet*G, *tet*H, *tet*S, *tet*T, *tet*X, *sul*1, *sul*2, *qnr*B, *erm*C) account for more than 70% of the total discharge of the 23 ARGs selected for quantification in both WWTPs (Fig. 3). Ten of these ARGs (*tet*B, *tet*G, *tet*H, *tet*S, *tet*T, *tet*X, *sul*1, *sul*2, *qnr*B, *erm*C) were enriched to a significantly higher extent than 16S rRNA ($p < 0.05$, Fig. 4). To quantify ARG and bacterial enrichment throughout the WWTPs the average enrichment ratios were calculated as the total discharge rate of ARGs (in the effluent plus waste DS) divided by the corresponding influent rate. ARG enrichment ratios ranged from 8 ± 1 (*tet*G) to 268 ± 248 (*tet*T), while the 16S rRNA resulted in 5 ± 2 (Fig. 3). The observed ARG enrichment underscores the need for improved fundamental understanding of how to manipulate WWTP operation variables (e.g., decrease the food-to-microorganisms ratio and increase contact time in anaerobic digesters) to hinder the ability of antibiotic resistant bacteria to harvest energy, and thus increase the metabolic burden of resistance plasmid replication, which is conducive to loss of antibiotic resistance (Rysz et al., 2013).

ARB were more resistant to disinfection by chlorination than total cultivable heterotrophs (Table S1). Specifically, removal efficiencies for cultivable total heterotrophs (grown on plates without antibiotics and counted as CFUs) were 85 ± 6% for the disinfection unit of WWTP1, and 88 ± 5% for WWTP2. In contrast, the corresponding removal efficiencies for various ARB were significantly lower ($p < 0.05$) for both WWTP1 (41 ± 5% for tetracycline-resistant, 42 ± 3% for sulfonamide-resistant, 69 ± 7% for ciprofloxacin-resistant and 55 ± 6% for erythromycin-resistant bacteria) and WWTP2 (79 ± 6% for tetracycline-resistant, 65 ± 5% for sulfonamide-resistant, 77 ± 8% for quinolone-resistant and 78 ± 9% for macrolides-resistant bacteria). Previous research has reported that some ARB are relatively resistant to chlorination (Al Jassim et al., 2015; Huang et al., 2011; Shi et al., 2013), although disinfection resistance is unlikely to be mechanistically related to antibiotic resistance. The resistance of some ARB to chemical disinfection implies that chlorination may contribute to increasing their relative abundance in the effluent microbial community (Al

Table 1
Antibiotic resistance gene flows (copy numbers per day) through two waste water treatment plants (WWTPs) in northern China. a. WWTP1. b WWTP2.

	①[a]	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩
16S	2.30E + 20	2.80E + 20	3.00E + 22	2.90E + 22	4.20E + 22	4.50E + 19	1.50E + 19	3.70E + 21	3.00E + 19	4.20E + 20
Tet	5.40E + 18	4.90E + 18	4.70E + 20	3.90E + 20	6.60E + 20	5.00E + 17	2.30E + 17	6.40E + 19	1.00E + 17	5.30E + 18
Sul	2.80E + 19	2.40E + 19	4.90E + 21	5.20E + 21	6.30E + 21	1.50E + 18	6.40E + 17	6.10E + 20	3.20E + 18	3.60E + 19
Qnr	8.70E + 16	5.70E + 16	1.30E + 19	1.60E + 19	3.80E + 19	3.30E + 15	1.70E + 15	3.70E + 18	2.10E + 16	1.00E + 17
Erm	9.50E + 17	6.00E + 17	9.90E + 19	1.10E + 20	1.80E + 20	3.10E + 15	2.10E + 15	1.50E + 19	1.10E + 17	9.30E + 17
	①[a]	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩
16S	2.30E + 19	1.70E + 21	5.60E + 22	4.60E + 22	9.90E + 22	6.50E + 19	7.10E + 18	2.30E + 22	2.20E + 22	2.9E + 20
Tet	8.80E + 18	1.10E + 20	1.10E + 21	9.40E + 20	1.70E + 21	2.90E + 18	8.40E + 17	4.70E + 20	3.20E + 20	9.4E + 18
Sul	1.10E + 19	1.80E + 20	1.80E + 21	1.60E + 21	2.70E + 21	4.10E + 18	1.20E + 18	7.00E + 20	4.50E + 20	1.4E + 19
Qnr	1.80E + 17	3.40E+ + 18	3.40E + 19	3.10E + 19	6.00E + 19	8.10E + 16	1.60E + 16	1.50E + 19	1.10E + 19	2.4E + 17
Erm	9.70E + 17	2.30E + 19	2.20E + 20	2.20E + 20	3.80E + 20	5.30E + 17	1.10E + 17	9.90E + 19	6.00E + 19	1.2E + 18

[a] For sampling locations, please refer to Fig. 1.

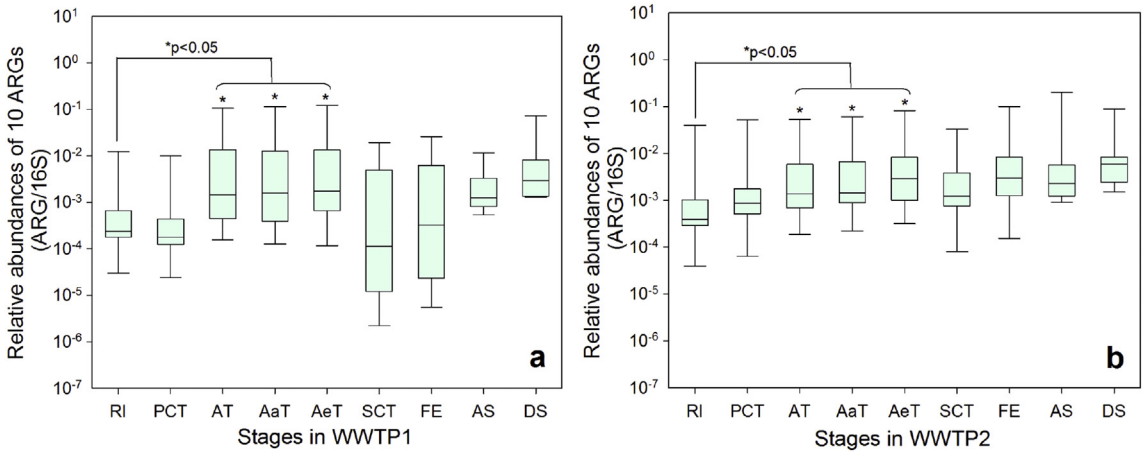

Fig. 4. Enrichment of 10 genes (*tet*B, *tet*G, *tet*H, *tet*S, *tet*T, *tet*X, *sul*1, *sul*2, *qnr*B, *erm*C) relative to 16S rRNA through each stage of (a) WWTP1 and (b) WWTP2. Asterisks indicate significant enrichment ($p < 0.05$).

Jassim et al., 2015).

The apparent higher resistance to chlorination of antibiotic resistant bacteria calls for technological innovation to more efficiently disinfect WWTP effluents. Although disinfection by UV alone may not be cost-effective due to the high irradiation intensities needed for ARG removal (McKinney and Pruden, 2012), one promising approach may be UV disinfection enhanced by photocatalysts that generate reactive oxygen species (Li et al., 2008). Potential advantages of this approach include simultaneous oxidation of recalcitrant organic compounds that are discharged from WWTPs, such as endocrine disruptors and pharmaceuticals (Choi et al., 2014; Miró et al., 2013), and avoidance of the formation of harmful disinfection byproducts associated with chlorination and ozonation (Hu et al., 1999; Mišík et al., 2011; Monarca et al., 2000; Schaar et al., 2010).

The effect of seasonal fluctuations on ARG discharges was also considered in this study. Several studies have reported a higher abundance of some ARGs in WWTP effluents during warm seasons (Goossens et al., 2005; Sui et al., 2011; Sun et al., 2012), while the opposite trend of higher abundance during the winter has also been observed (Yang et al., 2013). In this present study, however, no statistically significant seasonal shift of ARG abundance in WWTP effluent was observed.

3.3. Antibiotics contribute selective pressure for ARGs in WWTPs

ARG concentrations were normalized to the corresponding 16S rRNA gene concentrations to assess the effect of stress factors such as residual antibiotics concentrations or disinfection on the relative abundance of ARB. Although both WWTPs removed a portion of the antibiotics present in the raw influent (50–98% for sulfonamides, 58–96% for tetracyclines, 56–86% for quinolone, and 58–87% for macrolide), significant concentrations (up to 3.4 μg/L for oxytetracycline in WWTP2, for example) remained untreated (Fig. 5).

In this study, partial correlation analysis showed statistically significant correlations ($p < 0.05$) between residual antibiotic concentrations and the relative abundance of the corresponding ARGs in the final effluent of WWTPs (Fig. 6). Although other factors that influence biological activity may affect ARG abundance, these partial correlations between residual antibiotics and the relative abundance of ARGs in the effluent suggest that some antibiotics contribute selective pressure for ARG maintenance and proliferation in WWTPs. In this study WWTP2 exhibited higher influent and effluent antibiotic concentrations than WWTP1, as well as higher relative ARG abundance, corroborating that (through selective pressure) antibiotic residues may facilitate the maintenance and propagation of ARGs in WWTPs.

Previous studies have shown elevated levels of ARGs even in the

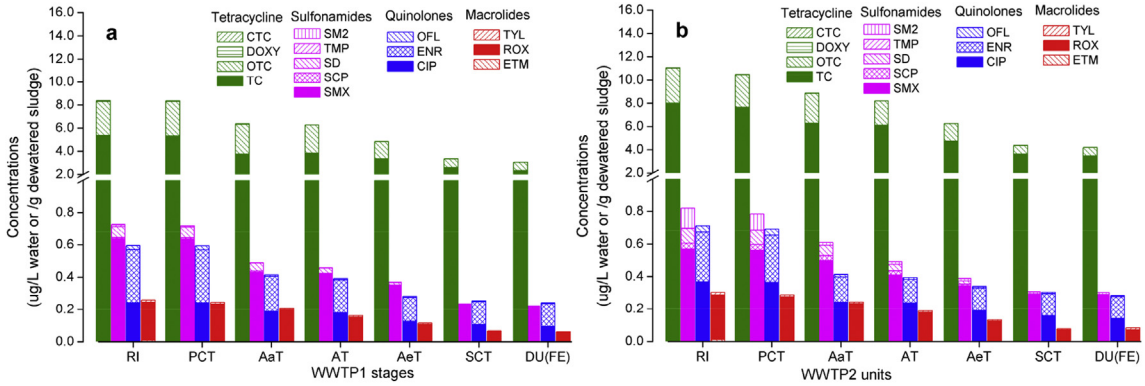

Fig. 5. Aqueous antibiotic concentrations through (a) WWTP1 and (b) WWTP2. Values represent averages of samples collected in November and December 2011. Sulfonamides: sulfamethoxazole (SMX), sulfachloropyridazine (SCP), sulfadiazine (SD), sulfamethoxazole (SM2), trimethoprim (TMP); Tetracyclines: oxytetracycline (OTC), tetracycline (TC), doxytetracycline (DOXY), chlorotetrcycline (CTC); Quinolones: ofloxacin (OFL), ciprofloxacin (CIP), enrofloxacin (ENR); Macrolides: etimicin (ETM), roxithromycin (ROX), tylosin (TYL).

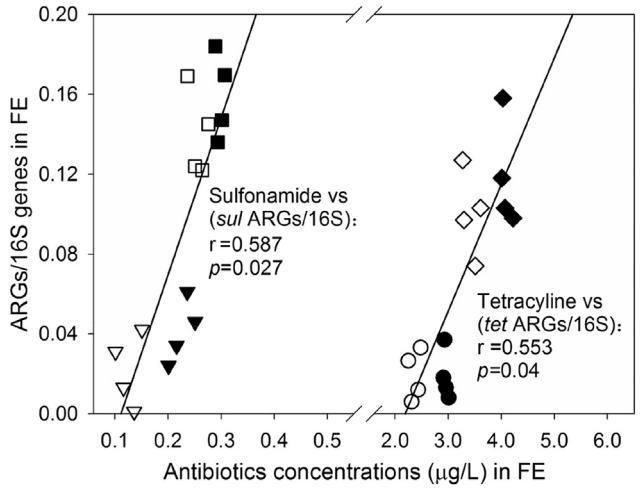

Fig. 6. Positive correlations between antibiotic concentrations and the relative abundance of the corresponding ARGs (ARG/16S rRNA genes) in the final effluent (FE) of two WWTPs by Partial Correlation analysis. The increase in the relative ARG abundance for sulfonamides (▼/▽ and ■/□) and tetracyclines (●/○ and ◆/◇) with the corresponding antibiotic concentrations suggest that residual antibiotics contribute selective pressure for maintenance and replication of ARGs. The symbols (▼/▽ and ●/○) indicate samples collected in WWTP1, and the symbols (■/□ and ◆/◇) indicate WWTP2. Dark symbols represent samples winter collected in November and December 2011, and white symbols correspond to summer samples collected in June and July 2012.

absence of antibiotics (Alonso et al., 2001; Baker-Austin et al., 2006; Wright et al., 2006), which could be due in part to effect of other factors such as heavy metals that induce a similar resistance mechanism as antibiotics (i.e., efflux pumps) and contribute selective pressure for ARG maintenance (Han et al., 2002; Ji et al., 2012; Knapp et al., 2011; Stepanauskas et al., 2006; Zhu et al., 2013). Heavy metal and antibiotic resistance co-occurrence has been previously reported within the same bacterial strain (Amachawadi et al., 2010; Chopra and Roberts, 2001; Knapp et al., 2011; Stepanauskas et al., 2006). Stepwise multivariate regressions (SMR) analyses on antibiotic and heavy metal concentrations (independent variables) and relative abundance of ARGs (dependent variables) in the final effluent of both WWTPs showed that relative abundances of ARGs were also correlated to heavy metal concentrations (Table 2). The beta coefficient (β) calculated in SMR was standardized to determine which of the independent variables have a greater effect on the dependent variable in the regression model. The greater the β value, the greater effect of the independent variables on the dependent variable. These correlations were also corroborated by partial correlation analysis. Significant correlations (adjusted $R^2 = 0.877$, $p < 0.01$) were determined between the concentrations of oxytetracycline ($\beta = 0.461$, $p < 0.01$) and zinc ($\beta = 0.591$, $p < 0.01$) and *tetG*, an efflux pump gene in *Pseudomonas* and *Salmonella* (Chopra and Roberts, 2001). Resistance via the efflux pump mechanism has been reported for zinc in *Thiobacillus ferrooxidans* (Choudhury and Srivastava, 2001). Significant correlations (adjusted $R^2 = 0.991$, $p < 0.01$) were also established between *tetB,* an efflux pump gene in *Moraxella and Erwinia* (Chopra and Roberts, 2001) and the concentrations of oxytetracycline ($\beta = 0.846$, $p < 0.01$) and arsenic ($\beta = 0.417$, $p < 0.01$). Efflux pump resistance mechanisms have previously been reported for arsenic in strains of *Agrobacterium* and *Pseudomonas* (Cai et al., 2009). The positive correlations observed in this study between *ARGs* and the concentrations of arsenic, zinc, lead and mercury (Table 2) indicate that the combined presence of heavy metals and antibiotic in sewage may facilitate the propagation of ARGs in WWTPs.

4. Conclusions

WWTPs can serve as breeding grounds and point sources of antibiotic resistant bacteria and associated ARGs. This represents a growing concern as the need for wastewater reuse increases and dewatered sludge is more commonly used to fertilize agricultural fields. A detailed analysis of the flow of 23 ARGs through each unit of two WWTPs in Northern China showed substantial proliferation and discharge of ARGs, especially through dewatered sludge. Significant ARG discharges also occurred through the effluent, which contained ARB that were also relatively resistant to chlorination. This underscores the need for improved microbial control

Table 2
Regression and correlation analysis of antibiotic (or heavy metal) concentrations versus the ARG relative abundance (i.e., ARG/16S rRNA genes) in the final effluent of both WWTPs.

Relative abundance (ARGs/16S)	Concentration (mg/L)	Stepwise multivariate regression			Partial correlation	
		Standardized coefficient (β)	Significance of coefficient (p)[a]	Adjusted R^2 and significance of model	Correlation coefficient (r)	Significance level (p)[a]
*sul*1	SCP	0.666	<0.01	$R^2 = 0.983$	0.869	<0.01
	Hg	0.459	<0.01	$p < 0.01$	0.963	<0.01
*tet*B	OTC	0.846	<0.01	$R^2 = 0.991$	0.997	<0.01
	AS	0.417	<0.01	$p < 0.01$	0.999	<0.01
*tet*G	Zn	0.591	<0.01	$R^2 = 0.877$	0.764	<0.01
	OTC	0.461	<0.01	$p < 0.01$	0.771	<0.01
*tet*H	Hg	0.740	<0.01	$R^2 = 0.864$	0.992	<0.01
	Ni	0.109	<0.05	$p < 0.01$	0.996	<0.01
*tet*S	Ni	0.838	<0.01	$R^2 = 0.982$	0.997	<0.01
	TC	0.412	<0.01	$p < 0.01$	0.959	<0.01
*tet*T	CTC	0.997	<0.01	$R^2 = 0.995$ $p < 0.01$	0.992	<0.01
*tet*X	Pb	0.638	<0.01	$R^2 = 0.997$	0.992	<0.01
	OTC	0.499	<0.01	$p < 0.01$	0.996	<0.01
*qnr*B	Pb	0.982	<0.01	$R^2 = 0.961$ $p < 0.01$	0.995	<0.01
*erm*C	ETM	0.999	<0.01	$R^2 = 0.999$ $p < 0.01$	0.998	<0.05

[a] p values less than 0.01 indicates significant correlation at the 99% confidence level. In this ANOVA test, antibiotic (or heavy metal) concentrations were set as independent variables, and the corresponding ARG ratios (ARG/16S rRNA genes) were set as dependent variables. In the partial correlation analysis, the overall consideration of physical/chemical/biological water quality parameters and sampling season were set as covariate variables.

approaches.

Significant correlations were found between the relative abundance of ARGs and the corresponding residual antibiotic concentrations and heavy metals in the effluents of WWTPs. Although correlation does not prove causation, this finding suggests that enhancing the removal of antibiotics or metals in pre-treatment units, so as to mitigate their release to sewage systems, may alleviate their selective pressure and mitigate the proliferation of ARGs in WWTPs.

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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.watres.2015.09.010.

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