Behavior of antibiotic resistance genes under extremely high-level antibiotic selection pressures in pharmaceutical wastewater treatment plants

Xinyan Guo, Zheng Yan, Yi Zhang, Weili Xu, Deyang Kong, Zhengjun Shan, Na Wang

Highlights

• The ARGs would proliferate or attenuate in different treatment units of pharmaceutical wastewater treatment plants (PWWTPs).
• A big part of the ARGs may be transported to the dewatered sludge.
• The bacterial abundance and antibiotic concentration within the PWWTPs influenced the fate of the associated ARG together.
• The intrinsic resistance mechanisms of corresponding ARGs play a key role in their fate.
• ARGs concentration in the wastewater from fermentation was significantly higher than chemical synthesis and preparation.

Abstract

Pharmaceutical wastewater treatment plants (PWWTPs), which receive wastewater containing extremely high levels of antibiotics, are regarded as potential hot spots for antibiotic-resistance development in the environment. Six sampling campaigns in six PWWTPs in Southeastern China were carried out to assess the prevalence and fate of antibiotic resistance genes (ARGs). Different genes were monitored in different PWWTPs (PWWTP A: lincosamides; PWWTP B: aminoglycosides and macrolides; PWWTP C: quinolones; PWWTP D: macrolides and quinolones; PWWTP E: cephalosporins; and PWWTP F: quinolones and macrolides) using real-time quantitative polymerase chain reactions (qPCR). The levels of typical ARG subtypes in the final effluents ranged from $(1.03 \pm 0.91) \times 10^1$ to $(6.78 \pm 0.21) \times 10^7$ copies/mL. The absolute abundance of ARGs in effluents accounted for 0%–577% of influents to the six PWWTPs with a median value of 6%. Most of the ARGs are transported to the dewatered sludge, with concentrations from $(1.38 \pm 0.21) \times 10^5$ to $(6.84 \pm 0.43) \times 10^{10}$ copies/g dry weight (dw). In different treatment units (before/after biological units), a clear trend of proliferation or attenuation was not observed for the ARGs, aside from a strong attenuation in moving bed biofilm reactor (MBBR) in PWWTP C. Through correlation analyses, this study demonstrated that the bacterial abundance and antibiotic concentrations within the PWWTPs influenced the fate of the associated ARGs, and this was possibly related primarily to the intrinsic resistance mechanisms of corresponding ARGs. Macrolide ARGs, which tend to locate in plasmids and...
transposons, positively correlate weakly with total macrolide antibiotic concentrations but positively correlate strongly with 16S rRNA concentrations. Furthermore, ARG concentrations in the wastewater from fermentation were significantly higher than in the wastewater from chemical synthesis and preparation. This is the first comprehensive study on the behavior of antibiotic resistance genes under extremely high-level antibiotic selection pressures in pharmaceutical wastewater treatment plants (PWWTPs) in Southeastern China.

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Fig. 1. The flow chart layouts of the treatment processes in the six pharmaceutical wastewater treatment plants and the sampling site location. The black dot represents the specific sampling location and the dotted line represents the recycled sludge. IC: internal circulation anaerobic reactor; SBR: sequencing batch reactor; VFST: vertical flow sedimentation tank; RFST: radial flow sedimentation tank; MBBR: moving bed biofilm reactor.
1. Introduction

Resistant microbes have the potential to rapidly spread across the entire planet, a process that is considered to be closely linked to the widespread misuse and overuse of antibiotics in humans, animals and agriculture (Walsh et al., 2011; Levy and Marshall, 2004). Recently, manufacturing sites have begun to be considered as a problematic pollution source, due to a much higher discharge level than that of any other source. The highest levels reported were 31 mg/L for ciprofloxacin in pharmaceutical wastewater treatment plant (PWWTP) effluent receiving wastewater from approximately 90 bulk drug manufacturers in Patancheru near Hyderabad, India (Larsson et al., 2007). Hou et al. (2015) reported that tetracyclines were discharged through the effluent of PWWTPs (up to 32.0 ± 6.0 mg/L) and dewatered sludge (up to 5481.1 ± 123.0 mg/kg). Qiu et al. (2016) from our team investigated two PWWTPs treating wastewater containing vancomycin (a glycopeptide antibiotic used for the treatment of serious, life-threatening infections by gram-positive bacteria) and reported a concentration of 0.24–0.50 mg/L in the effluent. Many reports have shown that high-levels of antibiotics exert selective pressure for the maintenance and propagation of antibiotic resistance genes (ARGs) (Kristiansson et al., 2011; Li et al., 2011; Li et al., 2010a; Huang et al., 2010). Therefore, it is not surprising that the maximum detected concentrations of ARGs in the final effluents of PWWTPs were 3.68 × 10^6 copies/mL, as reported by Wang et al. (2015) and 2.36 × 10^7 copies/mL, as reported by Zhai et al. (2016), both of which were much higher than the concentration of 9.50 × 10^5 detected in municipal wastewater treatment plants as reported by Mao et al. (2015).

As hot spots for antibiotic-resistance development, the control of ARG concentrations in full-scale pharmaceutical wastewater treatment plants should be given more attention. Although studies have characterized ARGs in various processes of wastewater treatment plants, the behavior of ARGs associated with high-level antibiotic residues would be extremely high-levels of antibiotics (Table S1 and Table S5). The flow chart layouts of the treatment plants that we studied are shown in Fig. 1, and the characteristics of the PWWTPs are listed in Table 1. Among the six different biological treatment processes, PWWTP A (Fig. 1A) used an anaerobic–oxic (A/O) process ef fluent, which are resistant to macrolides; and OXA and CTX, which are resistant to cephalosporins. We also explored the fate of various ARGs in different processing stages of pharmaceutical wastewater treatment systems by performing mass balance and correlation analyses. Furthermore, the influence of the antibiotic manufacturing technique (fermentation, chemical synthesis, and preparation) on the epidemiology of ARGs was investigated in this study. This is the first comprehensive study on the behavior of antibiotic resistance genes under extremely high-level antibiotic selection pressures in pharmaceutical wastewater treatment plants in Southeastern China.

2. Materials and methods

2.1. Pharmaceutical wastewater treatment plants

Six PWWTPs with different combinations of biological and physical/chemical technology processes in Southeastern China were investigated. All the PWWTPs that we studied treated wastewater containing extremely high-levels of antibiotics (Table S1 and Table S5). The flow chart layouts of the treatment plants that we studied are shown in Fig. 1, and the characteristics of the PWWTPs are listed in Table 1. Among the six different biological treatment processes, PWWTP A (Fig. 1A) used an internal circulation anaerobic reactor and sequencing batch reactor; PWWTP C (Fig. 1C) combined a moving bed bio-film reactor (MBBR) in an anoxic reactor and aerobic reactor. PWWTP B (Fig. 1B) and PWWTP E (Fig. 1E) utilized an anaerobic and aerobic operation, while an anaerobic–anoxic–oxic (A/A/O) operation was applied in PWWTP D (Fig. 1D). The processes of a deep well anaerobic system and activated sludge reactor were applied in PWWTP F (Fig. 1F).

2.2. Sample collection and pretreatment

The scheme of the PWWTPs and sampling locations is shown in Fig. 1. Three-day sampling was carried out in dry weather at the outlet of each treatment step in September 2015. To avoid confounding effects associated with hydraulic loading fluctuations, raw wastewater and process effluents were taken as 24 h flow-proportional composite samples. All water samples were collected in 10 L amber glass bottles. The sludge samples were generally taken at the outlet of every treatment step. Each sample was placed into a plastic container and immediately chilled in an icebox and transported under cool conditions to the

<table>
<thead>
<tr>
<th>PWWTs</th>
<th>Latitude and longitude</th>
<th>Wastewater treatment capacity (t/d)</th>
<th>Sludge treatment capacity (t/d)</th>
<th>Influent COD (mg/L)</th>
<th>Influent NH3-N (mg/L)</th>
<th>Antibiotics producing</th>
<th>Antibiotics Produced</th>
<th>Target ARGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>N 33°40′1″; E 116°58′26″</td>
<td>600</td>
<td>–</td>
<td>40,000</td>
<td>–</td>
<td>Lincomycin, Clindamycin</td>
<td>ImrA, ImrB, Inua</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>N 31°37′39″; E 102°23′7″</td>
<td>600</td>
<td>150</td>
<td>4000</td>
<td>120</td>
<td>Parnomycin, Ribostamycin</td>
<td>Spiramycin</td>
<td>qnrS, qnrD, oqxB</td>
</tr>
<tr>
<td>C</td>
<td>N 29°30′12″; E 102°54′42″</td>
<td>1.2</td>
<td>12</td>
<td>15,000</td>
<td>300</td>
<td>Levofoxacin</td>
<td>aac</td>
<td>ermC</td>
</tr>
<tr>
<td>D</td>
<td>N 30°8′40″; E 120°51′45″</td>
<td>1300</td>
<td>0.7</td>
<td>5000</td>
<td>100</td>
<td>Clarithromycin, Azithromycin, Roxithromycin, Ciprofloxacin, Enrofloxacin</td>
<td>qnrS, qnrD, oqxB, ermB, ermC, ermX</td>
<td>erfM, OXA, CTX</td>
</tr>
<tr>
<td>E</td>
<td>N 29°38′2″; E 120°49′28″</td>
<td>0.42</td>
<td>2</td>
<td>9000</td>
<td>700</td>
<td>Cefadine, Cephalexin</td>
<td>aac, aacD, aacG, ermB, ermC</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>N 31°44′1″; E 120°1′51″</td>
<td>480</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td>Roxithromycin, Levofoxacin, Lomefloxacin</td>
<td>qnrA, qnrS, qnrD, oqxB, ermB, ermC, ermA, ermA, ermX</td>
<td></td>
</tr>
</tbody>
</table>
laboratory and then stored in the dark at −20 °C until DNA was extracted (within 3 days).

### 2.3. Antibiotic analysis

The 14 targeted antibiotics obtained from the six PWWTPs are shown in Table 1. Samples were analyzed for antibiotics using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in the positive-ion mode with an Agilent 1290 UPLC system and ABSCIEX Triple Quad 4500MS/MS detector. The specific methods used are described in Guo et al. (2015) and Qiu et al. (2016). Details of the antibiotics analyses are presented in the Supplemental information.

### 2.4. DNA extraction

Sludge samples were centrifuged at 4000 × g for 10 min at room temperature, and 0.5 g of the pellet was used for DNA extraction. Excess sludge samples were divided into two subsamples: one was subjected to DNA extraction and the other was used to determine the moisture content for further ARG quantification. DNA was obtained using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer’s protocol. Water samples were first filtered with sterilized filter paper to separate undissolved substances, then filtered with a 0.22 μm nitrocellulose filter membrane (Millipore, Boston, USA) to collect bacteria. The total volume of water sample processed was recorded for subsequent analyses. All filter papers and membranes were stored at −80 °C. Total DNA was extracted from the sterilized filter papers together with the nitrocellulose filter membranes prepared previously using a Water DNA Kit (OMEGA, Norcross, GA, USA) according to the manufacturer’s instructions. The concentration and quality of the extracted DNA were determined by spectrophotometer analysis and 1% agarose gel electrophoresis.

### 2.5. Primer design

The ARGs, including four quinolone resistance genes (qnrA, oxyB, qnrS, and qnrD), four macrolide resistance genes (ermB, ermC, ermF and ermX), three aminoglycoside resistance genes (aph, aadD, and aac), three lincosamide resistance genes (lmrA, lmrB and lnuA) and two cephalosporin resistance genes (OXA and CTX) were analyzed in this study. Complete accession numbers of DNA sequences were obtained from the NCBI gene database, according to which the primers were designed by Primer 5.0 (Table S3 of Supplementary data). The specificity of the primers was tested by Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), after which the primers were synthesized by the biological company.

### 2.6. Construction of quantitative polymerase chain reaction (qPCR) standards

Amplification of the 16 ARGs and 16S rRNA were performed in a PCR machine (Bio-Rad, CA, USA). The PCR mixture (25 μL total volume) contained 1 × PCR buffer, 2.0 mmol/L MgCl₂, 1.0 mol/L dNTP mixture,
400 nmol/L each primer, 1.25 U of Taq DNA polymerase, and 100 ng template DNA. The temperature program initially denatured samples in a thermal cycler for 5 min at 94 °C and then subjected samples to 35 amplification cycles of 30 s at 94 °C, 30 s at different annealing temperatures and 45 s at 72 °C, followed by a final extension step of 6 min at 72 °C.

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. After the PCR products from ARGs were separated by 2% agarose gel electrophoresis, they were purified by an EasyPure Quick Gel Extraction Kit (TransGen Biotech, China). The purified PCR products were ligated into pEasy-T3 vector (TransGene Biotech, China) and then cloned into Trans1-T1 phage resistant chemically competent cells (TransGen Biotech, China). The sequences marked as positive clones using PCR standards were analyzed by the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/). Plasmids carrying the target genes were extracted using a Plasmid Miniprep Kit (Axygen, USA) and further used to prepare standardized products for real-time PCR.

2.7. Quantitative PCR

Real-time qPCR was applied to quantify ARGs and 16S rRNA genes in DNA extracted from samples. The qPCR reactions were conducted in 96-well plates. All real-time qPCR assays were performed in triplicate using the 2 × Ultra SYBR Mixture (CWBIO, China) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The real-time qPCR program was as follows: initial denaturing at 95 °C for 10 min, followed by 40 cycles of 10 s at 95 °C, 30 s at different annealing temperatures, and 30 s at 72 °C. The fluorescence data were acquired at 72 °C, and the final melting curve was constructed with the temperature ramping up from 65 to 95 °C. Tenfold serial diluted calibration curves for each ARG were tested in triplicate on the same PCR plate. For all standard curves, the linear coefficients (R²) were >0.990, and their amplification efficiencies were between 95% and 105%. The equations of standard curves are listed in Table S4 (Supplementary data).

2.8. Statistical analysis

A linear regression analysis was applied to plot the correlation between the concentrations of the 16s RNA and total resistance genes. Statistical evaluation of the data was conducted using SPSS Version 19.0. Shapiro-Wilk tests were used to determine whether the dataset (3 ≤ n ≤ 50) belongs to a normal distribution population. Nonparametric Kruskal-Wallis tests were conducted to determine significance. P values lower than 0.05 were considered to be significant. Correlations were conducted to assess the strength of the relationship between various subtypes of ARGs and residual antibiotic/16s RNA concentrations. Clustering analysis was performed using Heml 1.0 (Deng et al., 2014).

3. Results and discussion

3.1. Prevalence of ARGs in PWWTPs

Industry wastewater collection and treatment serves an essential role in the protection of human and environmental health. According to the mandatory standards for pharmaceutical industry effluent in China (GB 21904-2008 Discharge standards of water pollutants for pharmaceutical industry chemical synthesis products category and GB 21903-2008 Discharge standards of water pollutants for pharmaceutical industry fermentation products category), systems of traditional PWWTPs are designed to remove conventional pollutants, including suspended solids, nutrients (nitrogen and sometimes phosphorus), and organic matter, and are not designed for the removal of antibiotics or ARGs. Because the high levels of antibiotics in PWWTPS inevitably will exert strong selection for antibiotic resistance bacteria, abundant ARGs were widely present throughout the treatment processes of the six PWWTPs, and the concentrations of typical ARGs in raw influents and in final effluents ranged from (1.47 ± 0.02) × 10² to (2.96 ± 0.88) × 10⁸ copies/mL and (1.03 ± 0.91) × 10¹ to (6.78 ± 0.21) × 10⁷ copies/mL, respectively, while concentrations in the dewatered sludge ranged from (1.38 ± 0.21) × 10⁵ to (6.84 ± 0.43) × 10¹⁰ copies/g dw (Table S5).

3.2. Fate of ARGs in PWWTPs

3.2.1. The effect of different treatments

The variation in ARGs, 16S rRNA genes (a surrogate for total bacteria) and total antibiotic concentrations in the entire process flow, throughout various treatment units was determined to characterize the general trends within different treatment units (Fig. 2). In general, the abundance of 16S rRNA was significantly increased in biological treatments, followed by a decrease in physico-chemical treatments, while the total antibiotic concentration trended towards decreasing throughout all the processing stages, except for some antibiotics not in production. For ARGs, a variable but decreasing trend seemed to be apparent among factories (Fig. 2). The proliferation/attenuation proportions of ARGs in different treatment units compared with influents within the six treatment systems are shown in Fig. 3. Accordingly, proliferation and attenuation coexisted in all treatment units except for MBBR processing, which can increase ARG absorption and interception on biofilms (Breazeal et al., 2013). The abundance of ARGs in effluents accounts for −4.32–0.76 log proliferation of influents for the five PWWTPs, indicating relatively poor removal efficiency.

3.2.2. Mass balance analysis

To determine the proliferation of ARGs flowing into and out of each treatment unit of PWWTPs, two PWWTPs (B and C) were selected to perform mass balance analysis (copies/d) by multiplying the volumetric flow rates by the corresponding gene concentration. These sites were selected because the dewatered sludge was successfully obtained only in PWWTP B and PWWTP C. The results showed that the release of total ARGs was 9.01 × 10¹⁴ copies/d in influents and 5.04 × 10¹⁰ copies/d in dewatered sludge for PWWTP B. Compared with raw influents (1.67 × 10¹² copies/d), a significant proliferation was seen. These results are similar to results from a previous study by Wang et al. (2015), who reported that biological treatment units contribute significantly to proliferation. Unlike PWWTP B, the emission of total ARGs for PWWTP C was 2.29 × 10¹² copies/d in final effluents and 5.69 × 10⁹ copies/d in dewatered sludge, compared with 7.77 × 10¹² copies/d for raw influents. The phenomenon of PWWTP C indicates that attenuation may occur in pharmaceutical wastewater treatment systems, and this was presumed
to be caused by the removal of total antibiotics; see further discussion in Section 3.2.3.

3.2.3. Correlation analysis

In most previous studies, a positive correlation between the abundance of selected individual ARGs and 16S rRNA was observed, as shown in PWWTPs, municipal wastewater treatment plants, wastewater treatment systems of swine farms, etc. (Wang et al., 2015; Mao et al., 2015; Tao et al., 2014). Usually, the increase of 16S rRNA with microbial growth predicts significant replication of ARGs. However, the positive correlation between ARGs and 16S rRNA genes accounts for a smaller proportion in our work (Fig. 4 and Table S6), especially for PWWTP A, in which there was no positive correlation observed. In addition, only 2 out of 5 ARGs in PWWTP B, 1 out of 3 ARGs in PWWTP C, 4 out of 7 ARGs in PWWTP D, 1 out of 2 ARGs in PWWTP E and 4 out of 8 ARGs in PWWTP F showed significant positive correlations with 16S rRNA.

The role of antibiotics as selective agents is complex. Significant positive correlations between the concentrations of antibiotics and their corresponding ARGs were observed in several typical scenarios. Li et al. (2010b) suggested that the relative abundance of total quinolone resistance genes and the measured concentrations of total quinolones were significantly correlated in wastewater and soil ($r = 0.71, p < 0.05$). In some cases, the selective pressure for increasing ARGs was

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**Fig. 4.** The profile of the correlation coefficient $R$ for the ARGs and 16S rRNA, the ARGs and total antibiotics in the six PWWTPs.
applied not only from the corresponding classes of antibiotics but also from the noncorresponding classes of antibiotics; for example, Huang et al. (2015) observed that ribosomal protection protein genes showed significant correlation with tetracyclines in an improved anaerobic-anoxic-oxic wastewater treatment plant. However, some weak correlations were reported and probably contributed to the various environmental behavior and propagation mechanisms of ARGs and antibiotics in the environment (Ji et al., 2012; Gao et al., 2012). Many other studies, in which bacterial abundance was considered to be a more important factor for ARGs than antibiotics, normalized ARG concentrations to the corresponding 16S rRNA concentrations that were used to assess the effect of residual antibiotic concentrations (Wang et al., 2015; Mao et al., 2015). In pharmaceutical wastewater treatment systems, high concentrations of antibiotic residues throughout the treatment system seem to be inevitable, and they might contribute strong selective pressure for ARGs. The concentrations of total antibiotics (classified by families) in PWWTPs are shown in Table S5. The minimum inhibitory concentration (MIC) for some typical sensitive bacteria was obtained, for reference, from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Table 2). It is unlikely that the antibiotics inhibit or increase the microbiological activities in PWWTPs, since the detected antibiotic residues were much higher than the values of MICs. Therefore, we studied the correlations between antibiotics and the absolute concentrations of ARGs (Fig. 4 and Table S6). One out of 3 ARGs in PWWTP A, 2 out of 5 ARGs in PWWTP B, 2 out of 7 ARGs in PWWTP D, 1 out of 2 ARGs in PWWTP E and 2 out of 8 ARGs in PWWTP F were observed to have significant positive correlations with total corresponding antibiotic concentrations. These ARG subtypes did not positively correlate with 16S rRNA.

Fig. 4 shows the correlation coefficient R for corresponding ARGs and 16S rRNA/total antibiotic concentrations pairs. It was clear that 72.9% of corresponding ARGs showed significantly positive correlations with either 16S RNA or total antibiotic concentrations in each PWWTP; however, total quinolone ARG concentrations from PWWTP C showed significantly positive correlations with both 16S RNA and total quinolone antibiotic concentration. Consequently, it can be seen that 16S rRNA and total antibiotic concentrations were two key factors for corresponding ARG concentrations in each PWWTP, and, depending on which one dominates, might relate, in part, to the intrinsic resistance mechanisms of corresponding ARGs. Although classically attributed to chromosomal mutations, resistance in enterococci is most commonly associated with extra-chromosomal elements acquired from other bacteria in the environment (Michael and Stuart, 2007). When chromosomal mutations occur in bacteria, the level of corresponding ARGs more often relies on the existence and concentration of antibiotics (Zhang et al., 2011). This observation might explain the significantly positive correlations between several ARGs for lincosamides, quinolones, cephalosporins, and aminoglycosides and their corresponding antibiotic concentrations in our study. Normal susceptible bacteria can also acquire resistance to an antibiotic by acquiring a new characteristic through mutation of indigenous genes or the acquisition of resistance genes by horizontal gene transfer, mostly through the transfer of mobile genetic elements such as plasmids and transposons (Giedraitienė et al., 2011; Van et al., 2011; Soucy et al., 2015). The transmission of genetic material from one organism to another by mobile genetic elements can greatly contribute to the dispersal of antibiotic resistance, because it can occur between closely or distantly related species and in diverse environments (Aminov, 2011; Huddleston, 2014; Rossi et al., 2014; Wang et al., 2006). When horizontal gene transfer occurs in bacteria, the level of corresponding ARGs more often relies on 16S rRNA concentrations (a surrogate for total bacteria). Consistent with our findings, ARG concentrations for macrolides, which tend to locate in plasmids and transposons, rarely showed a positive correlation with total macrolide antibiotic concentrations, but did show a strongly positive correlation with 16S rRNA concentrations (Fig. 4, black box) (Ana et al., 2016; Wang et al., 2016; Gianluca et al., 2016; Naohiro et al., 2005). A similar observation from PWWTPs was shown in the study of Wang et al. (2015) in which the relative abundance of the ermB gene was high (8.76 × 10⁻³) in spite of the fact that their associated antibiotics were below the detection limit.

This phenomenon also explains why the ARGs did not proliferate in biological treatments from all the PWWTPs that we studied. Some ARGs, especially those that come from chromosomal mutations and that are under great selective pressure of antibiotics, would decrease with degradation of total antibiotics in final effluents. This finding suggests a good strategy for controlling the dissemination of ARGs in PWWTPs, i.e., by efficiently removing high residue levels of antibiotics.

### 3.3. Epidemiology of ARGs in PWWTPs dealing with wastewater from fermentation, chemical synthesis and preparation

Antibiotic manufacturing type plays an important role in wastewater characteristics, while wastewater from fermentation (Fig. S1) contains more abundant microbes. Similarly, the abundance of 16S RNA in the wastewater from fermentation (PWWTP A, PWWTP B and PWWTP C) was significantly higher than in wastewater from chemical synthesis (PWWTP D and PWWTP E) and preparation (PWWTP F) (p < 0.05); similar results were seen with ARGs (p < 0.05) (Fig. 5). Therefore, typical ARG subtypes for quinolones were selected from PWWTP C (fermentation), PWWTP D (chemical synthesis) and PWWTP F (preparation) for analysis. Similar trends were found as shown in a heatmap of ARG enrichment (Fig. 6). Despite having nearly the same levels of quinolone antibiotic residues in these three PWWTPs, the abundant ARGs in fermentation PWWTPs mainly relate to their abundant microbes.

For all the PWWTPs with high antibiotic residue levels that were far higher than relative MICs, we assume that their PWWTP systems share

![Table 2](image)

**Table 2.** The values of minimum inhibitory concentration of some typical sensitive bacteria.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sensitive bacteria</th>
<th>NOEC (μg/mL)</th>
<th>MIC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td><em>Serratia marcescens</em></td>
<td>0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td><em>Pasteurella multocida</em></td>
<td>0.002</td>
<td>0.016</td>
</tr>
<tr>
<td>Lincomycin</td>
<td><em>Staphylococcus aureus</em></td>
<td>0.064</td>
<td>0.5</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td><em>Coagulase negative</em></td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td><em>Actinomyces spp.</em></td>
<td>0.002</td>
<td>0.016</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td><em>Moraxella catarrhalis</em></td>
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<td>0.016</td>
</tr>
<tr>
<td>Azithromycin</td>
<td><em>Campylobacter coli</em></td>
<td>0.008</td>
<td>0.032</td>
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<tr>
<td>Azithromycin</td>
<td><em>Campylobacter jejuni</em></td>
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<td>0.032</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td><em>Haemophilus influenzae</em></td>
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<td>0.008</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td><em>Methicillin Staphylococcus aureus</em></td>
<td>0.004</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Data from the European Committee on Antimicrobial Susceptibility Testing.
locations are displayed in Fig. 1. The frame represents abundant microbes in wastewater from fermentation.

ARG levels.

with higher 16s RNA concentrations (fermentation) exhibit higher total resistance genes and 16s RNA was applied to data in all 37 samples. To test this hypothesis, a linear regression analysis between ARG concentrations in wastewater and membrane removal of antibiotic resistance genes, Water Res. 47, 130–140.

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4. Conclusions

Overall, this study summarizes how ARGs may become widespread in pharmaceutical wastewater treatment systems. With the relatively poor removal efficiency of ARGs, there are still abundant ARGs in final effluents; therefore, PWWTPs could be considered as point sources of antibiotic resistance. By performing a mass balance analysis and correlation analysis for each PWWTP, we found that ARGs from chromosomal mutations, under the great selective pressure of antibiotics, would decrease with degradation of total antibiotics in final effluents, instead of proliferating through biological treatment stages as previously reported. Additionally, ARGs, which tend to locate in plasmids and transpo-

sons, might be much more likely to show positive correlations with 16s RNA concentrations.

For all the PWWTPs studied, our assumption that their PWWTP systems share a common mechanism for determining how ARGs are related to microbe abundance was proven. ARG concentrations in wastewater from fermentation were significantly higher than in wastewater from chemical synthesis and preparation, due to their more abundant microbes. This observation is consistent with a linear regression analysis showing a positive correlation between ARGs and 16s RNA with an R value of 0.985 and p < 0.01 in PWWTPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.
doi.org/10.1016/j.scitotenv.2017.08.229.


