Occurrence of Antibiotic Resistance Genes in a Small Township Wastewater Treatment Plant and the Receiving River

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Abstract Six ARGs (tetX, sul1, qnrS, blaTEM, ermB and intl1) were quantified by qPCR, along with concentrations of inorganic nitrogen (ammonia, nitrite, nitrate), total phosphorous (TP) and chemical oxygen demand (COD). The sewage treatment facilities had elimination effects on the six target genes; absolute abundance decreased from $10^4$ to $10^7$ copies/mL in the influent to $10^3$ to $10^5$ copies/mL in effluent, and the removal efficiencies were 74.27–96.51%; the highest removal performance was for tetX. The main treatment units for eliminating ARGs were the aeration tank and the secondary sedimentation tank. Absolute abundances of ARGs in the effluent and downstream water were 3.24–18.83 and 1.86–8.55 times higher than that in the upstream river, respectively. The absolute abundances of different target ARGs were positively correlated ($R = 0.6762 \pm 0.1777$), indicating similar elimination mechanisms, and a positive correlation between ARGs and ammonia nitrogen ($R = 0.5025 \pm 0.2711$). Raw wastewater contained numerous ARGs, which were partially removed by the WWTP. However, there remained a high absolute abundance of ARGs in effluent, causing an increase in water-phase ARGs in the receiving river. Hence, effluent was an important pollution point source for the receiving river.

1. Introduction
In recent years, the extensive use of antibiotics has tremendously benefitted humans but also poses potential risks to human health and ecosystems. Antibiotics and antibiotic resistance genes (ARGs) in wastewater are derived from domesticated animals and humans, and eventually enter wastewater treatment plants (WWTPs) [1-3]. Wastewater treatment systems have some ability to remove antibiotics and ARGs [4-5]. However, biological processing units provide favorable conditions for the generation, proliferation and dissemination of ARGs, such as abundant microorganisms and mobile genetic elements (e.g. plasmid, transposons, etc.), antibiotic selection pressure conditions, nutrients and suitable growth environments [6-7]. Indeed, ARGs are prevalent in WWTP effluents around the world, and in many cases the ARG concentrations in effluents are higher than in influent [8-9]. Effluents from WWTPs containing abundant types and quantities of ARGs are continuously discharged into receiving aquatic environments and have become an important pollution point source affecting nearby ecosystems [10-11].

Currently, in light of ARGs in sewage treatment plants, researchers have focused on treatment facilities of hospital wastewater, pharmaceutical wastewater, livestock breeding wastewater and urban municipal wastewater [12-14]. However, relatively little attention has been paid to sewage treatment plants in small towns. The rapid development and improvement of rural environments in China have
led to a large number of new sewage treatment plants in small townships. In the context of increasing antibiotic use (in terms of both dosages and types), ARG pollution in township WWTPs may be as significant as in the aforementioned wastewaters. Some studies show that ARGs are present in sewage of small towns and villages [15-16]. Thus, the discharge from effluents containing ARGs from township WWTPs may have serious impacts on local ecosystems, since these WWTPs are usually built in the suburbs with relatively healthy ecosystems.

This study aims to investigate the occurrence and fate of typical ARGs in different treatment units in a small township WWTP, and the effect of the effluent on the receiving river. Six kinds of ARGs \([\text{tet, sul, qnr, bla, erm}],\) which are the resistance genes of tetracycline, sulfa, quinolone, Beta lactam and macrolide, respectively and a class 1 integron gene \((\text{int}1))\) are quantitated by real-time polymerase chain reaction (PCR) analysis. Furthermore, conventional contaminants [ammonia, nitrite, nitrate, total phosphorous (TP) and chemical oxygen demand (COD)] are determined to analyze the correlations with ARGs to provide insights into the relationship between conventional contaminants removal and ARGs change in the WWTP.

2. Materials and methods

2.1. Wastewater treatment process and sampling

A WWTP receiving municipal wastewater from a small town in Sichuan province, China, was chosen in this study, in which the core treatment units were an aeration tank (influent for 2 h, aeration for 6 h) and a constructed wetland (Figure 1). The designed flow rate of the sewage was 100 m\(^3\)/d. It is worth noting that ultraviolet disinfection used as the tertiary treatment process in this WWTP was not functioning normally at the sampling time in this study. Raw wastewater was treated in the aeration tank and constructed wetland units before being discharged into the receiving river.

![Figure 1. Flow chart of the treatment processes of the wastewater treatment plant (WWTP) and the sampling site locations (the treatment units sampling sites are shown in hollow triangles and the river sampling sites are shown in solid triangles).](image)

Water samples were collected in plastic containers from the treatment facilities and from upstream and downstream sites which were located 100 m from the effluent discharge pipe. Sample containers were washed with 75% alcohol and sterile water and air-dried before use. The water samples from the aeration tank were the supernatant of a mixture of wastewater and activated sludge that was allowed to settle for 30 min; samples were taken after aeration for 3 h. Three parallel samples were taken from each sample. Samples (250 mL) were passed through a 47-mm diameter makrolon filter (pore size = 0.25 μm) to concentrate the microbial biomass as soon as possible after collection.

2.2. Genomic DNA Extraction and quantification by qPCR

A soil genomic DNA rapid extraction kit (SK8233, Sangon Biotech, China) was used to extract microbial DNA from the filters. Extracted DNA was stored at –20 °C. The concentration and quality
of the extracted DNA were determined by NanoDrop 2000 (Thermo Scientific, USA) and agarose gel electrophoresis, respectively. The PCR reaction system included: 0.5 μL template DNA; 0.5 μL primer F (10 μM); 0.5 μL primer R (10 μM); 0.5 μL DNTP (10 mM); 2.5 μL Taq Buffer (10 x); 2 μL MgCl$_2$ (25 mM); 0.2 μL Taq enzyme (5 U/μL), and 18.3 μL H$_2$O. The PCR reaction was repeated 35 times under the following conditions: pre-denaturation at 95 ºC for 3 min; denaturation at 94 ºC for 30 s; annealing at 57 ºC for 30 s; extension at 72 ºC for 30 s, and; repair and extension at 72 ºC for 8 min. The following target genes were measured in this study: Six ARGs $[tetX, sul1, blaTEM, qnrS$ and $ermB$ and one integrase gene of class 1 integrons ($intI1$)]. The primers were presented in Table 1.

2.3. Analytical methods for conventional contaminants
Ammonia, nitrite, nitrate and TP were detected by standard methods (HJ 535-2009, GB 7493-87, HJ/T 346-2007 and GB 11893-89, respectively) using a spectrophotometer (TU-1901, Puxi, China). The COD was determined by the potassium dichromate method (GB 11914-89).

2.4. Data analyses
The absolute abundance of target genes indicated the gene copy number per milliliter of water sample, and the relative abundance was normalized by 16S rRNA gene copies. Data was analyzed and presented using Microsoft Excel 2010. Detrended correspondence analysis (DCA) was performed using Canoco software (version 5.0) to evaluate ARGs (based on absolute abundance) and conventional contaminant concentrations between samples.

Table 1. Primers used for detecting the antibiotic resistance genes (ARGs).

| Genes | ARG species | Size/bp | Primers | Sequence |
|-------|-------------|---------|---------|----------|
| tetX [10] | resistance to tetracyclines | 278 | tetX F–W | AGCCTTACCAATG
| | | | | GGTTGAAA
| | | | | TTCTTACCTTGAG
| | | | | ATCCCG
| | | | | tetX R–V
| sul1[17] | resistance to sulfonamides | 245 | Sul F1 | CTGAACGATATCCAAGGATYCC
| | | | | AAAATCCTCAGCCGC
| | | | | Sul R1
| blaTEM [18] | resistance to beta-lactams | 112 | bla TEM-F | TTCCGTGTTTTTGTCT
| | | | | CACCCAG
| | | | | bla TEM-R
| qnrS [19] | resistance to fluoroquinolones | 428 | qnrS-F | GCAAGTTCTATTGAAACGGGT
| | | | | TCTAAACCCCTGCAACGGCGCTG
| | | | | qnrS-R
| ermB [20] | resistance to macrolides | 364 | ermB-F | GATACCGTTTACGAAATGG
| | | | | GAATCGAGACTTGAAGTGATGC
| | | | | ermB-R
| intI1 [21] | the integrase gene of class I integrons | 280 | intI1-F | CCTCCGGCACGATGTAC
| | | | | TCCACGATCAGTCAGGC
| | | | | intI2-R
| Reference genes [22] | - | 230 | F357 | CCTACGGGAGCAAGC
| | | | | R518 | ATTACCGCGACTG
| | | | | CTGG

References:

- HJ 535-2009
- GB 7493-87
- HJ/T 346-2007
- GB 11893-89
- HJ/T 346-2007

Note: The primers and sequences are presented in Table 1.
3. Results

3.1. Removal of conventional pollutants

Continuous measurements of influent were conducted for one week, and the concentrations (mean ± standard deviation) of ammonia and COD were 52.32 ± 8.46 mg·L⁻¹ and 127.65 ± 18.34 mg·L⁻¹, respectively. The ammonia, nitrite, nitrate, COD and TP concentrations of influent were 57.20 mg·L⁻¹, 0.37 mg·L⁻¹, 25.00 mg·L⁻¹, 123.50 mg·L⁻¹ and 3.17 mg·L⁻¹, respectively (Figure 2), which were comparable to other rural wastewaters [23-24].

![Figure 2. Conventional pollutants removal performance of treatment units.](image)

Following the aerated reaction for 3 h, the ammonia and COD concentrations decreased by 22.53% and 10.44%, respectively, and the removal rates were 4.3 mg·L⁻¹·h⁻¹ and 10.19 mg·L⁻¹·h⁻¹, respectively. There was little change in nitrite or TP concentrations, showing that nitrite was not accumulated and that TP remained at a very low level. However, the nitrogen concentration increased by 6.88 mg·L⁻¹ compared with that of influent. This indicated that autotrophic nitrification and organic degradation were both occurring in the aeration tank, however, with relatively less efficacy than provided by aeration tanks from other urban municipal WWTPs, in which the ammonia and COD removal rates are generally 10–20 mg·L⁻¹·h⁻¹ and 20–30 mg·L⁻¹·h⁻¹, respectively [25-26]. This may be because the WWTP investigated in this study was relatively new and had been operational for only 10 months prior to the present study, and may also be due to the low temperature at the sampling time in November (i.e., winter).

The water in the secondary sedimentation tank was the effluent from the aeration tank, in which the ammonia, nitrite, nitrate, COD and TP concentrations were 16.03 mg·L⁻¹, 0.29 mg·L⁻¹, 12.28 mg·L⁻¹, 78.00 mg·L⁻¹, and 4.34 mg·L⁻¹, respectively. This indicated a high performance of nitrogen and organic removal in the aeration tank, however, it was lower than that of aeration tanks in the majority of urban WWTPs [25].

The effluent of the secondary sedimentation tank was further treated in a constructed wetland and disinfection channels (which were not functioning normally) and was finally discharged into nearby rivers. Ammonia, COD and TP concentrations in the effluent were 14.76 mg·L⁻¹, 65 mg·L⁻¹ and 3.76 mg·L⁻¹, respectively, and were reduced by only 1.27 mg·L⁻¹, 13 mg·L⁻¹ and 0.59 mg·L⁻¹, respectively, in the constructed wetland. This relatively poor removal performance may be due to the facility being new and having insufficient aquatic plants for treatment.

Overall, the aeration tank played the main role in the removal of conventional pollutants in this WWTP. However, the pollutant content in the effluent did not reach the level B standard of Discharge Standard of Pollutants for Municipal Wastewater Treatment Plant (GB 18918–2002) implemented by...
the WWTP. The main reasons for this are considered to be the relatively low professional technical level of operating staff, the treatment process not being appropriate for the target wastewater, and the management of the WWTP not being well implemented.

3.2. Absolute abundance attenuation of ARGs in WWTP facilities
All ARGs genes involved in this study were detected in the raw wastewater, and the absolute abundance of ARGs was $10^4$–$10^7$ copies/mL, which was similar to that of pig farm wastewater, urban wastewater and pharmaceutical wastewater in previous studies [27–29]. The absolute abundances of tetX ($1.19 \times 10^6$ copies/mL) and sul1 ($1.07 \times 10^6$ copies/mL) in influent were higher than that of qnrS ($2.80 \times 10^4$ copies/mL), blaTEM ($7.15 \times 10^4$ copies/mL) and ermB ($1.58 \times 10^5$ copies/mL) [Figure 3(A)]. In addition, intI1 had a higher absolute abundance ($1.45 \times 10^7$ copies/mL) than all the ARGs. Generally, intI1, sulfa resistance genes, and tetracycline resistance genes are the main ARGs in the water phase according to other studies on the distribution of ARGs in sewage treatment systems, which is consistent with the results of this study [30–32].

![Figure 3](image_url)

Figure 3. The antibiotic resistance genes (ARGs) removal performance of treatment units.
(A) absolute abundance of ARGs; (B) relative abundance of ARGs.

After treatment for 3 h in the aeration tank, the absolute abundances of all detected genes in the water phase were considerably reduced to $10^1$–$10^6$ copies/mL, which is 0.32–0.76 orders of magnitude lower than that in the influent, and the average removal rate was 66.95%. In addition, the removal efficiency of tetX genes was the highest, reaching 82.66%, while that of blaTEM genes was the lowest
(52.33%). The mixture (sewage and activated sludge) was pumped into the secondary sedimentation tank after 6 h aeration, and then deposited for 12 h. The absolute abundance of ARGs in the supernatant decreased by 0.40–91 orders of magnitude compared with the sample from the aeration tank, and the average removal efficiency reached 61.97%. Among the ARGs, the highest removal efficiency was of tetX (87.69%), and the lowest was of sul1 (60.43%). Subsequently, the aerated and precipitated sewage entered the constructed wetland for treatment and then went through the ultraviolet disinfection channel (which, as previously stated, was not in normal operation) before discharging to the environment. However, it was suggested in Figure 1(A) that the constructed wetland had little influence on the absolute abundance of ARGs.

Although there was a considerable reduction in the absolute abundance of ARGs following treatment in the aeration tank, the effluent contained ARGs residues in the range of $10^{3}–10^{6}$ copies/mL. Furthermore, the absolute abundance of intI1 reached $1.43 \times 10^{6}$ copies/mL, which was similar to the results obtained by other studies, and shows that the absolute abundance of ARGs in effluent from the sewage treatment system were 1-6 orders of magnitude [4,10,33,34]. Compared with the raw wastewater, intI1, tetX and sul1 were the main ARGs in effluent, and qnrS was present in only trace concentrations which could be considered negligible. Following treatment in the WWTP, the total removal efficiency of tetX (with the highest absolute abundance) reached 96.51%, while the lowest removal efficiency was of blaTEM (74.27%).

### 3.3. Relative abundance attenuation of ARGs in WWTP facilities

The relative abundance of target genes in water samples was $10^{-5}–10^{4}$ copies/16S rRNA gene [Figure 3(B)]. Among them, the relative abundance of tetX, sul1 and intI1 were an order of magnitude higher than qnrS, blaTEM and ermB. The aerobic tank substantially reduced the relative abundance of tetX and qnrS. Compared with raw influent, the relative abundances of tetX and qnrS were reduced by 59.88% and 49.00%, respectively, following 3 h aeration. Additionally, the relative abundance of ermB was reduced by 33.12%, while the relative abundances of sul1, blaTEM and intI1 did not change considerably. There was no difference between the relative abundance of ARGs in the supernatant in the secondary sedimentation tank and that in the aeration tank.

Subsequently, the water from the secondary sedimentation tank entered the constructed wetland. Among the target genes in the final effluent, the relative abundance removal efficiency of tetX was the highest (85.96%), followed by intI1 (45.70%), sul1 (34.55%) and qnrS (30.62%); BlaTEM and ermB were not markedly changed.

In general, the WWTP reduced the absolute and relative abundance of ARGs in sewage. The aeration tank and secondary sedimentation tank substantially reduced the absolute abundance of ARGs, while the constructed wetland had a relatively limited effect. The aeration tank and constructed wetland had a certain removal effect on the relative abundance of ARGs, however there was no effect of the secondary sedimentation tank.

### 3.4. ARGs pollution by effluent discharging into the receiving river

After passing through the constructed wetland, effluent was discharged directly into nearby rivers. In this study, all the target ARGs were detected upstream from the effluent discharge point, among which the absolute abundances of sul1 and intI1 genes were relatively high ($5.40 \times 10^{3}$ copies/mL and $4.03 \times 10^{3}$ copies/mL, respectively), and the absolute abundances of other genes were $10^{2}–10^{5}$ copies/mL, which were very low (Figure 4). In contrast, ARGs in effluent water were considerably higher than that in ambient water. The absolute abundances of tetX, sul1, qnrS, blaTEM, ermB and intI1 genes in the final effluent were 14.67, 3.24, 4.72, 18.83, 8.99 and 4.86 times higher, respectively, than the background concentrations in the river water. After effluent was discharged into the river, the absolute abundances of tetX, sul1, qnrS, blaTEM, ermB and intI1 genes detected downstream from the effluent discharge point increased considerably compared with those in the upstream river, and were 5.21, 2.11, 1.86, 8.55, 6.58 and 3.55 times higher, respectively, than background concentrations upstream.
Hence, it appears that effluent from sewage treatment plants is an important cause of increases in ARGs in natural water bodies.

In terms of relative abundance, the main ARGs in the upstream river were sul1 and intI1. Following effluent discharge into the river, the relative abundances of sul1 and qnrS in the downstream water decreased slightly, whereas the relative abundances of tetX, blaTEM, ermB and intI1 were 2.14, 3.51, 2.71 and 1.46 times higher, respectively.

**Figure 4.** The antibiotic resistance genes (ARGs) disturbance of wastewater treatment plant (WWTP) effluent on the receiving river. (A) absolute abundance of ARGs; (B) relative abundance of ARGs.

3.5. *Correlation analysis between ARGs, intI1 and conventional contaminants*

As shown in Figure. 5, The absolute abundances of ARGs in each treatment unit were positively correlated (R = 0.6762 ± 0.1777), which indicated that there were similarities in the elimination mechanisms of various ARGs in the sewage treatment system.

The DCA analysis was conducted to present an initial insight into the correlations between gene abundances and contaminant concentrations among all samples from this full-scale WWTP. It showed that the indexes could be clustered into two groups, i.e., ARGs with ammonia, and other conventional contaminants, indicating that ammonia was the only conventional pollutant index which had a relatively clear positive correlation with ARGs (R = 0.5025 ± 0.2711). This was possibly because the removal of both ammonia and ARGs in this WWTP mainly took place in the aeration tank, although the removal mechanisms of ammonia and ARGs were different.

4. *Discussion*

From the perspective of environmental ARG pollution caused by effluents from WWTPs, absolute abundance is more relevant than relative abundance. In this study, the aeration tank had obvious removal effects on the target ARGs from sewage. The reason for the elimination might be that the
microorganisms carrying ARGs perished and cracked under the high dissolved oxygen conditions in this treatment unit, and the resulting free DNA could be adsorbed and degraded by activated sludge. This process may have led to an increase in the ARGs carried by sludge in the aeration tank, which has been confirmed by other studies [29,34,35].

![Graph showing correlation between antibiotic resistance genes (ARGs) and conventional contaminants.](image)

**Figure 5.** Correlation between antibiotic resistance genes (ARGs) and conventional contaminants. (A) detrended correspondence analysis (DCA) ordination of absolute abundance of ARGs and concentration of conventional contaminants; (B) correlation coefficients between the absolute abundance of ARGs and concentrations of conventional contaminants.

The reduction effect of aerobic tanks on ARGs has been reported previously, but it was with uncertainty. For example, Du found that ARGs in anaerobic tanks and anoxic tanks decreased, while the ARGs in aerobic tanks increased in an A²/O-MBR sewage treatment system [30]. Chen et al. found that sul1 and qnrS increased following aerobic treatment in the wastewater treatment process on a farm in Jiangsu, China [36]. On the contrary, Mandy found that an aerobic oxidation pond had a remarkable removal effect on ARGs, although it could not remove all kinds of target genes [37].
Combined with the results of the absolute abundance of ARGs in the secondary sedimentation tank in the present study, it could be speculated that the primary causes of reduced ARGs were the adsorption and aerobic degradation of activated sludge. However, ARGs are transferred to the sludge phase, and ARG pollution of sludge remains a problem that requires attention. In general, extending precipitation time to reduce suspended solids (SS) in the effluent is conducive to the control of ARG pollution control.

Many studies have found that constructed wetlands are an ideal method to remove ARGs from municipal wastewater [38,39]. One study reported reductions in ARGs in rural wastewater by more than 99% [16]. However, other studies have reported very limited removal efficiency of ARGs by constructed wetlands [33]. This may be related to differences in region, technology, operation conditions and wastewater types. In this study, the removal of ARGs by the constructed wetland was not effective. The possible reasons were as follows: firstly, the mechanisms of ARG removal by constructed wetlands were mainly the interception and adsorption of plant roots and soil colloids in wetlands. The sedimentation time in the secondary sedimentation tank was sufficient and the concentration of SS of effluent was very low. Therefore, the effect of the constructed wetland was not considered relevant. Secondly, the sampling time was during winter and the wetland plants were removed due to wilting before the study in October, resulting in poor removal effects.

Sufficient precipitation could reduce the absolute abundance of ARGs in the water phase by reducing the concentration of SS, however, the relative abundance of ARGs could not be reduced.

Some studies reported that the discharge of sewage treatment effluent has caused increases in the concentrations of ARGs in receiving water bodies in many regions of the world. LaPara found that the quantities of tetA, tetX and tetW in water treated by a tertiary treatment process in the United States (U.S.) were 20 times higher than in nearby surface water [10]. Czekalski et al. studied the pollution associated with ARGs (including sul, tet and QNR genes) in sewage effluent in a freshwater lake in Switzerland, and found that the ARG concentration at the outlet was 200 times higher than in the center of the lake [40]. Li et al. found that the total ARG abundance increased by 0.1 orders of magnitude after the effluent from a sewage treatment plant in China was discharged into the receiving lake, and reported that the influence of the effluent on the receiving water was affected by the ARGs species, abundance, water type and diffusion of effluent [41]. One study has found that the ARG concentration in water samples collected from 1 km downstream were affected by effluent discharge [42]. Although the removal efficiency of the target ARGs by the sewage treatment units in this study reached 52.33–82.66%, the absolute abundance in effluent remained at $10^3$–$10^5$ copies/mL, which was considerably higher than that of the upstream river water. The results of this study indicated that the effluent from this township WWTP had become an important point source of ARG pollution for the receiving water body.

Due to the recent development and small scale of small township WWTPs, ARG pollution and the potential harm to the receiving environment have not previously been given due concern. However, this study shows that the WWTP effluent, as a ARGs point source, may pose potential risks to the surrounding environment (which is relatively unpolluted), and this requires attention.

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