Comparison of the elimination effectiveness of tetracycline and AmpC β-lactamase resistance genes in a municipal wastewater treatment plant using four parallel processes

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Abstract
Municipal wastewater treatment plants (mWWTPs), considered reservoirs of antibiotic resistance genes (ARGs), are selected to compare the contributions of technology and process to ARG removal. Fifteen ARGs (tetA, tetB, tetC, tetE, tetG, tetL, tetM, tetO, tetQ, tetX, MOX, CIT, EBC, and FOX) and two integron genes (intI1, intI2) were tracked and detected in wastewater samples from a large-scale mWWTP with four parallel processes, including three biological technologies of AAO (anaerobic-anoxic-oxic), AB (adsorption-biodegradation), and UNITANK, two different disinfection technologies, and two primary sedimentation steps. The results showed that ARGs were widely detected, among which tetA and tetM had the highest detection rate at 100%. AAO was the most effective process in removing ARGs, followed by the AB and UNITANK processes, where the separation step was critical: 37.5% AmpC β-lactamase genes were reduced by the secondary clarifier. UV disinfection was more efficient than chlorination disinfection by 47.0% in ARG removal. Both disinfection and primary sedimentation processes could effectively remove integrons, and the swirling flow grit chamber was a more effective primary settling facility in total ARG removal than the aerated grit chamber. The tet genes and AmpC β-lactamase genes were significantly correlated with the water quality indexes of BOD5, CODCr, SS, TP, TOC, pH and NH4+-N (p < 0.05). In addition, the correlation between efflux pump genes and AmpC β-lactamase genes was strongly significant (r² = 0.717, p < 0.01). This study provides a more powerful guide for selecting and designing treatment processes in mWWTPs with additional consideration of ARG removal.

Keywords Tetracycline resistance genes · AmpC β-lactamase genes · Municipal wastewater treatment plants · Process selection · ARG removal

Introduction
Tetracycline and β-lactam are two types of antibiotics predominately used in human welfare and livestock as prophylaxis and therapy, with residual antibiotics contaminating soil and water (Widyasari-Mehta et al. 2016; Zhang et al. 2015). Due to repeated exposure to antibiotics, environmental microorganisms develop resistance against antibiotics and survive, which exacerbates the problem of the emergence of antibiotic-resistant bacteria (ARB) and ARGs (Kim et al. 2018). To date, at least forty different tetracycline resistance genes have been detected in various environments, such as aquatic environments (Li et al. 2020), wetlands (Li et al. 2019), mangrove ecosystems (Liu et al. 2020), WWTPs (Chen, Zhang 2013a; b), hospital wastewaters (He et al. 2020), and livestock farms (Duan et al. 2019). Six urban lakes in Wuhan detected antibiotic efflux pumps and ribosomal protection protein genes (tetA, tetB, tetC, tetG and tetM, tetQ) (Yang et al. 2017). Microorganisms may use these pollutants in ecosystems to assimilate and transform (Gu. 2019). Different types of AmpC β-lactamase genes have also been found in many gram-negative bacteria, including Acinetobacter (Wang et al. 2008), Aeromonas caviae (Ye et al. 2010), Proteus mirabilis

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(Ibrahimagić et al. 2015), Providencia spp. (Mahrouki et al. 2015), Escherichia coli (Bajaj et al. 2015), and Klebsiella pneumoniae (Venieri et al. 2017). ARGs can disseminate within or among species via heredity and horizontal gene transfer (HGT), which means that ARGs can be transferred from one bacterial strain to another (Zhang et al. 2018). The frequency and rapid spread of acquired plasmid-mediated AmpC β-lactamases are increasing among Enterobacteriaceae worldwide, and infections caused by resistant Enterobacteriaceae due to AmpC have increased the morbidity and mortality rate compared with those caused by susceptible Enterobacteriaceae (Etemadi et al. 2020).

Higher concentrations of ARGs are easy to transfer to the human body, especially the human intestinal tract. In addition, mobile genetic elements such as conjugative plasmids frequently carry genes other than ARGs that contribute to microbial fitness (McInnes et al. 2020). When these pollutants are bio-enriched to a certain concentration, they will have a toxic effect on the entire ecology and affect the balance of the ecosystem. The induced ARGs, as emerging environmental contaminants, cause global threats to human society (Organization 2014).

Untreated wastewater usually contains a large number of pathogens. Souissi et al. (2018) isolated and identified Leuconostoc spp., Chryseomonas luteola, and Staphylococcus xylosus from WWTP wastewater. Medema et al. (2020) reported that SARS-CoV-2 viral RNA was first detected in the influent of WWTPs in the Netherlands. Fecal streptococci and pathogen Staphylococci groups contained serious β-lactams or aminoglycosides and acquired resistance (Souissi et al. 2018). New research has shown that the quantity of ARGs in effluents is related to the size of WWTPs, and the abundance of ARGs is highest in small WWTPs (Harnisz et al. 2020). Conventional WWTPs generally fail to effectively reduce ARGs, especially extracellular ARGs (Li et al. 2019). The AAO process, as a widespread biological nutrient removal configuration, can achieve simultaneous removal of organics (Huang et al. 2020). However, it was further revealed that tetA, tetB, tetC, tetE, tetM, tetO, and tetS were common and abundant in all reactors of the improved AAO in WWTPs (Huang et al. 2015). The AB process, also called the two-stage activated sludge process, has a high removal rate of organic substrates. The UNITANK process is an improvement of the sequencing batch reactor (SBR), in which wastewater is added to a single batch reactor and returning sludge is eliminated (Bashiri et al. 2018). However, it is prone to sludge deposition (Liu et al. 2018), which easily causes ARGs from wastewater to be transferred into sludge in both the AB and UNITANK processes. Higher amounts of some ARGs were detected during the pretreatment process, including influent and primary sedimentation in AAO, but decreased gradually via sequential treatment processes (Lee et al. 2017). Although both UV and chlorination disinfection could affect the reduction of ARGs, chlorination might augment the risk of ARG transfer in wastewater containing NH3-N (Sharma et al. 2016). The disadvantage of the AB process is that the A stage is prone to produce more sludge. The genes tetA, tetM, tetW, and tetX were a large proportion of the sludge samples. (Xu et al. 2020; Zhou et al. 2019; Zhang et al. 2019). However, in the presence of trace tetracycline, the relative abundances of efflux pumps, such as tetA and tetG, tended to remarkably increase in the UNITANK process (Liu et al. 2019). Overall, the conventional and advanced processes resulted in 0.03–2.40 log reductions for most ARG subtypes, such as intI1, qepA, strA, and strB, but neither treatment method affected the reduction of tetM (Hu et al. 2019). Traditionally, a combination of physical, chemical, and biological processes has been widely used in wastewater treatment plants to remove pollutants (Li et al. 2017), such as BOD5, CODCr, SS, TP, TN, etc. and ensure that the residual pollutants in the effluent conform to the legal requirements (Everage et al. 2014; Jaranowska et al. 2013), so the reduction of ARGs was not considered during the treatment process (De Sotto et al. 2016). WWTPs have been considered a reservoir of ARGs because a high abundance and diversity of ARGs were detected in WWTPs, such as quinolone resistance genes qnrS, qnrB, and acc(6’)-Ib-cr, AmpC genes, integrons intI1 and intI2 (Su et al. 2014; Kumar et al. 2020), tet genes tetM tetC tetK and tetA/P (Laht et al. 2014; Zhang, Zhang 2011), which might be released into the drinking water from the effluent and pose a potential risk to human health. Excessive residues of ARGs have caused serious threats to human and animal health due to the spread of antibiotics. To make the distribution of ARGs clear and raise the management level of ARGs, it is important to study the relationship between the present wastewater treatment process and the removal of ARGs.

In this study, a large-scale mWWTP in Guangzhou, which contains four different treatment processes, was selected to investigate the relationship between the treatment process and elimination of ARGs. Eleven tetracycline resistance genes (six efflux pump genes: tetA, tetB, tetC, tetE, tetG, tetL, four ribosomal protection genes: tetM, tetO, tetQ, tetS, one enzymatic modification gene: tetX), four family-specific AmpC β-lactamase genes (MOX, CIT, EBC, FOX), and two integron genes (intI1, intI2) were detected by using polymerase chain reaction (PCR) and real-time qualitative polymerase chain reaction (qPCR). According to the same properties of the influent and the same operation level in the plant, the results provide strong evidence for selecting the key process step and disinfection method to control ARG discharge, which will benefit the reduction of health and ecosystem risk.
Materials and methods

Municipal wastewater treatment plants

A specialized large-scale municipal wastewater treatment plant in Guangzhou was selected to collect samples. This plant treats the wastewater for an equivalent population of 2.96 million and has a maximum capacity of 560,000 m³/d, occupying an area of 390,000 m² and receiving the wastewater from the central city area of 228 km² belonging to the North Waterway of the Pearl River. It is constructed in four phases, and the basic information is shown in Table S1. The wastewater from the same conditioner with the same properties is pumped into four phases of proceedings, and effluent treated by different processes and disinfection methods is discharged through the same draining exit.

Sample collection and pretreatment

Twenty-one samples of water were collected from the influent, effluent, and each treatment unit of the mWWTP, as shown in Fig. 1. At each sampling location, a 2000 mL water sample was transferred to two sterile 1000 mL bottles and stored at 4 °C before pretreatment within 24 h. Chemical parameters of influent and effluent samples from four phases were detected and recorded (Table S2).

DNA extraction

One thousand milliliters of water samples were filtered through 50 mm cellulose ester membranes with pore sizes of 0.22 μm. The filters were stored at −20 °C to protect DNA before extraction. Total DNA was extracted using the E.Z.N.A.™ Water DNA Kit (Omerga Bio-tek, USA) according to the manufacturer’s protocol. The quality and concentration of the purified DNA were determined by spectrophotometer analysis (NanoDrop 1000, Thermo Scientific, USA) (He et al. 2017) and quantified by 1.5% agarose gel electrophoresis. The total DNA samples were stored at −20 °C pending further analysis.

Qualitative PCR

Fifteen ARGs (tetA, tetB, tetC, tetE, tetG, tetL, tetM, tetO, tetQ, tetS, tetX, MOX, CIT, EBC, FOX) and two integron genes (intI1, intI2) in all water samples were investigated by using conventional PCR with the primers listed in Table S3. The reaction volume of PCR was 25 μL consisting of 2.5 μL of 10 × PCR Buffer (Mg²⁺ Plus, TaKaRa, Japan), 2.0 μL of dNTP Mixture (each 2.5 mM, TaKaRa, Japan), 1.0 μL of each forward and reverse primer (10 μM), 0.125 μL of Taq polymerase (5 U/μL, TaKaRa, Japan), and 1 μL of DNA template, and 17.375 μL of ddH₂O. The PCR program was...

Fig. 1 Treatment process flow charts and sampling locations of the four phases with different process. A The influent of the grid screen as the influent of the main process. B1-2 Two different primary sedimentation tanks, Phase I(AAB process) and Phase II(UNITANK process) share a swirling flow frit chamber, and Phase III(Modified AAO process) and Phase IV(Modified AAO process) share an aerated grit chamber. Phase I: (C1) the mixture in the stage B aerobic tank, (C2) the effluent of the stage B clarifier. Phase II: (D1) the mixture in the A tank, (D2) the mixture in the B tank, (D3) the effluent of the UNITANK. Phase I-II, (R1) the disinfection effluent of Phase I and II. Phase III: (E1) the mixture in the pre-anoxic tank, (E2) the mixture in the anaerobic tank, (E3) the mixture in the anoxic tank, (E4) the mixture in the aerobic tank, (E5) the secondary clarifier effluent, and (E6) the disinfection by chlorination. Phase IV (Modified AAO process): (F1-5) same as Phase III and (F6) the disinfection by UV...
performed on a thermal cycler (Eppendorf, Germany) as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, 72 °C for 60 s, and a final extension of 72 °C for 7 min. PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel with ethidium bromide in 0.5× TBE buffer at 100 V for 40 min and visualized under UV transillumination. Ultrapure water was used as the negative control.

**ARG quantitative analysis**

The quantities of target ARGs, integrons, and 16S rRNA were detected by qPCR using the SYBR® Green approach and specific primers. The 20 µL qPCR reaction mixtures consisted of 7 µL of ddH2O, 10 µL of 2 × iTaq™ universal SYBR® Green supermix (Bio-Rad, USA), 2 µL of forward and reverse primers (10 µM of each type), and 1 µL of template DNA (Table S4). Amplification was conducted with CFX (Bio-Rad, USA) as follows: initial denaturation at 95 °C for 3 min, 40 cycles at 95 °C for 10 s, and 30 s with the plate read at the annealing temperature of 58 °C. Immediately after the qPCR assay, melting curve analyses were performed by increasing the temperature from 65 °C to 95 °C (0.5 °C/5 s) with continuous fluorescence recording according to Huang et al. (2017). Sterile ultrapure water was used as the negative control, and 16S rRNA was determined for each sample as the reference gene. Three independent samples were analyzed at each site, and each sample was quantified in triplicate to ensure reproducibility. The qPCR efficiency of each gene ranged from 90 to 110%, with $R^2$ values greater than 0.990 for all calibration curves.

**Statistical analysis**

Basic calculations were performed using Microsoft Excel 2016. Correlations between tet genes and AmpC β-lactamase genes were analyzed by SPSS 24.0 statistical software. A variable was considered statistically significant if $p < 0.05$ or $p < 0.01$. Data features were analyzed by Origin Pro 8.1 (OriginLab Co., MA, USA).

### Results and discussion

**Occurrence of ARGs in mWWTP from influent to effluent**

For the occurrence of the target ARGs, the detection frequency was 100% in the influent. The genes tetA, tetC, tetM and tetO showed strong persistence throughout the wastewater treatment plant, tetA and tetM were detected in all treatment units (100%), and the detection rate of tetO and tetC in water samples was 90.47% (19/21) (Table 1). However, the gene diversity in the samples was reduced

| Table 1 Occurrence of tet ARGs and AmpC β-Lactamase genes in the municipal WWTP with four different treatment phases |
|---------------------------------------------------------------|
| Sample sites   | Tetracycline resistance genes | AmpC β-lactamase genes | Sum |
|                | tetA | tetB | tetC | tetE | tetG | tetL | tetM | tetO | tetS | tetX | MOX | CIT | EBC | FOX |
| IN1-2-3-4      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AP1-2          | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | -   | +   | +   |
| AE1            | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | -   | -   | -   |
| CL1            | +    | -    | +    | -    | +    | +    | +    | -    | -    | +    | -   | -   | -   | -   |
| AN2            | +    | -    | +    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AN2            | +    | -    | +    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AE2            | +    | -    | +    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| CL2            | +    | -    | +    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| EF1-2          | +    | -    | +    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AP3-4          | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| PAN3           | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| ANA3           | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AN3            | +    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AE3            | +    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| SE3            | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| EF3            | +    | -    | -    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| PAN4           | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| ANA4           | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AN4            | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| AE4            | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| SE4            | ++   | -    | -    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| EF4            | +    | -    | -    | -    | +    | +    | +    | +    | +    | +    | +   | +   | +   | +   |
| Frequency index| 21/21| 4/21 | 18/21| 14/21| 10/21| 16/21| 21/21| 19/21| 18/21| 16/21| 17/21| 6/21| 16/21| 11/21|

+: Positive; -: Negative
during the treatment process. The quantity of gene types identified in the effluent of phase I-II, phase III, and phase IV were 9, 5, and 3, respectively. A total of 78.57% of the detected ARG types disappeared with the treatment of the whole process used in phase IV, which was the most efficient process for ARG type removal among the four parallel processes used in the mWWTP. Similar results were found that the detection frequencies of tetA, tetM, and tetO were higher than those of other tet genes in WWTPs (Storteboom et al. 2007), and the detection frequency of tetM was 100% (Zhang et al. 2017). The efflux genes (tetA, tetB, tetC, and tetE) and ribosomal protection protein genes (tetM and tetO) are frequently found in various environmental matrices, such as livestock farms (Duan et al. 2019), WWTPs (Xu et al. 2017) and ground water (Wu et al. 2020). The tetracycline resistance genes tetA, tetB, tetC, tetE, tetM, and tetO have a broad host range and are carried by several environmental matrices. Therefore, the strong persistence of these ARGs was due to their broad host range. The ARGs in wastewater were reduced, primarily based on the decrease in total biomass and selective removal of ARGs from bacterial cells (Bengtsson-Palme et al. 2016). Most of the available studies have concluded that WWTPs reduce the absolute numbers of both ARGs and total bacteria in wastewater (Pallares-Vega et al. 2019).

**Abundance of ARGs in mWWTP**

PCR-detectable tet genes and AmpC β-lactamase genes in each sample were normalized to the 16S rRNA as the relative abundance of genes. The results are shown in Fig. S1. The proportional results indicated that most relative abundances of tet genes were higher than AmpC β-lactamase genes, except for tetB and tetL. In all influent samples, the relative abundance of all resistance genes ranged from $10^{-3}$ to $10^{-5}$, in which tetX was the highest. However, the residual ARGs were different depending on the process. In Phase I-II, tetB, tetG, tetX, CIT, and FOX were undetected in the effluent, showing that these ARGs can be completely eliminated by the AAO process. For the Phase III effluent, most ARGs could be completely removed except tetA, tetE, tetM, tetO and tetS, while only tetA, tetM and tetS were detected in the effluent from Phase IV. Moreover, the average abundance of ribosomal protection protein genes was higher than that of both efflux pump genes and enzyme-modified genes, which was similar to Cheng et al. (2013). According to published studies, tetracycline resistance genes were found to be more prevalent in bacterial populations than AmpC β-lactamase genes because tetracycline resistance genes could be detected in both gram-positive and gram-negative bacteria (Chopra, Roberts 2001), while AmpC β-lactamase genes were only found in gram-negative bacteria (Jacoby. 2009). Moreover, tetracycline resistance genes could be found on bacterial chromosomes (e.g., tetQ), plasmids (e.g., tetC, tetE, tetK), and transposones (e.g., tetB, tetC, tetE, tetK) (Pazda et al. 2019). These tet genes with high abundance were always carried by mobile elements and could be transferred between bacteria in the environment (Agero et al. 2007; Huang et al. 2015; Ding et al. 2020).

**Comparison of ARG elimination efficiency by processes among AB, UNITANK, and Improved AAO**

By comparing phase I-II and phase III with different treatment processes (AB process, UNITANK process, improved AAO process), the gene removal efficiency was higher in phase III than in phase I-II (Fig. 2), e.g., the removal efficiency of tetO in phase I-II and phase III was 19.52% and 72.24%, respectively. However, it was intriguing that the relative abundance of genes in the clarifier of phase II was higher than that in the influent, such as tetA, which increased from $7.32 \times 10^{-3}$ to $1.23 \times 10^{-2}$, with a growth rate of 68.17%. This result was similar to the findings that the reduction magnitudes of tetO,
The removal rate observed for the tetM gene was an average log reduction of 2.53 ± 0.68 (Pallares-Vega et al. 2019). The tet genes include three types of resistance mechanisms: efflux pump mechanisms (genes encoding energy-dependent efflux proteins), target modification mechanisms (genes encoding ribosomal protection proteins, RPPs), and inactivating enzymes (Chen, Zhang 2013a; b; Huang et al. 2015; Pazda et al. 2019). According to the above results, the efflux pump genes of tetA and tetC and the ribosomal protection protein genes of tetM and tetO were difficult to remove, which may be due to the resistance mechanism (efflux pump mechanism and target modification mechanism). Zhang et al. (2018) also proved that the AAO process could reduce ARGs regardless of the relative abundance or absolute gene copies. This might be due to the proliferation of ARGs after conventional biological treatment processes, which had an influence on microbial growth (Wang et al. 2015). In addition, the plant containing individual process for sludge discharge such as AAO may tolerate to the complicated wastewater. The highest bacterial diversity was achieved in modified AAO process (Yan et al. 2019), which may imply the advantage in ARG removal. The tetracycline resistant bacteria (TRB) declined in the final effluent samples compared to the influent samples (Huang et al. 2015), which may be one of the reasons for the removal of ARGs. The results indicated that the removal efficiency of ARGs in the superior treatment process was better than that in the conventional treatment process, while the effect of the process was improved in the pattern of AAO process > AB process > UNITANK process.

AAO treatment also played an important role in reducing the relative abundance of resistance genes. As shown in Fig. 3, in the aerobic phase, the relative abundance of the four ARGs was effectively reduced, where the removal rates of the efflux pump of resistance genes, ribosome protection genes, enzyme modification genes, and AmpC β-lactamase genes were 29.61%, 63.66%, 67.46%, and 49.1%, respectively. In the anaerobic stage, the removal rate of the ARGs was also high, and the removal rate of tetX was as high as 73.07%. In the pre-anaerobic phase, most ARGs showed growth, in which the AmpC β-lactamase genes were increased by 1.23 times and the efflux pump genes were increased by 32.71%. In the anoxic phase, almost all types of ARGs had increased, with tetX increasing the most, reaching 22.74%, which meant that different ARGs may be attributed to dissolved oxygen or other nutrients. The removal of ARGs may also have a certain relationship with the difference of bacterial species because the oxygen content of each tanks is different. Therefore, in the AAO process stage, the ARG removal capability followed the order aerobic > anaerobic > preanaerobic > anoxic.

In addition, the removal of the ARGs in the secondary clarifier also played a crucial role, in which the removal rate of tetX could be as high as 100%, and the removal rate of AmpC β-lactamase genes was also 37.5%. It was found that the removal of the tetracycline resistance gene was more advantageous under aerobic conditions (Su et al. 2019). The ARGs were lower in aerobic tanks and higher in anaerobic tanks (Tao et al. 2014). The results shown in Fig. 3 suggest that biological treatments could more effectively reduce the abundance of tet genes and AmpC β-lactamase genes, which might be related to the high efficiency of WWTPs in reducing the bacterial population (Su et al. 2014). AmpC β-lactamase plays an important role in hydrolyzing all β-lactam antibiotics and contains two types of resistance mechanisms, i.e., chromosomal mediated and plasmid mediated (Mohamudha et al. 2012; Korzeniewska, Harnisz 2013), except cefepime and carbapenems (Maravić et al. 2013; Ebomah and Okoh. 2020). The genes encoding plasmid-mediated AmpC β-lactamase are harbored by mobile elements that could confer transmissible resistance to environmental bacteria and pathogens, which may accelerate cephalosporin resistance dissemination in the environment (Liu et al. 2015; Pazda et al. 2019). Therefore, the detection rates of MOX and EBC were very high (85.71%). In this study, tet genes and AmpC β-lactamase genes were widely found in mWWTP, which indicates a potential health risk in urban areas.

Fig. 3 Removal rate of antibiotic resistance genes in each step of modified AAO process. The number was the value of removal rate, which displayed by red or blue bar.
Comparison of ARG elimination efficiency by UV and chlorination disinfection

The relative abundances of ARGs in the effluent in phase III (improved AAO with sodium hypochlorite) and phase IV (improved AAO with ultraviolet disinfection) with the same superior treatment process were different because of different disinfection methods. The removal efficiency for each gene was determined by comparisons of relative abundance between them, which showed that UV disinfection was better than chlorination (Fig. 4). After two different disinfection, the AmpC β-lactamase resistance genes and inhibitory enzyme activity genes were completely removed. However, UV disinfection had removal rates of 99.99% and 97.23% for intI1 and intI2, respectively, and removal rates for intI2 and intI1 for chlorination were only 85.62% and 51.61%, respectively. Among them, UV had a removal rate of 100% for tetG, tetX, MOX, and EBC. Zhou et al. (2020) also found that UV radiation showed significant removal efficiency on ARGs. In phase III, the detection rate of the effluent resistance genes was 43.75% (7/16), while the detection rate of the effluent resistance genes in UV disinfection was 31.25% (5/16). The disinfection may adjust the relative abundance of the microbial genus to the ARGs, but low doses of chlorine stimulated HGT (Wang et al. 2020). However, Zhang et al. (2015) suggested that the removal efficiency of ARGs by chlorination was better than that with UV. This suggested that sodium hypochlorite might be used for a few stress-tolerant bacteria. A previous study supported this hypothesis that chlorination enriched ARGs and changed the microbial community structure (Shi et al. 2013).

Comparison of ARG removal by different primary sedimentation processes

The primary sedimentation tank is an important treatment for mWWTPs, which mainly removes suspended solids from wastewater. The AB process and UNITANK process share the swirling flow grit chamber, while another two different improved AAO processes share the aerated grit chamber. According to Fig. 4, the proportion of intI1 in the influent reached 54.4%, which was reported to be associated with multiantibiotic resistance (MAR) in various environments (Brooks et al. 2014). Integrons (intI1, intI2) are very important to the migration of ARGs (Zhou et al. 2020); therefore, the removal of integrons is beneficial to reduce the spread of ARGs in the environment. The aerated grit chamber could remove almost all intI1 of the influent, which was obviously more effective than that of the swirling flow grit chamber. However, for intI2, the removal rate by the swirling flow grit chamber was 69.99%, but intI2 in the effluent from the aerated grit chamber was 3 times higher than that in the influent. Jang et al. (2020) also indicated that most target ARGs (tetG, tetH, tetM, tetQ, tetX) showed a significant positive correlation with intI1. From the analysis of the quantity of ARGs and the total abundance, the swirling flow grit chamber was more advantageous than the aerated sand because the CIT was completely removed, and the removal rate of the ribosome protection genes reached 73.88%, which was 54.0% higher
Comparison of the elimination effectiveness of tetracycline and AmpC β-Lactamase resistance.  

**Table 2** Correlation matrix between *tet* ARGs and AmpC β-Lactamase genes in the municipal WWTP (Pearson correlation coefficients)

|       | tetA | tetB  | tetC  | tetE  | tetG  | tetL  | tetM  | tetO  | tetS  | tetX  | MOX  | CIT  | EBC  | FOX  |
|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|------|------|
| **tetA** | 1    | -0.218 | -0.288 | -0.434* | 0.124 | -0.572** | -0.460* | -0.362 | -0.094 | -0.276 | -0.006 | -0.393* | -0.213 | -0.195 |
| **tetB** | -0.218 | 1    | -0.250 | -0.025 | -0.122 | 0.316 | -0.236 | -0.070 | -0.083 | -0.104 | -0.260 | -0.118 | 0.604** | -0.036 |
| **tetC** | -0.288 | -0.250 | 1     | 0.519** | 0.330 | 0.265 | 0.782** | 0.942** | -0.305 | 0.734** | 0.204 | 0.621** | 0.367 | 0.756** |
| **tetE** | -0.434* | -0.025 | 0.519** | 1    | 0.334 | 0.673** | 0.701** | 0.632** | -0.132 | 0.497** | 0.382* | 0.860** | 0.340 | 0.409* |
| **tetG** | 0.124 | -0.122 | 0.330 | 0.334 | 1    | -0.020 | 0.216 | 0.287 | -0.128 | 0.392* | 0.654** | 0.447** | 0.303 | 0.262 |
| **tetL** | -0.572** | 0.316 | 0.265 | 0.673** | -0.020 | 1    | 0.618** | 0.387* | -0.254 | 0.305 | 0.334 | 0.665** | 0.262 | 0.252 |
| **tetM** | -0.460* | -0.236 | 0.782** | 0.701** | 0.216 | 0.618** | 1     | 0.820** | -0.233 | 0.617** | 0.262 | 0.828** | 0.288 | 0.627** |
| **tetO** | -0.362 | -0.070 | 0.942** | 0.632** | 0.287 | 0.387* | 0.820** | 1    | -0.204 | 0.736** | 0.171 | 0.675** | 0.531** | 0.735** |
| **tetS** | -0.094 | -0.083 | -0.303 | -0.132 | -0.128 | -0.254 | -0.233 | -0.204 | 1    | -0.235 | -0.182 | -0.145 | -0.310 | -0.226 |
| **tetX** | -0.276 | -0.104 | 0.734** | 0.497** | 0.392* | 0.305 | 0.617** | 0.736** | -0.235 | 1    | 0.371 | 0.629** | 0.358 | 0.730** |
| **MOX** | -0.006 | -0.260 | 0.204 | 0.382* | 0.654** | 0.334 | 0.262 | 0.171 | -0.182 | 0.371 | 1    | 0.448* | -0.093 | 0.004 |
| **CIT** | -0.393* | -0.118 | 0.621** | 0.860** | 0.447* | 0.665** | 0.828** | 0.675** | -0.145 | 0.629** | 0.448* | 1    | 0.266 | 0.499** |
| **EBC** | -0.213 | 0.0604** | 0.367 | 0.340 | 0.303 | 0.262 | 0.288 | 0.531** | -0.310 | 0.358 | -0.093 | 0.266 | 1    | 0.444* |
| **FOX** | -0.195 | -0.036 | 0.756** | 0.409* | 0.262 | 0.252 | 0.627** | 0.735** | -0.226 | 0.730** | 0.004 | 0.499** | 0.444* | 1    |
| **BOD₅** | -0.340 | -0.218 | 0.811 | 0.886 | 0.966* | 0.519 | 0.953* | 0.996** | -0.201 | 0.934 | -0.094 | 1.000** | 0.350 | 0.936 |
| **COD₅** | -0.327 | -0.233 | 0.820 | 0.879 | 0.962* | 0.506 | 0.957* | 0.995** | -0.188 | 0.931 | -0.083 | 1.000** | 0.335 | 0.931 |
| **SS**  | -0.319 | -0.237 | 0.820 | 0.877 | 0.961* | 0.504 | 0.958* | 0.994** | -0.193 | 0.934 | -0.091 | 1.000** | 0.332 | 0.930 |
| **TP**  | -0.311 | -0.262 | 0.839 | 0.864 | 0.953* | 0.478 | 0.965* | 0.991** | -0.153 | 0.918 | -0.049 | 0.999** | 0.307 | 0.919 |
| **NH₄⁺** | -0.367 | -0.189 | 0.795 | 0.900 | 0.973* | 0.544 | 0.943 | 0.998** | -0.221 | 0.937 | -0.110 | 0.999** | 0.378 | 0.947 |
| **TN**  | -0.410 | -0.094 | 0.722 | 0.935 | 0.988* | 0.626 | 0.904 | 0.997** | -0.336 | 0.967* | -0.226 | 0.988* | 0.463 | 0.971* |
| **TOC** | -0.330 | -0.229 | 0.818 | 0.881 | 0.963* | 0.509 | 0.956* | 0.995** | -0.192 | 0.932 | -0.087 | 1.000** | 0.339 | 0.932 |
| **pH**  | 0.178  | -0.760 | 0.998** | 0.421 | 0.607 | -0.112 | 0.937 | 0.733 | 0.376 | 0.599 | 0.407 | 0.799 | -0.288 | 0.529 |

**p < 0.01; *p < 0.05**
than that of the aerated grit chamber. In addition, the gene removal rates of inhibitory enzyme activity, AmpC β-lactamase, and integron reached 67.26%, 72.48%, and 83.29%, respectively. Total microorganisms, integrons, and organic matter removed in wastewater are conducive to the reduction of ARGs (Riquelme Breazeal et al. 2013). The swirling flow grit chamber could remove the ARGs more effectively than the aerated grit chamber, which might be due to the advantages of the low change in the flow rate and high efficiency of the kinetic energy. However, it is still unclear how the abundance and diversity of ARGs are affected by common wastewater treatment processes, especially in mWWTPs, so further investigations should be performed to better address this question.

**Correlation of ARGs in mWWTP**

The tet genes and the AmpC β-lactamase genes were significantly correlated with BOD₅, COD₅, SS, TP, TOC, pH and NH₄⁺-N (p < 0.05), of which tetO and all water quality parameters except pH were significantly correlated (p < 0.01) (Table 2). However, the efflux pump genes tetA, tetB, tetE, and tetL, ribosome protection gene tetS, and AmpC β-lactamase genes MOX and EBC were not significantly correlated with all water quality parameters (p > 0.05). This indicated that some kinds of ARGs in the wastewater were significantly correlated with some physical parameters. Yuan et al. (2018) also proved that most ARGs, including intI1 and tetA, positively correlated with wastewater nutrient (COD, NH₃-N, TN and TP) concentrations, and the relative abundance of some ARGs decreased as the quality of wastewater improved. Therefore, for mWWTPs, the removal rate of pollutants in sewage was an important factor affecting the change rate of ARGs.

There was a significant correlation between tet genes and AmpC β-lactamase genes (p < 0.05), in which tetC, tetE, tetM, tetO, tetX, tetO, and CIT were strongly and significantly correlated with FOX (p < 0.01), and tetE, tetL, tetM, and CIT had significant correlations with each other (p < 0.01) (Table 2), which was due to the large number of multidrug resistant bacteria carrying multiple resistance genes in mWWTP. Similarly, Huang et al. (2015) also found a strong significant correlation between tetM and tetO, as well as tetE and tetX (p < 0.01), in an improved AAO process. The total quantities of efflux pump genes, ribosomal protection protein genes, enzymatic modification genes, and AmpC β-actamase genes were significantly correlated, as shown in Table S5. The correlation between the quantity of efflux pump genes was found to be strongly significant with AmpC β-lactamase genes (r² = 0.717, p < 0.01) and enzyme-modified genes (r² = 0.523, p < 0.01). It was reported that there was a strong correlation among the total quantity of tet genes (Huang et al. 2015).

The correlation between ARGs was affected by not only their relative antibiotics (Cheng et al. 2013; Huang et al. 2015) but also the function of co-selection and cross-selection on resistance from antibiotics and heavy metals (Mckinney et al. 2010; He et al. 2017). Furthermore, water parameters, such as COD, DO, pH, and temperature, have been found to be related to ARGs, and correlations between the removal efficiency of ARGs and the removal efficiency of COD₅, BOD₅, nitrogen, and biomass were observed (Nõlvak et al. 2013; Yuan et al. 2016).

**Conclusions**

Overall treatment processes were carried out in four different phases to evaluate the tet genes and AmpC β-lactamase genes, especially in the effluent, which presented a lower abundance of ARGs. By comparing different processes, the removal efficiency of genes was most significantly improved by the AAO process, followed by the AB process and UNITANK process, while the aerobic tank played an important role in modifying the AAO of ARG reduction. The swirling flow grit chamber was more significant in ARG removal. In addition, ultraviolet disinfection was better than chlorination. The quantity between efflux pump genes and AmpC β-lactamase genes showed a strongly significant correlation (r² = 0.717, p < 0.01).

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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