RESEARCH ARTICLE

The fate of tetracycline resistance genes in different biological treatment processes of four wastewater treatment plants in various seasons

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Abstract: The widespread antibiotic resistance genes (ARGs) severely jeopardizing human health has been widely concerned. Wastewater treatment plants (WWTPs) are commonly considered the important sources and sinks of ARGs due to their roles in depositing ARGs and provoking ARGs' proliferation. Current WWTPs often use biological treatment to remove pollutants, whereas the purification efficacy of ARGs is unclear. Thus, this study investigated the seasonal changes of tetracycline resistance ARGs (*tet*-ARGs, including *tetA*, *tetC*, *tetM*, *tetO*, *tetQ*, *tetW*, and *tetX*, as representative) and a class of integron (*intI1*) in the sludge of different treatment units of four WWTPs to understand the role of WWPTs in ARGs spread. It is found that the abundance of *tet*-ARGs varied between summer and winter depending on the types of *tet*-ARGs and treatment units. The cumulative abundance of *tet*-ARGs in the same treatment unit showed no obvious difference between seasons. By comparison, the abundance of ARGs was generally lower in the anoxic unit than in the oxic unit. Moreover, the WWTPs with Cyclic Activated Sludge System (CASS) process reduced the amount of ARGs in sludge, while the WWTPs with Anaerobic-Anoxic-Oxic (A^2O) increased the abundance of *tet*-ARGs. In conclusion, this study demonstrated that the excess sludge still posed a risk for ARG spread after biological treatment.
Keywords: Antibiotic resistant genes, wastewater treatment plants, CASS, A^2O, sludge

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1 Introduction

Antibiotics have been widely used in human health care, animal husbandry, poultry farming, and aquaculture industries since the 1940s (Wen et al., 2016). Currently, antibiotics are extensively detected in soils, sediments, surface water, groundwater, and even drinking water systems (Mao et al., 2015). Due to the high concentration of antibiotics in the environment, antibiotic resistance genes (ARGs) and bacteria (ARB) are greatly induced, thus incurring potential risks to human and ecological health (Xu et al., 2015).

Normally, the wastewater treatment plants (WWTPs) are the final sites for receiving antibiotics. Due to the abundant nutrients and bacteria in WWTPs, antibiotics may pressure bacteria, motivate ARGs, and promote the expansion of ARGs because ARGs can be spread among different bacterial genera (Guo et al., 2014; Pazda et al., 2019; Cheng et al., 2020). In the past, most studies focused on ARGs removal from wastewater while ignoring the fate of ARGs in the sludge of WWTPs (Hiller et al., 2019). In fact, sludge, as the flocs of bacteria, might contain more abundant of ARGs than wastewater (Negreanu et al., 2012). For instance, previous studies reported that the abundance of ARGs in sludge is generally higher than that in effluent and occasionally even higher than that in influent (Liu et al., 2012; Jiao et al., 2018). On the other hand, ARGs in sludge could be more detrimental to the environment because excess sludge is often reused as fertilizer (Bibby et al., 2010; Berglund et al., 2015; Gaze et al., 2011; Amos et al., 2014). Therefore, it is necessary to investigate the fate of ARGs in sludge during wastewater treatment.

Although WWTPs could greatly reduce chemical pollutants (Sabri et al., 2020), the efficiency of removing biological pollutants such as ARGs is uncertain but highly potentially enhanced after WWTPs treatment (Huang et al., 2014; Sui et al., 2017). To date, there are many available biological treatments to remove pollutants in WWTPs, such as Anaerobic-anoxic-oxic (A^2O), Oxidation Ditch (OD), and

Cyclic Activated Sludge System (CASS). However, research on the fate of ARGs in different biological treatments is still lacking (Tong et al., 2019). Moreover, the efficiency of removing ARGs usually varies in different seasons due to the influence of temperature on microbial growth in WWTPs (Schages et al., 2020). However, seasonal changes in ARGs in WWTPs were relatively less reported (Guo et al., 2014). In this sense, it is worthwhile to investigate the changes in the abundance of ARGs in WWTPs using different treatments and in different seasons.

To understand the seasonal variations in removing ARGs from WWTPs with different biological treatment processes, this study chose four WWTPs with different treatment procedures to investigate ARGs' fate in each treatment unit of WWTPs. The secondary treatment procedures of four WWTPs are A^2O and CASS. Tetracycline is a widely used antibiotic and is frequently determined in WWTPs (Auerbach et al., 2007; Munir et al., 2011); thereby, the tetracycline-resistance genes (*tet*-ARGs), including *tetA*, *tetC*, *tetM*, *tetO*, *tetQ*, *tetW*, and *tetX* were chosen as the representative ARGs in this study. Additionally, mobile genetic genes (MGEs) such as the class 1 integron (*intI1*) in sludge can readily transfer between bacteria and promote the spreading of ARGs. Thus, this study also inspected *intI1* as a marker for indicating ARGs' horizontal gene transfer (HGT). Besides, seasonal variations of *tet*-ARGs and *intI1* abundance in the sludge of different treatment units in the four WWTPs were investigated to further clarify the role of WWTPs in removing ARGs.

2 Materials and Methods

2.1 Sampling sites

Sludge samples were collected from four WWTPs located in Chenghua (defined as F), Qionglai (defined as Q), Pengzhou (defined as P), and Xinjin (defined as X) in Chengdu, China (Figure 1). The details of each WWTP are presented in Table 1. The Q plant mainly receives industrial and domestic wastewater, while the F, P, and X plants mainly receive domestic wastewater (Table 1). The secondary treatment process of the F and Q plants was A^2O, while the P and X plants adopted CASS as the secondary treatment process. The sampling points were located at the end of each treatment unit of WWTP. The F plant's anaerobic, anoxic, and oxic tank was recorded as F-An, F-Ax, and F-O. Similarly, the Q plant's anaerobic, anoxic, and oxic tank was recorded as Q-An, Q-Ax, and Q-O. In the P plant, the aeration and precipitation phases (approaching anaerobic conditions) were recorded as P-O and P-A, respectively. Similarly, the aeration and the precipitation phase of the X plant were recorded as X-O and X-A, respectively. Three subsamples were taken at different treatment stages and mixed into one sample. Eventually, ten samples were collected from four WWTPs. To explore the seasonal variations of *tet*-ARGs and *intI1*, samples were collected in summer (July 2017) and winter (January 2018). After sampling, the sludge was put on ice and transferred into the lab as soon as possible. After that, sludge was stored at 4°C and -20°C for physicochemical property and DNA extraction for ARGs determination.

2.2 Tetracycline determination

2.2.1 Sample pretreatment

The lyophilized sludge sample of 1.00 g was weighed and placed in a centrifuge tube, and 10 mL of extraction solution (acetonitrile:formic acid water = 7:3) was added. After swirling for 1 min, ultrasonic extraction was performed for 15 min, centrifugation was performed at 10000 r min^{-1} for 10 min, and the supernatant was collected. After repeated extraction 2 times, the collected supernatant was combined and diluted to 200 mL with water. 0.5 g EDTA-Na_2 was added into the diluent, and the pH value of the diluent was adjusted to 3.0 by hydrochloric acid. All diluents were put through Oasis HLB solid phase extraction column (6 mL /150 mg) and washed with 5 mL water after loading. Then 5 mL methanol solution was used for elution. The elution was passed through an NH2 solid phase extraction column (6 mL /500 mg, Waters), and 6 mL acetone-methanol-formic acid (500:500:1) solution and 6 mL acetone-formic acid (1000:1) solution were used for elution. The eluent was collected and blow-dried under weak nitrogen flow, dissolved in 1 mL 5% methanol solution, eddied for 1 min, and transferred to a 2 mL sample bottle for UPLC-MS/MS determination.

Table 1. Process information and operational parameters of the four WWTPs

Plant	Process	Capacity (m^3 d^{-1})	Service area (km^2)	Service population (thousand)	Sewage source
F	A^2O	150,000	10	801	domestic sewage
Q	A^2O	20,000	10	100	industrial park produces wastewater and domestic sewage
P	CASS	30,000	27	193	domestic sewage
X	CASS	20,000	22	120	domestic sewage

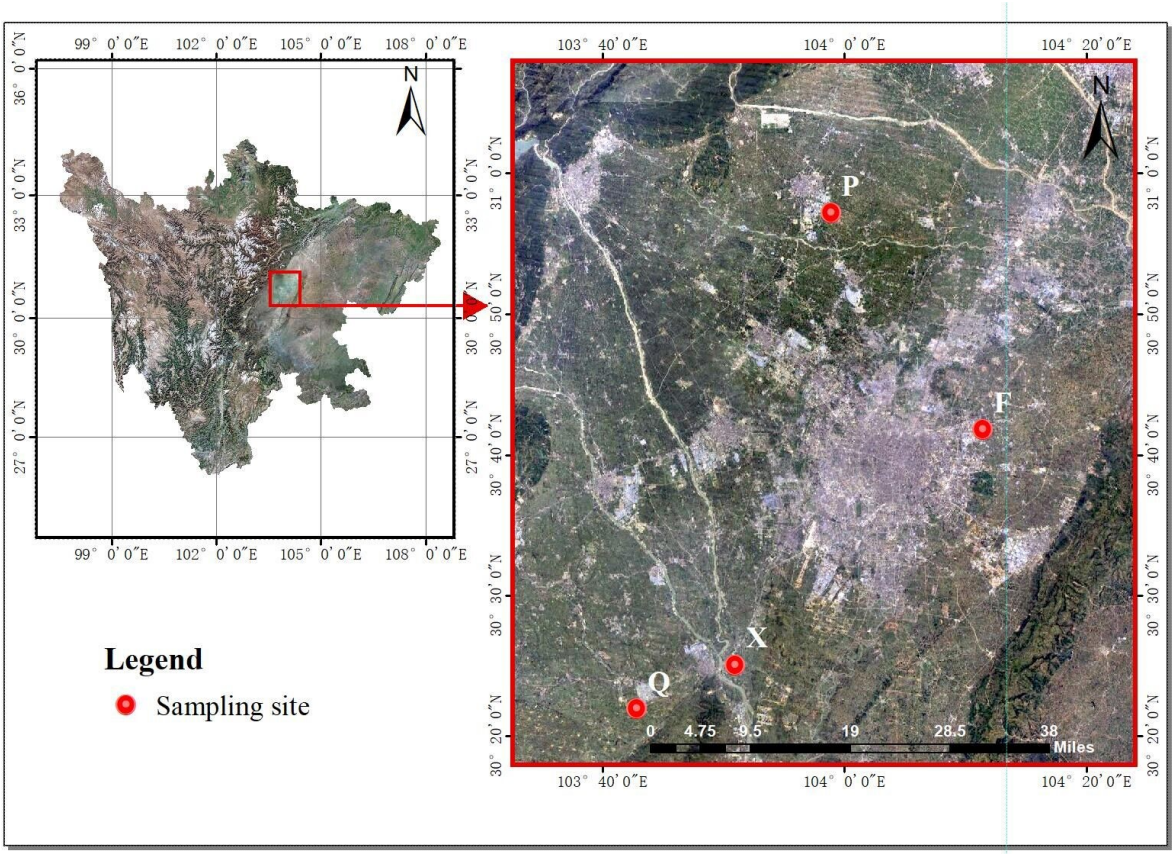

Figure 1. Sampling site of four wastewater treatment plants located in Chengdu, China

2.2.2 Tetracycline determination

The tetracycline content in the samples was determined by ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The column was Pro shell 120 EC-C_{18} (100 mm×4.6 mm×2.1 μm), mobile phase A: acetonitrile, mobile phase B: 2.5 mmol acetic acids. The ammonium and 0.1% formic acid aqueous solution were uniformly mixed. Gradient elution, flow rate 0.4 mL min^{-1}, injection volume: 20 μL, column temperature: 30°C. Mass spectrometry conditions were ion source, electrospray ion source; positive ion scan (ESI+), multiple reaction monitoring (MRM), electrospray voltage, 5500 V, atomizing gas pressure, 50 psi, air curtain gas pressure: 30 psi, auxiliary air 60 psi, ion source temperature, 500°C, collision gas, Medium. The monitoring ion parameters are ion pair m/z (415.0/410.0), de-clustered voltage DP, 120), collision gas energy CE, 27.

2.3 DNA extraction

Three samples were collected from each sampling site and were combined to form a representative sample for each sewage treatment plant (200 mL). The Power Soil ® DNA Isolation kit (MO BIO, USA) was applied to extract total DNA of the samples. Briefly, 0.1 g of freeze-dried sludge was weighed to extract DNA. The extraction was conducted according to the manufacturer's instructions. The concentration and quality of the extracted DNA were tested by Nano Drop-2000 spectrophotometer analysis (Thermo Scientific, Wilmington, DE, USA). DNA samples were stored at -20°C until further investigation.

2.4 Real-time quantitative PCR (qPCR)

The primers of target genes and the condition of PCR procedure are listed in Table S1. Plasmids carrying target genes were used to generate standard curves. As per the instructions of the manufacturer, the purified PCR products were ligated into the pMD19-T vector (TaKaRa) and then cloned into *Escherichia coli* DH5α (TaKaRa). Positive clones were screened by PCR to verify the cloning of the target genes and sequenced. The BLAST alignment tool (http://www.ncbi.nlm.nih.gov//) confirmed that all of the sequenced clones were matched with known genes. Plasmids carrying target genes were extracted according to a Plasmid Mini Kit (OMEGA). The plasmid carrying target genes was determined by NanoDrop 10-fold serial dilutions of a known copy number of the plasmid DNA, which were used to produce the standard curve. The Ct value of unknown samples was used to calculate the number of corresponding gene copies based on the standard curves. qPCR reaction was performed in a 10 μL volume mixture and conducted in 96 well plates containing 5 μL of SYBR Premix Ex Taq (OMEGA),

0.2 μL of ROX reference dye, 0.15 μL of each primer (10 μM), 2 μL of template DNA, and 2.5 μL of ddH_2O. The detailed protocol was as follows: 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at the annealing temperatures, 72°C extensions for 30 s, a fluorescence acquisition step at 72°C, and then a final melt curve stage with temperature ramping from 60 to 95°C. Each reaction was run in triplicate. R^2 values were more than 0.990 for all standard curves (Chen and Zhang 2013). The formula for calculating the copy number is as follows: Copies = ($6 \times 10^{23} \times$ (Plasmid concentration) $\times 10^{(-9)}$) / ((1943 + Target gene length) $\times$ 660).

2.5 Statistical Analysis

Copy numbers were log-transformed to normalize the distributions before statistical analysis. Data were analyzed by OriginPro 8.0 (OriginLab Corporation, Northampton, MA). Pearson correlation analysis was conducted to analyze the relationships related *tet*-ARGs to tetracycline and *intI1* with SPSS, version 19. $p < 0.05$ was considered statistically significant.

3 Results and Discussion

3.1 The abundance of *tet*-ARGs and *intI1* in WWTPs

As presented in Figure 2, it can be seen that the abundance of *tet*-ARGs/*intI1* varied in different seasons and treatment units of WWTPs. In the summer, the copies of *tetA*, *tetC*, *tetM*, *tetO*, *tetQ*, *tetW*, *tetX* and *intI1* in sludge of each sampling unit ranged from 4.47-6.91, 3.26-7.28, 4.46-5.82, 5.63-7.17, 3.93-6.53, 6.16-7.91, 4.73-6.81 and 6.03-7.31 logs g^{-1} dry sludge, respectively. In the winter, the abundances of *tetA*, *tetC*, *tetM*, *tetO*, *tetQ*, *tetW*, *tetX* and *intI1* in sludge of each sampling unit ranged from 4.31-6.56, 6.91-7.59, 4.15-6.17, 6.39-7.26, 4.39-6.22, 5.04-6.54, 5.93-7.10 and 6.83-7.64 logs g^{-1} dry sludge, respectively. In addition, the abundance of *tet*-ARGs/*intI1* in winter sludge (A_{winter}) and summer sludge (A_{summer}) was compared (ΔA = log A_{winter} - log A_{summer}, $\Delta A > 0$ indicating a higher abundance in winter, while $\Delta A < 0$ indicates a higher abundance in summer). The results showed that 57.5% of the samples had higher abundance of *tet*-ARGs/*intI1* in summer (Figure 3).

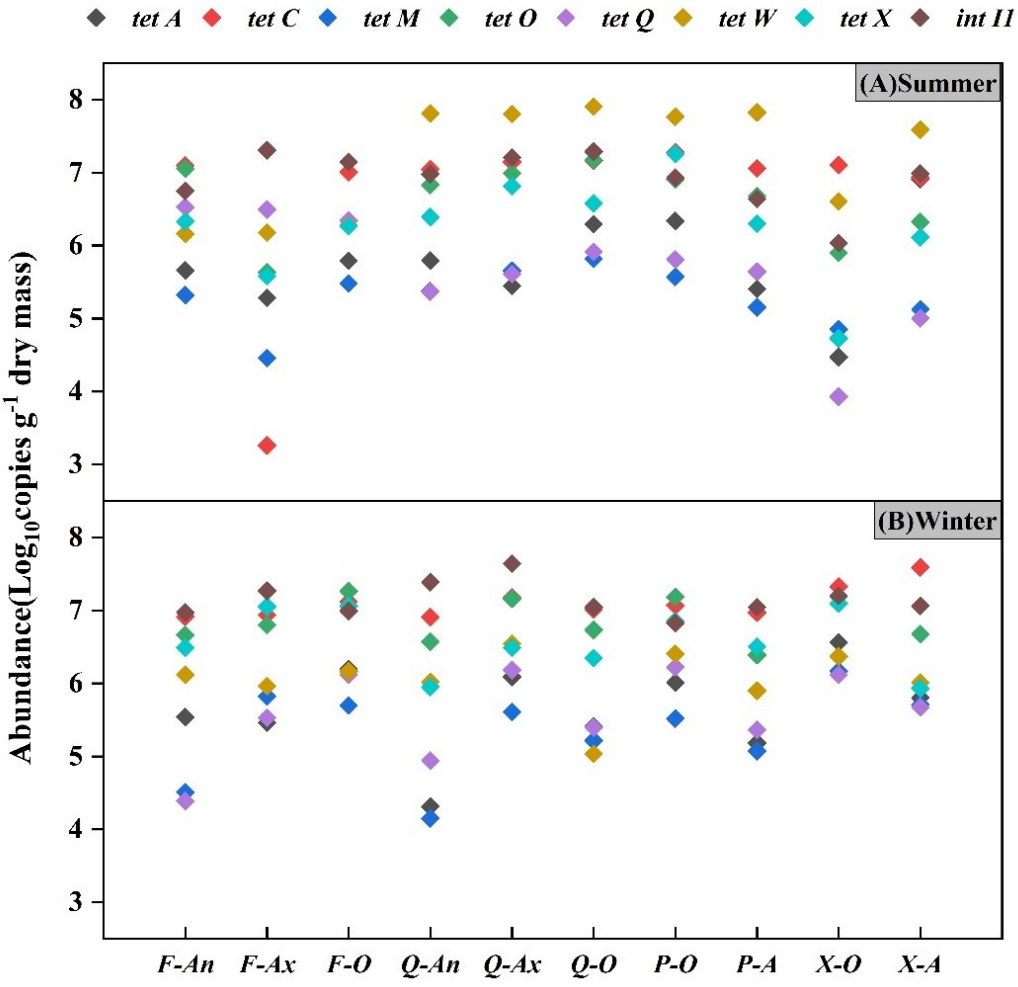

Figure 2. The abundance of *tet*-ARGs and *intI1* in different treatment units (A summer, B winter)

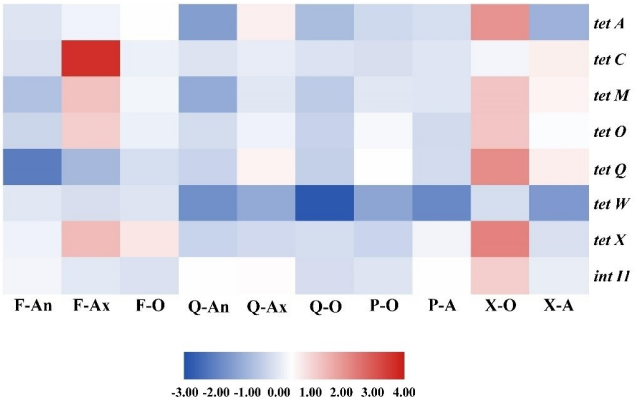

Figure 3. Seasonal variations heat map of *tet*-ARGs and *intI1* in different treatment units

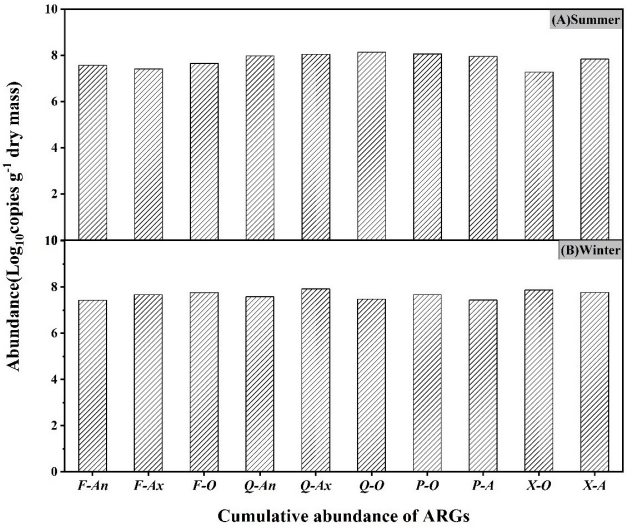

Figure 4. The accumulative abundance of *tet*-ARGs in sludge sampled from different treatment units (A summer, B winter)

In several previous studies, it was found that the abundance of ARGs of sludge in winter (January and February) was significantly higher than in summer (May, July, and August), while others reported higher ARGs level in summer (Mao et al., 2015; Schages et al., 2020). In this study, *tet*-ARGs and *intI1* showed no obvious changing trend between summer and winter. In general, it is difficult to explain the seasonal variations of ARGs because ARGs distribution in WWTPs can be affected by oxygen conditions, bacterial communities, interactions between antibiotics and ARGs, and so on (Chen and Zhang, 2013; Yang et al., 2014; Schages et al., 2020). More importantly, various ARGs can respond differently to these influential factors (Börjesson et al., 2010; Sui et al., 2017; Zhang et al., 2015). For example, the efflux pump genes *tetA* and *tetC* have been shown to increase with the concentration of tetracycline in the reactor (Zhang et al., 2016); the enzyme-modified gene *tetX* has been reported to decrease with the temperature increases in the reactor (Ghosh et al., 2009). Meanwhile, seasonal changes will cause changes in these influential factors and eventually lead to different seasonal changes in different ARGs. Notably, the similar accumulated ARGs in each unit between two seasons suggested that bacteria might adopt different resistance mechanisms.

To further study the causes of the variation in tested genes abundances, this study compared the accumulative abundance of *tet*-ARGs in sludge sampled from different treatment units (Figure 4). The accumulative abundance of *tet*-ARGs in F-Ax, F-An, and F-O was 7.41 logs, 7.57 logs, and 7.65 logs in summer, while 7.67 logs, 7.43 logs, and 7.76 logs in the winter (Figure 4). In the Q plant, the accumulative abundance of *tet*-ARGs in Q-O (8.14 logs) was more abundant than Q-Ax (8.05 logs) and Q-An (7.98 logs) in the summer, while Q-An and Q-Ax (7.58 and 7.92 logs) demonstrated higher accumulative *tet*-ARGs than Q-O (7.47 logs) in the winter (Figure 4). In the P plant, the accumulative abundance of *tet*-ARGs was higher in P-O than in P-A in both two seasons (Figure 4). In the X plant, the accumulative abundance of *tet*-ARGs in the sludge of X-A (7.84 logs) was higher than X-O (7.72 logs) in the summer, while *tet*-ARGs in X-O (7.87 logs) had a higher accumulative abundance than X-A (7.77 logs) in the winter (Figure 4). These results showed that the oxic treatment generally had higher *tet*-ARGs than anoxic/anaerobic treatment units besides the X plant.

ARG differences in sludges among oxic, anaerobic and anoxic treatment units might be closely related to microbial growth since the abundance of ARGs is generally correlated to the concentration of host bacteria (Lapara et al., 2011). The slow growth of the microbial community in anaerobic and anoxic treatment units would limit the increase of ARGs (Wang et al., 2015). For instance, the oxic treatment was speculated to probably provide suitable conditions for host bacteria to increase, leading to a higher abundance of ARGs. It was also reported that the anaerobic treatments showed significant efficiency in removing several *tet*-ARGs and *intI1* compared to oxic treatments (Diehl and Lapara, 2001). Previous studies revealed that *tet*-ARGs in *E. coli* lost faster under anaerobic conditions than under oxic conditions (Rysz et al., 2013). Thus, the proliferation and transfer of ARGs in sludge would be better inhibited by using an anaerobic process or placing an anaerobic stage at the end of biological treatment.

To further explore the fate of ARGs in sludge, the changes of *tet*-ARGs (ΔA) between anaerobic ($A_{initial}$, as the starting state) and oxic (A_{final}, as the end state) sludge were investigated ($\Delta A = \log A_{initial} \ \log A_{final}$, $\Delta A > 0$ indicates a decrease of *tet*-ARGs, while $\Delta A < 0$ indicates an increase in ARGs). The results are shown in Figure 5. In the summer, *tet*-ARGs in P plant showed a decreasing trend (except *tetW*), while the X plant showed the highest increase of *tetA* and *tetX*, with 2.43 logs and 1.38 logs increase, respectively (Figure 5). In the winter, the abundance of *tet*-ARGs in sludge generally increased along with the treatment processes in the F and Q plant. In contrast, the P and X plants showed a reduction of *tet*-ARGs, with 0.09-0.86 logs and

0.09-1.16 logs, respectively (Figure 5).

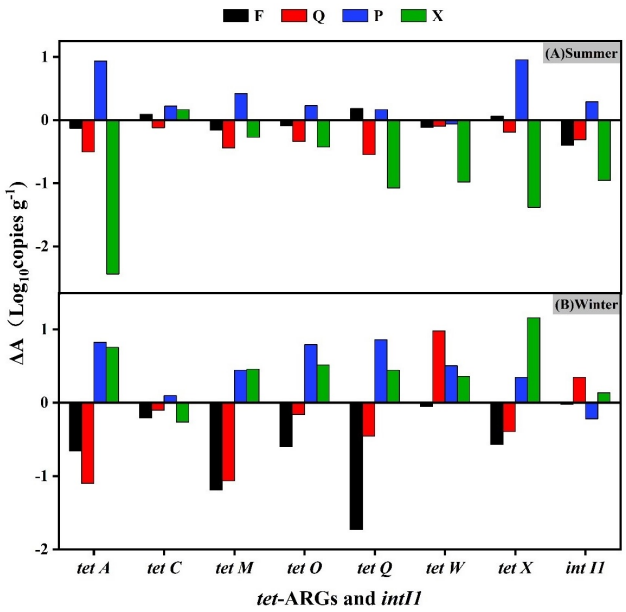

Figure 5. The removed amounts of *tet*-ARGs and *intI1* in biological treatment units of wastewater treatment plants (A summer, B winter)

According to previous research, the proliferation of the total number of bacteria in the oxic phase would increase ARGs concentration in sludge (Mao et al., 2015; Zhai et al., 2016; An et al., 2018). Therefore, the positions of the oxic phase in the treatment process of WWTPs would result in a different abundance of *tet*-ARGs in sludge. This study also supported that the CASS process could inhibit ARGs better than the A^2O process because the anaerobic phase in the CASS process follows the oxic phase (Figure 5). However, it is noteworthy that the inhibitory effect of the CASS process may be affected by seasonal change. For instance, the X plant did not reduce ARGs in winter as expected in summer and even increased. When comparing the fate of ARGs by the X plant between summer and winter, it was found that *intI1* showed a significant increase during the aeration phase in summer. The results indicated that the amount of MGEs might influence the inhibitory effect from plant X in summer, which agreed with the observation in previous studies (Tong et al., 2019). In conclusion, the inhibitory effect of the CASS process on the proliferation of ARGs in sludge was better than that of the A^2O process, but its inhibitory effect on the HGT of ARGs was not stable.

3.2 Relative proportions of each gene

The relative proportions of each gene to total tested *tet*-ARGs are presented in Figure 6 to understand the dominant resistance mechanism for microorganisms against tetracycline. In this study, seven genes were coded for different mechanisms of tetracycline resistance, including two efflux pump genes (*tetA* and *tetC*), four ribosome protective protein genes (*tetM*, *tetO*, *tetQ* and *tetW*), and one enzymatic modification gene (*tetX*) (Chopra and Roberts, 2001; Zhang et al., 2009). In Figure 6, the relative proportions of each gene in different wastewater treatment units varied widely, and ARGs in the same treatment units also showed seasonal variations. In the summer, the main ARGs of the Q plant were *tetC* and *tetO*, while *tetW* and *tetC* accounted for a large proportion of ARGs in the Q, P, and X plants. In particular, the relative proportion of *intI1* in F-Ax was up to 78% but not more than 40% in other sampling units (Figure 6). In the winter, the main ARGs in each treatment unit were *tetC*, *tetO*, and *tetX*, and the relative proportion was over 72%. In addition, the relative proportion of *intI1* in winter was generally higher than in summer (Figure 6).

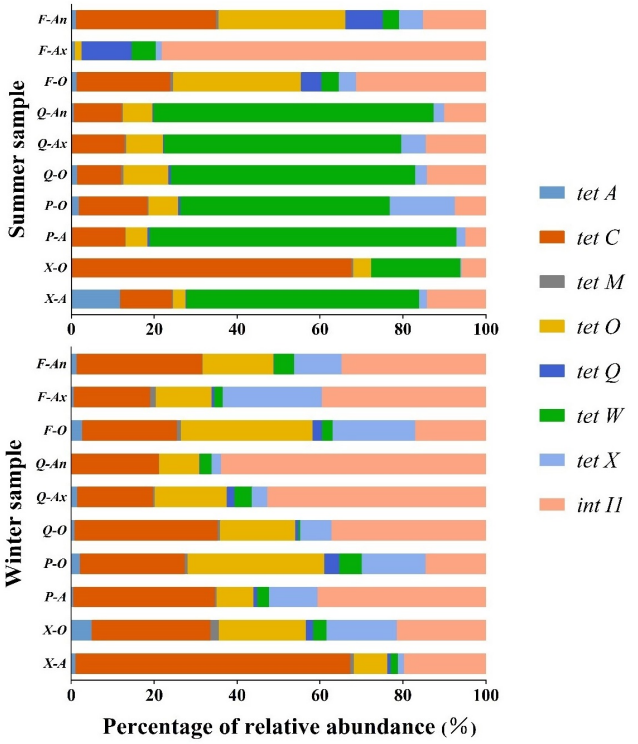

Figure 6. Relative ratio of *tet*-ARGs and *intI1* to the accumulative amount of *tet*-ARGs in different treatment units

According to the above results, the main ARGs in the tested sludge were *tetW*, *tetO*, and *tetC*, similar to previous research (Zhang et al., 2011; Chen and Zhang, 2013; Xu et al., 2015). However, the relative proportions of each ARG showed certain variations in different seasons. For instance, *tetC*, *tetO*, and *tetX* dominated in the winter, while *tetW*, *tetO*, and *tetC* dominated in the summer. The variation may cause by the seasonal changes in bacterial community structure. It was reported that the bacterial community structure at a genera level in sludge shifted significantly with seasonal change (Zhang et al., 2018). The amplification or attenuation of bacteria carrying different ARGs might change the distribution of ARGs in sludge. Previous studies have shown that efflux pump *tet* genes were generally most abundant among

all *tet* genes in biological systems (Zhang et al., 2019). Similarly, in the present study, the higher proportion of *tetC* in the winter demonstrated that the efflux pump was the primary tetracycline resistance mechanism. However, *tetW* and *tetO* accounted for a relatively more significant proportion in summer, implying that ribosome protective protein was the primary mechanism. On the one hand, it is probably attributed to the bacterial community change caused by different wastewater sources and different treatment processes. The different dominant bacteria in sludge might lead to instability of mainly tetracycline resistance mechanisms. Unfortunately, this study only determined part of the *tet* genes, and other not tested genes may have some influence.

intI1 was considered an important role in HGT (Bai et al., 2019). It should be noteworthy that the relative ratio of *intI1* was increased as the seasonal changes (summer to winter), speculating that HGT is more likely to occur in sludge in winter. Meanwhile, the relative ratio of *intI1* in anaerobic and anoxic treatment units was higher than oxic treatment in both summer and winter in these four WWTPs, which means the anaerobic and anoxic phases may provide suitable conditions for HGT of ARGs.

3.3 Correlations among tetracycline, *tet*-ARGs, and *intI1*

Table 2. Tetracycline concentration in sludge from different treatment units of wastewater treatment plants (ng mL^{-1})

	F-An	F-Ax	F-O	Q-An	Q-Ax	Q-O	P-O	P-A	X-O	X-A
Summer	2.1	4.8	2.5	3.3	2.1	5.1	2.5	3.3	9.8	2.4
Winter	10	4.3	6.9	82.3	4.4	17.1	95.5	60.4	29	634

Table 2 shows that the tetracycline concentration in each treatment stage of WWTPs in the winter was higher than in the summer. Previous studies have shown a similar phenomenon caused by the extensive usage of antibiotics in the winter (Schages et al., 2020). It was found that sludge adsorption was the primary removal mechanism of tetracycline in WWTPs (Gao et al., 2012; Zhang et al., 2013). A large amount of tetracycline was enriched in sludge by adsorption, affecting the abundance of *tet*-ARGs and *intI1*. Thus, it is necessary to explore the relationship between tetracycline and tested genes in sludge.

Table 3 demonstrates the correlations among tetracycline, *tet*-ARGs, and *intI1*. Previous studies have found a significant correlation between tetracycline and *tet*-ARGs, whereas some reported the opposite conclusion (Gao et al., 2012; Ji et al., 2012; Mao et al., 2015). As shown in Table 3, no significant correlation ($p > 0.05$) could be found between the tested *tet*-ARGs and tetracycline in this research. One reason is that only a part of representative *tet*-ARGs was analyzed in this study, and other *tet*-ARGs not considered in treatment units may influence the results (Gao et al., 2012). Another possible reason is that bacteria carrying *tet*-ARGs stayed longer in the sludge than in wastewater; thus, the change of antibiotic concentration in the sludge had no immediate effect on the abundance of *tet*-ARGs (McKinney et al., 2010). Moreover, another class of antibiotics or heavy metals may also affect the correlation by co-selection and cross-selection (Ji et al., 2012). In general, additional studies are needed to explore other factors' effects to understand better and characterize the correlation between antibiotics and ARGs.

Table 3 also summarizes the correlations among the tested *tet*-ARGs to explore the interactive effects of different *tet*-ARGs. There are four mechanisms for bacteria to withstand tetracycline pressure, including efflux pumps, ribosomal protection proteins, enzymatic inactivation of tetracycline, and antibiotic inactivation. Thus, it is speculated that the significant correlations among *tetM*, *tetO*, and *tetQ* are caused by the same resistance mechanism. However, *tetW* showed no significant correlation with *tetM*, *tetO*, and *tetQ* in this study, although the resistance mechanism was similar. The same situation was observed between *tetA* and *tetC*, probably because these *tet* genes were located on different MGEs such as transposons and plasmids or had different hosts. On the contrary, although some *tet* genes have different resistance mechanisms, they may be located on the same MGEs or have a co-host; therefore, there is a significant positive correlation between them (Bai et al., 2019; Zhang et al., 2019). For

Table 3. Pearson correlations among tetracycline, *tet*-ARGs, and *intI1* in wastewater treatment plants

	Tetracycline	*tet A*	*tet C*	*tet M*	*tet O*	*tet Q*	*tet W*	*tet X*	*int I1*
Tetracycline	-	0.943	0.405	0.587	0.855	0.993	0.315	0.447	0.909
tetA	-0.02	-	0.368	0.002	0.021	0.047	0.126	0.008	0.294
tetC	0.20	0.21	-	0.029	0.003	0.321	0.549	0.142	0.430
tetM	0.13	**0.66**	**0.49**	-	0.001	0.031	0.363	0.004	0.508
tetO	-0.04	**0.51**	**0.64**	**0.7**	-	0.036	0.615	0.000	0.193
tetQ	0.00	**0.45**	-0.23	**0.48**	**0.47**	-	0.919	0.045	0.045
tetW	-0.24	0.35	0.14	0.21	0.12	-0.02	-	0.574	0.644
tetX	-0.18	**0.58**	0.34	**0.62**	**0.76**	**0.45**	0.13	-	0.062
intI1	0.03	0.25	-0.19	0.16	0.30	**0.45**	-0.11	0.43	-

Note: Bold fonts indicate a significant correlation at $p < 0.05$. Pearson's coefficient R is in the lower-left corner, and p-value is in the upper right corner.

instance, it is reported that *tetQ* and *tetX* existed on the same conjugative transposon, resulting in a positive correlation between them (Zhang et al., 2013). The generation and propagation of ARGs in the environment are mainly through HGT, and *intI1* was one of the most common types of MGEs; thus, it is considered an important marker of HGT. In this present study, all tested *tet* genes except *tetQ* showed no significant correlation with *intI1*. It was speculated that the vertical transfer of genes between parents might be the primary way of *tet*-ARGs proliferation. The significant positive correlation between *tetQ* and *intI1* indicated that *tetQ* was likely amplified through HGT in this study (Auerbach et al., 2007).

4 Conclusion

There were seasonal differences in the abundance of *tet*-ARGs/*intI1* in the sludge of four WWTPs with different biological treatments, although no obvious seasonal trend was observed. The abundance of target ARGs in anaerobic tanks was generally lower than that in oxic tanks, suggesting that oxygen might be an important factor affecting the abundance of ARGs in WWTPs. By comparison, WWTPs adopted the CASS demonstrated the possibility to inhibit *tet*-ARGs in sludge relative to the A^2O treatment. Furthermore, this study found that none of the tested *tet*-ARGs significantly correlated with tetracycline, but some significant correlations among *tet*-ARGs were detected. Additionally, only *tetQ* was significantly correlated with *intI1*, indicating that the vertical transfer of genes between parents might be the ARG way for the tested ARGs. Since ARGs in the sludge may incur ecological and health risks, further study is required to investigate the approach of effectively removing ARGs from sludge.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by the author involved.

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