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Distribution of Antibiotic Resistance Genes and Their Association with Microbes in Wastewater Treatment Plants: A Metagenomics Analysis

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Abstract: The extensive use of antibiotics has resulted in the generation and accumulation of antibiotic resistance genes (ARGs) in the environment, and domestic wastewater is one of the main reservoirs of ARGs and resistant bacteria. In this study, Illumina high-throughput sequencing and network analysis were used to study the microbial community characteristics, ARGs' occurrence status, and resistance mechanism in the influent and effluent of a domestic sewage treatment plant in Changchun, China. A total of 29 phyla were found in the influent and effluent, including 23 bacterial phyla, 3 archaeal phyla, and 3 eukaryotic phyla. In total, 112 ARG subtypes were detected in the samples, and the dominant ARG subtypes were *Erm(35)* and *tet(W/N/W)*. In this study, ARGs related to tetracycline and macrolide accounted for a high proportion, and the resistance mechanisms of ARGs detected in the samples were mainly antibiotic inactivation and antibiotic efflux pumps. Co-occurrence maps of ARGs and microbes demonstrated by network analysis indicated that the resistance genes *kdpE*, *GES-5*, and *tetX* may easily bind to microbes, potentially making them antibiotic-resistant bacteria. Fifty-seven bacteria in the genera *Cupriavidus*, *Escherichia*, and *Collinsell* are potential hosts of multiple ARGs. The findings can increase our understanding of the distribution of ARGs and their association with microbes in wastewater treatment plants, and also provide a research foundation for controlling the diffusion of ARGs in the environment.

Keywords: biological wastewater treatment; metagenomics; ARGs; microbial community; co-occurrence network analysis

Citation: Shen, M.; Hu, X.; Li, M.; Lyu, C.; Hu, Y.; Bu, X.; Chen, T.; Cai, H.; Li, C.; Liu, J.; et al. Distribution of Antibiotic Resistance Genes and Their Association with Microbes in Wastewater Treatment Plants: A Metagenomics Analysis. *Water* **2023**, *15*, 1587. https://doi.org/10.3390/w15081587

Academic Editors: Weihua Zhao, Fangxu Jia, Xiaoxia Wang and Liangliang Shi

Received: 2 March 2023
Revised: 13 April 2023
Accepted: 15 April 2023
Published: 19 April 2023

1. Introduction

Antibiotics are widely used to treat and control bacterial infections and have revolutionized medicine and saved countless lives. However, the abuse of antibiotics in agriculture, animal husbandry, and medical care has led to the presence of antibiotic residues in the environment, humans, animals, and plants [1]. Antibiotics released into the environment can cause microorganisms to develop antibiotic resistance genes (ARGs). ARGs may replicate and spread with microorganisms in the environment through horizontal gene transfer (HGT) and vertical gene transfer (VGT), which causes even more microorganisms to develop resistance. Clearly, this poses a serious threat to human health and ecological security, causing ARGs to be recognized as emerging environmental pollutants [1–4].

Environmental sample monitoring results have shown that antibiotics and ARGs are present in various environmental media, such as natural water [5], sediment, soil [6], and domestic sewage. Wastewater treatment plants (WWTPs) are the main places to collect and treat municipal domestic sewage. The biological treatment process can remove most pollutants, such as N, P, and organic pollutants, in wastewater [7]. However, the growth and

reproduction of microorganisms during biological treatment provide convenient conditions for the spread of ARGs, resulting in the accumulation of a large number of antibiotics and ARGs in WWTPs [8,9]. Therefore, municipal wastewater treatment plants are important repositories of ARGs and sites for ARG propagation and diffusion [10,11]. The concentration, composition, and distribution characteristics of antibiotics and ARGs in WWTPs have become popular research topics. From an epidemiological perspective, the monitoring and analyzing of ARGs in WWTPs also are more beneficial to overcoming traditional surveillance limitations due to sample data only from hospitals [12]. Lee et al. [13] quantitatively detected the ARG concentration in the UV-disinfection effluent of two sewage treatment plants and found that up to 5.4×10^{16}–4.2×10^{18} copies/L of ARGs were discharged into the environment every day. Rodríguez et al. [14] used the shotgun metagenome and culture-based approaches to determine bacterial communities, the resistome and mobilome in a WWTP in Colombia, the results showed that the genes encoding resistance to macrolide-lincosamide-streptogramin, β-lactams, and those conferring multidrug resistance were the most abundant in the samples. In addition, tetracycline, sulfonamide, and quinolone ARGs are also highly detected in the WWTPs [1,15]. The research results above show that there are abundant and diverse ARGs with high concentrations in sewage treatment plants. In general, ARGs decrease significantly after sewage treatment. However, residual ARGs in sewage may still cause ecological risks. A study on the characteristics and risk assessment of ARGs in WWTPs in Hong Kong found that the ARGs' removal efficiency in WWTPs could reach a 2.34–2.43 log reduction rate. The investigated WWTPs had relatively high efficiency in eliminating ARGs compared with the global average. Despite this, ARG variants with high risk were still highly abundant in effluents, which deserves our attention [16]. Therefore, it is essential to study the distribution characteristics of ARGs during sewage treatment to analyze the horizontal transfer of ARGs and perform an accurate ecological risk assessment.

The species of ARGs have a strong co-occurrence relationship with the microbial community structure in the environment, in addition to extensive diffusion by microbial reproduction during pollutant removal. Zhao et al. [17] studied the correlation between bacteria and virus groups and ARGs subtypes, and found that in addition to bacteria, viruses, especially phages, also contributed to the spread of ARGs. They also found that *rpoB*, *drfE*, *gyrA*, and *parC* were significantly correlated with bacteria and phages. According to correlation analysis of microorganisms and ARGs in the activated sludge of sewage treatment plants, some environmental bacteria (such as *Clostridium* and *Nitrosomonas*) may be potential hosts for many ARGs [11]. Similarly, *Aeromonadaceae* and *Enterobacteriaceae* have also been found to have a significant association with some ARGs as *blaOXA-58* and *blaKPC* genes [14]. However, there is less research on the correlation analysis of the ARGs' subtype and microbes in terms of the resistance mechanism. Based on the resistance mechanism, the ARG subtype and microbial correlation can be analyzed to determine which resistance mechanism is common in each ARG subtype. This can be combined with the microbes to provide theoretical guidance for solving ARGs and antibiotic resistance bacteria (ARB) problems in different environments.

Previous studies have shown that PCR and metagenomics are effective tools for studying the abundance of ARGs [18]. However, PCR relies on available primers of known ARGs and is not suitable for the discovery of unknown ARGs in samples [19]. Metagenomics sequencing can overcome the shortcomings of PCR-based techniques [19,20] and provide comprehensive information about the diversity and abundance of ARGs. Although there have been some studies on ARGs in WWTPs using metagenomic sequencing, due to the differences in characteristics, composition, sources, and sewage treatment process in different regions, more extensive research is needed to obtain more comprehensive data. The wastewater plant in this study is in Changchun, China, which is located in the severe cold region of China with a permanent population of more than 9 million. Changchun is an important industrial base in northeast China, and there are many pharmaceutical enterprises. Therefore, it is necessary to explore the occurrence characteristics of ARGs

in wastewater in this region. In this study, the influent and effluent water samples of a domestic sewage treatment plant in Changchun, China, were collected, and the microbial community composition and ARG characteristics of the water samples were studied by Illumina NovaSeq and macrogenetic sequencing. The distribution characteristics of microbial communities and ARGs in water samples were analyzed, and the correlation between ARG subtypes and microbial was analyzed based on the resistance mechanism. The results provided a basis for further research on the environmental behavior of ARGs in WWTPs.

2. Materials and Methods

2.1. Sampling and DNA Extraction

The sewage plant in this study is located in Changchun, China. It is a conventional third-level treatment sewage plant, with an improved A^2/O + ozonation disinfection as the primary process. The designed daily treatment capacity was 100,000 m^3/d, and the primary sewage source was urban domestic sewage. Wastewater samples were collected at the influent and effluent of the sewage plant. The water samples were filtered by a 0.45 μm filter membrane, which was placed in a sterile centrifuge tube for measurements.

Genomic DNA extraction and metagenomics sequencing were completed by Shanghai Personalbio Technology Co., Ltd. DNA samples were extracted using an OMEGA Soil DNA Kit (D5625-01). A Qubit™ 4 Fluorimeter (Qubit™ 4 Fluorimeter with Wi-Fi: Q33238; Qubit™ assay tubes: Q32856; Qubit™ 1X dsDNA HS Assay Kit: Q33231) was used to assay the DNA concentration, and mass was determined by 1% agarose gel electrophoresis. The level of DNA concentration was about 100 ng/μL.

2.2. Metagenomics Sequencing and Data Analysis

Illumina NovaSeq whole genome shotgun DNA library construction and sequencing were carried out as follows. After DNA fragmentation, inserted fragment libraries of approximately 400 base pairs were constructed, one library per sample. These libraries were double-ended sequenced (2 × 150 bp). Quality control was performed on the obtained original sequence data, and sequence lengths < 50 bp and sequences containing fuzzy bases were removed. Kranken2 [21] was used for species annotation to obtain the species abundance table of each sample, and then the microbial diversity was analyzed. The gene sequences were annotated in the comprehensive antibiotic resistance database (CARD) database to obtain the abundance of ARGs in each sample. The ARGs whose ratio of protein length predicted by the sample gene sequence to that of the CARD database was 100 were screened. TPM (transcripts per kilobase per million mapped reads) was used as the standard to measure the gene abundance of each sample (Equations (1) and (2)). To visualize the correlations between major bacteria, archaea, and viruses and ARGs, Pearson correlations between microbial genera and ARGs with the top 60 relative abundances were calculated to construct a correlation matrix:

$$TPM = \frac{rg \times rl \times 10^6}{flg \times T} \quad (1)$$

$$T = \sum \frac{rg \times rl}{flg} \quad (2)$$

where *rg* is the number of reads aligned to the gene sequence, *rl* is the length of the read, and *f*lg is the length of the gene.

3. Results and Discussion

3.1. Diversity and Richness of Microbial Communities

After Illumina high-throughput sequencing was used to assemble and filter the data, the microbial community diversity of different samples was analyzed and compared. As shown in Figure 1, in both the influent and effluent samples, the sparse curves were close to the saturation trend, indicating that the sequencing depth in this study was sufficient for

classification coverage (Figure 1A). At the species level, the alpha diversity of microbes in the influent and effluent was analyzed, including the ACE richness indices and Shannon diversity indices. The ACE showed that microbial species richness was lower in the effluent than influent (Figure 1B). Shannon analysis showed that the diversity of microbial species in the effluent was lower than that in the influent (Figure 1C).

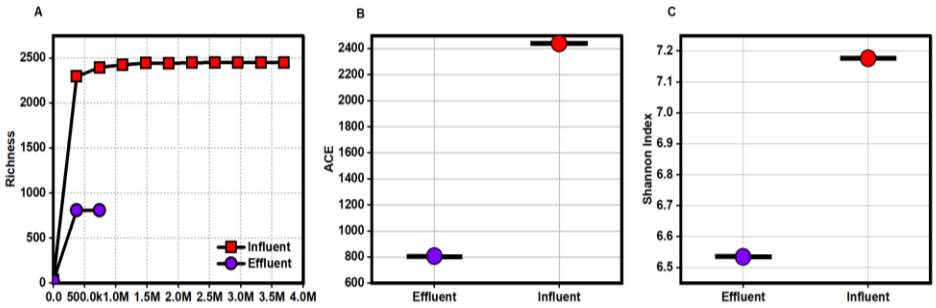

Figure 1. Analysis of metagenomics sequence diversity of samples, visualized by Origin 2022. (**A**) Sparsity curve analysis of influent and effluent samples; (**B**) ACE analysis of influent and effluent samples; and (**C**) Shannon analysis of influent and effluent samples.

To better understand the characteristics of microbial flora structures in the influent and effluent, Kranken2 was used to annotate the effective sequences of each sample from the phylum to species level (Table 1). Bacteria were the dominant microbes in both the influent and effluent, accounting for 99.12–99.61% in the influent and 97.45–99.36% in the effluent. Yasir et al. [22] studied the microbial community in a biological sewage treatment plant in Jeddah and also found that bacteria were the main microbial type in the influent and effluent (accounting for 98.9% of influent and 98.1% of effluent). Giwa et al. [23] analyzed the microbial community structure in the influent, activated, return sludge and effluent of the sewage plant, and found that bacteria were also dominant microbes in the influent. The analysis on the microbial community structure of the whole wastewater plant found that except bacteria, eukaryota had a high abundance, followed by archaea and viruses. In this study, except bacteria, archaea had a high abundance, followed by viruses and eukaryota, which was slightly different from the results of Giwa et al.

Table 1. Distribution of taxonomic units in influent and effluent samples [a] (%).

		Bacteria	Archaea	Virus	Eukaryota	Unassigned
Influent	Phylum	99.1243	0.00867	-	0.0030	0.7860
	Class	99.3440	0.00867	-	0.0023	0.5670
	Order	99.4580	0.0867	0.0103	0.0027	0.4423
	Family	99.4773	0.0857	0.0020	0.0073	0.4277
	Genus	99.6193	0.0870	0.0023	0.0020	0.2893
	Species	99.5957	0.0837	0.0043	0.0020	0.3143
Effluent	Phylum	97.4540	0.7853	-	0.1297	1.6310
	Class	97.8333	0.7803	-	0.1293	1.2570
	Order	98.0737	0.7827	0.0063	0.1327	1.0047
	Family	99.1380	0.7837	0.0050	0.1320	0.9413
	Genus	99.3617	0.7877	0.0000	0.1343	0.7163
	Species	98.5607	0.7710	0.0003	0.1897	0.4783

Note(s): [a]: The abundance in percentage was based on the taxonomic units using the identified DNA gene tags.

A total of 23 bacterial phyla, 3 archaeal phyla, 3 eukaryotic phyla, and 1 viral order were found in influent and effluent samples. At the species level, 2723 bacterial species were found in the influent samples, while only 971 bacterial species were found in the effluent samples. In the influent samples, 1 viral order, 4 viral families, 25 viral genera, and 15 viral species were identified. In the effluent samples, the number of taxonomic

units for viruses was significantly reduced. The number of taxonomic units for archaea and eukaryota showed little difference between influent and effluent samples (Table 2).

Table 2. Number of taxonomic units in influent and effluent samples.

		Bacteria	Archaea	Virus	Eukaryota
Influent	Phylum	23	3	-	3
	Class	40	4	-	5
	Order	94	6	1	7
	Family	195	9	4	11
	Genus	680	14	25	12
	Species	2723	26	15	9
Effluent	Phylum	19	3	-	3
	Class	38	4	-	9
	Order	88	6	1	11
	Family	174	8	3	14
	Genus	410	12	NA	11
	Species	971	17	1	10

Note(s): NA: Not annotated.

Bacteria are important vectors of ARGs, and ARGs may spread, transfer, and mutate through the reproduction of bacterial hosts [24]. At phylum level, *Proteobacteria* were the most abundant bacteria in this study, followed by *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Figure 2A). *Proteobacteria* accounted for 36.78% in the influent but increased to 52.85% in the effluent, in which it was dominant. Compared with the influent, the relative abundance of *Firmicutes* and *Bacteroidetes* decreased from 24.29% to 12.69% and from 34.34% to 17.24%, respectively. *Proteobacteria*, *Bacteroidetes*, and *Actinomyces* are considered to be common hosts of ARGs in the activated sludge of sewage treatment plants [25]. At the family level (Figure 2B), *Bacteroidaceae* (19.15%), *Campylobacteraceae* (14.91%), and *Prevotellaceae* (11.33%) were the dominant families in the influent, followed by *Lachnospiraceae* (9.04%) and *Ruminococcaceae* (7.23%). In the effluent samples, the dominant bacteria were *Enterobacteriaceae* (18.81%), *Bacteroidaceae* (11.27%), and *Nitrospiraceae* (9.61%). A previous study on *Enterobacteriaceae* in municipal sewage found that this type of bacteria could carry a variety of ARGs [26]. At the genus level, the top 6 genera were *Bacteroides* (accounting for 19.49% in the influent and 11.31% in the effluent), *Prevotella* (accounting for 11.23% in the influent and 3.53% in the effluent), *Arcobacter* (accounting for 11.22% in the influent and 3.99% in the effluent), *Acinetobacter* (accounting for 6.30% in the influent and 4.57% in the effluent), *Aliiarcobacter* (accounting for 3.63% in the influent and 1.34% in the effluent), and *Aeromonas* (accounting for 3.58% in the influent and 3.15% in the effluent) (Figure 2C).

Previous studies have shown that *Bacteroides*, *Acinetobacter*, *Aeromonas*, and *Pseudomonas* are all potential pathogens that may carry different types of multi-resistant ARGs [1,27]. This study showed that the influent of the WWTPs contained some potential host bacteria of ARGs. After treatment, the abundance of host bacteria such as *Bacteroides* in the sewage was reduced, thus reducing the risk of the spread of drug resistance. It is worth noting that ozonation was used in the wastewater plants sampled in this study, but in the effluent, some potential host bacteria of ARGs were still present. This may be due to the fact that ozonation disinfection often does not effectively inactivate bacteria in wastewater, and that cells in agglomerates, sludge flocs or particles of suspension may be shielded and protected against the action of a disinfectant [28]. Therefore, how to choose a more effective disinfection process may be the focus of future research on ARG removal. Previous studies have shown that incomplete mineralization by-products may be formed during ozonation of treated wastewater that is toxic to aquatic organisms. The toxicity class of treated wastewater may change from the completely non-toxic to very high hazard category [29]. Therefore, when selecting the disinfection process, in addition to considering the inactivation effect, the ecological safety of the disinfection method also needs our close attention.

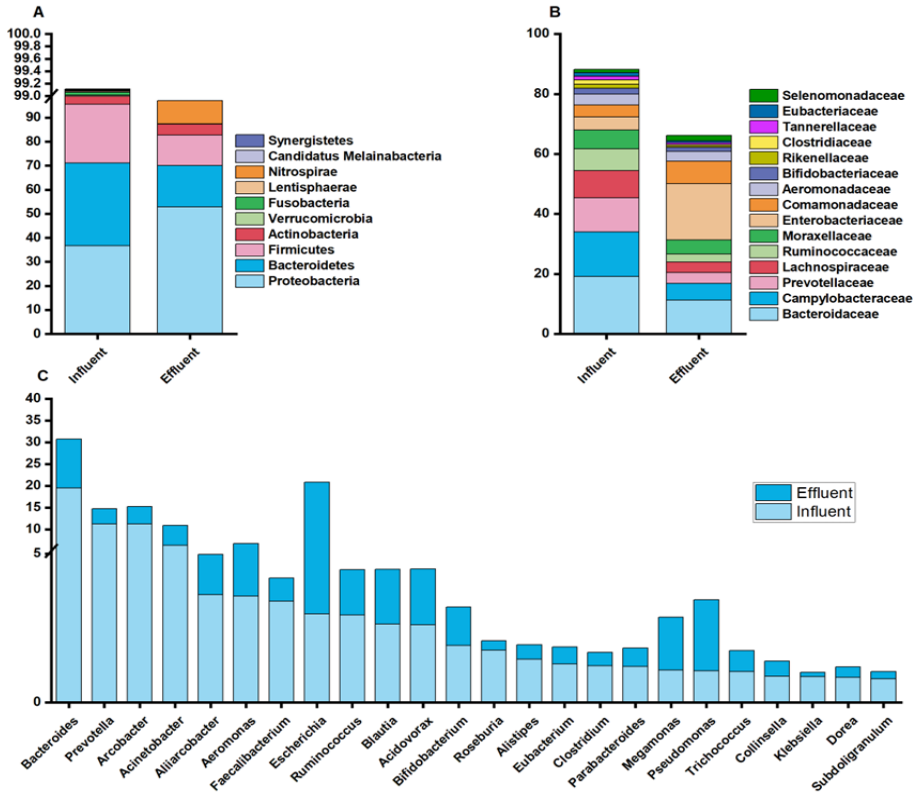

Figure 2. Metagenomics analysis of bacterial communities in influent and effluent, visualized by Origin 2022. The top 10 abundant phyla (**A**); the top 15 abundant families (**B**); and the top 24 abundant genera (**C**). Relative abundance is expressed as a percentage of the total valid sequences for each sample for each bacterial taxonomic units.

The characteristics of archaeal communities in influent and effluent samples were also studied. The results showed that *Euryarchaeota* accounted for 99.6% and 98.73% of all Archaea in the influent and the effluent, respectively, and was the dominant archaeal phylum, followed by *Thaumarchaeota* and *Crenarchaeota* (Figure S1). Li et al. [30] found that the dominant archaea in the receiving water of a domestic sewage discharge were *Euryarchaeota*, *Thaumarchaeota*, and *Crenarchaeota*, successively, which was consistent with the results of this study. *Euryarchaeota* can appear in various ecological niches and contain a variety of methanogenic archaea [31] that play an indispensable role in anaerobic wastewater treatment [32]. *Thaumarchaeota* contain most of the ammoxidation archaea, which participate in ammoxidation. *Euryarchaeota* and *Thaumarchaeota* play an important role in the nitrogen cycle of sewage treatment systems [33]. The dominant archaea at the genus level were *Methanothrix* (accounting for 45.24% of the influent and 12.06% of the effluent), *Methanosarcina* (accounting for 26.44% of the influent and 79.87% of the effluent), and *Methanobrevibacter* (accounting for 12.87% of the influent and 2.41% of the effluent) (Figure 3). Compared with the influent, except for *Methanosarcina*, *Methanothrix*, *Methanobrevibacter*, and *Methanomethylovorans* were significantly lower in the effluent. A previous study showed that *Methanothrix* and *Methanosarcina* are dominant methanogens in the anaerobic digestion [34]. In this study, *Methanothrix* had higher abundance than *Methanosarcina* in the influent. However, in the effluent, this genus was completely outnumbered by the metabolically versatile methanogens *Methanosarcinaceae*. The change of this dominant genus may be related to the concentrations of volatile fatty acids (VAFs) [35]. Both methanogens require VFAs in the process of anaerobic digestion. Based on K/r hypothesis, there might be a competitive relationship between them. High concentrations of VFAs may be more conducive to reproducing *Methanosarcina*, while low concentrations of VFAs may enable *Methanothrix* to remain the dominant genus. Perhaps the change in these substrate concentrations caused the change in the dominant genus in the present study.

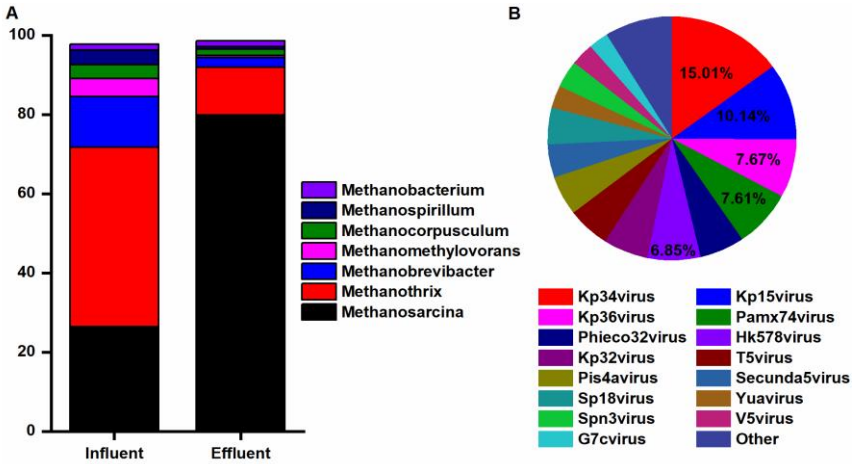

Figure 3. Metagenomics analysis of archaeal and viral communities at genus level, visualized by Origin 2022. The top 7 abundant archaeal genera (**A**) and the top 15 abundant viral genera (**B**). Relative abundance is expressed as a percentage of the total valid sequences for each sample for each archaeal/or viral taxonomic unit. Since viruses were not annotated at the genus level in the effluent, Figure 3B only shows the abundance of virus genera in the influent.

Next, the main types of viruses in sewage were studied. In the influent, at the viral family level, *Myoviridae*, *Siphoviridae*, and *Podoviridae* were dominant. At the genus level, *Kp34virus* (15.01%) and *Kp15virus* (10.14%) were dominant, followed by *Kp36virus* (7.67%), *Pamx74virus* (7.61%), and *Hk578virus* (6.85%), which belong to the order *Caudovirales*. Zhao et al. investigated the influent and effluent microbial communities in six decentralized facilities receiving livestock and household wastewater from rural areas of Changzhou City, Jiangsu Province, China. Consistent with this study, they found that the dominant viral structures in the influent and effluent were also *Myoviridae*, *Siphoviridae*, and *Podoviridae* [17]. Some studies have found that *Caudovirales* play a dominant role in virus-related sequences among sewage microbes [36], and *Caudovirales* are prone to horizontal gene transfer and may affect the ecological environment of sewage microorganisms through interactions with bacterial hosts [37]. The free ARGs in sewage may bind to *Caudovirales* through horizontal gene transfer, and viruses carrying AGRs may invade bacteria and eventually turn bacteria into ARBs.

3.2. Characteristics of ARGs

Based on the CARD database, this study identified 109 subtypes of ARGs in the influent samples and 97 subtypes of ARGs in the effluent samples after screening (the percentage length of reference sequence was 100%), with a total of 112 subtypes (Figure 4). The relative abundance of 26 ARG subtypes was >1%. The 26 ARG subtypes mainly included 7 macrolide resistance genes (*Erm(35)*, *mphE*, *mel*, *msrE*, *EreB*, *EreA2*, and *Mrx*); 6 tetracycline resistance genes (*tet(W/N/W)*, *tet(39)*, *tet(C)*, *tet(40)*, *tetX*, and *acrB*); and 4 cephalosporin resistance genes (*AAC(6′)-Ie-APH(2″)-Ia*, *OXA-333*, *acrB*, and *GES-5*). The ARG abundance calculation based on TPM showed that the total abundance of ARGs in the influent was 305 TPM, while that in the effluent was only 15 TPM, indicating that the wastewater treatment plant (A^2/O + ozonation) in this study had a good removal effect on ARGs. Hultman et al. [38] collected influent and effluent samples from two different municipal sewage treatment plants in Finland and found that the ARG content in the effluent decreased significantly after treatment. Pazda et al. [39] compared and analyzed the removal efficiency of quinolone, macrolide, sulfonamide resistance genes, and other ARGs by different sewage treatment processes and found that the number of ARG types in the treated effluent decreased significantly. Despite this significant decrease after treatment, Tang et al. [40] found that the emission of these low-dose "quasi-persistent" ARGs still posed ecological risks to the receiving water bodies.

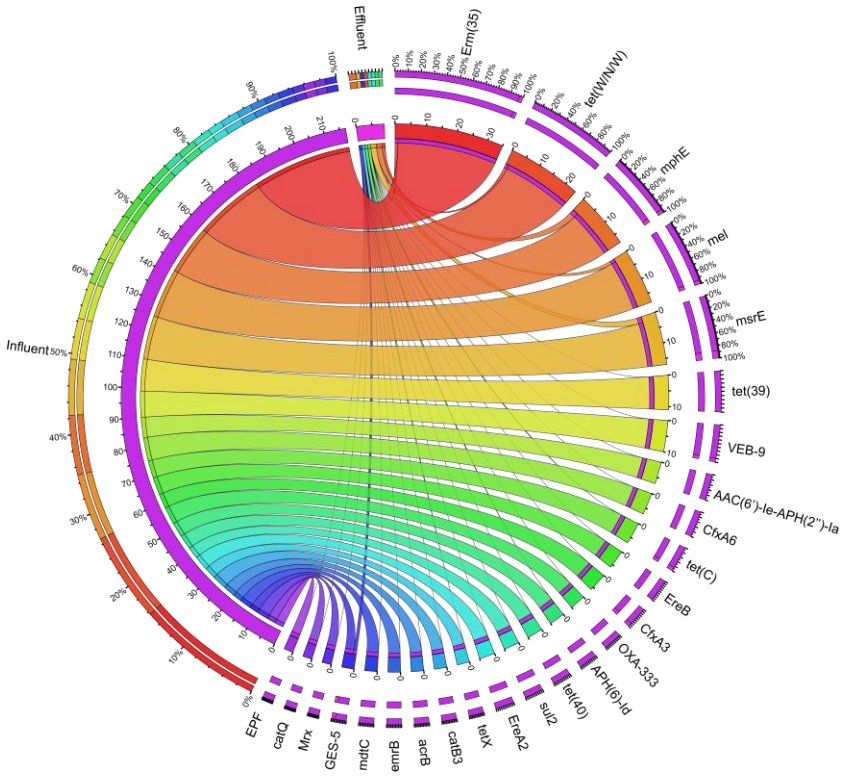

Figure 4. The distribution structure of ARG subtypes (relative abundance > 1%) in the influent and effluent samples, visualized by Circos software. The names of influent and effluent samples and ARG subtypes are listed in the outside circle. The connecting lines within the circles relate the ARG subtypes to the sample, and the width of each line indicates the abundance of each ARG subtype in the influent and effluent samples. The color of each line was used to distinguish the association between the sample and the ARG subtypes.

In this study, we observed a high abundance of ARG subtypes, including *Erm (35)* (macrolide/lincosamide/streptogramin antibiotic), *tet (W/N/W)* (tetracycline antibiotic), *mphE* (macrolide antibiotic), and *mel* (macrolide/streptogramin antibiotic). As shown in Figure 5B, ARGs associated with tetracycline and macrolides accounted for a higher proportion in the sample. In the influent, the abundance of ARGs associated with tetracycline was 17.77% and the abundance of ARGs associated with macrolides was 12.66%. In the effluent, the abundance of two types of ARGs was 12.43% and 19.43%, respectively. This may be related to two factors: tetracycline and macrolide are still the most commonly used antibiotics in China [41]; these two antibiotics are difficult to metabolize in vivo, so most antibiotics entering biological bodies end up in wastewater as unchanged parent compounds [42]. As a result, the content of tetracycline and macrolides in urban domestic sewage is high, which has led to the abundance of related ARGs. Of further concern in this study is that macrolide antibiotic abundance related to ARGs was higher in the antibiotic effluent compared with antibiotic influent, suggesting that macrolide ARGs were more difficult to remove than other types.

Previous studies have shown that the main mechanisms of microbial resistance to antibiotics include antibiotic inactivation, antibiotic target protection, and antibiotic efflux pumps [1,39]. The 112 subtypes of ARGs found in this study included 7 drug resistance mechanisms. In the influent, the highest abundance of ARGs with antibiotic inactivation mechanism was 118 TPM, followed by target protection mechanism, efflux pump mechanism, target alteration mechanism, target replacement mechanism, ATA-AEF, and ATA-AEF-RPA. In the effluent, ARGs with an antibiotic inactivation mechanism also had the highest abundance (7 TPM) (Figure 5A). The results showed that ARGs present in the wastewater treatment plant mainly caused microbial resistance via an antibiotic inactiva-

tion mechanism. Microbes often develop resistance to aminoglycosides, β-lactams, and macrolides through an antibiotic inactivation mechanism [40]. Antibiotic target protection, another antibiotic resistance mechanism in this study, is often related to microbial resistance to erythromycin antibiotics [43]. Antibiotic efflux has long been considered to be the main mechanism responsible for the development of simultaneous resistances to multiple antibiotics (e.g., tetracyclines, macrolides, and fluoroquinolones) [44].

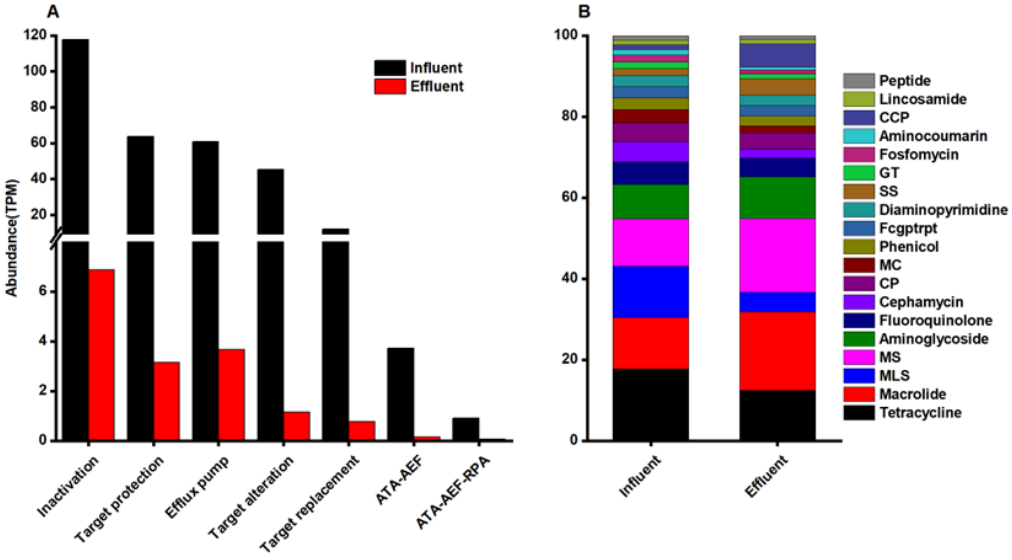

Figure 5. Abundance of antibiotic resistance genes, visualized by Origin 2022. (**A**) Abundance of ARGs classified by resistance mechanism in influent and effluent samples. ATA-AEF: antibiotic target alteration-antibiotic efflux; ATA-AEF-RPA: antibiotic target alteration-antibiotic efflux-reduced permeability to antibiotic; (**B**) Percent abundance (relative abundance > 1%) of influent and effluent samples classified by Drug Class in samples. MLS, MS, CP, MC, Fcgptrpt, SS, GT and CCP represent multi-resistance categories, as shown in Abbreviation.

3.3. Co-Occurrence of ARGs and Microbes

To further investigate the relationships between microbes, ARGs, and resistance mechanisms using correlation analysis, we used Gephi to visualize the co-occurrence patterns between ARG subtypes and microbial communities. ARGs co-occurring with bacteria (top 80 genera in influent samples), archaea, and viruses (top 40 genera in influent samples) were analyzed based on network analysis by Pearson correlation analysis. The results in Figure 6A show that the network of bacteria and ARGs consisted of 100 nodes (62 bacterial genera and 38 ARGs subtypes) and 109 edges. A total of 57 out of 62 bacterial genera may carry more than 2 ARG subtypes. Most of the 38 ARG subtypes were associated with antibiotic inactivation and antibiotic efflux mechanisms. Among these microbes, 14 harbored *kdpE* (antibiotic efflux), 12 harbored *GES-5* (antibiotic inactivation), and 11 harbored *tetX* (antibiotic efflux). Although the abundances of these three ARG subtypes were not high, many microbial species carried these three ARG subtypes, indicating that microorganisms may have been more likely to serve as hosts of these three ARG subtypes. The 62 bacterial genera were mainly classified into *Proteobacteria* and *Firmicutes* phyla, among which *Anaerostipes*, *Fusicatenibacter*, and *Cupriavidus* carried more diverse ARG isoforms than the other genera (5, 4, and 4 species, respectively). In Figure 6B, the network of archaea with viruses and ARGs contained 71 nodes (13 Archaeal genera, 22 viral genera, and 36 ARG subtypes) and 62 edges. Among the 36 ARG subtypes, the antibiotic inactivation and antibiotic efflux mechanisms were still dominant. *Methanosarcina* carried four species (*msrE*, *tet(39)*, *QnrS2*, and *cphA5*), and three resistance mechanisms (antibiotic inactivation, antibiotic efflux, and antibiotic target protection) (Figure 6B). Analysis of the microbial community structure showed that the abundance of *Methanosarcina* was high in both influent and effluent, and its abundance in the effluent was higher than that in the influent, indicating

that the removal effect of *Methanosarcina* by A^2/O + ozonation process in this study was lower than in the case of other archaeal genera.

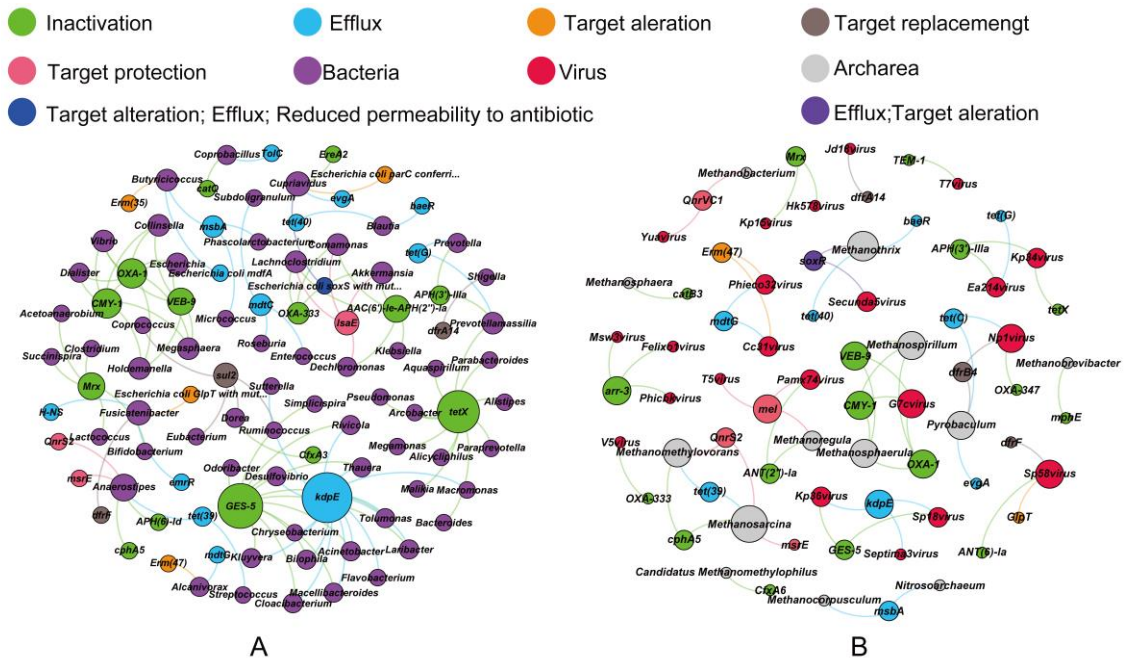

Figure 6. ARGs and microbial co-occurrence network based on correlation analysis, visualized by Gephi 0.9. Nodes represent ARG subtypes or species, and lines represent *p*-values < 0.05 and a strong correlation. The size of a node is proportional to its number of connections. Nodes are colored by the type of resistance mechanism of ARG subtypes and by the type of microorganism. Bacterial and ARG co-occurrence network (**A**). (**B**) Archaeal co-occurrence network with viruses and ARGs.

Therefore, *Methanosarcina* needs to be focused on as a potential ARG host. *Bacteriophages*, the most abundant organisms on Earth, play an important role in the composition and dynamics of microbial communities, biogeochemical cycles, and bacterial evolution in ecosystems around the world. *Bacteriophages* and their mediated transduction may play an important role in the enrichment and diffusion of ARGs. In this study, three kinds of phages (*sp58virus*, *G7cvirus*, and *Np1virus*) carried three kinds of ARGs. Among them, *sp58virus* may have carried *GlpT*, *dfrF*, and *ANT(6)-Ia*; G7cvirus may have carried *VEB-9*, *OXA-1*, and *CMY-1*; *Np1virus* may have carried *tet(C)*, *OXA-347*, and *dfrB4*.

4. Conclusions

In this study, metagenomics sequencing and network analysis were used to explore the community structure characteristics of bacteria, archaea, and viruses in the influent and effluent of urban wastewater treatment plants and to determine correlations between microbial communities and ARGs. The results showed that bacteria were the dominant microorganisms in both the influent and effluent. *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* were the most abundant bacterial phyla in samples. ARGs in WWTPS conferred microbial resistance mainly through an antibiotic inactivation mechanism. Among bacteria, *Anaerostipes*, *Fusicatenibacter*, and *Cupriavidus* carried more diverse ARG isoforms than other genera. Among archaea, *Methanosarcina* was the dominant genus in the effluent and possibly carried four ARGs, which requires special attention in future research. Among viruses, *Siphoviridae* and *Myoviridae* showed significant positive correlations with ARGs. Ozonation was used in the sewage plants sampled in this study, but there were still some potential host microbes of ARGs in the effluent, and the potential ecological risks caused by residual ARGs cannot be ignored. Therefore, how to choose a more effective disinfection process may be the focus of future research on ARGs' removal. In the future, it is necessary

to conduct long-term monitoring of ARGs in WWTPs, and to carry out tracking monitoring ARGs in receiving water to assess the risks caused by ARGs to human health at a long-term and large-scale level.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15081587/s1, Figure S1: The top 3 abundant archaeal phyla in influent and effluent, visualized by Origin 2022.

Author Contributions: Conceptualization, M.S. and C.L. (Chenyang Li); methodology, M.L., Y.H. and C.L. (Chen Lyu); validation, X.B., T.C. and H.C.; writing—original draft preparation, X.H.; writing—review and editing, J.L. and M.S.; supervision, J.L. and K.F.; funding acquisition, M.S. and C.L. (Chenyang Li). All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by Science and Technology Development Plane of Jilin Province (Grant no. 20210203177SF), Education Department of Jilin Province (Grant No. JJKH 20230350KJ), and the Bureau of Science and Technology of Yulin.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviation

Abbreviation	Full Name
MLS	macrolide antibiotic; lincosamide antibiotic; streptogramin antibiotic
MS	macrolide antibiotic; streptogramin antibiotic
CP	cephalosporin; penam
MC	monobactam; cephalosporin
FCGPTRPT	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan
SS	sulfonamide antibiotic; sulfone antibiotic
GT	glycylcycline; tetracycline antibiotic
CCP	carbapenem; cephalosporin; penam

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