RESEARCH ARTICLE

Distribution of antibiotic resistance genes and their association with bacteria and viruses in decentralized sewage treatment facilities

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HIGHLIGHTS

• Distribution of ARGs in decentralized sewage facilities were investigated.

• Bacitracin-ARGs were most predominant ARGs in rural wastewater.

• ARGs were identified in bacterial and viral community.

• ARGs of *rpoB, drfE, gyrA* and *parC* were both correlated with bacteria and phages.

• More attention should be paid to the risk of spreading ARG by phages.

GRAPHIC ABSTRACT
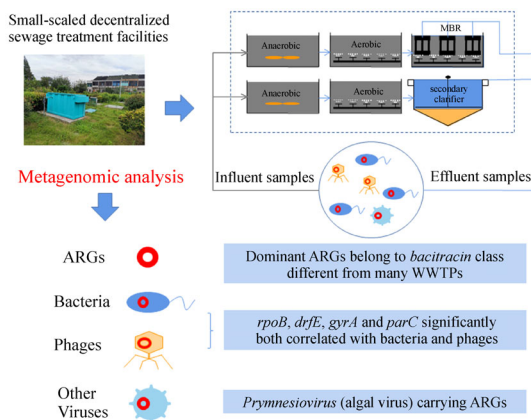
ABSTRACT

The distribution of antibiotic resistance genes (ARGs) has been intensively studied in large-scale wastewater treatment plants and livestock sources. However, small-scale decentralized sewage treatment facilities must also be explored due to their possible direct exposure to residents. In this study, six wastewater treatment facilities in developed rural areas in eastern China were investigated to understand their risks of spreading ARGs. Using metagenomics and network analysis tools, ARGs and bacterial and viral communities were identified in the influent (INF) and effluent (EFF) samples. The dominant ARGs belonged to the bacitracin class, which are different from most of municipal wastewater treatment plants (WWTPs). The dominant hosts of ARGs are *Acidovorax* in bacterial communities and *Prymnesiovirus* in viral communities. Furthermore, a positive relationship was found between ARGs and phages. The ARGs significantly correlated with phages were all hosted by specific genera of bacteria, indicating that phages had contributed to the ARG's proliferation in sewage treatment facilities. Paying significant concern on the possible enhanced risks caused by bacteria, viruses and their related ARGs in decentralized sewage treatment facilities is necessary.

1 Introduction

The recent spread of the coronavirus across the community has urgently warranted the detailed understanding of distribution and proliferation of viruses in ambient environment (Zhang et al., 2020b). With reports of detecting such virus in municipal wastewater in China, Australia, Netherlands and other regions, the risks of sewer and sewage facilities to support virus amplification have been a concern (Ahmed et al., 2020; Medema et al., 2020; Randazzo et al., 2020). Concurrently, sewage biological units also amplify the risk of antibiotic resistance genes (ARGs). ARGs that are often expressed in antibiotic resistance bacteria (ARB), has been intensively studied to

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understand their risks to human health and ecosystem safety in clinical therapy, livestock breeding, fish farming and so on (Guo et al., 2017; Wang et al., 2018b; Chen et al., 2019). Municipal wastewater treatment plants (WWTPs) have been identified as an important reservoir of ARGs and a hot spot of ARG propagating and disseminating (Rizzo et al., 2013; Guo et al., 2017; Ju et al., 2019; Osińska A et al., 2020). The high level of horizontal genes transfer intensity between ARG hosts and fresh recipients in the dense microbial flocs in the bioreactors aroused great concerns for the high spreading risks of ARGs via wastewater treatment to environmental water bodies (Yang et al., 2014). Thus, ARGs and viruses in wastewater must be carefully studied to evaluate and control their risks.

Extant previous studies have already revealed the prevalence of ARB and ARGs and their removal efficiencies through the physical, biological and chemical treatment processes in WWTPs (Chen and Zhang, 2013; Wang et al., 2018a; Sharma et al., 2019; Li et al., 2020). However, understanding the behavior of ARGs and viruses is very limited in small-scaled and decentralized sewage treatment facilities. Two reasons exist why decentralized facilities must be concerned. First, the effluent of decentralized facilities is usually recycled in the local area, which increases exposure risks (Han et al., 2019; Liu et al., 2020). Second, the treatment performance of decentralized facilities is usually worse than those of large-scale WWTPs due to insufficient maintenance. Therefore, the behavior and risks of ARGs and viruses in decentralized sewage treatment facilities in rural areas need more attention.

Bacteria and viruses both relate with ARGs in municipal WWTPs (Zhou et al., 2017; Debroas and Siguret, 2019). Thus they should also be concerned in decentralized facilities. The phenotype ARB, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, or *Stenotrophomonas maltophilia* (Martinez, 2008), which usually acquire ARGs through mutation (e.g., changes in antibiotic targets), vertical gene transfer (VGT) cell division, or horizontal genes transfer (HGT) through conjugation, transduction and transformation (Blair et al., 2015; Soucy et al., 2015). The correlation of ARGs and ARB has been widely observed and discussed in the consortium of soil, wastewater, human and animal manure, surface water and reclaimed water (Forsberg et al., 2012; Forsberg et al., 2014; Li et al., 2015; Chopyk et al., 2020). Besides bacteria, viruses are ubiquitously distributed in the environment, with an estimated abundance ranging from 10^9–10^{10} viruses per liter in seawater and 10^8–10^9 viruses per gram in human feces, respectively (Fuhrman, 1999; Kim et al., 2011). Bacteriophages, a typical class of virus, can transfer ARGs horizontally between different species through transduction, which was considered an essential way of ARG diffusion (Subirats et al., 2016; Gunathilaka et al., 2017; Debroas and Siguret, 2019). There existed direct evidence of ARG transduction from donors to the human pathogenic bacteria, *Staphylococcus aureus*, via its prophage (Haaber et al., 2016). Moreover, phages were found capable of promoting ARG dissemination in the natural environment by co-carrying ARGs with their targeted bacteria (Wang et al., 2018a; Yang et al., 2018). The role of bacteriophages as vehicles of ARGs transmission to bacteria has been used to explain the frequent gene communication in bacterial communities (Yang et al., 2018; Debroas and Siguret, 2019). Therefore, ARGs and viruses may seriously threaten human health and ecosystem safety, especially given that certain ARGs are shared by ARB and viruses (Debroas and Siguret, 2019). Thus, the links between ARGs and viruses in wastewater treatment facilities must be understood.

In this study, we investigated the ARGs, bacteria and viruses in the INF and EFF of six decentralized sewage treatment facilities (one facility has MBR). Using the 214.5G metagenomic raw data from the Illumina Hiseq4000 platform, we utilized network analysis tools to distinguish the correlation of microbial taxa with antibiotic resistome. The results revealed the sharing ARGs between bacteria and bacteriophages, indicating bacteriophages (as a reservoir of ARGs) involved and directly contributed to the ARG proliferation in the decentralized facilities under investigation.

2 Material and method

2.1 Sampling

Six decentralized facilities were investigated in rural zones of Changzhou city, Jiangsu Province of China and the names were abbreviated as DS, TJT, DDJ, WL, BTC and MA. These facilities received livestock and household wastewater in rural areas. During sampling, no incidence of flu in the catchment was reported before sampling. The process description and average water quality are shown in Table 1.

In April 2019, 1 L of INF and 3 L of EFF were collected from each facility. The samples were transported to the laboratory in a thermostat box with ice for cooling. The samples were filtrated through a 0.22 μm membrane filter paper within 24 h after sampling. Then, the enrichment one membrane was stored in a –80°C refrigerator for cryopreservation before sending for metagenomic analysis.

2.2 DNA extraction and sequencing

DNA for metagenomics was extracted using the EZNA® DNA Kit (Omega Bio-Tek, Norcross, GA, US) in accordance with the manufacturer's instructions. DNA quality was examined with the gel electrophoresis (1% agarose) and the concentration and purity were quantified using TBS-380 and NanoDrop2000, respectively.

Table 1 Process description and water quality of six investigated facilities in this study

Plant ID	DS	TJT	DDJ	WL	BTC	MA
Process design	AO	AO	AO	AO	AO + MBR	AO
Flow rate (m³/d)	30	40	55	24	24	60
Population equivalent	4126	500	722	120	230	791
Average water quality of influent/effluent						
COD (mg/L)	198.16/28.91	263.67/57.33	210.89/48.93	208.67/50.86	220.50/33.99	256.33/58.67
NH₃ (mg/L)	20.57/2.47	55.06/41.73	27.95/3.80	25.49/4.13	20.97/2.89	66.01/16.96
TN (mg/L)	16.47/3.48	57.32/42.15	24.32/5.89	22.82/4.38	20.59/5.77	68.26/20.15
TP (mg/L)	2.14/0.65	3.65/3.52	2.19/0.58	2.25/0.82	1.83/0.47	3.65/3.52

After fragmentation, a paired-end library with an average size of about 300 bp was constructed. Adaptor-appended fragments were sequenced using the Illumina HiSeq4000 platform (Illumina Inc., San Diego, CA, US) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). As for quality filtering, 3′ and 5′ ends were stripped using SeqPrep. Low-quality reads (length < 50 bp or with a quality value < 20 or having N bases) were removed using Sickle.

After quality control, the clean reads were assembled into contigs employing SOAPdenovo assembler (Li et al., 2010). K-mers, varying from 1/3–2/3 of read length, were tested to obtain the minimum scaffold number and the maximum value of N50 and N90. Then, scaffolds with the length of over 500 bp were extracted and broken into contigs without gaps. Contigs (the length>300bp) were used for further gene prediction and annotation.

2.3 Metagenomic taxonomy classification

Open reading frames (ORFs) were predicted from the assembled contigs using MetaGene. The predicted ORFs with lengths over 100 bp were retrieved and translated to protein sequences based on the NCBI translation table. All complete translated sequences were clustered with a 95% sequence identity (90% coverage) by the CD-HIT, then cluster sequences were constructed into non-redundant gene catalogs. Meanwhile, the high-quality reads were mapped against the non-redundant gene catalogs by SOAPaligner using the criterion of "identity>95%," and the mapped reads of each gene were counted as gene abundance.

Taxonomic classification of contigs was conducted by searching non-redundant gene catalogs against the NCBI NR database using BLASTP with an e-value cutoff of 1E-5. To differentiate the bacteria– and virus–ARGs, the contigs were divided into bacterial and viral contigs based on the results of taxonomic annotation, and then the bacterial and viral contigs were clustered as the bacterial and viral non-redundant gene catalogs for further ARGs annotation. Antibiotic resistance annotations were conducted using the BLASTP search (Version 2.2.28 +) against the Antibiotic Resistance Genes Database (ARDB) and the Comprehensive Antibiotic Resistance Database (CARD) with an e-value cutoff of 1E-5 (Liu and Pop, 2009; Jia et al., 2017). According to the functions, the sequences can be divided into four categories on the Majorbio platform (developed by Majorbio Bio-Pharm Technology Co., Ltd, China): Antibiotic Resistance (AR) refers to genes directly resisting to antibiotics; Antibiotic Sensitive (AS) refers to genes endowed with antibiotic resistance through mutation, knockout and so on.; Antibiotic Target (AT) refers to genes binding with antibiotic action target; Antibiotic Biosynthesis (ABS) refers to the genes related to antibiotic biosynthesis.

2.4 Statistics analysis

Reads Per Kilobase Million (RPTM) is used to calculate the gene relative abundance (Lawson et al., 2017), which can eliminate the influence of sequencing quantity and gene length difference on the abundance calculation results.

$$RPKM = \frac{R_i \times 10^6}{L_i \times \Sigma_1^n(R_j)}, \tag{1}$$

R_i represents the abundance value of Gene$_i$ in a sample, that is, the number of reads compared to Gene$_i$ in the sample; L_i represents the nucleotide length of Gene$_i$; $\Sigma_1^n(R_j)$ represents the sum of reads corresponding to all genes in the sample.

The microbial taxa and ARGs type in total annotated sequences in all samples were visualized by Circos (Krzywinski et al., 2009). Differential discriminant analysis of Lefse was performed to find the ARGs with a significant difference from abundance. Additionally, the Mann–Whitney U test was used to analyze the significant difference of ARGs between INF and EFF groups ($n = 6$) (Hu et al., 2013).

2.5 Network analysis

To visualize the correlation of the main bacteria and viruses with ARGs, a correlation matrix was constructed by computing all possible pairwise rank of spearman

correlations between microbial genera and the genotype of ARG with the top 40 relative abundance. This preliminary screening step can effectively identify the main microorganisms and ARG types and thus reduced the artificial association bias (Li et al., 2015). In addition, a robust correlation determined by the correlation coefficient of spearman's rank was>0.7 and the P-value was < 0.01 (Su et al., 2018; Chen et al., 2019), which measured the degree of correlation of microbial taxa and ARGs. To reduce the probability of obtaining false positive results, Benjamini – Hochberg method was used to adjust the P-value through multiple testing connection (Benjamini and Hochberg, 1995). When the correlation coefficient reaches the threshold, ARGs and microorganisms will be connected by lines to investigate related patterns, which can be used to evaluate the possible hosts of ARGs (Zhao et al., 2018b; Zhang et al., 2020a). Moreover, to improve the liability of the ARGs possible hosts derived from related patterns, whether the related genes and microorganisms in the bipartite network were on the same contigs was identified by retrieving the taxanomic and ARGs annotation information. Network analysis was performed in the python environment using the Networkx package. Cytoscape, an open sources software platform, was employed to visualize complex networks.

3   Results and discussion

3.1   Structure of bacterial and viral community

The microbial community in the INF and EFF samples are summarized in Fig. 1. The structure of bacterial and viral communities was shown in the taxonomic annotation at the genus level with relative abundance higher than 1%. Bacterial species accounted for 95.6% (INF) and 94.7% (EFF) of total species, which were overwhelming the existence of virus (only 3% in both INF and EFF).

The dominant phyla of bacteria were *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*, which are typical hosts of ARGs in activated sludge in WWTPs (Fig. S1) (Tong et al., 2019; Zhang et al., 2020a). In the influent samples, dominant bacterial genera included *Flavobacterium* (14.3%), *Acidovorax* (10.2%) and *Arcobacter* (10.1%), which were followed by *Limnohabitans* (8.6%) and *Pseudomonas* (7.5%). In the EFF samples, the dominant bacterial species were *Acidovorax* (14.5%), *Mycobacterium* (12.4%), *Flavobacterium* (9.8%) and *Pseudomonas* (8.5%). All these species were often found antibiotic-resistant in literature (Pazda et al., 2019; Yu et al., 2020). Furthermore, *Mycobacterium* and *Pseudomonas* were listed in urgent new therapy by the World Health Organization (WHO).

Using aqueous membrane filters to trap the suspended solids from water samples according to the literature (Subirats et al., 2016; Wang et al., 2018b; Yang et al., 2018;

Petrovich et al., 2020), could concentrate the viruses that were adsorbed on the cell surface and that invaded inside the cells. The dominant species of viruses in influent and effluent samples were *Myoviridae*, *Siphoviridae* and *Podoviridae*, which belong to the order of *Caudovirales* (Fig. 1). The result was similar to previous studies in the literature (Moon et al., 2020; Petrovich et al., 2020). It is interesting to note that a particular kind of algal infectious virus such as *Chlorovirus*, was found in the samples. The hosts of *Chlorovirus* are normally symbionts and often classified as zoochlorellae (Van Etten and Dunigan, 2012).

3.2   Features of bacteria-related ARGs

On the basis of the ARDB database, 66 kinds of ARGs were identified in INF samples and 64 kinds in EFF ones, among which 26 kinds of ARGs had a relative abundance greater than 1%. The corresponding antibiotics of some detected ARGs are included as the critically important drugs for human health by WHO (Collignon et al., 2016), e.g., aminoglycoside, cephalosporins, glycylcyclines, macrolides, penicillins and quinolones. Some other detected ARGs refer to antibiotics considered as highly important drugs, such as lincosamides, tetracyclines and sulfonamides.

The dominant ARGs in INF samples include bacitracin, tetracycline, sulfonamide and penicillin-related ARGs, with relative abundances of 27.8%, 8.7%, 6.2% and 3.9%, respectively. Similarly, dominant ARGs in the EFF samples also included bacitracin (36.8%), tetracycline (4.3%), sulfonamide (7.4%) and penicillin (4.1%) classes (Fig. 2(a)). Meanwhile, in typical municipal WWTPs, dominant ARGs mainly belong to beta-lactams, quinolone, aminoglycoside, tetracycline and sulfonamide classes (Tang et al., 2016; Guo et al., 2017; Ng et al., 2019; Wang et al., 2020), *bacitracin*-ARG was not frequently identified. Bacitracin is a kind of antibiotics to treat skin infection and often used as an additive in chicken feed (Ma et al., 2017), which may be the reason why this kind of ARG is relatively abundant in decentralized sewage facilities. Furthermore, the alteration of antibiotic resistome after the treatment process was analyzed as shown in Fig. 2(b). The relative abundance of macrolide-ARG in effluent significantly reduced compared with the INF ($p < 0.05$), but the relative abundance of ARG types of bacitracin, sulfonamide, penicillin, fluoroquinolone, streptomycin, aminoglycoside and β-lactam increased in EFF samples. Especially, the relative abundance of bacitracin-ARG in EFF samples was 9.74% higher than those in INF ones. Bacitracin-ARG was persistent in the wastewater, which was hardly removed by ultraviolet disinfection (Hu et al., 2016). A previous study reported that bacitracin-ARG was hosted in 153 bacterial species, making it inherent and widespread in the environment (Hu et al., 2013). Therefore, more attention should be paid to bacitracin resistance genes.
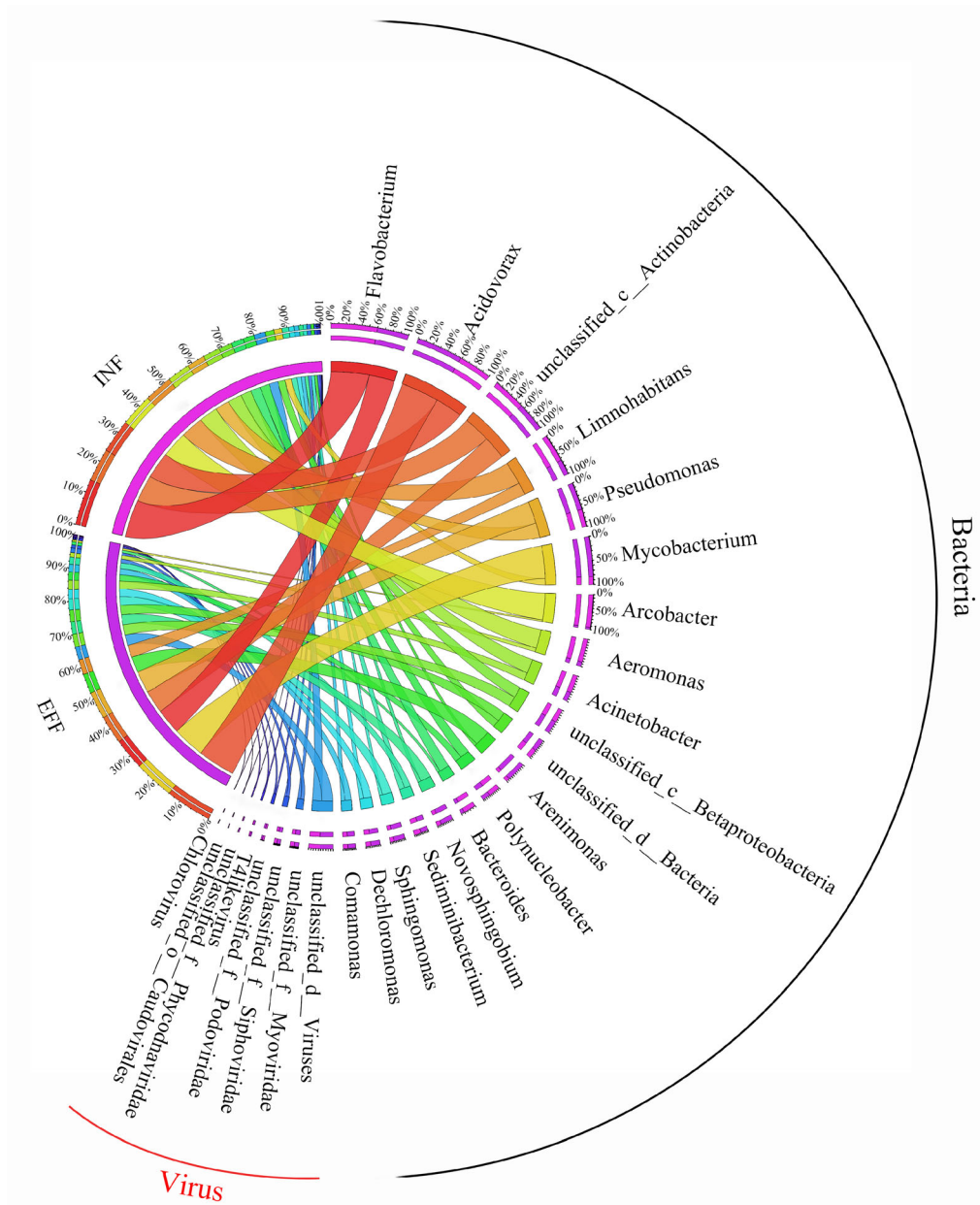
Fig. 1 Bacterial and viral community composition in influent (INF) and effluent (EFF) samples at the genus level. The inner circle lists names of INF and EFF samples and microbial genera. The connecting lines inside the circle link the genera to the samples, and the width of each line indicates the relative abundance of each genera in the INF and EFF sample. The color of each line was used to distinguish the links between samples and microbial genera.

Furthermore, CARD database was used to classify the detected 825 ARGs in bacterial communities into four ARG functional groups: AR, AS, AR/AT and ABS. As shown in Fig. 3(a), the relative abundance of the top 19 bacteria-related genotypes confers resistance to lipoptide, rifampin, fluoroquinolone, aminocoumarin antibiotics and so on. The detailed information is shown in Table S1. 80.8% (667 out of 825) ARGs belonged to AR group, which also had the highest total abundance of 64%. The ARGs in AS-type occupied 7.8% (65 out of 825) with an

abundance of 26.6%. The AR/AT group had 85 kinds of ARGs, and only 5 kinds of ARGs belonged to ABS type.

A similar abundance trend existed between the ARGs and microbial taxa because of some specific microbial taxa carrying ARGs(Forsberg et al., 2014), which may explore how the bacteria hosted the specific ARGs. According to Fig. 3(b), the bacterial genera showed similar correlations to ARG types in all the samples, indicating that a certain genus of bacteria can host different kinds of ARGs and held the potential of multi-resistance. The relatively higher
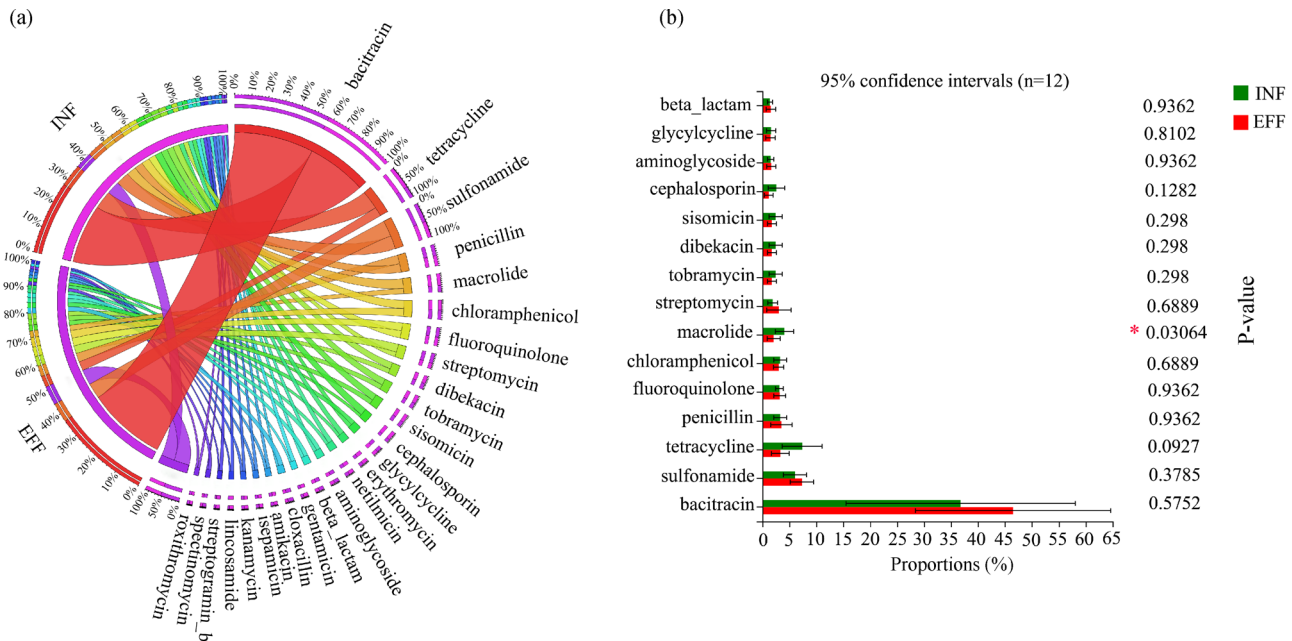
Fig. 2 Distribution of bacteria-related ARGs in wastewater. (a) Relative abundances of ARGs conferring resistance to antibiotics detected in the influent (INF) and effluent (EFF) samples. (b) The differences between proportion values of ARGs in INF and EFF samples based on the Wilcoxon rank-sum test. *means statistically significant difference (p-value < 0.05) between INF and EFF samples. Error bars indicated standard deviation (n = 12).

risks of multi-ARG hosted bacteria were *Actinobacteria*, *Acidovorax* and *Flavobacterium*.

3.3　Features of virus-related ARGs

Viruses were also important reservoirs of ARGs in the environment (Debroas and Siguret, 2019). On the basis of CARD database, the distribution and diversity of ARGs carried by the viruses were analyzed in the INF and EFF samples. Fig. 4(a) shows 19 virus-related genotypes conferring resistance to trimethoprim, rifampin, lipopeptide, aminocoumarin, fluoroquinolone and pyrazinamide antibiotics, which were not completely the same as the main bacteria-related ARGs. The high abundance was contributed by the dominant ARGs of *drfE*(trimethoprim-ARG) and *rpoB*(rifampin-ARG). Based on the classification of virus-related ARGs, *dfrE* (trimethoprim-ARG) with the highest abundance (with an average relative abundance of 66% and 54% in INF and EFF samples) belonged to AR and AT categories, the remaining 13 kinds of ARGs belong to AS category and 5 kinds belong to AR category respectively. The average relative abundance of *ropB* (rifampin-ARG) in INF and EFF samples were 6% and 14%. The detailed information is shown in Table S2. Especially, *ropB* belonging to AS category was also abundant in bacterial communities with an average relative abundance of 10% and 11% in INF and EFF samples (Fig. 3(a)), indicating that *rpoB* can spread between bacteria and viruses.

Compared with bacteria-related ARGs (826 ARG subtypes), only 19 ARG subtypes were detected in the viral communities. On the one hand, viruses such as phages rarely encode ARGs (Enault et al., 2016), but could acquire ARGs mainly by phage transduction or incorrect replication of the host DNA. Given the low probability of these two ways, phages cannot obtain all kinds of ARGs of their hosts. On the other hand, the inherent potential defects of viral metagenome that a large number of unclassified viruses and the database limitations cause the emergence of a mass of unassigned viral sequences (Petrovich et al., 2020), indicating the viral diversity exceeds what can be currently represented by taxonomic annotation.

Similarly, we further explored the contribution of viral communities to different categories of antibiotic resistance. Figure 4(b) exhibits that *Pymnesiovirus*, *T4likevirus,* unclassified *Myoviridae* and *Phycodnaviridae* were the main contributors to AR, AT and AS categories. The total contribution rates of these viruses to AR, AT and AS were 95.9%, 100% and 97.5% in INF samples, and 89.2%, 100% and 94.8% in EFF samples. The *Chlorovirus* and unclassified *Mimiviridae* only contributed to the AR category, and *Mimivirus* only contributed to the AS category. It's worth noting that the high contribution of unclassified *Myoviridae* to AR, AT and AS categories indicated that ARGs could spread by phage transduction. However, the reasons for the differences in ARG contribution of viral communities must be further studied.
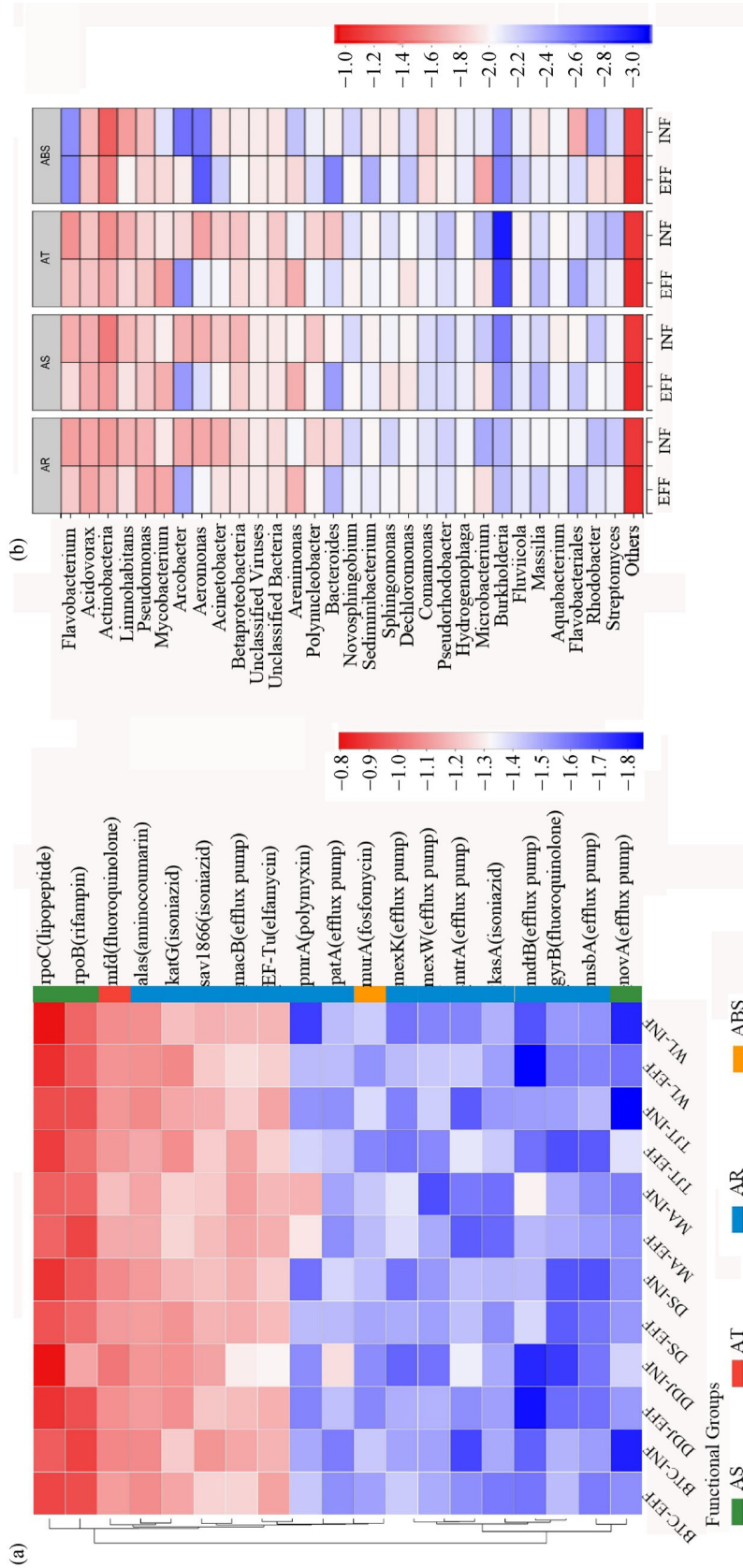
Fig. 3 Different kinds of ARGs and their distribution in bacterial communities (the color intensity in each panel indicates log10-transformed values of relative abundance).(a) The top 20 abundant bacterial ARGs based on the RPKM method. Larger positive value means higher relative abundance. AR is antibiotic resistance, AS is antibiotic sensitive, ABS is antibiotic biosynthesis and AT is antibiotic target; (b) Contribution of bacteria at genus level to the four antibiotic resistance types (AR, AS, AT, ABS).
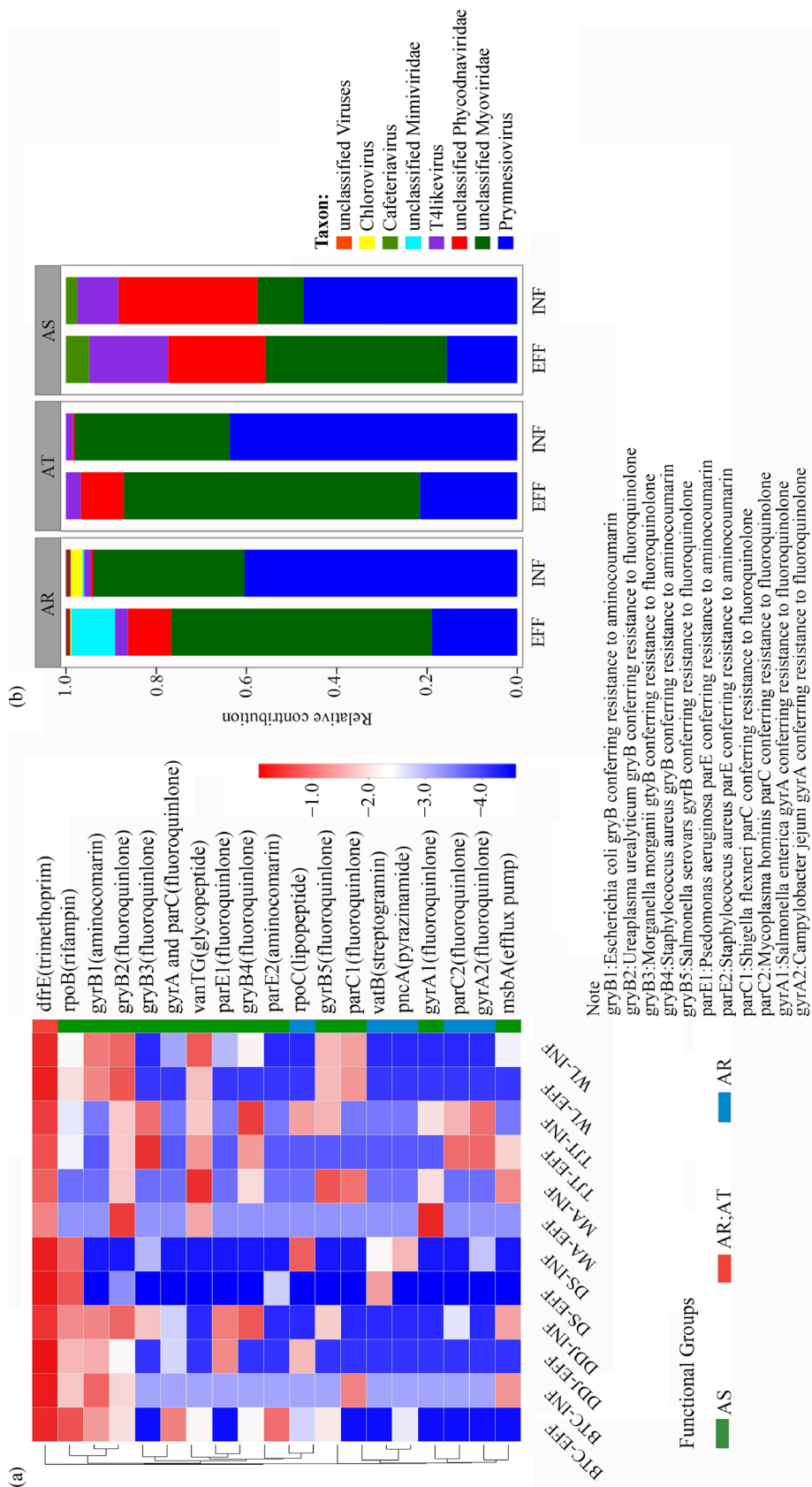
Fig. 4 Different kinds of ARGs and their distribution in viral communities. (a) Top 20 abundant ARGs based on the RPKM method (the color intensity in each panel stands for log10-transformed value of relative abundance), along with the antibiotics that each ARG confers resistance to (in parentheses). And colorful bars of the right side represent different categories. (b) Contribution of viral compositions at genus level to the three antibiotic resistance types (AR, AT, AS) in influent (INF) and effluent (EFF) samples.

3.4   Correlation between ARGs subtypes and microbial taxa

The variation of microbial community composition was the main factor affecting the change of ARGs, which is the potential driving factor of antibiotic resistance (Zhou et al., 2017; Zhao et al., 2018a). Moreover, the non-random correlation pattern between ARGs and microbial taxa constructed on the basis of their abundances can explain the possible host information of ARGs (Li et al., 2015). In this study, a bipartite network of bacterial /viral taxa and ARG subtype was constructed on the basis of the correlation coefficient matrix (ρ>0.7, *P < 0.01) selection (Su et al., 2017; Chen et al., 2019).

The bipartite network of bacteria and ARGs comprised 49 nodes (23 genera and 26 ARG subtype) and 103 edges (Fig. 5). Moreover, the related genes (bacterial taxa and ARGs) were identified in the same contigs, indicating that all the bacteria (23 genera) in the network were the possible hosts of ARGs. Most of the 23 genera belong to *Proteobacteria*, *Acidovorax*, *Hydrogenophage*, *Aeromonas* and *Aquabacterium*, carried more diverse ARG subtypes (15,12, 10 and 10, respectively) than other genera, indicating that they were the main possible hosts of ARGs. Some of these possible ARG hosts have been identified in previous studies. For instance, (i) *Pseudomonas*, the potential host of *vanR* and multidrug-related ARGs (*mexF*), has been verified by previous studies (Guo et al., 2017; Ma et al., 2017); (ii) *Hydrogenophage* carried the *mdtK* and antibiotic efflux ARGs (Zhang et al., 2019a; Zhang et al., 2020a) (iii) *Aeromonas* significantly correlated with ARGs and contributed to the increase of *qnrS* abundance (Zhao et al., 2018a; Zhang et al., 2019a).

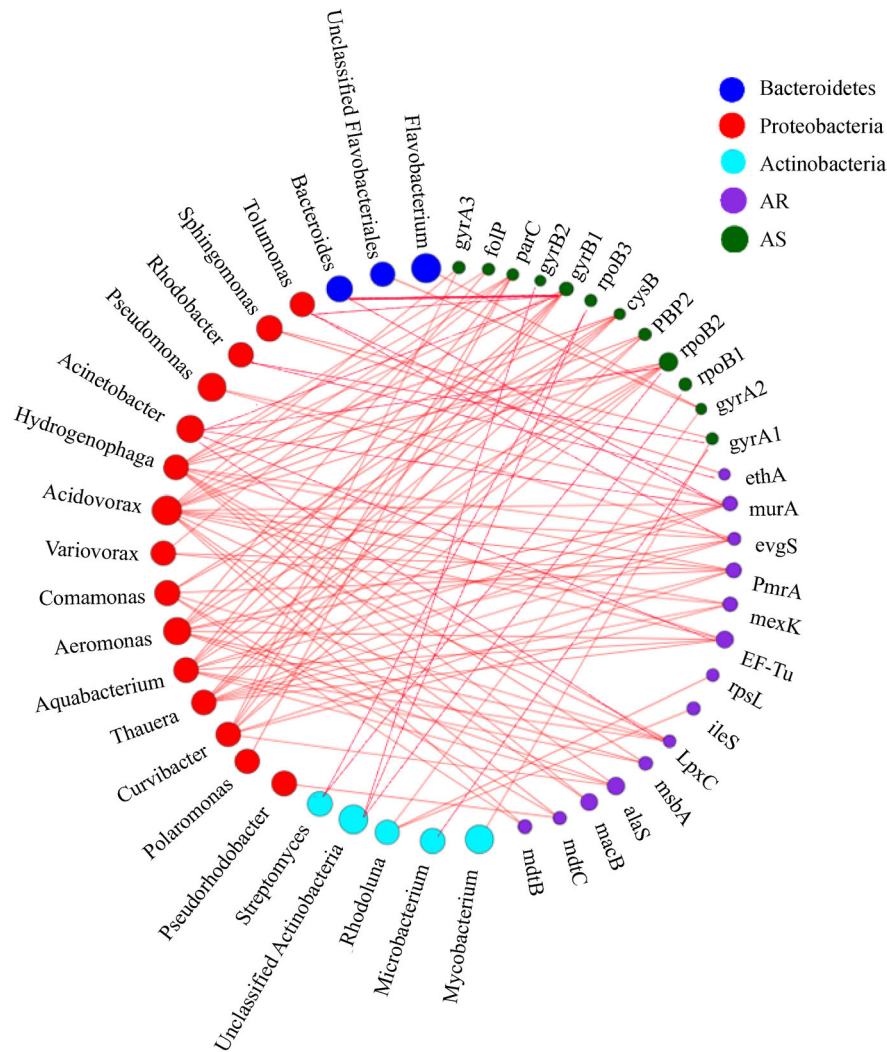The bipartite network showed that 23 and 4 kinds of viral genera and ARGs subtype exist, respectively (Fig. 6).

Fig. 5 The bipartite network of bacterial genera and ARGs. The colorful nodes refer to various ARG categories and bacterial genera, and the sizes of nodes represent the relative abundance of ARGs and bacteria. The red lines represent positive correlations between ARGs and bacteria.

Phages were the main virus in the bipartite network, such as *T4likevirus* and *T5likevirus*. However, unlike bacteria, phages rarely encode ARGs (Enault et al., 2016). Besides, none of the related genes (phages and ARGs) were identified in the same contig. Given that phages are important vehicles for ARG spread, a significantly positive relationship existed between phage and ARGs as shown in the bipartite network. Furthermore, one related gene (*rpoB* and *Prymnesiovirus*) was found in the same contigs, indicating that the *Prymnesiovirus* was the carrier of *rpoB*. *Prymnesiovirus* belongs to algal virus, however, studies on the mechanism of algal virus carrying ARGs remain limited. Terrestrial plants can absorb antibiotics and ARGs in the soil applied with organic fertilizer (Peng et al., 2017; Chen et al., 2019; Zhang et al., 2019b). Meanwhile, the structure, initial infection stage and many genes of algal viruses (e.g., *Chlorovirus*) were similar to phages (Van Etten and Dunigan, 2012). Therefore, algae may absorb antibiotics and related ARGs from the water environment, resulting in the accumulation of ARGs in algae, and then ARGs in algae may transmit into algal viruses through a certain way, such as transduction.

3.5 Risk of spreading ARG by phages in sewage treatment

Bacteriophage as ubiquitous virus infects bacteria and widely exists in wastewater treatment facilities (Calero-Cáceres., 2019). Phages have been confirmed to carry various ARGs in sewage, and can be transmitted through transduction or as transfer vector (Soucy et al., 2015; Ji et al., 2021). Moreover, phages are more likely to survive than bacteria in wastewater (Balcázar, 2018). Compared with ARGs carried by bacteria, the phage-related ARGs have stronger resistance under different temperatures, pH and disinfectant conditions (Calero-Cáceres and Muniesa, 2016). Therefore, more concern should be alloted to the risk of ARG spread by phages in wastewater treatment.

Figure 7 presents that *rpoB*, *drfE, gyrA* and *parC* were significantly correlated with bacteria and phages (Spearman correlation coefficient >0.7 and *$P < 0.01$), indicating that phages have the risk of spreading ARGs. The ratio of relative abundance of ARGs correlated with phages to that with bacteria can be used to evaluate the spreading ability of ARGs through transduction (Wang et al., 2018a; Wang et al., 2018b) . The ratios of *rpoB*, *drfE*, *gyrA* and
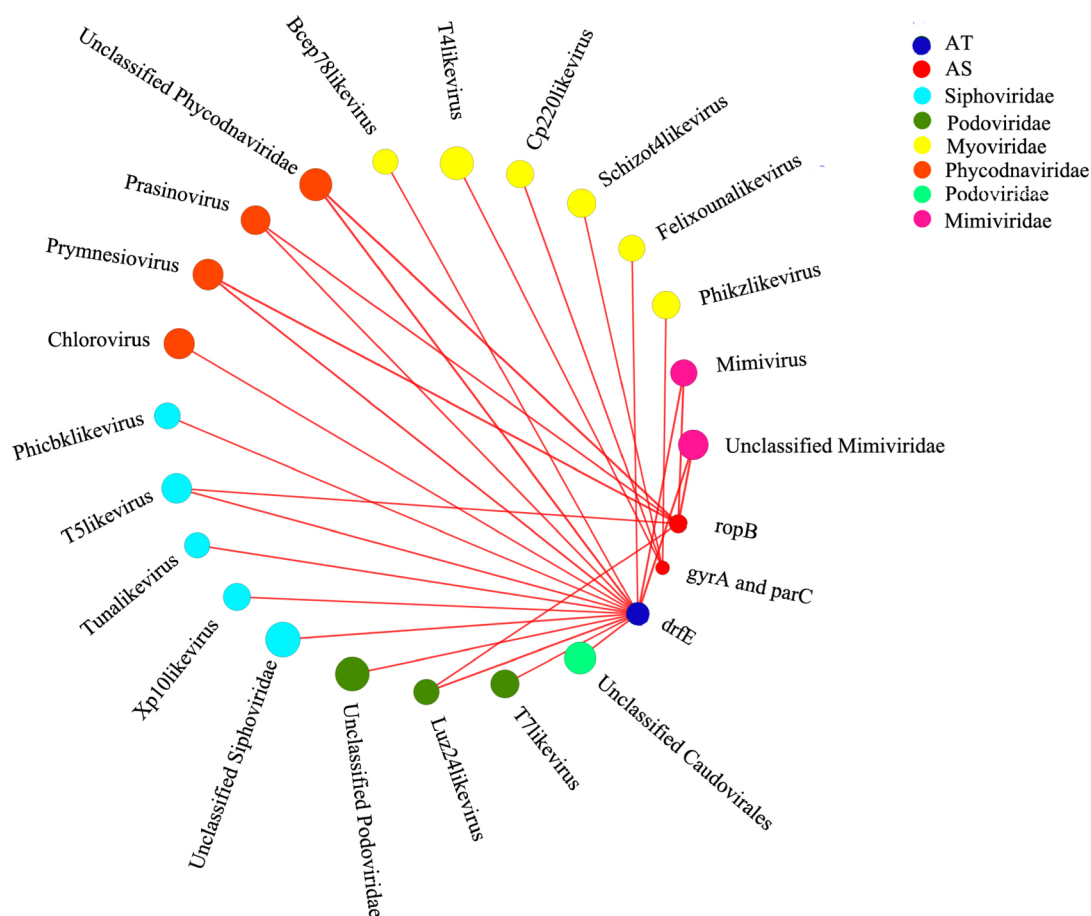

Fig. 6 The bipartite network of viral genera and ARGs. The colorful nodes refer to various ARG categories and viral genera and the sizes of nodes represent the relative abundance of ARGs and virus. The red lines represent positive correlations between ARGs and viral genera.
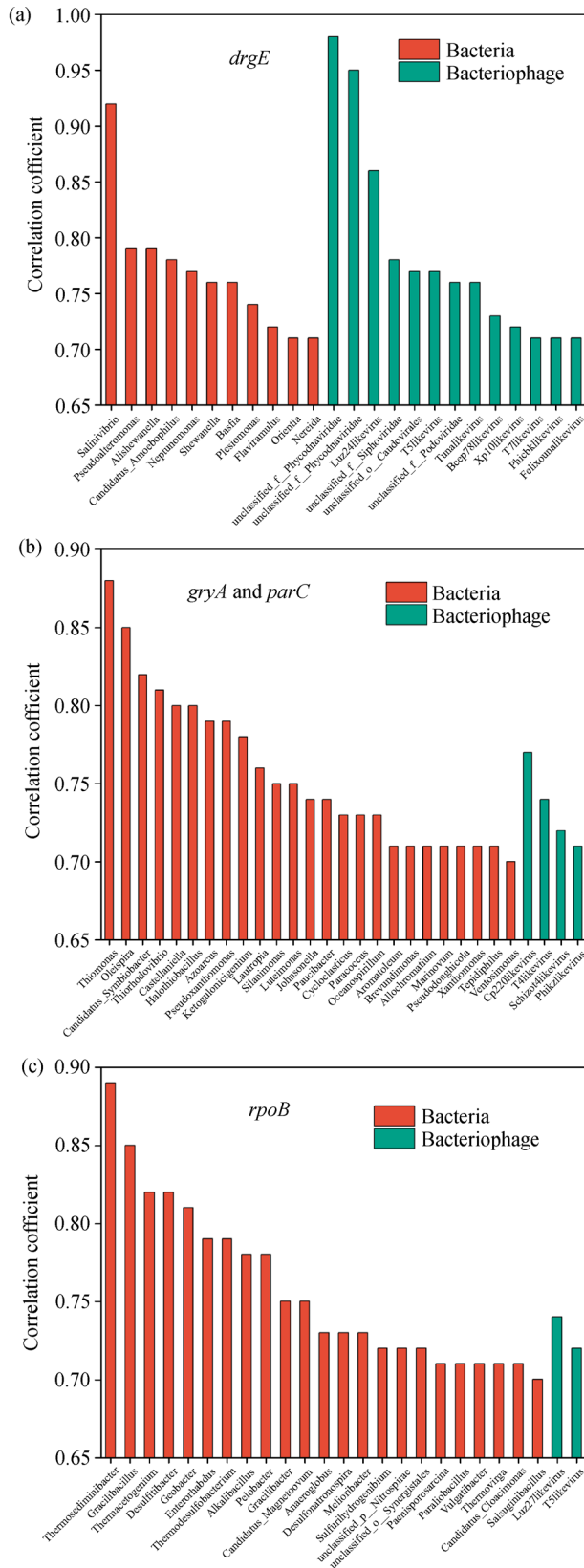
Fig. 7 Correlations between ARGs and bacteria or bacteriophages. (a) *drfE*; (b) *gryA* and *parC*; (c) *rpoB*.

parC were calculated as shown in Table 2. According to the results, the order of spreading ability of ARGs was *rpoB* > *drfE*>*gyrA* and *parC*, and their spreading capacity values were 59.15, 39.18 and 8.49, respectively, which indicated that *rpoB* has the greatest risk of spreading through phage. The previous analysis results also showed that *rpoB* was highly abundant in bacterial and viral communities. In future, more attention should be paid to ARGs spreading by phages in decentralized sewage treatment facilities.

Table 2 The spreading ability of shared-ARG by phage

ARG genotype	Relative abundance of ARGs correlated with phage (RA1)	Relative abundance of ARGs correlated with bacteria (RA2)	Spreading ability (RA1/RA2)
ropB	0.2767	0.004678	59.15
drfE	0.2971	0.007582	39.18
gryA & *parE*	0.0278	0.003276	8.49

4   Conclusions

Metagenomic and network analyses distinguished the features and correlations of bacterial and viral taxa with antibiotic resistome in decentralized sewage treatment facilities. A total of 825 bacterial and 19 viral genotypes were identified in the rural wastewater. The bacterial ARGs mainly belong to the AR category, whereas most of viral ARGs belong to the AS category. Bacitracin-ARG was the most dominant ARG type, which differed from municipal wastewater. Besides bacteria, viruses, especially phages, also were also contribute to ARG spreading, it was found that ARGs of *rpoB*, *drfE*, *gyrA* and *parC* significantly correlated with bacteria and phages. Therefore, more attention should be paid to the risk of spreading ARGs through phages.

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