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Spatiotemporal dynamic changes of antibiotic resistance genes in constructed wetlands and associated influencing factors☆

Ling Zhang [a,b], Changzhou Yan [a,*], Dapeng Wang [a], Zhuo Zhen [a]

[a] Key Laboratory of Urban Environment and Health, Fujian Key Laboratory of Watershed Ecology, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, 361021, China
[b] University of Chinese Academy of Sciences, Beijing, 100049, China

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ABSTRACT

A better understanding of the spatiotemporal dynamics and influencing factors of sulfonamide antibiotic resistance genes (ARGs) distribution in subsurface flow constructed wetlands is essential to improve the ARGs removal efficiency. The spatiotemporal dynamics of sulfonamide ARGs were explored in the vertical upflow subsurface flow constructed wetland (VUSFCW). The results showed that the absolute abundance of ARGs presented a trend of bottom layer > middle layer > top layer. The relative abundance of ARGs decreased significantly from the bottom layer to the middle layer, but increased in the top layer. The bottom layer was the main stage to remove ARGs. The absolute abundance of ARGs at each point in summer was significantly higher than that in winter. Based on the spatiotemporal distribution of ARGs, the internal mechanism of ARGs dynamic change was explored by the partial least square path analysis model. The results showed that physical-chemical factors, microorganisms and antibiotics indirectly affected the spatiotemporal distribution of ARGs mainly through mobile genetic elements. The indirect influence coefficients of physical-chemical factors, microorganisms and antibiotics on the spatiotemporal distribution of ARGs were 0.505, 0.221 and 0.98 respectively. The direct influence coefficient of MGEs on the spatiotemporal distribution of ARGs was 0.895. The results of network analysis showed that the potential host species of ARGs in summer were more abundant than those in winter. The selection mode of sulfonamide ARGs to potential hosts was nonspecific. There is a risk of sulfonamide ARGs infecting pathogens in VUSFCW. Fortunately, VUSFCW has proven effective in reducing the absolute abundance of ARGs and the potential risk of pathogens carrying ARGs. These findings provide a model simulation and theoretical basis for effectively reducing the threat of ARGs.

1. Introduction

Antibiotic resistance genes (ARGs) have attracted considerable attention as emerging pollutants in recent years. With the abuse of antibiotics, the residual antibiotics entering natural water can exert selective pressure on bacteria, resulting in an increase in the population of antibiotic resistant bacteria and with it ARGs (Liang et al., 2018). ARGs can not only persist, transfer and spread in the environment (Zhu et al., 2019), but also have rapid expansibility, which is different from conventional pollutants. With the rapid development of the global economy, ARGs can spread rapidly around the world during processing, preservation and global transportation, and spread to humans through the food chain (Q.L. Chen et al., 2019), which poses a great threat to public safety. How to effectively control the diffusion of ARGs in water has become an urgent problem to be solved.

In recent years, many researchers have paid attention to the removal of antibiotics and ARGs in water using vertical subsurface flow constructed wetlands (VSFCWs). VSFCWs have significant advantages in treating refractory organic wastewater because of their unusual structure with anaerobic zone at the bottom and aerobic zone at the top (Huang et al., 2017b; Tang et al., 2020). Many studies have been carried out on the removal of antibiotics and their ARGs in VSFCWs. Some researchers have optimized the operating parameters of constructed wetlands to improve the pollutant removal efficiency (Ávila et al., 2014; Huang et al., 2017a). Some previous studies reported that different substrates and plant configurations lead to great differences in the

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* Corresponding author.
E-mail address: czyan@iue.ac.cn (C. Yan).

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removal of antibiotics and ARGs (Abou-Kandil et al., 2021; Chen et al., 2016a; Huang et al., 2019). Some scholars have also been committed to revealing the relationship between antibiotics and ARGs (Huang et al., 2015; Song et al., 2018b), or the relationship between mobile genetic elements (MGEs) and ARGs in constructed wetlands (Li et al., 2019). However, to date, little has been studied regarding the dynamic mechanism of ARGs distribution in constructed wetlands, especially regarding quantification of the influencing factors. Moreover, it is well known that sulfonamides are widely used as broad-spectrum antibiotics, and their corresponding ARGs (*sul1, sul2, sul3*) are the most frequently and abundantly detected in the environment (Su et al., 2012). However, many studies have shown that the clinical correlation between sulfonamides and their corresponding ARGs is small (Li et al., 2019), which makes it difficult to control sulfonamide ARGs diffusion by controlling the input of sulfonamides. If ARGs in the environment are combined with pathogenic bacteria, antibiotics may lose their efficacy against pathogens, and the health risk of ARGs will increase. Therefore, it is particularly necessary to pay more attention to this kind of "ancient" and common ARGs and conduct a comprehensive exploration. Based on the spatiotemporal distribution of sulfonamide ARGs in vertical upflow subsurface flow constructed wetland (VUSFCW), this study attempts to explore the spatiotemporal differences and dynamic mechanism of ARGs distribution driven by environmental factors (physical-chemical factors, antibiotics, microorganisms and MGEs) and the selection mode of ARGs to the host. Whether ARGs and pathogenic bacteria can be effectively removed by VUSFCW was also evaluated.

The objectives of this study were: 1) to explore the spatiotemporal dynamics of ARGs in VUSFCW; 2) to reveal the dynamic mechanism of ARGs distribution driven by environmental factors; and 3) to identify the host selection mode of ARGs and the effectiveness of VUSFCW in removing ARGs and pathogenic bacteria.

2. Materials and methods

2.1. Experimental equipment and sampling settings

The constructed wetland was built in October 2019 at the Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China. The dimensions are 1250 mm × 750 mm × 700 mm (L × W × H). The substrate of constructed wetland consists of three layers, the upper layer is soil-sand mixture (specific surface area: 5.1 m^2/cm^3), the middle layer is bio-ceramic (specific surface area: 15.2 m^2/cm^3, STCMOET, China), and the bottom layer is gravel (specific surface area: 0.94 m^2/cm^3, Jinfeng, China). Their thicknesses are 100 mm, 200 mm and 300 mm, respectively. The particle size of the soil-sand mixture is less than 1 mm, that of bio-ceramic is 2–4 mm, and that of gravel is 10–20 mm.

Iris and *Scirpus validus Vahl* were planted in the constructed wetland. The planting density of both *Scirpus validus Vahl* and *Iris* was 6 clumps/m^2, and the two plants were mixed. They were planted in early 2020. Due to the low temperature in the early stage, the plants reached adulthood in June 2020. During sampling in summer (July and August), the plants grew well. During sampling in winter (December), the plant growth status was poor, and there were withered branches and leaves (timely removal every day).

According to the pre-experimental results, the hydraulic retention time was set to 24 h. The influent flow of the constructed wetland used was 124 L/d, the hydraulic load was 0.138 m/d, and the influent mode was continuous influent. A number of water sample collection devices were set inside the constructed wetland to regularly collect water samples. The constructed wetland was divided into three stages: stage 1 (gravel layer), stage 2 (bio-ceramic layer) and stage 3 (soil-sand layer). Each stage consists of two sampling points: stage 1 (CD-1 and CD-2), stage 2 (CZ-1 and CZ-2), and stage 3 (CS-1 and CS-2) (Fig. 1).

The water samples were collected by multi-point and multi-times sampling method. Each constructed wetland was provided with two sampling sections, 9 sampling points were distributed in each section, and three sampling points were distributed in each stage (layer) of each sampling section (Fig. S1a, Supporting Information). During sampling, the samples of three sampling points at the same stage (layer) were mixed into one sample. In this way, six mixed samples were obtained from each constructed wetland (Fig. S1b, Supporting Information). 200 mL water samples were collected. The influent was taken from the internal river of the Institute of Urban Environment, Chinese Academy of Sciences. A certain amount of methanol, sodium acetate, sodium nitrate, ammonium sulfate, potassium dihydrogen phosphate and sulfonamide antibiotics were added to the influent. The chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP) and antibiotic concentrations of influent water are shown in Table 1.

Table 1
Physicochemical parameters and pollutant concentration of the influent.

Index	Concentration	Unit
COD	200–287	$mg·L^{-1}$
NH_4–N	9.75–11.16	$mg·L^{-1}$
TN	16.69–17.24	$mg·L^{-1}$
TP	3.47–3.74	$mg·L^{-1}$
SD	19.4–20.3	$μg·L^{-1}$
SM1	19.6–21.1	$μg·L^{-1}$
SM2	19.2–20.5	$μg·L^{-1}$
SMZ	19.0–20.2	$μg·L^{-1}$
pH	6.0-6.7	–

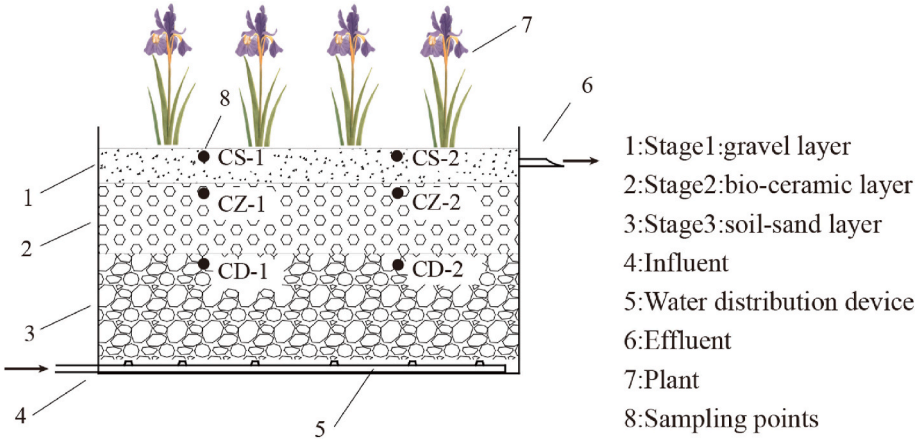

Fig. 1. The schematic design of the VUSFCW.

2.2. The detection of physical-chemical factors and antibiotics

In this study, physical-chemical factors mainly included dissolved oxygen (DO), temperature, pH, TN, TP, NH_4–N and COD. The DO, temperature and pH of the water samples were detected by a portable water quality detector (h40q, HACH, USA). The concentrations of TN, TP, NH_4–N and COD in the water samples were analyzed using the National Standard Methods of China (Ministry of Environmental Protection of China, 2002). The measurements were performed in triplicate for each sample.

The sulfonamides to be detected in this experiment were SD, SM1, SM2 and SMZ, and their detection methods were based on our group's previous studies (Wang et al., 2019). Using LC-20 liquid chromatograph (Shimadzu, Kyoto, Japan) and tandem ABI6500 mass spectrometer (Applied Biosystems, Foster City, CA, USA), the internal standard method was used for detection. The detailed parameters are shown in Table S1 (Supporting Information). To ensure detection accuracy, the solvent blank, program blank and standard solution were run next to each batch of samples in order to check the background pollution and evaluate the performance of the instrument.

The solid-phase extraction process of sulfonamide antibiotics in water was as follows: 500 mL water sample was filtered with 0.22 μm filter membrane. The pH of the filtered water sample was adjusted to 3.0, and 0.2 g EDTA was added to eliminate the interference of heavy metals. An HLB solid phase extraction column was used to enrich antibiotics in water. The HLB column was activated by adding 10 mL methanol and 10 mL ultrapure water. Then the water sample was passed through the column at a flow rate of 5–10 mL/min. The column was cleaned with 10 mL ultrapure water and the water was removed by vacuum. The antibiotics on the HLB column were eluted with 6 mL pure methanol, and the eluent was collected in a test tube. The eluent was blown to near dryness with nitrogen at an appropriate flow rate. The sample was diluted to 1 mL with 20% methanol water solution and filtered with 0.22 μm filter membrane. The filtered sample was subjected to further analysis.

2.3. The detection and quantification of ARGs

The 300 mL water sample was filtered through a 0.22 μm aseptic filter membrane, shredded with sterile tweezers and scissors, and placed in a tube provided by the MP Soil DNA separation kit (MP Bio, USA). The extraction steps followed the scheme provided by the reagent manufacturer. The concentration and quality of extracted DNA were determined by a Nanodrop ND -1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE) and gel electrophoresis.

Real time fluorescent quantitative PCR (Roche light cycler 480II) was used to detect common sulfonamide resistance genes, including *sul1, sul2* and *sul3*. A class of integrons *intI1* and 16Sr RNA were also detected. The primer sequences for the respective target gene are shown in Table S2 (Supporting Information). Roche Light Cycler 480II real-time fluorescent quantitative PCR system used SYBR Green qPCR kit to quantify the abundance of the target gene. Each run included a positive control and a negative control (milli-q water). A total of 40 cycles were applied to improve the chance of product formation from a low initial template concentration. The PCR volume was 20 μL. The details are shown in Table S3 (Supporting Information). The quantitative temperature program of ARGs includes pre-denaturation at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min for a total of 40 cycles. The detailed PCR procedure is shown in Table S4 (Supporting Information). Negative controls were conducted using sterile water to exclude any possible contamination. The correlation coefficients (R^2) values of the plasmid standard curves were higher than 0.99 and the PCR amplification efficiencies ranged from 90% to 110%, which demonstrated the linearity and sensitivity of each qPCR assay. PCR amplification was performed in triplicate for each sample.

2.4. Bacterial 16S rRNA gene sequencing

The Illumina MiSeqpe 300 high-throughput sequencing platform was used for microbial community sequencing. The primers for 16S rDNA v3-v4 were 338F (5′- ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT -3′). PCR system: 12.5 μL 2 × Taq PCR Master Mix, 3 μL BSA (2 ng/μL), 2 μL Primer (5uM), 2 μL template DNA, and 5.5 μL ddH_2O. Reaction parameters: pre-denaturation at 95 °C for 5 min; denaturation at 95 °Cfor 45 s, annealing at 55 °C for 50 s, extension at 72 °C for 45 s, 32 cycles; Extend at 72 °C for 10 min. The original sequence was uploaded to the SRA database of NCBI.

The data were filtered, spliced and removed by QIIME (v1.8.0) software. Sequences with scores less than 20, base ambiguity, primer mismatch or sequencing lengths less than 150 bp were removed. According to barcodes, the sequence information of each processing group was classified in terms of operational taxonomic units (OTUs) for species classification, and OTU similarity was set to 97%. By comparison with the Silva database, the species classification information of each OTU was obtained.

2.5. Statistical analysis

The relative abundance of ARGs was determined by normalizing the absolute gene copy numbers (GCN) of ARGs to GCN of the 16S rRNA gene (Huang et al., 2015; Song et al., 2018a). The calculation method is as follows: the relative abundance of ARG = abundance of ARG/abundance of 16S rRNA gene.

The removal rate formulas of ARGs are as follows:

$$MR_u(\%) = \frac{C_i - C_u}{C_i} \times 100\%$$

where $MR_u(\%)$ represents the ARGs removal rate of the sampling points in stage 1, C_i represents the influent ARGs copy number, and C_u represents the ARGs copy number of water in stage 1.

$$MR_b(\%) = \frac{C_u - C_m}{C_i} \times 100\%$$

where $MR_b(\%)$ represents the ARGs removal rate of the stage 2 or stage 3, C_u represents the ARGs copy number of sampling points in the stage 2 or stage 3, and C_m represents the ARGs copy number of sampling points in the pre sequence stage.

The Excel 2010 (Microsoft Corp. Redmond, WA, USA) was used for data processing and calculation. The plots of antibiotic concentrations and ARGs abundance were prepared by Origin (ver.2017, Origin Lab Corporation, USA). Partial least squares discriminant analysis (PLS-DA) of the microbial community was performed by R software (3.6.1), based on the mixOmics package. Linear discriminant analysis effect size (LEfSe) was completed online based on microbial abundance data (http://huttenhower.sph.harvard.edu/galaxy/). The calculation of node level topology characteristics (such as degree, betweenness, closeness, and eigenvector) in network analysis was based on the "igraph" R package, and network visualization and modular analysis were performed with Gephi version 0.9.2 (Xue et al., 2018). Spearman correlation analysis and significance analysis were performed by SPSS (ver. 23; SPSS Inc, Chicago, IL, USA).

The partial least squares path analysis model (PLSPM) is a statistical analysis method used to study the complex multivariable relationship between explicit variables (which can be measured directly) and implicit variables (which are difficult to measure directly). In the PLSPM network, the connection of the network represents a causal process. The process flows in one direction (circulation is not allowed). After constructing the network, one of the goals is to quantify the relationship between variables. PLSPM was performed by R software (3.6.1) based on the plspm package (Wang et al., 2021). In this analysis, the factors influencing the distribution of ARGs were divided into four modules:

physical-chemical factor module (including temperature, pH, DO, COD, TN and TP), microbial community module, antibiotic module (including SD, SM1, SM2 and SMZ), and MGEs module.

3. Results

3.1. The spatiotemporal dynamics and removal of ARGs

The spatiotemporal dynamics of sulfonamide ARGs in the VUSFCW were investigated in this experiment. The spatiotemporal distribution of three genes (*sul1*, *sul2* and *sul3*) and one MGE (*intI1*) in winter and summer are shown in Fig. 2. In summer, the absolute abundance of ARGs in stage 1 was significantly higher than that in stage 2 and stage 3 at one sampling section. For instance, the absolute abundance of *intI1* in CD-1 was 4.5×10^6 GCN/mL, while that in CS-1 decreased to 8.3×10^5 GCN/mL. The points in winter also had similar trend, but the absolute abundance was significantly lower than that in summer. The relative abundance of ARGs decreased from stage 1 to stage 2 in summer and winter, but increased in stage 3. The correlation analysis results showed that there was a lack of correlation between the total antibiotic concentration and ARGs in summer and winter (Table S5, Supporting Information). Therefore, the absolute abundance of ARGs in the VUSFCW decreased stage by stage, but the soil layer (stage 3) had the risk of increasing the relative abundance of ARGs.

In this study, the removal rates of ARGs in summer were 89.79%–96.22%, as well as the removal rates of ARGs in winter were 78.96%–94.47% (Fig. S2, Supporting Information). Moreover, stage 1 was the main stage of ARGs reduction in summer, and stage 1 and stage 2 were the main stages of ARGs reduction in winter (Fig. 3a and Fig. 3b).

3.2. The spatiotemporal dynamics of microbial community composition

The spatiotemporal distribution characteristics of microorganisms were studied in this experiment. Fig. 4a shows that *Proteobacteria* (phyla) were dominant at each point, and their relative abundance was between 28% and 58% in summer and winter. The relative abundance of *Bacteroidota*, *Firmicutes* and *Desulfobacterota* decreased from stage 1 to stage 3 in summer and winter. However, the relative abundance of *Actinobacteriota*, *Myxococcota* and *Acidobacteriota* increased from stage 1 to stage 3. At the genus level, the microorganisms in stage 3 were mainly aerobic and facultative anaerobic microorganisms, such as *Thermomonas*, *Dyella* and *Klebsiella* (Fig. S3a, Supporting Information). The

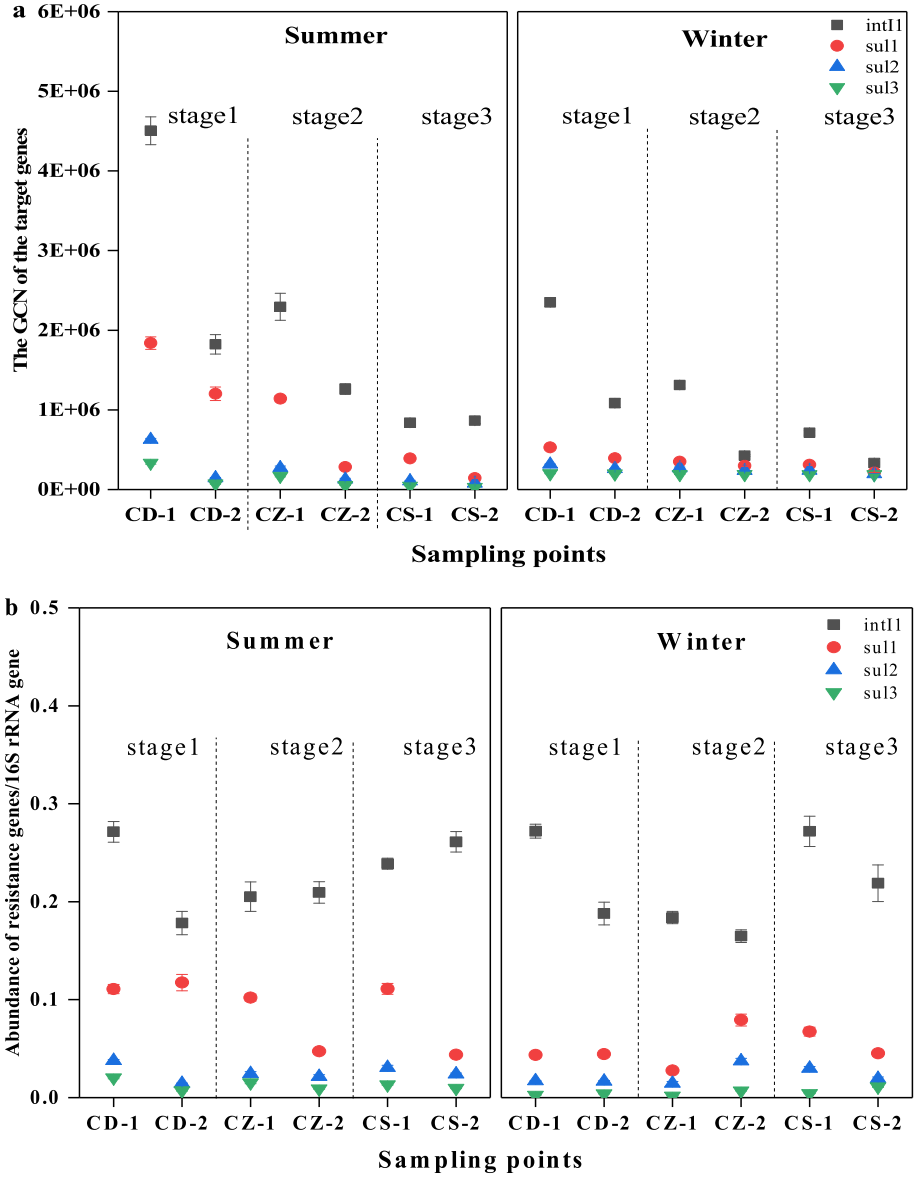

Fig. 2. The spatiotemporal distribution of ARGs in the VUSFCW. (a) The absolute abundance of ARGs. (b) The relative abundance of ARGs.

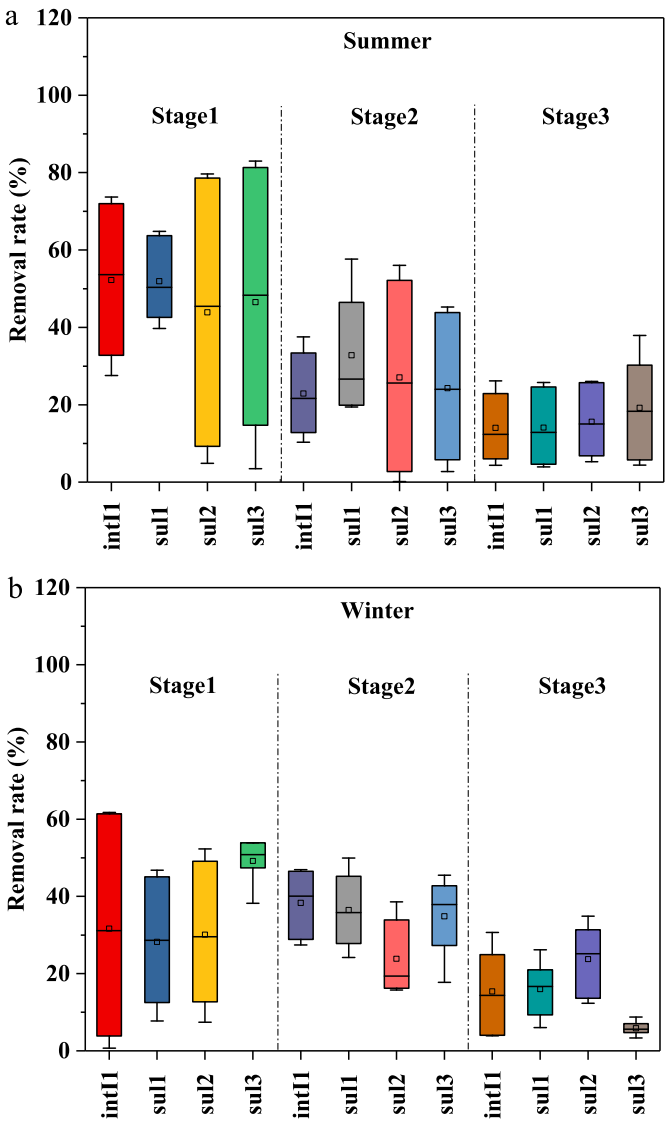

Fig. 3. The removal rate of ARGs at each stage (a) The removal rate of ARGs in summer. (b) The removal rate of ARGs in winter.

microorganisms in stage 1 and stage 2 were mainly anaerobic and facultative anaerobic microorganisms, such as *Truepera*, *Rivibacter* and *Desulfovibrio*.

Linear discriminant analysis effect size was used to identify the most differentially abundant microbial taxa in summer and winter (Fig. 4b). The microbial species with significance thresholds greater than 3.0 were recorded in linear discriminant analysis (LDA) (Fig. S3b, Supporting Information). The results showed that there were more microbial species in summer than in winter. *Anaerolineae*, *Holophagales*, *Chalmydiae* and *Methylococcales* were abundant in summer, and *Lachnospirales* and *Prevotellaceae* were abundant in winter. These taxa can be used as biomarkers of the corresponding constructed wetlands. In addition, the PLS-DA results of OTU data in different seasons showed that the sampling sites tended to gather together in the same season (Fig. S3c, Supporting Information), which meant that there were significant differences in the composition of microbial communities in different seasons (Adonis: $p < 0.05$).

3.3. The driving factors of ARGs spatiotemporal dynamics and horizontal gene transfer

Based on the PLSPM model, this study evaluated the influences of physical-chemical factors, microorganisms, antibiotics and MGEs on the distribution of ARGs in the VUSFCW. The goodness-of-fit (GoF) index of the analysis model was 0.712, which means that the prediction ability of the model was 71.2%, and the models had good prediction performance (GoF >0.7).

In this study, the physical-chemical factors mainly affected the distribution of ARGs through indirect paths. As shown in Fig. 5a, physical-chemical factors have a significant effect on the distribution of ARGs through the indirect path of antibiotics and MGEs. The results showed that the indirect influence coefficient of physical-chemical factors on the distribution of ARGs was 0.5 (Fig. 5b). Therefore, indirect effects are the main way for physical-chemical factors to affect the distribution of ARGs.

The influence of antibiotics on the distribution of ARGs is mainly realized through indirect effects. Antibiotics affect the distribution of ARGs by affecting microorganisms and MGEs (Fig. 5a), and the indirect coefficient of antibiotics on ARGs was 0.98 (Fig. 5b). The direct effect coefficient of antibiotics on the distribution of ARGs was only 0.021.

The influence of microorganisms on the distribution of ARGs is mainly indirect. Microorganisms indirectly affect the distribution of ARGs through MGEs (Fig. 5a). The indirect coefficient of microorganisms on ARGs distribution was 0.221 (Fig. 5b), and the direct coefficient was only 0.16.

MGEs had the greatest effect on the positive standardization of ARGs in the VUSFCW, reaching 0.895 (Fig. 5a). Previous studies have also shown that the *sul1* gene is often integrated into *intI1* with other resistance genes (Luo et al., 2010), which means that sulfonamide ARGs exist in microorganisms with MGEs as carriers. Therefore, MGEs had a strong direct effect on ARGs in this study.

3.4. The potential hosts of ARGs and their selection model for the microbial community

The correlation matrix between the total ARGs (*sul1*, *sul2*, *sul3* and *intI1*) and the microbial community in different seasons was built in the study. The correlation was visualized by network analysis (screening the elements with $R^2 > 0.7$ and $p < 0.05$). The results showed that *intI1* and *sul1* were the two largest nodes both in summer and winter. At the genus level, 23 genera were detected in summer and winter, 35 genera existed only in summer and 27 genera existed only in winter. *Azohydromonas*, *Acidovorax*, *Desulfovibrio* and *Lentimicrobium* were all highly correlated with the four ARGs in summer, *Dechloromonas, Anaerolinea, Lentimicrobium* and *Sulfuritalea* were highly correlated with the four ARGs in winter. At the phylum level, *Proteobacteria*, *Bacteroidota*, *Patescibacteria* and *Firmicutes* were the main microbial species (phyla) having high correlation with the total ARGs in summer, accounting for 44.17%, 12.5%, 9.17% and 8.33% respectively (Fig. 6a). In winter, the microbial species (phyla) with high correlations with the total ARGs were mainly *Proteobacteria*, *Bacteroidota*, *Chloroflexi* and *Desulfobacterota* (Fig. 6b), accounting for 28.37%, 16.31%,10.64% and 9.93% respectively. This result meant that these microbes may be the main potential hosts of ARGs. A number of additional differences in ARGs and microbial diversity were observed. In summer, among the main potential hosts of *intI1* and *sul1*, *Proteobacteria* and *Bacteroidota* accounted for approximately 40% and 14% (Table S6, Supporting Information), which was similar to that of the total ARGs.

The correlation between ARGs and pathogens bacteria was investigated in this study. The sequencing results showed that five pathogenic bacteria (genera) were found in this study (*Legionella, Escherichia, Staphylococcus, Mycobacterium and Klebsiella*). Among them, *Klebsiella* had a high relative abundance. Correlation analysis showed that *Klebsiella* and ARGs were significantly correlated (positive) in summer (Table 2). In winter, *Klebsiella* had a significant positive correlation with *intI1* and *sul1* (Table 2). This result implied that *Klebsiella* may be a potential host of ARGs.

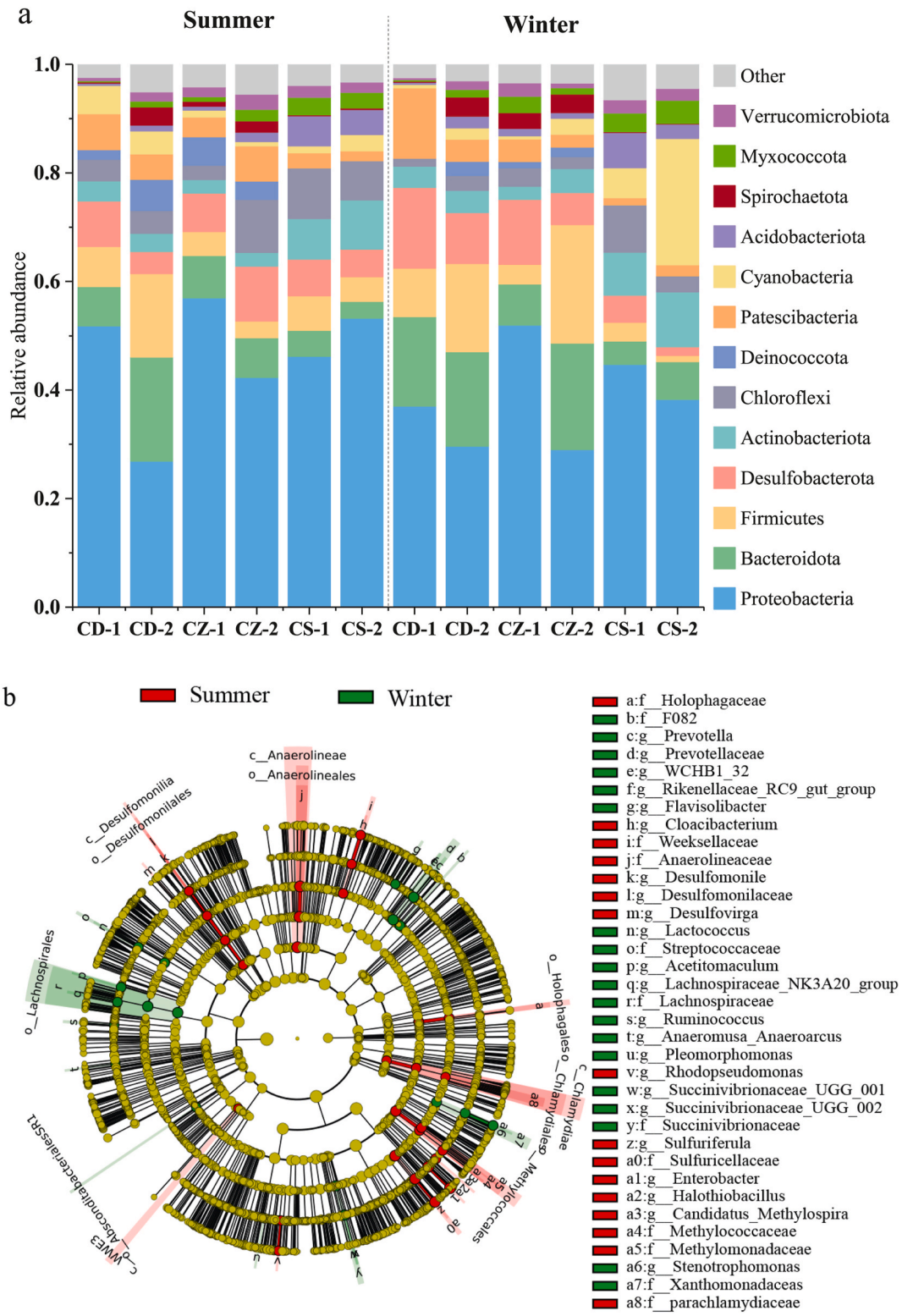

Fig. 4. Microbial community structure. a: Relative abundance of microorganisms (phyla) in summer and winter; b: LEfSe analysis results of constructed wetlands in summer and winter.

4. Discussion

In subsurface flow constructed wetland, physical-chemical factors, antibiotics, microbial community and MGEs are regarded as the factors influencing the spatiotemporal dynamics of ARGs. The results of this study showed that there is a significant correlation between MGEs and ARGs, which mainly influences the spatiotemporal dynamics of ARGs through direct pathway. Physical-chemical factors and antibiotics indirectly influence the spatiotemporal dynamics of ARGs through MGEs. However, the direct and indirect influences of microorganisms on the spatiotemporal dynamics of ARGs were relatively small.

Physical-chemical factors mainly influence the spatiotemporal

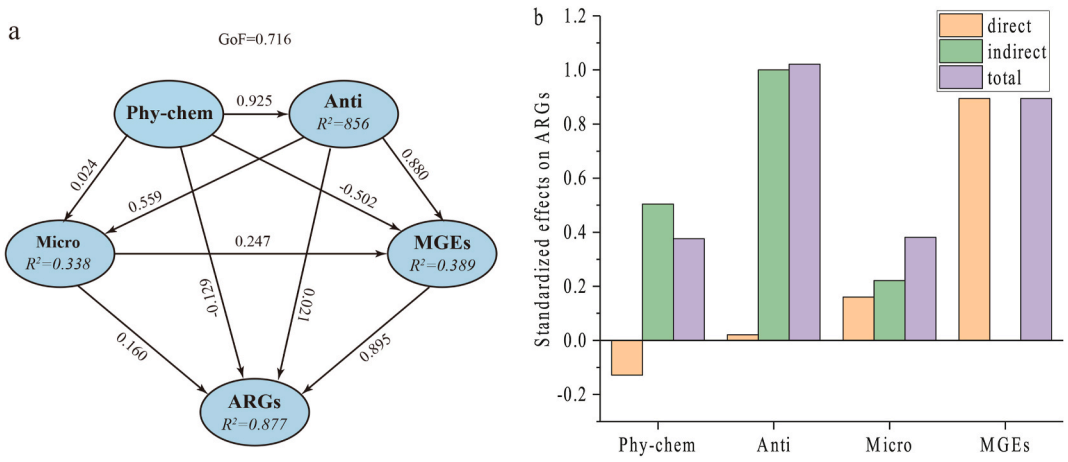

Fig. 5. The influencing factors of ARGs distribution. a. Analysis results of PLSPM in the VUSFCW; b. Standardized direct and indirect effects on the ARG abundance in the VUSFCW. "Phy-chem" stands for the physical-chemical factor module; "Anti" stands for the antibiotic module; "Micro" stands for the microbial community module and "MGEs" stands for the mobile genetic elements' module.

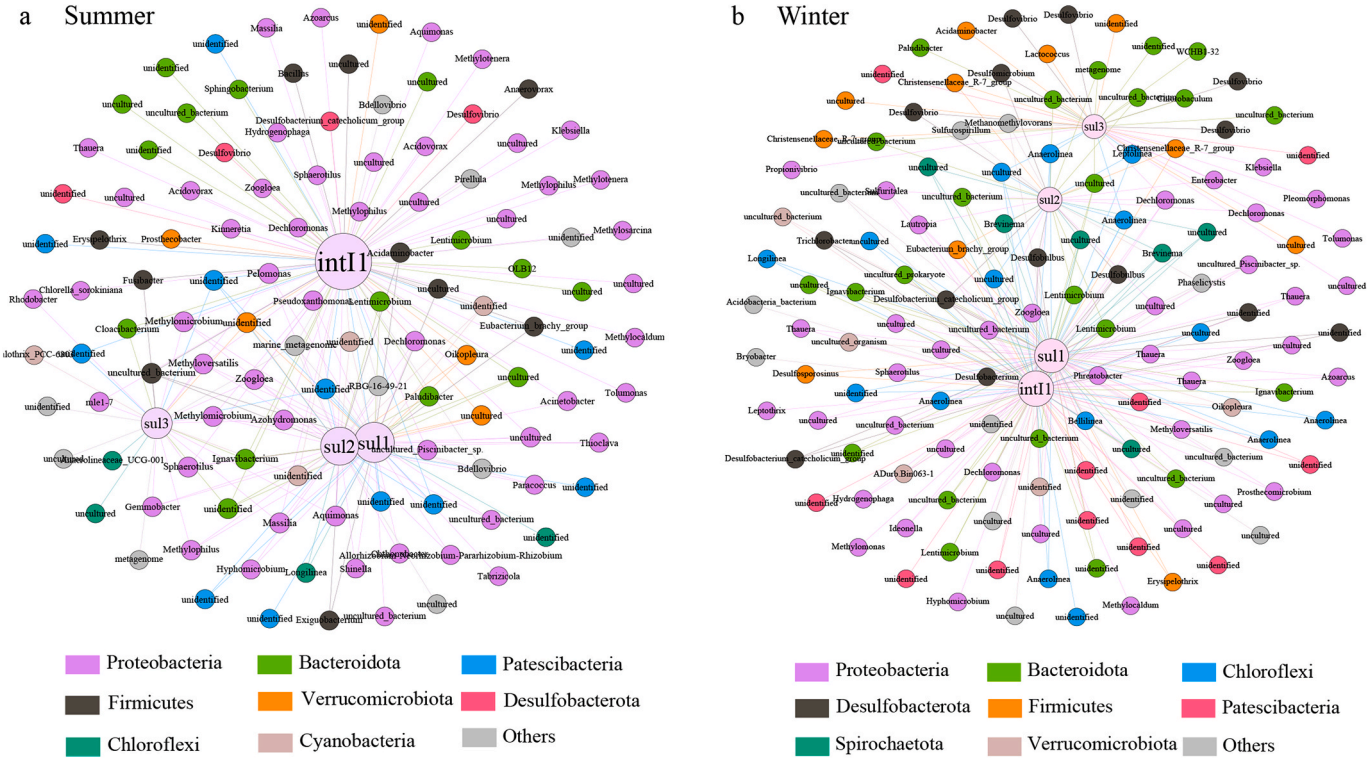

Fig. 6. Co-occurrence between ARGs and their potential microbial hosts.

dynamics of ARGs through indirect pathways, while the influence on the spatiotemporal dynamics of antibiotics occurs through direct pathways. The physical-chemical factors inside the constructed wetland influence the dynamic changes in antibiotics. pH, ORP and temperature influence the substrate adsorption of antibiotics and antibiotic hydrolysis in constructed wetlands, and nutrients, temperature and ORP influence the microbial degradation of antibiotics (Zhang et al., 2022). For example, stage 3 was in an oxidative environment, while stage 2 and stage 1 were mostly in a reductive environment or weakly oxidative environment in this study (Fig. S4, Supporting Information). Stage 3 was dominated by aerobic microorganisms, while stage 2 and stage 1 were dominated by anaerobic and facultative anaerobic microorganisms (Ishii et al., 2014; Padilla-Córdova et al., 2018). Therefore, the physical-chemical factors have a significant effect on the spatiotemporal dynamics of antibiotics.

In this study, antibiotics significantly affected the spatiotemporal dynamics of MGEs. However, antibiotics have no direct influence on the spatiotemporal dynamics of ARGs. Similar to previous studies, there was no significant correlation between antibiotics and ARGs. This result may be due to the following factors: 1) antibiotics can exert selective pressure on bacteria, resulting in an increase in the population of antibiotic resistant bacteria and with it ARGs However, an antibiotic concentration that is too high may lead to the death of microorganisms carrying ARG (Li et al., 2019); 2) heavy metals in soil can also induce ARGs diffusion (Wu et al., 2015). Therefore, antibiotics seem unlikely to directly influence the dynamics of ARGs in this study.

MGEs have a significant direct influence on the spatiotemporal dynamics of ARGs. Previous studies also showed that *intI1* plays the most important role in affecting dynamics of ARGs (Bengtsson-Palme et al.,

Table 2
The correlation between ARGs and pathogenic bacteria.

	ARGs		Klebsiella	Legionella	Escherichia	Staphylococcus	Mycobacterium
Summer	**intI1**	R^2	0.886**	−0.543	0.372	0.500	−0.771*
		p	0.009	0.133	0.234	0.156	0.036
	sul1	R^2	0.943**	−0.600	0.541	0.294	−0.543
		p	0.002	0.104	0.134	0.286	0.133
	sul2	R^2	0.943**	−0.600	0.372	0.500	−0.829*
		p	0.002	0.104	0.234	0.156	0.021
	sul3	R^2	1.000**	−0.771*	0.541	0.500	−0.714
		p	0.000	0.036	0.134	0.156	0.055
Winter	**intI1**	R^2	0.829*	−0.812*	0.131	−0.393	−0.812*
		p	0.021	0.025	0.402	0.221	0.025
	sul1	R^2	0.657*	−0.812*	0.131	−0.393	−0.812*
		p	0.038	0.025	0.402	0.221	0.025
	sul2	R^2	0.657	−0.812*	0.131	−0.393	−0.812*
		p	0.078	0.025	0.402	.221	0.025
	sul3	R^2	0.371	−0.870*	−0.131	0.131	−0.986**
		p	0.234	0.012	0.402	0.402	0.00

2018; Hu et al., 2020). The reason is mainly that MGEs mediated horizontal gene transfer is the main mechanism to promote the spread of ARGs in different aquatic ecosystems (Ju et al., 2019; Liao et al., 2018). In this study, the absolute abundance of *sul1* was much higher than that of *sul2* and *sul3*. *Sul1* is part of a conserved fragment in *intI1*, which can exist in plasmids or transposons (He et al., 2018). This feature may be the theoretical basis for the direct influence of MGEs on ARGs. Therefore, MGEs had a strong direct effect on ARGs in this study.

The microbial community had no direct influence on the dynamic changes in ARGs in this study. This finding may be related to the selection mode of ARGs for microbial hosts. This study found that the distribution of potential host microorganisms was basically consistent with the distribution of the major microorganisms in UVSFCW (Table S7, Supporting Information). There was no significant difference between the main microbial species of the microbial community structure and the main potential host microorganisms ($p > 0.05$). This means that the selection pattern of ARGs for potential hosts may be non-specific. The non-specific selection mode of ARGs to potential hosts may be related to the ubiquitous presence of *intI1* in microbial communities. Because of the close relationship between ARGs and *intI1*, integrons carrying recombinant gene cassettes are randomly inserted into transposons or conjugated plasmids (Liu et al., 2014), and move in different bacteria to spread drug resistance. Additionally, integrons can capture and excise gene cassettes (Hall and Collis, 1995). One integron can capture one or more gene cassettes, and the same gene cassettes can be integrated into different integrons, which leads to the movement of gene cassettes and the rapid propagation and proliferation of ARGs among various microbial categories through horizontal gene transfer (Bondarczuk and Piotrowska-Seget, 2019; Hou et al., 2021). This may be the main mechanism of non-specific selection of ARGs to potential hosts. The non-specific selection mode of ARGs to microbial hosts indicates that even dynamic changes in the microbial community make it difficult to cause dynamic changes in ARGs. Therefore, the direct and indirect influences of microbial community on the dynamic changes in ARGs are small.

It should be noted that the soil-sand layer (stage 3) has the risk of increasing the relative abundance of ARGs. Previous studies have also reached similar conclusions (Huang et al., 2017b; Song et al., 2018b). This risk may be related to the following factors: First, the release of rhizosphere oxygen and secretions increases the survival rate of pathogens in this area, thereby increasing the abundance of ARGs (Vivant et al., 2016). Second, microorganisms are rich in the soil, and ARGs may spread to other microorganisms through horizontal gene transfer (Tang et al., 2020). Third, in addition to sulfonamides, heavy metals such as Cu, Hg and Cd in soil can induce ARGs (Wu et al., 2015), because many ARGs and heavy metal resistance genes are located on the same MGEs (Sun et al., 2019). Fourth, the concentration of antibiotics in stage 3 was relatively low, and a low concentration of antibiotics (clearly below known MICs) is more conducive to promoting an increase in the population of antibiotic resistant bacteria and with their ARGs (Jutkina et al., 2018). Therefore, the soil-sand layer has the risk of increasing the relative abundance of ARGs. However, our results suggested that VUSFCW has a good ability to remove ARGs and can fully meet the need of controlling ARGs diffusion. Stage 1 and stage 2 are the main stages of ARGs removal. The main reasons may be as follows: On the one hand, stage 1 and stage 2 have relatively low dissolved oxygen levels. Long-term low DO operating conditions may inhibit the emergence and distribution of ARGs by inhibiting plasmid reproduction (Ma et al., 2018). On the other hand, the processes of bacterial removal in constructed wetlands include filtration, adsorption and aggregation of substrates, biofilm microorganisms and macrophytes (Wu et al., 2016), and filtration through constructed wetland substrates has been shown to be a significant removal process for faecal indicator bacteria (Santos et al., 2019). It is worth noting that plants can affect the removal of ARGs. Plants release secretions through their roots, which can provide nutrients for the survival and reproduction of microorganisms or regulate microbial populations (Hijosa-Valsero et al., 2011; Huang et al., 2019; Liu et al., 2014). Plants also remove some ARGs and nutrients, but relevant studies have shown that the contribution is small (Chen et al., 2016b; Vymazal et al., 2020). Previous studies also suggested that plant roots affect ARGs removal mainly through indirect ways, that is, by changing the physical and chemical properties of rhizosphere (Huang et al., 2019). Therefore, there are still some controversies about the influence of plants on ARGs in constructed wetlands. Unfortunately, the influence of plants on ARGs has not been studied separately in this paper, and we will conduct this study in the future.

Based on the mechanism of multiple influencing factors affecting the distribution of ARGs through MGEs and the co-metabolism mechanism of nutrients and antibiotics, improving the nutrient removal rate may be an effective way to control ARGs (Y. Y. Chen et al., 2019; Wang et al., 2021). Previous research results showed that compared with other types of constructed wetlands, VUSFCW has significant advantages in removing nutrients and ARGs (Chen et al., 2016b). Providing sufficient hydraulic retention time can also ensure the removal effect of nutrients (Chen et al., 2016a). In addition, adsorption is a main way to remove ARGs. The selection of substrates with large specific surface areas can provide a large number of adsorption sites for ARGs hosts. A multi-layer substrate may be selected as a suitable substrate for removing ARGs due to its high adsorption capacity, effectively preventing the blockage of the constructed wetland (Ding et al., 2018). It is worth noting that the specific improvement measures need to be further studied because the removal of ARGs by constructed wetland is a complex process with many influencing factors.

5. Conclusion

Based on the spatiotemporal dynamics of ARGs, we analyzed the driving factors of ARGs dynamics change and the selection mode of ARGs by hosts. The results showed that the absolute abundance of ARGs in the VUSFCW decreased stage by stage, but the soil layer (stage 3) had the risk of increasing the relative abundance of ARGs. Physical-chemical factors, antibiotics and microorganisms indirectly influenced the spatiotemporal dynamics of ARGs through MGEs. The selection mode of ARGs to microbial hosts was non-specific. Using VUSFCW, choosing substrates with large specific surface area and providing sufficient hydraulic retention time may be effective measures to control the diffusion of ARGs.

Credit author statement

Ling Zhang: Software, Methodology, Writing – original draft, Investigation, Visualization, Writing – original draft preparation. **Changzhou Yan**: Conceptualization, Writing – review & editing, Project administration, Funding acquisition. **Dapeng Wang**: Resources. **Zhuo Zhen**: Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.119176.

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