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RESEARCH ARTICLE

Effect of the ultraviolet/chlorine process on microbial community structure, typical pathogens, and antibiotic resistance genes in reclaimed water

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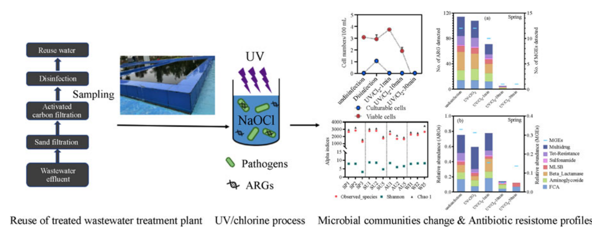
HIGHLIGHTS

- UV/chlorine can effectively remove VBNC pathogens, ARGs and MGEs in reclaimed water.
- Microbial community was changed with reduced diversity during UV/chlorine process.
- CRBs-carried MGEs were the predominant groups during UV/chlorine process.
- No direct co-selection strategy was shared between UV/chlorine and resistome.

GRAPHIC ABSTRACT



ABSTRACT

Urban wastewater contains a wide range of pathogens and antibiotic resistance genes (ARGs), which are a serious concern if reusing treated wastewater. However, few studies have explored the microbial communities in reclaimed water using ultraviolet (UV)/chlorine treatment and assessed the changes of the resistome. This study investigated the occurrence of typical pathogens, ARGs, and bacterial communities in UV/chlorine-treated reclaimed water samples. The numbers of culturable and viable but non-culturable pathogens were effectively reduced to 0 CFU/mL within 1–10 and 10–30 min after UV/chlorine treatment, respectively. Meanwhile, the physicochemical indices of water quality were not affected. UV/chlorine treatment could significantly change the bacterial community structure of reclaimed water, showing a decrease in bacterial abundance and diversity. Chlorine-resistant *Acinetobacter* and *Mycobacterium* were the dominant bacterial genera (>50%) after UV/chlorine treatment. Moreover, the number of ARGs and mobile genetic elements (MGEs) decreased with an increase in UV/chlorine exposure. However, eight ARGs and three MGEs were consistently detected in more than three seasons, making these major concerns because of their potential role in the persistence and dissemination of antibiotic resistance. Overall, the results of this study suggest that UV/chlorine treatment can potentially improve the microbiological safety of reclaimed water. And more attention should be paid to the pathogens that are both chlorine-resistant and carry MGEs because of their potential for resistance transmission.

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1 Introduction

Reusing treated wastewater (RTWW) is an important solution to solve the water shortage issue (Becerra-Castro et al., 2015; Sorinolu et al., 2021). Reclaimed water has widespread applications, including agricultural irrigation, water recharge, and use as non-potable urban water (e.g., for landscape irrigation use) (Hong et al., 2018; Sorinolu et al., 2021). Previous studies have shown that wastewater treatment plants can not completely remove emerging contaminants, such as pathogenic bacteria and antibiotic resistance genes (ARGs) (Pallares-Vega et al., 2019; Beattie et al., 2020; Sabri et al., 2020), which are of considerable concern for wastewater reuse because of their transmission potential (Gideon et al., 2009; Hong et al., 2018). For example, it has been shown that reusing water for spray irrigation might pose an additional risk of pathogens, including *Pseudomonas aeruginosa* (Evans et al., 1996) and *Staphylococcus aureus* (Wertheim et al.,

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2005). Unfortunately, for pathogens, only *Escherichia coli* is used as an indicator of fecal contamination; this may underestimate the risk of environmental pathogens due to their low correlation with other pathogens (Wang et al., 2012; Mraz et al., 2021). More specific detection of pathogenic bacteria is necessary for microbial safety evaluation of reclaimed water. In addition, it is difficult to remove ARGs with relatively high environmental persistence (Dodd, 2012; Zhang et al., 2021b), such as emerging contaminants, which are not adequately accounted for existing water reuse guidelines and standards. Given the risk that non-potable urban water from RTWW may come into contact with the human body directly or through aerosol generation (Goldmann, 2000), the potential microbial health risks posed by pathogens and bacterial antibiotic resistance should not be ignored.

Effective disinfection is a prerequisite for the microbial safety of reclaimed water, for which chlorination and ultraviolet (UV) radiation are widely used. Chlorination can not inactivate chlorine-resistant bacteria (CRB) completely, which may enrich this type of pathogen (e.g., *Pseudomonas*, *Acinetobacter*, and *Aeromonas*) (Shekhawat et al., 2020; Luo et al., 2021a, 2021b). Similarly, it has been reported that chlorine-resistant *Bacillus cereus* spores were only reduced by 1-log after 8.5 mg/L sodium hypochlorite (NaClO) treatment, whereas the inactivation rate of *E. coli* was more than 4-log (Zeng et al., 2020). Furthermore, the durability of UV irradiation is weak, and disinfection is sometimes incomplete, which might lead to the reactivation/regrowth of microorganisms (Tosa et al., 2003; Jungfer et al., 2007; McKinney and Pruden, 2012). In addition to the above mentioned shortcomings of disinfection in response to microbial contamination, both chlorination and UV irradiation have also been reported to induce pathogens into a viable but non-culturable (VBNC) state, where pathogens can be resuscitated and pose an infection risk (Zhang et al., 2015; Chen et al., 2018; Guo et al., 2019; Ye et al., 2020). It has been suggested that ARGs remain bioactive even though the host bacteria are fully inactivated (Dodd, 2012). For ARG elimination, an extremely high disinfection dose is required to achieve high removal efficiency (McKinney and Pruden, 2012; Iakovides et al., 2019; Wang et al., 2020). Hence, the currently used single disinfection technology is not economically friendly in the full-scale RTWW treatment process.

As a synergistic disinfection process that can increase disinfection efficiency (Liu et al., 2019; Wang et al., 2020), photolysis of chlorine can produce a variety of radicals (e.g., HO• and reactive chlorine species) (Fang et al., 2014), which have relatively strong pathogenic bacteria- and ARG- removal effects (Liu and Hu, 2020; Wang et al., 2020). Recent studies have also confirmed the excellent performance of UV/chlorine in eliminating DNA (Zhang et al., 2019b; Phattarapattamawong et al., 2021). Furthermore, several studies have confirmed that the UV/chlorine process shows excellent oxidation performance in removing chemical pollutants (Matamoros and Salvadó, 2013; Tian et al., 2020). Early studies on treating microorganisms using UV/chlorine have mostly focused on model bacteria (*P. aeruginosa* and *E. coli*) (Jennie et al., 2008; Wang et al., 2021), pure cultures (Li et al., 2018), or simulated systems (Liu et al., 2019). Unfortunately, very little is known about the changes in microbial communities in reclaimed water and the effects of effluent on microorganism removal during UV/chlorine treatment. In addition, the evaluation of ARGs in previous studies was limited to the selection of specific target genes. Nevertheless, the throughput was too low to comprehensively evaluate the occurrence of ARGs in reclaimed water. Fortuitously, recent studies have demonstrated the effectiveness of high-throughput quantitative PCR (HT-qPCR) in solving this issue (Wan et al., 2019; Yang et al., 2020). However, mechanistic insights into the performance of UV/chlorine treatment in the microbiological safety control of reclaimed water remain lacking.

This study aimed to investigate the efficiency of UV/chlorine treatment on pathogen inactivation and elimination of ARGs as well as the spatiotemporal changes in the bacterial communities in actual reclaimed water. Culturable and total pathogens were detected by heterotrophic plate counting (HPC) and qPCR methods, respectively. VBNC pathogens were identified by combining the results of the two methods. High-throughput sequencing (HTS) was used to investigate the bacterial community structure and its relationship with the UV/chlorine process. Furthermore, to achieve an integrated evaluation of the occurrence of ARGs and mobile genetic elements (MGEs) in reclaimed water, HT-qPCR with 296 validated primers was used to analyze the abundance and diversity of the resistome in the water samples. This investigation could improve the understanding of the feasibility of UV/chlorine treatment for microbial safety control of reclaimed water.

2 Materials and methods

2.1 Plant configuration and sample collection

In this study, a full-scale wastewater treatment plant with a daily sewage capacity of 3000 t in Fujian Province, China, was selected. The source of sewage was domestic wastewater from the approximately 20000 residents. The following treatment tanks were used sequentially: regulating tank, anoxic tank, biological contact oxidation tank, and inclined-tube sedimentation tanks (wastewater effluent). Advanced treatment processes were also used, including sand filtration, biological activated carbon

(BAC) filtration, and UV-ClO_2 disinfection (UV dose: 20 mJ/cm^2; residence time = 30 min). After the advanced treatment processes, reclaimed water effluent was mainly used for landscape irrigation. The details of the wastewater reuse process and sampling points are shown in Fig. S1. Considering the possible differences in the spatiotemporal variation of the bacterial community structure and antibiotic resistome in reclaimed water during the UV/chlorine process, sample collection was carried out in winter (January), spring (March), summer (May), and autumn (November) 2020. All water samples were pretreated within 24 h prior to the measurements. Undisinfected reclaimed water (BAC filtration effluent, 20 L) was sampled for UV/chlorine disinfection. Undisinfected reclaimed water and reclaimed water effluent (i.e., UV-ClO_2 effluent, 5 L) were used as the blank control and disinfection efficiency control, respectively. UV/chlorine-treated samples were collected at different reaction times (1, 10, and 30 min).

2.2 Experimental procedures for the UV/chlorine process

Undisinfected reclaimed water was used for the UV/chlorine disinfection experiment, which was conducted in a quartz container (Wang et al., 2021). The UV fluence rate was 0.2 mW/cm^2, measured by a UV-C irradiance meter (TN-2254, TRINR, China). Briefly, 250 mL of undisinfected reclaimed water was added to 6 mg/L (final concentration) NaClO solution. The reaction solutions were simultaneously exposed to UV irradiation (15 W, 254 nm; Philips, Netherlands) for 1, 10, and 30 min, and the system was quenched with 1 g/L $Na_2S_2O_3$.

2.3 Water quality analysis and culturable bacteria counting

Undisinfected reclaimed water, reclaimed water effluent, and UV/chlorine-treated water samples for 30 min were pretreated using a 0.45 μm microporous membrane filter (HAWG047S6, Merck Millipore Limited, Cork, Ireland) and analyzed for pH as well as NO_3^--N, NO_2^--N, NH_4^+-N, and total organic carbon (TOC) contents. The water pH was measured using a multiparameter meter (pH700, Hach Company, Loveland, CO, USA). The TOC was determined using a TOC instrument (TOC-V WP, Shimadzu, Kyoto, Japan). Other physicochemical indices were measured in the laboratory following the Methods for Monitoring and Analysis of Water and Wastewater (Fourth Edition) (China Environmental Science Press, 2002).

The total culturable bacteria were assayed using the HPC method on nutrient agar (NA) and Reasoner's 2A (R_2A) medium (Hopebio, China) at 37°C for 48 h and 28°C for 5 days in the dark, respectively. Two parallel groups were used in each sample. Pathogen culture medium was used to enumerate culturable fecal coliforms (MFC agar), *E. coli* (mTEC agar), *P. aeruginosa* (CN agar), *Shigella* spp. (SS agar), *Legionella pneumophila* (GVPC agar), and *S. aureus* (*Staphylococcus* agar) (Hopebio, China) (Guo et al., 2021). Fecal coliforms and *E. coli* were incubated at 45°C for 24 h in the dark with *P. aeruginosa* and *Shigella* spp. *S. aureus* and *L. pneumophila* were cultured at 37°C for 24 h in the dark.

2.4 DNA extraction and quantitative PCR

The water samples (1 L) from different treatment groups were concentrated using a 0.22 μm microporous membrane filter (GSWG047S6, Merck Millipore Limited, Cork, Ireland) and stored at −20°C prior to DNA extraction. Total genomic DNA was extracted using the FastDNA SPIN Kit (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. DNA concentration was quantified using a B-500 fluorometer (B-500, Metash Instruments Co., Ltd., China). Pathogenic primers were selected (Table S1) (including one total bacteria (16S rRNA) and nine representative waterborne pathogens, i.e., *P. aeruginosa*, *S. aureus*, *E. coli*, *Salmonella* spp., *L. pneumophila*, *Shigella* spp., *Yersinia*, *Vibrio cholerae*, and *Mycobacterium avium*) for qPCR analysis. The qPCR system contained 10 μL of 2 × PerfectStart Green qPCR SuperMix (PerfectStart Green qPCR SuperMix, TransGen Co., Ltd., China), 0.4 μL of each primer (10 μM), 2 μL of template DNA, and 7.2 μL of DNA-free water (final volume: 20 μL). The program consisted of a pre-denaturation step at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 60 s using an ABI Q6 system (ABI Q6, Life Technologies Holdings Pte., Ltd., Singapore). Each target gene was run in triplicate. The standard curves were pretreated from 10-fold serial dilutions of plasmid standards that carried the target genes (Table S1). For comparison, a negative control was set using DNA-free water as the DNA template. Conversion of specific gene copies to viable cell numbers was established by quantitatively correlating the copy numbers of specific genes and bacterial cell numbers in a series of diluted bacterial cultures (Table S2) (Guo et al., 2021).

2.5 High-throughput sequencing

Sequencing analysis of samples was performed using the Illumina NovaSeq platform (Illumina, San Diego, CA, USA). Briefly, quality-controlled genomic DNA (1 ng/μL) was amplified using bacteria-specific primers (338 F/806 R) containing a barcode. The PCR products were detected by electrophoresis on a 2% agarose gel and recovered using a gel recovery kit (Qiagen, Hilden, Germany). A TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina) was used to construct the library. After the library was qualified, NovaSeq6000 was used for sequencing (Novogene Science and Technology Co., Ltd., Nanjing, China).

2.6 High-throughput qPCR

HT-qPCR was performed to quantify the profiles of ARGs and MGEs using SmartChip Real-Time PCR System (WaferGen Bio-systems, Fremont, CA, USA), which can simultaneously process 5184 qPCR reactions. A total of 296 primer sets (Table S3) was used to amplify the target genes (283 ARGs, 12 MGEs, and a 16S rRNA gene) (Zhu et al., 2013). The HT-qPCR system contained a LightCycler 480 SYBR Green I Master (Roche Applied Sciences, Indianapolis, IN, USA), DNA templates, primers, and nuclease-free water. A SmartChip Multi-Sample NanoDispenser (TaKaRa Bio Inc., Shiga, Japan) was used to dispense the system into a 5184-well chip. The HT-qPCR program consisted of a pre-denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, and annealing at 60°C for 30 s using SmartChip Cycler (TaKaRa Bio Inc., Japan) (Wan et al., 2021). Negative controls consisted of a nuclease-free water template. Melting curve analysis was automatically generated using WaferGen software (WaferGen Bio-systems, USA). Each target gene was run in triplicate. The HT-qPCR results were analyzed using SmartChip qPCR software (version 2.7.0.1). Multiple melting peaks and amplification efficiencies beyond the range (1.8–2.2) were discarded, and a threshold cycle of 31 was set as the detection limit. The HT-qPCR results were used for further data analysis only if both conditions were met. Relative gene copy numbers were calculated as follows: relative ARG/MGE copy numbers = gene copy numbers of each ARG or MGE/gene copy number of 16S rRNA. The absolute abundance of 16S rRNA was determined by the standard curve method of quantification (Roche 480, Indianapolis, IN, USA). Absolute abundance of ARG/MGE = relative abundance of each ARGs or MGE * absolute abundance of 16S rRNA (Wan et al., 2021).

2.7 Statistical analysis

The data were plotted in Prism 8.0 and processed using *R* studio, SPSS 16.0, and Prism 8.0 software. For the HTS results, UPARSE v7.0.1001 was used for OTU clustering. Principal coordinates analysis (PCoA) was used to study community differences. Mothur was used for species annotation according to the SSUrRNA database, and Tax4Fun was used for functional annotation.

3 Results and discussion

3.1 Conventional water quality changes during UV/chlorine treatments

The physicochemical parameters of undisinfected reclaimed water, reclaimed water effluent, and UV/chlorine 30 min-treated water samples are summarized in Table S4. On the one hand, compared with the actual disinfection effect (UV-ClO_2), the average removal of TOC decreased significantly (36.3%) after UV/chlorine treatment, indicating that UV/chlorine treatment can further reduce the level of organic matter in reclaimed water. On the other hand, UV/chlorine treatment also showed better performance in nitrogen removal (e.g., NO_2^--N, NO_3^--N, and NH_4^+-N). Thus, conventional water quality can not be affected by UV/chlorine technology in practical applications. The effective removal of carbon- and nitrogen-containing nutrients contributes to controlling the biological stability of drinking water (Hijnen et al., 2018). The good performance of UV/chlorine in removing chemical contaminants has been demonstrated in previous studies (Michael et al., 2012; Matamoros and Salvadó, 2013), which also supports our results.

The HPC method was used to count culturable bacteria, which is the most common water quality indicator in microbiology. The inactivation of culturable total bacteria and six typical opportunistic pathogens (OPs) during UV-ClO_2 and UV/chlorine treatment is shown in Fig. 1. The pathogenic bacteria in reclaimed water was low (i.e., total culturable cells: 2–3 log CFU/mL; culturable pathogens: 0–2 log CFU/100 mL). After UV-ClO_2 treatment, there was only a slight decrease in culturable total bacteria, only within an order of magnitude of change. In contrast, UV/chlorine inactivated almost all culturable total bacteria within 1 (spring and summer samples) and 10 min (autumn and winter samples). In particular, all six selected OPs reached 0 CFU during the UV/chlorine process within 10 min (*L. pneumophila* was not detected in any of the samples, data not shown). In previous studies, good performance of the UV/chlorine process in the removal of OPs has been reported in simulated drinking water distribution systems (Liu et al., 2019). Wang et al. (2021) reported a similar result for the inactivation of *P. aeruginosa* in phosphate buffer solution system. Our results further confirmed that UV/chlorine had a superior sterilization effect on the OPs in reclaimed water. Notably, in the autumn samples, the CFU of *P. aeruginosa* increased even after UV-ClO_2 treatment. This problem of CRB has been reported in many studies (Zhang et al., 2019a; Zeng et al., 2020; Luo et al., 2021b). Comparatively, *P. aeruginosa* was inactivated by UV/chlorine treatment, indicating its high efficiency in CRB removal. *L. pneumophila* was not detected in reclaimed water in the present study as a commonly detected bacterial species in water supply networks (Garrison et al., 2016).

It should be clarified that the antibacterial effect of UV/chlorine treatment should be evaluated objectively. The experimental laboratory conditions of the actual reclaimed water samples are controllable and relatively stable, but actual sewage treatment plant management might be defective and non-standard. In the discussion that follows, the possible application of UV/chlorine is discussed under set experimental conditions, and it is noted that the

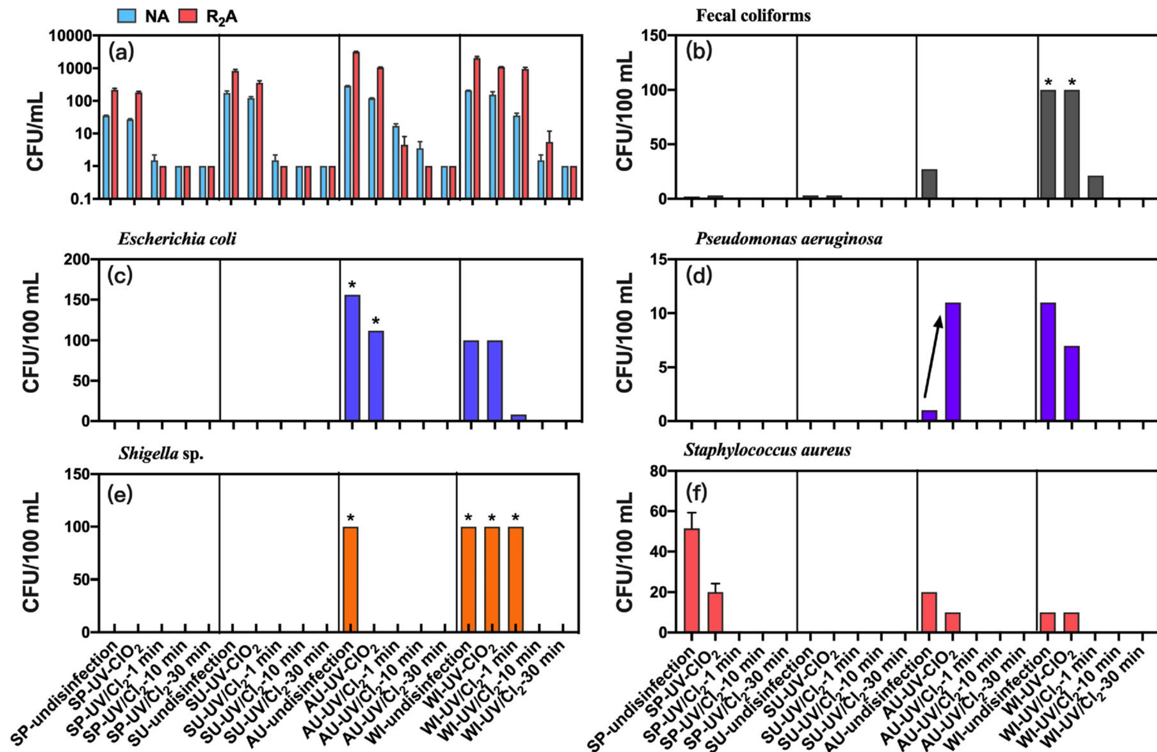

Fig. 1 Removal efficiency of culturable pathogens by UV-ClO_2 and UV/chlorine process. (a) total culturable bacteria (NA and R_2A), (b) culturable Fecal coliforms (MFC agar), (c) *E. coli* (mTEC agar), (d) *P. aeruginosa* (CN agar), (e) *S. aureus* and (f) *Shigella* spp. (SS agar). The "*" indicates that CFU/100 mL > 100. *L. pneumophila* was not detected in any of the samples (Figure no shown). Conditions: $[Cl_2]_0$ = 6 mg/L. SP: spring samples; SU: summer samples; AU: autumn sample. WI: winter samples.

practical application of UV/chlorine needs to be further verified by field experiments.

3.2 The occurrence of pathogenic microorganisms during UV-ClO_2 and UV/chlorine treatments

In addition to the culturable pathogens, the occurrence of nine typical waterborne pathogens during UV-ClO_2 and UV/chlorine treatments was investigated by qPCR (Fig. 2). The absolute abundance of pathogens was calculated based on the standard curves (Table S1). Figure 2(a) shows that all pathogens were detected at different levels in undisinfected reclaimed water and UV-ClO_2 effluent, except for *L. pneumophila* and *M. avium*. In particular, *E. coli*, *Shigella* spp., and *V. cholerae* were detected in all four seasons. Various pathogens exist in reclaimed water, and conventional disinfection can not effectively remove them. This phenomenon suggests that the health risks posed by pathogenic bacteria in the RTWW system should receive more attention. Moreover, the highest levels of *E. coil* (6.52 log copies/100 mL), *Shigella* spp. (5.33 log copies/100 mL), *P. aeruginosa* (5.16 log copies/100 mL), and *S. aureus* (4.76 log copies/100 mL) were found in the WI samples in UV-ClO_2 effluent. This indicates that UV-ClO_2 disinfection may increase the abundance of specific pathogens. In this study, UV/chlorine treatment effectively reduced the absolute abundance of most pathogens (Fig. 2(a)). In particular, 30 min of UV/chlorine treatment could reduce the number of pathogenic bacteria below the qPCR detection limit, except for *E. coli*. The disinfection performance of UV/chlorine differed for the reclaimed water samples in different seasons. However, there was no seasonal regularity, which was more closely related to the initial levels of bacteria, which will be discussed in detail in the following section.

In contrast to the trend of absolute abundance, the relative abundance of OPs showed an increasing trend following UV/chlorine disinfection (e.g., *P. aeruginosa* and *E. coli*) (Fig. 2(b)). In other words, some pathogenic bacteria may be chlorine-resistant and are more likely to survive in the community after UV/chlorine treatment. For example, Jia et al. (2015) reported that the relative abundance of *Pseudomonas* and *Sphingomonas* increased in drinking water disinfected via chlorination. A similar result was observed using 2 and 5 mg Cl_2/L in the reverse osmosis treatment system of municipal wastewater (Luo et al., 2021a). However, the "enrichment" of pathogenic bacteria by the disinfection process might also lead to misjudgment of disinfection efficiency. The absolute number of OPs is the basis for causing disease; the

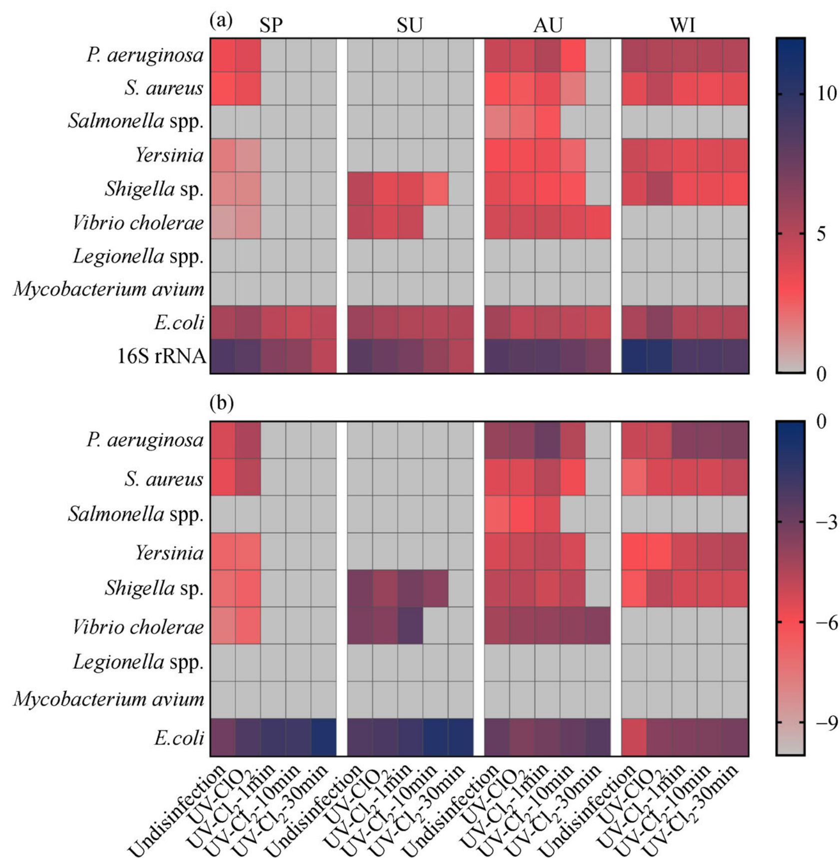

Fig. 2 Quantitative PCR results on 16S rRNA gene and genes of nine typical pathogens with UV-ClO_2 and UV/chlorine treatment. (a) Absolute quantitative results. Value = lg (gene copies/100 mL); (b) Relative quantitative results. 16S rRNA gene is the internal reference, and value = lg (pathogenic gene copies/16S rRNA gene copies). Conditions: $[Cl_2]_0$ = 6–7 mg/L. SP: spring samples, SU: summer samples, AU: autumn samples. WI: winter samples. All values are expressed by three parallel mean values.

absolute number of OPs is more appropriate as the evaluation standard from the perspective of disease prevention and control. In fact, the absolute number of OPs, which is a better indicator for evaluating the performance of UV/chlorine processes, was significantly reduced after treatment.

3.3 Survival of VBNC bacteria during UV-ClO_2 and UV/chlorine treatments

The relationship between the specific gene copy and culturable bacteria numbers was assessed to define the standard curve (Table S2) in the current study. We used the curve to convert the target-specific gene copy numbers of OPs to viable cell numbers. The number of VBNC bacteria discussed was defined by comparing the difference between viable and culturable cells (Guo et al., 2019, 2021). According to the results shown in Fig. 3, only autumn and winter samples were individually identified as culturable *E. coli*, *P. aeruginosa*, and *Shigella* spp. (blue column) in undisinfected reclaimed water and UV-ClO_2 effluent. However, high concentrations of viable OPs were ubiquitous in almost all water samples in different seasons, indicating that these OPs were almost in the VBNC state. Specifically, the numbers of VBNC *E. coli*, *P. aeruginosa*, and *Shigella* spp. in undisinfected reclaimed water were ~5–6 log cells/100 mL, 2–3 log cells/100 mL, and 3–4 log cells/100 mL (black circle), respectively. UV-ClO_2 treatment had little effect on removing VBNC OPs, and in some cases, the levels of WI-*E. coli*, SP-*P. aeruginosa* and WI-*Shigella* spp. increased after UV-ClO_2 treatment. Conventional disinfection seems to pose serious hidden risks in the control of VBNC pathogens.

As shown in Fig. 3 (red column), UV/chlorine treatment led to a significant reduction in VBNC *P. aeruginosa*, *S. aureus*, and *Shigella* spp. levels, except in winter samples. Increasing the UV/chlorine treatment time decreased the number of VBNC cells below 0 CFU. Comparatively, UV/chlorine did not have a good removal efficiency for VBNC *E. coli* and *P. aeruginosa* in the WI samples, and similar results were found with UV-ClO_2 disinfection. Combined with previous results on the occurrence of pathogenic

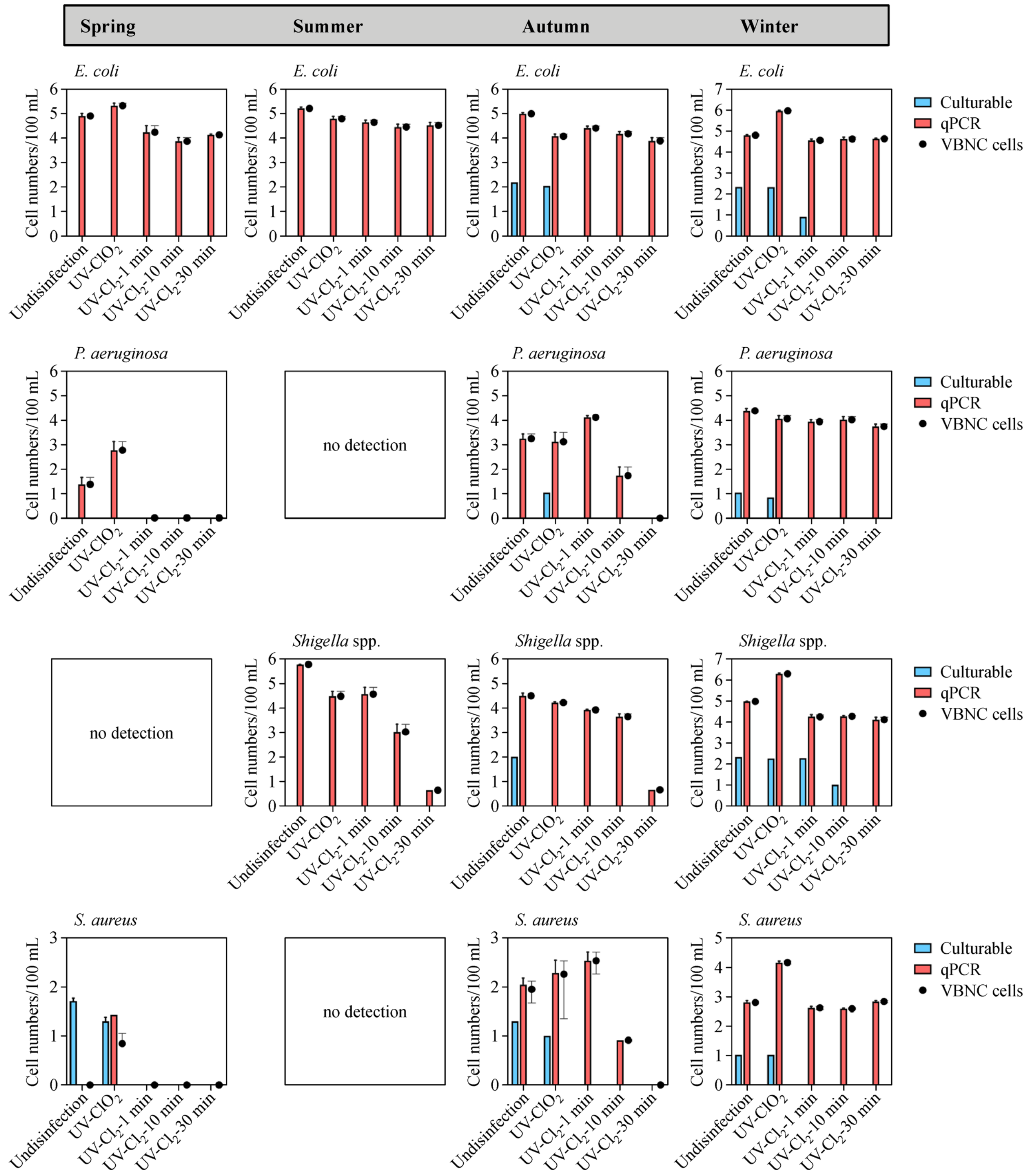

Fig. 3 Enumeration of VBNC *E. coli*, *P. aeruginosa*, *Shigella* spp. and *S. aureus* with UV-ClO_2 and UV/chlorine treatments. The culturable cells were detected using the selective medium. Viable cells were converted to the target specific gene copies of OPs from reclaimed water. VBNC OPs numbers = viable cell numbers- culturable cell numbers.

bacteria (Fig. 2), the reasons for this were summarized as the following two aspects: 1) the total amount of bacteria was large (i.e., 10–11 log gene copies/100 mL, Fig. S2) in winter samples, which was 1–2 orders of magnitude higher than that from the spring, summer, and autumn samples; and 2) the water quality in winter was inferior. Both these factors contributed to accelerated chlorine decay (Fig. S3). As a result, radical formation was reduced, and synergistic disinfection could not work well, resulting in the low removal efficiency of OPs and VBNC cells. Therefore, to

ensure the effective removal of VBNC OPs, a certain amount of residual chlorine and appropriate bacterial numbers are prerequisites.

3.4 Dynamic changes of microbial communities during UV-ClO_2 and UV/chlorine treatment

HTS was conducted to obtain more information on the dynamic changes in microbial communities during UV-ClO_2 and UV/chlorine treatments. A total of 1628023 qualified sequences were obtained for each sample, ranging from 60848 (SU-UV/Cl_2-30 min) to 105171 (WI-undisinfected) after HTS, paired-end read assembly, and quality control. After the UV/chlorine process, the sequence of samples SP-UV/Cl_2-30 min, SU-UV/Cl_2-30 min, and AU-UV/Cl_2-30 min was significantly reduced. The analyzed results of the relative abundance of the bacterial community, PCoA, alpha index statistics (OTUs, Shannon index, and Chao 1 index), and Tax4Fun feature annotation are shown in Fig. 4. UV-ClO_2 treatment had little effect on changing the community composition, as indicated by the similarities of the communities in undisinfected reclaimed water and UV-ClO_2 effluent for all seasons (Fig. 4(a)). PCoA analysis based on weighted UniFrac further confirmed this phenomenon, namely, the nodal distance between undisinfected reclaimed water and UV-ClO_2 effluent samples for all seasons was very short (Fig. 4(b)), although seasonal factors determined bacterial community composition in reclaimed water.

In contrast, UV/chlorine treatment led to significant changes in the community structure (e.g., SP-UV/Cl_2-1 min, SU-UV/Cl_2-30 min, and AU-UV/Cl_2-30 min,

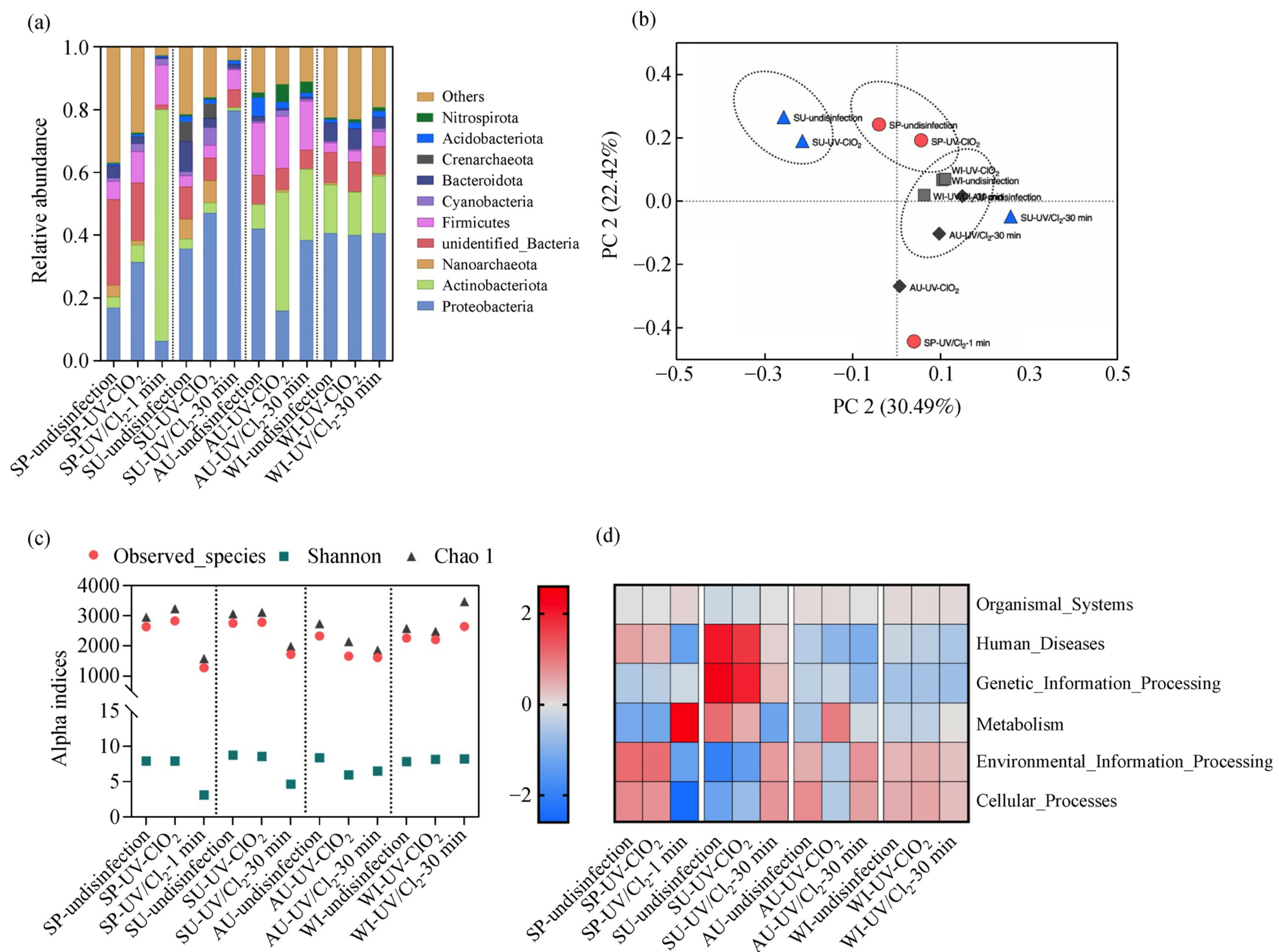

Fig. 4 Taxonomic profiles of microbial communities with UV-ClO_2 and UV/chlorine treatments. (a) Relative abundance of bacterial phyla. "Others" represent the sum of the relative abundance of all the phyla except the top ten phyla. (b) PCoA analysis based on Weighted Unifrac. The closer the distance between the samples are, the more similar the composition of the community is. (c) Alpha Indices statistics. Observed_species: Number of species (OTUs). Shannon: The total number of categories in the sample and their proportion. The higher the community diversity is, the higher the Shannon index is. Chao 1: Estimate the total number of species in the community. (d) Clustering heat map based on Tax4Fun feature annotation. The heat map value = (the relative abundance of the samples-the average relative abundance of all the samples)/the standard deviation of all the samples. Conditions: $[Cl_2]_0$ = 6 mg/L. SP: spring samples, SU: summer samples, AU: autumn samples. WI: winter samples. Since the DNA contents of SP-UV/Cl_2 10, 30 min were too low to meet the HTS requirements, we have used SP-UV/Cl_2 1 min as the UV/chlorine treatment group.

Fig. 4(a)). At the phylum level, Proteobacteria and Actinobacteria had the highest abundance during UV/chlorine treatment in all samples, accounting for more than 50% of the community (58.9%–80.6%). However, as shown in Fig. S4, the abundance of bacteria became uneven, with the proportion of *Mycobacterium* (73.3% in SP-UV/Cl_2-1 min) and *Acinetobacter* (53.1% in SU-UV/Cl_2-30 min) exceeding 50% of the community following UV/chlorine treatment. This was attributed to the fact that most bacteria that remained during disinfection were CRB (Zhang et al., 2019a; Zeng et al., 2020;Luo et al., 2021b). As the community was much simpler, the alpha indices of the community were significantly reduced after UV/chlorine treatment. The number of species (OTUs) decreased from 2650 to 1247, 2719 to 1684, and 2297 to 1581 in spring, summer, and autumn samples, respectively (Fig. 4(c)). Both Shannon index and Chao 1 analyses showed the same decreasing trend, indicating the superior removal efficiency of UV/chlorine on most species of bacteria. In addition, based on Tax4Fun feature annotation analysis, we also found that UV/chlorine could reduce related biological functions, such as “human diseases” and “genetic information processing” (Fig. 4(d)). These results suggest reduced levels of pathogens after UV/chlorine treatment. This phenomenon was consistent with the results obtained from HPC and qPCR, further proving the effective removal of OPs by UV/chlorine treatment.

3.5 Dynamics of the antibiotic resistome during UV-ClO_2 and UV/chlorine treatments

Using HT-qPCR, the detected ARGs of antibiotics, commonly administered to humans and animals, were observed to confer resistance to eight major classes of antibiotics, including fluoroquinolone, quinolone, florfenicol and chloramphenicol; amphenicol; aminoglycosides; *β*-lactams; macrolide-lincosamide-streptogramin B; sulfonamides; tetracycline; multiple drugs, and MGEs (Fig. 5 and Table S3). For the undisinfected reclaimed water samples, a total of 126, 88, 91, and 101 genes in ARG species were detected (Figs. 5(a), 5(c), 5(e), and 5(g)) for the UV/Cl_2-1 min treatment of spring, summer, autumn, and winter samples, respectively. The result was similar to that of the UV-ClO_2 effluent, with a range of 72 to 120 ARGs (Figs. 5(a), 5(c), 5(e), and 5(g)), indicating that the actual process did not effectively reduce the levels of ARGs. Previous studies have reported similar results of high levels of ARG species in secondary effluent from municipal wastewater treatment plants after chlorination (Lin et al., 2016) and in BAC filters in drinking water treatment plants (Xu et al., 2016; Zhang et al., 2018). These results indicate that the prevalence of ARGs is the pain point of current water treatment processes and also proved that RTWW is an important reservoir for ARGs (Beattie et al., 2020; Sabri et al., 2020). Beta-lactam resistance genes (10–29 types) had the highest detection rate in undisinfected reclaimed water, followed by aminoglycoside (16–21 types) and tetracycline resistance genes (13–14 types), which are frequently detected in aquatic environments (Lin et al., 2016; Wan et al., 2021). With an increase in UV/chlorine exposure, the number of ARGs decreased. UV/chlorine-1 min treatment reduced 4–45 ARGs (Figs. 5(a), 5(c), 5(e), and 5(g)). After 30 min of treatment, only 2–88 ARGs remained. The absolute abundance of ARGs showed a similar decline trend (Fig. S5). Thus, the UV/chlorine process is very effective in controlling both the number and absolute abundance of ARGs and MGEs. There was no specific trend for the removal of ARG species, suggesting the non-selectivity for ARG removal by UV/chlorine treatment. Although the removal efficiency varied across different seasons, the best ARG removal efficiency occurred during UV/chlorine-30 min treatment, implying that synergistic disinfection decreased ARG abundance.

Compared with UV-ClO_2, the effect of UV/chlorine treatment might have increased the relative abundance of ARGs in this study. As shown in Figs. 5(b), 5(d), 5(f), and 5(h), the relative abundance of ARGs in the samples (SP-UV/Cl_2-1 min, SU-UV/Cl_2-10 min, AU-UV/Cl_2-1 min) increased after UV/chlorine treatment for a short time (1–10 min). In particular, almost all selected ARGs were enriched in the AU-UV/Cl_2-1 min sample (Fig. 5(f)). This result was similar to that of the chlorinated disinfection system (Shi et al., 2013; Lin et al., 2016), so we surmised that this was because the combined disinfection effect had not been exploited, i.e., the residual chlorine in the reaction system was rapidly consumed, and only a chlorine-treated system could be formed. This once again indicates that only the effective residual chlorine level can ensure radical generation, which may achieve the control of persistent pollutants (such as ARGs). For example, the relative abundance and species of ARGs in the spring samples of UV/chlorine 10- and 30-min treatment in the present study were significantly reduced after UV/chlorine treatment, and the DNA content of these samples did not meet the requirements for HTS, which was attributed to the presence of residual chlorine in the system (Fig. S3).

Overall, the underlying mechanism for the effects of UV/chlorine treatment on ARGs is unclear. The mainstream view is that ARGs are carried by bacteria as a result of bacterial community succession. For example, Jia et al. (2015) found a strong correlation between CRB and residual ARGs after chlorine disinfection. Moreover, after UV/chlorine treatment, the relative abundance trend of MGEs was similar to that of ARGs. Pearson correlation analysis of all samples showed that there was a significant positive correlation between ARGs and MGEs ($p < 0.05$) (Fig. S6), suggesting that no direct co-selection strategies are shared between UV/chlorine and antibiotic resistance, and the enrichment of ARGs and their potential health threats of horizontal gene transfer (HGT) should not be exaggerated.

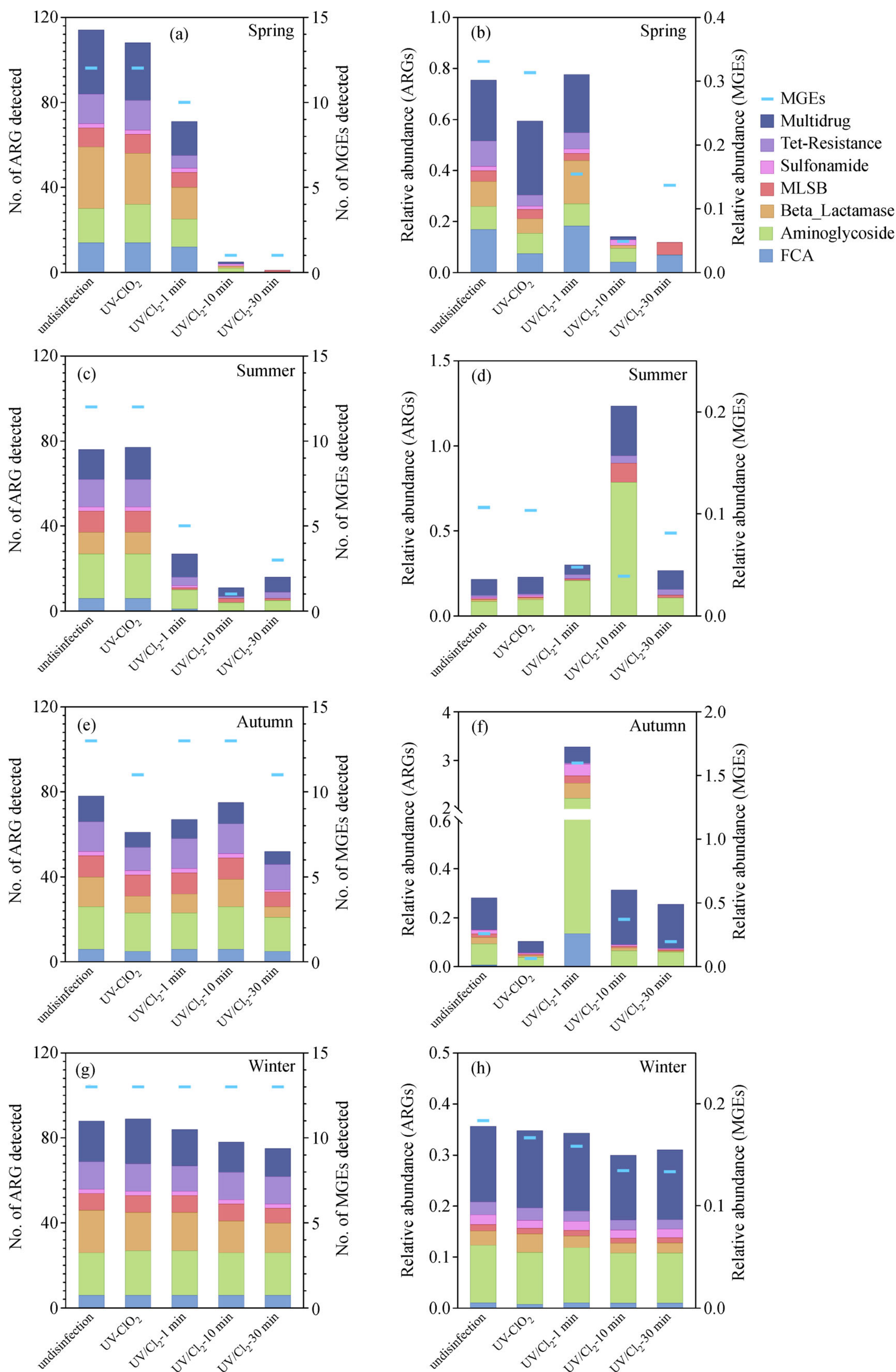

Fig. 5 Resistome profiles of undisinfected reclaimed water, UV-ClO_2 effluent, UV/Cl_2 1 min, UV/Cl_2 10 min, and UV/Cl_2 30 min. Detected number of ARGs and MGEs (a), (c), (e), (g); Relative abundance of ARGs and MGEs (b), (d), (f), (h). Multiple melting peaks and amplification efficiencies beyond the range (1.8–2.2) were discarded and the threshold cycle of 31 was set as the detection limit. Relative gene copy numbers were calculated as follows: Relative ARG/MGE copy numbers = Gene copy numbers of each ARG or MGE/gene copy numbers of 16S rRNA.

3.6 Shared resistome during UV-ClO_2 and UV/chlorine treatment

In the present study, the bipartite association network was used to analyze the shared resistome (Fig. 6), which could better illustrate the temporal variation of ARGs in reclaimed water during UV-ClO_2 and UV/chlorine treatments. ARG removal in the four seasons differed, corresponding to four degrees. Specifically, compared with undisinfected reclaimed water, the residual ARGs constituted 3.2%, 19.3%, 67%, and 88.2% in the UV/Cl_2-30 min treatment samples in spring, summer, autumn, and winter, respectively (Fig. 5). Eleven persistent genes were consistently detected in three or more seasons including two aminoglycosides (*aac*(6')-II and *aac*A_*aph*D), three tetracyclines (*tet*A-02, *tet*PB-03, and *tet*R-02), three multidrugs (*mex*F, *qac*Edelta1-01, and *qac*Edelta1-02), and three MGEs (*int*I-1(clinic), *int*I-1LC, and *tnp*A-05). These genes are persistent and contain a high proportion of MGEs, which may increase the frequency of HGT and should be considered a major concern. For example, the three shared MGEs (*int*I-1(clinic), *int*I-1LC, and *tnp*A-05) were reported to play important roles in HGT (Yang et al., 2020; Zhang et al., 2021a). As mentioned above (Fig. 3), *Acinetobacter* and *Mycobacterium* were the main bacterial genera in the community (> 50%) after UV/chlorine disinfection, and considerable attention should be paid to them. *Acinetobacter* is not only a CRB but also has an extensive antibiotic resistance spectrum (Chen et al., 2011). Moreover, *Acinetobacter* and *Mycobacterium* are both potential hosts for the *tnp*A gene (Zhang et al., 2021a). "Superbugs" may be generated when OPs carrying MGEs acquire ARGs via HGT. Therefore, more attention should be paid to the removal efficiency of these OP types. Furthermore, the persistent ARGs and MGEs proved that UV/chlorine has no selectivity for genes but can only remove bacteria containing the corresponding antibiotic resistome.

4 Conclusions

This study investigated the occurrence of pathogens and antibiotic resistome in reclaimed water during UV/chlorine treatment. The following conclusions were drawn:

- UV/chlorine can effectively remove culturable (1–10 min) bacteria from reclaimed water. However, for the removal of VBNC pathogens, a longer reaction time (10–30 min) isrequired as long as the residual chlorine was maintained. At the same time, the physicochemical indices

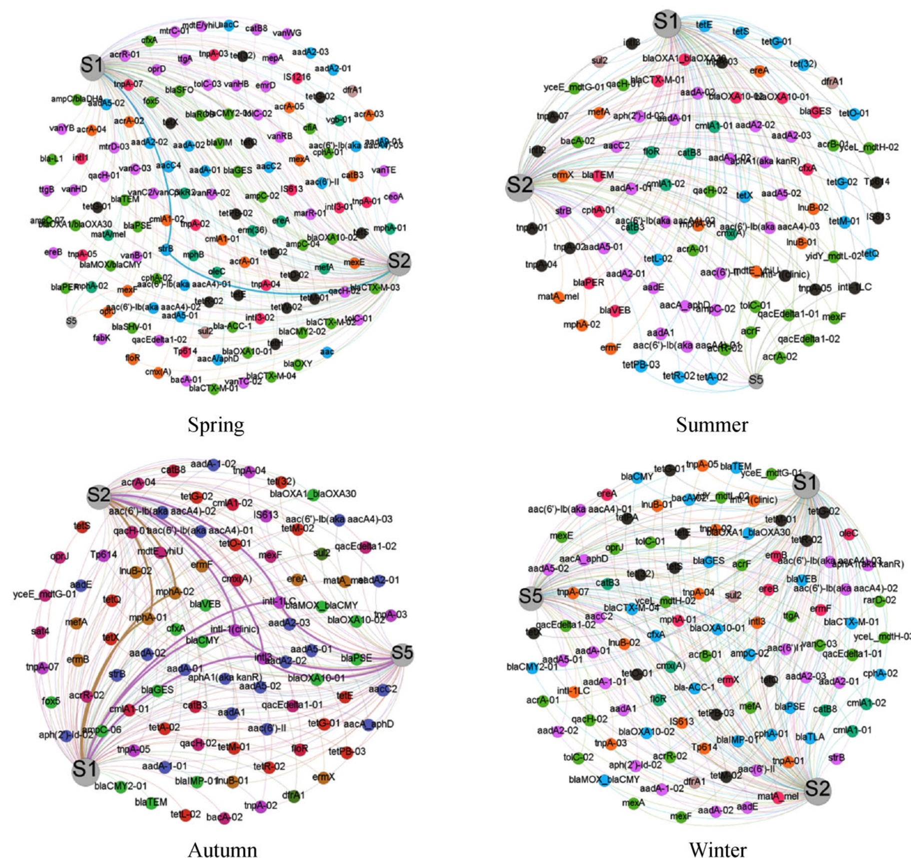

Fig. 6 Shared resistome with UV-ClO_2 and UV/chlorine treatment. Representative resistome was selected (ARG subtypes that occurred in 100% of the total samples) to generate the network.

of water quality are not affected.

- Total bacteria and water quality are two important factors that affect UV/chlorine treatment efficiency, mainly by accelerating the chlorine decay process. Insufficient residual chlorine results in low radical formation, due to which synergistic disinfection can not occur.
- The absolute abundance of typical pathogens decreased significantly after UV/chlorine treatment, but their relative abundance increased with no seasonal regularity. This suggests that OPs are also CRB, which are more likely to survive in the community after oxidation disinfection.
- With an increase in UV/chlorine exposure, the number and absolute abundance of ARGs and MGEs decreased. However, 11 persistent genes were consistently detected in three or more seasons. These genes are persistent and contain a high proportion of MGEs, which may increase the frequency of HGT and should be considered a major concern.
- *Acinetobacter* and *Mycobacterium* were the main bacterial genera in the community (> 50%) after UV/chlorine treatment, and both are potential hosts for the *tnp*A gene (MGEs). Therefore, more attention should be paid to this type of chlorine-resistant and pathogenic bacteria that carry MGEs.

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