



Occurrence and removal of antibiotics, antibiotic resistance genes, and bacterial communities in hospital wastewater

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Abstract

Hospital wastewater contains a variety of human antibiotics and pathogens, which makes the treatment of hospital wastewater essential. However, there is a lack of research on these pollutants at hospital wastewater treatment plants. In this study, the characteristics and removal of antibiotics and antibiotic resistance genes (ARGs) in the independent treatment processes of hospitals of different scales (primary hospital, H1; secondary hospital, H2; and tertiary hospital, H3) were investigated. The occurrence of antibiotics and ARGs in wastewater from three hospitals varied greatly. The first-generation cephalosporin cefradine was detected at a concentration of 2.38 µg/L in untreated wastewater from H1, while the fourth-generation cephalosporin cefepime had the highest concentration, 540.39 µg/L, at H3. Ofloxacin was detected at a frequency of 100% and had removal efficiencies of 44.2%, 51.5%, and 81.6% at H1, H2, and H3, respectively. The highest relative abundances of the β-lactam resistance gene *bla_{GES-1}* (1.77×10^{-3} copies/16S rRNA), the quinolone resistance gene *qnrA* (8.81×10^{-6} copies/16S rRNA), and the integron *intI1* (1.86×10^{-4} copies/16S rRNA) were detected in the treated wastewater. The concentrations of several ARGs were increased in the treated wastewater (e.g. *bla_{OXA-1}*, *bla_{OXA-10}*, and *bla_{TEM-1}*). Several pathogenic or opportunistic bacteria (e.g. *Acinetobacter*, *Klebsiella*, *Aeromonas*, and *Pseudomonas*) were observed at high relative abundances in the treated wastewater. These results suggested the co-occurrence of antibiotics, ARGs, and antibiotic-resistant pathogens in hospital wastewater, and these factors may spread into the receiving aquatic environment.

Keywords Antibiotics · β-lactams · Antibiotic resistance genes · Hospital wastewater treatment plants · Pathogenic bacteria · Disinfection

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Introduction

Antibiotics have been widely used to cure infectious diseases since they were discovered in the 1930s. In the environment, antibiotics are chemical pollutants that can exert toxic effects and are able to exert selection pressure on bacteria (González-Pleitera et al. 2019; Pazda et al. 2019). The excessive use and abuse of antibiotics have contributed immensely to the emergence of antibiotic-resistant bacteria (ARBs), such as vancomycin-resistant enterococci (Hocquet et al. 2016) and methicillin-resistant *Staphylococcus aureus* (Knight et al. 2012). Hospitals accumulate large amounts of antibiotics and human-related pathogens (Andersson and Hughes 2014). For example, *Acinetobacter baumannii* and *Citrobacter freundii*, which cause serious hospital-associated infections, have shown resistance to multiple antibiotics (Davies and Davies 2010). In addition, horizontal gene transfer of antibiotic resistance genes (ARGs) has aggravated the

potential risk of antibiotic resistance evolution in recent years (Levy and Marshall 2004; Sorensen et al. 2005). Without suitable treatment, the discharge of ARBs and ARGs in hospital wastewater poses ecological and ARB evolution risks to aquatic environments and humans.

Large amounts of antibiotics are consumed in Chinese hospitals, especially β -lactams, quinolones, and trimethoprim (Boeckel et al. 2014; Zhang et al. 2015a; Klein et al. 2018). However, their occurrence in hospital wastewater is not yet understood. In recent years, studies have found several kinds of antibiotics (e.g. ofloxacin and cefalexin) in untreated hospital wastewater in the city of Xinxiang in central China (Wang et al. 2018). A similar result also showed that ofloxacin was detected at a high level in treated hospital wastewater (Rodriguez-Mozaz et al. 2015). In addition, most hospital wastewater is treated by independent hospital wastewater treatment plants (HWWTPs) and then discharged into the aquatic environment or downstream municipal wastewater treatment plants (MWWTPs). In recent years, many studies have focused on MWWTPs that receive wastewater from hospitals, communities, and industry (Lee et al. 2017; Quintela-Baluja et al. 2019). However, studies focusing on the hospital wastewater treatment process are limited, leaving a knowledge gap related to the occurrence of antibiotics, from traditional to last-line antibiotics, and their corresponding ARGs after hospital wastewater treatment protocols. Studying independent HWWTPs could help us understand the discharge of clinical antibiotics and ARGs and reveal the potential risks of hospital wastewater to the aquatic environment and receiving MWWTPs.

Hospitals can be divided into primary, secondary, and tertiary hospitals according to their medical capabilities and facilities (National Health Commission of the People's Republic of China (NHCPRC) 1989; Jamison et al. 2006; Moore et al. 2014). Primary hospitals provide mostly basic health care for the community, while comprehensive and specialist health services are provided in tertiary hospitals (e.g. teaching hospitals, chest hospitals, and infectious disease hospitals) (World Health Organization (WHO) 1978; National Health Commission of the People's Republic of China (NHCPRC) 1989). Differences in health services, bed capacity, antibiotic usage, amount of wastewater, and HWWTPs among hospitals of different scales may lead to different patterns of pollution of hospital wastewater with antibiotics, ARGs, and bacterial communities. In addition, antibiotics can sometimes be categorized into different generations according to their antimicrobial properties. For example, cephalosporins are grouped into the first, second, third, and fourth generations (Ribeiro et al. 2018). Revealing the contamination characteristics of different kinds of antibiotics and ARGs in different-scale hospitals could help us to understand the pollution status of antibiotics and the evolution of ARGs in hospital wastewater sources. To the best of our knowledge, this fundamental information has

not yet been reported. Therefore, this study aims to (1) reveal the pollution characteristics of a variety of antibiotics and ARGs at different-scale hospitals in East China; (2) clarify the removal effectiveness of the conventional hospital wastewater treatment process for the target antibiotics and ARGs; and (3) evaluate the characteristics of the total microbial community and cultivable bacteria in untreated and treated hospital wastewater.

Materials and methods

Sample collection

Samples were collected in triplicate in January and May 2019 from three different hospitals with different sizes and properties (primary, secondary, and tertiary hospitals, abbreviated H1, H2, and H3, respectively) in East China. These three hospitals had different independent treatment processes, with wastewater volumes of 80, 100, and 727 m³ per day. Briefly, H1 adopted a simple chlorine disinfection process because of its small scale and wastewater amount. At H2, the wastewater was first treated by a hydrolysis acidification tank and then by an aerobic contact tank, a secondary settling tank, and chlorine disinfection. H3 used an aerobic contact tank followed by a secondary settling tank and then chlorination treatment. The treated wastewater from these three hospitals was then discharged into receiving MWWTPs. Detailed information (e.g. hospital bed capacity, governance, and wastewater quality) of the three hospitals is listed in Table A.1. The samples were untreated wastewater and treated wastewater from the three HWWTPs, as shown in Fig. 1. The raw wastewater was a mixture of the medical ward and domestic wastewater. To avoid the possible degradation of β -lactam antibiotics, grab samples were collected in 5-L brown glass bottles on weekdays between 8:30 a.m. and 10:30 a.m., returned to the laboratory on ice in coolers, and analysed immediately. The containers were washed with methanol, water, and sterilized deionized water before wastewater collection. These included 18 samples for each season's sampling campaign. Wastewater NH₃-N and total phosphorus (TP) were analysed by a UV-Vis spectrophotometer (HACH DR 5000), and the chemical oxygen demand (COD) was measured by potassium dichromate titration, as shown in Table A.2. The concentrations of free chlorine were detected by the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method (APHA 2017). The detailed chemicals and reagents that were used in this study are listed in Text A.1.

Quantification of antibiotics in hospital wastewater

Samples were analysed in triplicate for the determination of the target antibiotics following established methods (Gros

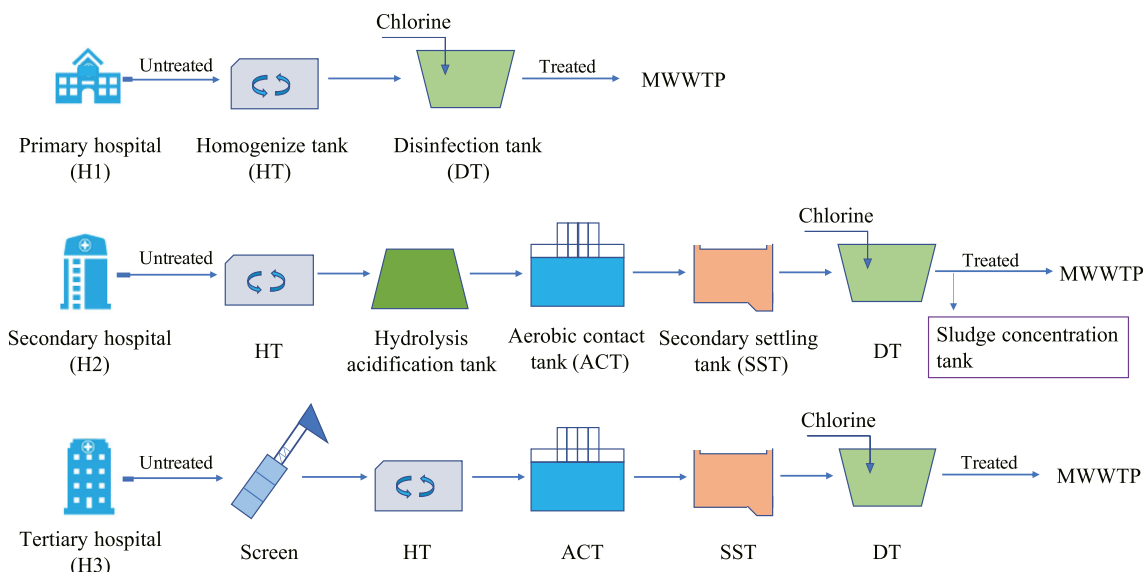


Fig. 1 Schematic diagram of three hospital wastewater treatment plants (HWWTPs). The wastewater from these three hospitals was eventually discharged to the receiving municipal wastewater treatment plants (MWWTPs)

et al. 2013; Cheng et al. 2015), with some modifications. Briefly, hospital wastewater was vacuum-filtered through a 0.7- μm glass fibre filter (Whatman GF/F, UK), followed by a 0.22- μm cellulose acetate membrane filter (Anpel, Shanghai, China). A suitable volume (1 mL) of a Na_2EDTA solution (10 g/L) was added to the different types of water to achieve a final concentration of 0.1 g/L, and the pH of the samples was adjusted to 2.5 with hydrochloric acid. Water samples were automatically extracted using Oasis HLB cartridges (Wilford, MA, USA) for hospital wastewater matrices. SPE cartridges were activated with 6 mL of methanol followed by 6 mL of ultrapure water flowing at a gravity-dependent rate. Next, 100 mL of hospital wastewater was loaded onto the cartridge at a flow rate of 0.6–1 mL/min. After sample pre-concentration, the cartridges were rinsed with 6 mL of HPLC grade water at a flow rate of 2 mL/min and vacuum-dried for 5 min to remove excess water. Following this, the cartridges were eluted with 10 mL of pure methanol at a flow rate of 1 mL/min. Then, the eluents were evaporated to approximately 100 μL under a gentle nitrogen stream and redissolved in 1 mL of methanol/water (1:1, v/v) solution.

The samples were finally analysed by liquid chromatography with tandem mass spectrometry (Shimadzu, LCMS-8050) using a Nexera X2 HPLC system equipped with a binary solvent manager and an autosampler, an electrospray ionization (ESI) interface, and LCMS LabSolutions software (Version 5.89). MS/MS parameters were optimized in multiple reaction monitoring (MRM) mode. An analytical Shim-pack GISS C18 column (2.1 \times 100 mm, 1.9 μm , Shimadzu) was used at a flow rate of 0.4 mL/min, and the column oven temperature was set at 40°C. Mobile phase A (ultrapure water with 0.05% (v/v) formic acid) and mobile phase B (methanol) were used in stepwise gradient mode. The elution gradient

started with 5% B, increased to 50% B over 4 min, then increased further to 80% B over 2 min, and finally increased to 95% B over 2 min; it was held at 95% B for 1 min and then brought back to the initial value over 0.1 min, where it was maintained for 2 min until the next injection. Mass spectra were acquired in positive ion mode with a nebulizer flow of 3 L/min, heating gas flow of 10 L/min, interface temperature of 250°C, desolvation line temperature of 150°C, heating block temperature of 400°C, and dry gas flow of 10 L/min.

To compare the characteristics of antibiotics in different-scale hospitals, typical antibiotics, such as first-, second-, third-, and fourth-generation cephalosporins, penicillins, carbapenem, quinolones, and trimethoprim, were selected. Standard first-generation cephalosporins (cefalexin (99%), cefalotin (99%), cefazolin (99%), cefradine (99%)), second-generation cephalosporins (cefoxitin (99%)), third-generation cephalosporins (ceftazidime (99%)), fourth-generation cephalosporins (cefepime (99%)), penicillins (amoxicillin (99%), ampicillin (99%), penicillin G (99%)), carbapenems (meropenem (99%)), quinolones (ofloxacin (99%), norfloxacin (99%)), and trimethoprim (99%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). For accurate quantification, stable isotope $^{13}\text{C}_3$ -caffeine (99%) (Augsburg, Germany) was spiked into the samples prior to SPE, and total recoveries were determined (n=3). The standard curve for each antibiotic was constructed with at least five concentrations ($R^2 > 0.99$). The precursor ions, product ions, limits of detection (LODs), limits of quantification (LOQs), recoveries, linear R^2 values, and reproducibility and repeatability results are shown in Table A.3. The low recovery values of β -lactam antibiotics may be attributed to their instability in hospital water matrices, and the detection of

some antibiotic metabolites may benefit from the method used (Gros et al. 2013).

Characterization of bacteria and quantification of target genes

Hospital wastewater samples were serially diluted in 1× phosphate-buffered saline (PBS), and 100 µL volumes of samples were spread-plated in triplicate and incubated on lysogeny broth agar at 30°C for 48 h to determine the concentration of cultivable bacteria at the sampling site (Le et al. 2016). Colonies in the range of 30 to 300 cells were counted to minimize errors caused by the presence of too many colonies to count. One hundred millilitres of hospital wastewater was filtered through a 0.22-µm pore size cellulose nitrate membrane (Anpel, Shanghai, China). Total DNA was extracted from filters using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. DNA concentration and purity were measured using a NanoDrop spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Wilmington, DE, USA). The isolated DNA was stored at -20°C until subsequent analysis. Community characterization of total bacteria was performed by 16S amplicon sequencing at Sangon Biotech (Shanghai) Co., Ltd. (NCBI accession number: PRJNA646019). The V4 region of the 16S rRNA genes was amplified with the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') and sequenced, and the diversity of the total bacteria was analysed. Specifically, the Illumina MiSeq raw sequencing data contained barcode, primer, and linker sequences. The primer and linker sequences were removed, and then the paired reads were merged into a sequence according to the relationship between paired-end reads. Samples were identified according to the individual barcodes to obtain data for each sample. The denoising process is described in the supporting information (Text A.2). Quality filtering of reads, trimming of paired ends, the denoising process, and the alpha diversity values from the bacterial diversity analysis are provided in the supplemental document (Table A.4, Table A.5, and Table A.6). In addition, for quantitative PCR (qPCR) analysis, DNA samples were diluted tenfold with nuclease-free water to minimize background interference in the qPCR reactions. qPCR was used to measure the abundances of 10 specific ARGs: the six β-lactam resistance genes *bla_{OXA-1}*, *bla_{OXA-10}*, *bla_{TEM-1}*, *bla_{DHA-1}*, *bla_{SHV-1}*, and *bla_{GES-1}* and the four quinolone resistance genes *qnrA*, *qnrS*, *qnrD*, and *qepA*. The 16S rRNA gene and *intI1* were analysed to determine the relative abundances of ARGs and the transferability of ARGs, respectively. All runs were performed with positive and negative controls. The positive controls were obtained by cloning target DNA into plasmids at different dilutions, and nuclease-free water was used as the negative control, as described in a previous study (Hu

et al. 2019a). The details of the primers, annealing temperatures, and amplicons for the target genes were the same as those in our previous study and are listed in Table A.7 (Hu et al. 2019a). The detailed qPCR conditions were the same as those in our previous study and are listed in Text A.3 (Hu et al. 2019b).

Statistical analysis

The results are presented as the means ± standard deviations (SDs), and all diagrams were generated using R 3.6.1 (The R Foundation for Statistical Computing). The correlation analysis of antibiotics and ARGs was carried out using R 3.6.1 software. Correlation analysis of the target ARGs and mobile genetic elements was carried out using SPSS 25.0. Two-tailed $P < 0.05$ was considered to indicate significance.

Results and discussion

Analysis of hospital wastewater quality

The wastewater quality indices from the three hospitals are summarized in Table A.2. The treated wastewater quality indices met the active hospital wastewater pretreatment standard (Discharge Standard of Water Pollutants for Medical Organization, GB 18466-2005) (Table A.2). NH₃-N, TP, and COD values decreased after HWWTP treatment. In particular, the NH₃-N values ranged from 10.4 to 50 mg/L in the treated wastewater. NH₃-N in wastewater can rapidly react with free chlorine to form chloramine, thereby reducing the concentration of free chlorine and the disinfection effect (Huang et al. 2012; Kevin 2016). Therefore, the breakpoint dosage (a mass ratio of chlorine to NH₃-N of 7.6:1) of free chlorine was required in the presence of NH₃-N in wastewater (Zhang et al. 2015b).

Occurrence of antibiotics in hospital wastewater

The pollution characteristics of antibiotics in hospital wastewater are shown in Fig. 2. The statistically significant differences in antibiotic pollution between the two sampling seasons were also analysed in Fig A.1, which revealed a nonsignificant difference. To provide a real picture of the pollution of antibiotics in hospital wastewater, the concentrations of the target antibiotics in the untreated wastewater and the removal efficiencies of the HWWTPs are summarized in Table 1. Thirteen antibiotics were detected in the wastewater of the three hospitals, but penicillin G was not detected. β-Lactams such as first-, second-, third-, and fourth-generation cephalosporins, penicillins, and carbapenem were widely detected in hospital wastewater. The diversity of antibiotics in the wastewater from the three hospitals varied greatly. For example,

Table 1 The concentration and removal efficiency of different kinds of antibiotics at hospital wastewater treatment plants (HWWTPs) in this study and in recent studies

Antibiotics	This study						Literature data ^a		
	H1		H2		H3		HWWTPs		
	Untreated (µg/L)	Removal efficiency (%) ^b	Untreated (µg/L)	Removal efficiency (%)	Untreated (µg/L)	Removal efficiency (%)	Untreated (µg/L)	Removal efficiency (%) ^c	References
Cefalexin	0.09~0.56	100	0.03~0.10	57.3	0.03~0.88	100	n.d. ^c	- ^d	Gros et al. (2013)
							2.23	-	Li and Lin (2015)
Cefalotin	0.02~0.05	100	0.06~0.11	100	0.03~0.06	100	-	-	-
Cefazolin	n.d.	-	n.d.	-	0.45~5.01	100	n.d.	-	Gros et al. (2013)
							4.91	-	Li and Lin (2015)
							0.83	-	Rodriguez-Mozaz et al. (2015)
Cefradine	0.37~2.38	100	1.12~1.65	20.2	0.90~2.27	100	0.17	-	Li and Lin (2015)
Cefoxitin	n.d.	-	0.85~8.17	75.1	0.36~8.96	100	-	-	-
Ceftazidime	0.14~0.34	100	0.31~7.27	51.4	4.74~31.21	100	3.66	-74.0	Szekeres et al. (2017)
Cefepime	n.d.	-	n.d.	-	106.76~540.39	100	8.52	-	Szekeres et al. (2017)
Amoxicillin	0.04~0.84	100	n.d.	-	0.02~1.43	100	0.11	-	Dinh et al. (2017)
							0.22	-	Gros et al. (2013)
Ampicillin	0.18~0.51	100	0.14~0.67	36.2	0.15~0.50	100	-	55.0	Szekeres et al. (2017)
							n.d.	-	Gros et al. (2013)
Penicillin G	n.d.	-	n.d.	-	n.d.	-	-	-	-
Meropenem	n.d.	-	n.d.	-	0.02~0.20	100	1.07	-	Le et al. (2016)
Ofloxacin	2.38~9.23	44.2	1.39~25.65	51.5	6.95~49.47	81.6	0.94	54.0	Li et al. (2016)
							16.8	38.0	Liu et al. (2019)
							10.37	-	Gros et al. (2013)
							7.26	-	Li and Lin (2015)
							14.38	-	Rodriguez-Mozaz et al. (2015)
							17.9	-	Dinh et al. (2017)
Norfloxacin	0.05~0.06	82.1	0.19~0.46	69.3	0.18~0.61	90.5	0.78	42.0	Li et al. (2016)
							0.61	45.0	Liu et al. (2019)
							12.1	-	Dinh et al. (2017)
							0.24	-	Li and Lin (2015)
							0.33	-	Gros et al. (2013)
Trimethoprim	n.d.~0.02	100	0.12~0.31	100	n.d.~0.50	100	30.3	81.0	Szekeres et al. (2017)
							0.84	8.0	Liu et al. (2019)
							0.08	-	Li and Lin (2015)
							0.94	-	Dinh et al. (2017)

^a The concentration and removal efficiency in current literatures in hospital wastewater treatment plants

^b The average of triplicate experiments

^c Not detected

^d No available da

ofloxacin (2.38–9.23 $\mu\text{g/L}$) and the first-generation cephalosporin cefradine (0.37–2.38 $\mu\text{g/L}$) were detected at high concentrations in the wastewater from H1. The concentration of cefradine was higher than the concentration of cefradine (0.17 $\mu\text{g/L}$) reported in Taiwan, which may be attributed to its low clinical consumption (Li and Lin 2015). The main antibiotic types detected in the wastewater from H2 were quite different from those in the wastewater from H1. High concentrations of the second-generation cephalosporin cefoxitin (0.85–8.17 $\mu\text{g/L}$) and the third-generation cephalosporin ceftazidime (0.31–7.27 $\mu\text{g/L}$) were detected in wastewater from H2. The greatest diversity of antibiotics was detected in H3 compared with H1 and H2. H3 is the largest central hospital, receiving approximately 700,000 patients every year, and has independent intensive care units (ICUs). Thirteen kinds of antibiotics, including ten cephalosporins, two quinolones, and trimethoprim, were detected in wastewater from H3. Strikingly, the fourth-generation cephalosporin cefepime was detected only in wastewater from H3, with the highest concentration being 540.39 $\mu\text{g/L}$. The concentration of cefepime in this study was significantly higher than that reported in Romanian hospitals (8.52 $\mu\text{g/L}$). This might be explained by the smaller population served and the lower consumption of these antibiotics (providing service to 30,000 inhabitants) in the Romanian hospital (Szekeres et al. 2017). The carbapenem antibiotic meropenem, which is generally used as the last line of defence, was also detected in samples from H3 at a concentration of 0.20 $\mu\text{g/L}$. A higher concentration of meropenem (1.07 $\mu\text{g/L}$) was detected at a tertiary hospital that had 1500 beds (Le et al. 2016). Furthermore, the concentrations of the second-generation cephalosporin cefoxitin and the third-generation cephalosporin ceftazidime in wastewater from H3 were also higher than those in wastewater from H1 or H2. Notably, we found third-generation cephalosporin ceftazidime at all three hospitals; it is frequently used in Chinese hospitals according to the Status Report on Antimicrobial Administration and Antimicrobial Resistance in China, 2018 (National Health Commission of the People's Republic of China, www.nhc.gov.cn).

As shown in Fig. 2, ofloxacin was detected in all samples (including untreated and treated samples from the three hospitals) with high concentrations of 9.23 $\mu\text{g/L}$, 25.65 $\mu\text{g/L}$, and 49.47 $\mu\text{g/L}$ in the samples from H1, H2, and H3, respectively. Similar results showed a high concentration of ofloxacin in a 360-bed hospital in the Essonne District, France (Dinh et al. 2017). However, the concentrations were higher than those in wastewater from Xinjiang Province, China, where the concentration of quinolones (e.g. ofloxacin, norfloxacin) ranged from 0.45 to 0.94 $\mu\text{g/L}$ in untreated hospital wastewater (Li et al. 2016). Trimethoprim is often used with sulfonamide antibiotics to improve antibacterial properties. In this study, trimethoprim was detected at H1, H2, and H3 at concentrations of 0.02 $\mu\text{g/L}$, 0.31 $\mu\text{g/L}$, and 0.50 $\mu\text{g/L}$, respectively. The low

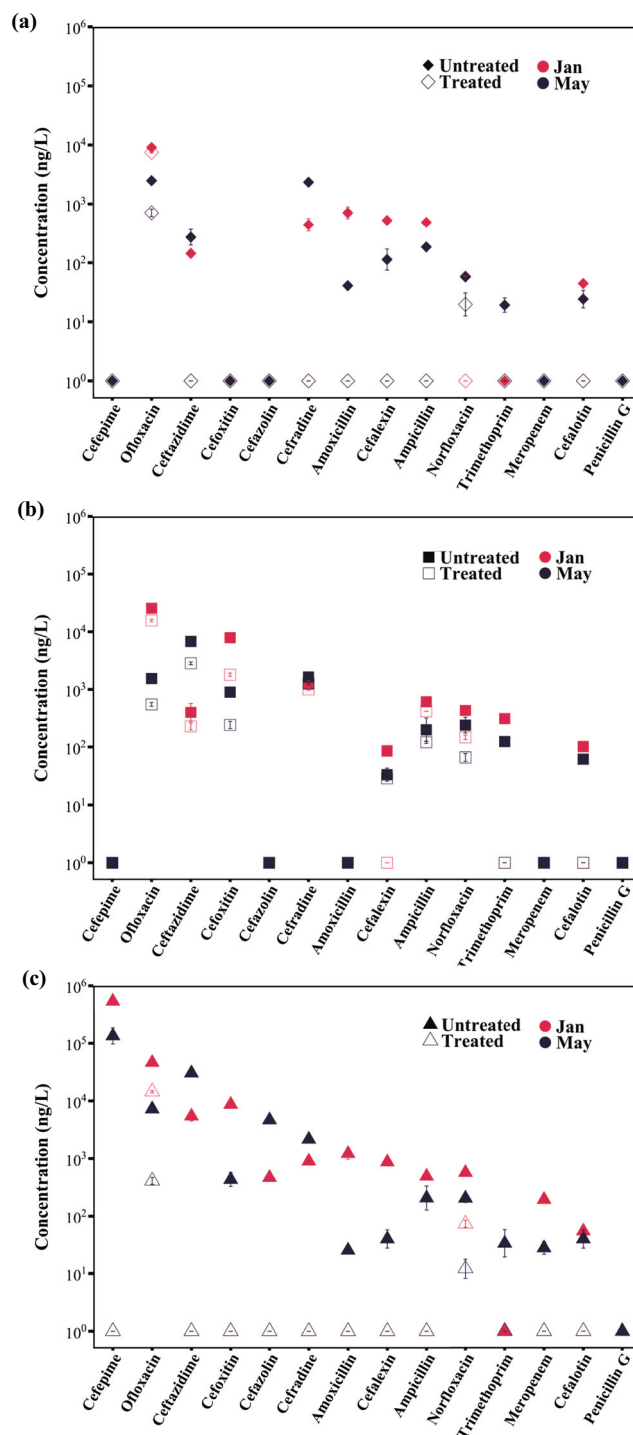


Fig. 2 The residue concentrations (ng/L) of target antibiotics in the untreated and treated wastewater from primary-scale hospital H1 (a), secondary-scale hospital H2 (b), and tertiary-scale hospital H3 (c) in Jan and May

concentrations of trimethoprim might be attributed to the small percentage of sulfonamides (e.g. sulfamethoxazole and sulfadiazine) that were used as human medicines in China. A recent study also showed that trimethoprim was detected at 0.78 to 0.84 $\mu\text{g/L}$ in a hospital in Beijing, China (Liu et al.

2019). In recent years, total antibiotic consumption in many low- and middle-income countries was higher than that in high-income countries, with a rapidly increasing trend (Klein et al. 2018). The hospital consumption of antibiotics in China is the second largest in the world (Boeckel et al. 2014). Therefore, we suggest strengthening the management of antibiotics at hospitals and using antibiotics reasonably to reduce their discharge from hospital sources.

Removal of antibiotics at different HWWTPs

Considering that many kinds of antibiotics are widely detected in untreated hospital wastewater, the removal of antibiotics at different HWWTPs is further discussed. The removal rates of the target antibiotics at different HWWTPs ranged from 20.2 to 100% (Table 1). Specifically, the removal rates of β -lactams (including cephalosporins, penicillins, and carbapenem) reached 100% at the independent HWWTPs H1 and H3. Similar results showed high removal efficiencies of β -lactam antibiotics in biological wastewater treatment processes (84.4–99.5%) (Tran et al. 2016). It has been reported that the removal efficiency of antibiotics in hospital wastewater treatment processes ranges from –74.0 to 81.0% (Szekeres et al. 2017). High concentrations of β -lactams remained in the treated wastewater from H2, with the highest concentration being 2.95 $\mu\text{g/L}$. This differential result may be attributed to the fact that the removal of β -lactams is strongly affected by chemical or hydraulic retention times (Hou and Poole 1971; Le-Minh et al. 2010). The concentration of chlorine at H2 was relatively lower than those at H1 and H3 (Table A.2). It is also generally observed that increasing the removal efficiency of β -lactams demands more oxidizing agents (chlorine) in natural water matrices (Acero et al. 2010). The third-generation cephalosporin ceftazidime exhibited a removal efficiency of 51.4%. The release of critical β -lactam antibiotics (ceftazidime) may enhance antibiotic resistance in aquatic environments.

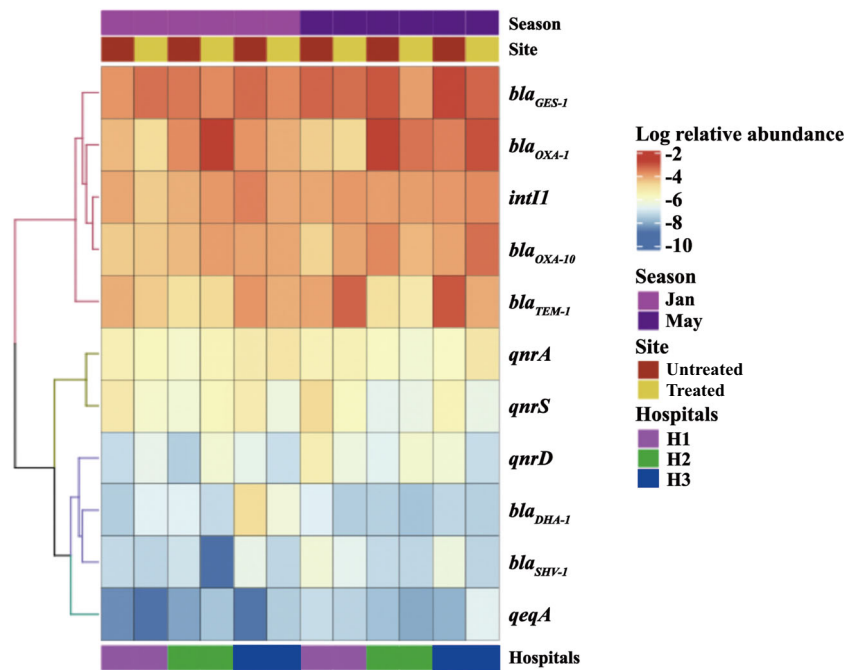
Ofloxacin was recalcitrant and detected with a frequency of almost 100% in the treated effluents from the three hospitals. Many studies have reported that ofloxacin is detected more frequently than other antibiotics in hospital wastewater (Rodriguez-Mozaz et al. 2015; Wang et al. 2018). The removal efficiencies of ofloxacin at the HWWTPs of H1 and H2 were 44.2% and 51.5%, respectively. Although the removal efficiency of ofloxacin at the HWWTP of H3 was improved (68.8%), this antibiotic still presented a high concentration of 14.90 $\mu\text{g/L}$ in the treated wastewater. This result was consistent with those of previous studies that proved that traditional hospital wastewater treatment systems applying biological processes, biological anoxic reactors, and membrane filtration methods exhibited limited removal efficiency for ofloxacin (Kovalova et al. 2012; Dinh et al. 2017). The low removal efficiency of ofloxacin may be attributed to the chemically stable piperazinyl ring and its lack of biodegradation

vulnerabilities (Turiel et al. 2005; Hapeshi et al. 2013). Considering that the conventional hospital treatment process was not designed to remove antibiotics, emerging powerful options for the treatment of antibiotics have been studied in recent years. For example, quinolones can be efficiently removed by simulated solar radiation systems (Babic et al. 2013). The emerging electro-Fenton process has shown good removal of antibiotics and has the advantages of low cost and reusable solid catalysts as sources of Fe^{2+} and Cu^{2+} ions (Barhoumi et al. 2016; Barhoumi et al. 2017).

Occurrence of ARGs in hospital wastewater

The prevalence of ARGs (normalized to 16S rRNA and log transformed) in hospital wastewater is shown in Fig. 3. The relative abundances of the analysed ARGs are listed in Table A.8. Fig. A.2 shows the statistically significant differences in ARGs pollution between the two sampling seasons, and a nonsignificant difference was found. The order of the average relative abundances of target ARGs and *intI1* was as follows: H3 (1.35×10^{-4} copies/16S rRNA gene) > H2 (1.30×10^{-4} copies/16S rRNA gene) > H1 (6.25×10^{-5} copies/16S rRNA gene) (Fig. 3). Therefore, the pollution of ARGs at tertiary hospital H3 was more serious than that at primary hospital H1 and secondary hospital H2. The most abundant ARGs in the wastewater from H1, H2, and H3 were *bla*_{TEM-1}, *bla*_{GES-1}, and *bla*_{OXA-1}, respectively. The relative abundances of the carbapenem resistance gene *bla*_{GES-1} in the wastewater from H1, H2, and H3 were 6.21×10^{-5} , 1.77×10^{-3} , and 9.44×10^{-4} copies/16S rRNA gene, respectively. In addition, the cephalosporin resistance gene *bla*_{OXA-1} showed a high relative abundance of 1.27×10^{-3} copies/16S rRNA gene at three hospitals. *bla*_{OXA-1} is frequently detected (57.9%) in *Escherichia coli* resistant to cefotaxime in hospital wastewater (Adegoke et al. 2020). Mobile genetic elements or plasmid-associated OXA β -lactamase genes were found to be significantly enriched in the plasmid reads of secondary wastewater samples (Barlow and Hall 2002; Martin et al. 2020). To provide a real picture of ARG pollution in wastewater, the ARG pollution in this study and data from other parts of the world is summarized in Table A.9. A previous systematic review also found the presence of antibiotics (e.g. β -lactam, quinolone, trimethoprim, and multidrug efflux genes) in municipal, hospital, and industrial wastewater in different countries (e.g. USA, Canada, and China) (Pazda et al. 2019). The class 1 integron displayed a relative abundance of 1.17×10^{-4} copies/16S rRNA gene in untreated hospital wastewater. The class 1 integron is a representative mobile gene element that can sometimes enhance the HGT of ARGs. The removal rate for the class 1 integron at H3 was significantly higher than those at H1 and H2 (Fig. 4). In previous work, less than 1.2 log of *intI1* was removed by hospital wastewater treatment, and *intI1* remained detectable at 8×10^{-5}

Fig. 3 Heat map of the relative abundances of antibiotic resistance genes (ARGs) at the three hospital wastewater treatment plants (HWWTPs). The concentrations of ARGs were normalized to the 16S rRNA level and log transformed



copies/16S rRNA gene (Timraz et al. 2017). Forty-three ARG cassettes mediated by *int11* were detected with great diversity in hospital wastewater (Yuan et al. 2021). As indicated in Table A.10, the presence of the last-line antibiotic (carbapenem) resistance gene *bla_{GES-1}* was strongly associated with the presence of *bla_{OXA-10}*. In addition, the presence of *bla_{OXA-10}*, *bla_{DHA-1}*, *bla_{SHV-1}*, *qnrA*, and *qnrS* was significantly correlated with the presence of *int11* at the 0.01 level. This

phenomenon may be attributed to the presence of these genes in the same gene cassettes that could enhance the risk of ARG transfer, which was also proven by some studies (Ma et al. 2017; An et al. 2018).

Quinolone ARGs decreased in abundance in the order *qnrA* > *qnrS* > *qnrD* > *qeqA*, which was consistent with the results of a previous study showing that *qnrA* was the quinolone ARG with the highest concentration (Wang et al. 2018). The

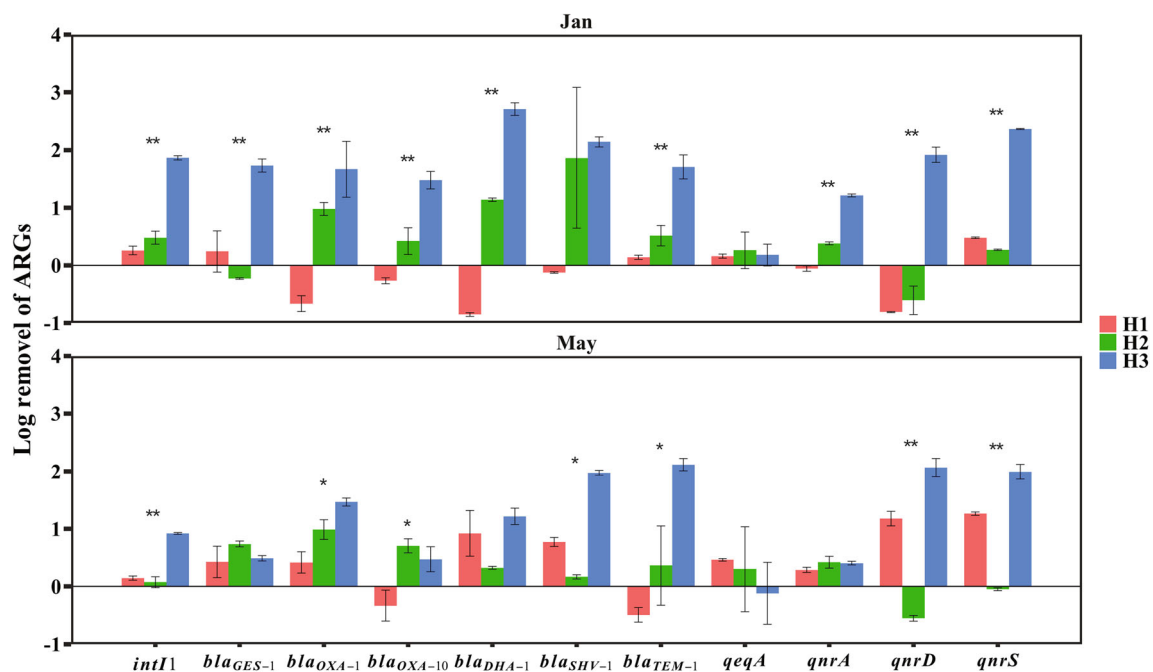


Fig. 4 Log removal of antibiotic resistance genes (ARGs) at three hospital wastewater treatment plants (HWWTPs). Log removal of ARGs = $\log(C_{\text{untreated}}/C_{\text{treated}})$. A log removal value <0 means that the hospital wastewater treatment processes increased the concentration of the target ARG

concentration of *qnrA* (1.44×10^5 copies/mL) in this study was one magnitude higher than that in the above study (1.58×10^4 copies/mL). In addition, the average concentration of β -lactam resistance genes (4.87×10^6 copies/mL) was two orders of magnitude higher than that of quinolone resistance genes (5.38×10^4 copies/mL), which were present at 1.53×10^{-6} copies/16S rRNA gene in treated wastewater from the three hospitals. Therefore, these findings suggested that the risk of β -lactam antibiotics and the related ARGs should be given more attention and considered to improve the clearance efficiency for ARGs in HWWTPs that have the possibility of harbouring the targeted ARGs (Ju et al. 2016).

In addition, correlations of all the targeted types of antibiotics with their corresponding ARGs were further evaluated (Fig. A.3). A significantly positive correlation was revealed between *bla_{GES-1}* and the total β -lactam concentration ($P < 0.01$) and between *bla_{OXA-1}* and the total β -lactam concentration ($P < 0.05$). This result suggested that the high concentration and variety of β -lactams may be responsible for the high concentrations of β -lactam ARGs. However, *bla_{SHV-1}*, *bla_{TEM-1}*, and *qnrD* had weak correlations with the corresponding antibiotics. A strong correlation between *qnrA* and the total quinolone concentration ($P < 0.05$) and between *qnrS* and the total quinolone concentration ($P < 0.05$) was observed. A previous study showed a significant correlation between the total concentration of plasmid-mediated quinolone resistance genes and the corresponding antibiotics in wastewater and soil samples (Li et al. 2012). Similarly, strong correlations between the *qnrS* gene and ofloxacin and ciprofloxacin were observed (Rodríguez-Mozaz et al. 2015). Exposure to antibiotics could exert selective pressure on bacteria and increase the concentration of ARGs in the environment. Although we have demonstrated a significant correlation between several types of antibiotics and ARGs, the environmental influencing factors and other pollutants in the hospital wastewater matrix need to be further evaluated to provide a better understanding of the co-occurrence of these pollutants.

Removal of ARGs at different HWWTPs

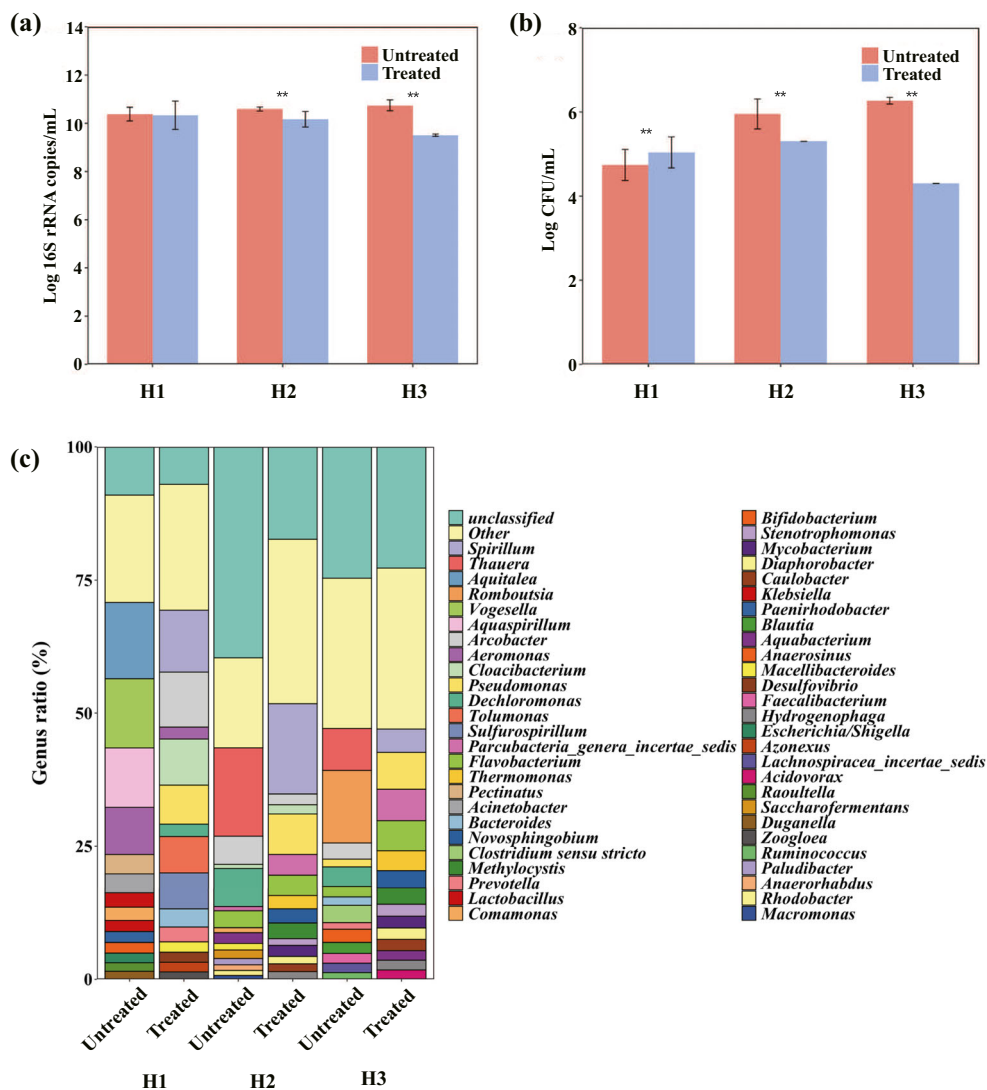
The removal efficiencies of ARGs at different HWWTPs are shown in Fig. 4. The removal rates of the target ARGs at the three HWWTPs ranged from -0.85 to 2.71 log in both sampling campaigns (Fig. 4). Specifically, the log reductions were the highest for *qnrS*, *bla_{SHV-1}*, and *bla_{DHA-1}* among the target ARGs. Similar to the results of a previous study, *qnrS* and *bla_{SHV}* were reduced by approximately one order of magnitude at traditional wastewater treatment plants (Laht et al. 2014; Rodríguez-Mozaz et al. 2015). The traditional wastewater treatment process cannot efficiently remove target ARGs from hospital wastewater. Notably, the abundances of five β -lactam ARGs (*bla_{OXA-1}*, *bla_{OXA-10}*, *bla_{DHA-1}*, *bla_{SHV-1}*, and *bla_{TEM-1}*) and two quinolone ARGs (*qnrA* and *qnrD*)

increased after treatment at H1 in Jan. Several studies have proven that the chlorination process can increase ARG concentrations. For example, the levels of aminoglycoside-resistant genes (*aac(6')-II* and *aacC2*) and tetracycline-resistance genes (*tetR* and *tetX*) increased from 0.22 to 2.23 log (Wang et al. 2020). Several ARGs (e.g. *qnrB*, *tetM*, and *tetW*) were also increased after disinfection with sodium hypochlorite (Hu et al. 2019a). The increases in ARGs may be attributed to an inadequate chlorine dosage, inducing the formation of more pili for conjugative transfer (Guo et al. 2015). The horizontal transfer of ARGs was easily induced at a non-lethal dose of chlorine (0.5 mg/L) (Wang et al. 2020). Therefore, the enhanced disinfection efficiency and the inhibition of ARG transferability in hospital wastewater need to be considered. In the present study, the relative abundances of ARGs were between 1.01×10^{-9} and 1.77×10^{-3} copies/16S rRNA gene in the treated wastewater. The residual ARBs and ARGs in the treated wastewater are further sent to MWWTPs and become potential sources of gene propagation and horizontal transfer (Xu et al. 2015; Bengtsson-Palme et al. 2016). These results suggested that the HWWTPs had a poor removal efficiency for ARGs and that it is necessary to improve hospital wastewater treatment facilities to remove these ARGs and mobile genetic elements. In recent years, potential methods that have shown good removal ability for these novel pollutants, such as bioelectroreduction, UV/chlorination, and radiation, have been applied in hospital wastewater treatment after careful testing (Liang et al. 2019; Zhang et al. 2019).

Analysis of bacteria and community characterization in hospital wastewater samples

Figure 5 shows the results of bacterial abundance and community characterization in the untreated and treated wastewater of the three hospitals considered in this work, namely H1, H2, and H3. In Fig. 5a, abundance values are expressed as the 16S rRNA gene copy number per millilitre, and as shown, the concentration of the 16S rRNA gene ranged from 2.93×10^9 to 8.85×10^{10} copies/mL in the wastewater, which were higher than the values reported in a previous study (Szekeres et al. 2017). This may be attributed to the smaller number of people served (around 30,000 inhabitants) in Cluj County, Romania (Szekeres et al. 2017). The removal rates of the 16S rRNA gene showed a declining trend, with removal ranging from 0.05 to 1.24 log. The number of culturable bacteria in the untreated wastewater increased in the order $H1 < H2 < H3$ (Fig. 5b), while it increased in the order $H3 < H1 < H2$ in the treated wastewater, which may be explained by the low total chlorine level at H2 (Table A.2). The culturable bacterial concentration ranged from 2.0×10^4 to 2.0×10^5 CFU/mL in the treated hospital wastewater. A similar result showed that live bacteria were still present at a density of 2.6×10^4 CFU/mL in the treated wastewater (Lee et al. 2017). Cefazidime-resistant

Fig. 5 Bacterial abundances expressed as the 16S rRNA gene copy number per millilitre (a), log density (CFU/mL) of cultivable bacteria in liquid lysogeny broth (b), and microbial community structure at the genus level in untreated and treated hospital wastewater (c)



bacteria ranged from 10^4 to 10^6 CFU/mL at two hospitals in Singapore (Le et al. 2016). β -Lactam-resistant bacteria can destroy even novel penicillins and cephalosporins (Levy and Marshall 2004). It has been reported that chlorine disinfection cannot completely inactivate ceftazidime-resistant bacteria, and there remains a potential risk of releasing the bacteria to the environment (Beattie et al. 2020). These results indicated that the treated hospital wastewater still contained high concentrations of microorganisms, especially live bacteria. These surviving bacteria may further proliferate and pose a risk for receiving MWWTPs.

The communities in the untreated and treated H1, H2, and H3 hospitals were also analysed. The bacterial compositions in hospital wastewater differed at the genus level (Fig. 5c). For example, sequences affiliated with the genera *Aquitalea*, *Thauera*, and *Romboutsia* were detected at the highest abundance in the untreated wastewater from H1, H2, and H3,

respectively. Notably, several genera related to (opportunistic) pathogens, such as *Acinetobacter* (3.59%), *Klebsiella* (2.07%), *Aeromonas* (8.84%), and *Pseudomonas* (7.60%), were found at relatively high abundances in hospital wastewater. This was similar to the results of a previous study, which showed that *Acinetobacter* and *Aeromonas* were the dominant (opportunistic) pathogens in untreated hospital water (Wang et al. 2018). *Acinetobacter* is a leading emerging opportunistic pathogen that is frequently reported at hospitals. For example, multiple antibiotic-resistant forms of *Acinetobacter baumannii* were discharged from hospital sites and hospital wastewater into receiving urban wastewater (Kovacic et al. 2017; Music et al. 2017). *Aeromonas* species showed resistance not only to most cephalosporins and penicillins but also to carbapenems, which are usually used as the last reliable antibiotic treatment. In recent years, the opportunistic pathogen *Aeromonas* sp. has frequently been reported to

contain both genomic and plasmid-mediated carbapenem resistance genes (Mathys et al. 2019).

In addition, the sequences affiliated with the genera *Pseudomonas* and *Spirillum* were dominant in treated wastewater from all three hospitals, accounting for 4.37–16.94% of the total genera. *Pseudomonas aeruginosa* is a common opportunistic human bacterium that is frequently detected in hospital wastewater; it has been reported to convey resistance to third- and fourth-generation cephalosporins and carbapenems and to persist after treatment with oxidizing chemicals (Polotto et al. 2012; Hou et al. 2019). Another typical human bacterium, *Escherichia coli*, which is strongly related to some human diseases, was also present in the treated wastewater from primary hospital H1. *Mycobacterium* is also a typical human pathogen with a unique cell wall that survives under long-term exposure to many antibiotics, oxidants, and even chlorine, and it presents higher relative abundances in the treated wastewater from H2 and H3 than in that from H1. Pathogens from hospital wards, ICUs, and toilets are discharged into HWWTPs, where the pathogens may be further propagated and extensively spread. A recent study showed that the abundance of multi-ARBs in hospital wastewater was significantly greater than that in non-hospital wastewater (Moges et al. 2014). Furthermore, it has been shown that hospitals are the main source of carbapenem-resistant pathogens in the environment (Lamba et al. 2017).

In addition, the presence of long-term chlorine disinfectants makes it possible for microorganisms to develop chlorine resistance (Luo et al. 2021). Considering that hospital wastewater contains a variety of clinically related antibiotics and pathogens, pathogens that show both antibiotic and chlorine resistance may enhance the potential risk of transmission to the aquatic environment and humans. Given the severe impact of the outbreak of COVID-19 in 2020, hospital wastewater also became a potential reservoir of the virus (Saguti et al. 2020). As discussed in the present study, the occurrence of antibiotics and the incomplete disinfection of ARGs and ARBs in HWWTPs indicate the potential release of these pollutants. Therefore, novel effective disinfection techniques for ARBs, ARGs, or even viruses in hospital wastewater matrices warrant further attention.

Conclusion

This study investigated the occurrence and removal of antibiotics, their corresponding ARGs, and human-related pathogens in different-scale hospitals. The occurrence of antibiotics and ARGs in wastewater from three hospitals varied greatly. Wastewater from primary hospital H1 frequently contained traditional antibiotics (e.g. ofloxacin and cefradine), while wastewater from tertiary hospital H3 contained new antibiotics (e.g. the fourth-generation cephalosporin cefepime and

the carbapenem meropenem). HWWTPs were not efficient at removing all ARGs, and the concentrations of several ARGs were increased in the treated wastewater. Antibiotics and ARGs have the potential risk of spreading into downstream MWWTPs and aquatic environments from hospital wastewater. The treated hospital wastewater still contained high concentrations of microorganisms, especially live bacteria. Several opportunistic or pathogenic bacteria (e.g. *Acinetobacter*, *Klebsiella*, *Aeromonas*, and *Pseudomonas*) had high proportions in the treated wastewater. These results provide the basis for the co-occurrence of new antibiotics, ARGs, and antibiotic-resistant pathogens in hospital sources and highlight the need to eliminate these contaminants in the hospital source.

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Author contribution The manuscript was reviewed and approved for publication by all authors. CC and SY conceived and designed the experiments. SY and QY performed the experiments. YH and JY analysed the data. SY and CC wrote the paper. SY, JY, QY, YH, TZ, LJ, SM, KL, and CC reviewed and revised the paper.

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Data Availability The availability of data and materials is on the base of personal request.

Declarations

Ethical approval The manuscript was reviewed and ethical approved for publication by all authors.

Consent to participate The manuscript was reviewed and consents to participate by all authors.

Consent to publish The manuscript was reviewed and consents to publish by all authors.

Conflict of interest The authors declare no competing interests.

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