

Occurrence and Removal of Antibiotics, Antibiotic-Resistance Genes and Bacterial Communities in Hospital Wastewater

Shijie Yao

East China University of Science and Technology

Jianfeng Ye

Shanghai Academy of Environmental Sciences

Qing Yang

Shanghai Academy of Environmental Sciences

Yaru Hu

East China University of Science and Technology

Tianyang Zhang

Tongji University

Lei Jiang

National Engineering Research Center of Urban Water

Salvator Munezero

East China University of Science and Technology School of Materials Science and Engineering

Kuangfei Lin

East China University of Science and Technology

changzheng cui (✉ cuichangzheng@ecust.edu.cn)

East China University of Science and Technology

Research Article

Keywords: Antibiotics, β -Lactams, Antibiotic resistance genes, Hospital wastewater treatment plants, Pathogenic bacteria, Disinfection

Posted Date: February 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-192739/v1>

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Abstract

Hospital wastewater contains a variety of human-related antibiotics and pathogens, which makes the treatment of hospital wastewater essential. However, there is a lack of research on these pollutants in hospital wastewater treatment plants. In this study, the characteristics and removal of antibiotics and antibiotic resistance genes (ARGs) in 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 of H1, while the fourth-generation cephalosporin cefepime had the highest concentration, 540.39 µg/L, in H3. Ofloxacin was detected at a frequency of 100% and had removal efficiencies of 44.2%, 51.5%, and 81.6% for 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), quinolone resistance gene *qnrA* (8.81×10^{-6} copies/16S rRNA), and integron *int11* (1.86×10^{-4} copies/16S rRNA) were detected in the treated wastewaters. 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 with high relative abundances in the treated wastewater. These results suggested the co-occurrence of novel antibiotics, ARGs, and antibiotic-resistant pathogens in hospital wastewater that may spread into the receiving water environment.

1 Introduction

Antibiotics have been widely used for curing infectious diseases since they were discovered in the 1930s. However, the excessive use and abuse of antibiotics has contributed immensely to the emergence of antibiotic-resistant bacteria (ARB), such as vancomycin-resistant enterococci (Hocquet et al. 2016) and methicillin-resistant *Staphylococcus aureus* (Knight et al. 2012). Hospitals accumulate great amounts of antibiotics and human-related pathogens (Andersson and Hughes 2014). For example, *Acinetobacter baumannii* and *Citrobacter freundii*, which cause serious hospital-linked 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 ARB and ARGs from hospital wastewater poses ecological and ARB evolution risks to water environments and human beings.

Large amounts of antibiotics are consumed in Chinese hospitals, especially β-lactams, quinolones and trimethoprim (Klein et al. 2018; Boeckel et al. 2014; Zhang et al. 2015a). However, their occurrence in hospital wastewater is not yet very clear. 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 water environment or downstream municipal wastewater treatment plants (MWWTPs). In the past few years, many studies have focused on municipal wastewater treatment plants 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 gap of knowledge on the occurrence of antibiotics, from the traditional to the last-line antibiotics, and their corresponding ARGs in hospital wastewater treatment protocols. Studying independent HWWTPs could help us understand the discharge of medical-related antibiotics and ARGs and reveal the potential risks of hospital wastewater to the water environment and receiving MWWTPs.

Hospitals can be divided into primary, secondary and tertiary hospitals according to their medical capabilities and facilities (Jamison et al. 2006; Moore et al. 2014; NHCPRC 1989). The 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 hospitals) (NHCPRC 1989; WHO 1978). The different health services, bed capacity, antibiotic usage, amount of wastewater and HWWTPs in different-scale hospitals may lead to different pollution patterns of hospital-related 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 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 not yet very clear. Therefore, this study aims to (1) reveal the pollution characteristics of a variety of antibiotics and ARGs in 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.

2 Materials And Methods

2.1 Sample collection

Samples were collected in triplicate in January and May of 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 cubic metres per day. Briefly, H1 adopted a simple chlorine disinfection process because of its small scale and wastewater amount. In 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 of 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 three HWWTPs, as shown in Fig. 1. The raw wastewater was a mixture of the medical ward and domestic wastewater. Fresh mixed water samples were collected in 5 L brown glass bottles, returned to the laboratory on ice in coolers and analysed immediately. Containers were washed with methanol, water, and sterilized deionized water before wastewater collection. These included 18 samples in each season's sampling campaign. The wastewater $\text{NH}_3\text{-N}$ 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).

2.2 Quantification of antibiotics in hospital wastewater

Samples were analysed in triplicate for the determination of the target antibiotics following established methods (Cheng et al. 2015; Gros et al. 2013) with certain 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 of a Na_2EDTA solution 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 preconcentration, 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, 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% in 4 min, then increased further to 80% in 2 min, and finally increased to 95% in 2 min; it was held at 95% for 1 min and then brought back to the initial value in 0.1 min, where it was maintained for 2 min until the next injection. Acquisition of mass spectra was conducted 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 compounds of first-generation cephalosporins (cefalexin, cefalotin, cefazolin, cefradine); second-generation cephalosporins (cefoxitin); third-generation cephalosporins (ceftazidime); fourth-generation cephalosporins (cefepime); penicillins (amoxicillin, ampicillin, penicillin G); carbapenems (meropenem); quinolones (ofloxacin, norfloxacin); and trimethoprim were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Ultrapure water was prepared using a Milli-Q water system (Millipore, USA). For accurate quantification, stable isotope $^{13}\text{C}_3$ -caffeine 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, linearities R^2 , reproducibilities and repeatabilities 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 developed (Gros et al. 2013).

2.3 Characterization of bacteria and quantification of target genes

Hospital wastewater samples were serially diluted in 1× phosphate-buffered saline (PBS), and 100 μ L 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 in the sampling site. Colonies in the range of 30 to 300 cells were counted to minimize the error caused by the too-numerous-to-count problem. One hundred millilitres of hospital wastewater was filtered through a 0.22- μ m pore size cellulose nitrate membrane (Anpel, Shanghai, China). Total DNA was extracted 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 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. In addition, for quantitative PCR (qPCR) analysis, DNA samples were diluted tenfold with nuclease-free water to minimize background interferences of the qPCR reactions. qPCR was used to measure the abundances of 10 specific ARGs, including 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 *int1* were analysed to determine the relative abundances of ARGs and the transferabilities of ARGs, respectively. All runs were performed with positive standards and negative controls. The positive standards 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. 2019). The details of the primers, annealing temperatures, and amplicons for the target genes are listed in Table A.4.

2.4 Statistical analysis

The results are presented as the means \pm standard deviations (SDs), and all diagrams were generated using R 3.6.1 (The R Foundation for Statistical Computing). Two-tailed $p < 0.05$ was judged as significant.

3 Results And Discussion

3.1 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). $\text{NH}_3\text{-N}$, TP, and COD values decreased after HWWTP treatment. In particular, the $\text{NH}_3\text{-N}$ values ranged from 10.4 mg/L to 50 mg/L in the treated wastewater. $\text{NH}_3\text{-N}$ in wastewater can rapidly react with free chlorine to form chloramine, thereby reducing the concentration of free chlorine and the disinfection effect (Kevin 2016; Huang et al. 2012). Therefore, the breakpoint dosage (a mass ratio of chlorine to $\text{NH}_3\text{-N}$ of 7.6:1) of free chlorine was required in the presence of $\text{NH}_3\text{-N}$ in wastewater (Zhang et al. 2015b).

3.2 Occurrence of antibiotics in hospital wastewater

The pollution characteristics of antibiotics in hospital wastewater are shown in Fig. 2. 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 by 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, ofloxacin and the first-generation cephalosporin cefradine were detected at high concentrations in the wastewater of H1. The latter was higher than the concentration of cefradine (0.17 μ 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 of H2 were quite different from those in H1. High

concentrations of the second-generation cephalosporin cefoxitin and the third-generation cephalosporin ceftazidime were detected in H2 wastewater. Ceftazidime-resistant bacteria ranged from 10^4 CFU/mL to 10^6 CFU/mL in two hospitals in Singapore (Le et al. 2016). β -Lactam-resistant bacteria can destroy even novel penicillins and cephalosporins (Levy et al. 2004). It had 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). The greatest diversity of antibiotics was detected in H3 compared with H1 and H2. H3 was the largest central hospital, receiving approximately 700,000 patients every year, and had independent intensive care units (ICUs). Thirteen kinds of antibiotics, including ten cephalosporins, two quinolones and trimethoprim, were detected in wastewater from H3. Strikingly, fourth-generation cephalosporin cefepime was only detected in H3 wastewater, with the highest concentration of 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 lower consumption of these antibiotics (providing service to 30,000 inhabitants) in the Romanian hospital (Szekeres et al. 2017). The carbapenem antibiotic meropenem, generally used as the last line of defence, was also detected in H3 samples at a concentration of 0.20 $\mu\text{g/L}$. A higher concentration of meropenem (1.07 $\mu\text{g/L}$) was detected in a tertiary hospital that had 1,500 beds (Le et al. 2016). Furthermore, the concentrations of the second-generation cephalosporin cefoxitin and the third-generation cephalosporin ceftazidime in H3 wastewater were also higher than those in H1 and H2 wastewaters. Notably, we found the third-generation cephalosporin ceftazidime in 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).

Table 1

The concentration and removal efficiency of different kinds of antibiotics in hospital wastewater treatment plants in this study and in current studies

Antibiotics	This study						Literatures data ^a			
	H1		H2		H3		HWWTPs		References	
	Untreated (µg/L)	Removal efficiency (%) ^b	Untreated (µg/L)	Removal efficiency (%)	Untreated (µg/L)	Removal efficiency (%)	Untreated (µg/L)	Removal efficiency (%) ^c		
Cefalexin	0.09 ~ 0.56	100	0.03 ~ 0.10	57.3	0.03 ~ 0.88	100	n.d. ^c 2.23	- ^d -	Gros et al. 2013 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. 4.91	- -	Gros et al. 2013 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	

^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 data.

Antibiotics	This study						Literatures data ^a		
	H1		H2		H3		HWWTPs		References
	Untreated (µg/L)	Removal efficiency (%) ^b	Untreated (µg/L)	Removal efficiency (%)	Untreated (µg/L)	Removal efficiency (%)	Untreated (µg/L)	Removal efficiency (%) ^c	
							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 data.									

As shown in Fig. 2, ofloxacin was detected with a high frequency of 100% and high concentrations of 9.23 µg/L, 25.65 µg/L and 49.47 µg/L in the samples from H1, H2 and H3. 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 µg/L to 0.94 µg/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 in H1, H2 and H3 at concentrations of 0.02 µg/L, 0.31 µg/L, and 0.50 µg/L, respectively. The low 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 µg/L to 0.84 µg/L in a

hospital in Beijing, China (Liu et al. 2019). However, trimethoprim was detected with the highest concentration of 30.3 µg/L in hospital wastewater of Romania (Szekeres et al. 2017). In recent years, total antibiotic consumption in many low- and middle-income countries was higher than that in high-income countries, showing a rapid increasing trend (Klein et al. 2018). The hospital consumption of antibiotics in China was the second-largest source in the world (Boeckel et al. 2014). Therefore, we suggest strengthening the management of antibiotics in hospitals and using antibiotics reasonably to reduce their discharge from hospital sources.

3.3 Removal of antibiotics in different HWWTPs

Considering that many kinds of antibiotics are widely detected in untreated hospital wastewater, the removal of antibiotics in different HWWTPs is further discussed. The removal rates of the target antibiotics in different HWWTPs ranged from 20.2–100% (Table 1). Specifically, the removal rates of β-lactams (including cephalosporins, penicillins, and carbapenem) reached 100% in the independent HWWTPs of 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 – 74.0–81.0% (Szekeres et al. 2017). High concentrations of β-lactams remained in the treated wastewater of H2, with the highest concentration being 2.95 µg/L. This differential result may be attributed to the fact that the removal of β-lactams is highly affected by chemical or hydraulic retention times (Hou and Poole 1971; Le-Minh et al. 2010). The concentration of chlorine in H2 was relatively lower than those in H1 and H3 (Table A.2). It is also generally observed that increasing the removal efficiency of β-lactams demands more oxidizing agent (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 water environments.

Ofloxacin was recalcitrant and detected with a frequency of almost 100% in the treated effluents in 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 in the HWWTPs of H1 and H2 were 44.2% and 51.5%, respectively, and the residual amounts of ofloxacin in the treated wastewaters of H1 and H2 reached 7.62 µg/L and 16.18 µg/L, respectively. Although the removal efficiency of ofloxacin in the HWWTP of H3 was improved (68.8%), this antibiotic still presented a high concentration of 14.90 µg/L in the treated wastewater. This result was consistent with those of previous studies that proved that the traditional hospital wastewater treatment systems applying biological processes, biological anoxic reactors and membrane filtration methods exhibited limited removal efficiency for ofloxacin (Dinh et al. 2017; Kovalova et al. 2012). The low removal efficiency of ofloxacin may be attributed to the chemically stable piperazine ring and its lack of biodegradation vulnerabilities (Hapeshi et al. 2013; Turiel et al. 2005). 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 Fe²⁺ and Cu²⁺ ions (Barhoumi et al. 2016; Barhoumi et al. 2017).

3.4 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 order of the average relative abundances of target ARGs and *int1* 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 in the tertiary hospital H3 was more serious than that in the primary hospital H1 and the secondary hospital H2. The most abundant ARGs in the wastewaters of 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 wastewaters of H1, H2 and H3 were 6.21×10^{-5} , 1.77×10^{-3} and 9.44×10^{-4} copies/16S rRNA gene, respectively. This indicated that hospital wastewater was seriously polluted by carbapenem resistance genes that pose great health risks to humans and the environment. Carbapenems show the broadest antibacterial spectrum with the strongest antibacterial activity and have become some of the most important antimicrobial drugs for the treatment of severe bacterial infections in hospitals. Unfortunately, carbapenem resistance genes are persistent in hospital wastewater, and carbapenemase-producing *Enterobacteriaceae* propagate and proliferate worldwide (Nordmann et al. 2011). In addition, the cephalosporin resistance gene *bla*_{OXA-1} showed a high relative abundance of 1.27×10^{-3} copies/16S rRNA gene in three hospitals. *bla*_{OXA-1} is frequently detected (57.9%) in *Escherichia coli* resistant to cefotaxime in hospital wastewater (Adegoke et al. 2020). The OXA resistance gene encodes a class of β-lactamases that can move from chromosomes to plasmids (Barlow and Hall 2002). It seems that the abundance of β-lactam resistance genes in this study was higher than those in groundwater, surface water and drinking water but less than that in pharmaceutical wastewater treatment systems (Hu et al. 2019; Wu et al. 2020; Zhai et al. 2016). The class 1 integron displayed the highest abundance of 2.16×10^7 copies/mL in untreated

hospital wastewater. Similar results in which a high abundance of *int1* ($1.51\text{--}4.57\times 10^7$ copies/mL) was detected in untreated hospital wastewater have been reported (Wang et al. 2018). *int1* provided the opportunity to transfer the embedded ARGs to new genera by horizontal gene transfer. As indicated in Table A.5, the last-line antibiotic (carbapenem) resistance gene *bla*_{GES-1} was strongly associated with *bla*_{OXA-10}. In addition, *bla*_{OXA-10}, *bla*_{DHA-1}, *bla*_{SHV-1}, *qnrA* and *qnrS* were significantly correlated with *int1* 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).

The 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 concentration of *qnrA* in this study was one magnitude higher than that in the above study, indicating serious pollution in this study. In addition, the average number of β -lactam resistance genes was two orders of magnitude higher than that of quinolone resistance genes, which were present at 1.53×10^{-6} copies/16S rRNA gene in treated wastewater from the three hospitals. This result suggested that the high concentration of β -lactams caused serious development of ARB in hospital and hospital wastewater. 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 concerned ARGs (Ju et al. 2016).

In addition, correlations of all the concerned types of antibiotics and their corresponding ARGs were further evaluated (Fig. A.1). 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 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 (Rodriguez-Mozaz et al., 2015). Exposure to antibiotics could exert selective pressure on bacteria and increase ARGs in the environment. Although we have demonstrated a significant correlation between several types of antibiotics and ARGs, the environmental influencing factors or other pollutants in the hospital wastewater matrix need to be further evaluated to provide a better understanding of the co-occurrence of these pollutants.

3.5 Removal of ARGs in different HWWTPs

The removal efficiencies of ARGs in different HWWTPs are shown in Fig. 4. The removal rates of the target ARGs in the three HWWTPs ranged from -0.85 log to 2.71 log (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 in traditional wastewater treatment plants (Laht et al. 2014; Rodriguez-Mozaz et al. 2015). The ARGs were not so efficiently reduced by the treatment process. The traditional wastewater treatment process cannot efficiently remove target ARGs in hospital wastewater, causing their spread and gene transfer to wild bacteria. 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 in H1. Several studies have proven that the chlorination process can increase ARGs. For example, aminoglycoside-resistant genes (*aac(6)-II* and *aacC2*) and tetracycline-resistance genes (*tetR* and *tetX*) increased from 0.22 log to 2.23 log (Wang et al. 2020). It was also observed that chlorination had no significant ARG removal effect (Liu et al. 2019). Several ARGs (e.g., *qnrB*, *tetM* and *tetW*) were also increased after disinfection with sodium hypochlorite (Hu et al. 2019). 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 nonlethal dose of chlorine (0.5 mg/L) (Wang et al. 2020). Therefore, the enhanced disinfection efficiency and the inhibition of transferability of ARGs in hospital wastewater need to be considered. 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 in H3 was significantly higher than those in H1 and H2 (Fig. 4). In previous work, less than 1.2 log of *int1* was removed by hospital wastewater treatment, and *int1* remained detectable at 8×10^{-5} copies/16S rRNA gene (Timraz et al. 2017). Forty-three ARG cassettes mediated by *int1* were detected with great diversity in hospital wastewater (Yuan et al. 2021). 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 wastewaters. The residual ARB and ARGs in the treated wastewaters are further sent into the MWWTPs and become potential sources of gene propagation and horizontal transfer (Bengtsson-Palme et al. 2016; Xu et al. 2015). It was observed that the impact and contribution of ARGs and ARB in hospital wastewater on the receiving MWWTPs and water environment was up to 11.49% (Beattie et al. 2020; Rodriguez-Mozaz et al. 2015). 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 developing 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).

3.6 Analysis of bacteria and community characterization of 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 copies/mL to 8.85×10^{10} copies/mL in the wastewater, higher than the values in a previous study (Szekeres et al. 2017). This may be attributed to the lower number of people served around (30,000 inhabitants) in Cluj County, Romania (Szekeres et al. 2017). The removal rates of 16S rRNA gene showed a declining trend, with the removal ranging from 0.05 log 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 in H2 (Table A.2). The culturable bacteria concentration ranged from 2.0×10^4 to 2.0×10^5 CFU/mL in the treated hospital wastewaters. A similar result showed that live bacteria still presented 2.6×10^4 CFU/mL in the treated wastewater (Lee et al. 2017). 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 wastewaters of H1, H2 and H3, respectively. Notably, several genera related to (opportunistic) pathogens, such as *Acinetobacter*, *Klebsiella*, *Aeromonas* and *Pseudomonas*, 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 in 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. was frequently 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 all three treated hospital wastewaters, 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 persists 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, also existed in the treated wastewater in primary hospital H1. Recently, various *Escherichia coli* samples from different aquatic environments were proven to contain *bla*_{NDM-1} or *bla*_{KPC} which are typical ARGs related to invincible bacteria (Le-Vo et al. 2019). *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 wastewaters of H2 and H3 than in that of H1. Pathogens from hospital wards, ICUs, and toilets were discharged into HWWTPs, where the pathogens may be further propagated and extensively spread. A recent study showed that the presence of multi-ARB 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 make it possible for microorganisms to obtain chlorine resistance (Luo et al. 2021). Considering that hospital wastewater contains a variety of clinically related antibiotics and pathogens, the pathogens showed that both antibiotic and chlorine resistance may enhance the potential risk of transmission to the water environment and human beings. 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 novel antibiotics and the incomplete disinfection of ARGs and ARB in

HWWTPs indicate the potential release of these pollutants. Therefore, novel effective disinfection techniques for ARB, ARGs or even viruses in hospital wastewater matrices are worth further attention.

4 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. Primary hospital H1 wastewater frequently exhibited traditional antibiotics (e.g., ofloxacin and cefradine), while tertiary hospital H3 wastewater exhibited new antibiotics (e.g., the fourth-generation cephalosporin cefepime and the carbapenem meropenem). HWWTPs were not efficient in removing 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 water 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 relative abundances 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 the need to eliminate these contaminants in the hospital source.

Declarations

Acknowledge

This work was supported by the Science and Technology Commission of Shanghai Municipality (No. 20dz1200800), and China National Major Science and Technology Program for Water Pollution Control and Treatment (No. 2017ZX07402003).

Availability of data and materials

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

Funding

The Science and Technology Commission of Shanghai Municipality (No. 20dz1200800), and China National Major Science and Technology Program for Water Pollution Control and Treatment (No. 2017ZX07402003) .

Authors' Contributions

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 analyzed the data. SY and CC wrote the paper. SY, JY, QY, YH, TZ, LJ, SM, KL and CC reviewed and revised the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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.

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Figures

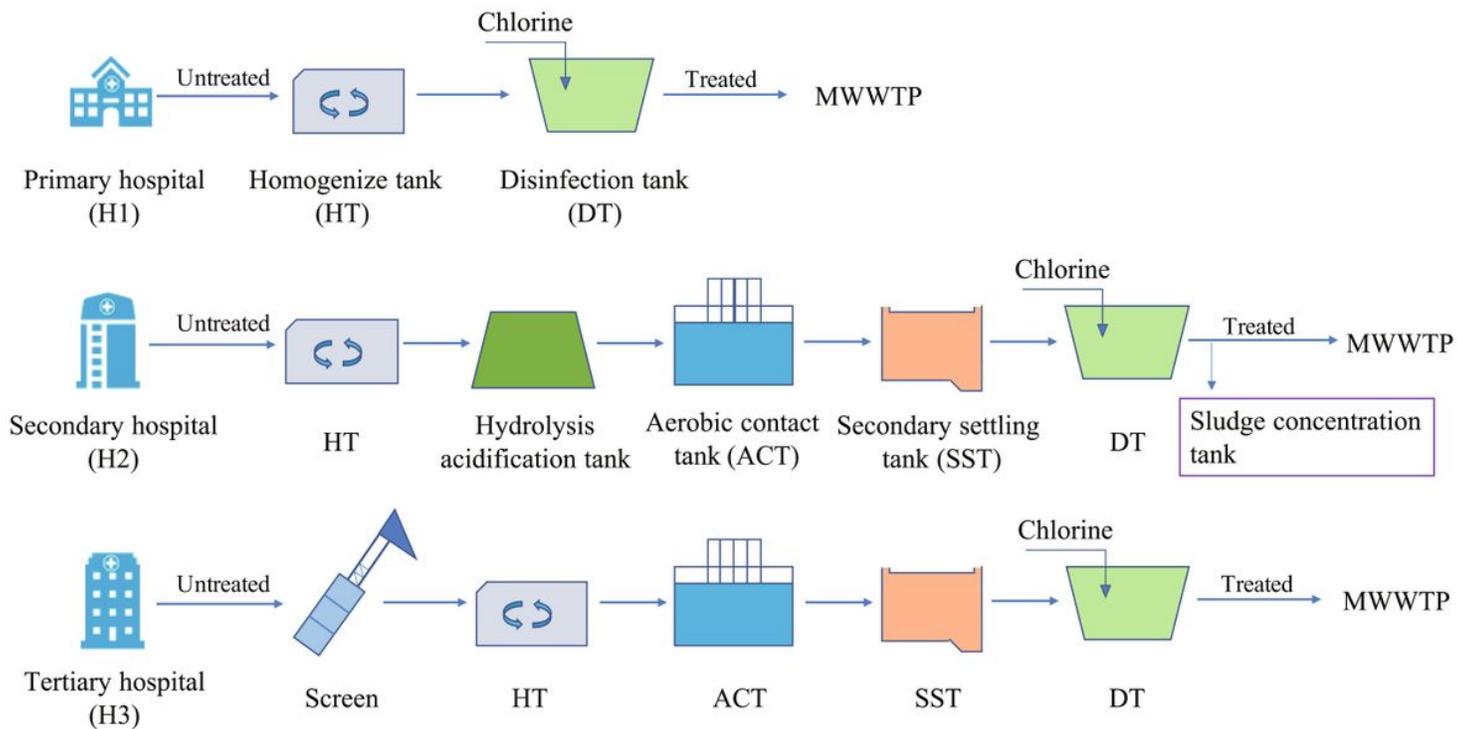


Figure 1

Schematic diagram of three hospital wastewater treatment plants. The wastewaters of these three hospitals were eventually discharged to the receiving municipal wastewater treatment plants (MWWTPs).

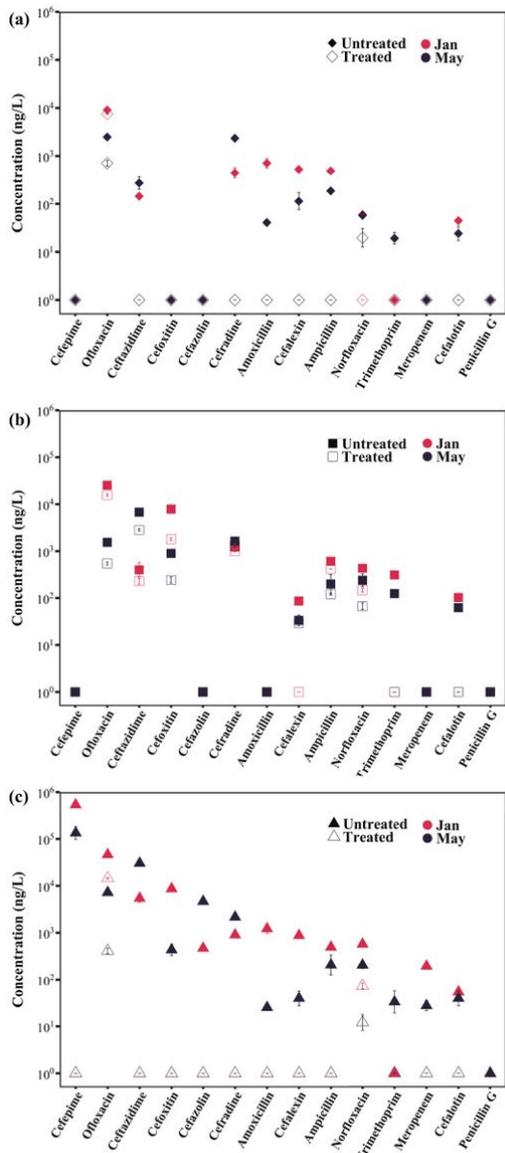


Figure 2

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

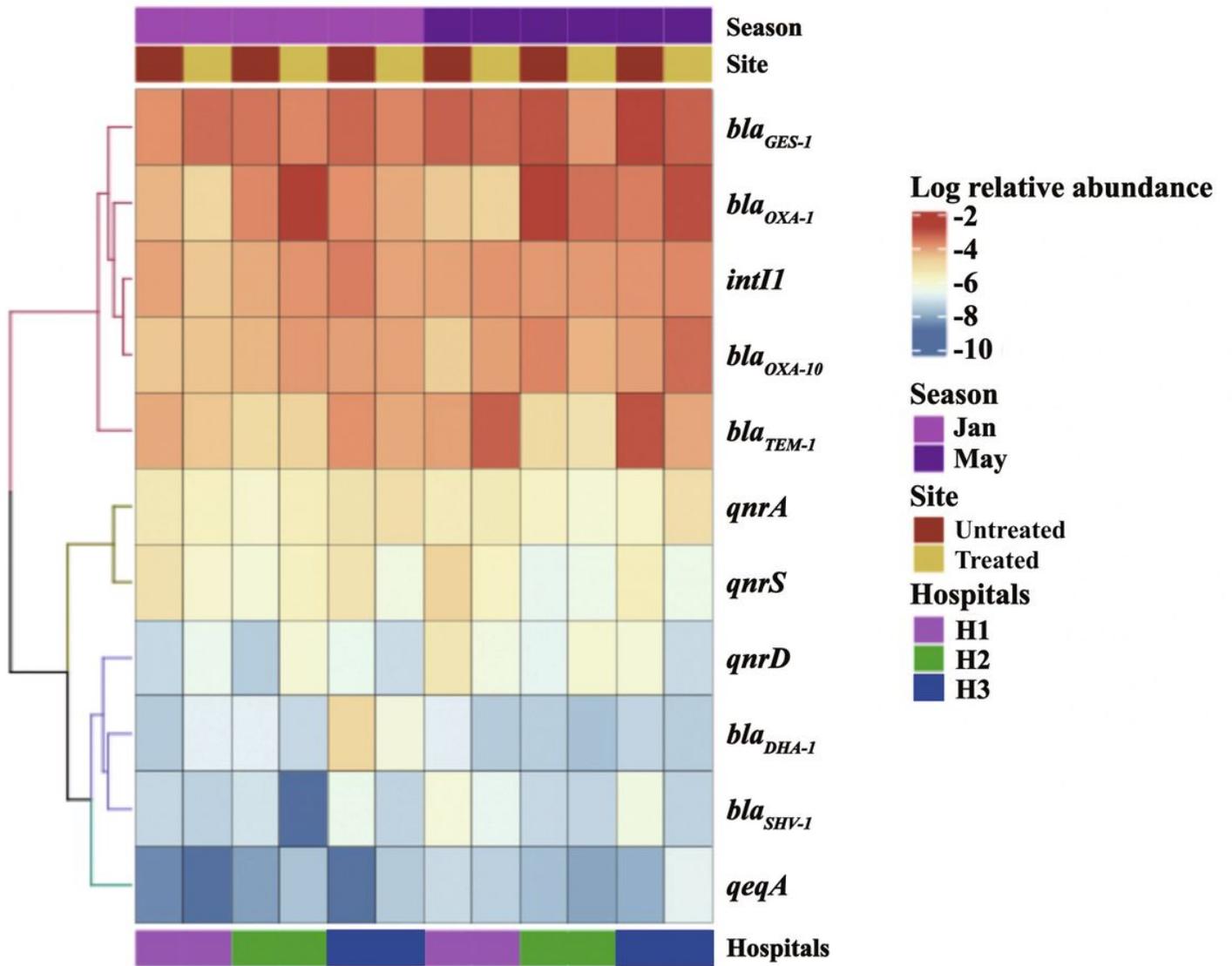


Figure 3

Heat map of the relative abundances of ARGs in the three HWWTPs. The concentrations of ARGs were normalized to the 16S rRNA level and log transformed.

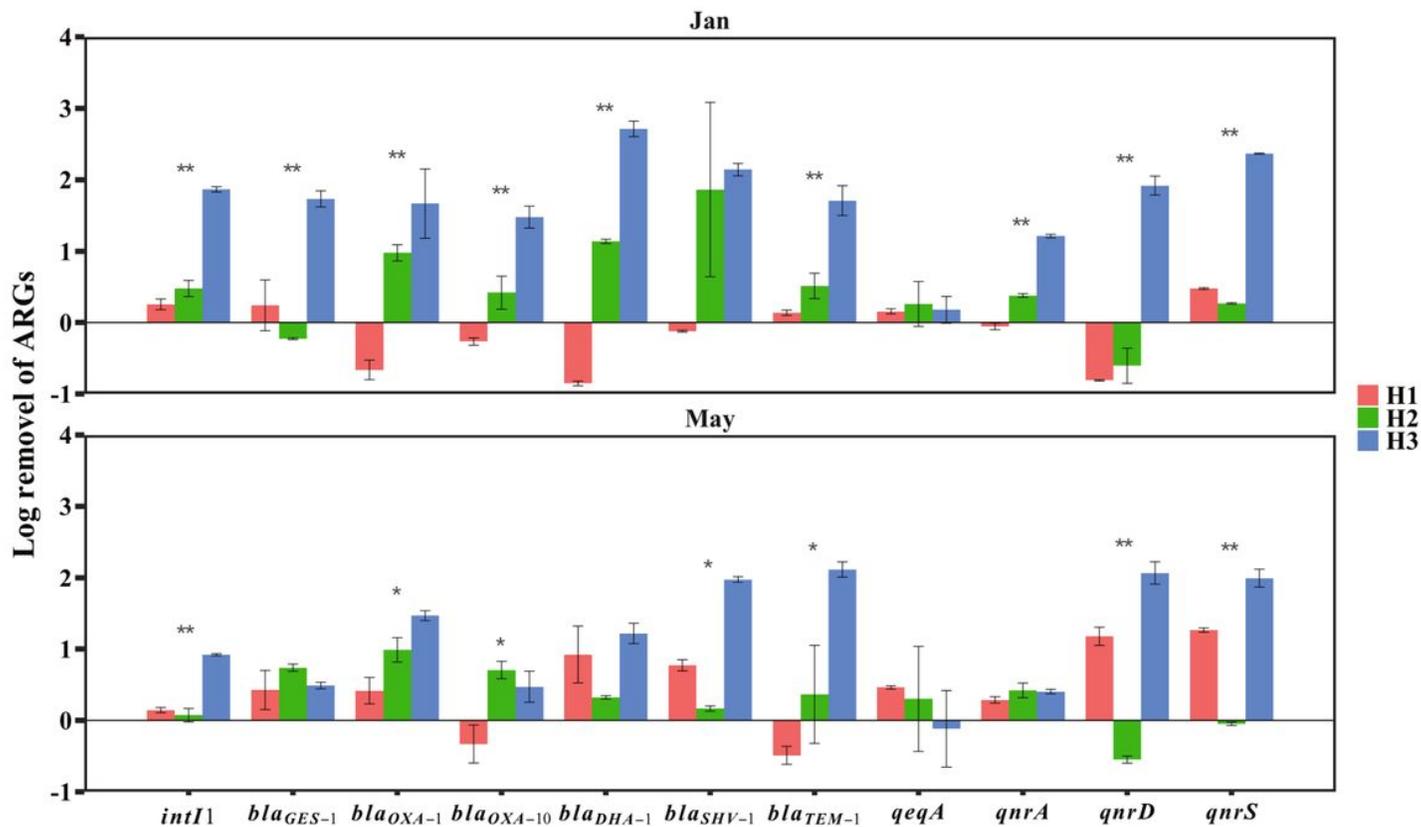


Figure 4

Log removal of ARGs in three hospital wastewater treatment plants. Log removal of ARGs = $\log(\text{C}_{\text{untreated}}/\text{C}_{\text{treated}})$. A log removal value <0 means that hospital wastewater treatment processes increased the concentration of target ARG.

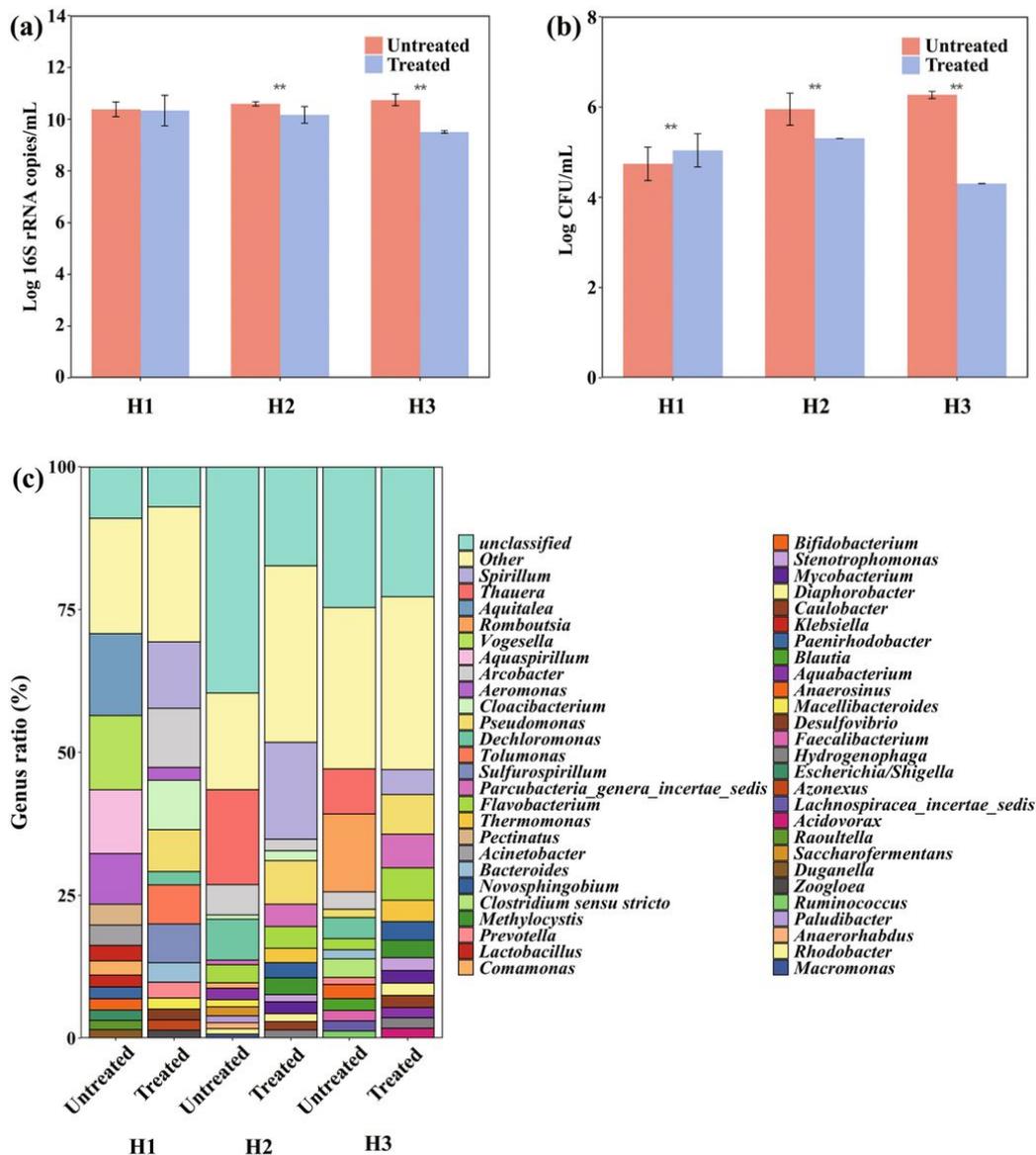


Figure 5

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupportingInformation.docx](#)