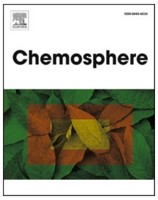
Chemosphere 283 (2021) 131224

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere



Metagenomic profiles and health risks of pathogens and antibiotic resistance genes in various industrial wastewaters and the associated receiving surface water

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ARTICLE INFO

Handling Editor: A. Gies

Keywords:
Pathogens
Antibiotic-resistance genes
Metagenomic analysis
Virulence factors
Health risk

ABSTRACT

The aquatic environment may represent an essential route for transmission of antibiotic resistance to opportunistic human pathogens. Since industrial wastewater is discharged into the river after treatment, understanding the distribution of antibiotic resistance genes (ARGs) in river systems and the possibility of pathogens acquiring antibiotic resistance are challenges with far-reaching significance. This work mainly studied distribution profiles of pathogens and ARGs, and compared their health risk in various industrial wastewater with that of river water. Results showed that 166 pathogens were concurrently shared by the six water samples, with *Salmonella enterica* and *Pseudomonas aeruginosa* being the most abundant, followed by *Fusarium graminearum* and *Magnaporthe oryzae*. The similar composition of the pathogens suggests that pathogens in river water may mainly come from sewage discharge of slaughterhouses and that changes in water quality contribute significantly to the prevalence of these pathogens. Of the 57 ARG types detected, bacitracin was the most abundant, followed by sulfonamide, chloramphenicol, tetracycline, and aminoglycoside. Strikingly, the wastewater from a pharmaceutical factory producing Chinese medicine was also rich in *bacA*, *sul1*, *mexW*, *mexB*, *mexF* and *oprn*. It can be seen from the co-occurrence patterns that pathogens and the main ARGs have strong co-occurrence. Higher abundance of offensive virulence factors in industrial wastewater and their strong correlation with pathogens containing ARGs suggest higher microbiological risk. These findings highlight the need to assess ARG acquisition by pathogens in the surface water of human-impacted environments where pathogens and ARGs may co-thrive.

1. Introduction

Globally, urbanization and industrialization are usually associated with the dissemination of antibiotic resistance of microorganisms owing to the increased input of antibiotics due to human activities (Marano et al., 2019; Pruden et al., 2012). Considerable evidence shows that overuse and misuse of antibiotics in clinical medicine, aquaculture and livestock breeding (as food additives and growth promoters) can enter aquatic systems through different routes including municipal sewage plants, animal husbandry and landfill leachate (Ben et al., 2019; Watts et al., 2017). Moreover, antibiotic residues that remain in various environmental systems, especially in wastewater, might pose a powerful selection pressure on natural and human microbial systems, resulting in the widespread occurrence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Pan and Chu, 2018; Qiao et al., 2018). Unfortunately, the prevalence of ARGs and ARB, as new emerging pollutants, has caused great concern for human beings, becoming an emerging challenge worldwide. This is mainly because ARGs may recombine with clinically important pathogens and evolve to create multiple antibiotic resistance bacteria and even super-bacteria, leading to the failure of conventional drug treatments and posing a potential risk to both animal and human health (Marano et al., 2019).

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https://doi.org/10.1016/j.chemosphere.2021.131224
Received 1 March 2021; Received in revised form 6 June 2021; Accepted 11 June 2021

Besides antibiotics, adverse stimulation from the water environment also has a great influence on the development of ARGs (Bengtsson-Palme et al., 2018). For example, heavy metals can not only enrich metal-resistance genes, but also contribute to the proliferation of ARGs (Imran et al., 2019; Xu et al., 2017). Other external environmental stimuli, such as herbicides, fungicides, insecticides, light irradiation and natural minerals, can also activate the bacterial stress system, thereby producing certain resistance to antibiotics (Chen et al., 2019; Hawkins et al., 2019; Li et al., 2020; Zhou et al., 2017). Wastewater containing antibiotics, heavy metals and other pollutants from hospitals, wastewater treatment plants, slaughterhouses and pharmaceutical plants are thus all considered as the hot spots of ARGs (Larsson et al., 2018; Michael et al., 2013).

Previous studies have demonstrated that wastewater significantly contributes to the prevalence and diversity of ARGs and pathogens in the environment (Czekalski et al., 2014). For example, the *strB*, *ermB* and *sul1* genes were greatly enriched by combining sewage, runoff from agricultural areas and tail water from urban wastewater treatment plant (Wu et al., 2019a). Furthermore, since ARGs always exist in the natural aquatic environment, and ARG-containing industrial wastewater is eventually discharged into rivers, the natural surface water system is basically considered as the bank and hub of ARGs (Rizzo et al., 2013; Stoll et al., 2012; Xu et al., 2020). By exploring the relationship between ARG profiles and human activities, Pruden et al. found that the upstream capacities of wastewater treatment plants and animal feeding operations were correlated with *sul1* (Ben et al., 2017; Pruden et al., 2012). Moreover, according to metagenomic analyses, the pathogens in human environments share almost the same resistome with those in nature systems, primarily because ARGs can be readily transferred across bacterial species (Harwood et al., 2014). To date, however, the pollution profiles of ARGs and pathogens in various wastewater environments, as well as the characterization of their impacts on ARGs and pathogens in the receiving water, has not received extensive attention. In addition, although Ashbolt et al. has revealed that industrial wastewater influences the composition of ARGs and pathogens in the effluent of advanced drinking water treatment systems (Ashbolt et al., 2013), no reports have addressed the mutual influence between ARGs and pathogens in different water environments.

With the ubiquity of antibiotic resistance in the natural environment, growing attention has focused on the risk evaluation of antibiotic resistance transferred to the human environment (human beings are directly exposed to ARB, or exposed to commensal human pathogens that acquire resistance through horizontal transfer) (Ashbolt et al., 2013). However, human health risk evaluation of antibiotic resistance has been principally associated with antibiotic residues in drinking water, soil, food, air and dust (Ben et al., 2019). Research on the transfer of ARGs or ARBs in the natural aquatic environment to human-related environments is very lacking. Recently, attempts have been made to evaluate the daily intake of ARGs via the inhalation of bioaerosols (Liang et al., 2020a), drinking water, $PM_{2.5}$ and ingestion of soil particles (Xie et al., 2018). However, to the best of our knowledge, the public health challenges related to both ARGs and ARB in wastewater has yet to be adequately addressed. To evaluate the probability of illness and death from ARB-associated infection, more studies are necessary that consider human exposure to ARB and ARGs.

The main objectives of this work were to: (1) compare the diversity and relative abundance of pathogens and ARGs in wastewater from a food processing plant (FPP), slaughterhouse (SH), Chinese medicine pharmaceutical factory (CMPF), hospital (HP), wastewater treatment plant (WWTP) with the those in river water (RW) through metagenomic analysis; (2) analyze the impact of external environmental factors (water quality and heavy metals) and human activities (WWTP and industrial wastewater from FPP, SH, CMPF and HP) on the distribution and abundance of ARGs in the water samples; (3) attempt to assess the possible health risk and dissemination risk of ARG-containing pathogens and ARGs from industrial wastewater and WWTPs to the surface water. These results provide comprehensive profiles of ARGs and pathogens in various water environments, which could be a useful reference for water treatment optimization, water quality management and public risk control.

2. Materials and methods

2.1. Sampling sites and sample collection

Water samples (1 L each) were collected on July 16, 2019 at six distinct sites, specifically RW and five discharge ports from FPP, SH, CMPF, HP and WWTP located in south China. To ensure representation, the water was collected from three random places at each sampling site and quickly transferred to laboratory on ice. For the follow-up experiment, three random samples from each site were mixed and then the six mixed samples were used for further treatment. It is worth mentioning that before being discharged, the four industrial wastewaters were treated as follows: the wastewaters from FPP and CMPF were purified by hydrolysis acidification after coagulation and sedimentation, and then oxidized using contact oxidation technology; the SH wastewater was first oxidized and then treated by hydrolysis acidification in a pulse tank; the HP wastewater was first passed through an adjustment tank to regulate water quality, then flowed into a biological contact oxidation tank, and finally disinfected with chlorine dioxide (Yu et al., 2014). All these preliminarily treated wastewaters were further purified in WWTP employing the anaerobic-anoxic-oxic treatment technology before being discharged into the river.

2.2. DNA extraction and quality control

Each water sample (0.5 L) was first filtered through a 10-μm membrane (PVDF, JINTENG, China) to remove impurities, and then the filtrate was passed through a 0.22-μm membrane (PVDF, JINTENG, China) to obtain the microorganisms (Mao et al., 2014). After removing the extracellular DNA using primers (as shown in Table S1 and the methods described in Supporting Information (SI)), the intracellular DNA (iDNA) was extracted with the E.Z.N.A.® DNA Kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's protocol, which is provided in the SI. The iDNA concentration and purity were determined using both spectrophotometry (NanoDrop2000) and agarose gel electrophoresis. The extracted iDNA with an $OD_{260/280} > 1.8$ was stored at -80 °C for further analysis.

2.3. Analysis of environmental water quality

Water temperature, dissolved oxygen and pH were measured with portable instruments (ST300D; ST3100, OHAUS, USA). While the total phosphorus (TP), total organic carbon (TOC) and chemical oxygen demand (COD) were analyzed via ammonium molybdate spectrophotometry, combustion oxidation nondispersive infrared absorption and fast digestion spectrophotometry, respectively, according to the national standard method of China for water quality (HJ501–2009, GB11893-89, HJ/T399-2007). The concentrations of four heavy metals (Zn, Cu, Pb and Cd) were analyzed using inductively coupled plasma mass spectrometry (ICP-MS; PerkinElmer ELANDRC-e, USA). The detailed procedures of microwave digestion and the ICP-MS operating parameters are described in SI and Table S2. Detection limits and recoveries of the four heavy metals were in the range of 0.006–0.030 mg L^{-1} and 87.5%–97.6%, respectively.

2.4. Library preparation, sequencing and assembly

To ensure high quality data, the following criteria were observed: the DNA concentration of each sample must be greater than or equal to 10 ng μL^{-1}; the total amount of DNA in each sample must be greater than or equal to 1 μg; and the DNA must be free from degradation and contamination. The DNA concentration was quantified with the TBS-380 fluorometer. The DNA quality was examined by 1% agarose gel electrophoresis. The DNA was fragmented for paired-end library construction to an average size of approximately 300 bp using the Covaris M220 ultrasonicator (Gene Company Limited, China). The paired-end library was prepared using the TruSeq™ DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Adapters containing a full complement of sequencing primer hybridization sites were ligated to the blunt-end fragments and sequenced on the Illumina HiSeq4000 platform (Illumina Inc., San Diego, CA, USA) using the HiSeq 3000/4000 PE Cluster Kit and HiSeq 3000/4000 SBS Kit according to the manufacturer's instruction (www.illumina.com). Sequencing was performed at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Low-quality bases (length $<$ 50 bp, quality value $<$ 20 or presence of N bases) were discarded using Sickle (https://github.com/najoshi/sickle) according to reference (Noguchi et al., 2006). The clean data obtained for each sample (8.33 Gbp on average) were deposited into the National Center for Biotechnology Information (NCBI) sequence reads archive (SRA) under accession number SUB7750109.

The de Brujin graph-based assembler SOAPdenovo (http://soap.genomics.org.cn, version 1.06) was employed to assemble short reads. K-mers, varying from $1/3 - 2/3$ of the read length, were tested for each sample. Scaffolds with a length over 500 bp were retained for statistical tests. Specifically, we evaluated the quality and quantity of scaffolds generated by each assembly and finally chose the best K-mer that yielded the minimum scaffold number and the maximum N50 and N90 values. Then, scaffolds with a length over 500 bp were extracted and broken into contigs without gaps. These contigs were used for further gene prediction and annotation.

2.5. Pathogens, virulence factor and ARG analysis

Open reading frames (ORFs) were predicted from the contigs using MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/). The predicted ORFs longer than 100 bp were translated into amino acid sequences using the NCBI translation table (Noguchi et al., 2006). Pathogen annotations were conducted by aligning amino acid sequences with the PHI database (http://www.phi-base.org/index.jsp) through BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using an e-value cutoff of 10^{-5}. The abundance of a taxonomic group was calculated via the reads number analytical method by summing the abundance of genes annotated to a feature (Yan et al., 2017). Furthermore, ORFs were compared against the virulence factor (VF) database (VFDB; http://www.mgc.ac.cn/VFs/) using a BLASTP search to identify VF genes. An ORF was annotated as a virulence gene if the BLASTP alignments exceed 95% identity, over 90% of the length of ORF, and over 50% of gene in the VFDB (Moustafa et al., 2018). The ORFs were also checked for ARGs against the widely accepted ARG database (ARDB) using BLASTP (Version 2.2.28+). A read was annotated as an ARG fragment if the similarity of the sequence was $>$90% and the alignment length was $>$25 amino acids. The resulting ARGs were shown as types (antibiotics to which the genes are resistant) and subtypes (resistance genes). The proportions of different types of ARG sequences in the total ARG sequences and total sequences were defined as the percentage (%) and abundance, respectively, according to the reported method (Wang et al., 2018).

2.6. Statistical analysis

A Venn diagram reflecting the number of shared pathogens and ARGs among all samples was generated using the Venn diagram viewer tool termed Jvenn (Bardou et al., 2014). Regression analysis, NMDS and Fisher's analysis were implemented using various packages within the R software. Variations were considered significant at a P-value $<$ 0.05. In addition, the underlying co-occurrence pattern between ARGs and pathogens was explored by constructing a correlation matrix to express all possible pairwise Spearman's rank correlations.

3. Results and discussion

3.1. Pathogen: diversity, abundance and the influence of environmental factors

Metagenomic sequencing was applied to assay the composition and function of pathogenic microorganisms in the six water samples, and total of 174, 182, 176 and 177 kinds of pathogens were respectively identified from the FPP, SH, CMPF and HP water samples (classified as industrial wastewater samples) (Fig. 1a), while 175 and 174 kinds of pathogens were identified in the WWTP and RW samples, respectively (Fig. 1b). Among them, 162 pathogens were concurrently shared by the industrial wastewater samples, while 166 were shared between the three kinds of water (industrial wastewater, WWTP and RW. This indicates that the diversity of pathogens within the industrial wastewaters was not significantly different due to the shared species accounting for more than 90% in each industrial wastewater, and that they are also very similar to the WWTP and RW. In addition, the distribution patterns of pathogens were explored using NMDS analysis based on Bray-Curtis distance. The results showed that no distinct difference of pathogens composition was observed between the SH and RW samples (Fig. S1a), indicating that pathogenic bacteria in the RW may primarily come from the SH wastewater. A previous study detected a high amount of total coliform and *Escherichia coli*, which are present only in faeces, in a river contaminated by human and animal waste (Olayinka et al., 2013). However, the composition of pathogens in water samples from the FPP, CMPF, WWTP and HP was clearly separated (Fig. S1a), indicating that there was a significant difference in pathogen composition.

In total, 16,357 species were identified based on a 97% sequence similarity. *Pseudomonas alcaligenes* was the most abundant microbial species in the CMFP with a relative abundance of 80.50%. In the HP, *Rhodoluna lacicola* (30.50%), *Leptothrix cholodnii* (12.60%) and *Limnohabitans* sp. *Rim47* (13.40%) were dominant, while Mediterranean phage uvMED dominated the SH and WWTP (Fig. S2). Furthermore, by mapping their species-level microbial compositions against the PHI database, we found that the most abundant pathogens, on average, in these six water samples were *Salmonella enterica* (8.78%) and *Pseudomonas aeruginosa* (8.21%), followed by *Fusarium graminearum* (4.45%), *Magnaporthe oryzae* (4.35%), *Staphylococcus aureus* (4.33%) and *Klebsiella pneumoniae* (3.66%) (Fig. 1c). Comparatively, the abundance of *S. enterica* (11.68%), which can cause gastroenteritis or septicemia (in the case of some serovars), was the highest in the FPP, while the CMFP had a higher abundance of *P. aeruginosa* (14.27%), which is considered as one of the most common opportunistic pathogens in clinical practice. A previous study demonstrated that *S. enterica* often appears in poultry meat processing plants and is a common food-borne pathogen during foodstuff processing (Galie et al., 2018). Conversely, *P. aeruginosa* is extensively present in nature as well as normal human skin, respiratory tract and intestine (Verchere et al., 2015). Overall, high levels of pathogens, especially *S. enterica* and *P. aeruginosa*, in these tested water samples indicate that they can survive these commonly employed wastewater treatment technologies, thereby posing potential risks for propagating pathogenicity.

The six water samples provided varying conditions to compare the relative and net influence of different wastewater inputs on pathogens. As Fig. S3a shows, the COD contents (3.22–107.31 mg L^{-1}) were high, especially at the CMPF site. In contrast, the TP contents were low, ranging from 0.01 to 0.71 mg L^{-1}. However, the temperature and pH in the six water samples were almost unchanged. The Cr concentration in the SH (101.83 mg L^{-1}) was 8–10 times higher than that in the other samples, while the As concentration was much higher in the WWTP (72.84 mg L^{-1}) (Fig. S3b and S3c). These results agree with previous studies that traditional Chinese medicine industries produce medium-concentration wastewater with a large volume of COD, and SH wastewater may contain chromium, which is toxic to aquatic life (Bustillo-Lecompte and Mehrvar, 2017; Vanerkar et al., 2005). Considering that

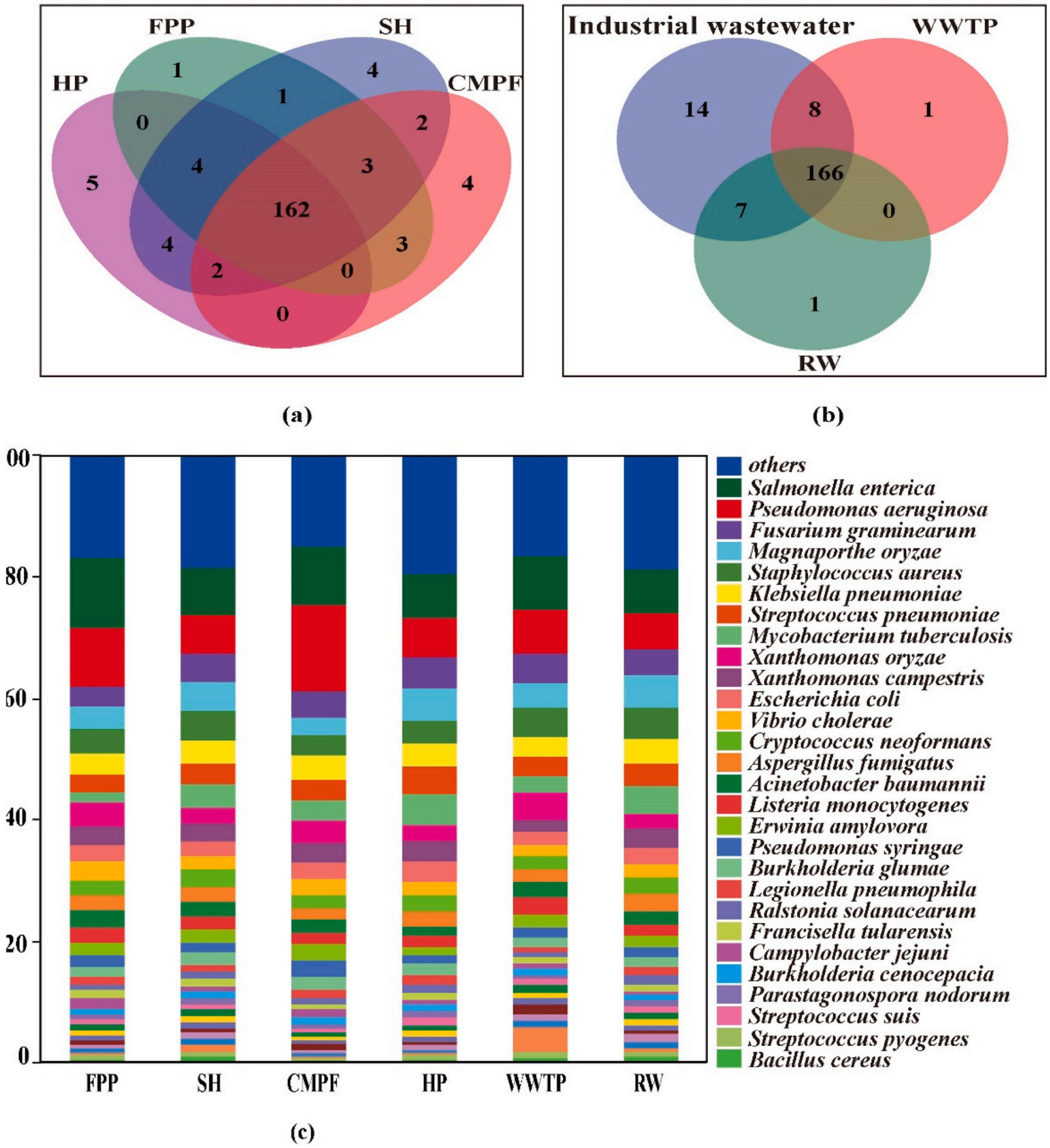


Fig. 1. The diversity and relative abundance of pathogens in the six water samples. Venn diagram showing the number of shared and unique pathogens among industrial wastewater including FPP, HP, CMPF and SH (a); as well as WWTP, RW and industrial wastewater (b); Relative abundance of pathogens at species level (merge the pathogens with relative abundance less than 1% into others) (c).

some environmental factors were autocorrelated, the variance inflation factor (VIF) analysis was employed for autocorrelation screening prior to correlation analysis between environmental factors and pathogens. As Table S3 shows, TOC, COD, pH, Cd and Pb with less interaction were reserved for redundancy analysis (RDA). Among the nine important pathogens, four (*S. aureus*, *Mycobacterium tuberculosis*, *Xanthomonas campestris* and *K. pneumoniae*) were positively correlated with pH and TOC, and three (*S. enterica*, *Xanthomonas oryzae* and *P. aeruginosa*) were positively correlated with COD, while the other six pathogens were negatively correlated with COD (Fig. 2a). This is because TOC can provide the necessary energy for microorganisms to maintain life activities (Bai et al., 2019). In contrast to the other three screening indicators (TOC, COD, pH), Cd and Pb were both negatively correlated with the nine pathogens in this study. This may be due to the toxic effect of heavy metals (Gadd and Griffiths, 1977). Microorganisms are particularly sensitive to changes in heavy metal pollution; for example, Verrucomicrobia and Crenarchaeota have been significantly negatively correlated with Cd and Pb (Zhao et al., 2020a). Therefore, the above-mentioned results demonstrate that the factors providing the necessary substances for life activities have a positive impact on some pathogens. Nevertheless, the pollutants that inhibit the growth of the community have a negative influence.

Except for these six environmental factors, other stimuli, such as antibiotics, will also accelerate the evolution of bacterial antibiotic resistance and reduce their fitness cost through early emerged stress responses (Guest and Raivio, 2016; Yin et al., 2019, 2020). In the absence of antibiotics, antibiotic resistance usually has an adverse effect on bacteria, which is usually observed as reduced growth and survival rates due to changes in essential genes and/or changes in the use of resources that are termed as fitness cost (Martinez and Rojo, 2011). That is, stimuli will increase ARB and decrease susceptible pathogenic populations by changing the expression of essential genes and/or the use of resources. Similarly, previous studies have reported that antibiotic-resistant pathogens reduce virulence due to fitness cost (Beceiro et al., 2013). Another study showed that reducing the bacterial fitness cost can inhibit the emergence of antibiotic resistance (Zuo et al., 2021). In addition, strains with increased antimicrobial resistance were slower to grow, which is likely a fitness cost due to enhanced

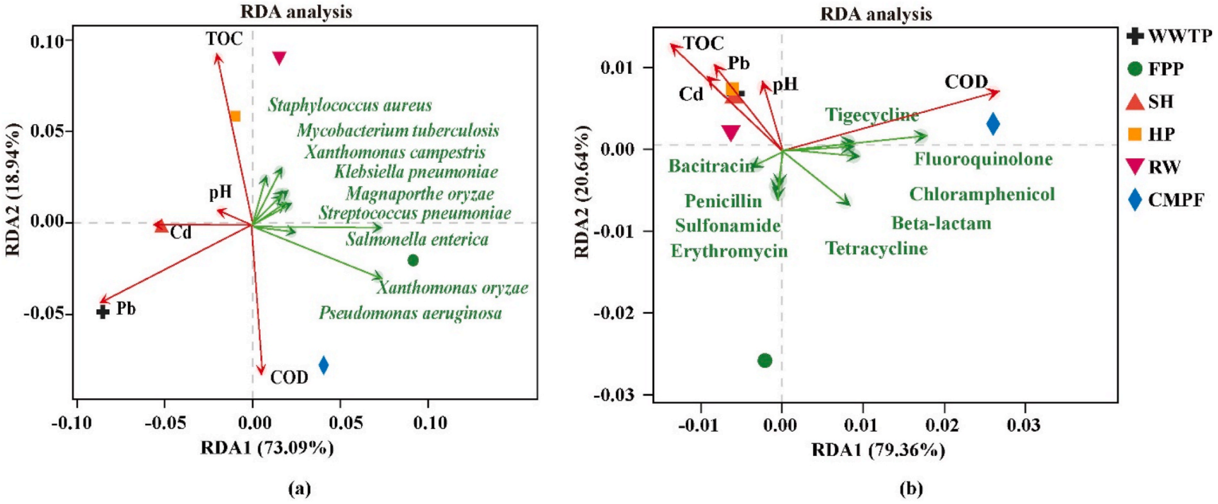


Fig. 2. Redundancy analysis based on Bray-Curtis distance showing the correlation between environment factors and pathogens (a) as well as ARG types (b) in six water samples.

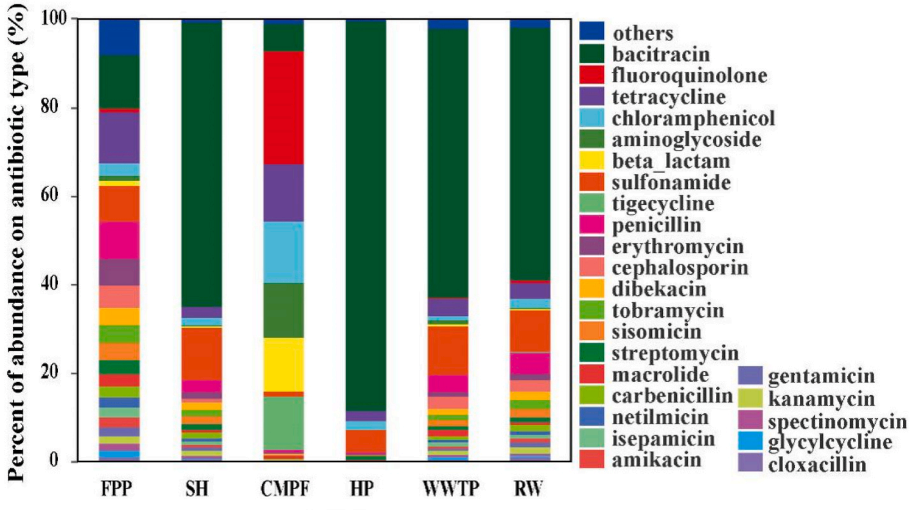
antimicrobial resistant activity (Torres et al., 2021). Therefore, in future studies, more representative water quality parameters, including broader antibiotic types and multi-phase resistome screening, need to be considered.

3.2. *Abundance and diversity of ARGs*

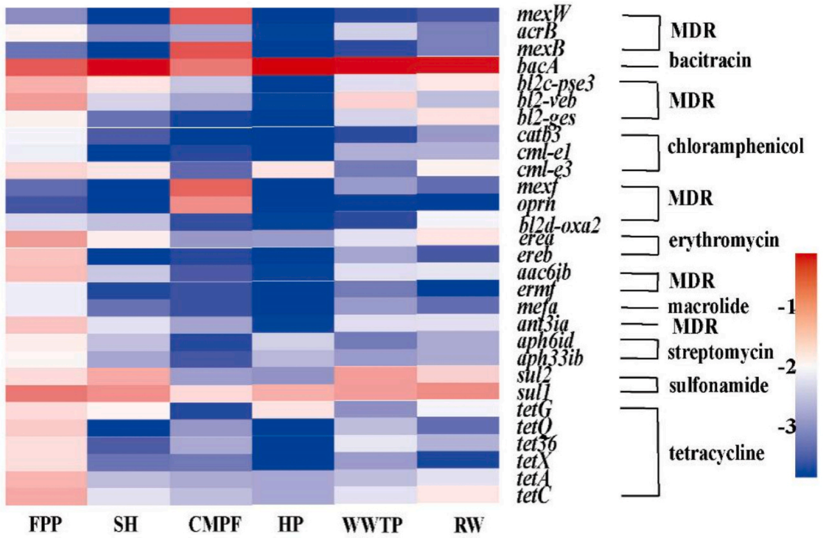
Metagenomic sequencing was applied to evaluate ARG profiles in these industrial wastewaters and the receiving surface water. In total, 57 ARG types were detected, with bacitracin resistance genes being the most abundant in the HP, SH, WWTP, and RW samples, accounting for 88.20%, 63.96%, 60.80% and 57.13%, respectively, of the total ARG types (Fig. 3a). This result suggests that bacitracin is intensively used in the HP and SH, and that the WWTP cannot effectively remove bacitracin resistance genes. A previous study also demonstrated that bacitracin is currently used as a topical therapy for necrotic enteritis and as a growth promoter in animals (Charlebois et al., 2012). In contrast, the CMFP tended to be dominated by ARGs conferring resistance to fluoroquinolone (25.65%), chloramphenicol (13.66%), tetracycline (13.07%), aminoglycoside (12.32%), beta-lactam (12.32%) and tigecycline (12.15%). In the FPP, bacitracin (12.04%), tetracycline (11.47%), penicillin (8.50%) and sulfonamide (8.05%) were the four most abundant ARGs types. Previous studies also found these ARG types in different water environments. For example, in an urban river, ARG types presented resistance to aminoglycoside, beta-lactams, fluoroquinolone, chloramphenicol, sulfonamide, macrolide, tetracycline and vancomycin, among which aminoglycoside and beta-lactams resistance were most abundant (Zhou et al., 2017). In coastal industrial mariculture systems, ARGs related to sulfonamide, tetracycline, bacitracin, or chloramphenicol are commonly distributed (Wang et al., 2018). Furthermore, in the leachate of food waste, the genes conferring multidrug, beta-lactam aminoglycoside and tetracycline resistance were the most abundant (Liang et al., 2020b). The ubiquitous existence of ARGs in various systems indicates that human activities might significantly impact ARG development.

A total of 107 ARG subtypes were found in the six water samples. Among them, the bacitracin resistance gene *bacA* and the sulfonamide resistance gene *sul1* were the most abundant ARG subtypes (Fig. 3b). To be specific, the relative abundance of *bacA* in the SH, HP, WWTP and RW was as high as 74%, 89%, 74% and 72%, respectively. The SH, WWTP and RW were also dominated by *sul1* with a relative abundance of 9%, 7% and 9%, respectively. These results indicate that the community in the SH, HP, WWTP and RW might experience selective pressure from bacitracin and sulfonamide. Recently, *bacA* and *sul1* were found to be the most abundant ARG subtypes in a WWTP (Ng et al., 2019), while *sul1* was present in a WWTP that mainly treated hospital wastewater (Rolbiecki et al., 2020). Surprisingly, although traditional Chinese medicines are usually broad-spectrum bactericidal medicines with no specific target, the CMPF was rich in *bacA*, *sul1* and multi-drug resistance genes *mexW*, *mexB*, *mexF* and *oprn*. Therefore, we speculate that the high ARG abundance in the CMPF may be due to their selection pressure on pathogens. However, until now, no report has detected the ARG profile in CMFP polluted wastewater or other environments, and only one paper has reported that the addition of Chinese herbal medicine residues to chicken manure compost increases the abundance of *sul1* and *intl1* (Wu et al., 2019b). Moreover, *Stenotrophomonas maltophilia* isolated from a university hospital practicing traditional Chinese medicine showed resistance to sulfamethoxazole with *qac△E1*, *sul2* and *Intl1* as the dominant ARGs (Zhao et al., 2017). This suggests that Chinese medicine may play an important role in antibiotic resistance development, similar to other stimuli, by influencing the stress response system, as well as the corresponding gene expression patterns and cell physiology of bacteria (Parnanen et al., 2019; Yin et al., 2020). Comparatively, the diversity and abundance of ARG subtypes in the FPP was higher than the other five samples, with *bacA*, *sul1*, *tetA*, *tetC*, *tetO*, *bl2c-pse3*, *bl2-veb*, *ereA* and *ereB* as the most dominant, indicating that FPPs may be an important hub for ARGs. Similarly, Li et al. reported that 60.7% of isolates (102) from a meat processing plant showed resistance to tetracycline; 57.7% (97) showed resistance to trimethoprim/sulfamethoxazole; and *tetA*, *tetC*, *sul1* and *sul2* were found at a frequency of 6.0%, 4.8%, 17.9% and 38.1%, respectively (Li et al., 2016). The *sul1*, *sul2*, *tetC*, *ermB* and *IntI1* genes were also found in a treatment plant for restaurant food waste (He et al., 2019). Therefore, the presence of ARGs in industrial wastewater is a noteworthy issue since they can greatly contribute to the antibiotic resistance in natural systems.

The differences in ARG composition in the six water samples were further investigated by NMDS analysis. As Fig. S1b shows, ARGs from the SH, HP, WWTP and RW clustered together and were clearly separated from those from the CMPF and FPP, indicating that no distant difference in ARGs was observed among the samples from the SH, HP, WWTP and RW, while a significant shift of the ARG profile occurred at the CMFP and FPP. The similar ARG composition between the RW and SH, as well as the HP, suggests that ARG pollution in the RW might be due to the contribution of the SH and HP. This is well supported by previous studies indicating that livestock and sanitation facilities have a great influence on the total abundance of ARGs in a river basin due to the selection pressure of antibiotics used in both humans and animals (Bai et al., 2019; Cairns et al., 2018). By evaluating the similarity of ARG



(a) ARGs type



(b) ARGs subtype

Fig. 3. Relative abundance of the ARG types (a) and subtypes (b) in six water samples. Other types represent the ARG subtypes with abundance less than 1%; MDR denotes multiple antibiotic resistance. The color transition from blue to red in the heatmap represents relative abundances from low to high. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compositions between industrial wastewater and the WWTP using Fisher's analysis, we found that the deviation of ARG composition between the WWTP and HP, as well the SH, was lower than that of the FPP and CMPF, further confirming that the HP and SH are important contributors to the ARG profiles in the WWTP sample (Fig. S4a-d). Moreover, a high similarity of the ARG composition was found between the WWTP and RW, demonstrating that the anaerobic-anoxic-oxic treatment method in the WWTP can only reduce the abundance of ARGs to some extent, resulting in plenty ARGs discharging into the river (Fig. S4e). A study carried out in a lake impacted by different contamination sources showed that hospital and municipal sewage were the key ARG sources for the receiving water body (Czekalski et al., 2014).

RDA revealed that water quality was also significantly correlated with the elevated ARG levels in the six water samples (Fig. 2b). To be specific, TOC, Pb, Cd and pH were positively correlated with the most dominant ARG subtype bacitracin, but negatively with other ARG subtypes. COD was positively correlated with tigecycline, fluoroquinolone, chloramphenicol, beta-lactam and tetracycline, but negatively correlated with the dominant bacitracin, penicillin, sulfonamide and erythromycin. Similarly, a negative correlation was obtained between sulfonamide resistance genes and COD in leachate (Liang et al., 2020b). Furthermore, the *sul* genes have been significantly correlated with heavy metals (Zhang et al., 2016b). Considering the complex composition of wastewater, besides the factors described above, other stimuli such as antibiotics and other contaminants in industrial wastewater could also exert selective pressure on the community and result in ARB enrichment (Barancheshme and Munir, 2018). These factors create a positive feedback mechanism of promoting antibiotic resistance in the persistence and proliferation of ARGs, thereby leading to the occurrence of antibiotic-resistant pathogens in RW.

3.3. Co-occurrence patterns of ARGs with pathogens

As a useful way to track the potential ARG hosts in water environments (Pehrsson et al., 2016), co-occurrence patterns of ARGs with pathogens were further explored using network analysis based on the significant (P-value < 0.05) and robust (Pearson's $r > 0.8$) correlations. A total of 34 nodes (including 18 pathogens and 16 ARGs) and 161° were obtained in the network (Fig. 4). More details on the node centrality analysis showing a complex pattern of interrelationships between ARGs

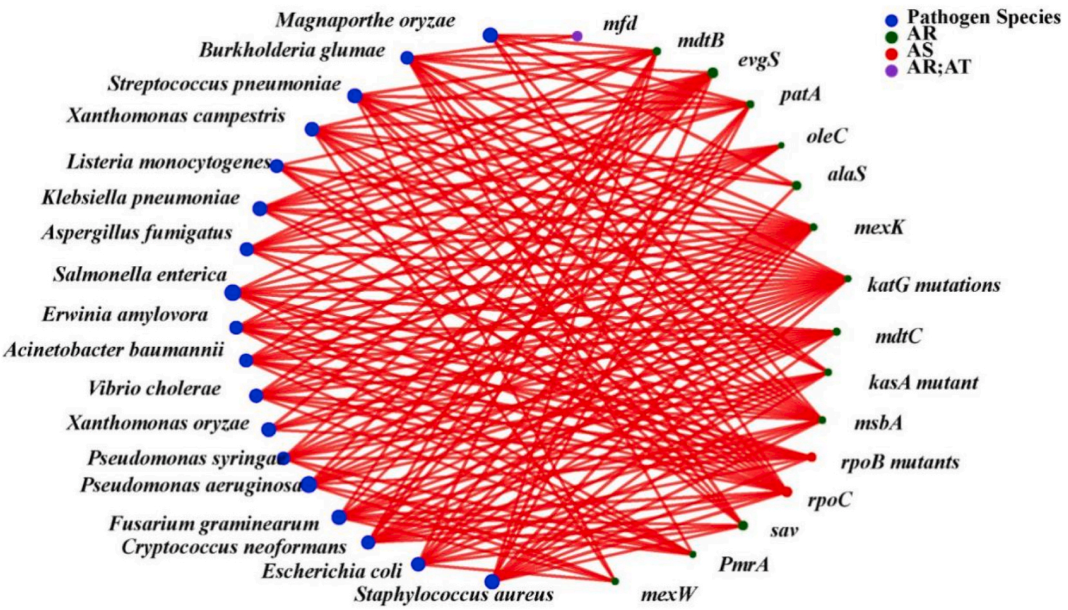


Fig. 4. Network analysis revealing the co-occurrence patterns among ARGs and pathogens with significant Spearman's correlation coefficient ≥0.80 (P-value ≤ 0.01). The nodes were colored according to resistance mechanism. Edges and node size weighted were based on the correlation coefficient and the relative abundance of ARGs and pathogens, respectively. AR: antibiotic resistance; AS: antibiotic sensitive; AT: antibiotic target.

and pathogens are provided in Table S4. To be specific, ARGs related to efflux pump-encoding genes (*mexK*, *msbA* and *evgS*), lipopeptide (*rpoC*) and isoniazid (*katG*) have higher closeness centrality than other ARGs; while *Burkholderia glumae*, *F. graminearum*, *S. aureus*, *Cryptococcus neoformans*, *Streptococcus pneumoniae*, *E. coli* and *X. campestris* were the typical pathogens with higher closeness centrality.

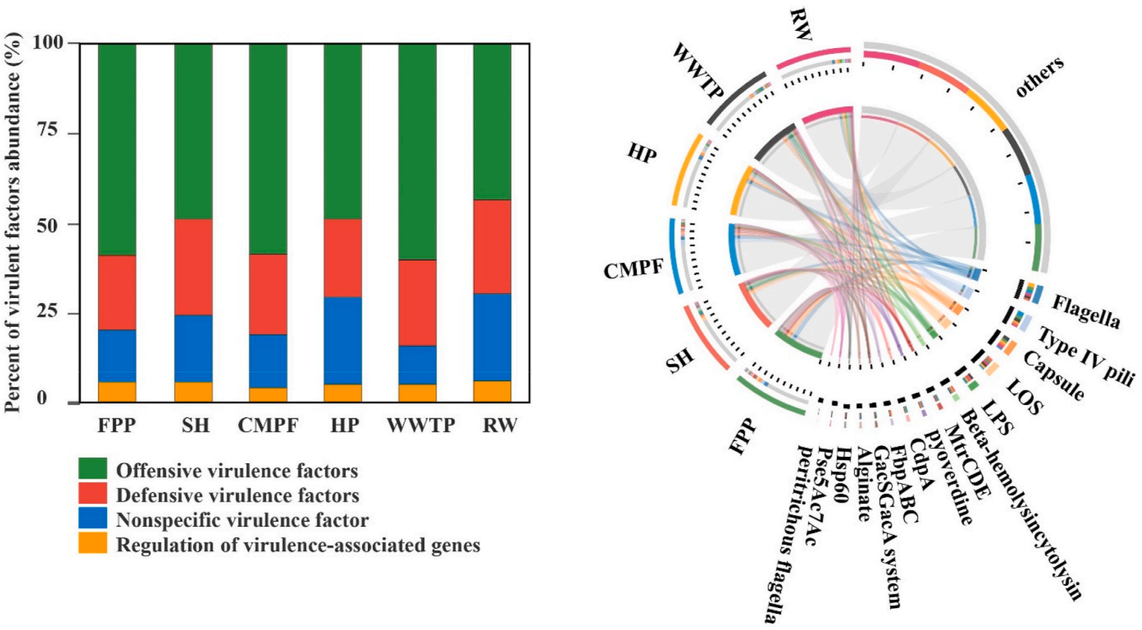
Free ARGs in the water environment do not pose a direct risk to cause antibiotic resistance; only when they enter a bacterial cell and are expressed can lead to the emergence of antibiotic resistance. (Zhang et al., 2016a). Accordingly, this study focused on the link between ARGs and emerging pathogenic bacteria. *Burkholderia glumae*, *C. neoformans*, *F. graminearum* and *S. aureus* were speculated as the potential hosts of 11 kinds of ARGs (Fig. 4). As a common food-borne pathogenic bacterium, *S. aureus* is associated with *mdtB*, *evgS*, *patA*, *mexK*, *katG* mutations, *metC*, *kasA* mutations, *msbA*, *rpoC*, *sav* and *rpoB* mutations (Dehkordi et al., 2019). The riskiest opportunistic pathogens *S. pneumoniae* and *E. coli* were speculated as the potential hosts of 10 kinds of ARGs, namely *mdtB*, *patA*, *alaS*, *katG* mutations, *kasA* mutations, efflux pump-encoding genes (*mexK*, *msbA)*, *rpoC*, *sav* and *mexW* mutations. The macrolide and fluoroquinolone resistance of *S. pneumoniae* may be mediated by efflux pump-encoding genes (Gill et al., 1999; Reinert, 2009).

Since the main function of the bacterial community could reveal the contribution of the pathogens acquired resistance, the metabolic potential and antibiotic resistance of the community was analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2019). As Fig. S5 shows, the main function of the bacterial community was metabolism and genetic information processing. A previous study also demonstrated that amino acid and carbohydrate metabolisms were the two largest gene categories in the multidrug-resistant pathogen *Chryseobacterium arthrosphaerae* strain ED882-96, which was resistant to all tested antibiotics, including beta-lactams, aminoglycosides, fluoroquinolones and tetracycline (Liang et al., 2019). Moreover, transport proteins (which can help most hydrophilic compounds, such as ions and antibiotics, to pass through the hydrophobic barrier formed by the lipid bilayer around a cell or organelle (Li et al., 2019)) and quorum sensing-encoding genes (which is considered to be able to regulate various community behaviors, including drug resistance and toxin production (Zhao et al., 2020b)), were also dominant. In particular, the abundance of ABC transporters was the highest among all the transport proteins (Fig. S6). Considering that ABC transporters are efflux transporters and that the efflux pump is the most important resistance mechanism of ARB, we concluded that they might be crucial to form antibiotic resistance (Pohl et al., 2011; Sun et al., 2015). The second most dominant transport proteins belong to the resistance-nodulation-cell division (RND) superfamily. Li et al. revealed that multidrug pumps of the RND superfamily, particularly AcrAB-TolC and Mex pumps, not only mediate intrinsic resistance and acquire multidrug resistance, but they are also associated with the bacterial stress response and pathogenicity (Li et al., 2015). This suggests that efflux-mediated antibiotic resistance is ubiquitous in the pathogens from the six environments, thus presenting a great challenge for the development of antibiotics.

3.4. Microbiological risk assessment based on virulence factors and pathogens with ARGs

VFs include bacterial toxins, cell surface proteins regulating bacterial adhesion, proteins protecting bacteria themselves, carbohydrates on the cell surface and hydrolases with bacterial pathogenicity. They are derived from microorganisms and could aid bacteria in invading the host, causing disease and evading host defense (Durso et al., 2011). They are also found to be important evaluation factors in health risk assessment (Unc et al., 2012). By predicting the abundance of VFs using the virulence factor database (Geisinger and Isberg, 2017), we found that among the four main VF types, offensive VFs were dominant (>48% on average) in all samples, with dominant subtypes including adherence, secretion systems and toxins (Fig. 5a). Higher abundance of offensive VFs in the FPP, CMPF and WWTP suggest a highly aggressive capability, considering that they can confer infection ability of the VF hosts. A lower proportion of defensive VFs in the FPP, CMPF, HP and WWTP revealed a lower resistance to immunoreactions of humans/animals due to the attenuated endurance capacity against host immunoreactions. Hence, the FPP, CMPF, HP and WWTP exhibit higher risk. These results highlight the importance of wastewater as a reservoir for VFs responsible for determining well-known pathogenic mechanisms.

Spearman correlation analysis revealed a strong correlation between pathogens and VFs (Fig. S7). Specifically, the potential ARG hosts *F. graminearum*, *M. oryzae*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *E. coli*



(a) Virulent factors at the level 1

(b) Virulent factors

Fig. 5. The relative abundance of virulent factors (VFs) at the level 1 (a) and the distribution of VFs in the six water samples (b). The data was visualized via Circos software (http://circos.ca/) and others represent VFs with abundance less than 2%.

and *B. glumae*, which were highly abundant in the water samples, showed significant positive correlations with offensive VFs. This suggests that these bacteria might be involved in producing VFs, thereby causing illness and infectious diseases. Previous studies have demonstrated that *S. aureus*, the most common pathogenic bacterium on human skin, can produce various VFs, including immune-modulatory factors, toxins, and exoenzymes, resulting in food poisoning, suppurative diseases, pneumonia, and toxic shock syndrome (Lin et al., 2020; Oogai et al., 2011). Some serotypes of *E. coli* can also produce toxins, adhesins, iron acquisition factors, polysaccharide capsules, lipopolysaccharides and invasins, causing gastrointestinal infections in humans and animals, urinary tract infections, arthritis, meningitis and septic infections (Knopp and Andersson, 2018). The above results suggest that particular attention should be paid directly towards the VFs when assessing microbiological risk.

Flagella, type IV pili, capsule, lipo-oligosaccharides, and lipopolysaccharides, implicated as important VFs, were also dominant in the six samples (Fig. 5b). This is alarming regarding these VFs. For instance, lipo-oligosaccharide molecules can aid in the evasion of host immune defenses, while lipopolysaccharides are useful for maintaining structural integrity and membrane localization as well as inducing host responses (Misra et al., 2021). Therefore, different enzymes or precursors involving in the biosynthetic pathway of cell-bound polysaccharides in bacteria can act as VFs to cause pathogenesis in the host cell, which could be a good target to develop antibiotics against. Overall, VFs carried by pathogens help to effectively infect the host, while ARGs carried by pathogens help them to avoid the bactericidal effect of antibiotics. Therefore, we concluded that pathogens with ARGs and high virulence pose a higher risk.

4. Conclusions

In this study, the pollution characteristics of the ARGs and pathogens, as well as their associated health risks, were investigated in six water samples using metagenomic sequencing. Among the 177, 174, 182 and 176 pathogens identified from the HP, FPP, SH and CMPF, respectively, 162 pathogens were concurrently shared, indicating that the diversity of pathogens among industrial wastewaters was comparable. Moreover, the similar pathogen composition indicates that pathogenic bacteria in the RW may primarily come from the SH wastewater. The abundance of *S. enterica* and *P. aeruginosa* was highest in the FPP and CMFP water, respectively. Physicochemical parameters COD, pH and TOC of the water were positively correlated with pathogens, while heavy metals showed a negative correlation. Of the 57 ARG types and 107 subtypes detected in the six water samples, bacitracin resistance genes, *bacA* and *sul1* were the most abundant. It is worth noting that the CMFP had a high abundance of fluoroquinolone, chloramphenicol, tetracycline, aminoglycoside, beta-lactam and tigecycline resistance genes, which might be due to the selection pressure exerted by Chinese medicine on the pathogens. The dominant bacteria *B. glumae*, *C. neoformans*, *F. graminearum* and *S. aureus* were speculated as the potential hosts of 11 kinds of ARGs, while the ABC transporters might play an important role in forming antibiotic resistance. Higher abundance of offensive VFs in the FPP, CMPF and WWTP, and their strong correlation with pathogens containing ARGs, suggests a higher microbiological risk. Overall, this study reveals that industrial wastewaters are dominated by ARGs and pathogens, and that they will pollute the receiving water, causing higher health risks to the surrounding residents.

Author statement

Yao Tang: Methodology, Formal analysis, Writing – original draft, **Zhishu Liang**: Methodology, Formal analysis, **Guiying Li**: Writing-Reviewing and Editing, **Huijun Zhao**: Supervision, **Taicheng An**: Conceptualization, Supervision.

Declaration of competing interest

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

Acknowledgements

This work was supported by the National Natural Science Foundation of China (41877363, U1901210 and 41807405), Key Project of

Guangdong-Guangxi Joint Fund (2020B1515420002), Natural Science Foundation of Guangdong Province (2019A1515010599), and Guangdong Provincial Key R&D Program (2020B1111350002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.131224.

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