Science of the Total Environment 973 (2025) 179132

Contents lists available at ScienceDirect

Science of the Total Environment

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

An integrated analysis of the use and potential risks of pharmaceutical drugs in hospital wastewater: Consumption and occurrence by suspect screening analysis, and antibiotic resistance

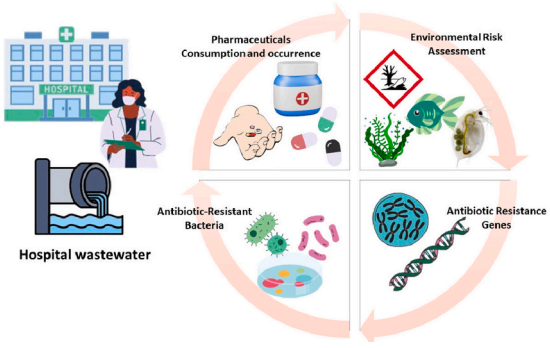
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HIGHLIGHTS

- 113 drugs identified in hospital wastewater and antibiotics were the most detected.
- Antibiotic resistant bacteria were detected in two Brazilian hospital wastewaters.
- ARG profile and Multidrug-resistance (*florR*) in wastewater varied between hospitals.
- Drugs identified in hospital wastewaters present high environmental risks.
- Potential environmental risks for ampicillin, escitalopram, and topiramate

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Jay Gan

Keywords:
Pharmaceuticals
Qualitative suspect screening
High resolution mass spectrometry

ABSTRACT

Hospital wastewater (HWW) contains pharmaceutical drugs, antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARG), and pathogens, posing potential risks to environmental and public health. However, data on the occurrence of these contaminants are scarce, especially in low- and middle-income countries. This study employed LC-QTOF MS for a qualitative suspect screening of 1922 pharmaceutical drugs and metabolites in HWW (n = 8 samples). Results were used in an environmental risk assessment applied with the support of *in silico* tools, to predict potential risks to aquatic organisms. ARB were analyzed using plate count agar method and ARG

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https://doi.org/10.1016/j.scitotenv.2025.179132
Received 14 September 2024; Received in revised form 11 March 2025; Accepted 11 March 2025
Available online 18 March 2025
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Antimicrobial resistance
Risk assessment

by Real-time PCR. 113 drugs were identified in HWW, and antibiotics were the most frequently detected (15 compounds). Environmental risk assessment indicated that 29 compounds have the potential to cause toxic effects to aquatic fauna, among which 4 compounds (ampicillin, escitalopram, iopromide, and topiramate) may pose a very high potential risk (RQ > 2). In addition, ARB resistant to azithromycin and cefazolin (10^6–10^7 CFU/mL) were abundant in HWW samples, while low levels of bacteria resistant to meropenem (10^5 CFU/mL) were detected. The relative number of copies of *blaKPC*, *catA1*, *sul1*, *ermB*, *qnrS*, *vanB*, *floR*, and *aadA* were higher in HWW from one of the hospitals. However, there was no significant difference for *blaOXA* (p value = 0.1271) or *mecA* genes (p value = 0.6077) in HWW samples. These results call attention to potential risks posed by the discharge of HWW and reveal the need to raise data for improving management practices for environmental protection.

1. Introduction

Drinking water consumption in hospitals can reach 400 to 1200 L per patient per day (Boillot et al., 2008), leading to substantial volumes of hospital wastewater (HWW) being generated. HWW contains organic matter mixed with chemicals, detergent, disinfectants, surfactants, pharmaceuticals drugs, metals, radionuclides, radioactive tracers, iodinated contrast media, antiseptics, solvents, biological fluids, bacteria, virus, multi-resistant bacteria, fungi and other pathogens (Boillot et al., 2008; Khan et al., 2020).

Pharmaceutical drugs concentrations in HWW are higher than in domestic sewage. In Turkey, Ulvi et al. (2022) estimated levels in HWW 4 to 9-fold higher than in domestic sewage sampled at the inlet of a municipal treatment plant. Similarly, a review by Verlicchi et al. (2010) reported that HWW may contain 2 to 150-fold more pharmaceuticals than urban wastewater. However, only a few studies report the occurrence of pharmaceutical drugs in HWW for hospitals located in low- and middle-income regions (Becker et al., 2020) where drug consumption profiles differ from other regions. Antibiotics deserve special attention in this context as their release in the environment contributes to the selection of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Della-flora et al., 2020), an issue of global concern (WHO, 2018a).

Moreover, the direct discharge of HWW without any previous treatment in the sewer system is a current practice in some low- and middle-income countries, such as Brazil, where antibiotic consumption is high (Castro et al., 2020). Considering direct discharge practices and the inability of conventional wastewater treatment plants (WWTP) to remove pharmaceutical drugs and their metabolites, ARB and ARGs from sewage (Ibáñez et al., 2021), healthcare facilities contribute with the spread of antibiotics and antimicrobial resistance in the environment. This is even more critical when in places where municipal wastewater treatment is incipient. For instance, wastewater treatment coverage in Brazil is lower than 50 % of total produced wastewater (SNIS, 2021).

Thereafter, (i) scarcity of data on the occurrence of pharmaceutical drugs in HWW, (ii) indiscriminate use of antibiotics, and (iii) inadequate sanitation infrastructure intensify threats posed by antibiotic resistance in low- and middle-income countries (Rossi, 2011). In this context, it is critical to develop knowledge on the contributions of HWW discharge into domestic sewage systems to the spread of pharmaceutical drugs, ARB and ARGs in these regions (Batista et al., 2023) to subsidize control measures.

Considering the current need to raise data related to sources that contribute to environmental contamination by pharmaceutical drugs, there is increasing demand for analytical methodologies which enable the qualitative assessment of various compounds simultaneously without mandatory use of analytical standards, namely "suspect screening analysis". These techniques have been successfully applied in different studies (Ccanccapa-Cartagena et al., 2019; Di Francesco et al., 2024; Pinasseau et al., 2019; Su et al., 2022; Wang et al., 2019; Wiest et al., 2021) and regularly explore advanced techniques, such as gas/liquid chromatography coupled with high resolution mass spectrometry (HRMS). The accurate-mass full-spectrum data from HRMS provides valuable information about molecular ion and fragmentation profile of each compound (Hernández et al., 2019a, 2019b; Nikolopoulou et al., 2023).

Suspect screening analysis is a qualitative method supported by a database containing the elemental formula and exact mass of molecular ion and fragmentation profiles of target compounds. This enables simultaneous evaluation of thousands of analytes and allows for a retrospective analysis (Ibáñez et al., 2017). The application of such strategy is advantageous as it guides the selection of target analytes prior to the development of assertive quantitative methods, thus reducing costs, optimizing time, and covering a wider spectrum of contaminants compared to traditional approaches. This is even more valuable when data to subsidize the selection of target analytes are scarce, such as for the occurrence of pharmaceutical drugs in HWW located in low and middle-income countries.

In addition, HWW threatens aquatic ecosystems as it contains compounds that are toxic to various taxonomic groups (Gautam et al., 2007; Mubedi et al., 2013). Once a certain drug is detected by suspect screening analysis in an environmental sample, its quantification allows for the evaluation of potential environmental risks to biota, and human health, through an environmental risk assessment (ERA). ERA enables the identification of priority compounds (Länge and Dietrich, 2002; Vestel et al., 2016) and was previously applied to evaluate risks posed by HWW (Becker et al., 2020; Escher et al., 2011; Serna-Galvis et al., 2022). The *in silico* (Quantitative) Activity Structure Relationship ((Q)SAR) model may be used in an ERA to support the estimation of predicted environmental concentration (PEC) of target analytes and estimate their ecotoxicity to different taxonomic groups (Mansour et al., 2020). Even though the (Q)SAR model has some limitations related to the accuracy of input data, the selection of descriptors, the complexity in the representation of chemical structures and the simplification of complex biological interactions, as well model validation (De et al., 2022; Veerasamy, 2022), it is an accepted and applicable tool to allow for a comprehensive risk assessment.

In this context, this work aimed to assess the consumption of pharmaceutical drugs in hospitals in Brazil and to investigate their occurrence in HWW by qualitative suspect screening analysis (LC-QTOF MS). Besides, results obtained by qualitative analysis supported an ERA carried out to estimate potential environmental risks posed by these chemicals, and ARB and ARG occurrences in HWW were analyzed as human-health relevant indicators.

2. Materials and methods

2.1. Chemicals

Analytical standards of 19 pharmaceutical drugs used to confirm the presence of some compounds were purchased from different suppliers (Table S1, Section S1, Supplementary Material). Methanol - LC-MS grade (Merck, Darmstadt-Germany), ultrapure water (18.2 MΩ.cm, Millipore, USA), and formic acid (98 % purity, Sigma-Aldrich, St. Louis-USA) were used in LC-QTOF MS analyses.

2.2. Consumption of pharmaceutical drugs and hospital wastewater sampling

The two hospitals under study are both tertiary level facilities-central hospitals located in Belo Horizonte- Minas Gerais, southeast of Brazil with highly qualified staff which provide specialized regional services (GLOBAL-PPS, 2022). Hospital A has a total area of 65,000 m^2 and 650 beds, while hospital B has 68,010 m^2 and 504 beds. Drug consumption data corresponded to 12 months (2020) for hospital A and 36 months (2020 to 2022) for hospital B. These data were provided by the hospitals' pharmacy departments and correspond to the mass of pharmaceutical drugs consumed per year (kg $year^{-1}$). Data was made available after confidentiality agreements aiming to protect hospitals' identity.

HWW samples were taken by following a composite scheme (1 L of sample collected manually every hour for 24 h, mixed on site) every two months per hospital and on working days in each hospital between November/2021 and August/2022, thus totaling four samples per hospital (n = 8). Sampling points in both hospitals receive raw wastewater from ambulatories, ward settings, and medical units, yet there is no influence of wastewater from the kitchen, laundry nor sterilization sectors. Samples were taken and stored in amber glass bottles and preserved under cooling conditions (4 °C) during sampling, transportation and prior to characterization and extraction, which took place immediately within 24 h from sampling. Physicochemical parameters: pH, chemical oxygen demand (COD), total suspended solids (TSS), conductivity, total dissolved solids (TDS), turbidity, alkalinity, biochemical oxygen demand (BOD), dissolved organic carbon (DOC), chloride, phosphate, nitrate, bromide, fluoride, and sulphate were analyzed in samples according to APHA (2017).

2.3. Sample preparation, extraction, and instrumentation

Qualitative suspect screening analysis was performed by liquid chromatography coupled with a quadrupole time of flight mass spectrometry (LC-QTOF MS) according to the method described by Cardoso et al. (2022). Details related to method instrumentation and identification strategy are presented in Section S2 (Supplementary Material).

Solid phase-extraction (SPE) was carried out prior to chromatographic analysis according to the method described by Hernández et al. (2015) to allow for the qualitative screening of the highest number of compounds possible in HWW samples. Oasis® HLB cartridges (200 mg, 6 mL) were used in SPE, using a vacuum manifold (Waters®). Cartridges were first conditioned with 6.0 mL of methanol, followed by 6.0 mL of ultrapure water (Milli-Q®). 200 mL of sample (pre-filtered with fiberglass filter 0.45 μm) were percolated through the cartridge. Cartridges were washed with 6 mL of ultrapure water, and vacuum dried for 20 min. The analytes were eluted with 2 × 4 mL of methanol. The extract was evaporated and reconstituted with 500 μL of H_2O:methanol (90:10). The extract was filtered using a 0.22 μm PVDF syringe filter and analyzed by LC-QTOF MS.

2.4. Qualitative suspect screening workflow

The quality of LC-QTOF MS data was guaranteed by some procedures like calibrant (ammonium formate) injection before each sample, allowing mass calibration of every sample; analysis of a mix of standards in two different concentrations to verify the constancy of peak areas in the extracted ion chromatogram as well as standards retention time and molecular and fragmentation profiles (errors <5 ppm were accepted). Besides, blank samples were analyzed between experiments to exclude possible cross contamination. After analysis by the LC-QTOF MS, all raw data were processed using Data Analysis software (v. 4.2, Bruker Daltonics, EUA).

To perform qualitative screening analysis, an extended database was constructed with data available in the literature and freely available platforms for identification of the pharmaceuticals and metabolites (www.mzcloud.org; https://massbank.eu/MassBank/). The database contains information of molecular formula, *m/z*, and fragmentation profile of each compound. A total of 1922 compounds were added to the database, including compounds frequently used in hospitals. The identification followed the workflow presented in Fig. 1.

The first step of data treatment stage was the identification of the molecular ion of investigated compounds with a mass error below 5 ppm and signal to noise ratio (S/N) higher than ten. The identification of compounds for which analytical standards were available was confirmed by the use of additional parameters (retention time, characteristic fragments). These compounds were only designated as "confirmed" if, besides initial parameters, they presented retention times within ±0.2 min error from standards, and at least two characteristic fragments with errors <5 ppm and mSigma <50 (parameter that confirms isotopic profile).

Pharmaceuticals and metabolites for which no analytical standards were available were considered as "suspect" in samples in case exact mass, molecular formula, fragmentation and isotopic profile data matched information obtained in the scientific literature and free databases. In these cases, the following criteria were adopted: exact mass (error <5 ppm in relation to the theoretical exact mass); the presence of at least 2 characteristic fragments obtained at the same retention time as the molecular ion, and mSigma <50.

2.5. Environmental risk assessment (ERA)

The risk quotient pertaining to each analyte - RQ_i (Eq. (1)) was based on the ratio between the predicted environmental concentrations (PEC) of pharmaceutical drugs identified in HWW and predicted no effect concentrations (PNEC). This approach was defined considering that qualitative screening does not allow for the quantification of target compounds, so PEC values were used to calculate RQ_i. This analysis was restricted to pharmaceuticals drugs and did not include metabolites or amino acids (11/113 compounds: 2-hydroxyquinoline; 5-aminosalicylic acid; clopidogrel carboxylic acid; cotinine; hydroxytestosterone; losartan carboxylic acid; *o*-desmethyldinortramadol; theobromine; theophylline; tyrosine and valdecoxib) identified in HWW samples due to lack of data for PEC estimation for these compounds.

PEC calculation followed Eq. (2) (adapted from Becker et al., 2020) where: m is the total consumption of pharmaceuticals drugs per year (kg $year^{-1}$) in each hospital; % exc is the excretion rate of each pharmaceutical (%); V is the volume of wastewater generated per year (m^3 $year^{-1}$), and 1,000,000 is the transformation factor to μg L^{-1}. Excretion rates used to estimate PEC for each of the identified compounds were based on values reported in the scientific literature as shown in Table S9. Monthly water consumption in each hospital averaged 23,000 m^3 (hospital A) and 18,000 m^3 (hospital B). For compounds identified in samples which lacked consumption data from hospitals, a worst-case scenario considering the highest occurrence concentrations reported in the literature for each compound were applied for PEC estimation (Table S9).

$$RQ_i = \frac{PEC_i}{PNEC} \quad (1)$$

$$PEC = \frac{\frac{m\ X\%exc}{100}}{V} \times 1000000 \quad (2)$$

PNEC values were estimated by *in silico* (Quantitative) Activity Structure Relationship ((Q)SAR), considering the ratio of acute ecotoxicity to three different taxonomic groups (*algae*, *producer*; *Crustaceans - Daphnia magna*, *primary consumer*; fish, secondary/tertiary consumer) and an assessment factor of 1000 (Escher et al., 2011; Mansour et al., 2020). The chosen assessment factor considered that input data corresponds to acute toxicity and is 100 times higher than the value indicated for chronic toxicity by EMEA (2024) (assessment factor of 10). PNEC values were predicted using VEGA QSAR v. 1.2.0 software (Benfenati

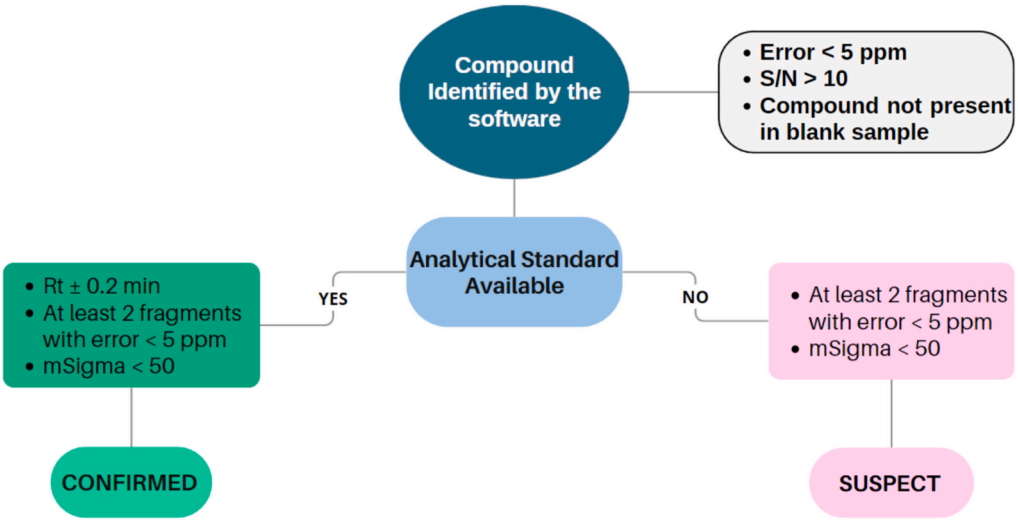


Fig. 1. Qualitative suspect screening workflow used in this study to detect drugs in hospital wastewater samples.

et al., 2013) by applying three specific models of acute toxicity: Algae Acute (EC_{50}) Toxicity model (IRFMN) 1.0.1; *Daphnia magna* LC_{50} 48 h (EPA) 1.0.8; and fish Acute (LC_{50}) Toxicity model (IRFMN) 1.0.1.

Risk quotients associated with the mixture (RQ_{mix}) of compounds occurring in each HWW were also determined by considering the sum of individual RQ_i for compounds identified in each sample (Eq. (3)) as performed by Bhandari et al. (2021) and Finckh et al. (2022). Although this approach does not consider interactions between different compounds, it avoids the underestimation of ecological risks promoted by presenting only the individual risks for each compound.

$$RQ_{mix} = \sum RQ_i \quad (3)$$

2.6. Microbiological analysis, ARB and ARG assessments

Microbiological analyses were carried out immediately after sampling to ensure the accuracy and reliability of results. Two indicators of fecal contamination, total coliforms (TC) and *Escherichia coli* (*E. coli*) were identified and quantified by the rapid-Colilert method *Colilert®-18/Quanti-Tray®2000* (IDEXX, 2008). Briefly, samples were diluted in sterile distilled water (10^{-7} and 10^{-6}) and the Colilert media was added to quantification trays and incubated for 24 h at 35 °C prior to observation of color in trays. The Plate Count Agar method was used to quantify ARB, as this method targets species of human-health relevance (Rocha et al., 2019). Samples dilutions varied for each antibiotic and are reported in Table S2 (Section S3, Supplementary Material). Diluted HWW samples (Table S2) were spread onto petri plates containing non-selective agar medium (Plate Count Agar - PCA, Kasvi) (9215-B) (APHA, 2017) for the quantification of total heterotrophic bacteria (THB). PCA medium enriched with antibiotics at their minimum inhibitory concentrations (MICs) (Table S2) (CLSI, 2020) were used for the quantification of cultivable ARB in diluted HWW (Table S2). Target antibiotics were chosen the WHO priority list for tackling antibiotic-resistant organisms (WHO, 2024) and also owing to relatively high consumption of these drugs in both hospitals under study (Section 3.1). All microbiological analyses were performed in duplicates.

Total DNA extraction was performed using the FastDNA® Spin Kit for Soil protocol (MP Biomedicals). Real-time PCR was performed to check the relative number of copies of ARGs (*blaKPC*, *blaOXA-48*, *dfrA17*, *rdxA*, *sul1*, *ermB*, *qnrS*, *vanB*, *floR*, *mphA1*, *aadA*, *mecA*, and *catA1*) in HWW samples. DNA was quantified by Qubit 3.0 Fluorometer (Invitrogen by Life Technologies), and 10 ng were used in each reaction. The relative number of copies of ARG was detected by the GoTaq qPCR Master Mix (Promega - A6002) using the StepOne™ Real-Time PCR System (Applied Biosystems). Samples were tested in duplicate for each primer pair (Table S3, Section S3, Supplementary Material). Conditions used for amplification were 95 °C for 10 min, 40 denaturing cycles at 95 °C for 30 s, followed by annealing and extension at 60 °C for 1 min. After 40 cycles, the temperature gradually increased, by 1 °C increments, from 60 °C to 95 °C, resulting in the generation of a melting curve. Relative quantification was carried out by $2^{-\Delta Ct}$ method and data was normalized by the 16S rDNA reference gene. Graphs were built on GraphPad Prism 5.0 program (GraphPad Inc., San Diego, CA, United States) and statistical analysis between ARG occurrence in HWW from hospitals A and B was performed by the *t*-test.

3. Results and discussion

3.1. Pharmaceuticals consumption profile

Antibiotics and analgesics were the most consumed classes of drugs in both hospitals in 2020 (Table S4, Supplementary Material). However, there are notable differences in drug consumption profiles of each hospital (Fig. S1, Supplementary Material), such as the higher consumption of diuretics and antivirals in hospital B. Another significant difference is that hospital B did not report the consumption of any antihypertensive, stimulant, or antirheumatic drugs during this period. Consumption comprehended a total of twenty-two different classes of drugs in hospital B (Table S4).

The profile of pharmaceuticals drugs consumption in hospital A included sixteen different classes and the class of antibiotics (193.81 kg, 88.9 %; cefazolin alone corresponded to 60.84 kg) was the most. Similarly to hospital B, the class of analgesics was the second most consumed (13.36 kg, 6.13 %; paracetamol accounted for 10.22 kg) in hospital A. Most used antibiotics in hospital A in 2020 include: cefazolin, meropenem, ceftriaxone, ampicillin, and vancomycin (Fig. 2). These results are consistent with Porto et al. (2020) who reported that the most prescribed antibiotics to treat infections in Brazil (2017) were meropenem, vancomycin, piperacillin, polymyxin B or E, and amikacin.

3.2. Hospital wastewater physicochemical characterization

HWW samples showed the typical physicochemical profile observed for wastewater from other hospitals in Brazil (Table S5, Section S4, Supplementary Material) (Chagas et al., 2011; Minetto et al., 2012; Perini et al., 2018; Silva et al., 2011; Souza et al., 2017) and municipal wastewater (Camelo et al., 2024). HWW from both facilities are slightly alkaline (pH: 6 to 9), present low alkalinity (170–350 mg $CaCO_3$ L^{-1})

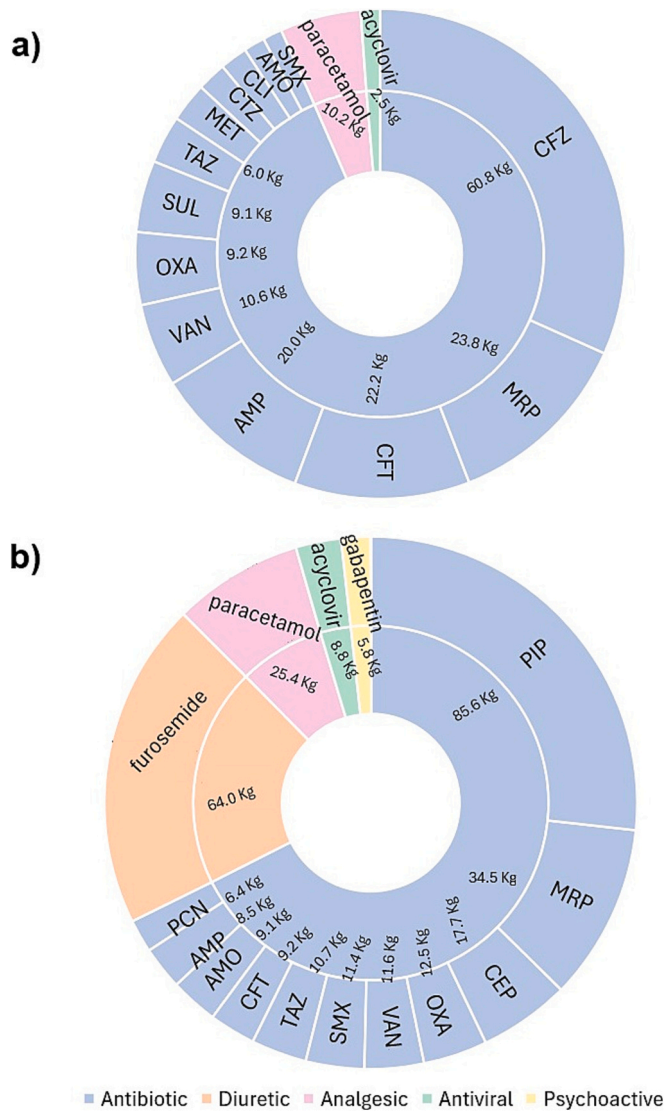


Fig. 2. Chart showing the most consumed pharmaceutical drugs (>5 kg) in 2020 at (a) hospital A and (b) hospital B, both located in Belo Horizonte, Brazil. Piperacillin - PIP, cefazolin - CFZ, meropenem - MRP, ceftriaxone - CFT, ampicillin - AMP, cefepime - CEP, vancomycin - VAN, oxacillin - OXA, sulbactam - SUL, penicillin - PCN, tazobactam - TAZ, metronidazole - MET, ceftazidime - CTZ, clindamycin - CLI, amoxicillin - AMO, and sulfamethoxazole – SMX. Numbers represent the mass consumption for each drug in kg.

and high electrical conductivity (570–2030 μS cm^{-1}). HWW samples presented moderate organic load as COD and DOC concentrations ranged between 194 and 479 mg O_2 L^{-1} and 104–204 mg L^{-1}, respectively.

Among evaluated anions, only chloride was detected in pronounced concentrations in HWW samples from both hospitals sampled in August and March (417 and 274 mg L^{-1}, respectively). High chloride concentrations in HWW were also reported by Minetto et al. (2012) and Diniz (2015) (132 and 434 mg L^{-1}), respectively. Salts (chlorides and sulphates) present in HWW are associated with physiological solutions, urine, and cleaning products. High levels of salinity, especially chloride, may limit the growth of ARB carrying *sul2*, *tetG* and *amrB* in activated sludge (Liu et al., 2018). On the other hand, high levels of salinity also has the potential to inhibit microbial activity in activated sludge and hinder biomass acclimation due to water loss from bacterial cells and inhibition of enzymatic activity (Song et al., 2018). According to Linaric et al. (2013), chloride concentrations below 10 g L^{-1} of NaCl may inhibit microbial activity without compromising the acclimation process. This is relevant considering that wastewater from both hospitals, and many others in Brazil, are directly discharged in the sewer system without any previous treatment, which may influence biological treatment efficiency in WWTP.

3.3. Occurrence of pharmaceuticals in HWW samples

Qualitative suspect analysis of 1922 compounds in eight HWW samples identified the presence of 113 compounds, as shown in Table 1. These compounds were classified in two different classes: "confirmed compounds" and "suspect compounds", according to availability and comparison to analytical standards as described in Section 2.4. 94 compounds were classified as suspects while 19 were confirmed (Table S6, Section S4, Supplementary Material). It is important to highlight that qualitative screening does not allow for the quantification of confirmed compounds, yet for their confirmation in HWW samples.

Considering all drugs (93) identified in HWW from hospital A, 37 did not occur in samples from hospital B. Two of these drugs are restricted to hospital usage: ifosfamide (chemotherapy) and iopromide (contrast agent). In contrast, 20 out of 76 identified compounds in HWW sample from hospital B did not occur in samples from hospital A. Meanwhile, 56 compounds were identified in HWW samples from both health care facilities. An average of 50 compounds were identified in all HWW samples from hospital A while an average of 47 compounds were identified in all samples from hospital B. These differences may be attributed to variations in patient profiles, treatment protocols and resource allocation between the hospitals, as hospital B serves more patients, yet with potentially limited resources, including more complex treatments and emergencies.

Among drugs identified in HWW from both hospitals, atenolol, furosemide, hydrochlorothiazide, losartan potassium, metformin, metoprolol, and propranolol are provided by the federal government as part of the primary health care program. This program includes pharmaceutical drugs considered as essential for the treatment of common health conditions, including chronic diseases, and are provided free of charge or at a reduced cost by the Brazilian government. Thus, contributing to increased consumption by population and higher chances of discharge in the environment.

A total of 31 different pharmaceutical classes were identified in samples, 30 classes were detected in samples from hospital A, while 23 classes were detected for hospital B. The class of antibiotics prevailed in both hospitals as the most detected class (15 compounds, 13–17 % detection frequency), followed by anti-inflammatories (14 compounds, 5–11 % detection frequency for hospital A and 7–12 % detection frequency for hospital B), analgesics (8 compound, 5–8 % detection frequency for hospital A and, 7–12 % detection frequency for hospital B) and antihypertensives (8 compounds, 8–13 % detection frequency for hospital A and 10–15 % detection frequency for hospital B) (Fig. 3). It is important to note that the frequency of occurrence of each of these classes in HWW from both facilities showed no significant differences ($\alpha = 0.05$), except for antihistamine ($p = 0.05$) and statin ($p = 0.02$) for which frequencies were significantly higher in wastewater from Hospital A.

Considering detection frequency, 19 compounds (10 confirmed and 9 suspect) from 11 different classes were identified in all samples: analgesic (acetaminophen and tramadol); antiarrhythmic (practolol); antibiotics (ciprofloxacin, levofloxacin, ofloxacin and trimethoprim - TMP); antidepressant (desvenlafaxine); antifungal (fluconazole); antihypertensive (atenolol, losartan and losartan carboxylic acid); anti-inflammatory (naproxen); antiseptic (benzododecinium); diuretic (hydrochlorothiazide); psychoactive (cotinine) and stimulants (caffeine, theobromine and theophylline). These results are consistent with the profile of the most frequently consumed compounds in hospital B. Once again, antibiotics constitute the main class, as it is the class that shows more compounds (four) detected in all samples among all classes. These results are in accordance with the frequent detection of antibiotics in

Table 1
Pharmaceuticals compounds identified in HWW samples from hospitals A and B in Belo Horizonte – Brazil. Highlights correspond to compounds identified in all eight samples (yellow, 100 % detection frequency), in seven samples (orange, 88 % detection frequency), or those identified exclusively in hospital A (green) or B (blue).

N	Compound	Sample							
		Hospital A				Hospital B			
		Nov/21	Jan/22	Mar/22	May/22	Dec/21	Feb/22	Apr/22	Aug/22
1	17-Alpha-estradiol	X							
2	17-Alpha-hydroxyprogesterone	X	X			X		X	
3	2-Hydroxyquinoline							X	X
4	2-Phenethylamine	X	X					X	X
5	5-Aminosalicylic acid							X	
6	8-Hydroxyquinoline			X	X	X	X	X	X
7	Acetaminophen*	X	X	X	X	X	X	X	X
8	Aciclovir			X					
9	Alprenolol			X		X	X		
10	Amantadine	X							
11	Ampicillin			X					
12	Anastrazole			X					
13	Androstenedione			X		X			
14	Antipyrine			X	X	X	X	X	X
15	Apronalide			X	X	X			
16	Atenolol*	X	X	X	X	X	X	X	X
17	Azithromycin		X						
18	Benzododecinium	X	X	X	X	X	X	X	X
19	Bisoprolol				X				
20	Bufexamac	X							
21	Butylscopolamine	X		X				X	
22	Caffeine*	X	X	X	X	X	X	X	X
23	Caproylresorcinol	X	X	X		X	X	X	
24	Carbamazepine			X		X	X	X	X
25	Chlortalidone			X					
26	Cimetidine				X				
27	Ciprofloxacin*	X	X	X	X	X	X	X	X
28	Citalopram	X		X		X		X	
29	Clarythromycin	X	X			X			X
30	Clindamycin	X							
31	Clopidogrel		X		X				
32	Clopidogrelcarboxylic acid			X			X		
33	Cocaine		X						X
34	Codeine		X						
35	Corticosterone	X		X	X	X	X	X	X
36	Cortisol/hydrocortisone				X				
37	Cortisone					X			
38	Cotinine	X	X	X	X	X	X	X	X
39	Desvenlafaxine	X	X	X	X	X	X	X	X
40	Diclofenac*	X				X		X	X
41	Diphenhydramine*	X	X	X					
42	Ephedrine	X	X	X					X
43	Escitalopram	X		X	X	X		X	
44	Esomeprazole	X	X						
45	Etodolac	X					X		X
46	Fexofenadine	X	X		X		X		
47	Fluconazole*	X	X	X	X	X	X	X	X
48	Furosemide*	X		X		X	X		
49	Gabapentin			X		X		X	X

N	Compound	Sample							
		Hospital A				Hospital B			
		Nov/21	Jan/22	Mar/22	May/22	Dec/21	Feb/22	Apr/22	Aug/22
50	Guaifenesin		X		X				
51	Hydrochlorothiazide	X	X	X	X	X	X	X	X
52	Hydrocodone		X						
53	Hydroxytestosterone					X	X	X	X
54	Ibuprofen*					X		X	X
55	Ifosfamide			X					
56	Iopromide		X						
57	Ioversol			X	X				
58	Ketoprofen	X	X	X	X				
59	Lamotrigine	X							
60	Levetiracetam						X		
61	Levofloxacin*	X	X	X	X	X	X	X	X
62	Lidocaine*	X	X		X	X	X	X	X
63	Lincomycin	X							
64	Losartan*	X	X	X	X	X	X	X	X
65	Losartan carboxylic acid	X	X	X	X	X	X	X	X
66	Mdppp	X							
67	Meclofenamicacid	X		X				X	
68	Mefenamicacid						X		X
69	Memantine			X					
70	Mesalazine							X	
71	Metformin	X						X	
72	Methenolone						X		
73	Metoclopramide*	X				X			
74	Metoprolol*	X		X	X	X	X	X	X
75	Metronidazole*	X	X	X	X			X	
76	Morphine							X	
77	Mycophenolicacid					X	X		
78	Naproxen*	X	X	X	X	X	X	X	X
79	Nicotine					X			
80	nm-2-ai	X	X						
81	Norfloxacin						X		
82	o-Desmethyldinortramadol							X	X
83	Ofloxacin*	X	X	X	X	X	X	X	X
84	Omeprazole					X			
85	Ondansetron		X						
86	Orphenadrine					X			
87	Oxcarbazepine					X	X	X	X
88	Pantoprazole				X				
89	Phenylephrine								X
90	Piracetam			X					
91	Practolol	X	X	X	X	X	X	X	X
92	Pravadoline		X						
93	Prednisolone					X			
94	Propafenona	X		X					
95	Propranolol*								X
96	Pseudoephedrine	X	X	X					X
97	Quetiapine	X							
98	Rosuvastatin		X	X					
99	Sertraline						X		
100	Sulfamethoxazole			X					
101	Sulfapyridine					X		X	X
102	Terbutaline								X

N	Compound	Sample							
		Hospital A				Hospital B			
		Nov/21	Jan/22	Mar/22	May/22	Dec/21	Feb/22	Apr/22	Aug/22
103	Testosterone			X		X	X	X	X
104	Theobromine	X	X	X	X	X	X	X	X
105	Theophylline	X	X	X	X	X	X	X	X
106	Topiramate		X			X		X	
107	Tramadol	X	X	X	X	X	X	X	X
108	Trimethoprim*	X	X	X	X	X	X	X	X
109	Tropisetron	X							
110	Tyrosine				X				
111	Valdecoxib		X						
112	Valsartan	X	X	X		X	X	X	X
113	Venlafaxine		X	X		X	X	X	X
Total (number of compounds)		**54**	**46**	**54**	**37**	**51**	**42**	**49**	**46**

*Confirmed compounds = analytical standards were available.

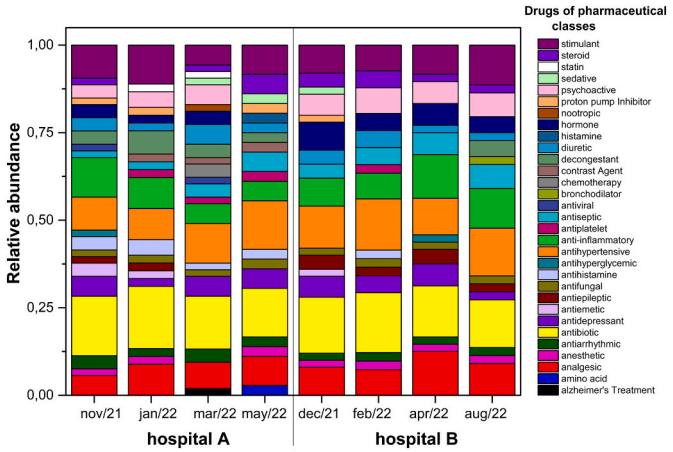


Fig. 3. Pharmaceutical classes of drugs identified in HWW samples (November 2021–August 2022) from two hospitals A and B located in Belo Horizonte, Brazil.

HWW elsewhere (Al-Maadheed et al., 2019; Cardoso et al., 2022; Carraro et al., 2016). This is a matter of public health concern as, once in the environment, antibiotics contribute to AMR spread (Nappier et al., 2020) by selecting ARB and bosting ARG transfer (Amarasiri et al., 2020). Thus, these results support the need to investigate antibiotics, ARB and ARG in HWW samples, especially in regions where such data are scarce.

The screening analysis also allowed for the identification of eight suspect metabolites present in samples: 2-hydroxyquinoline, clopidogrel carboxylic acid, cotinine, hydroxytestosterone, losartan carboxylic acid, *o*-desmethyldinortramadol, theobromine, theophylline. Except for 2-hydroxyquinoline, parent compounds corresponding to all suspect metabolites were also identified in HWW samples, thus supporting their occurrence. For instance, theobromine and theophylline are caffeine metabolites, a stimulant that is present or added to food, beverages and pharmaceuticals and which was also identified in HWW samples. In contrast, the detection of 2-hydroxyquinoline in the absence of quinolones points out to the use of quinolones in hospitals. This reveals the relevance of performing a screening analysis for both, pharmaceuticals and metabolites, in complex samples such as HWW.

3.4. Environmental risk assessment

Individual RQ were classified from negligible (RQ < 1) to low (1 ≤ RQ < 1), medium (1 ≤ RQ < 2) and very high risk (RQ ≥ 2) (Franquet-Griell et al., 2015; Gosset et al., 2021) and transformed to logarithm scale in Fig. 4. RQ values obtained for all compounds are presented in Section S5 (Tables S7, S8, S9 and S10 - Supplementary Material).

Despite high use of anti-inflammatories and analgesics in various treatments in hospitals and their percentages of excretion in the original form range (2–95 %) (Moratalla et al., 2022), this study detected a low risk for ibuprofen for all three taxonomic groups (Fig. 4a). Even so, potential risks to environmental integrity associated with the occurrence of anti-inflammatories and analgesics in wastewater and surface water are real as nonsteroidal anti-inflammatory drugs exhibit toxicity to aquatic organisms, for example to *V. fischeri* (<20 mg L^{-1} in 5 min) (Cleuvers, 2004).

Considering individual RQs, 73 drugs presented negligible risk for all the evaluated taxonomic groups. Among these, 13 were antibiotics (azithromycin; caproylresorcinol; clarythromycin; clindamycin; levofloxacin; lincomycin; metronidazole; mycophenolic acid; norfloxacin; ofloxacin; sulfamethoxazole; sulfapyridine and trimethoprim). These results can possibly be attributed to the low consumption of these compounds as indicated by drug consumption data provided by hospitals.

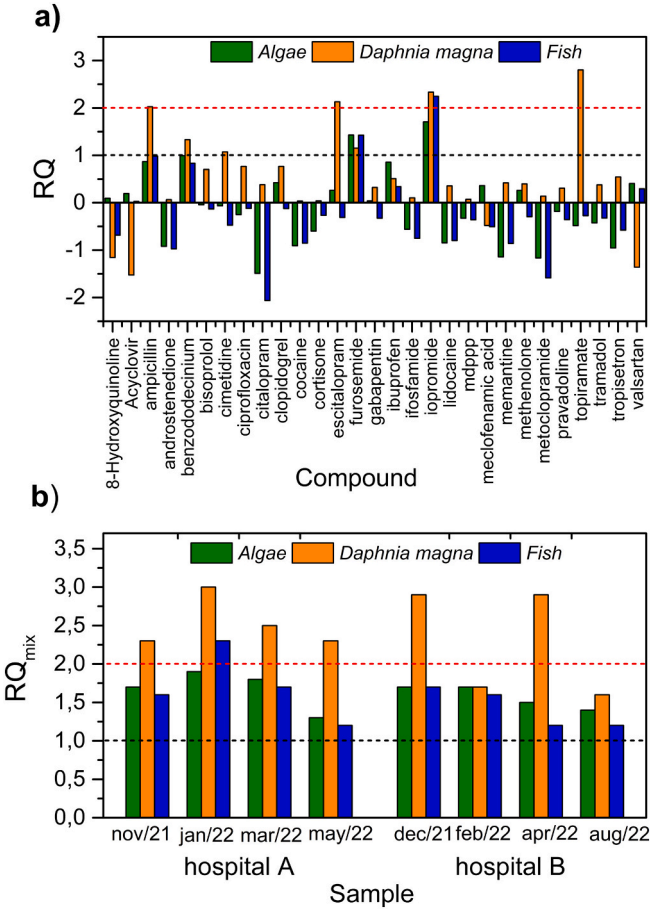


Fig. 4. Pharmaceuticals drugs that presented potential environmental risk ($RQ_i > 1$) to at least one taxonomic group (a) and risks associated to the mix (RQ_{mix}) (b) of pharmaceutical drugs detected in HWW from hospitals A and B. RQ were classified from low (0 ≤ RQ < 1), to medium (black line, 1 ≤ RQ < 2) and very high risk (red line; RQ ≥ 2). Logarithm scale was used for better view of results.

Nevertheless, 29 compounds represented potential risk to at least one of the taxonomic groups evaluated (Fig. 4a). 25 out of the 29 of these compounds presented potential low risk to at least one trophic level while 4 compounds presented potential medium risk to at least one trophic level (benzododecinium, cimetidine, furosemide and iopromide) and 4 compounds presented potential very high risk to at least one trophic level (ampicillin, escitalopram, iopromide and topiramate). Among these, ampicillin is the only antibiotic, while the others are, respectively, an antidepressant, an X-ray contrast, and anticonvulsant.

Although individual risks are generally low, it is critical to observe that these compounds do not occur alone in samples and that wastewater discharged to the environment often contains a mixture of compounds which can influence matrix toxicity. Thus, RQ_{mix} of each sample is presented in Fig. 4b. As demonstrated in other studies with HWW, the mixture of pharmaceutical drugs in this matrix represents serious risks for the environment (Becker et al., 2020; Gosset et al., 2021). In this study, six out of the eight samples analyzed showed very high risk to at least one trophic level when considering the RQ_{mix}. Although no interactions between identified pharmaceuticals were evaluated, these results demonstrate a very high potential risk of these samples to aquatic species from different taxonomic groups.

According to Fig. 4b, wastewater samples from hospital A represent higher risks to the environment compared to hospital B, since all samples from hospital A showed very high risk to at least one taxonomic group and presented the higher RQ_{mix} to *Daphnia magna* (897.9)

compared to hospital B (with RQ_{mix} to *Daphnia magna* = 834.1). This may be associated with the higher number of beds in hospital A (650) when compared to hospital B (504). The potential risk of all compounds identified in HWW combined in one value (RQ_{mix}) provides for a better understanding of environmental risks and may support decision-making policies related to the management of hospital wastewater (Coors et al., 2018; Gosset et al., 2020, 2021).

3.5. Occurrence of antibiotic-resistant bacteria (ARB) and genes (ARG) in HWW

HWW showed higher levels of TC and *E. coli* (10^7–10^{10} and 10^6–10^9 MPM/100 mL, respectively) (Fig. S2, Section S6, Supplementary Material) compared to typical values reported in the literature, which range between 10^5–10^8 and 10^3–10^6 MPM/100 mL for TC and *E. coli*, respectively (Chagas et al., 2011; Kumari et al., 2020; Perini et al., 2018). Although hospital B showed higher levels of TC and *E. coli* than hospital A (Fig. S2), there was no significant difference at a significance level of 0.05 (p-value = 0.4681 for TC; p-value = 0.2843 for *E. coli*).

Considering water consumption, the contribution load in terms of TC and *E. coli* is 1.89×10^{16} and 5.52×10^{14} MPN per day^{-1}, and 2.47×10^{16} and 1.88×10^{15} MPN per day^{-1}, for hospitals A and B, respectively. Taking *per capita* contribution of raw domestic sewage for TC and *E. coli* (10^9–10^{13} and 10^9–10^{12} MPN hab^{-1} day^{-1}, respectively) (Von Sperling, 2014), these hospitals account for a population equivalent of 2177 ± 414 (hospital B) and 1224 ± 951 (hospital A) inhabitants. Thus, representing significant contribution of TC and *E. coli* and, potentially, other pathogens, such as ARB, to domestic sewage.

There was no significant difference (p-value = 0.072) in total heterotrophic bacteria (THB) levels between HWW from each hospital (Fig. S3) and detected concentrations (1.1×10^6 to 4.1×10^7 CFU/mL) corroborate with those reported by Proia et al. (2018) (10^7 and 10^{10} CFU/mL) for HWW from Belgium. Regarding ARB, median concentrations in HWW from hospital B were significantly higher than in HWW from hospital A (p-value = 0.028), possibly due to the higher complexity of treatments employed and greater number of patients treated at hospital B compared to hospital A.

ARB resistant to SMX + TMP were present at low levels in all samples (Fig. 5), being significant only in HWW from April/22 and August/22 (hospital B) (3.15×10^6 and 9.40×10^5 CFU/mL, respectively). This may possibly be attributed to the lower consumption of these antibiotics compared to CFZ, CFT and MRP, as discussed in Section 3.1. Abundance of SMX + TMP resistant bacteria in this work was lower than that reported elsewhere (Chagas et al., 2011; Prado, 2007).

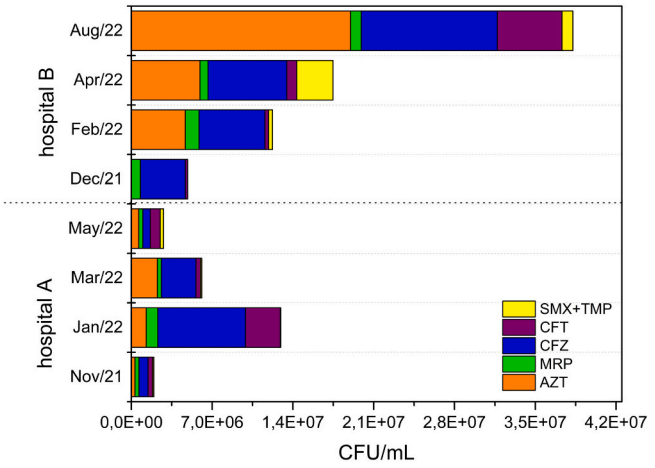


Fig. 5. Concentrations of ARB showing resistance to sulfamethoxazole + trimethoprim (SMX + TMP), ceftriaxone (CFT), cefazolin (CFZ), meropenem (MRP), and azithromycin (AZT) in hospital wastewater samples from two hospitals in Belo Horizonte – Brazil (Hospital A and hospital B).

The HWW sample from January/2022 showed the highest levels of CFZ- and CFT-resistant bacteria (10^6 CFU/mL) This results is probably associated with the increased use of these antibiotics in Belo Horizonte during the Sars-CoV-2 pandemics (SES, 2022), with hospital B reporting a rise in consumption from 4.26 kg of CFZ and 9.23 kg of CFT in 2020 to 10.55 kg of CFZ and 10.95 kg of CFT in 2022. CFZ-resistant bacteria represented 31–69 % and 16–58 % of THB in hospitals B and A, respectively, aligning with findings by Schwartz et al. (2003) (58 % of THB).

ARB resistant to azithromycin were abundant in HWW samples taken from February to August/22 (10^6 CFU/mL). As AZT was widely used to treat Covid-19 infections, this may have increased risks promoted by bacteria resistant to this antibiotic (Kamel et al., 2022; Khoshnood et al., 2022). The low levels of MRP-resistant bacteria in HWW from both hospitals align with the literature (Chagas et al., 2011), as MRP is reserved only for severe cases in Intensive Care Units (Casals et al., 2014).

All evaluated ARGs were detected in HWW samples, except for *rdxA*. ARGs *mecA*, *blaOXA-58*, and *blaKPC* were previously detected in HWW from Portugal (Ferreira et al., 2022). Although the relative number of copies of ARG seems higher in HWW samples from hospital B (Fig. 6) there was no significant difference for *blaOXA* nor *mecA* genes (significance level of 0.05), which provide resistance to β-lactams and are associated with MRSA (methicillin-resistant *Staphylococcus aureus*), respectively. It is important to highlight that MRSA is listed as a high priority pathogen by the WHO (2024).

The relative number of copies of *mphA1* (linked to MLSB resistance) and *dfrA17* (associated with trimethoprim resistance), was higher in HWW from hospital A, while *blaKPC*, *catA1*, *sul1*, *ermB*, *qnrS*, *vanB*, *floR*, and *aadA* were higher in HWW from hospital B. The high levels of *blaKPC* corroborate with the fact that carbapenems are the most consumed antibiotic class in Brazil (WHO, 2018b) and in both hospitals under study (Section 3.1). Chagas et al. (2011) reported that genes encoding β-lactamases were the most prevalent ARGs in ARB isolates from Brazilian HWW as they occurred in 40 % of isolates (n = 221 isolates).

Sul1 was the second most abundant ARG detected in HWW samples, reflecting sulfonamides' widespread use in Brazil (WHO, 2018a). The *qnrS* and *ermB* genes, which have been detected in HWW samples from several countries (Kayali and Icgen, 2020; Rodriguez-mozaz et al., 2015), showed a similar relative number of copies in HWW from both facilities. The presence of MDR (*florR*) indicates that HWW samples contain multidrug-resistant bacteria. This is a concern considering that HWW produced in both hospitals are discharged directly in the sewer system without any previous treatment, a common practice in Brazil. Critically, wastewater treatment in the country accounts only for 52 % of total generated volume, which is also a reality in other low- and middle-income countries, thus justifying the need for more studies exploring potential contribution of HWW to the spread of antimicrobial resistance in these regions.

4. Conclusions

This study investigated HWW from Brazilian hospitals, assessing drug consumption, occurrence of pharmaceutical drugs by qualitative suspect screening, as well as ARB, and ARG. Antibiotics and analgesics were the most consumed drugs in both hospitals and consumption patterns were statistically similar. The occurrence of pharmaceutical drugs in HWW samples were also similar between hospitals and antibiotics was the most frequently detected class, thus reflecting consumption profiles. The environmental risk assessment identified that 29 of 113 compounds have the potential to cause toxic effects to aquatic fauna, and four compounds may pose high risk (8-hydroxyquinoline, acyclovir, ampicillin, androstenedione, benzododecinium, bisoprolol, cimetidine, ciprofloxacin, citalopram, clopidogrel, cocaine, cortisone, escitalopram,

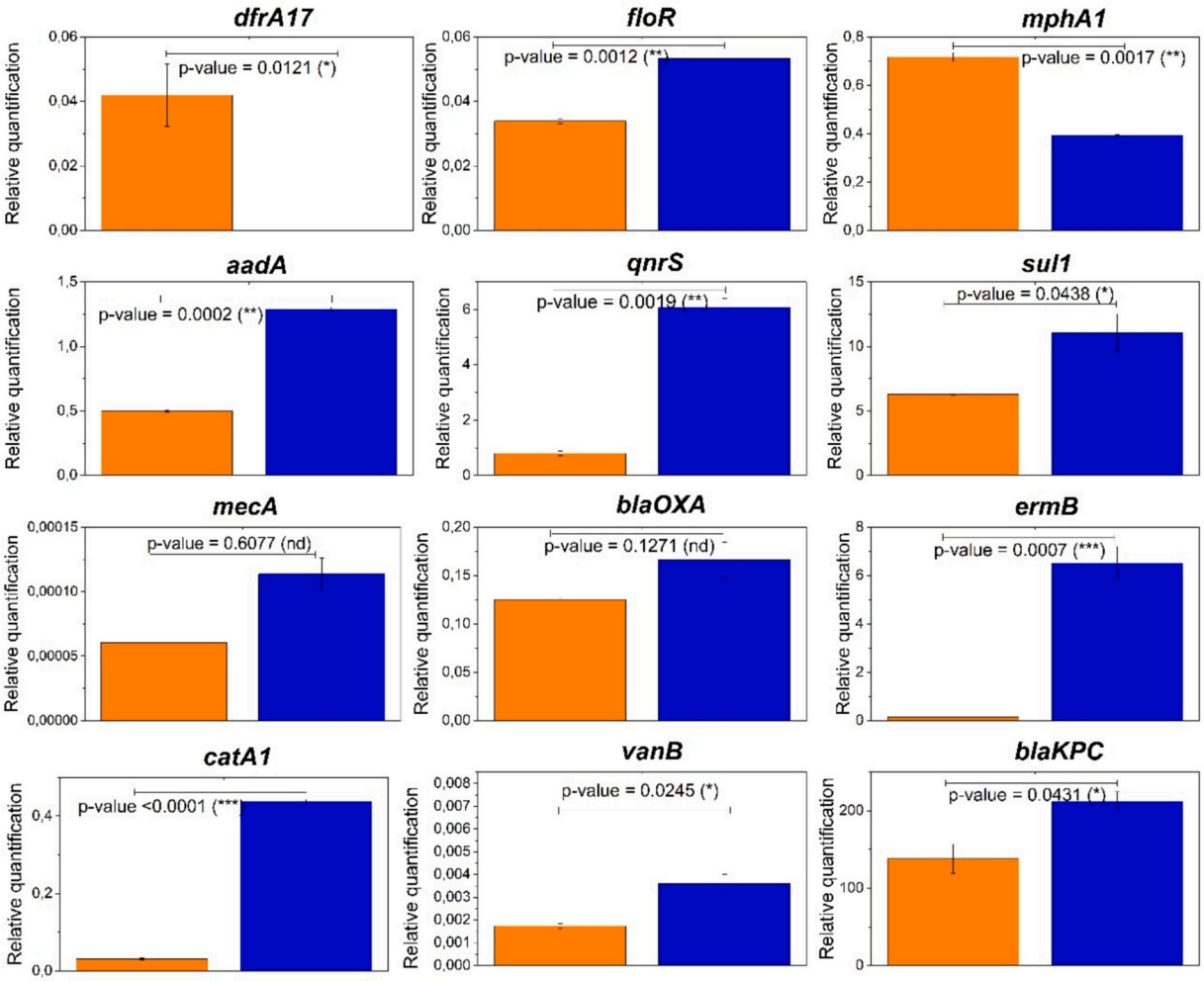


Fig. 6. Relative number of copies of ARGs in hospital wastewater samples from two hospitals in Belo Horizonte – Brazil. In orange (hospital A) and in blue (hospital B). Bars represent the confidence interval, and the p-value displayed above bars indicate the result of the *t*-test at a significance level *0.05, **0.01, and ***0.001. Relative quantification of each resistance gene was carried out by $2^{-\Delta Ct}$ method normalized by the 16S rDNA reference gene.

furosemide, gabapentin, ibuprofen, ifosfamide, iopromide, lidocaine, mdppp, meclofenamic acid, memantine, methenolone, metoclopramide, pravadoline, topiramate, tramadol, tropisetron, valsartan). The occurrence of cultivable ARB in HWW samples varied between hospitals probably owing to drug consumption patterns and external factors. As for ARGs, *blaOXA* and *mecA* levels were similar in HWW from hospitals, yet *blaKPC*, *catA1*, *sul1*, *ermB*, *qnrS*, *vanB*, *floR*, and *aadA* were significantly more abundant in one of the hospitals. This study highlights the link between hospital drug use and their presence in HWW, emphasizing potential environmental and public health risks associated to the occurrence of antibiotics, ARB, and ARG in this matrix. As data on the occurrence of these contaminants in HWW is scarce in low- and middle-income countries, these data may subsidize the development of HWW management strategies in these regions aiming at environmental and public health protection.

CRediT authorship contribution statement

Eloísa Stéphanie da Silva: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Raquel W. Becker:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Maria Clara V.M. Starling:** Writing – review & editing, Visualization, Resources, Conceptualization. **Elayne C. Machado:** Writing – review & editing, Visualization, Resources, Conceptualization. **Ana Paula V. Reis:** Writing – review & editing, Investigation, Conceptualization. **Ananda Pereira Aguilar:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Tiago Antônio de Oliveira Mendes:** Writing – review & editing, Resources, Funding acquisition, Formal analysis, Conceptualization. **Carla Sirtori:** Writing – review & editing, Resources, Funding acquisition, Formal analysis, Conceptualization. **Camila C. Amorim:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Funding sources

This work was supported, in the whole, or in part, by the Bill & Melinda Gates Foundation, Seattle, WA [grant number OPP1193112]. Under the grant conditions of this Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission. Prof. Carla Sirtori thanks CNPq (Process 310717/2020-5) for her research productivity grant. This study was financed in part by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) – Finance [Code 001]. Prof. Camila Amorim would like to thank the Federal University of Minas Gerais (UFMG), Foundation of Support to Research in Minas Gerais (FAPEMIG) [grant number PPM 00489 17], Coordination of Improvement of Superior Education (CAPES) [Code 001], and the National Council for Scientific and

Technological Development (CNPq) [grant numbers, 436902/2018 4, 314642/2021 8].

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

The authors would like to thank the Federal University of Minas Gerais (UFMG), to the two hospitals that provided important information and allowed the collection of wastewater samples, Bill & Melinda Gates Foundation, Foundation of Support to Research in Minas Gerais (FAPEMIG), Coordination of Improvement of Superior Education (CAPES), National Council for Scientific and Technological Development (CNPq), and Foundation of Support to Research in Minas Gerais (FAPEMIG).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179132.

Data availability

Data will be made available on request.

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