Research Paper

The balance between treatment efficiency and receptor quality determines wastewater impacts on the dissemination of antibiotic resistance

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HIGHLIGHTS

- The highest antibiotic resistance removal rate is observed after secondary treatment.
- UV and ozone have limited capability to remove antibiotic resistance genes (ARGs).
- Upstream river contamination and high flow rates may mask UWTPs effects.
- Some emerging ARGs (e.g. bla_{IMP}, bla_{OXA-48}, bla_{OXA-58}) were disseminated in the river.
- Hospital effluents (HEs) may be a source of emerging ARGs (e.g., bla_{VIM}, bla_{KPC}).

GRAPHICAL ABSTRACT
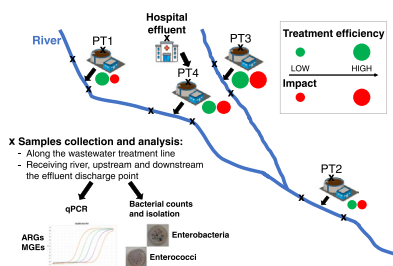
ARTICLE INFO

ABSTRACT

This study investigated the balance between treatment efficiency and impact caused by urban wastewater treatment plants (UWTPs) on the dissemination of antibiotic resistance. Four full-scale UWTPs (PT1-PT4) and the receiving river were sampled over four campaigns. The 16 S rRNA gene, two mobile genetic elements (MGEs), eight antibiotic resistance genes (ARGs), and culturable bacteria were monitored over different treatment stages and in hospital effluent. The bacterial and antibiotic resistance load was not significantly different in the inflow of the four UWTPs (p > 0.01). Biological treatment promoted ARGs reduction values up to 2.5 log-units/mL, while UV (PT1, PT2) or sand filtration/ozonation (PT3) led to removal values < 0.6 log-units/mL. The final effluent of PT3, with the highest removal rates and significantly lower ARGs abundance, was not significantly different from the receiving water body. Emerging ARGs (e.g., bla_{VIM}, bla_{OXA-48}, and bla_{KPC}) were sporadically detected in the river, although more frequent downstream. Hospital effluent might contribute for the occurrence of some, but not all these ARGs in the river. A major conclusion was that the impact of the UWTPs on the river was not only determined by treatment efficiency and final effluent quality, but also by the background contamination of the river and/or dilution rate.

1. Introduction

Urban wastewater treatment plants (UWTPs) are both receptors and sources of antibiotic resistance. Receptors, because receive the human

sewage, which along with human excreta is where most of the antibiotic resistance contaminating the environment is loaded (Manaia et al., 2016). Sources, because treatment systems, although removing part of the anthropogenic bacteria entering the system, and therefore also

antibiotic resistant bacteria and antibiotic resistance genes (ARB & ARGs), are not efficient enough to produce final effluents exempt of these biological contaminants (Fernandes et al., 2019; Narciso-da-Rocha et al., 2018; Novo and Manaia, 2010). Indeed, it has become increasingly evident that ARB & ARGs released by well-functioning UWTPs may have a negative impact on the environment, representing a threat for humans and/or ecosystems (Berendonk et al., 2015; Makowska et al., 2016; Manaia et al., 2016). From the UWTPs, ARB & ARGs can be disseminated to the receiving water, to terrestrial environments such as plants and soil, with the risk of entering the animal and human food chains (Becerra-Castro et al., 2015; Karkman et al., 2018; Scaccia et al., 2021). There is evidence that *Escherichia coli* (*E. coli*) isolated from origins as distinct as hospitalized patients, wastewater, surface water or seagulls belong to closely related genetic lineages (Varela et al., 2015). Also, the spread and persistence of ARGs in aquatic environments with presumable origin in sewers, intensive agriculture or UWTPs discharges have been evidenced (Bueno et al., 2018). Different quantitative PCR (qPCR) or metagenomics studies have reported that dozens to hundreds of antibiotic resistance and genetic recombination genes and determinants may be discharged in the final effluent of UWTPs (Che et al., 2019; Ju et al., 2019; Lira et al., 2020; Pärnänen et al., 2019). Other studies have suggested that these emissions may exceed 10^{14}-10^{18} ARG copies released per day to the receiving environment (Manaia et al., 2016; McConnell et al., 2018). Different studies designed to assess the impact of UWTPs in the receiving environment revealed the non-negligible impact of human activities. From urban areas, to agriculture, through hospital effluents, the discharge of antibiotic resistance is noticed in the downstream water bodies, being the risks of persistence higher in already polluted water systems (Ben et al., 2017; Lorenzo et al., 2018; Proia et al., 2018). The impact of UWTPs on the surrounding environment is influenced by a complex interplay of factors that include the characteristics of the raw sewage, the treatment efficiency, and the characteristics of the receiving water bodies. These aspects guided the design of this study focused on four full-scale UWTPs (named PT1 to PT4) located in Northern Portugal, in a ratio of 38 kilometres, all discharging into the same river basin. The selected UWTPs differed on the i) type of inflow received that besides home sewage might include untreated hospital effluent or textile industry effluent, ii) combination of treatment processes (secondary treatment and disinfection) or iii) river transect where the final effluent was discharged. Wastewater treatment efficiency and UWTPs impacts were assessed based on the load of ARB, mobile genetic elements (MGEs), and ARGs. Specifically, it was aimed to i) assess if the inflow of distinct UWTPs in the same region could be influenced by hospital or industrial inputs, more than by home sewage; ii) compare the efficiency of distinct treatment systems on the removal of ARB & MGEs & ARGs; and iii) assess the impact of the discharge of the final effluent of each UWTP on the receiving river.

2. Methods

2.1. Sample collection and processing

Four full-scale UWTPs, herein designated PT1, PT2, PT3, and PT4 (Table 1 and Table S1), located in Northern Portugal and all discharging to the same river basin, were selected for this study. PT1, PT2, and PT4 receive 70–80% of domestic effluents, including raw hospital wastewater and PT3 receives up to 60% of industrial effluents (mainly textile industry). These plants include a preliminary treatment, primary treatment, and activated sludge-based secondary treatment. PT1 and PT2 include UV disinfection that in PT2 is supplemented by chlorination, NaOCl (2 ppm). In PT2 this disinfection process is operated only during the bathing season (June to September). PT3 includes sand filtration and ozonation. In PT4, coagulants are added to the biological reactor to remove colour due to textile industry effluents. Samples were collected over four campaigns in summer and autumn 2018 and in winter and spring 2019 at different treatment stages (in PT2, samples after UV disinfection were collected in two campaigns when that process was operating), and in the receiving river, upstream and downstream the discharging point at distances where sampling was possible, 90–950 m and 325 m - 1 500 m, respectively (Table 1). Twenty-four hours composite samples were collected with the aid of automatic samplers at each of the sampling points of the UWTPs. Hydraulic retention time-equivalent samples were collected downstream of the raw wastewater sampling point, being also taken into consideration the feasibility of processing and analysis after transport and samples' delivery in the laboratory. Hence, final treated wastewater samples were collected about 48 h after collecting the raw wastewater of each UWTP. Hospital effluent grab samples were collected at the same day as the raw wastewater of the respective UWTP (PT4). River water was collected at the same day as samples of final treated wastewater at both upstream and downstream the area of discharge of each UWTP. The latter were collected manually as grab samples, whenever necessary using an extensible sampler to reach about 2–3 m from the margins of the river and 20 cm deep. Samples were transported refrigerated and analysed within 12 h after collection. Sample processing consisted of membrane filtration through cellulose nitrate membranes (0.22 μm porosity; Sartorius Stedim Biotech, Göttingen, German) or polycarbonate membranes (0.22 μm porosity; Whatman, England) for bacteria enumeration or DNA extraction, respectively.

For bacteria enumeration volumes of 1, 10, and 100 mL or of 10-fold serial dilutions were filtered, and for DNA extraction samples were filtered 50 mL of raw wastewater, 150–250 mL of treated wastewater after secondary treatment, the same volume of wastewater after UV disinfection or sand filtration, 250–400 mL after ozonation, and 250–300 mL of river water. These volumes corresponded to the best balance between the filtration capacity of the membrane before collapsing, total DNA extraction yield, and DNA needed for genes quantification by qPCR. Filtrations were all made in triplicate. After filtration, the membranes for microbial culture analysis were processed

Table 1
Characterization of the urban wastewater treatment plants (UWTPs) located in Northern Portugal and discharging to the same river basin.

UWTP	Population equivalent (inhabitants)	Daily flow (range values in campaigns 1–4 in m^3)	Distance of receiving water body to UWTP (meters)		Tertiary treatment		
			Up River	Down River	UV disinfection	Sand filtration	Ozonation (gO$_3$/m^3)
PT1	22 684	1 548 – 3 661	125	650	32.76 W/m^2	no	no
PT2	257,000	12,500 – 17,280	950	1 500	38.00 mJ/cm^2 (+ NaOCl, 2 ppm)	no	no
PT3	187,087	14,120 – 22,387	90	325	no	yes	19.6 – 26.0
PT4[a]	170,513	22,839 – 25,237	450	850	no	no	no

[a] This UWTP (PT4) receives the untreated hospital effluent analysed in this study serving a population equivalent to 252,365 inhabitants. PT4 has a secondary treatment with addition of coagulants to the biological reactor to remove colour.

immediately, and for DNA extraction were stored at − 80 °C.

2.2. Enumeration and characterization of cultivable bacteria

Enterobacteria were enumerated on the culture medium Chromogenic Coliform Agar (CCA, VWR Chemicals) or on this medium supplemented with ciprofloxacin (2 mg/L), cefotaxime (2 mg/L) or meropenem (2 mg/L). Enterococci were enumerated on m-Enterococcus Agar medium (mEnt, Difco DB) or on this medium supplemented with ciprofloxacin (2 mg/L) or vancomycin (6 mg/L). Enumerations were made on cultures incubated for 24 h at 37 °C (for total enterobacteria and presumably resistant to ciprofloxacin or cefotaxime) or at 30 °C (for enterobacteria presumably resistant to meropenem), and for 72 h at 37 °C (for enterococci and presumably resistant to ciprofloxacin or vancomycin). Presumably meropenem-resistant enterobacteria were incubated at 30 ºC based on previous assays that demonstrated that carbapenem-resistant enterobacteria were uncommon in that environment, and it was expected that cultures would be under strong stress due to the presence of meropenem. Counts were expressed as colony forming units/mL (CFU/mL). In situations where no colonies or a single colony was observed corresponding to filtered volumes of 1 mL, 10 mL, and 100 mL of sample, it was considered as a value close to the limit of quantification (LOQ, 1 CFU/100 mL, −2.0 log-units).

Randomly selected isolates, presumable *E. coli* or *Klebsiella* spp. (according to manufacturer's instructions) isolated on CCA or on CCA supplemented with ciprofloxacin, cefotaxime or meropenem over the first two sampling campaigns were isolated and characterized. Isolates were preliminarily characterized based on Random Amplified Polymorphic DNA (RAPD) and antibiotic resistance phenotypes, as previously described (Ferreira da Silva et al., 2007). Possible repetitions (isolates recovered in the same sampling campaign, on the same culture medium plate, with the same RAPD genotype and similar antibiotic resistance pattern) were removed from further analysis. The identification of the isolates was made based on 16 S rRNA gene sequencing, and supported by the database EZBioCloud (Yoon et al., 2017). The presence of the ARGs bla_{KPC}, bla_{OXA-48}, bla_{OXA-58}, and bla_{CTX-M} was screened by conventional PCR (Gootz et al., 2009; Poirel et al., 2011; Woodford et al., 2006; Weill et al., 2004) and amplicon sequence analysis.

2.3. DNA extraction and genes quantification

The DNA was extracted in triplicate from each sample using the DNeasy PowerWater Kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions. DNA extracts were stored at − 20 °C before quantification by qPCR of the 16 S rRNA gene, a measure of total bacteria; *intI1* gene and replicon type incF as indicators of MGEs; and genes encoding for resistance to the following beta-lactams, penicillin's (bla_{TEM}), cephalosporins (bla_{CTX-M}), carbapenems (bla_{IMP}, bla_{VIM}, bla_{OXA-48}, bla_{OXA-58}, and bla_{KPC}), and methicillin MRSA (methicillin resistant *Staphylococcus aureus*, *mecA*). The quantification was based in the standard curve method as described by Brankatschk et al. (2012) in a StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The qPCR results were analysed and validated based on the following parameters: standard curves for each gene with reaction efficiencies from 90% to 110%, authenticity indicated by the expected melting temperature (single peak), and quantification values in samples within the range defined in the calibration curve described in Rocha et al. (2020) (Table S2).

2.4. Statistical analysis

Data were expressed as logarithm of CFU per volume of sample ($\log_{10}$ (CFU/mL)) and of gene copy number per volume of sample ($\log_{10}$ (gene copy/mL)) or per 16 S rRNA gene copy number ($\log_{10}$ (gene copy/16 S rRNA)). The one-way analysis of variance (ANOVA) and the Tukey's and Bonferroni post hoc tests were used to infer statistically significant differences ($p < 0.01$) in the abundance of total bacteria and presumably antibiotic resistant enterobacteria and enterococci, and of the genes analysed by using SPSS Statistics for Windows v.24.0 (IBM Corp., Armonk, NY, USA).

Removal values of bacteria or genes were expressed as log-units. For genes was considered the abundance of 16 S rRNA gene or of each gene analysed, and also the sum of abundance of ARGs (bla_{TEM}, bla_{CTX-M}, bla_{IMP}, bla_{VIM}, bla_{OXA-48}, bla_{OXA-58}, bla_{KPC}, and *mecA*), and the sum of abundance of genes associated to MGEs (*intI1* and replicon type incF). The log-removal values were calculated between each sampling site and the site immediately before, i.e. upstream receiving river water (up River) vs. raw wastewater (RWW); RWW vs. secondary treatment (sTWW); sTWW vs. UV disinfection (uvTWW); sTWW vs. sand filtration (sandTWW); sand filtration (sandTWW) vs. ozonation (ozTWW) and after treatment for discharge into the river vs. downstream receiving river water (down River). Calculations used the formula: log-removal = logA1 – logA2, where A1 and A2 correspond to two sampling sites contiguous in treatment process, with A2 succeeding A1. Log-removal values of 0 indicated no effect on the evaluated parameters; log-removal > 1 indicated a decrease of the evaluated parameters; and log-removal < 1 indicated an increase of the evaluated parameters. The removal values were interpreted as a measure of treatment efficiency in the UWTPs or dilution effect due to the discharge into the receiving river.

3. Results

This section is organized to address specific questions: i) if raw wastewater entering each UWTP and the hospital effluent presented distinct abundance or prevalence of antibiotic resistance; ii) if treatment efficiency was identical in the four UWTPs and how it influenced the quality of the final effluent; and iii) if the different UWTPs had identical impacts on the receiving river, since all discharge into the same river basin.

3.1. Raw wastewater

The abundance of the 16 S rRNA gene per volume of raw wastewater (7.9 log-units/mL) was non-significantly different in the four UWTPs (p > 0.01), being slightly and significantly (p < 0.01) lower in the hospital effluent (7.4 log-units/mL) (Fig. 1a). The amplicons associated with MGEs, *intI1* and incF, ranged 6.3–7.3 and 3.8–4.8 log-units/mL, respectively, in the same order of magnitude as in hospital effluent (6.0 and 4.7 log-units/mL, respectively) (Fig. 1 and Fig. S1). In general, the abundance of ARGs did not differ significantly in raw wastewater of the four UWTPs, ranging from 4.1 to 5.1 log-units/mL for the most abundant ($bla_{TEM} > bla_{OXA-58} > bla_{CTX-M}$) to 2.1–3.2 log-units/mL for the least abundant (bla_{IMP}). The abundance of carbapenem-resistance encoding genes bla_{IMP}, bla_{VIM}, and bla_{KPC} was about 2 log-units/mL higher in hospital effluent than in the UWTPs (p < 0.01) (Fig. 1a). Curiously, the hospital effluent did not have significantly higher abundance of the genes bla_{OXA-48} and bla_{OXA-58}. The *mecA* gene, detected only occasionally, was quantified in raw wastewater of PT1, PT2, and hospital effluent with abundance values ranging 0.7–1.7 log-units/mL. The abundance of ARGs and MGEs per 16 S rRNA gene copy number (prevalence) was also non-significantly different among the inflow of the UWTPs (p > 0.01). The genes bla_{IMP}, bla_{VIM}, bla_{OXA-58}, and bla_{KPC} were significantly more prevalent in hospital effluent (p < 0.01) than in the UWTPs inflow (Fig. 1b).

Culture enterobacteria and enterococci ranged 5.8–6.3 log-units CFU/mL and 4.4–5.0 log-units CFU/mL, respectively, in the inflow of all UWTPs (Fig. S2). Presumably resistant bacteria, cultured on media supplemented with antibiotics, presented counts of 1–2 log-units lower, ranked as ciprofloxacin > cefotaxime > meropenem for enterobacteria and ciprofloxacin > vancomycin for enterococci (Fig. S2). Compared with the UWTPs inflow, hospital effluent presented significantly lower
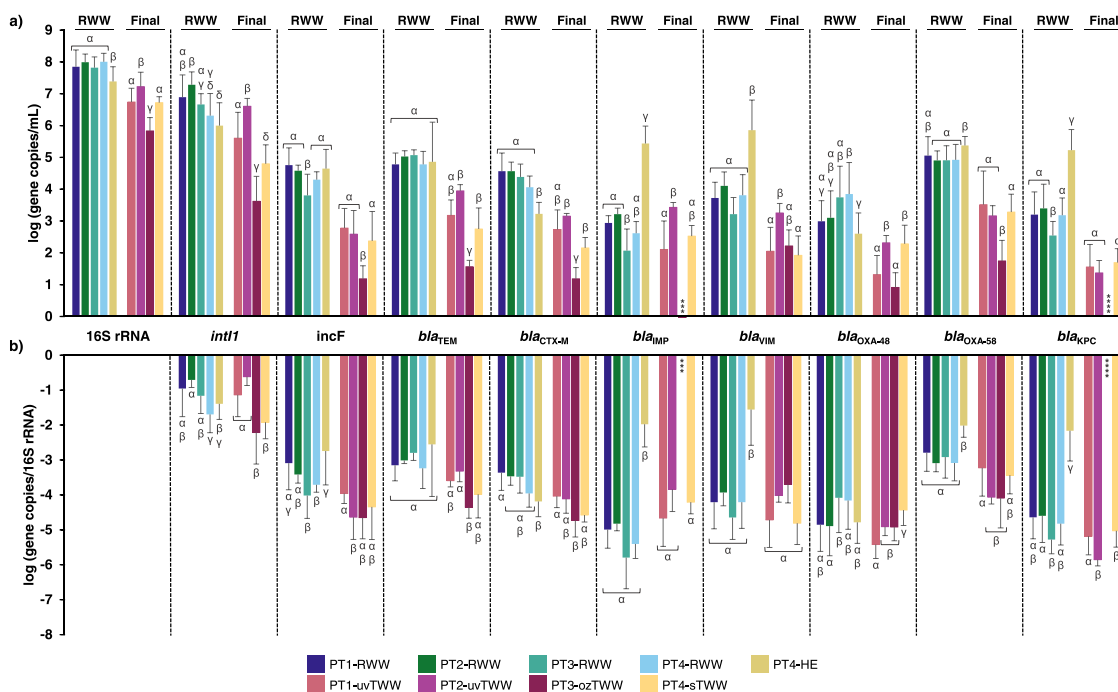
Fig. 1. Genes abundance and prevalence over raw wastewater (PT1-RWW, PT2-RWW, PT3-RWW, PT4-RWW) and final treated wastewater (PT1-uvTWW, PT2-uvTWW, PT3-ozTWW, PT4-sTWW) from four UWTPs (PT1 to PT4), and hospital effluent discharging to PT4 (PT4-HE). **a)** Abundance (log (gene copies/mL of sample)) and **b)** prevalence (log (gene copies/16 S rRNA copy number)) of the analysed genes in four UWTPs. Gene *mecA* was quantified in raw wastewater from PT1, PT2, and in hospital effluent, and in other samples abundance values were below the limit of detection. RWW: raw wastewater; uvTWW: wastewater collected in PT1 and PT2 after UV disinfection wastewater treatment; ozTWW: wastewater collected after ozonation wastewater treatment; sTWW: wastewater collected after secondary wastewater treatment. Data refer to average values of four sampling campaigns, except in uvTWW of PT2 that refer to average values of two sampling campaigns. α, β, γ, and δ indicate significantly (p < 0.01) different Tukey's groups comparing the raw wastewater or final treated wastewater between UWTPs. * ** and * ** * indicate values below the limit of detection obtained in 3 and 4 campaigns from the total of 4 sampling campaigns, respectively.

abundance of culturable enterobacteria, although identical or significantly higher percentage of counts on medium supplemented with ciprofloxacin (~25% in UWTPs vs. 63% in hospital), cefotaxime (~3% in UWTPs vs. 15% in hospital) or meropenem (~6% in UWTPs vs. 26% in hospital) (Fig. S2a). For culturable enterococci, no significant differences were observed between the UWTPs inflow and the hospital effluent, although as for enterobacteria, higher presumable resistance percentage values were observed in the latter for both ciprofloxacin (~5% in UWTPs vs. 37% in hospital) and vancomycin (~5% in UWTPs vs. 10% in hospital) (Fig. S2b).

3.2. Secondary treatment

Secondary treatment promoted reduction values of the 16 S rRNA gene that ranged 1.1–1.3 log-units/mL in PT1, PT3, and PT4 and of 0.7 log-units/mL in PT2 (Fig. 2). The reduction values observed for MGEs ranged 1.3–1.5 log-units/mL in PT1, PT3, and PT4 and 0.9 log-units/mL in PT2, while ARGs registered reduction values of 2.5 log-units/mL in PT3 and considerably lower in PT1, PT2, and PT4 where values ranged 1.3–1.7 log-units/mL (Fig. 2). In general, these reduction values corresponded to statistically significant decreases (p < 0.01), except in a few cases observed for *bla*$_{IMP}$ in PT1, PT2, and PT4 and *bla*$_{OXA-48}$ in PT2. Assuming that MGEs or ARGs and the bacterial biomarker 16 S rRNA gene would be removed during secondary treatment, all at the same rate, it would be expected the same prevalence values (per 16 S rRNA gene abundance) before and after the activated sludge step. However, this was not observed, suggesting that some bacterial populations harbouring the measured genes, or the genes themselves, were removed more extensively than others. Also, the pattern of removal was distinct in different UWTPs. A significant (p < 0.01) decrease of prevalence of the MGE incF and of the ARG *bla*$_{KPC}$ was observed in PT1 and PT2, of the ARG *bla*$_{TEM}$ in PT2 and PT3, of the

ARG *bla*$_{CTX-M}$ in all except in PT4, and of ARG *bla*$_{OXA-58}$ in all except PT1 and PT4. The only situation where ARG prevalence was observed to significantly increase after secondary treatment was for *bla*$_{IMP}$ in PT4 (Fig. S1).

In all UWTPs, secondary treatment contributed to reduce the abundance of culturable bacteria in 1–3 log-units CFU/mL and, in general, did not change resistance prevalence (Fig. S3). The only exception was for enterobacteria presumably resistant to ciprofloxacin that decreased from 25% (inflow) to 15% (secondary effluent).

3.3. Tertiary treatment

Disinfection was available in PT1 and PT2 through UV radiation, and in PT3 through ozonation after sand filtration. Disinfection led to reductions of the 16 S rRNA gene (log (gene copy/mL)) of 0.02 in PT1, 0.36 in PT2, and 0.63 in PT3 (removal of sand filtration + ozonation), being the latter the only one where a significant reduction was observed (after sand filtration) (Fig. S1). Sand filtration was also associated to the significant reduction (log (gene copy/mL)) of the *intI1* gene, while ozonation led to the significant reduction of the ARGs *bla*$_{TEM}$ and *bla*$_{OXA-48}$ (Fig. S1). UV did not cause significant variations in any of the measured genes, except in PT2 where a significant increase in the abundance and prevalence of the MGE incF was observed (Fig. S1).

Disinfection was accompanied by reductions of culturable bacteria in 0.2–2.4 log-units CFU/mL, with slight reductions on the prevalence of some ARB (Fig. S3). Specifically, ciprofloxacin-resistant enterococci reduced from 6% to 3% in PT2 and from 2% to < 0.01% in PT3, while vancomycin-resistant enterococci decreased from 2% to 0.01% in PT2. In general, these results suggested the limited potential of the disinfection processes for removing bacteria, MGEs or ARGs.
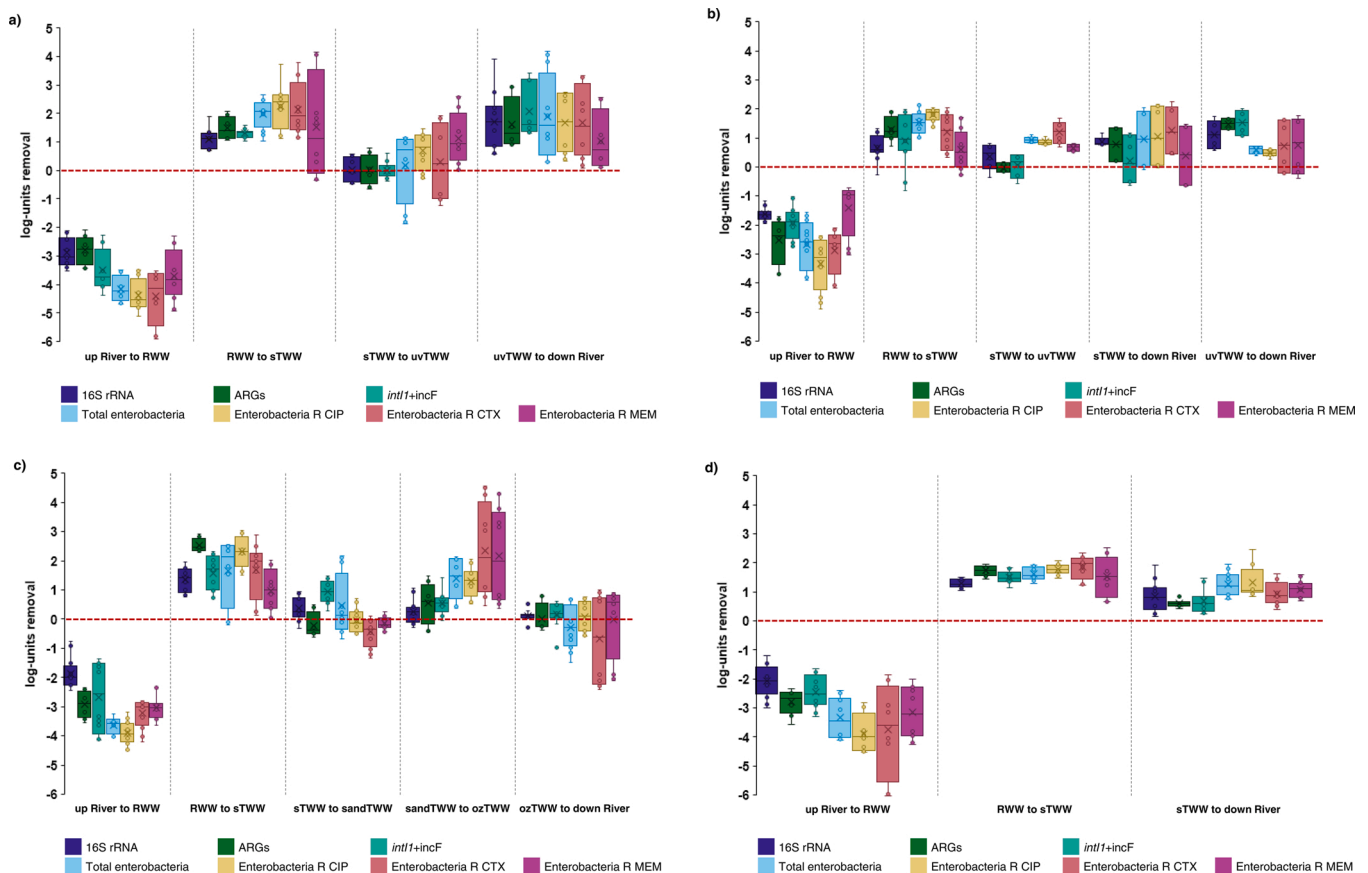
Fig. 2. Log-removal values calculated based on the abundance values of: 16 S rRNA gene, sum of ARGs (bla_{TEM}, bla_{CTX-M}, bla_{IMP}, bla_{VIM}, bla_{OXA-48}, bla_{OXA-58}, bla_{KPC}, and $mecA$), sum of amplicons associated to MGEs ($intI1$ and incF), presumptive total enterobacteria, presumable enterobacteria resistant to ciprofloxacin (enterobacteria R CIP), cefotaxime (enterobacteria R CTX) or meropenem (enterobacteria R MEM) in raw wastewater (RWW), secondary treatment (sTWW), after UV disinfection (uvTWW), after sand filtration (sandTWW), after ozonation (ozTWW), and upstream (up River) and downstream (down River) the receiving river from a) PT1, b) PT2, c) PT3, and d) PT4. Data refer to values of four sampling campaigns, except in uvTWW of PT2 that refer to values of two sampling campaigns.

3.4. Treatment efficiency

The overall treatment efficiency is summarized in Table 2. As expected, the abundance of the majority measured genes (16 S rRNA, $intI1$, incF, bla_{TEM}, bla_{CTX-M}, bla_{VIM}, bla_{OXA-48}, bla_{OXA-58}, bla_{KPC}) and culturable bacteria was reduced after treatment in the four UWTPs. For each plant, removal values were ranked as culturable enterobacteria > ARGs > 16 S rRNA, suggesting that the latter may be the most sensitive analyte to assess treatment efficiency. The UWTPs could be ranked according to their capacity to remove the housekeeping gene 16 S rRNA and ARGs as PT3 > PT4 ~ PT1 > PT2 according to the average reduction values. PT3, equipped with sand filtration and ozonation, demonstrated the highest efficiency, although the most efficient step was the secondary treatment. The reduction of specific genetic determinants is probably a result of the dynamics of the respective bacterial hosts, dictated by the set of conditions imposed during treatment. Indeed, different determinants showed distinct removal rates that was the highest for $intI1$, incF, bla_{TEM}, bla_{CTX-M}, and bla_{OXA-58}, with average reductions ~2 log-units (Table 2). In contrast, other genetic determinants showed much lower removal rates, as was the case of bla_{IMP} (Table 2).

3.5. Impact of treated wastewater discharge from UWTPs

The variations of gene or bacteria abundance between the raw wastewater and its delivery into the river are represented in Fig. 2, where the first column measures the difference between upstream river and sewage and the last one the impact of the UWTP in the downstream river. As showed above, although the load of bacteria and genetic

determinants received in the different UWTPs is not significantly different (Section 3.1.), the fact that treatment efficiency differs among UWTPs (Section 3.4.) would be sufficient to expect distinct impacts on the receiving water body. However, as Fig. 2 shows, the impacts were also determined by the receiving environment. Indeed, PT3, the UWTP with highest removal rates, was the one generating the highest impact in the river as can be concluded based on the limited dilution of the measured biomarkers.

The downstream river samples were not all collected at the same distance of the discharging point (due to technical limitations), being this PT3 < PT1 < PT4 < PT2. However, this analysis suggested that the impacts caused by the different UWTPs, due to dilution effect, could be ranked as PT3 > PT4 > PT2 > PT1, and highlights the importance of the receiving environment, to be considered in parallel with treatment efficiency. UWTPs discharges caused significant increases of the MGE incF downstream PT1 and of ARGs bla_{OXA-48}, bla_{OXA-58}, and bla_{KPC} downstream PT4 (Fig. 3a-d). PT4 is the UWTP that receives the effluents of the largest hospital in the area, which may be a relevant source of ARGs into the receiving river. Curiously, PT3 discharges were accompanied by significant (p < 0.01) decreases of incF, and the same effect was observed for 16 S rRNA gene in PT2. The conductivity and Chemical Oxygen Demand values (Table S1) in the river where PT2 and PT3 discharge suggest high levels of contamination that may explain these observations. Indeed, overall, it is suggested that the river main already contain a considerable level of ARGs contamination that explains an apparent limited impact (Tables S3 and S4).

Table 2
Differences in the abundance of enterobacteria, total or presumably resistant to ciprofloxacin, cefotaxime or meropenem (log (CFU/mL)), and the abundance of genes (log (gene copy/mL)) between raw wastewater and final treated wastewater from PT1-PT4 (log-removal values).

UWTP	Total bacteria 16S RNA	MGEs		ARGs							Average log-removal genes	Culturable enterobacteria				Average log-removal bacteria
		$intI1$	$incF$	bla_{TEM}	bla_{CTX-M}	bla_{IMP}	bla_{VIM}	bla_{OXA-48}	bla_{OXA-58}	bla_{KPC}		Total	Ciprofloxacin	Cefotaxime	Meropenem	
PT1	1.11	1.29	1.98	1.75	1.83	0.83	1.67	1.67	1.54	1.65	1.56	2.15	2.84	2.41	2.63	2.51
PT2	0.82	0.88	2.08	1.12	1.24	+ 0.16[a]	1.22	0.40	1.67	1.43	0.99	1.99	2.22	1.80	0.92	1.73
PT3	1.99	3.28 b	2.62	3.50	3.20b	> 2.58[c]	1.00	2.83b	3.17b	2.05	2.62	3.51 b	3.54	3.58	3.01	3.41
PT4	1.29	1.51	1.92	2.03	1.91	0.09	1.89	1.56	1.64	1.49	1.52	1.62 b	1.78	1.85	1.53	1.69
Average log-removal all UWTPs	1.30	1.74	2.15	2.10	2.04	0.83	1.45	1.62	2.00	1.65		2.32	2.60	2.41	2.02	

MGEs, mobile genetic elements; ARGs, antibiotic resistance genes.
[a] abundance values of bla_{IMP} gene higher in the final treated effluent compared to the raw wastewater from PT2.
[b] indicate significantly (p < 0.01) different Tukey's groups comparing values from differences in abundance of genes or cultivable bacteria between PT3 and the UWTP PT1, PT2, and PT4. The total enterococci and enterococci presumably resistant to ciprofloxacin or vancomycin were not included in these analyses as values after secondary treatment and tertiary treatment were, in general, below the limit of quantification that would underestimate their removal.
[c] abundance values of bla_{IMP} gene below limit of detection in final treated effluent from PT3.

3.6. Multidrug-resistant Enterobacteriaceae

Bacteria harbouring the genes bla_{KPC} or bla_{CTX-M} (n = 33), 10 from PT1, 11 from PT2, 5 from PT3, and 7 from PT4 were characterized (Table 3). Twelve of these isolates were from river, 3 upstream and 9 downstream. In 6 out of 33 isolates, identified as *Klebsiella* spp. (n = 5) or *Enterobacter sichuanensis* (n = 1), all from treated wastewater or river, was detected the gene bla_{KPC-3} (n = 4) or bla_{KPC-2} (n = 2). Twenty-nine isolates yielded the gene bla_{CTX-M}, two of which also harboured the gene bla_{KPC-2}. Samples from which were recovered the isolates harbouring the genes bla_{KPC} or bla_{CTX-M} presented values that ranged 0.5–5.2 log-units/mL of bla_{KPC} and 0.9–4.6 log-units/mL of bla_{CTX-M}, suggesting that qPCR can assess the occurrence of culturable ARB. However, qPCR fails to offer a perspective of dissemination paths as was exemplified by the recovery of two isolates identified as *Klebsiella quasivariicola* that harboured the gene bla_{KPC-2}, one with origin in the secondary effluent of PT1 and the other from the respective downstream river. These results suggest the persistence of these bacteria during treatment, with implications in the consequent environmental contamination.

4. Discussion

Urban wastewater treatment plants are major barriers to attenuate the impacts of human water uses on the environment, with important reduction of carbon, nitrogen, and phosphorus nutrients as well as microbial load (Tchobanoglous et al., 2003). However, the increasing occurrence of contaminants of emerging concern in wastewaters seriously limits the capacity of existing wastewater treatment systems to produce safe effluents, specifically exempt of ARB & ARGs (Krzeminski et al., 2019). Studies conducted worldwide show that UWTPs operating according to recommended guidelines promote the removal of 1–3 log-units of bacterial cells, assessed based on the 16 S rRNA gene, and similar or slightly higher removal values of antibiotic resistance determinants (Pallares-Vega et al., 2019; Pärnänen et al., 2019). These values can be considered low if it is taken into consideration that 4–6 log-units of MGEs and ARGs are discharged into the environment per millilitre of treated wastewater (Krzeminski et al., 2019; Pallares-Vega et al., 2019; Pärnänen et al., 2019). While it is clear from the literature available that the removal of MGEs and ARGs during wastewater treatment is quite variable, numerous studies have explored the factors that may influence such discrepancies (McConnell et al., 2018; Novo et al., 2013; Pallares-Vega et al., 2021). The load of MGEs and ARGs in the raw inflow is one of the factors that may influence the treatment efficiency. This was one of the hypotheses tested in this study, supported by the fact that we were comparing raw inflows with a broad variation of physicochemical parameters and load of industrial effluents (in PT3 and PT4) (Table 1 and Table S1). Contrary to this expectation, the raw wastewater of the four UWTPs did not evidence significant differences on the abundance of MGEs or ARGs (Fig. 1a), suggesting that the antibiotic resistance load is fairly stable in the sewage produced in a given region. These observations are in agreement with previous publications that suggest that sewage samples, even in a limited number, can be representative of the antibiotic resistance profiles of a large urban population and with good correlation with clinical surveillance data (Aarestrup and Woolhouse, 2020; Hutinel et al., 2019; Pärnänen et al., 2019).

In contrast, it has been shown that final effluents that underwent wastewater treatment present distinct profiles of antibiotic resistance and bacterial communities (Fernandes et al., 2019; Lira et al., 2020). Probably this effect is due to the shifts that are imposed to microbial communities during wastewater treatment (Narciso-da-Rocha et al., 2018; Tong et al., 2019). Indeed, the association between microbial community and antibiotic resistance was demonstrated at the phylum level based on the analysis of more than 650 samples of wastewater, human and animal gut, among other, collected worldwide (Li et al.,
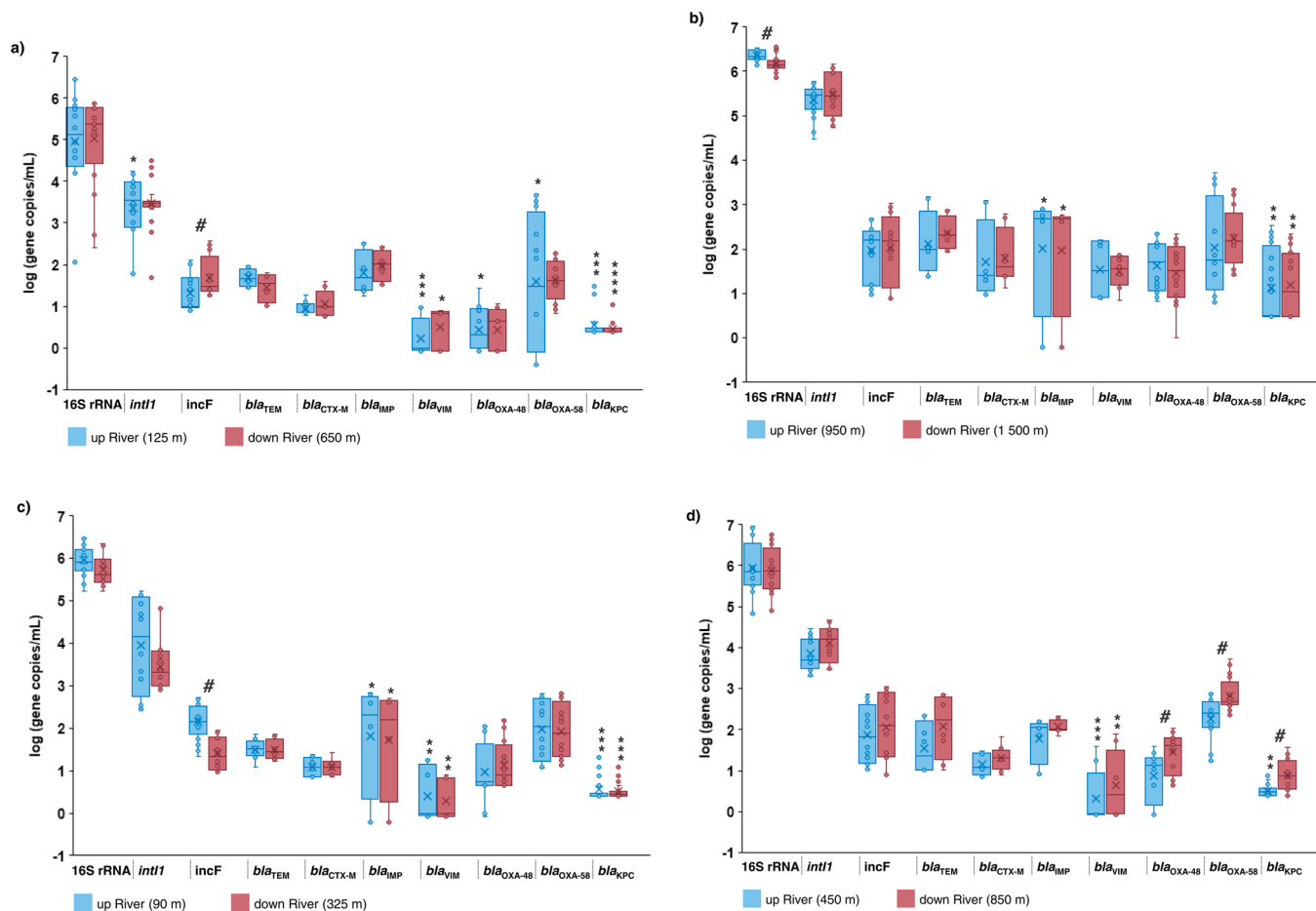
Fig. 3.. Abundance (log (gene copies/mL of sample)) of the genes analysed in river samples collected upstream (up River) and downstream (down River) the discharge points (distances in meters of sampling sites in parenthesis) of each UWTP, **a)** PT1, **b)** PT2, **c)** PT3, and **d)** PT4. To perform data analysis, the value below the limit of detection was assumed as missing values. Significant differences of genes abundance between up River and down River for each UWTP were tested by t-test and indicated with cardinal ($p < 0.01$). *, **, ***, and **** indicate the presence of values below the limit of detection obtained in 1, 2, 3 or 4 campaigns from 4 sampling campaigns, respectively.

2018). Nevertheless, the drivers that impose microbial community rearrangements and antibiotic resistance removal are not very well understood and can hardly be explained by a well-defined set of factors, such as the occurrence of other contaminants, geographic region or organic matter (Fernandes et al., 2019; McConnell et al., 2018; Novo et al., 2013; Tong et al., 2019; Pallares-Vega et al., 2019; Pärnänen et al., 2019). Indeed, the stochasticity of the fate of bacteria and genes during wastewater treatment has been increasingly demonstrated (Lira et al., 2020). While understandable from the ecology point of view, this fact represents a major drawback to design and recommend improved methods to treat wastewater aiming at an almost resistance-free final effluent.

Increasingly, the production of wastewater of high quality is a major ambition to preserve the environment, the human health, and also to encourage a sustainable water reuse. Advanced treatment, with different installation and operation costs, from chemical (e.g., chlorination, ozone) and physical (e.g., UV) disinfection to membrane bioreactors, has been proposed as a complement to produce high quality water (Ng et al., 2019; Rizzo et al., 2020). Despite the high potential of adequate membrane bioreactors to remove antibiotic resistance, these methods still release unwanted contaminants (Ng et al., 2019) and may be not affordable in every world region. Indeed, the most commonly used methods are UV, ozone, and chlorination, all with recognized disinfection capacity (Dodd, 2012) but with limited capacity to extensively remove resistant bacteria in real effluents (Rizzo et al., 2020). The use of low UV fluence as was the case of PT1 and PT2 (30–40 mJ/cm^2) and an

excess of suspended particulate matter that may interfere with radiation absorption by bacteria may be responsible for the reduced effect of this disinfection process (Guo et al., 2013; Narciso-da-Rocha et al., 2018). Pilot system studies have suggested that ozone can be an effective disinfectant of treated wastewater with log-removal values of bacteria and ARGs of 3 and 2, respectively (Alexander et al., 2016; Wei et al., 2020). The present full-scale study conducted in PT3 showed smaller removal values, and sand filtration contributed more for 16 S rRNA and MGEs, and ozone contributed more for ARGs log-removal (0.4, 0.9, and 0.5, respectively) (Fig. 2c). However, it can be expected that milder effects as those observed in full-scale UWTPs, as in the current and previous studies (Narciso-da-Rocha et al., 2018), may avoid regrowth, which is considered a major unintended effect of disinfection (Alexander et al., 2016; Di Cesare et al., 2016). In summary, the scale-up from pilot to full-scale systems, the control of external interfering factors, and minimization of unintended effects are important aspects to consider on the design and implementation of cost-effective solutions to produce treated wastewater with adequate quality.

Improved wastewater treatment is regarded as a major step to attenuate the impact of UWTPs in the receiving environment. These impacts have been demonstrated worldwide, irrespective of the quality of wastewater treatment (Bueno et al., 2018). Also, in this study was possible to demonstrate such impacts. The presence of the MGE *intI1* and of the ARGs *bla*$_{TEM}$ and *bla*$_{CTX-M}$ in the examined river basin, even upstream the UWTPs, was not surprising given the widespread distribution of these genes, previously reported in aquatic environments (Amos et al.,

Table 3

Resistance phenotype and genotype characteristics of non-repetitive enterobacteria isolates obtained in different urban wastewater treatment plants (UWTPs) over the first two sampling campaigns and respective abundance of bla_{KPC} and bla_{CTX-M} in each sample determined with qPCR.

UWTP	Sample type	Gene abundance (log (no. copies/mL))* bla_{KPC}	bla_{CTX-M}	Identification	Resistance genes bla_{KPC}	bla_{CTX-M}	AML	TIC	CP	CAZ	MEM	CT	CN	STR	TET	CIP	SUL	SXT
PT1	RWW	3.2 ± 0.7	4.6 ± 0.6	*Shigella flexneri*	–	+	R	R	R	I	S	S	R	R	S	S	R	R
PT1	RWW	3.2 ± 0.7	4.6 ± 0.6	*Klebsiella pneumoniae* subsp. *pneumoniae*	–	$bla_{CTX-M-15}$	R	R	R	R	S	S	S	R	S	R	R	R
PT1	sTWW	1.5 ± 0.6	2.7 ± 0.3	*Klebsiella quasivariicola*	bla_{KPC-2}	$bla_{CTX-M-15}$	R	R	R	R	R	S	R	S	R	R	R	I
PT1	uvTWW	1.6 ± 0.7	2.7 ± 0.6	*Klebsiella variicola* subsp. *variicola*	–	$bla_{CTX-M-15}$	R	R	R	S	S	S	R	R	R	I	R	R
PT1	Down River	0.5 ± 0.1[a]	1.1 ± 0.3	*Klebsiella quasivariicola*	bla_{KPC-2}	$bla_{CTX-M-15}$	R	R	R	R	R	S	R	R	S	R	R	I
PT1	Up River	0.5 ± 0.3[a]	0.9 ± 0.2	*Klebsiella quasivariicola*	–	$bla_{CTX-M-15}$	R	R	R	S	S	R	R	S	I	R	R	R
PT1	RWW	3.2 ± 0.7	4.6 ± 0.6	*Klebsiella pneumoniae* subsp. *ozaenae*	–	$bla_{CTX-M-15}$	R	R	R	R	S	S	R	I	R	R	R	R
PT1	sTWW	1.5 ± 0.6	2.7 ± 0.3	*Klebsiella pneumoniae* subsp. *pneumoniae*	–	$bla_{CTX-M-15}$	R	R	R	S	S	S	R	I	S	R	R	R
PT1	sTWW	1.5 ± 0.6	2.7 ± 0.3	*Escherichia coli*	–	+	R	R	R	S	S	S	S	I	S	R	S	S
PT1	Down River	0.5 ± 0.1[a]	1.1 ± 0.3	*Klebsiella variicola* subsp. *variicola*	–	$bla_{CTX-M-15}$	R	R	R	S	S	S	R	R	S	R	R	R
PT2	RWW	3.4 ± 0.8	4.6 ± 0.3	*Klebsiella pneumoniae* subsp. *ozaenae*	–	$bla_{CTX-M-15}$	R	R	R	S	S	R	I	S	R	R	R	
PT2	sTWW	1.8 ± 1.0[a]	2.9 ± 0.5	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*	–	$bla_{CTX-M-15}$	R	R	R	R	S	S	R	R	S	I	R	R
PT2	sTWW	1.8 ± 1.0[a]	2.9 ± 0.5	Not identified	–	+	R	R	R	R	S	S	R	R	R	R	R	R
PT2	uvTWW	1.4 ± 0.4	3.1 ± 0.1	*Escherichia coli*	–	+	R	R	R	R	S	S	S	I	R	R	R	R
PT2	Down River	1.9 ± 0.2[a]	1.8 ± 0.6	*Escherichia coli*	–	+	R	R	R	R	S	S	S	S	R	S	R	R
PT2	Down River	1.9 ± 0.2[a]	1.8 ± 0.6	*Escherichia coli*	–	+	R	R	R	R	S	S	R	R	R	R	R	R
PT2	RWW	3.4 ± 0.8	4.6 ± 0.3	*Klebsiella pneumoniae* subsp. *ozaenae*	–	$bla_{CTX-M-15}$	R	R	R	R	S	S	R	R	S	I	R	R
PT2	sTWW	1.8 ± 1.0[a]	2.9 ± 0.5	*Escherichia marmotae*	–	+	R	R	R	I	S	S	S	R	R	R	R	R
PT2	uvTWW	1.4 ± 0.4	3.1 ± 0.1	*Shigella flexneri*	–	+	R	R	R	R	S	S	S	R	R	I	R	R
PT2	uvTWW	1.4 ± 0.4	3.1 ± 0.1	Not identified	–	$bla_{CTX-M-15}$	R	R	R	I	S	S	R	I	S	R	R	R
PT2	Down River	1.9 ± 0.2[a]	1.8 ± 0.6	*Escherichia coli*	–	+	R	R	R	S	S	S	R	R	R	R	R	R
PT3	sandTWW	0.9 ± 0.6[a]	1.7 ± 0.6	*Klebsiella pneumoniae* subsp. *ozaenae*	bla_{KPC-3}	–	R	R	R	R	R	S	S	I	S	R	R	I
PT3	sandTWW	0.9 ± 0.6[a]	1.7 ± 0.6	*Klebsiella variicola* subsp. *variicola*	–	$bla_{CTX-M-15}$	R	R	R	R	S	S	R	R	S	I	R	R
PT3	RWW	2.5 ± 0.4	4.4 ± 0.4	*Klebsiella pneumoniae* subsp. *ozaenae*	–	$bla_{CTX-M-15}$	R	R	R	I	S	S	R	R	R	R	R	R
PT3	RWW	2.5 ± 0.4	4.4 ± 0.4	*Escherichia coli*	–	+	R	R	R	R	S	S	R	R	R	R	R	R
PT3	Up River	0.6 ± 0.3[a]	1.1 ± 0.2	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*	–	$bla_{CTX-M-15}$	R	R	R	I	S	S	R	R	R	I	R	R
PT4	Hospital effluent	5.2 ± 0.6	3.2 ± 0.4	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*	–	$bla_{CTX-M-15}$	R	R	R	R	I	S	R	R	S	R	R	R
PT4	Down River	0.9 ± 0.4	1.3 ± 0.3	*Klebsiella variicola*	bla_{KPC-3}	–	R	R	R	R	R	S	R	R	S	I	R	R
PT4	Down River	0.9 ± 0.4	1.3 ± 0.3	*Escherichia coli*	–	+	R	R	R	R	S	S	S	I	R	S	S	S
PT4	Down River	0.9 ± 0.4	1.3 ± 0.3	*Escherichia coli*	–	+	R	R	R	R	S	S	S	I	R	S	S	S
PT4	sTWW	1.7 ± 0.4	2.1 ± 0.3	*Enterobacter sichuanensis*	bla_{KPC-3}	–	R	R	R	R	R	S	S	R	R	R	R	R
PT4	Up River sediment	n.d.	n.d.	*Klebsiella pneumoniae* subsp. *ozaenae*	bla_{KPC-3}	–	R	R	R	R	R	S	S	S	I	R	S	S

The antibiotics tested according to the standard disc diffusion method were: amoxicillin (AML, 25 μg), ticarcillin (TIC, 75 μg), cephalothin (CP, 30 μg), ceftazidime (CAZ, 30 μg), meropenem (MEM, 10 μg), colistin (CT, 50 μg), gentamicin (CN, 10 μg), streptomycin (STR, 10 μg), tetracycline (TET, 30 μg), ciprofloxacin (CIP, 5 μg), sulfamethoxazole (SUL, 25 μg), and sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 μg). Genes blaOXA-48 and blaOXA-58 were not detected by PCR-based method. * , Data correspond to the average abundance values of blaKPC and blaCTX-M genes of four sampling campaigns. a, Presence of values below the limit of detection in two to four campaigns from four sampling campaigns. n.d. - not done. RWW, raw wastewater; sTWW, wastewater collected after secondary wastewater treatment; uvTWW, wastewater collected in PT1 and PT2 after UV disinfection wastewater treatment; sandTWW, wastewater collected after sand filtration; ozTWW, wastewater collected after ozonation wastewater treatment; Hospital effluent, hospital effluent discharging to PT4; river samples collected upstream (up River) and downstream (down River) the discharge point of each UWTP.

2014, 2020). ARGs such as bla_{OXA-48}, bla_{OXA-58}, and bla_{KPC} associated with carbapenem resistance and of recognized clinical relevance are considered emerging threats. These genes are increasingly spread in aquatic environments and may be enriched during wastewater treatment (Bengtsson-Palme et al., 2016; Teixeira et al., 2022). These previous studies confirm the current results that show that those genes were already present in the natural environment and were sometimes increased downstream the UWTPs discharge. An increase of antibiotic resistance load in rivers downstream UWTPs has been consistently shown worldwide (Cacace et al., 2019; Quintela-Baluja et al., 2019; Raza et al., 2021; Thornton et al., 2020). However, such impacts must be interpreted as a result of a compromise between the quality of the effluent and the robustness of the receiving water body, and efforts should not be placed solely on the treatment side. Even high removal rates of ARGs, as those that can be obtained with microfiltration systems (up to 7 log-units/volume in the whole train), do not avoid the release of up to ~3 log-units/mL of ARGs in the final effluent (Le et al., 2018). The need for this compromise was illustrated in this study, with the UWTP with the highest average removal rate (PT3) being the one discharging an effluent that in average presented an antibiotic resistance load identical to the receiving water body. Critical factors that determine the impacts of UWTPs discharges into rivers are the dilution potential and the capacity of the receiving environment to attenuate or enhance the effects of exogenous microbiota. The antibiotic resistance discharged by UWTPs can be outcompeted by the autochthonous microbiota or nurtured by other nonpoint sources of pollution, as those emitted by agriculture or by other activity sectors (Bueno et al., 2018; Ribeirinho-Soares et al., 2022; Storteboom et al., 2010). This compromise between emissions intensity and receptor attenuation capacity may be the key to control antibiotic resistance emitted by UWTPs.

5. Conclusions

The abundance of 16 S rRNA gene, MGEs, and ARGs was not significantly different in the inflow of the four UWTPs, in spite the fact that they had different inputs of industrial effluents and physicochemical parameters.

Secondary treatment, based on activated sludge, contributed to the most extensive removal of ARGs, up to 2.5 log-units/mL. However, distinct genes behaved differently in different UWTPs, suggesting the importance of the complex interplay of multiple, and mostly unknown, factors that determine the success of a treatment process.

The reception of hospital sewage by PT4, where represented < 1% of the inflow, may not have an impact on the abundance of resistance genes in the inflow or effluent. However, it may represent an important path for the introduction of emerging resistance genes in the environment and increased impacts on the receiving environment.

The impacts of the UWTPs should not be assessed simply based on the final effluent quality, as they are strongly influenced by the dilution and potential synergic pollution sources occurring in the receiving environment.

Culture-based methods, although laborious and sometimes hampered by uncultivability, are still powerful tools to track antibiotic resistance across distinct environmental compartments.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128933.

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