Antibiotic resistance in wastewater: Occurrence and fate of *Enterobacteriaceae* producers of Class A and Class C β-lactamases

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Antibiotics have been intensively used over the last decades in human and animal therapy and livestock, resulting in serious environmental and public health problems, namely due to the antibiotic residues concentration in wastewaters and to the development of antibiotic-resistant bacteria. This study aimed to access the contribution of some anthropological activities, namely urban household, hospital and a wastewater treatment plant, to the spread of antibiotic resistances in the treated wastewater released into the Mondego River, Coimbra, Portugal. Six sampling sites were selected in the wastewater network and in the river. The ampicillin-resistant *Enterobacteriaceae* of the water samples were enumerated, isolated and phenotypically characterized in relation to their resistance profile to 13 antibiotics. Some isolates were identified into species level and investigated for the presence of class A and class C β-lactamases. Results revealed high frequency of resistance to the β-lactam group, cefoxitin (53.5%), amoxicillin/clavulanic acid combination (43.5%), cefotaxime (22.7%), aztreonam (21.3) cefpirome (19.2%), ceftazidime (16.2%) and to the non-β-lactam group, trimethoprim/sulfamethoxazol (21.1%), tetracycline (18.2%), followed by ciprofloxacin (14.1%). The hospital effluent showed the higher rates of resistance to all antibiotic, except two (chloramphenicol and gentamicin). Similarly, higher resistance rates were detected in the wastewater treatment plant (WWTP) effluent compared with the untreated affluent. Regarding the multidrug resistance, the highest incidence was recorded in the hospital sewage and the lowest in the urban waste. The majority of the isolates altogether are potentially extended-spectrum β-lactamases positive ($ESBL^+$) (51.9%), followed by $AmpC^+$ (44.4%) and $ESBL^+/AmpC^+$ (35.2%). The most prevalent genes among the potential ESBL producers were bla_{OXA} (33.3%), bla_{TEM} (24.1%) and $bla_{CTX\text{-}M}$ (5.6%) and among the AmpC producers were bla_{EBC} (38.9%), bla_{FOX} (1.9%) and bla_{CIT} (1.9%). In conclusion, the hospital and the WWTP activities revealed to have the highest contribution to the spread of multidrug resistant bacteria in the study area. Such data is important for future management of the environmental and public health risk of these contaminants. This is the first embracing study in the water network of Coimbra region on the dissemination of antibiotic resistance determinants. Moreover, it is also the first report with the simultaneous detection of multiresistant bacteria producers of AmpC and ESBLs β-lactamases in aquatic systems in Portugal.

Keywords: AmpC, antibiotic-resistant bacteria, antibiotic-resistant genes, *Enterobacteriaceae*, environmental contaminants, ESBL, hospital wastewater, multidrug resistance, public health, WWTP.

Introduction

The antibiotic (AB) era with about 70 years is considered as a relatively recent event in evolutionary terms. It is characterized by an exponential increase of antimicrobial-resistant bacteria as a result of a large-scale AB use. Up to date, the drugs with most successful application belong to the AB group, used in the treatment and prevention of bacterial infections of humans, animals and plants. Additionally, despite the use of AB as growth promoters in intensive livestock farming is not allowed in Europe, these can still be used as feed additives in the aquaculture industry and in the poultry production.[1–3]

Antibiotics are daily excreted into the environment through urine and feces, as a mixture of unchanged xenobiotic compounds and bioactive forms partially

metabolized by humans and animals. This release, together with antibiotic-resistant bacteria (ARB), can occur by several routes, the major of which are the network of municipal sewers and the soil farm fertilization with manure or sewage sludge. Besides the chemical pollution by AB themselves, their long-term permanence in most water systems, pressures selection at sub-inhibitory concentrations upon microorganisms, resulting in the development of ARB and antibiotic resistance genes (ARG).[1,3–5] Consequently, the wastewater from multiple human activities, which contains high levels of organic and inorganic matter and also high concentrations of microorganisms, including pathogenic, commensal and environmental bacteria convert the sewage into an ecological niche especially adapted to the growth and spread of antibiotic resistances.[6]

The wastewater treatment plant (WWTP) is therefore the meeting point of most of these resistance determinants, particularly in those with activated sludge or percolator biological filters for the biological treatment, acting as an important reservoir of *Enterobacteriaceae* carrying resistance genes potentially transferable. At these locations, where bacterial density is high, there is a selective pressure for resistance and increased availability of nutrients; accordingly, the optimal conditions are gathered for the horizontal ARG transfer between the newly arrived bacterial population and the resident, thus favoring the increase of bacterial resistance.[7-11] Some recent studies on the evaluation of the role played by the biological WWTP on the levels of resistance determinants showed higher percentages of multiresistant bacteria in the effluent than in the affluent of treated wastewater.[5,7–10]

The extended-spectrum β-lactamases (ESBL) are enzymes that hydrolyze the AB of the *β*-lactams class, such as penicillins, cephalosporins of the first, second and third generation and aztreonam. Among the AB group, this class is the most representative for human consumption in most of the countries.[5,8] Nowadays, the increasing prevalence of bacteria carrying ESBLs represent a worldwide major clinical problem, which may cause a variety of infections and increase the risk of therapy failure.[12–14]

Although many species of Gram negative bacteria could host ESBLs, they are mainly *Enterobacteriaceae*.[14] More than 200 different genes encoding for ESBLs are identified, and the TEM and SHV families were the most prevalent worldwide until the mid-1990s. Over the last 15 years, these groups have been replaced by the CTX-M genes family. There are however other β-lactamases that have ESBL phenotypes, such as some members of the OXA family.[12,15,16] The recent emergence of Gram-negative bacteria harboring other type of β-lactamases known AmpC mediated by plasmids raises new problems.

Originally these enzymes were described as chromosomal and inducible in *Enterobacteriaceae*, however currently plasmidic genes are described in seven families: bla_{ACC}, bla_{CMI}, bla_{DHA}, bla_{FOX}, bla_{ACT}, bla_{MOX} and bla_{EBC}.[17] The distinction between AmpC and ESBLs lies on the capacity of AmpC, unlike ESBL, to hydrolyze cefoxitin and cefotetan and not letting themselves to be inhibited by β-lactamases inhibitors. The AmpC plasmidic genes, which generally co-inhabit mobile genetic elements together with ESBL and other resistance genes from other classes of AB, are horizontally transferred to sensitive receptor bacteria. Infections caused by co-producing ESBL/AmpC bacteria are increasing all over the world.[18] Consequently, all the multidrug resistance phenotypes impose further limitations on the therapeutic options currently representing a major public health issue.[14,19–23]

The environmental problem here exposed is exacerbated on one hand by the increasing need of food production to meet the world population growth and on the other hand by water scarcity for agriculture. Therefore, the treated wastewater irrigation has benefits for all countries, but mainly in arid and semiarid regions.[24] The wastewater treatment, aiming the removal of those contaminants from the effluent and consequently the mitigation of potential negative impacts on water resources, is therefore a crucial point for environmental management. The choice of the type of treatment to be used will depend on the nature of the wastewater and their final use. However, although there has been improvements in wastewater treatment technologies, there are many problems that still remain to be solved, particularly safety issues.[3,5,25,26] The search for local solutions to avoid the environmental dissemination of these pollutants requires the knowledge of the specific residues and the resistant determinants present in that wastewater.

The general objective of this study is the assessment of the pattern of AB resistance contribution of some anthropological activities: household, hospital and Wastewater Treatment Plant (WWTP) to the treated wastewater quality released into the Mondego River in the Coimbra metropolitan area, Portugal.

Materials and methods

Study area

The Choupal WWTP (40°13'29"N and 8°27'3"W) in Coimbra is the largest WWTP exclusively with a conventional treatment system by trickling filter in Portugal. It is responsible for the sewage treatment of the University Hospital of Coimbra (HUC), of various domestic urban areas and some industrial wastewater effluents, representing about 220,000 inhabitants-equivalents. This infrastructure performs a biological treatment and it is dimensioned to treat an average daily flow rate of about 40,000 m^3, in a sequence of organs comprising three phases of treatment.

The liquid phase includes the following organs: preliminary wastewater treatment (three rows of thick and thin disking, two lines of grit removal and degreasing, two

primary clarifiers, four trickling filters and two secondary clarifiers. The solid phase is composed by a gravitic thickener, two heated anaerobic digesters, a centrifugal, seven drying beds and a sludge park. The gas phase (biogas production), has two mobile summit gasometers, a heating sludge system and a turbine powered by biogas for electricity production. The treated effluent is daily unloaded into the Lower Mondego River that runs for about 40 kilometers, an extremely fertile alluvial plain with the most productive rice fields of Europe.[27,28]

Sample collection

The six sampling sites selected for this study (Fig. 1) include the affluent (AWWTP) and effluent (EWWTP) of the Coupal WWTP; the wastewater collector boxes of the city urban area (CWW) and the hospital (HWW); and the surface water of the Mondego River, upstream (URW) and downstream (DRW) the WWTP treated effluent discharge. Sampling was carried out between April and July, in three different periods. All samples were collected in 1L sterile plastic bottles and maintained at 4°C until the microbiological processing, which was always carried out within 4 h after sampling. Water river samples were collected at a depth of 0.5 m and a distance of 2 m from the river bank.

Physicochemical parameters

The physicochemical parameters of river and wastewater samples included the temperature (°C), determined by thermometry; pH, determined by potentiometry; water turbidity, measured in nephelometric turbidity units (NTU); Water Flow (m^3/h); Total Suspended Solids (TSS), measured by Gravimetry; Biochemical Oxygen Demand (BOD) (mg/L) determined by a manometric method and the Chemical Oxygen Demand (COD_5), determined by potentiometric titration and expressed as the dissolved O_2 before and after 5-day incubation at 20°C in the dark (mg/L). These analyses were carried out in the laboratory of Águas do Mondego.

Enumeration, detection and identification of bacteria

The microbiological analysis of the water samples began with the determination of its microbial load. In order to obtain the number of colonies per filtration recommended for counting (20-80), decimal dilutions were prepared in sterile saline 0.9% NaCl. Then 100 mL of each dilution were filtered through cellulose membranes of 0.45 μm in pore size (Millipore, Bedford, MA, USA) under a vacuum system and the filters aseptically placed on the surface of selective medium for *Enterobacteriaceae* VRBG (Violet Red Bile Glucose) agar (Oxoid, Hampshire, England) supplemented with 20 μg/mL ampicillin (AppliChem, Darmstadt, Germany). After aerobic incubation of the plates for 24 h at 37°C, all the *Enterobacteriaceae* isolates resistant to ampicillin (AMP^r) were counted. The colonies with different morphological types were selected, pricked out three times and its purity microscopically confirmed. The species identification of some isolates was performed by biochemical tests using the standard API 20E galleries (BioMerieux, Marcy-l'Etoile, Lyon, France) according to the manufacturer recommendations.

Antimicrobial susceptibility testing

The isolates were tested for the antimicrobial susceptibility using the disk diffusion Kirby-Bauer method on Mueller-

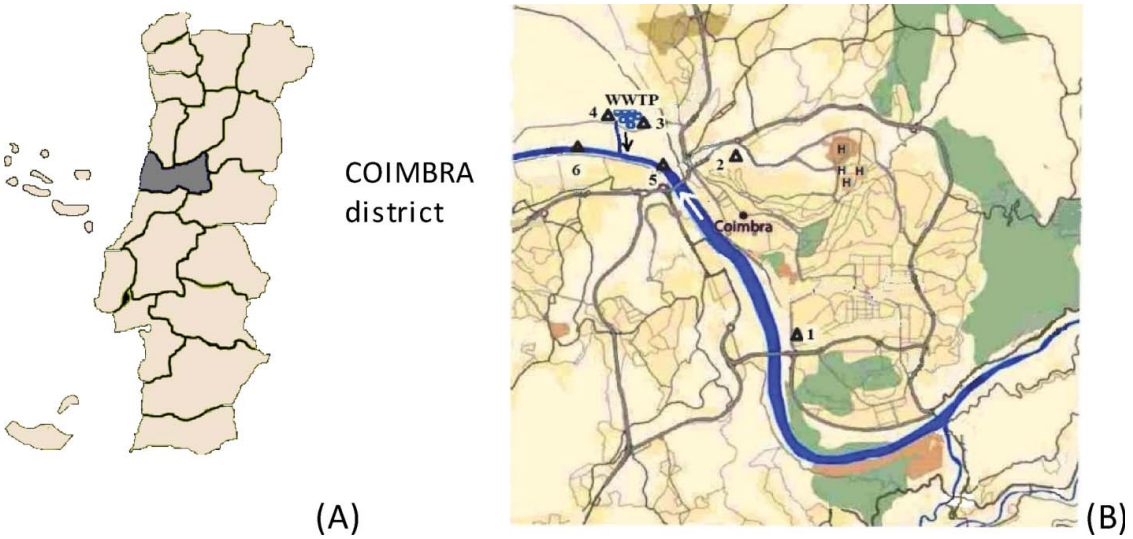

Fig. 1. Location of sampling sites in: (A) the Portugal map (approximate scale 1:11,000,000) and (B) the urban area of Coimbra city (approximate scale 1:170,000). 1- City Wastewater (CWW); 2- Hospital Wastewater (HWW); 3- Affluent of the WWTP (AWWTP); 4- Effluent of the WWTP (EWWTP); 5- Mondego River, 500m upstream the WWTP discharge (URW); 6- Mondego River, 500 m downstream the WWTP discharge (DRW).

Hinton agar (Oxoid, Hampshire, England) with AB disks (Oxoid, Hampshire, England), in agreement to the Clinical Laboratory Standards Institute tests.[29] According to the main AB classes used in Portugal in particular, β-lactams, quinolones, chloramphenicol, sulphonamides, tetracyclines, aminoglycosides, carbapenems, the following ABs were selected: amoxicillin/clavulanic acid combination (AMC) 30 μg/10 μg, respectively; ceftazidime (CAZ) 30 μg; cefotaxime (CTX) 30 μg; cefpirome (CPO) 30 μg; aztreonam (ATM) 30 μg; cefoxitin (FOX) 30 μg; imipenem (IPM) 10 μg; meropenem (MEM) 10 μg; chloramphenicol (CHL) 30 μg; tetracycline (TET) 30 μg; gentamicin (GEN) 10 μg; trimethoprim/sulfamethoxazol (SXT) combination (1:19) and ciprofloxacin (CIP) 5 μg. The isolate *Escherichia coli* ATCC25922 (Liofilchem S.R. L., Roseto degli Abruzzi, Italy), *E. coli* J53 (Az^R) and *E. coli* HB101 (Stp^R) were used as quality controls in the minimal inhibitory concentration (MICs) assays.

Determination of MAR index

The Multiple Antibiotic Resistance (MAR) index per sampling sites was calculated according to Krumperman[30] to compare the contribution of each human activity upon the environmental and public health risk. This calculation considered 13 of the tested AB, as ampicillin was excluded since the medium to select the isolates was AMP supplemented. The sites with an index equal or below 0.2 are considered at low risk of contamination, where AB are rarely or never used, while sites with index above 0.2 are considered at high risk of contamination, due to the constant and high AB exposure.

Detection of ESBL and AmpC genes

The presence of the most frequent *Enterobacteriaceae bla* genes in the isolates under study was determined by different multiplex PCR. These included the bla_{TEM}, bla_{SHV}, bla_{OXA} and $bla_{CTX\text{-}M}$ gene group encoding various ESBLs and the variants of bla_{ACC}, bla_{FOX}, bla_{MOX}, bla_{DHA}, bla_{CIT} and bla_{EBC} genes coding for the family of β-lactamase AmpC. Some isolates were selected, according to their phenotypic resistance profile, for total DNA extraction and used as a template in the PCR amplifications. PCR reactions were performed in a thermocycler (iCycler, Biorad, Thermal Cycler, Hercules, CA, USA) with primers and under conditions described by Dallenne et al.[17] All PCR reactions were performed in 25 μL volume containing DNA (1 μL), 1x PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), deoxynucleoside triphosfate (200 mM), $MgCl_2$ (1.5 mM), *Taq* DNA polymerase (0.5U, Invitrogen) and a variable concentration of group specific primers (0.4 pmol/μL for ESBL PCR and 0.2 pmol/μL for AmpC PCR). The cycling conditions included 10 min of denaturation at 94°C; 30 cycles of 94°C for 40s, 60°C for 40s and 72°C for 1min; followed by a final extension at 72°C for 7 min. Strains used as positive controls in PCR amplifications were: *E. coli* HY0401091 (for $bla_{CTX\text{-}M\text{-}2}$), *E. coli* UB0402407 (for $bla_{CTX\text{-}M\text{-}9}$ and bla_{TEM}), *K. pneumoniae* HY0301692 (for $bla_{CTX\text{-}M\text{-}1}$, bla_{TEM} and bla_{SHV}), *K. pneumoniae* AA0404346 and *K. pneumoniae* MR0500681 (for bla_{SHV}). Negative controls without DNA were always included. The amplification products were separated by electrophoresis on a 1% agarose gel (BioRad) stained with ethidium bromide (125 μg/mL) and visualized under a UV transillumination (Vilber Lourmat, France).

Results and discussion

Physicochemical and microbiological parameters

The physicochemical parameters of the river and wastewater samples are shown in Table 1. The average temperature observed in the samples ranged between 16–17°C. Based on these data, the physicochemical parameters of the river water quality was described as satisfactory and is consistent with the results advised by Marecos do Monte and Albuquerque.[31]

The quantification of the Amp^R *Enterobacteriaceae* in wastewater samples collected at the WWTP entrance and departure, showed a decrease to about half (7.7%, 2.0×10^6 cfu/mL) (Table 1). These results are in agreement with those reported by Marecos do Monte and Albuquerque[31]: a decrease of total coliforms in untreated water of 10^7 to 10^9 and a reduction in treated water of 10 to 100. Similar results had been found by Korzeniewska et al.[5]: a 2 log reduction of the mean values of *E. coli*. In this study, the highest values were recorded in the urban and hospital wastewaters, $3.6 \times 10^6 \pm 1.64$ cfu mL^{-1} and $5.8 \times 10^6 \pm 0.53$ cfu mL^{-1}, which is not surprising since these are highly populated areas. In contrast, in the river samples there were a 44.5-times increase (9375%, 4.5×10^4 cfu mL^{-1}) between samples collected upstream and downstream WWTP effluent discharging point. Similar results were obtained by Li et al.,[32] who found a 1 log increase of the total number of cells between river sampling sites upstream and downstream of the WWTP discharge point.

Antibiotic resistance profile

A total of 383 Amp^R isolates of the *Enterobacteriaceae* family were obtained from the six sampling sites: 72 (AWWTP), 67 (EWWTP), 84 (CWW), 81 (HWW), 40 (URW) and 39 (DRW). A comprehensive analysis of results reveals that these isolates have a particularly high frequency of resistance to the β-lactam group, FOX (53.5%), AMC (43.5%), CTX (22.7%), ATM (21.3) CPO (19.2%), CAZ (16.2%) and to the non-β-lactam group,

Table 1. Physicochemical and microbiological parameters of wastewater and river water of six sampling sites, collected in April, May and July 2012.

Sampling Sites	*AWWTP*	*EWWTP*	*CWW*	*HWW*	*URW*	*DRW*
Temperature (°C)[§]	16.7 ± 1.5	16.7 ± 0.6	15.7 ± 0.6	16.3 ± 1.2	15.7 ± 0.6	17.3 ± 1.2
pH[§]	7.6 ± 0.1	7.6 ± 0.1	ND	ND	ND	ND
Turbidity (NTU)[§]	ND	ND	260.3 ± 12.7	186.3 ± 17.2	1.0 ± 0.0	1.0 ± 0.0
TSS (mg/L)[§]	274.3 ± 30.1	33.7 ± 0.6	ND	ND	ND	ND
BOD_5 (mg/L O_2)[§]	381.7 ± 9.5	31.3 ± 0.6	ND	ND	< 2.0	ND
COD (mg/L O_2)[§]	758.7 ± 23.4	120.3 ± 2.5	ND	ND	< 30.0	ND
Water Flow (m^3/h)[5]	ND	1696.0−982.0	ND	ND	*177000 ± 52300	*177000 ± 52300
Amp^R *Enterob.* (cfu/mL)[5]	0.7×10^6−4.3×10^6	1.3×10^5−2.4×10^5	1.1×10^6−4.9×10^6	5.2×10^6−6.6×10^6	2.5×10^2−7.0×10^2	3.7×10^4−5.3×10^4
Amp^R *Enterob.* (cfu/mL)[§]	2.2×10^6 ± 1.40	1.7×10^5 ± 0.47	3.6×10^6 ± 1.64	5.8×10^6 ± 0.53	4.8×10^2 ± 2.13	4.5×10^4 ± 0.56

Sampling Sites: AWWTP, Affluent of the WWTP; EWWT, Effluent of the WWTP; CWW, City Wastewater; HWW, Hospitals Wastewater; URW, Mondego River 500 m upstream the WWTP discharge; DRW, Mondego River 500 m downstream the WWTP discharge. [§]average ± standard deviation; [5]range values; cfu, colony forming unit; TSS, Total Suspended Solids; BOD_5, Biochemical Oxygen Demand after 5-day incubation at 20°C; COD, Chemical Oxygen Demand; *Sistema Nacional de Informação de Recursos Hídricos (http://snirh.apambiente.pt/).

SXT (21.1%), TET (18.2%), followed by CIP (14.1%) (data not shown). The high β-lactams resistance might be explained by its intensive use in Portugal, as according to the INFARMED[33] report, the β-lactams were still the AB group mostly sold, reaching total of 33.3% of all AB packages sold in the country.

Within the non-β-lactam group, fluoroquinolone (CIP) and sulfonamide (SXT) had a significant number of sales. The former, was the AB with the highest consumption increase over the last decade, being Portugal the 6th-largest consumer of fluoroquinolones in Europe, and currently accounting for about 9% of the total ambulatory AB consumption.[33-35] In addition, the intensive use of TET in veterinary practice justifies the high spread of resistance to this AB in water resources.[36,37]

Comparing the AB resistance profiles from the different sampling sites, the hospital effluent showed the higher rates of resistance to all AB, having only two (CHL and GEN) a resistance rate lower than 15% (Fig. 2A). Similarly, Korzeniewska et al.[5] reported higher rate of AB-resistant *E. coli* in the wastewater of hospital effluent in relation to the urban area. As expected, these data indicate that the clinical activity is a major route of environmental spread of AB-resistant *Enterobacteriaceae*, once large doses of such drugs are daily used on patients, thereby increasing the selective pressure and thus the emergence of resistant bacteria.

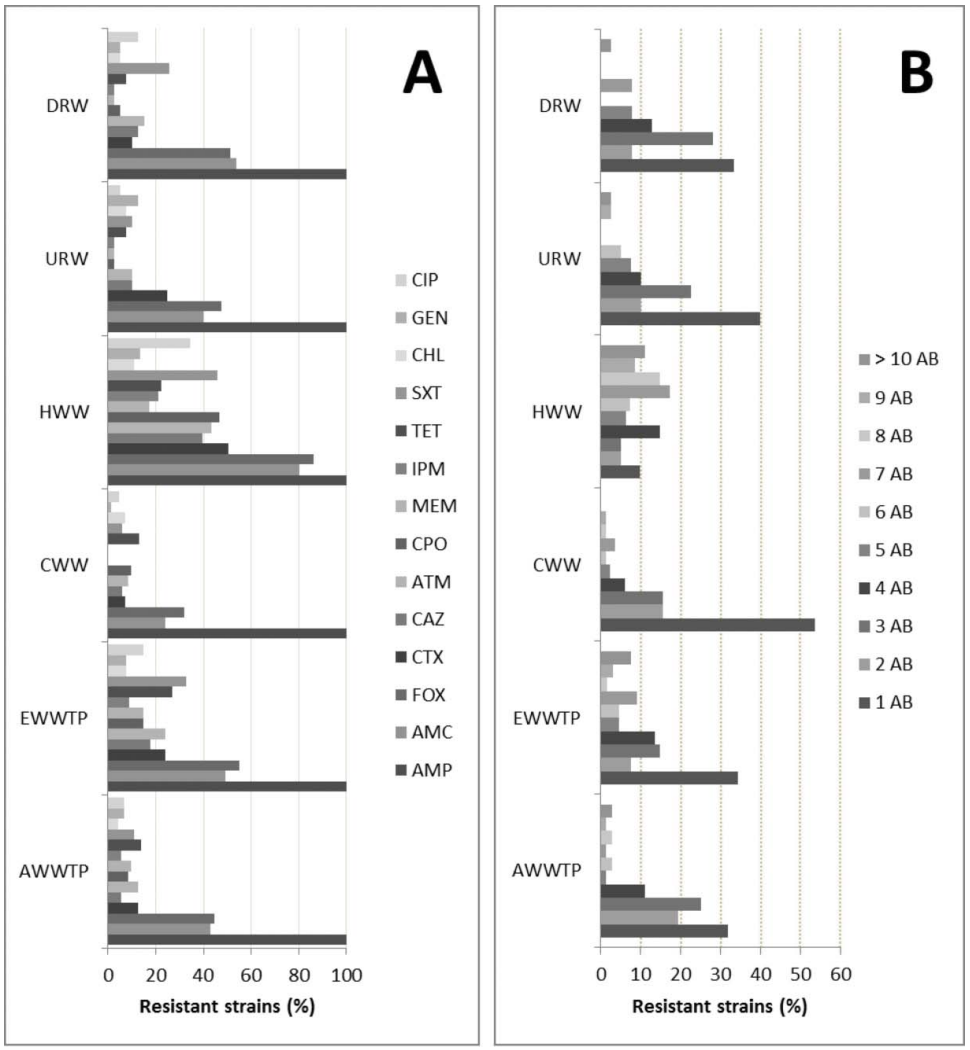

Fig. 2. Antibiotic resistance of *Enterobacteriaceae* isolates obtained from six sampling sites A) Relative frequency of resistance patterns exhibited by resistant and intermediate resistant isolates to 14 AB (13 tested + 1 used for bacteria screening); B) Relative frequency of isolates exhibiting multiple AB resistance. Tested AB: nine β-lactams: ampicillin (AMP), amoxicillin/clavulanic acid (AMC), ceftazidime (CAZ), cefotaxime (CTX), cefpirome (CPO), aztreonam (ATM), cefoxitin (FOX), imipenem (IPM), meropenem (MEM) and non-β-lactams: chloramphenicol (CHL), tetracycline (TET), gentamicin (GEN), trimethoprim/ sulfamethoxazol (SXT) and ciprofloxacin (CIP). Sampling sites: AWWTP, Affluent of the WWTP; EWWTP, Effluent of the WWTP; CWW, City Wastewater; HWW, Hospitals Wastewater; URW, Mondego River 500 m upstream the WWTP discharge; DRW, Mondego River 500 m downstream the WWTP discharge.

The isolates from the urban domestic wastewater exhibited the highest resistance rates to AMC (23.8%), FOX (32.1%) and TET (13.1%); however, no resistance was detected to MEM and IPM (Fig. 2A). The highest resistance to these two AB (17.3% and 21.0%, respectively) was registered in the isolates from hospital effluents. This result can be explained, once both AB of the carbapenems class, are mainly used in hospitals as a last resort, often in combination with other AB to treat patients in hospital with severe infections.[38]

Curiously, the isolates from the WWTP effluent have higher rates of resistance to all tested AB, with emphasis on FOX (55.2%), AMC (49.3%), CTX (23.9%), ATM (23.9%), TET (26.9%) and SXT (32.8%), when compared with those obtained from the WWTP entrance sampling site. The higher resistance values at this site were to AMC (43.1%) and FOX (44.4%) (Fig. 2A). Similar results were reported by Li et al.[32] in a WWTP receiving a pharmaceutical industry effluent, which produce penicillin G. These authors found significantly higher levels of resistance to almost all tested AB in the isolates obtained downstream the WWTP compared to those collected upstream the plant; and pointed as a putative explanation for these results, the biological treatment plant system used as the secondary treatment, the trickling bed filter. In fact, it appears that this WWTP ecosystem is an important reservoir of bacteria containing ARG that potentially can be horizontally transferred to the newcomer bacteria, thus favoring the increase of bacterial resistance.[8,9-11]

Additionally, the river isolates sampled after the WWTP discharge point, have higher AB resistance (with highlight for AMC, 53.8%; FOX, 51.3% and SXT, 25.6%) when compared to those sampled upstream the WWTP, exception for CTX (10.3%), CHL (5.1%) and GEN (5.1%) (Fig. 2A). These results reinforce the importance of appropriate treatments in the WWTP aiming reducing the spread to the environment of resistance determinants, namely ARB and ARG. However, it should be noted that the presence of ARB in the water river sampled upstream the WWPT discharge point, is not surprising, as some previous studies have pointed that even in nonpolluted environments there may be some AB resistance.[32,39]

Higher AB resistance was detected in the WWTP effluent samples compared with those sampled in the river downstream the plant, except for AMC. Depending on the AB, the reduction rate of resistant isolates ranges from 7.1% (FOX) to 82.0% (MEM). This might be due to the dilution factor, quite high in the river water. However, the resistance profile of the two sites, mainly for the CIP, GEN, CHL, SXT and TET, is quite proportional, which may indicate that the discharge of this treated wastewater can contribute to the spread of these resistant bacteria in aquatic environment, as already reported in Poland by Koczura et al.[40] in 2012.

Regarding the multidrug resistance among the 383 isolates, 66.6% were resistant to more than one of the 13 AB tested, 55.3% of which are resistant to at least three AB. The occurrence rate of resistant isolates to least three AB, per sampling site is: HWW (86.9%), DRW (59.0%), EWWTP (58.2%), URW (50.0%), AWWTP (48.6%) and CWW (31.0%). The sampling site with the highest number of AB resistant isolates was the hospital sewage and with the lowest was the urban waste (Fig. 2B).

There was an increase of multiresistant isolates between the WWTP entrance and departure, which is in agreement with the results of other studies in WWTP, that identified mobile genetic elements in bacteria isolated either from the wastewater or from the biological treatment, demonstrating that even with the reduction of the microbial load in treated wastewater, there is a proportional increase in multidrug-resistant bacteria.[5,8-10]

Moreover, there was an increased incidence of multidrug-resistant isolates between the water river sampled before and after the treatment plant discharge point. As mentioned above, the proportion of multiresistant isolates is very similar among the WWTP effluent and the downstream river samples. These results agree with those obtained by Koczura et al.,[40] who isolated bacteria resistant to an average of seven different AB, both in the treated WWTP effluent and in the river downstream the WWTP discharge.

Concurrently, the multiple antibiotic resistance (MAR) indices were determined according to Krumperman[30] (Table 2). The MAR Index provides a method that assesses the risk of contamination in aquatic environments.

The MAR indices of the hospital effluent (0.42) and the WWTP effluent (0.27) are higher than the threshold index of 0.2, indicating their high risk of environmental contamination and consequently rising serious problems of water quality and public health. The index of the river after the WWTP discharge (0.22) may indicate that this aquifer resource is not the most adequate for the rice fields irrigation, located downstream of the treatment plant.

Table 2. Multiple Antibiotic Resistance (MAR) index of wastewaters and river water.

	Sampling sites	*n. isolates*	*n. AB*	*MAR index*
Wastewaters	AWWTP	72	207	0.21
	EWWTP	67	252	0.27
	CWW	84	184	0.16
	HWW	81	479	0.42
River Water	URW	40	113	0.20
	DRW	39	119	0.22

Sampling sites, for codes, *cf.* legend of Fig. 1; n. isolates, number of isolates; n. AB, sum of the number of antibiotic resistances per isolate.

Screening of ESBL e AmpC genes

For the screening of the most frequent *bla* genes of class A or class C among *Enterobacteriaceae*, 54 isolates were selected, through the susceptibility profiles, according to their β-lactams resistance. *Enterobacter cloacae* was the most prevalent species (51.3%), followed by *Klebsiella pneumoniae* (12.8%), *Raoultella planticola* (10.3%), *Serratia liquefaciens* (7.7%). Only two isolates were identified as *Escherichia coli* (5.1%), which was therefore not the most representative species in this study, conversely to what is reported by many authors (Table 3).

The majority of the isolates are potentially $ESBL^+$ (51.9%), 44.4% are $AmpC^+$ and 35.2% are $ESBL^+$/ $AmpC^+$. The most representative species, *Enterobacter cloacae,* belongs to the latter genotype. However, *Enterobacter sakazaki* was the species that harbored the majority of the *bla* genes (TEM, OXA, CTX-M-1, EBC). This percentage of isolates co-producers of β-lactamases AmpC and ESBL, supports the idea that this type of microorganisms, especially *Enterobacteriaceae,* are becoming increasingly common.[41,42] In addition, as 33.3% of the tested isolates were negative for all genes detected, it suggests that the enzymes involved in the phenotypic resistance are different from those searched in this study, being therefore necessary to search for other less common genes, including bla_{EBV}, bla_{PER}, bla_{GES}, bla_{IMP}, bla_{VIM} and bla_{KPC}.

The most prevalent genes among the potential ESBL producer isolates were bla_{OXA} (33.3%) and bla_{TEM} (24.1%) (Table 4). No bla_{SHV} gene was detected, and only three isolates (5.6%) harbored the $bla_{CTX\text{-}M}$ gene: two of the CTX-M-1 group and one of the CTX-M-9. The result that these frequencies differ from other reports suggested the prevalence of the $bla_{CTX\text{-}M}$ gene, in particular of the $bla_{CTX\text{-}M\text{-}15}$, which encodes for one of the most widespread ESBL, in isolates obtained from hospitals,[22,43] agro-ecosystems[44,45] or sewerage water.[5,42]

However, our results are partly in agreement with those obtained by Diallo et al.,[13] who reported among the ESBL-producing *E. coli*, isolated from urban wastewater, the $bla_{CTX\text{-}M\text{-}14}$ gene (11.1%), the $bla_{TEM\text{-}52}$ gene (66.6%) and from the slaughterhouse and WWTP effluents the $bla_{CTX\text{-}M\text{-}1}$ (22.2%). All those isolates also contained $bla_{TEM\text{-}1}$ and none contained bla_{SHV}.

The bla_{OXA} gene was recorded in 33.3% of the ESBL-producer isolates. This result is consistent with that obtained by Sana et al.[46] in a study conducted in Lebanon, showing a high prevalence of the bla_{OXA} gene (45.2%) among the 73 ESBL-producer clinical isolates of *E. coli* analyzed.

Other studies about the AB resistance of clinical isolates obtained from Portuguese hospitals, including the Coimbra hospital under study, revealed the spread of *Enterobacteriaceae* containing a combination of $bla_{CTX\text{-}M\text{-}15\text{-}1}$, $bla_{OXA\text{-}1}$ and $bla_{TEM\text{-}1}$ genes with others, coding for aminoglycosides and fluoroquinolones resistance.[47,48] The former authors also revealed results similar to our findings, but instead of $bla_{OXA\text{-}1}$ they detected $bla_{OXA\text{-}30}$, all belonging to the same $bla_{OXA\text{-}1\text{-}like}$ group. Moreover, it appears that all OXA isolates (except three) are resistant to at least one carbapenem, suggesting the presence of β-lactamase OXA carbapenemase (Table 3). Identical results were recently reported from 21 Spanish WWTP, with prevalence of $bla_{CTX\text{-}M}$, high frequency (47%) of bla_{TEM} and lower frequency (8.3%) of bla_{OXA} genes in their effluents.[42]

It is noteworthy that both isolates harboring $bla_{CTX\text{-}1}$ group, to which $bla_{CTXM\text{-}15}$ gene belongs, exhibit ciprofloxacin resistance (Table 3). This result is consistent with other reports, which had revealed an association between fluoroquinolone resistance and the presence of $bla_{CTXM\text{-}15}$ gene.[42,49,50]

The AB resistance profile of the isolates reveals their clear multiresistance (Table 3). Nevertheless, sequencing will be required to confirm whether all putative ESBL phenotypes are really ESBL genotypes, although ESBLs are often associated with the multidrug resistance.[12,19] Tacão et al.[51] also observed ESBL strains isolated from Portuguese rivers with high levels of non-β-lactam resistance, such as tetracycline, aminoglycosides and fluoroquinolones. Once 59.3% of the studied isolates were resistant to at least one carbapenem, it would also be interesting to search for the presence of bla_{IMP}, bla_{KPC} and bla_{VIM} genes, responsible for resistance to carbapenems.

The co-production of plasmid-encoded *bla* genes has been reported in *Enterobacteriaceae*, mainly related to the ESBL and carbapenemases.[52] Moreover, the association of the AmpC gene with the acquisition of additional resistance genes for non-β-lactam AB has been well documented;[53,54] although, the lower frequency of studies about the occurrence of plasmid-mediated ESBL and AmpC co-producer isolates.[55]

The majority of the isolates carrying AmpC β-lactamases, encoded by plasmid genes (94.4%) were resistant to both cefoxitin and amoxicillin associated with clavulanic acid (Table 3). This association has been interpreted as simple indicative of AmpC producers. In fact, no AmpC genes were detected in the three isolates sensitive to those β-lactams, which for those with a FOX-resistant phenotype might be explained by an overexpression of the chromosomal AmpC.[44]

The most prevalent genes of this β-lactamase type belong to the bla_{EBC} gene family (38.9%) (includes bla_{ACT} and $bla_{MIR\text{-}1}$), followed by the gene families bla_{FOX} (includes $bla_{FOX\text{-}1}$ to $bla_{FOX\text{-}5}$), bla_{DHA} (includes $bla_{DHA\text{-}1}$ and $bla_{DHA\ \text{-}2}$) and bla_{CIT} (includes $bla_{CMY\text{-}2}$ to $bla_{CMY\text{-}7}$, $bla_{CMY\text{-}12}$ to $bla_{CMY\text{-}18}$, $bla_{CMY\text{-}21}$ to $bla_{CMY\text{-}23}$, $bla_{LAT\text{-}1}$ to $bla_{LAT\text{-}3}$ and $bla_{BIL\text{-}1}$) with only one isolate each (1.9%). These results do not confirm the numerous references that indicate $bla_{CMY\text{-}2}$ as the most prevalent gene in *Enterobacteriaceae* encoding for the AmpC enzymes plasmid-mediated and its widely worldwide occurrence in

Table 3. List of AB resistance profile and β-lactamases genes harbored per isolate and sampling site.

			β-lactamases genes type	
Sampling sites	*Species*	*Resistance Phenotype*[a]	*AmpC*	*ESBL*
AWWTP	*Enterobacter sakazaki*	AMP, AMC, FOX, CAZ, CPO, CHL, SXT, TET	EBC	
	Enterobacter sakazaki	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, CHL, CIP, GEN, SXT	EBC	TEM, OXA, CTX-M-1
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, MEM, SXT		OXA
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Raoultella plantícola	AMP, AMC, CTX, ATM, IPM, MEM, CIP, GEN		
	Serratia liquefaciens	AMP, AMC, FOX, CTX, CPO,ATM, IPM, MEM, SXT	EBC	OXA
	ND	AMP, AMC, FOX, MEM, TET	EBC	TEM, OXA
	ND	AMP, AMC, FOX, MEM	CMY	OXA
	ND	AMP, AMC, FOX, ATM, IPM, MEM	EBC	OXA
	ND	AMP, CTX, CAZ, CPO, ATM, SXT		TEM, CTX-M-9
EWWTP	*Klebsiella pneumoniae*	AMP, AMC, FOX, CAZ, ATM, MEM		
	Escherichia coli	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, CIP, SXT		
	Enterobacter cloacae	AMP, AMC, FOX, CTX,CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA, CTX-M-1
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, SXT	EBC	OXA
	Citrobacter koseri	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Raoultella plantícola	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, GEN		TEM
	Raoultella plantícola	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, IPM, MEM, CHL, CIP, GEN		
	Raoultella plantícola	AMP, AMC, FOX, CTX, CAZ, CPO, IPM, MEM, CHL, CIP, GEN		
	Serratia liquefaciens	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, IPM, MEM, CHL, CIP	EBC	
	ND	AMP, AMC, FOX, CTX, CAZ, ATM, MEM		
	ND	AMP, AMC, FOX, CTX, CAZ, ATM, MEM		
CWW	*Citrobacter freundii*	AMP, AMC, FOX, CAZ, CTX, CPO, ATM		
	Escherichia coli	AMP, CHL, CIP, GEN, SXT		TEM
	Klebsiella oxytoca	AMP, AMC, FOX, CTX, CPO, ATM, SXT, TET		
	Enterobacter cloacae	AMP, AMC, FOX, CAZ, CTX, CPO, ATM,CHL, TET		
	ND	AMP, AMC, FOX, CAZ, CTX, CPO, ATM	EBC	
	ND	AMP, AMC, FOX, CAZ, CTX, ATM	EBC	
HWW	*Enterobacter cloacae*	AMP, AMC, FOX, CPO, IPM, CHL, CIP, SXT, TET		TEM
	Enterobacter cloacae	AMP, AMC, FOX, CAZ, CTX, CPO, IPM, CHL, CIP, SXT		TEM
	Enterobacter cloacae	AMP, AMC, FOX, CAZ, CTX, ATM, IPM, CIP, SXT	ND	ND
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, ATM, MEM, SXT	ND	ND
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Raoultella plantícola	AMP, AMC, FOX, CTX, CAZ, ATM, CHL, CIP, SXT, TET	DHA	TEM
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CHL, CIP, GEN, SXT	FOX	TEM

	Enterobacter cloacae	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, IPM, MEM, CIP, GEN, SXT	EBC	TEM
	Klebsiella pneumoniae	AMP, AMC, FOX, CAZ, CTX, CPO, ATM, IPM, CIP,TET		
	Klebsiella pneumoniae	AMP, AMC, FOX, CAZ, CTX, CPO, ATM, MEM, CHL		
	Klebsiella pneumoniae	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, CIP, GEN	ND	ND
	Serratia liquefaciens	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP	ND	ND
	ND	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, CIP, TET		
	ND	AMP, AMC, FOX, CTX, CAZ, IPM, GEN, SXT	EBC	
	ND	AMP, AMC, FOX, CTX, CPO, ATM, IPM, MEM, CIP	EBC	OXA
URW	*Klebsiella pneumoniae*	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, CHL, SXT, TET		TEM, OXA
	Enterobacter cloacae	AMP, AMC, FOX, CTX, GEN		
	Enterobacter cloacae	AMP, AMC, FOX, CTX, IPM, MEM, CIP, GEN, SXT		
	ND	AMP, AMC, FOX, CTX, CAZ, ATM		
DRW	*Enterobacter cloacae*	AMP, AMC, FOX, CTX, CAZ, CPO, ATM, IPM, MEM, CIP, SXT	EBC	OXA
	Enterobacter cloacae	AMP, AMC, FOX, CTX, CAZ, ATM, GEN		
	ND	AMP, AMC, FOX, CTX, ATM, CHL, SXT		TEM
	ND	AMP, AMC, FOX, ATM, SXT		TEM
	ND	AMP, AMC, FOX, CTX, CAZ, CPO, SXT		

Sampling sites: AWWTP, Affluent of the WWTP; EWWTP, Effluent of the WWTP; CWW, City Wastewater; HWW, Hospitals Wastewater; URW, Mondego River, 500 m upstream the WWTP discharge; DRW, Mondego River, 500 m downstream the WWTP discharge. a) AB: AMP, ampicillin; AMC, amoxicillin/clavulanic acid; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CPO, cefpirome; ATM, aztreonam; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; GEN, gentamicin; CHL, chloramphenicol; SXT, trimethoprim/ sulfamethoxazol; TET, tetracycline.

Table 4. Frequency of isolates with AmpC and ESBL β-lactamases genes per sampling site.

β-lactamase group	*AWWTP N (%)*	*EWWTP N (%)*	*CWW N (%)*	*HWW N (%)*	*URW N (%)*	*DRW N (%)*	*Total N (%)*
ESBL							
TEM	3(30%)	1(9.1%)	1(16.7%)	5(27.8%)	1(25%)	2(40%)	13(24.0%)
OXA	7(70%)	4(36.4%)		5(27.8%)	2(50%)	1(20%)	18(33.3%)
CTX-M-1	1(10%)	1(9.1%)					2(3.7%)
CTX-M-9	1(10%)						1(1.9%)
AmpC							
CIT	1(10%)						1(1.9%)
DHA				1(5.6%)			1(1.9%)
EBC	6(60%)	5(45.5%)	2(33.3%)	7(38.9%)		1(20%)	21(39.0%)
FOX				1(30%)			1(1.9%)

For sampling sites codes, *cf*. legend of Fig. 1; N (%), absolute and (relative) frequency of isolates carrying the gene.

human clinical isolates[20,53,56–58] in healthy or diseased animals[44,59,60] and in environmental waters.[54]

In some geographical regions, the AmpC β-lactamases especially those encoded by the $bla_{\text{CMY-2}}$ gene, have become more prevalent than the ESBL,[61] despite in other regions the bla_{DHA} and bla_{FOX} are also highly prevalent.[57] For example, in Chinese pediatric hospitals the *E. coli* isolates revealed $bla_{\text{DHA-1}}$ as the most prevalent β-lactamase (93.2%), compared to $bla_{\text{CMY-2}}$, with 6.8%.[62] Also in Indian hospitals, Mohamudha et al.[63] reported bla_{DHA} as the predominant (38.1%) among the AmpC producing isolates.

The results published by Al-karawyi et al.[64] are similar those obtained in our study. These authors analyzing isolates collected from hospital urine samples of Iraqi patients with urinary tract infections found a distribution frequency of the AmpC of: 50% bla_{EBC}, 44.4% bla_{FOX}, 38.9% bla_{CIT} and 27.8% bla_{DHA}. Additionally, in the USA the β-lactamase ACT-1 (EBC family) and FOX-5 were reported in the majority of clinical isolates, and interestingly a minority of CMY-2 and DHA-1 producers.[65] Moreover, with exception to the predominance of CMY-2, Galán-Sánchez et al.[58] also observed, among the clinical isolates of a Spanish hospital, a AmpC susceptibility pattern of 78.2% : bla_{TEM} (51.9%), bla_{SHV} (6.3%), bla_{OXA} (3.8%) and $bla_{\text{CTX-M}}$ (3.8%).

In our study, no genes of the bla_{ACC} and bla_{MOX} families were detected (Table 4); conversely to Hussain et al.,[22] who detected CIT and MOX family genes, as the most prevalent in the clinical isolates. Moreover, none of the isolates harbored more than one family of AmpC, although such occurrence has already been registered in India by Shahid et al.[66] Synthetizing, the higher diversity of AB resistance phenotypes observed in the sampling sites HWW, AWWTP and EWWTP is in agreement with the higher diversity of genes coding for β-lactamases (Tables 3 and 4).

Conclusion

To our knowledge, this is the first study on the dissemination of AB resistance determinants in urban and hospital wastewaters, in the receptor WWTP and their distribution in water resources in Coimbra. Moreover, this paper reports the simultaneous detection of multiresistant bacteria producers of AmpC and ESBLs β-lactamases in aquatic systems in Portugal.

This study has demonstrated that the high spread of multidrug-resistant bacteria detected is a consequence of the anthropogenic activities, particularly in urban and clinical environments. Moreover, the wastewater treatment can further contribute to a frequency increase of these bacteria in aquatic ecosystems; since the treatment plant is an important reservoir of *Enterobacteriaceae* containing transferable resistance genes. This environmental spread of AB resistance determinants becomes a concerning matter when the treated wastewater is reused for crop irrigation, as in the Lower Mondego region, where rice and other food crops are grown. It would be important to improve combined treatments for disinfection, investing in the development of cheapest and easy to apply to inactivate and prevent the spread of unwanted biological contaminants.

Given this situation, there is an urgent need for efficient management of hospital wastewaters, since they are a major source of environmental contamination. In Portugal, the legislation does not impose limits to AB residues or to ARB in hospital wastewaters. Consequently, in the absence of specific legislation, there is no accurate information regarding those effluents, which is the required basis to plan and implement an adequate and specific treatment of these wastewaters.

In many countries, including Portugal, there are regulations regarding the use of treated wastewater in agriculture. However, this regulation only sets quality requirements upon some chemical characteristics and thresholds to the presence of pathogenic microorganisms, including the indicator microorganisms that may cause risk to public health. Currently, it would be important that legislator entities include in the regulations additional criteria for wastewater quality regarding to the supervision and control of AB multidrug-resistant bacteria.

If all these measures, including the rationalization of AB use, the investment in the production of new AB and the

public awareness of this serious problem, are not taken seriously, mankind risks of back to the pre-AB era, where the mortality rate was high due to numerous and common bacterial infections that we have considered controlled. The consequences will be also extended to the environment, with long-term negative impacts, including microbiota imbalances in environmental ecosystems, surface and ground waters and soils. These environmental and public health risks bring high challenges to this issue.

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