# High prevalence of multidrug-resistant *Escherichia coli* and *Enterococcus* spp. in river water, upstream and downstream of a wastewater treatment plant

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## ABSTRACT

In this study, microbial quality and antimicrobial resistance of faecal bacteria from a Portuguese river were assessed. River water samples collected upstream and downstream of a wastewater treatment plant, throughout a 3-month period, were used for the enumeration of *Escherichia coli* and *Enterococcus* spp. The highest numbers found for *E. coli* and enterococci were $1.1 \times 10^4$ and $1.2 \times 10^4$ colony forming units (CFU)/100 ml, respectively. In total, 144 isolates of *E. coli* and 144 of enterococci were recovered and tested for antimicrobial susceptibility; 104 *E. coli* and 78 *Enterococcus* spp. showed resistance to one or more antimicrobial drugs. Overall, 70 and 32 different resistance patterns were found for *E. coli* and enterococci, respectively. One *E. coli* showed resistance to imipenem and 29 isolates were extended spectrum $\beta$-lactamase-producers. Multidrug-resistant *E. coli* belonged mostly to groups A, B1 and group D. *Enterococcus* spp. were mostly resistant to rifampicin, tetracycline, azithromycin and erythromycin; six isolates showed resistance to vancomycin, presenting the VanA phenotype. The high levels of *E. coli* and enterococci and the remarkable variety of antimicrobial resistance profiles, reinforces the theory that these river waters can be a pool of antimicrobial resistance determinants, which can be easily spread among different bacteria and reach other environments and hosts.

**Key words** | faecal contamination, multidrug-resistant *E. coli*, river water, vancomycin-resistant enterococci

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## INTRODUCTION

Microbial water quality is assessed by quantifying the presence of *Escherichia coli* and enterococci, which are normally present in the intestinal tracts of warm-blooded animals and are released to the environment through their faeces. For that reason these microorganisms are considered suitable indicators of faecal contamination and can also signal the presence of intestinal pathogens such as *Salmonella* spp., *Shigella* spp., pathogenic *E. coli*, enteroviruses and *Giardia* (McLellan 2004; Carlos *et al.* 2010).

*E. coli* strains have been classified into three types: commensal, intestinal pathogenic or extra-intestinal pathogenic (Russo & Johnson 2000) and also into four different phylogenetic groups (A, B1, B2, and D) (Clermont *et al.* 2000). Strains of each group share phenotypic and genotypic features. Commensal *E. coli* strains usually belong to groups A and B1 whereas the pathogenic extra-intestinal strains belong mainly to phylogenetic group B2 and, to a minor extent, to group D (Duriez *et al.* 2001; Pereira *et al.* 2013). Therefore, the phylogenetic classification may give information about the origin and pathogenicity of the strain.

*Enterococcus faecalis* and *E. faecium* are two important enterococcal species frequently used as suitable indicators of faecal contamination in water (Suzuki *et al.* 2012). However, many other *Enterococcus* species (*E. casseliflavus*, *E. durans*, *E. gallinarum*) can be found in surface waters. Enterococci are opportunistic pathogens that have been associated

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with nosocomial infections and play an important role in the dissemination of antibiotic resistance, particularly because they exhibit resistance to a wide variety of antimicrobial drugs and have the capacity to exchange genetic information by conjugation (Jackson *et al.* 2004).

The presence of enteric bacteria in aquatic environments is a current issue in terms of public health, especially due to the fact that such bacteria can carry resistances to antibiotics and, therefore, aquatic systems can be a vehicle to spread antibiotic resistance (Ash *et al.* 2002; Pereira *et al.* 2013). Among those aquatic systems, rivers are the main receptors of bacterial contaminants from human or animal origins coming from discharge from wastewater treatment plants (WWTP), and livestock and poultry production farms. These bacteria present in river waters are subjected to many selective pressures exerted by well-known factors such as antibiotics and other substances (e.g. heavy metals) (Tello *et al.* 2012; Cantas *et al.* 2013) that may facilitate the selection of antimicrobial-resistant bacteria or the acquisition of resistance genes by horizontal gene transfer (Martinez 2012).

The general assessment of river water quality can help to drive environmental improvements by dealing with the main sources of chemical pollutants as well as contaminant micro-organisms (Kittinger *et al.* 2013). In this study we assessed the microbial quality of a Portuguese river, generally known as being a polluted river, by quantifying the presence of faecal contaminants during a 3-month period and by determining their antimicrobial resistance profile.

## MATERIALS AND METHODS

### River features and sample collection

Ave River, in the north of Portugal, was taken as a water sample source for this study since the current knowledge about its microbial quality is scarce. This river has an 85-km course, its headwaters are in the Mountain Cabreira (Portugal) and it flows into the Atlantic Ocean. It crosses a highly populated region and several cities, with many industries and livestock farms in its surroundings. Samples were collected in the part of the river that crosses a city, Santo Tirso, near a WWTP (Figure 1). This WWTP is a tertiary treatment facility and

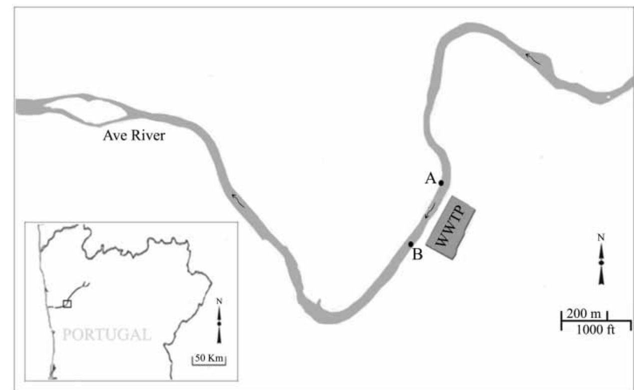


**Figure 1** | Location of study site and points (A and B) of sample collection in Ave River.

receives effluent from an urban population of approximately 165,873 people, including effluent from a large industrial area. The treatment uses the activated sludge process. The average flow rate of treated wastewater is 24,881 $m^3$/day.

Between September and December 2010, at six time points, two river water samples were collected from the Ave River, 50 m upstream (sample point A – N41°21′01.30″/W8°27′47.62″) and 50 m downstream (sample point B – N41°20′58.87″/W8°27′49.78″) of the WWTP, respectively. Each sample was collected in a 1.5-l plastic bottle, transported to the laboratory in an ice box and maintained at 4 °C until required.

### Isolation and enumeration of *E. coli* and *Enterococcus* spp.

Water samples with volumes of 1, 10 and 100 ml were filtered through 0.45 μm-pore-size membrane filters (Millipore Corporation, USA), which were then placed on Tryptone Bile X-glucuronide agar (TBX) (BioKar Diagnostics, Beauvais, France) and on Slanetz and Bartley agar (SB) (Oxoid, Basingstoke, UK) for *E. coli* and enterococci enumeration, respectively. The plates were incubated at 37 °C for 24 h (*E. coli*) or 48 h (enterococci). *Enterococcus* spp. colonies were confirmed in Kanamycin Aesculin Azide agar (KAA) (Liofilchem, Roseto degli Abruzzi, Italy), incubated at 44 °C for 4 h. Biochemical confirmatory methods were subsequently performed. Moreover, *E. coli* and enterococci isolates were also obtained in the adequate antibiotic-supplemented media, as subsequently described. Water samples with volumes of 50 to 100 ml were directly filtered through 0.45-μm pore size

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membrane filters and then placed on TBX agar (for *E. coli*) supplemented with cefotaxime (2 μg/ml), ampicillin (8 μg/ml) and ciprofloxacin (4 μg/ml) (Sigma-Aldrich, St Louis, MO, USA), and on SB agar (for enterococci) supplemented with vancomycin (6 μg/ml), ampicillin (8 μg/ml) and ciprofloxacin (4 μg/ml). The plates were incubated at 37 °C for 24–48 h. For further antimicrobial susceptibility testing, a maximum of five colonies of *E. coli* and enterococci were picked up from each antibiotic-supplemented and non-supplemented medium.

### Antimicrobial susceptibility testing

The resistance patterns of 144 *E. coli* and 144 *Enterococcus* spp., obtained from the non-supplemented and antibiotic-supplemented media, were determined by the agar disk-diffusion method on Mueller-Hinton agar (BioKar Diagnostics, Beauvais, France) following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2007). For *E. coli*, the following 19 antimicrobial agents were used: cephalothin (KF, 30 μg), cefoxitin (FOX, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), amoxicillin/clavulanic acid (AMC, 30 μg), ampicillin (AMP, 10 μg), aztreonam (ATM, 30 μg), imipenem (IPM, 10 μg), gentamicin (CN, 10 μg), kanamycin (K, 30 μg), tobramycin (TOB, 10 μg), amikacin (AK, 30 μg), streptomycin (S, 10 μg), nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (C, 30 μg), tetracycline (TE, 30 μg), nitrofurantoin (F, 300 μg) and sulfamethoxazole/trimethoprim (SXT, 25 μg). In the case of *Enterococcus* spp., 12 antimicrobial agents were used: ampicillin (AMP, 10 μg), gentamicin (CN, 120 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (C, 30 μg), tetracycline (TE, 30 μg), nitrofurantoin (F, 300 μg), vancomycin (VA, 30 μg), teicoplanin (TEC, 30 μg), erythromycin (E, 15 μg), azithromycin (AZM, 15 μg), rifampicin (RD, 5 μg) and quinupristin-dalfopristin (QD, 15 μg). All antimicrobial agents were from Oxoid (Oxoid, Basingstoke, UK). *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used as reference strains. The extended spectrum *β*-lactamase (ESBL) phenotype in *E. coli* culture was observed on plate according to the method of disk approximation test (Cormican *et al.* 1996; Drieux *et al.* 2008).

### *E. coli* phylogenetic group determination

*E. coli* isolates that presented resistance to six or more antimicrobial drugs were selected for phylogenetic group determination. DNA was extracted using the InstaGene matrix (BioRad Laboratories, CA, USA). Phylogenetic groups A, B1, B2 and D were determined according to Clermont *et al.* (2000), by multiplex polymerase chain reaction (PCR) of the genes *chuA* and *yjaA* and the DNA fragment TspE4.C2. Samples of PCR products were analysed by electrophoresis in a 1.5% agarose gel at 150 V for 45 min. Gels were stained with ethidium bromide and photographed under ultraviolet (UV) light. Appropriate positive and negative controls were included in the assay.

### *Enterococcus* species identification and vancomycin resistance genotype

*Enterococcus* spp. isolates presenting resistance to vancomycin, tetracycline or rifampicin or to four or more antimicrobial drugs were used for species identification. The genomic DNA of *Enterococcus* spp. was extracted by enzymatic treatment with lysostaphin (1 mg/ml; Sigma-Aldrich Chemie, Saint-Quentin Fallavier, France) for 2 h and followed by treatment with lysozyme (50 mg/ml; AppliChem GmbH, Darmstadt, Germany) and proteinase K (20 mg/ml; Bioron, Ludwigshafen, Germany) for another 2 h. A multiplex PCR was then performed for *Enterococcus* species identification. Amplification of the genes related to the species-specific identification of *E. fecalis*, *E. faecium*, *E. flavescens*, *E. durans*, *E. casseliflavus*, *E. gallinarum*, *E. avium*, *E. cecorum*, and *E. hirae* among other species was performed as described by Jackson *et al.* (2004). Subsequently, isolates that showed vancomycin resistance ($n = 6$) in the antimicrobial susceptibility testing assay were used for the evaluation of glycopeptides resistance genotype described for enterococci (*vanA*, *vanB*, *vanC-1*, *vanC-2* and *vanC-3*), following the procedure of Dutka-Malen *et al.* (1995).

## RESULTS

### Quantification of *E. coli* and *Enterococcus* spp. over time

The enumeration of *E. coli* and *Enterococcus* spp. recovered from samples collected at points A and B at the six time points is shown in Figure 2. Differences in counts

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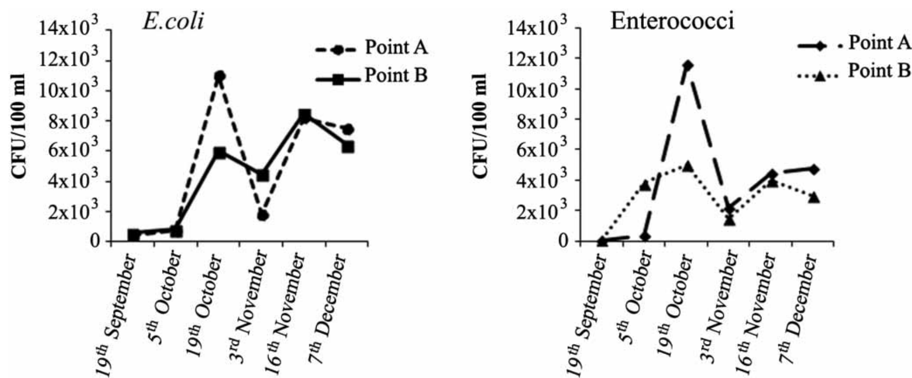


**Figure 2** | *E. coli* and *Enterococcus* spp. enumeration in river water samples collected from points A and B at six different time points.

between point A and B were particularly evident at some time points (19th October and 3rd November, in the case of *E. coli*; 5th and 19th October, in the case of enterococci). The fluctuation observed in the enumeration throughout time followed the same trend both in *E. coli* and *Enterococcus* spp.

The isolates recovered in the media supplemented with antibiotics were also counted in order to observe the proportion of 'potential' resistant strains at point A and B. These results permitted an initial selection of possible resistant strains of *E. coli* and *Enterococcus* spp.

In total, 144 *E. coli* and 144 enterococci (72 isolates from each sample point) were recovered and used to carry out the study. Twenty-four out of the 144 were collected from the non-supplemented media (TBX and SB for *E. coli* and enterococci isolates, respectively) and the remaining from the antibiotic-supplemented media. From the TBX supplemented with ampicillin, ciprofloxacin and cefotaxime, it was possible to recover 24, 36 and 60 isolates of *E.* coli, respectively. The same numbers, 24, 36 and 60 of enterococci isolates were recovered from SB supplemented with ampicillin, ciprofloxacin and vancomycin, respectively.

## Characterization of antimicrobial-resistant *E. coli* and *Enterococcus* spp. isolates

Overall, 40 out of the 144 *E. coli* isolates and 66 out of the 144 *Enterococcus* spp. isolates were susceptible to all antimicrobial drugs tested. The remaining 104 *E. coli* and 78 *Enterococcus* spp. isolates showed resistance to one or more antimicrobial drugs. The percentage of resistance to each antimicrobial drug, among the antimicrobial-resistant isolates, was then calculated and is shown in Table 1. Regarding *E. coli* isolates, very high percentages of resistance to some antibiotics were observed, for instance, approximately 61.8% (89 out of 144) were resistant to ampicillin. A huge heterogeneity of resistance patterns was found among the isolates. Overall, about 70 and 32 different resistance patterns were found for *E. coli* and *Enterococcus* spp., respectively. The multidrug resistance patterns to six or more antimicrobials found for *E. coli* are shown in Table 2. Also for *E. coli*, the number of multidrug-resistant isolates to 10 or more antibiotics was rather common. Additionally, it was observed that 29 isolates with different antimicrobial resistance patterns were ESBL-producing *E. coli*, 14 were isolated from sample point A and 15 from point B. It must be highlighted that an *E. coli* resistant to imipenem was also isolated. As this type of resistance phenotype is very unusual, especially in isolates from the environment, an API 20E was performed to ascertain that it was indeed an *E. coli* strain, which was confirmed. Poirel *et al*. (2012) have characterized this isolate, which was resistant to all $\beta$-lactams, including all carbapenems, fluoroquinolones and aminoglycosides and susceptible only to tetracycline, fosfomycin, and colistin. The molecular analysis revealed the presence of the *bla*KPC-2 $\beta$-lactamase gene. Another interesting and atypical phenotype with the resistance pattern, AMP FOX TE CAZ AMC KF, was also found; it showed resistance to four β-lactam antibiotics, but was not an ESBL-producing bacteria.

*Enterococcus* spp. were mostly resistant to rifampicin, tetracycline, azithromycin and erythromycin. Six isolates

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**Table 1** | Antimicrobial resistance of *E. coli* and *Enterococcus* spp. isolated from sample points A and B. The percentage of antimicrobial resistance shown here was calculated among isolates that presented resistance towards at least one antimicrobial drug

| Point of sampling | Antimicrobial drugs tested and respective numbers (%) of resistant *E. coli* isolates | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **AMP** | **FOX** | **IPM** | **CIP** | **CN** | **F** | **TE** | **CTX** | **ATM** | **CAZ** | **AMC** | **KF** | **AK** | **S** | **NA** | **C** | **TOB** | **SXT** | **K** |
| A ($n = 50$) | 45 (90) | 7 (14) | 1 (2) | 18 (36) | 7 (14) | 1 (2) | 33 (66) | 14 (28) | 8 (16) | 7 (14) | 4 (8) | 27 (54) | 0 (0) | 21 (42) | 28 (56) | 9 (18) | 4 (8) | 22 (44) | 8 (16) |
| B ($n = 54$) | 44 (81.5) | 5 (9.3) | 0 (0) | 19 (35.2) | 4 (7.4) | 0 (0) | 34 (62.9) | 10 (18.5) | 9 (16.7) | 3 (5.6) | 4 (7.4) | 28 (51.8) | 1 (1.8) | 15 (27.8) | 24 (44.4) | 11 (20.4) | 3 (5.6) | 18 (33.3) | 2 (3.7) |

| Point of sampling | Antimicrobial drugs tested and respective numbers (%) of resistant enterococci isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **AMP** | **QD** | **TE** | **RD** | **E** | **CN** | **VA** | **AZM** | **TEC** | **C** | **F** | **CIP** |
| A ($n = 41$) | 14 (34.1) | 5 (12.2) | 14 (34.1) | 19 (46.3) | 15 (36.6) | 6 (14.6) | 4 (9.7) | 16 (39.0) | 0 (0) | 0 (0) | 1 (2.4) | 17 (41.4) |
| B ($n = 37$) | 11 (29.7) | 10 (27.0) | 18 (48.6) | 13 (35.1) | 19 (51.3) | 1 (2.7) | 2 (5.4) | 21 (56.8) | 0 (0) | 2 (5.4) | 3 (8.1) | 17 (45.9) |

of enterococci showed resistance to vancomycin and all harboured the *vanA* gene (data not shown) that confers resistance to high levels of vancomycin and also to teicoplanin. Accordingly, these isolates showed high resistance to vancomycin and intermediate resistance to teicoplanin in the antimicrobial susceptibility testing assay (data not shown). The most relevant resistant patterns found for *Enterococcus* spp. are shown in Table 3.

In a few cases, isolates recovered from antibiotic-supplemented media did not present resistance to the same antibiotic in the antimicrobial susceptibility testing assay.

### Diversity of *E. coli* phylogenetic groups and *Enterococcus* species

Phylogenetic analysis of *E. coli* showed that the isolates with patterns of antimicrobial resistance to six or more drugs belonged mostly to groups A and B1, but also to group D. No isolate was found to belong to group B2 (Table 2). *E. coli* from each group were equally distributed at the two points of sample collection.

Regarding the *Enterococcus* species identification, it was found that the drug-resistant enterococci were mainly *E. faecalis* and *E. faecium* (Table 3). Among 34 antibiotic-resistant isolates, only one was *E. casseliflavus*. All isolates resistant to vancomycin were identified as being *E. faecalis* and *E. faecium*.

## DISCUSSION

High levels of *E. coli* and enterococci (around $10^4$ CFU/100 ml) were present in the river water at some time points. Such high numbers are normally found in sewage and not in river water. The fluctuation shown in the numbers of *E. coli* and *Enterococcus* spp. isolated during the 3-month period from the two sample points might be explained mainly by surface runoff (Farnleitner *et al.* 2010), especially taking into account that during the period of sample collection there was a lot of variation in rainfall and, in accordance, the highest number of CFUs was from samples collected after days of intensive rainfall (on 19th October). Accordingly, Ratajczak *et al.* (2010) observed that hydrological conditions, such as a wet or dry period,

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**Table 2** | Antimicrobial resistance profile and phylogenetic group of 40 multidrug-resistant *E. coli* isolates and their distribution per sample points and time points (1–6) of sample collection

| Antimicrobial resistance profile | Isolation media | ESBL-producing bacteria | Phylogenetic group | Number of isolates per sample and per sampling points | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | |
| | | | | A | B | A | B | A | B | A | B | A | B | A | B |
| AMP TE CTX ATM CAZ KF | TBX + CTX | Yes | A | I | | | | | | | | | | | |
| AMP TE CTX KF NA SXT | TBX + CTX | Yes | A | II | | | | | | | | | | | |
| AMP FOX TE CTX CAZ KF S C | TBX + CTX | No | A | | | I | | | | | | | | | |
| AMP CIP TE NA SXT K | TBX + CIP | No | A | | | | | I | | | | | | | |
| AMP CIP TE KF S NA C SXT | TBX + AMP | No | A | | | | | | | I | | | | | |
| AMP CIP CN TE S NA SXT | TBX + CIP | No | B1 and A | | | | | | | II | | I | | | |
| AMP FOX CIP CN TE KF S NA C TOB SXT K | TBX + CTX | No | B1 | | | | | | | I | | | | | |
| AMP FOX CIP F TE KF S NA C SXT K | TBX + CTX | Yes | B1 | | | | | | | I | | | | | |
| AMP FOX CIP CN TE AMC KF S NA C TOB SXT K | TBX + CTX | No | A | | | | | | | I | | | | | |
| AMP CIP TE KF S NA SXT | TBX + CIP | No | D | | | | | | | I | | | | | |
| AMP CIP TE S NA SXT | TBX + CIP | No | A and B1 | | | | | | II | | | I | | | |
| AMP TE CTX ATM KF S SXT K | TBX + CTX | Yes | A | | | | | | | | | I | | | |
| AMP FOX CAZ AMC KF NA | TBX + CTX | No | D | | | | | | | | | I | | | |
| AMP CIP TE CTX ATM KF NA SXT | TBX + CTX | Yes | A | | | | | | | | | I | | | |
| AMP CIP TE CTX ATM KF NA | TBX + CIP | Yes | D | | | | | | | | | | | I | |
| AMP CIP TE S NA C SXT | TBX + CIP | No | D | | | | | | | | | | | II | |
| AMP CIP CTX ATM CAZ KF NA TOB SXT K | TBX + CTX | No | A | | | | | | | | | | | I | |
| AMP FOX IPM CIP CTX ATM CAZ AMC KF S NA C SXT K[a] | TBX + CTX | Yes | A | | | | | | | | | | | I | |
| AMP CIP TE CTX ATM KF S NA C SXT K | TBX + CTX | Yes | A | | | | I | | | | | | | | |
| AMP CIP TE NA C SXT | TBX + CIP | No | A | | | | I | | | | I | | | | |
| AMP FOX TE ATM KF S STX | TBX + CTX | No | A | | | | I | | | | | | | | |
| AMP CIP TE KF NA SXT | TBX + CIP | Yes | B1 | | | | | | I | | | | | | |
| AMP CN CTX ATM CAZ KF | TBX + CTX | Yes | A | | | | | | I | | | | | | |
| AMP CIP CN TE KF S NA TOB SXT | TBX + CIP | No | B1 | | | | | | | | I | | | | |
| AMP CIP TE CTX KF S NA | TBX + CTX | Yes | B1 | | | | | | | | I | | | | |
| AMP CIP CN TE KF S NA SXT | TBX + CIP | No | D | | | | | | | | | | I | | |
| AMP CIP TE KF NA C TOB SXT | TBX + CIP | Yes | D | | | | | | | | | | I | | |
| AMP CIP TE S NA C | TBX + CIP | No | A | | | | | | | | | | I | | |
| AMP FOX TE CAZ AMC KF | TBX + CTX | No | A | | | | | | | | | | I | | |
| AMP CTX ATM KF S SXT | TBX + CTX | Yes | A | | | | | | | | | | I | | |
| AMP FOX CIP AMC KF NA | TBX + CIP | No | A | | | | | | | | | | | | I |
| AMP TE CTX KF C SXT | TBX + CTX | Yes | A | | | | | | | | | | | | I |
| AMP CN CTX ATM CAZ KF S NA C TOB SXT K | TBX + CTX | Yes | D | | | | | | | | | | | | I |

[a]Resistance phenotype of the imipenem-resistant isolate. 1: 19th September; 2: 5th October; 3: 19th October; 4: 3rd November; 5: 16th November; 6: 7th December.

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**Table 3** | Antimicrobial resistance profiles and respective enterococcal species of 34 isolates and their distribution per sample points and time points (1–6) of sample collection

| | | | | Number of isolates per sample and per sampling points | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | |
| **Antimicrobial resistance pattern** | **Isolation media** | **Vancomycin resistance genotype** | **Species** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** | **A** | **B** |
| TE | SB | | *E. faecalis* | I | | I | II | | | | | | | | |
| AMP RD E AZM CIP | SB + CIP | | *E. faecium* | | | I | | | I | | | I | | | |
| AMP TE RD E CN AZM CIP | SB + CIP | | *E. faecalis* | | | | | I | | | | | | | |
| AMP RD E CN AZM CIP | *SB* + AMP | | *E. faecium* | | | | | | | | | | I | | |
| AMP RD E CN VA AZM CIP | SB + VA | VanA | *E. faecium* | | | | | I | | | | | | | |
| AMP TE E VA AZM | SB + VA | VanA | *E. faecium* | | | | | | | I | | | | | |
| AMP QD TE E VA AZM | SB + VA | VanA | *E. faecium* | | | | | | | | | I | I | | |
| QD TE VA AZM | SB + VA | VanA | *E. faecalis* | | | | | | | | | I | | | |
| AMP RD E VA AZM CIP | SB + VA | VanA | *E. faecium* | | | | | | I | | | | | | |
| QD TE RD E CN AZM CIP | SB + CIP | | *E. faecium* | | | | | | | | | I | | | |
| QD TE E CN AZM CIP | SB + VA/SB + CIP | | *E. faecalis* | | | | | | | II | | | | I | |
| AMP TE E AZM | SB + AMP | | *E. faecium* | | | | | | | | | | | I | |
| QD TE RD AZM C | SB + CIP | | *E. faecalis* | | | | | I | | | | | | | |
| AMP TE RD E AZM CIP | SB + CIP | | *E. faecium* | | | | | I | | | | | | I | |
| TE E CN AZM CIP | SB + CIP | | *E. faecium* | | | | | | | | | | | I | |
| RD | SB + VA | | *E. casseliflavus* | | I | | | | | | | | | | |
| QD RD | SB | | *E. faecalis* | | | | I | | | | | | | | |
| QD TE | SB + VA | | *E. faecalis* | | | | I | | | | | | | | |
| AMP TE E AZM CIP | SB + AMP | | *E. faecium* | | | | | | I | | | | | | |
| QD TE E AZM CIP | SB + CIP | | *E. faecalis* | | | | | | II | | | | | | I |
| AMP RD E AZM CIP | SB + CIP | | *E. faecium* | | | | | | | | II | | | | |
| AMP E AZM CIP | SB + AMP | | *E. faecalis* | | | | | | | | | | | | I |

1: 19th September; 2: 5th October; 3: 19th October; 4: 3rd November; 5: 16th November; 6: 7th December.

can cause structural and numerical changes in the population of *E. coli*. Moreover, the day that preceded the last sample collection, from which was recovered a high variety of resistant strains, was the most rainy day of the month (http://www.ipma.pt/resources.www/docs/im.publicacoes/edicoes.online/20110111/gsdvtvtuIXrMgkFtHTzG/cli_20101201_20101231_pcl_mm_co_pt.pdf – in Portuguese), with the river water rising over the riverbanks and reaching the soil of diverse cultivation areas. In the previous months of sample collection (from May to September – dry season) those cultivation areas had been fertilized with farmyard manure. Thus, the first rainfalls (19th October) dragged the faecal bacteria to the river water, explaining the high CFU numbers upstream; yet, the lower CFU number observed at the same time point downstream may be explained by a sort of 'dilution effect' due to the discharge of the effluent from the WWTP. After the first intensive rainfall (from 3rd November on), the enumeration of *E. coli* and enterococci bacteria in water samples did not differ appreciably between upstream (point A) and downstream (point B) of the WWTP. Moreover, we may say that the disinfection process used at the WWTP before effluent disposal was effective, not allowing the release of significant amounts of bacteria to the stream. Nevertheless, the disinfection step,

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despite reducing the number of bacteria, sometimes can lead to a positive selection of isolates with antimicrobial resistance patterns (Galvin *et al.* 2010; Łuczkiewicz *et al.* 2010). However, that seems not to have happened since the percentage of antimicrobial resistance of *E. coli* isolates from point B was, in general, lower than from point A (Table 1).

Several isolates recovered from selective media (supplemented with antibiotics) proved not to be resistant to the same selective antibiotic in the antimicrobial susceptibility testing assay. This is probably because the bioburden placed in the selective medium was very heterogeneous and included bacteria that were able to produce enzymes that could degrade the antibiotic present in the medium, thus enabling the growth of susceptible strains. Nonetheless, the presumed antibiotic resistance given from CFU counts in the antibiotic-selective media appeared to be a good indicator of general resistance levels, for both *E. coli* and *Enterococcus* spp., as similarly reported by others (Alexander *et al.* 2008).

Approximately three-quarters of total *E. coli* isolates and more than half of *Enterococcus* spp. isolates showed resistance to multiple antimicrobial drugs simultaneously, which is a worrying number, revealing that this river can be a vehicle for the spread of antibiotic-resistant bacteria. The large heterogeneity in the antimicrobial resistance phenotypes was recorded throughout time, with little recurrence of the same resistance pattern, which emphasizes the instability and the environmental pressures that the river endures.

Among multidrug-resistant *E. coli* isolates, the major part showed resistance to ampicillin (85%), which reflects the confirmed large use of aminopenicillins in Portugal (European Surveillance of Antimicrobial Consumption Network – ESAC-Net, http://ecdc.europa.eu/en/activities/surveillance/ESAC-Net/database/Pages/consumption-by-group.aspx), followed by tetracycline (64%) and cephalothin (53%). The first two antibiotics are largely used in medicine and tetracycline is also used in animal production (Chopra & Roberts 2001). Cephalothin resistance was mainly associated with ESBL production; these enzymes hydrolyse oximino β-lactams, including the third generation cephalosporins (Moore *et al.* 2010). The presence of such a high number of cephalothin-resistant *E. coli* as well as a considerable number of ESBL-producing *E. coli* (28%) in river water may indicate that these isolates have a clinical origin and are associated with human-impacted environments. According to the study by Morris *et al.* (2009), some ESBL variants detected in the outflow of the WWTP generally correspond to the most common variants associated with clinical infection in the population. Additionally, one *E. coli* isolate showing resistance to 16 antimicrobials, including to imipenem (normally used as last treatment option to treat severe nosocomial infections) was also recovered from sample point A, suggesting that the river could be contaminated by effluents from a hospital source. Poirel *et al.* (2012) have searched for more carbapenem-resistant enterobacterial isolates in the same river, however, with no success, leading to the hypothesis that the aquatic environment could actually contain KPC-producing bacteria, which might represent the source of a future human colonization.

Several multidrug-resistant *E. coli* belonged to group D, which normally includes virulent extra-intestinal strains. This result emphasizes the potential risk factor for public health that represents the presence of these virulent strains in the river water.

Regarding the antibiotic resistance profiles of *Enterococcus* spp., many isolates displayed resistance to antibiotics infrequently used in the treatment of common bacterial infections, such as rifampicin, tetracycline, azithromycin and erythromycin. Resistance to rifampicin is a good indicator of river contamination via a human source, once this antibiotic had an almost exclusive use in human infections, particularly in the treatment of tuberculosis. The incidence of notified cases of tuberculosis in Portugal was 21/100,000 inhabitants in 2012 (http://www.portaldasaude.pt/NR/rdonlyres/8E0DFF04-F030-43B4-80EB-A71AD96F3718/0/relatorio_tuberculose_2012.pdf – in Portuguese). Despite a decrease having been registered over recent years, Portugal is still one of the European countries with higher incidence numbers. Therefore, there has been a continuous and long-term use of rifampicin, leading to the emergence of resistant bacteria. On the other hand, the resistance to tetracycline (41%), an antibiotic largely used in animal production (Lim *et al.* 2013), suggests bacterial contamination via an animal source, which is very likely to happen due to the presence of several animal husbandries and livestock farms throughout the course of the river, especially upstream of the sample points.

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The low proportion of ampicillin-resistant *Enterococcus* spp. contrasts with the high percentage of *E. coli* resistant to the same antibiotic. Martins da Costa *et al.* (2006) suggested as a possible explanation the exceptionally low capacity of ampicillin-resistant enterococci for epidemic spread. Six isolates showed resistance to vancomycin, presenting the VanA phenotype. Vancomycin-resistant enterococci are one of the most important causes of nosocomial infections (Méndez-Alvarez *et al.* 2000). However, glycopeptide-resistant *Enterococcus* spp. have been found not only in hospitals but also outside of them, namely in European commercial animal husbandry, where the glycopeptide avoparcin was used as growth promoter in the past. Therefore, the dissemination of drug-resistant *Enterococcus* spp., especially those resistant to vancomycin, gentamicin and ampicillin, reduces dramatically the therapeutic possibilities in enterococcal infections (Klare *et al.* 2003).

The 34 drug-resistant enterococci isolates evaluated for species identification belong to three different species: *E. faecium* ($n = 18$), *E. faecalis* ($n = 15$) and *E. casseliflavus* ($n = 1$). The predominance of the first two species was not surprising, suggesting both human and animal faecal contamination. Furthermore, *Enterococcus faecium*, according to the Infectious Disease Society of America, is one of the so-called ESKAPE pathogens (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), which highlights its capability to escape from the actions of available, effective therapies. An additional cause of concern is the fact that it is spreading globally (Molton *et al.* 2013).

## CONCLUSIONS

In conclusion, the main remarks of this study are the presence of high levels of indicators of faecal contamination and the great diversity of antimicrobial resistance profiles found among the major part of the *E. coli* and enterococci isolates, revealing that this river represents an important reservoir of antimicrobial-resistant bacteria and genes that can be disseminated among human pathogens, and commensal and environmental microorganisms. Similar results have been reported for other rivers, such as the San Pedro River in Mexico (Ramírez Castillo *et al.* 2013). The spread of antimicrobial resistance through surface waters and its inherent threat to human and animal health is becoming a worldwide concern.

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