River water analysis using a multiparametric approach: Portuguese river as a case study

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ABSTRACT

The Ave River in northern Portugal has a history of riverbanks and water quality degradation. The river water quality was assessed by physicochemical, biological (macroinvertebrates) and microbiological (*Enterococcus* spp. and *Escherichia coli*) parameters in six locations (A–F, point A being the nearest to the source) throughout its course during a year. Epilithic biofilms were studied through PCR-DGGE. Antimicrobial susceptibility testing helped with selecting isolates ($n = 149$ *E. coli* and $n = 86$ enterococci) for further genetic characterization. Pursuant to physicochemical and macroinvertebrates-based parameters, the river water was of reasonable quality according to European legislation (Directive 2000/60/EC). However, the microbiological analysis showed increased fecal contamination downstream from point C. At point D, four carbapenem-resistant *E. coli* isolates were recovered. Paradoxically, point D was classified as a point of Good Water Quality according to macroinvertebrates results. Point F presented the highest contamination level and incidence of multidrug-resistant (MDR) isolates in the water column (13 MDR enterococci out of 39 and 33 MDR *E. coli* out of 97). Epilithic biofilms showed higher diversity in pristine points (A and B). Thus, biological and microbiological parameters used to assess the water quality led to divergent results; an outcome that reinforces the need for a holistic evaluation.

Key words | *Enterococcus* spp., epilithic biofilms, *Escherichia coli*, macroinvertebrates, multidrug-resistant isolates, river water

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INTRODUCTION

The assessment of water quality is an essential measure within environmental monitoring. When water quality is poor, it affects not only the aquatic life but also the surrounding ecosystems. Rivers are unquestionably important parts of the hydrological cycle, mainly because they are fluxes of water and not reservoirs of water. Rivers, along with water, drag off sediments and other suspended materials (biotic and abiotic) that ultimately will reach all the other aquatic environments (Davies-Colley 2013). In 2000, the European Parliament and the Council of the European Union approved the Directive

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2000/60/EC (http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02,000L0060-20141120&qid=1506068744209&from=EN), which provided the guidelines to be followed by all member states in order to improve the ecological quality of the European Community surface waters. These guidelines included the simultaneous analysis of physical and chemical factors as well as biological parameters.

River water quality is routinely assessed using physico-chemical parameters, including temperature, conductivity, pH, dissolved oxygen, and total suspended solids (see www.epa.ie/water/wm/rivers/; www.epa.ie/pubs/reports/water/waterqua/Water%20Quality%20in%202016%20An%20Indicators%20Report.pdf). Furthermore, biological water quality assessment using macroinvertebrates is also an approach usually contemplated (Sharma & Rawat 2009; Bobori *et al.* 2018). The biodiversity of benthic macroinvertebrates present in an aquatic ecosystem, and their relative abundances, help to make inferences about the pollution burden, since they are good indicators of habitat/hydro-morphological alterations (Azrina *et al.* 2006; Balderas *et al.* 2016). Nevertheless, incorporation of microbiological parameters should also be included systematically in the assessment of water quality. It has been reported by others that the combination of different methods, such as physicochemical parameters and microbiological indicators, can provide a better estimation of pollution distribution (González-Fernández *et al.* 2010).

The microbiological quality of water is frequently assessed by quantifying indicators of fecal contamination (*Escherichia coli* and *Enterococcus* spp.), which can also signal the presence of other intestinal pathogens (Field & Samadpour 2007). Given the global problem of antimicrobial resistance, monitoring the aquatic environment, especially rivers, for the presence of resistant bacteria is of interest and may help in understanding the weight these environments have in the spread of antibiotic resistance.

The microbial consortia within a river is very complex, composed of both planktonic and attached bacteria, which are part of epilithic biofilms. Therefore, microbiological analysis of planktonic and attached bacteria should be simultaneously performed in order to provide more data that will help construct a more accurate snapshot of the environmental quality involving river water (Araya *et al.* 2003; Lyautey *et al.* 2005), in spite of being ignored by the above-mentioned EU directive.

The Ave River, located in the north of Portugal, has been considered one of the most polluted rivers both in Portugal and Europe (Soares *et al.* 1999), and for that reason it has been the focus of several studies (Poirel *et al.* 2012; Bessa *et al.* 2014; Kieffer *et al.* 2016). Such studies had exclusively assessed the microbial quality of the river, reporting worrying levels of antimicrobial resistance in fecal bacteria obtained from the river water.

Aiming at a more holistic evaluation of the Ave River water quality, in the present study a toolbox of parameters, including physicochemical, biological and microbiological, were used in spatial and temporal scales. From June 2014 to May 2015, five water samples and four epilithic biofilm samples were collected from six locations throughout the course of Ave River. Moreover, macroinvertebrate communities were also studied in three surveys coincident with three out of the five water samples collected.

MATERIALS AND METHODS

Study area and surveys

Six locations throughout the river course were selected for sampling. As shown in Figure 1, the six sites were not equidistant, because they were carefully chosen based on their location, either near a wastewater treatment plant (WWTP) or downstream of main tributaries. Contrarily to points A and B (upper part of the river), which may be deemed pristine site points, C and D (middle part) and E and F (lower part) are surrounded by a higher population density.

Physicochemical and microbiological parameters were assessed in the water samples collected in two 1-L sterile bottles from each location visited five times during a year (June 2014, September 2014, November 2014, February 2015 and May 2015). Epilithic biofilms were collected from submerged rocks at the same locations except during the May 2015 survey. Visible biofilms were scraped from the rocks using a brand new toothbrush into a sterile

Figure 1 │ Location of the six sites sampled along the Ave River in the north of Portugal. Wastewater treatment plants (WWTP) and hospitals situated in the surroundings of the river are also shown on the map.

100-mL flask and the volume was adjusted to 30 mL of sterile distilled water.

The assessment of the benthic macroinvertebrate communities was performed during three surveys (June 2014, November 2014 and May 2015). The procedure for the collection of macroinvertebrates followed the protocol established by a Portuguese entity (INAG 2008), which takes into account the recommendations of the European Directive 2000/60/EC.

All samples collected were kept at 4 °C, for no longer than 24 h, until further treatment in the laboratory.

The values of the precipitation on the day of each survey were retrieved from the site of 'Instituto Português do Mar e da Atmosfera', the Portuguese meteorological, seismic, sea and atmospheric organization (www.ipma.pt/pt/).

Physicochemical and environmental parameters

Temperature, pH, conductivity, total suspended solids (TSS) and dissolved oxygen were measured *in situ*, using a multiparameter instrument (Multiparameter pH/ORP/

EC/DO/Temperature Waterproof Meter, Hanna Instruments, Portugal). The concentration of dissolved nutrients such as phosphate (PO_4^{3-}), nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) were determined using a Multiparameter Bench Photometer C 200 (Hanna Instruments, Portugal) according to the instructions from the manufacturer. The Biochemical Oxygen Demand (BOD_5) was also determined according to the Winkler method (Winkler 1888).

Macroinvertebrates

Macroinvertebrate samples were sorted in the laboratory using sieves of 1 and 0.5 mm in order to be separated according to their size and to facilitate their visualization. Sieve contents were washed with running tap water and placed in a white tray to sort the organisms, which were then examined under a stereo microscope (Stemi 205, Zeiss, Germany). Finally, the macroinvertebrates were kept in plastic tubes containing 70% ethanol. Each macroinvertebrate was subsequently identified with the help of

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dichotomous keys from Tachet *et al.* (2003). Taxonomic resolution at a class-level was considered for Oligochaeta, and the remaining macroinvertebrates were identified at the family-level.

The analysis of macroinvertebrate communities was performed based on the determination of several indices and metrics, which allowed the evaluation of the ecological quality of the stream. The following metrics were determined: Shannon–Weaver Diversity Index; Pielou Evenness Index; *Índice Português de Invertebrados do Norte* (IPtIN), which is a Portuguese multimetric index (INAG 2009); the biotic index IBMWP (Iberian Bio Monitoring Working Party), which is highly sensitive to water quality and has been extensively applied in the Iberian Peninsula (Bonada *et al.* 2004); the Average Score per Taxon (ASPT), which is simply the IBMWP score divided by the number of scoring taxa in the sample, and ranges from 0 to 10; the Belgian Biotic Index (IBB) (De Pauw & Vanhooren 1983); and other metrics based on enumerations such as % EPT (Ephemeroptera, Plecoptera, Trichoptera), EPT/C (EPT/Chironomidae), % DF (Dominant Family), % ETO (Ephemeroptera, Tricoptera, Odonata, % FC (Filterer Collectors) (Balderas *et al.* 2016).

Isolation and quantification of *Escherichia coli* and *Enterococcus* spp.

The membrane filtration method was used for the isolation and quantification of indicators of fecal contamination, *E. coli* and *Enterococcus* spp., from water samples (Bessa *et al.* 2014). Each biofilm suspension was subjected to disaggregation in an ultrasonic water bath (Sonorex RK 52, Bandelin, Germany) for 15 min, prior to being processed as described above for water samples.

The media used, for both water and biofilm samples, were Tryptone Bile X- glucuronide agar (TBX) (BioKar Diagnostics, Beauvais, France), and Slanetz and Bartley agar (SB) (Oxoid, Basingstoke, UK) for *E. coli* and enterococci enumeration, respectively. Media supplemented with antibiotics were also used for the isolation of resistant isolates: TBX agar (for *E. coli*) supplemented with cefotaxime ($2 \mu g\ mL^{-1}$), ampicillin ($8 \mu g\ mL^{-1}$), ciprofloxacin ($4 \mu g\ mL^{-1}$) and imipenem ($2 \mu g\ mL^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA), and SB agar (for enterococci) supplemented with

vancomycin ($6 \mu g\ mL^{-1}$), ampicillin ($8 \mu g\ mL^{-1}$) and ciprofloxacin ($4 \mu g\ mL^{-1}$).

Antimicrobial susceptibility testing

A maximum of three colonies of *E. coli* and enterococci were picked up from each specific antibiotic-supplemented and non-supplemented medium for further antimicrobial susceptibility testing, which was carried out by the agar disk diffusion method as previously described (Bessa *et al.* 2014). Regarding *E. coli* isolates, 19 antimicrobial agents were used: ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid (AMC, 30 μg), cephalothin (KF, 30 μg), cefoxitin (FOX, 30 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), imipenem (IPM, 10 μg), aztreonam (ATM, 30 μg), gentamicin (CN, 10 μg), kanamycin (K, 30 μg), amikacin (AK, 30 μg), streptomycin (S, 10 μg), tobramycin (TOB, 10 μg), nalidixic acid (NA, 30 μg), tetracycline (TE, 30 μg), ciprofloxacin (CIP, 5 μg), nitrofurantoin (F, 300 μg), sulfamethoxazole/trimethoprim (SXT, 25 μg) and chloramphenicol (C, 30 μg). While for *Enterococcus* spp., 11 antimicrobial agents were used: ampicillin (AMP, 10 μg), gentamicin (CN, 120 μg), ciprofloxacin (CIP, 5 μg), tetracycline (TE, 30 μg), nitrofurantoin (F, 300 μg), vancomycin (VA, 30 μg), teicoplanin (TEC, 30 μg), erythromycin (E, 15 μg), rifampicin (RD, 5 μg), linezolid (LZD, 30 μg) and doxycycline (DO, 30 μg). All antimicrobial disks were purchased from Oxoid. The detection of extended spectrum β-lactamase (ESBL)-producing *E. coli* was based on agar plates observation according to the disk approximation test (Drieux *et al.* 2008). The interpretative criteria for antimicrobial susceptibility determination followed the CLSI recommendations (CLSI 2014).

Isolates were classified as multidrug-resistant (MDR – resistant to at least one agent in three or more antimicrobial categories), extensively drug-resistant (XDR – resistant to at least one agent in all but two or fewer antimicrobial categories) and pandrug-resistant (PDR – resistant to all agents in all antimicrobial categories) (Magiorakos *et al.* 2012).

Escherichia coli phylogenetic group determination and enterococci species identification

Escherichia coli isolates that presented an ESBL phenotype and/or resistance to six or more antimicrobial drugs were

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selected for DNA extraction and subsequent phylo-group assignment (Clermont *et al.* 2013). Accordingly, seven phylo-groups were considered (A, B1, B2, C, D, E, F). Generally, commensal strains belong to phylo-groups A and B1, while pathogenic strains usually fit into phylo-groups B2 and D.

The species of enterococci isolates that presented resistance to vancomycin and/or teicoplanin or to four or more antimicrobial drugs were then identified by performing a multiplex-PCR already described (Jackson *et al.* 2004).

Characterization of river biofilms by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE)

The bacterial DNA of the biofilm was extracted after centrifugation at $2,655 \times g$ for 10 min of 1 mL of biofilm suspension previously disaggregated in the ultrasonic bath for 15 min. The DNA extraction was carried out using the Powersoil DNA Isolation Kit (Mo Bio laboratories, USA), according to the instructions from the manufacturer.

PCR amplification of the variable regions V3–V5 of the 16S rRNA gene was performed with the primers 907R (5′-CCG TCA ATT CMT TTG AGT TT-3′) and 341F (5′-CCT ACG GGA GGC AGC AG-3′) with a 40 bp GC sequence clamped to its 5′ end (Lyautey *et al.* 2003). The 25-μL reaction mixture contained 200 μM of each nucleotide, 0.5 mM MgCl$_2$, 1× Complete KCl reaction buffer (Bioron GmbH, Germany), 0.4 μM of each primer, 1.25U of DFS-Taq DNA polymerase (Bioron GmbH, Germany) and 80–100 ng template DNA. The PCR was carried out in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using a program previously described (Schauer *et al.* 2000). PCR products (550 bp) were checked by electrophoresis in 1.5% agarose gels at 100 V for 1 h.

The DGGE was performed in a DGGE-4001 system (CBS Scientific Co, San Diego, CA, USA), using a 40–60% denaturing gradient (100% denaturing gradient was 7 M urea and 40% deionized formamide) in vertical polyacrylamide gels (6% w/v acrylamide in 0.5× TAE buffer). Electrophoresis was carried out on 0.5× TAE buffer at 61 °C for 16 h at 60 V. Gels were stained with GreenSafe Premium (NZYTech, Lisbon, Portugal) for 15 min as specified by the manufacturer. Image analysis was carried out

using the BioNumerics 7.5 software (Applied Maths, Sint-Martens-Latem, Belgium). For each sample, a densitometric profile was generated to determine the relative contribution of each band to the total signal in the sample lane. A UPGMA Jaccard distances dendrogram was generated from the DGGE profiles of the biofilm samples collected. Moreover, based on the relative intensities of the bands, diversity indices such as the Shannon–Wiener and Simpson indices were calculated.

Statistical analysis

Descriptive statistics were computed using IBM SPSS Statistics (23.0) for categorical and continuous variables. Relative and absolute frequencies were calculated for categorical variables, and maximum, minimum, median and interquartile range for continuous variables. The assessment of normality and homoscedasticity was completed as part of the testing assumptions. None of the continuous variables showed a normal distribution. Kruskal-Wallis 1-way ANOVA was applied to compare bacterial counts between different collection points and surveys. The Spearman correlation coefficient was used to assess the association between the counts of fecal bacteria found in the water column and biofilm. In both tests, the significance level was set at 0.05.

RESULTS

Analysis of physicochemical parameters

The results of all physicochemical and environmental parameters determined upon the Ave River water samples are shown in Table 1. Generally, no drastic changes occurred in each parameter analyzed along the river. The lower part of the river presented the most alkaline values of pH. The upper part of the river, being located in a mountain section, presented the lowest temperatures in relation to the other locations. The maximum variation in oxygen saturation was around 30%. Variations in this parameter occurred regardless of the location and the survey. An abnormal and rather high conductivity (213 μS cm^{-1}) was registered in point B in the survey of September 2014 and also, in the same survey, the highest values of BOD$_5$ were recorded.

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Table 1 | Physicochemical data recorded at the six locations of Ave River in five time points during a year

Sampling sites	Date of sampling	Water temperature (°C)	Conductivity (μS cm^{-1})	pH	Dissolved O$_2$ (ppm)	O$_2$ saturation rate (%)	BOD$_5$	NO$_3^-$ (mg L^{-1})	NO$_2^-$ (mg L^{-1})	NH$_4^+$ (mg L^{-1})	PO$_4^{3-}$ (mg L^{-1})
A	R1	17	14	7.31	8.36	92.30	2.60	0	0	0	0
	R2	18.50	15	6.86	8.51	84	1.30	0.15	0	0.30	1.01
	R3	10.73	7	7.45	9.20	85	1.45	0.07	0	0.22	1.09
	R4	6.99	9	5.98	11.07	95	0.57	0	0	0	0.83
	R5	18.38	32	8.90	8.59	95.10	0.71	0.30	0	0	0.13
B	R1	22.18	48	7.50	7.28	79	3.20	0.00	0	0.09	1.75
	R2	26.60	213	6.40	9.74	81.10	3.05	0.44	0	0.19	0.37
	R3	13.26	44	7.08	9.20	85	1.17	1.85	0	0.21	1.25
	R4	10.68	77	7.56	10.47	84.40	1.42	0	0	0	0.93
	R5	20.91	74	7.95	8.80	99	2.10	0.80	0	0	0.09
C	R1	22.10	75	6.98	6.29	73	4.15	0.02	0	0.13	1.86
	R2	23	48	6.60	10.40	65.30	5.12	0	0	0.32	0.13
	R3	13.33	42	7.46	9.52	88.60	1.32	3.10	0	0.15	0.19
	R4	10.07	47	7.43	10.28	90.40	0.33	7.68	0	0	0.13
	R5	21.30	94	7.57	8.03	91	2.03	0.93	0	0	0.15
D	R1	20.10	139	7.04	6.90	69.40	3.90	0.30	0	0.28	14.17
	R2	22.80	99	6.80	10.55	80	4.02	1.33	0	0.88	0.45
	R3	13.40	108	7.83	9.60	88.30	1.70	3.18	0	0.19	0.17
	R4	10.49	109	7.70	10.21	93.70	0.04	4.73	0	0	0.28
	R5	19.40	170	7.55	8.75	93	2.88	1.50	0.01	0.16	0.31
E	R1	21	141	8.02	6.72	72	1.98	0.15	0	0.66	33.00
	R2	22.50	193	7.50	10.13	70.20	4.24	1.03	0	0.44	1.53
	R3	13.56	148	8.53	9.73	89.50	1.50	0	0	0.23	0.37
	R4	10.80	133	8.09	10.73	98	2.49	8.01	0.02	0.01	0.48
	R5	20.68	222	6.66	8.33	91	2.22	1.80	0.01	0.10	0.86
F	R1	23.78	245	8.60	7.28	83.50	0.91	2.81	0.00	0.22	12.00
	R2	22.40	227	7.30	9.63	72.20	3.55	1.92	0.00	0.37	1.60
	R3	13.32	135	8.18	10.28	92.60	2.26	4.36	0.01	0.10	0.66
	R4	10.96	107	7.63	10.35	94.50	1.38	13.59	0.00	0.12	0.93
	R5	19.74	274	8.50	8.01	88	0.91	2.03	0.02	0.22	0.98

R1: June 2014; R2: September 2014; R3: November 2014; R4: February 2015; R5: May 2015.

The dissolved inorganic nutrients evaluated did not present a coincident variation among them throughout time and along the six sampling sites, as initially anticipated. Phosphates, represented by PO$_4^{-3}$, were particularly high in June 2014 in all the sampling sites analyzed. The highest values of ammonia were found in the lower part of the river. Nitrites were detected in very low concentrations (<0.025 mg L^{-1}), occasionally in the lower part of the river.

Macroinvertebrates-based metrics

From the analysis of the various metrics determined (Table 2), it was possible to infer on the ecological quality of the river points studied. The progression of the calculated indices throughout the sites is very similar. The Shannon–Weaver diversity index indicated a decrease in the water quality in the following order: A > D > B > F > E > C; A and C being the points where there was higher and lower diversity within the macroinvertebrate population, respectively. The Pielou equitability index suggested a similar trend on the water degradation: A > D > C > F > B > E. The results from the IPtIN index were alike (A > D > F > B > C > E), classifying point A as having a 'Good ecological status', points D, F and B as 'reasonable' and points C and E as 'medium'.

Equally, all the other metrics, except % FC, attributed to point A the highest ecological quality, followed by points D and F, which indeed presented a reasonable number of different individuals (Ephemeroptera, Plecoptera, Trichoptera and

Table 2 | Metrics retained from the biological (macroinvertebrates) assessment of samples collected over a year (R1: June 2014; R2: November 2014; R3: May 2015) at the six locations of Ave River

Sampling sites	Date of sampling	Shannon-Weaver	Pielou	IPtIN	IBB	IBMWP	ASPT	% EPT	EPT/C	% DF	% ETO	% FC
A	R1	1.48	0.46	0.80	10	169	6.76	42	1.04	44	41	9
	R3	2.55	0.79	0.82	10	159	6.36	44	1.24	35	27	27
	R5	2.28	0.74	0.92	10	179	8.14	62	2.58	24	56	14
B	R1	0.18	0.16	0.48	4	25	8.33	2	0.02	96	4	47
	R3	1.37	0.60	0.37	6	45	4.50	16	0.26	60	16	58
	R5	1.04	0.32	0.63	9	122	4.88	21	0.28	74	21	27
C	R1	0.64	0.92	0.35	4	10	5.00	33	0.50	67	33	24
	R3	0.54	0.28	0.35	5	32	4.57	8	0.09	85	8	58
	R5	0.62	0.24	0.37	6	56	4.31	7	0.08	89	7	49
D	R1	1.20	0.44	0.53	9	79	5.27	4	0.07	65	6	12
	R3	1.49	0.56	0.50	8	68	4.86	75	0.48	38	18	39
	R5	1.39	0.44	0.60	8	89	3.87	9	0.21	43	9	22
E	R1	0.63	0.39	0.37	5	24	4.80	5	0.10	52	5	24
	R3	0.87	0.42	0.41	7	40	5.00	8	0.13	65	8	23
	R5	0.56	0.18	0.20	3	18	3.60	0	0.00	69	0	14
F	R1	0.42	0.20	0.45	7	33	4.13	4	0.05	92	4	24
	R3	1.87	0.63	0.67	8	89	4.68	32	0.76	42	32	28
	R5	1.74	0.55	0.51	7	58	3.63	39	0.90	43	39	26

IPtIN: *Índice Português de Invertebrados do Norte* (Portuguese index); IBB: Belgian Biotic Index; IBMWP: Iberian Bio Monitoring Working Party; ASPT: Average Score per Taxon; EPT: Ephemeroptera, Plecoptera, Trichoptera; EPT/C: EPT/Chironomidae; DF: Dominant Family; ETO: Ephemeroptera, Tricoptera, Odonata; FC: Filterer Collectors.

Odonata) that indicate a good ecological environment. The % FC throughout all river points was notably higher in the survey of November 2014, which was also a time point presenting generally high levels of fecal contamination.

An additional outcome, which could be an indication of the decreasing quality of the water and habitat of the river, was the fact that an invading species of bivalve, *Corbicula fluminea*, was detected in all the three macroinvertebrates surveys, from point C downstream. It was most prevalent at point D: June 2014 ($n = 4$), November 2014 ($n = 2$) and May 2015 ($n = 120$).

Enumeration of the indicators of fecal contamination

Enterococci enumeration in the water column at different collection points revealed an increase from A to F. The contamination increased significantly ($p < 0.05$) from B to C. The same trend was observed regarding the levels of contamination by *E. coli*, in particular from B to C, although a significant difference was also noticed between point A and point B. The contamination levels of the water column by both fecal bacteria at different collection times did not differ significantly ($p > 0.05$).

The Spearman coefficient indicates a positive correlation between the number of fecal bacteria present in the water column and in the biofilm. This correlation was more evident in the case of *E. coli* (Spearman's rho = 0.770; $p < 0.001$), compared to enterococci (Spearman's rho = 0.579; $p = 0.003$).

Spatially, it was visible that site C seems to be a turning point, since it was from that location that the counts of both *E. coli* and enterococci obtained by the culture method started to be more substantial in the water stream (Figure 2) and also in the biofilm. Regarding the variation throughout the timeframe of a year, it was possible to observe that the fluctuations in the CFU numbers did not follow a pattern coincident with the rainfall. It is noteworthy that *E. coli* and *Enterococcus* spp. counts were significantly higher in the river biofilm than in the streaming water.

Antimicrobial resistance, diversity of *E. coli* phylogenetic groups and *Enterococcus* species

In total, from the four surveys (June 2014, September 2014, November 2014 and February 2015), and of both water and biofilm samples, 149 *E. coli* and 86 enterococci isolates were
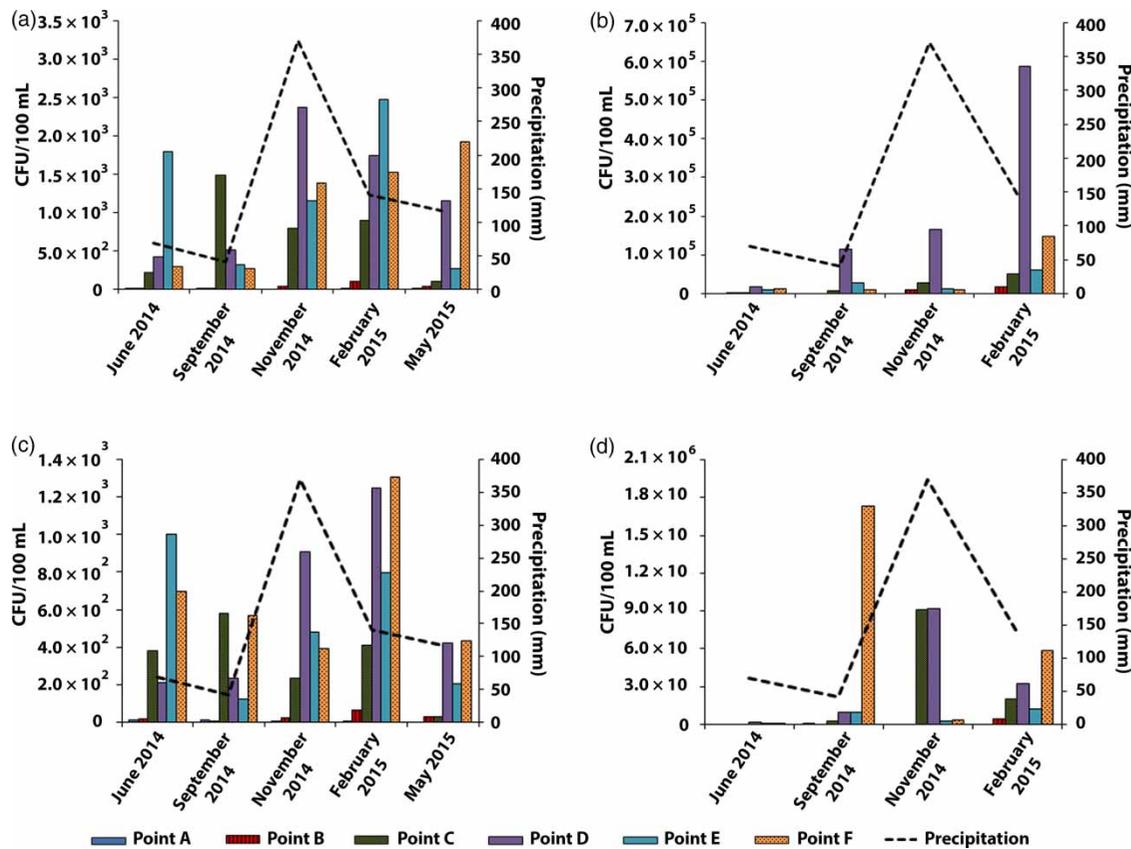
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Figure 2 │ *E. coli* and *Enterococcus* spp. enumeration in the river water samples (a and c, respectively) and in the epilithic biofilms (b and d, respectively) collected from the six collection points at different time points throughout a year. The values (in mm) of the precipitation recorded in the day of each survey were retrieved from the site of 'Instituto Português do Mar e da Atmosfera' (www.ipma.pt/pt/) and are shown in each graph.

selected for further studies after analysis of the antimicrobial susceptibility results. When isolates obtained from the same sample had the same antimicrobial resistance profile, only one was selected. In a few cases, isolates obtained from antibiotic supplemented media end up not presenting resistance to the concrete antibiotic in the medium.

The most relevant profiles as well as the origin of 52 MDR *E. coli* isolates amongst the 149 isolates studied are shown in Table 3. All the 149 *E. coli* isolates presented resistance to AMP (ampicillin) and to TE (tetracycline), and the majority (77.9%) was MDR, one was XDR and the other was PDR. Four carbapenemase-producing *E. coli* were obtained from the water sample collected from point D in February 2015. As shown in Figure 3, the majority of the MDR isolates was obtained from the water samples. Overall, the collection points D, E and F were those from which more MDR *E. coli* isolates were recovered. Though MDR isolates started to appear at point B, it was at point

C that such occurrence may lead to foreseeing a tendency of increase.

Escherichia coli isolates presenting an ESBL phenotype were obtained from all sampling sites and in all collection times. Overall, 67 ESBL-producing *E. coli* were obtained. The major part of MDR *E. coli* isolates belonged to the phylogenetic groups A or B1 (Table 3). The PDR isolate belonged to the phylo-group E, which is closely related to phylo-groups D and B2 that are known to enclose mostly pathogenic strains.

The numbers of MDR enterococci were lower than the counts of MDR *E. coli*. Besides that, MDR enterococci were, as were MDR *E. coli*, detected in higher numbers in the water than in the epilithic biofilms. The incidence of MDR enterococci was more marked in water samples from points D and F, especially in the survey of June 2014 (Figure 4). Curiously, at point E a breakout in the trend was observed. Nonetheless, point C could also be deemed

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Table 3 | Antimicrobial resistance profiles, origin and phylogenetic group of 52 multidrug-resistant (MDR) E. coli isolates selected among the 149 isolates studied

Date of sampling	Sampling sites	Sample	Antimicrobial resistance profiles	No. of isolates	Phylogenetic group
R4	D	Water	AMP FOX IPM CIP CN F TE KF K SXT CAZ CTX AK S NA C TOB AMC ATM[a]	1	E
R4	D	Water	AMP FOX IMP CIP CN TE KF K SXT CAZ CTX AK S NA TOB AMC ATM[b]	1	A
R4	D	Water	AMP FOX CIP CN TE KF K SXT CAZ CTX S NA C TOB AMC[c,d]	1	F
R4	D	Water	AMP FOX CIP CN KF K SXT CAZ S NA TOB AMC ATM[d]	1	E
R4	C	Bofilm	AMP CIP CN TE KF K SXT CTX S NA TOB ATM[c]	1	A
R1	E/F	Biofilm	AMP CIP CN TE S NA C TOB SXT K[c]	2	A
R1	C/E	Water	AMP FOX CIP CN TE AMC KF S NA[c]	2	B1
R1	B/D	Water	AMP CIP TE KF S NA SXT[c]	4	A
R3	E/F	Water	AMP CIP TE KF CTX S NA[c]	3	B1/A
R4	C/F	Water	AMP TE KF SXT CTX S NA[c]	2	B1/A
R1	C	Water	AMP CIP TE NA SXT K	2	B1/A
R1	F	Water	AMP FOX TE AMC KF NA[c]	2	B1/A
R3	B/C/D/F	Water/ Biofilm	AMP CIP TE SXT S NA	6	B1/A
R3/R4	C/E	Water	AMP TE KF SXT CTX S	3	B1/A
R1/R2/R4	E/F	Water/ Biofilm	AMP CIP TE NA	4	A/B1
R1	D	Water	AMP TE KF NA[c]	2	F
R1/R2	D/E/F	Water	AMP CIP NA SXT	4	A
R2/R3/R4	D/E/F	Water	AMP TE CTX KF[c]	4	A
R1/R2	D/F	Water	CIP TE NA	4	A
R3	F	Water	AMP CN TE KF SXT CTX S NA TOB[c]	1	B2
R4	F	Biofilm	AMP TE KF NA	1	B2
R2	D	Water	AMP CTX KF	1	D

[a]PDR: Pandrug-resistant.
[b]XDR: Extensively drug-resistant.
[c]ESBL producers.
[d]These isolates presented intermediate resistance to imipenem. R1: June 2014; R2: September 2014; R3: November 2014; R4: February 2015. AMP: Ampicillin; FOX: Cefoxitin; IPM: Imipenem; CIP: Ciprofloxacin; CN: Gentamicin; F: Nitrofurantoin; TE: Tetracycline; KF: Cephalothin; K: Kanamycin; SXT: Sulfamethoxazole/Trimethoprim; CAZ: Ceftazidime; CTX: Cefotaxime; AK: Amikacin; S: Streptomycin; NA: Nalidixic Acid; C: Chloramphenicol; TOB: Tobramycin; AMC: Amoxicillin/Clavulanic Acid; ATM: aztreonam.

a turning point, from where MDR enterococci started to appear. Exceptionally, at point A, one MDR *Enterococcus* spp. was recovered from the water sample collected in November 2014.

The most frequent antibiotic resistance found among the enterococci was towards tetracycline (TE – 52.3%). Resistance to AMP, nitrofurantoin (F), and chloramphenicol (C) were less frequent. Some of the most relevant profiles amongst the 59 antimicrobial-resistant enterococci isolates and their respective origins are presented in Table 4. Isolates displaying simultaneous resistance to ciprofloxacin (CIP), teicoplanin (TEC), and vancomycin (VA) were consistently found in June and September 2014 at points D, E and F.

Overall, 17 isolates presenting phenotypic resistance to vancomycin (VA) were obtained; 15 from water samples and two from biofilms. They were only detected in samples from points D, E and F. In the last survey, in February 2015, only one vancomycin-resistant *Enterococcus* spp. was obtained and it was from point F.
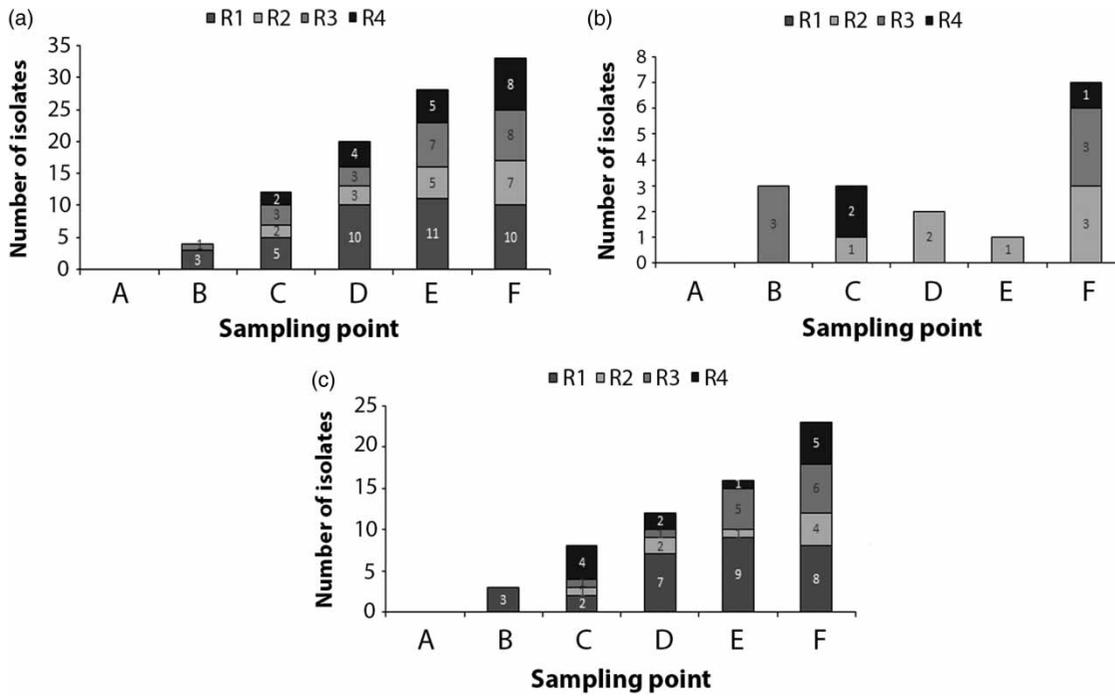
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Figure 3 | Distribution of MDR *E. coli* isolates in the water column (a) and in the biofilm (b), and of ESBL-producing *E. coli* in the water column (c). R1: June 2014; R2: September 2014; R3: November 2014; R4: February 2015.
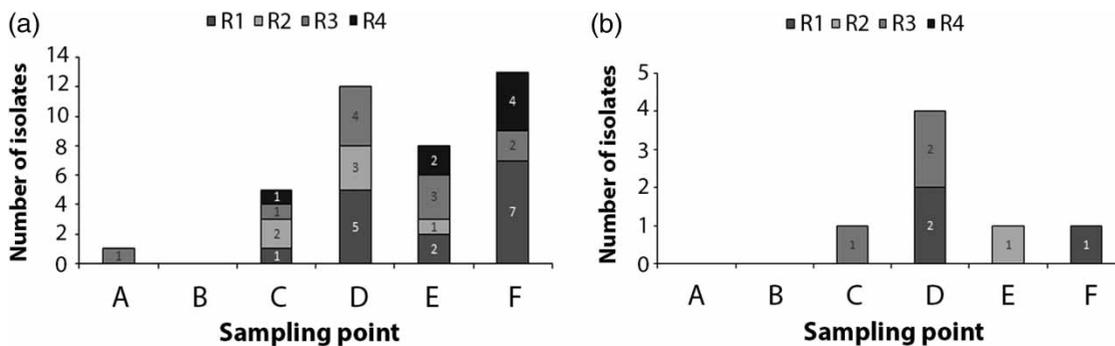

Figure 4 | Distribution of MDR enterococci isolates per sampling point and survey, in the water column (a) and in the biofilm (b). R1: June 2014; R2: September 2014; R3: November 2014; R4: February 2015.

Regarding the identification of the species of *Enterococcus*, it was found that the most common species were *Enterococcus faecalis* and *Enterococcus faecium*, followed by *Enterococcus durans* and *Enterococcus mundtii*.

Analysis of the bacterial communities within the biofilms

Diversity in the composition of bacterial epilithic biofilms collected at three surveys (June 2014, September 2014 and November 2014) was explored by DGGE of the PCR-amplified 16S rRNA gene fragments. Based on the DGGE profiles, an UPGMA dendrogram from cluster analysis using the Jaccard coefficient was constructed (Figure 5). Spatial and temporal differences within the epilithic biofilms microbial community could be clearly observed. The closest related genetic fingerprints presented a similarity of 70%. Overall, the genetic fingerprints obtained from the survey of November 2014 were notably distinct from those obtained from June and September 2014. Assuming that

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Table 4 | Antimicrobial resistance profiles, origin and species identification of 34 enterococci isolates selected among the 59 isolates studied

Date of sampling	Sampling sites	Sample	Antimicrobial resistance profiles	N° of isolates	Species identification
R1	D	Water	AMP TE RD E DO CN CIP TEC VA	1	*E. feacalis*
R1	F	Water	AMP RD E CN LZD CIP VA	1	*E. faecium*
R1	F	Water	TE RD E DO CN CIP C	1	*E. faecalis*
R3	E	Water	AMP TE RD E CIP TEC VA	1	*E. faecalis*
R4	C/E/F	Water	AMP TE RD E CIP	4	*E. faecium/E. faecalis*
R1	C/D/E	Water/Biofilm	AMP RD E CIP	6	*E. faecium/E. faecalis*
R1	F	Water	TE DO CIP TEC VA	1	*E. faecium*
R2/R3	C/D/E/F	Water	AMP RD E CIP	4	*E. faecalis/E. faecium/E. hirae*
R1	D/F	Water/Biofilm	AMP RD E CIP	2	*E. faecium/E. faecalis*
R1	D	Water	TE DO TEC VA	1	*E. faecalis*
R1/R2	D/E/F	Water	CIP TEC VA	6	*E. durans/E. mundtii/E. faecium/ E. faecalis*
R2	D	Water	TE CIP VA	1	*E. durans*
R3	E	Water	TEC F VA	1	*E. faecalis*
R1	F	Biofilm	F VA	1	*E. faecium*
R1	E	Water	TEC VA	1	*E. faecalis*
R2	D	Biofilm	CIP VA	1	*E. mundtii*
R4	E	Biofilm	TEC F	1	*E. faecium*

R1: June 2014; R2: September 2014; R3: November 2014; R4: February 2015.
AMP: Ampicillin; TE: Tetracycline; RD: Rifampicin; E: Erythromycin; DO: Doxycycline; CN: Gentamicin (120 μg); LZD: Linezolid; CIP: Ciprofloxacin; TEC: Teicoplanin; VA: Vancomycin; F: Nitrofurantoin.
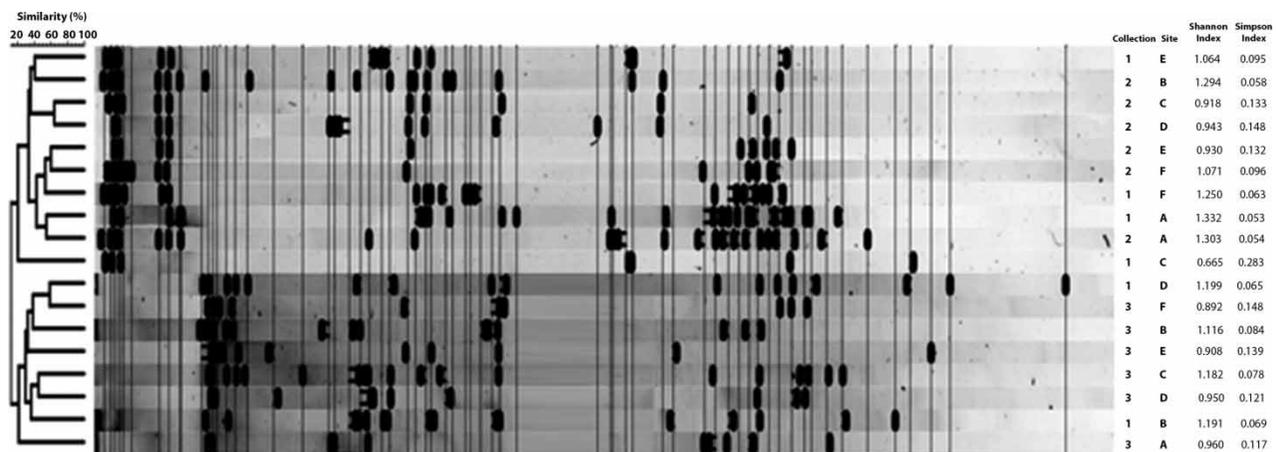
Figure 5 | UPGMA Jaccard distances dendrogram of DGGE profiles obtained after PCR amplification of bacterial 16S rRNA of the biofilms collected at the six sampling sites in the first three surveys.

each band represents an operational taxonomic unit (OTU), the profiles obtained from samples collected in June and September 2014 from point A presented higher similarities between each other and a higher number of OTU in comparison to the sample collected from the same site in November 2014. The genetic fingerprint obtained from

point C in June 2014 was the one that showed the lowest number of OTU (six bands), while the samples collected from point A in June and September 2014 originated profiles with the highest number of OTUs registered, i.e. 22 OTUs.

The values of the calculated diversity indices are shown in Figure 5. Higher values obtained for the Shannon–Wiener index represent higher diversity. The values of the Shannon–Wiener index ranged between 0.8918 and 1.332. Equally, the Simpson index (that ranges between 0 and 1) indicates that the closer to 0 the values, the higher diversity is present in the community. Thus, the two diversity indices were in accordance. The biofilms collected from point A, both in June and in September, were the most diverse in their composition, while the biofilms collected from point C, in the same dates, presented lower diversity.

DISCUSSION

Through the years, the Ave River has not been in compliance with the requirements needed to attest its water quality (Costa 2008). For that reason, many campaigns and actions to clean up Ave River have been carried out by competent national entities, namely TRATAVE (entity responsible for the wastewater treatments on Ave River and its tributaries). The physicochemical quality of the water has improved, however, some issues regarding its microbiological quality are still continuing, as demonstrated by our results.

Despite suffering occasional variations, probably due to punctual discharges into the river, all the physicochemical parameters analyzed throughout time in all sampling sites were within the values recommended to classify the river water as having reasonable quality, according to the European water framework directive (Directive 2000/60/EC). Only the concentration of phosphates consistently surpassed the limit values (0.10 mg L^{-1}), particularly in the summer time, indicating a possible detergent contamination from sewage water.

Macroinvertebrates are used in the biomonitoring of surface waters since they are good indicators of habitat/hydromorphological alterations (Balderas *et al.* 2016). The analysis of the macroinvertebrate metrics studied showed that the ecological quality of the river did not diminish throughout

its course (from point A to point F). Indeed, point D was deemed by the macroinvertebrate-based monitoring metrics as a point of 'Good Water Quality'.

Therefore, physicochemical and macroinvertebrates-based parameters retrieved results that could classify the river water of, at the least, satisfactory quality (Directive 2000/60/EC; INAG 2009). However, on the contrary, microbiological parameters, which are not included in the European Directive, revealed an increase in fecal contamination, especially downstream from point C, where the levels of contamination by *E. coli* (CFU 100 mL^{-1}) have increased by about 17.5 times in relation to point B. At point D the levels of fecal contamination were rather high. There, in the total of the four surveys, in the water column, 20.6% (20 out of 97) MDR *E. coli* and 30.8% MDR enterococci (12 out of 39) were obtained. Thus, the high numbers of fecal indicators obtained, around 1,000 CFU 100 mL^{-1} or higher, are not reliable with the apparent good quality of the river making the river water not appropriate for either irrigation or treatment aimed at human consumption, according to the existing legislation (Directive 2000/60/EC).

This increase in the number of fecal contamination indicators throughout the river is not unexpected, once the population density surrounding the river also increases, especially from point C onward, and moreover the agriculture environment gives place to industrial activities. Thus, the entry of non-treated waters (originated from agriculture, animal farming and urban waters) can occur and explain the increase in fecal contamination throughout the river.

The contamination of a water stream by *E. coli* is intimately correlated with hydrological conditions, such as rainfall and its consequent dragging effects (Ratajczak *et al.* 2010). However, that was not verified in this study; the peak of rainfall occurred in the third sampling collection, on November 2014, while the highest contamination by *E. coli* was observed in the fourth collection (February 2015), when the precipitation was relatively low. At point C, the highest number (CFU 100 mL^{-1}) of *E. coli* was registered in June 2014, when there was dry and hot weather. This contamination may be explained by some occasional discharges into the river and/or by the 'concentration effect' occasioned by the reduction in water level during the summer time, which is usually quite marked in Portugal

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due to the low rainfall in that season of the year. An association between rainfall and increase of enterococci CFU counts was also not observed. Furthermore, the progression of contamination by *E. coli* and by enterococci did not overlap. Overall, the CFU numbers of *E. coli* were higher than those of *Enterococcus* spp., which is not surprising since *E. coli* predominate over enterococci in the gastrointestinal tract (Dubreuil 2012).

When comparing the CFU numbers (both of *E. coli* and enterococci) obtained in the river water and in the biofilm, it becomes clear that those numbers are much greater in the biofilm than in the water, which is in accordance with previous studies (Craig *et al.* 2004; Balzer *et al.* 2010). Nevertheless, a positive correlation was still detected between the number of fecal bacteria present in the water column and in the biofilm, especially in the case of *E. coli*. This was an interesting outcome that will help to outline the risk management of the river.

At each point, the variation of % FC throughout time followed the same progression as the fecal contamination, which may suggest that the proliferation of filterer collectors may be due to the increase of fecal contamination.

Significant numbers of MDR isolates were also obtained in this study. The appearance of MDR *E. coli* at point B, deemed as pristine, could be a worrying fact, nonetheless, its appearance was not consistent throughout time (they were only recovered in June 2014 and November 2014). The incidence of MDR *E. coli* and enterococci was particularly noticeable in water samples from points D, E and F. In the surroundings of these points of the river, there are WWTPs, whose treated effluents are launched into the river. However, it is known that WWTPs are not 100% effective in eliminating bacteria. Previous studies have demonstrated that WWTPs in Portugal could only reduce and not totally eliminate the bacterial load in the outflow (Martins da Costa *et al.* 2006, 2007). Moreover, they also concluded that those WWTPs could select MDR strains and favor the accumulation of genes of antibiotic resistance. Indeed, WWTPs can receive in the same tank not only municipal wastewater, but also wastewater from hospitals and industries, allowing the contact of antibiotic residues with bacteria and consequently favoring the occurrence of horizontal transference of resistance genes.

In 2010, a KPC-producing *E. coli* was isolated from this river (Poirel *et al.* 2012), being the first report of that kind of carbapenemase-producing *E. coli* in Portugal. Now, in our study of February 2015, four other carbapenemase-producing *E. coli* (one PDR, one XDR and two MDR) were isolated approximately 9.5 km upstream of point D. A molecular analysis confirmed that the PDR and XDR isolates harbored the bla_{IMP-8} and bla_{VIM-1} genes, respectively, and that the two MDR isolates, showing intermediate resistance to imipenem, harbored one of the two genes, bla_{IMP-8} and bla_{VIM-34} (Kieffer *et al.* 2016).

MDR enterococci were also frequent in water samples collected from point D, including isolates that presented resistance to vancomycin (mainly *E. faecalis*).

The presence of carbapenemase-producing *E. coli* and vancomycin-resistant enterococci impelled us to contact the hospitals nearby in that region in order to know if they had had cases of infections by bacteria with similar profiles of antimicrobial resistance. None of the hospitals had reports of cases of infection by carbapenemase-producing *E. coli* in the timeframe of this study and it is known that vancomycin-resistant enterococci have been rarely found in the clinical setting of that region. Although the national scenario of the epidemiological situation of carbapenemase-producing *Enterobacteriaceae* has worsened from 2013 to 2015 from 'sporadic occurrence' to 'sporadic hospital outbreaks', respectively (Albiger *et al.* 2015), the regional hospitals near Ave River do not contribute to such statistics. That fact is also corroborated by the scarcity of *E. coli* belonging to the phylo-groups B2 and D found amongst the set of MDR *E. coli* isolates obtained in this study. Therefore, as apparently these isolates were not originated in the hospital setting, their presence in the river remains quite enigmatic. It can be hypothesized that low concentrations of these last-resort antibiotics or others to which they are resistant by co-selection mechanisms could be in the water and thus allow the development of resistance or the enrichment of resistant strains in the water. Another hypothesis could be the presence of some chemicals or pollutants that had the capacity to allow/favor the acquisition of these kind of antibiotic resistances, especially to carbapenems, since the carbapenemase-producing *E. coli* found in Ave River in 2010 and 2015 harbored different genes (Poirel *et al.* 2012; Kieffer *et al.* 2016).

The presence of MDR isolates in the epilithic biofilms was rather lower than those detected in the water column. These differences account for the risk evaluation of dissemination of resistance. The resistant bacteria floating in the water column represent a high risk for the rapid dissemination of resistance, since such bacteria will quickly spread downstream. The resistant strains present within the biofilm can contribute to the dissemination prolonged in time, representing a temporal risk and favoring the horizontal transfer of genes conferring antibiotic resistance among bacteria. Biofilms can also work as suppliers of resistant bacteria to the water column, especially when there are torrents and increased flow. The fact that higher levels of antibiotic resistance were found in the water column than in the biofilm reveals the difficulty of resistant strains in integrating multispecies biofilms.

Considering that the bacterial community existing in the water column and in the river biofilms are substantially different (Beier *et al.* 2008), and based on the premise that the changes in the community within the biofilm are indicative of changes in the aquatic ecosystem (Lear *et al.* 2008), the variations in those communities through the PCR-DGGE approach were also analyzed. Overall, for each sampling point, the results showed that the bacterial composition of the epilithic biofilms collected in June and September 2014 were quite similar, however changing considerably in November 2014. This was probably due to rainfall that occurred in the month of November, previously to the survey, in which the river flow increased, causing resuspension of the sediment and thus altering the composition of the microbial communities within the epilithic biofilms (Lear *et al.* 2008).

Through the analysis of the diversity indices calculated, we verified that the upper part of the river generally presented higher diversity in their bacterial communities in respect to points D, E and F. At point C, there was little diversity in the first two surveys and more variability in the last one. This loss of diversity in the bacterial community at points D, E and F match with the increase of fecal indicators of contamination in the biofilms at those points, which may led us to infer that the fecal bacteria are able to dominate over some of the original environmental bacteria. Furthermore, several other factors account for changes in the community composition, for instance, an increase in the temperature of the water has great influence on the metabolism of some bacterial families that are less thermotolerant and may not survive in such conditions (Araya *et al.* 2003).

CONCLUSIONS

This study confirms the need of evaluating simultaneously multiple parameters to fully assess the quality of river water. Nowadays more than ever, the quantification of indicators of fecal contamination, and the determination of their antimicrobial susceptibility profiles may be important parameters to complement the physicochemical and biological ones. Changes in the biological communities, either microbial or of macroinvertebrates, portray alterations suffered by the watercourse due to climatic changes or pollution and anthropogenic activities.

Though macroinvertebrate-based monitoring metrics may be helpful in obtaining an ecological assessment of the river, they may not be adequate to mirror the overall water quality. More specific studies in the critical points of the river identified in this study still have to be performed in order to better understand the microbial community and the river water dynamics, as well as studies on the relation between fecal contamination and functional feeding groups under a controlled environment.

The fecal contamination increased downstream as the population density and industrial, livestock and agricultural explorations increased. Point F presented the highest contamination level and presence of MDR *E. coli* (including ESBL phenotypes) and enterococci (including vancomycin-resistant *E. faecalis* and *E. faecium*). However, point D was considered the most worrisome in terms of isolates showing resistance to last-line antibiotics exclusively for human use, such as imipenem (IMP). Pinpointing throughout the river the presence of isolates presenting resistance to last-line antibiotics and being able to analyze the distribution of isolates with a huge heterogeneity in their resistance profiles, for both *E. coli* and enterococci, is of great importance to understanding the critical points in the river and gain awareness that rivers can be the means of dissemination and sources of emergence of MDR strains.

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