Incidence of enterococci resistant to clinically relevant antibiotics in environmental waters and in reclaimed waters used for irrigation

Silvia Monteiro and Ricardo Santos

ABSTRACT

Treated wastewater discharged into the environment or reused in different activities can be a major vehicle for the transmission of antibiotic-resistant bacteria and antibiotic-resistance genes. In this study, environmental and wastewater samples, collected at different stages of treatment, were studied to identify the possibility of a positive selection of antibiotic-resistant organisms in wastewater treatment plants (WWTPs). Enterococci were isolated, characterized into the main human species, and subjected to the Kirby–Bauer test using seven antibiotics (five classes): ampicillin, chloramphenicol, ciprofloxacin, gentamicin, linezolid, tetracycline, and vancomycin. Furthermore, vancomycin-resistant enterococci (VRE), a major cause of nosocomial infection, was identified, and the genes *vanA* and *vanB* detected directly in the samples and in all confirmed VRE. Data showed that WWTPs were able to reduce the levels of antibiotic resistance, although 72% of the disinfected wastewaters still presented antibiotic-resistant enterococci. VRE were detected in 6% of the samples, including in reclaimed waters. UV disinfection was not effective at removing VRE and multiple antibiotic-resistant (MAR) enterococci, most commonly *Enterococcus faecalis*. The use of reclaimed water containing VRE and MAR enterococci in crop production, irrigation of urban gardens, and street cleaning increases immensely the potential risk to human health.

Key words | antibiotic resistance, enterococci, environmental waters, Kirby–Bauer test, vancomycin

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HIGHLIGHTS

- 72% of disinfected wastewater with antibiotic-resistant enterococci.
- Vancomycin-resistant enterococci detected in reclaimed water.
- UV disinfection ineffective at removing vancomycin-resistant and multiple antibiotic-resistant enterococci.
- Vancomycin-resistant enterococci isolates increase in the WWTP in comparison to total number of enterococci.

GRAPHICAL ABSTRACT

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INTRODUCTION

Antimicrobial substances are one of the most important developments in modern medicine, contributing to the steep decrease in mortality and morbidity. Modern medicine relies heavily on the effectiveness of antimicrobial substances, including antibiotics, for the prevention and treatment of infections (Banin *et al.* 2017). Due to the vast misuse and overuse of antibiotics, bacteria started developing resistances other than the natural ones, which was first documented in the early days of the antibiotic era (Kon & Rai 2016). Antibiotic resistance is the capacity of bacteria to resist the action of a given antibiotic, action to which they were previously sensitive. The resistance to antibiotics is intensifying worldwide with new mechanisms emerging and spreading, threatening the capacity to treat regular infectious diseases and ultimately leading to an increase in the health risk to the population. In regions of the globe where antibiotics can be freely acquired without a prescription, where standard treatment guidelines are non-existent, and where counterfeit antibiotics can be bought on the streets, the emergence of antibiotic resistance dramatically increases (Patterson 2014; WHO 2017a). The World Health Organization (WHO) published a priority list of antibiotic-resistant bacteria (ARB; WHO 2017b). The list is represented by a total of 12 bacterial families considered to represent a risk to human health and stratified into three categories: critical, high, and medium (Table 1).

An issue that was once thought to be restricted to clinical environments has now, for a long time, crossed the barrier into the environment. Water is one of the most important reservoirs for the dissemination and one of the main routes of transmission of antibiotic resistance (Wellington *et al.* 2013; Karkman *et al.* 2019), particularly because wastewater treatment plants (WWTPs) receiving sewage from domestic dwellings and hospitals are unable to fully remove ARB and antibiotic-resistance genes (ARGs) (Li *et al.* 2015; Rodriguez-Mozaz *et al.* 2015). This can pose a serious problem since insufficiently treated wastewater is released into environmental waters and may be reused as irrigation water (Rodriguez-Mozaz *et al.* 2015). Enterococci, widely dispersed in the environment, are known to be a multidrug carrier, contributing to their resistance to treatment (Arias *et al.* 2010; Ahmed & Baptiste 2018). Vancomycin-resistant enterococci (VRE) represent one of the most important pathogens in clinical infections. As a result, VRE is included in the WHO list as a high priority pathogen (WHO 2017b).

The aim of this study was to investigate the prevalence of multidrug-resistant enterococci and VRE in environmental sources (throughout the different wastewater treatments, in reclaimed wastewater used for municipal park irrigation and street cleaning and in the receiving waters) and to determine the antibiotic-resistance profiles of the enterococci, using the Kirby–Bauer's disk diffusion test. This test was chosen over other tests because it has been widely used and standardized, and it is the test recommended by the WHO (Khan *et al.* 2019). This is one of the few studies addressing the issue of the presence of multiple antibiotic-resistant (MAR) enterococci in reclaimed water currently used for the irrigation of city gardens and in street washing.

Table 1 | WHO priority pathogen list

WHO priority pathogen list for new antibiotics research
Priority 1: Critical
Acinetobacter baumannii, carbapenem-resistant
Pseudomonas aeruginosa, carbapenem-resistant
Enterobacteriaceae, carbapenem-resistant, third-generation cephalosporin-resistant
Priority 2: High
Enterococcus faecium, vancomycin-resistant (VRE*fm*)
Staphylococcus aureus, methicillin-resistant, vancomycin-intermediate and -resistant
Helicobacter pylori, clarithromycin-resistant
Campylobacter, fluoroquinolone-resistant
Salmonella spp., fluoroquinolone-resistant
Neisseria gonorrhoeae, third-generation cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: Medium
Streptococcus pneumoniae, penicillin-non-susceptible
Haemophilus influenzae, ampicillin-resistant
Shigella spp., fluoroquinolone-resistant

MATERIALS AND METHODS

Sample collection

Environmental samples were collected from three WWTPs situated across Portugal. All WWTPs consist of a conventional biological secondary treatment and disinfection treatment by UV radiation (Table 2).

In WWTP2 and 3, the system is bipartite with a part of the treated wastewater relocated to further disinfection treatment (including UV disinfection and filters) for usage in municipal park irrigation and street cleaning (Figure 1(a)).

Additionally, two sampling sites upstream (receiving water 1, RW1) and downstream (receiving water 2, RW2) of WWTP1 were collected and analyzed (Figure 1(b)). At each sampling location, 500 mL of water was collected in

Table 2 | Description of the locations, including the WWTPs, sampled in this study

Location	Population served (×1000)	WWTP flow (×1000 m^3/day)	Sampling location	No. of samples	Mean concentration enterococci (CFU/100 mL)	Total no. of isolates	No. of isolates per sampling location
WWTP1	28	4	Post-filtration	5	4.10×10^2	73	24
			Treated wastewater	5	1.49×10^2		30
			Disinfection	5	1.00×10^1		19
RW1				5	3.19×10^2	9	9
RW2				5	3.60×10^2	19	19
WWTP2	760	181	Raw wastewater	5	8.46×10^6	46	16
			Treated wastewater	5	3.24×10^5		9
			Disinfection	5	8.56×10^4		10
			Reclaimed water	5	1.40×10^1		11
WWTP3	211	54	Raw wastewater	5	1.20×10^7	39	10
			Disinfection	5	3.00×10^0		10
			Reclaimed water	5	4.00×10^0		19

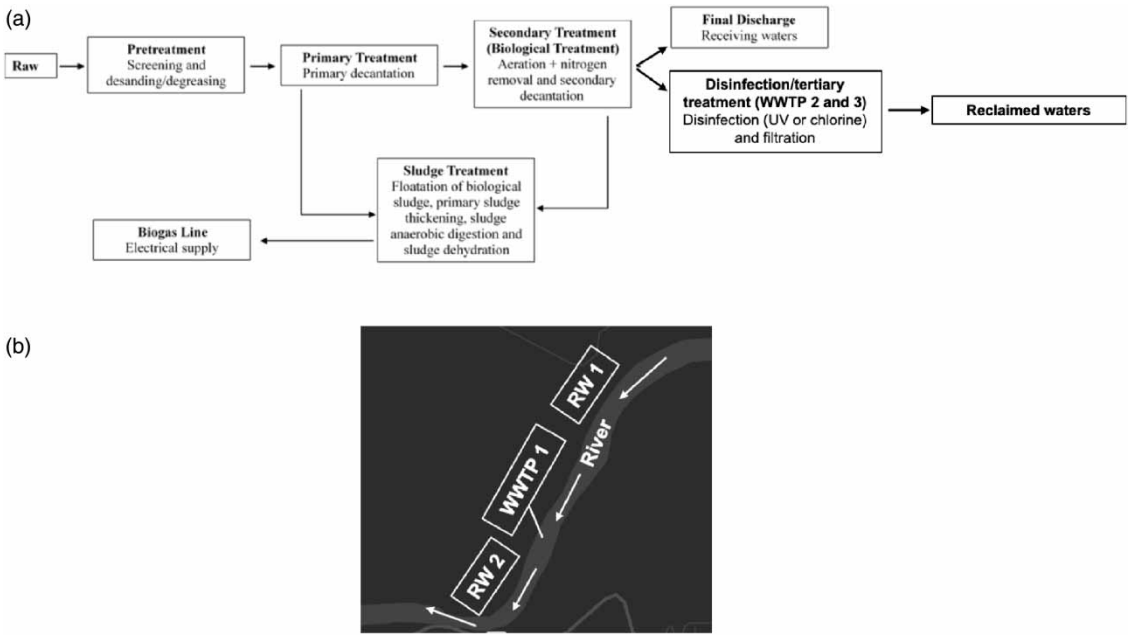

Figure 1 | (a) Description of the various treatment stages of a typical Portuguese WWTP (*Source*: SimTejo 2013) and (b) design of WWTP1 in relation to the two RW points sampled in this study. The RW1 site was collected before the WWTP1 discharge and the RW2 site collected after the discharge of WWTP1.

sterile plastic bottles. The samples were transported to the laboratory at 5 ± 3°C and analyzed within 8 h of collection. Enterococci enumeration and isolation were performed according to ISO 7899-2:2000 (International Organization for Standardization 2000), and the samples were tested in duplicate. Briefly, the samples (direct or diluted, depending on the sample location) were filtered using 0.45 μm sterile cellulose acetate membrane filters (Whatman, UK), and the membranes were placed on Slanetz and Bartley medium (Oxoid, UK). Dilutions with counts inferior to 10 CFU/plate were chosen preferably to proceed with the tests. Following incubation at 37 ± 1°C for 44 ± 4 h, all red to maroon colonies were counted and considered presumptive enterococci. The membranes were then transferred to Bile Aesculin Agar (Oxoid, UK) and incubated at 44 ± 5°C for 2 h. The colonies showing a tan to black color in the surrounding media were confirmed fecal enterococci. Five colonies from the plates containing counts above five were picked and further isolated on tryptic soy agar (TSA; Oxoid, UK) and incubated at 37 ± 1°C for 24 h. For plates containing less than five colonies, all colonies were isolated on TSA as previously described.

Antibiotic-susceptibility testing

All isolates identified as enterococci were subjected to antibiotic-susceptibility testing using the Kirby–Bauer's disk diffusion test (Clinical and Laboratory Standards Institute 2014). Briefly, an enterococci suspension of 0.5 McFarland was inoculated in Mueller–Hinton agar (BD, USA), and the different antibiotic disks were then placed over the inoculated suspension. Plates with antibiotics disks were incubated overnight at 37 ± 1°C, and the antibiotic inhibition growth was measured. Isolates were tested for their sensitivity to seven antibiotic agents: ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (120 μg), linezolid (30 μg), tetracycline (30 μg), and vancomycin (30 μg) (BD, USA). The antibiotics were chosen based on their respective class and on the human usage in hospital and ambulatory settings.

Extraction of enterococci *vanA* and *vanB* genes

The detection of *vanA* and *vanB* genes was performed in two steps: directly in the original samples and in all confirmed VRE following the antibiotic-susceptibility test.

Genomic DNA extraction from isolates and raw wastewater for enterococci genotyping and detection of resistance genes

The GenoLyse kit (Hain LifeScience BMGH, Germany) was used to extract DNA from the isolates following the manufacturer's instructions. Briefly, a suspension with a concentration of enterococci of approximately 10^8 CFU/mL was prepared fresh and from the suspension, and two mL were centrifuged at 12,000×g for 10 min. For raw wastewater, one mL of sample was centrifuged in the same conditions as described for the enterococci suspension. The supernatant was then removed, and the pellet was resuspended in 50 μL of the lysis buffer. The isolates were incubated at 95 ± 5°C for 5 min. Following incubation, 50 μL of the neutralization buffer was added. The extracted isolates were stored at −80 °C until further processing.

PCR amplification

PCR amplification was conducted in an Applied Biosystems Veriti thermal cycler (Thermo Fisher Scientific, USA). Genotyping of the isolates and detection of vancomycin-resistance genes were performed using the *ddl* gene present in enterococci and the *vanA* and *vanB* genes with PCR primers (Table 3) as described previously by Dutka-Malen *et al.* (1995). Each PCR was composed of 12.5 μL of 2× Maxima Hot Start Green PCR Master Mix (Thermo Fisher Scientific), 5 μL of DNA template, 800 nM of each primer, and nuclease-free water to a final volume of 25 μL. Additionally, 10- and 100-fold dilutions of every DNA extract were analyzed to limit amplification inhibition. Positive and negative controls were also performed with each run. PCR conditions were: 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. Amplicons were visualized after electrophoresis on 2.5% agarose gel and ethidium bromide staining.

Statistical analysis

All the data analysis was performed with Microsoft Excel 2016 or SPSS 25 (IBM, USA). The MAR index was calculated and determined according to Krumperman (1983)

Table 3 | Primers used for the identification of enterococci species and the detection of vancomycin-resistance genes

Gene	Primers	Sequence (5′–3′)	Amplicon size (bp)	Reference
$ddl_{E.\ faecalis}$	$E_{faecalis1}$	ATCAAGTACAGTTAGTCT	941	Dutka-Malen *et al.* (1995)
	$E_{faecalis2}$	ACGATTCAAAGCTAACTG		
$ddl_{E.\ faecium}$	$E_{faecium1}$	GCAAGGCTTCTTAGAGA	550	
	$E_{faecium2}$	CATCGTGTAAGCTAACTTC		
vanA	Van_{A1}	GGGAAAACGACAATTGC	732	
	Van_{A2}	GTACAATGCGGCCGTTA		
vanB	Van_{B1}	ATGGGAAGCCGATAGTC	635	
	Van_{B2}	GATTTCGTTCCTCGACC		

using the following equation:

$$\text{MAR} = \frac{\text{number of antibiotics to which enterococci were resistant to}}{\text{total number of antibiotics tested}} \quad (1)$$

MAR indexes greater than 0.2 are indicative of high-risk contamination with antibiotics used regularly (Krumperman 1983).

RESULTS

Prevalence and typing of enterococci in wastewater samples

A total of 186 enterococci isolates were obtained: 158 isolates from the WWTPs and 28 isolates from the receiving waters (RW1 and RW2).

Enterococci isolates were further characterized using the *ddl* gene into *Enterococcus faecalis*, *Enterococcus faecium* or *Enterococcus* spp. Further attempts to identify other species of enterococci beside *E. faecium* and *E. faecalis* were not performed since these were the species of interest for this study because they have become particularly important etiological agents of nosocomial infections with particular intrinsic resistance to aminoglycosides and cephalosporins and acquired resistance to others, prominently to vancomycin (Byappanahalli *et al.* 2012). From the 186 isolates, 98 isolates were determined as *E. faecium* (53%), 20 isolates were assigned as *E. faecalis* (11%) and the remaining were determined and are described from here forward as *Enterococcus* spp. (36%). Generally, *E. faecium* were detected more frequently and at higher concentration than the remaining enterococci, exception made for RW1, RW2 and WWTP3, being found in percentages ranging from 31% in RW2 to 63% at WWTP1. *E. faecalis* were less frequently detected from WWT3 (5%), and the highest prevalence was determined at 16% in RW2. The recovery of *E. faecium* from WWTP1 was in deep contrast with that from the respective receiving waters, with values of 31, 33, and 63% for RW1, RW2, and WWTP1, respectively. In WWTP3, the number of isolates determined as *E. faecium* and *Enterococcus* spp. was similar (46 and 49%, respectively).

Antibiotic-resistance distribution in *Enterococcus* species and in the environment

Ciprofloxacin (34%), tetracycline (40%), and linezolid (41%) were the antibiotics to which enterococci showed the highest levels of resistance (Table 4). On the other hand,

Table 4 | Antibiotic resistance of enterococci (%)

Antibiotic (concentration)	RW1	WWTP1	RW2	WWTP2	WWTP3	Total
AMP (10)	0	10	37	20	18	16
C (30)	11	4	16	4	5	6
CIP (5)	**44**	26	37	**46**	31	34
GEN (120)	0	1	0	2	0	1
LIN (30)	33	38	42	41	**46**	**41**
TET (30)	0	**47**	**63**	43	21	**40**
VAN (30)	0	**4**	**5**	**9**	**8**	**6**

AMP (10), ampicillin 10 μg; C (30), chloramphenicol 30 μg; CIP (5), ciprofloxacin 5 μg; GEN (120), gentamycin 120 μg; LIN (30), linezolid 30 μg; TET (30), tetracycline 30 μg; and VAN (30), vancomycin 30 μg. The highest percentage of enterococci resistance is highlighted in bold.

gentamycin was the antibiotic to which enterococci isolates presented the lowest level of resistance (1%). Resistance to vancomycin was found at 6%, a result similar to chloramphenicol. Resistance to ampicillin was determined in a relatively low percentage of the isolates (16%).

Resistance to linezolid was kept at the same levels in the different sampling locations (Table 4; Figure 2). WWTP1 contributed largely to the appearance of ampicillin, tetracycline, and vancomycin resistances in the environment (Table 4 and Figure 2), with enterococci isolated in receiving waters prior to the discharge of the WWTP being susceptible to these three antibiotics. Following the discharge site, high levels of resistances were encountered particularly for ampicillin (37%) and tetracycline (63%), with 5% of the isolates resistant to vancomycin into environmental waters.

The antibiotic-resistance patterns varied among the different WWTPs (Figure 2). WWTP1 showed lower levels of susceptibility to linezolid and tetracycline (38 and 47%), whereas WWTP2 showed resistance in higher percentages to ciprofloxacin, linezolid, and tetracycline, and WWTP3 mostly to linezolid. Nonetheless, the largest variation was observed for RW1 with a lower percentage, in general, of antibiotic-resistant enterococci.

Ampicillin-resistant enterococci (ARE) were detected in all WWTPs at different percentages, varying between 10% in WWTP1 and 20% in WWTP2. Highly gentamycin-resistant enterococci were scarcely detected throughout the study (WWTP1 and WWTP2). The impact of WWTP1 in the receiving waters is extremely evident in Figure 2 where, overall, the percentage of antibiotics that

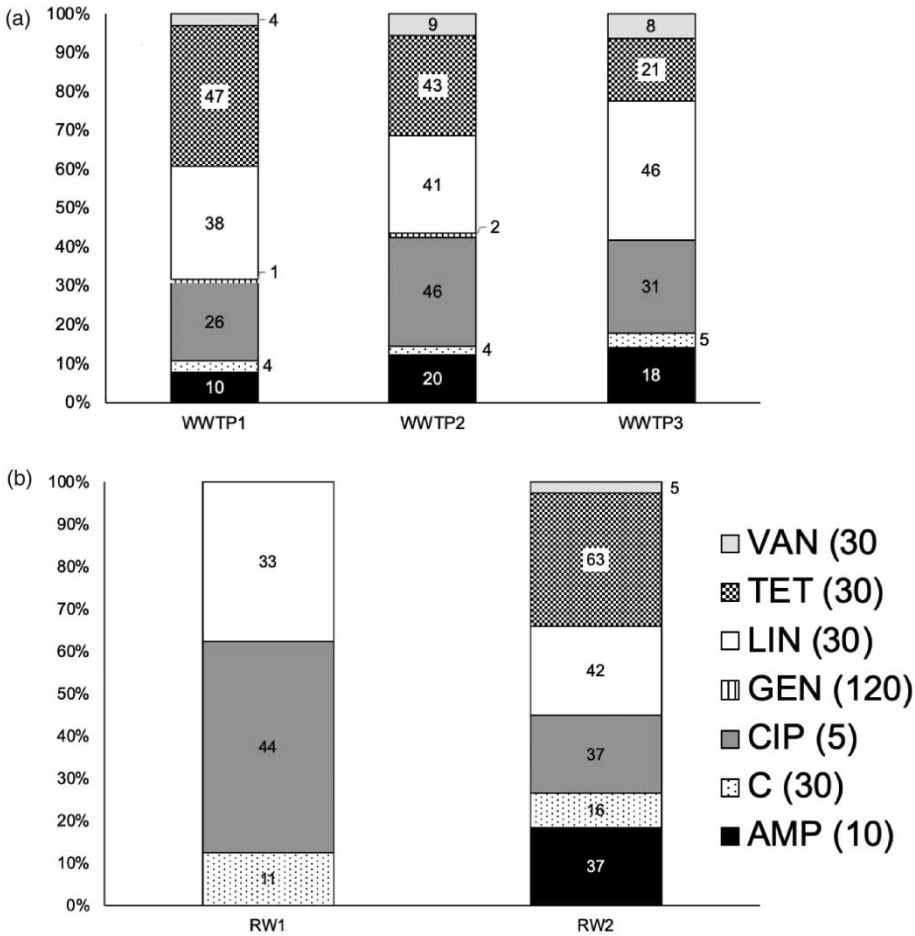

Figure 2 | Breakdown of antibiotic-resistance patterns in (a) the three WWTPs and (b) receiving waters RW1 and RW2. AMP (10), ampicillin 10 μg; C (30), chloramphenicol 30 μg; CIP (5), ciprofloxacin 5 μg; GEN (120), gentamycin 120 μg; LIN (30), linezolid 30 μg; TET (30), tetracycline 30 μg and VAN (30), vancomycin 30 μg.

existed prior to the discharge increased following the discharge point.

Multiple antibiotic-resistance patterns

An MAR index for each enterococci isolate was calculated in accordance with Equation (1). Results for the MAR index varied from 0 (where the isolate was susceptible to all antibiotics tested) to 0.71 (when the isolate was resistant to five antibiotics). No enterococci isolate was resistant to all antibiotics. MAR to three or more antibiotics was 17%, where 1% of the isolates showed resistance to five antibiotics. Data showed that almost half of the isolates had an MAR index superior to 0.20 (41%), with 16% displaying a high MAR index (above 0.43) (Figure 3).

The MAR index displayed different patterns depending on the environmental compartment tested. All WWTP had a greater prevalence of enterococci isolates displaying one or two antibiotic resistances (MAR indexes of 0.14 and 0.29). WWTP2 had the highest percentage of enterococci isolates with three or more antibiotic resistances (22% with an MAR index of 0.43 or above), followed by WWTP3 at 13%. WWTP2 receives wastewater from different large hospitals, whereas WWTP3 receives not only wastewater from hospitals but also farm waste. Therefore, the input and impact of the hospitals and farm waste in the presence of certain resistances in the WWTPs cannot be overlooked and may explain the highest percentage of isolates with a higher number of antibiotic resistances. The influence of WWTP1 in the receiving waters is also noticeable regarding the introduction of multiple-resistant enterococci, since half of the isolated enterococci in RW1 were susceptible to all antibiotics tested and only 22% showed multiple resistances (to two or three antibiotics). The pattern changes greatly after the WWTP discharge, with only 21% of the isolates susceptible to the antibiotics chosen, and with the appearance of enterococci displaying resistance to four antibiotics (MAR of 0.57) and a total of 69% of the isolates showing multiple resistance to antibiotics (MAR superior to 0.20). As for WWTP1, half of the isolated enterococci were resistant to two or more antibiotics with only 5% of the isolates being susceptible to all antibiotics.

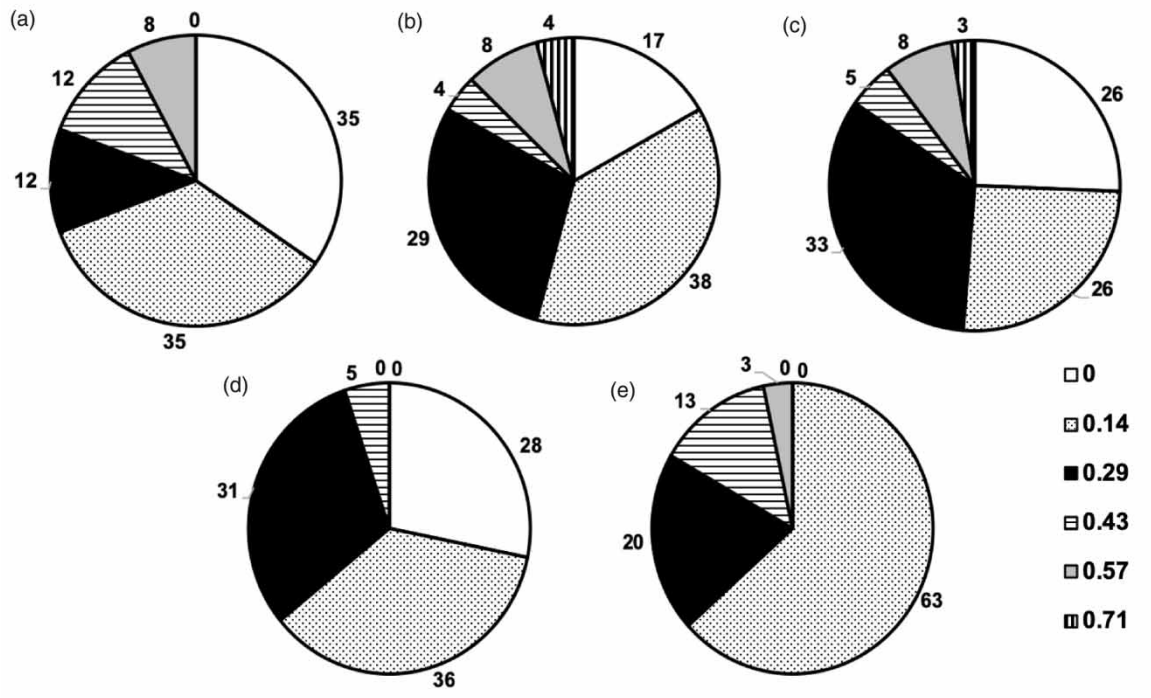

Figure 3 | MAR index in enterococci isolates by the WWTP sampling location. The results are in percentage to the total number of enterococci isolates in each WWTP sampling location. (a) raw wastewater; (b) post-filtration; (c) treated wastewater (following secondary treatment), (d) following UV disinfection and (e) reclaimed water.

The distribution of antibiotic resistances in the different stages of the WWTPs was also analyzed (Figure 3 and Table 5). The antibiotic-resistance pattern varied considerably according to the sampling location within the WWTPs. Overall, the level of resistance to three or more antibiotics remained stable in raw wastewater, post-filtration, in treated wastewater and in reclaimed water (20, 16, 16 and 16%, respectively). The samples after the UV disinfection stage have a maximum index of 0.43, in deep contrast to the remaining sampling points that have high percentages of MAR indexes above 0.43.

The main difference occurred after the UV disinfection stage, where the highest MAR index determined was 0.43. Raw wastewater had the highest percentage of enterococci isolates susceptible to every antibiotic tested, and the lowest was recorded in reclaimed water, with all isolates presenting at least one resistance, and a selection mainly towards the presence of resistance to one antibiotic.

Conversely, the percentage of enterococci isolates resistant to two antibiotics increased in post-filtration, in treated wastewater and UV disinfection stages (29, 33 and 31%, respectively), with a lower prevalence in the reclaimed waters (20%) and in raw wastewater (12%).

In WWTP1, the prevalence rate of enterococci resistant to three or more antibiotics was 16 and 11% for post-filtration wastewater and in treated wastewater, respectively. In the respective disinfected waters, the vast majority of the isolated enterococci were resistant to one antibiotic (47%), though none of the isolates were resistant to three or more agents. In WWTP2, raw and treated wastewater revealed the presence of 32 and 33% of isolates demonstrating resistance to three or more antibiotics, respectively. The percentage of isolates with resistance to three or more antibiotics decreased sharply to 10% following disinfection.

The treatment stages at WWTP2 appeared to positively select for the presence of enterococci with resistance to two antibiotics, with percentages of 44 and 50% in treated wastewater and disinfected waters, respectively. In reclaimed waters, the vast majority of the isolated enterococci were resistant to just one antibiotic, but still a moderate percentage of isolated enterococci were resistant to two and three antibiotics, including ampicillin, ciprofloxacin, linezolid, tetracycline and vancomycin. In WWTP3, the disinfected waters showed the presence of enterococci resistant to three antibiotics, although no such isolates were found in raw wastewater. A similar result was found in the reclaimed waters. Nonetheless, in these waters, the vast majority of the isolates were resistant to only one antibiotic (58%).

Although being detected in lower frequency, *E. faecalis* massively contributed to the existence of multiple resistances in these waters. Half of the isolated *E. faecalis* were resistant to two or more antibiotics, whereas *E. faecium* and *Enterococcus* spp. had a lower prevalence of this category (43 and 38%, respectively). The difference further deepens when considering just resistance to three or more antibiotics, with 40% being *E. faecalis*, 13% *E. faecium*, and 15% *Enterococcus* spp.

Table 5 | Enterococci isolates MAR index percentages per WWTP and sampling location

		% MAR index					
WWTP	**Sampling location**	**0**	**0.14**	**0.29**	**0.43**	**0.57**	**0.71**
RW1	Before WWTP1 discharge	**45**	**33**	**11**	**11**	**0**	**0**
RW2	After WWTP1 discharge	**21**	**10**	**26**	**32**	**11**	**0**
1	Post-filtration	17	38	29	4	8	4
	Treated wastewater	30	30	30	7	3	0
	Disinfection	26	47	26	0	0	0
2	Raw wastewater	31	31	6	19	13	0
	Treated wastewater	11	11	44	0	22	11
	Disinfection	20	20	50	10	0	0
	Reclaimed water	0	73	18	9	0	0
3	Raw wastewater	40	40	20	0	0	0
	Disinfection	40	30	20	10	0	0
	Reclaimed water	0	58	21	16	5	0

*The most relevant results are highlighted in bold

Presence of VRE and detection of *vanA* and *vanB* resistance genes

In this study, the presence of *vanA* and *vanB* genes was determined in concentrated raw wastewater and in the isolated enterococci. Data from isolated bacteria were in agreement with that from raw wastewater, showing the complete absence of these genes. Nonetheless, a total of 6% of the enterococci isolated still showed resistance to VRE using the antibiotic-susceptibility test (Table 4). VRE were detected post-filtration and disinfection stages in WWTP1 with prevalence rates of 3 and 1%, respectively. In

WWTP2, VRE were found in raw wastewater (4%), treated wastewater (2%) and in reclaimed waters (2%), and in WWTP3, VRE were only detected in reclaimed waters (8%). Half of the detected VRE were found in reclaimed waters, which are currently being used for irrigation of the WWTPs and city gardens and for street washing. In addition, 90% of the VRE also presented an MAR index superior to 0.20 (Figure 4), with the vast majority being resistant to three or more antibiotics.

The PCR revealed that the highest prevalence rate of resistance to vancomycin was found for *E. faecalis* at 3%, followed by *E. faecium* (2%) and *Enterococcus* spp. (1%). Considering just the number of isolates per species, the difference in the number of vancomycin resistances between the species steeply increases compared with the total number of isolates (*E. faecalis*, *E. faecium* and *Enterococcus* spp.). *E. faecalis* isolates exhibiting vancomycin resistance comprised 30% of the total number of confirmed *E. faecalis*, whereas VRE constituted just 3% of the total number of *E. faecium* and *Enterococcus* spp. isolates. The concentration of enterococci decreased along the treatment steps at the WWTP, but the percentage of VRE out of the total enterococci actually increased for WWTP2 and WWTP3 (Figure 5).

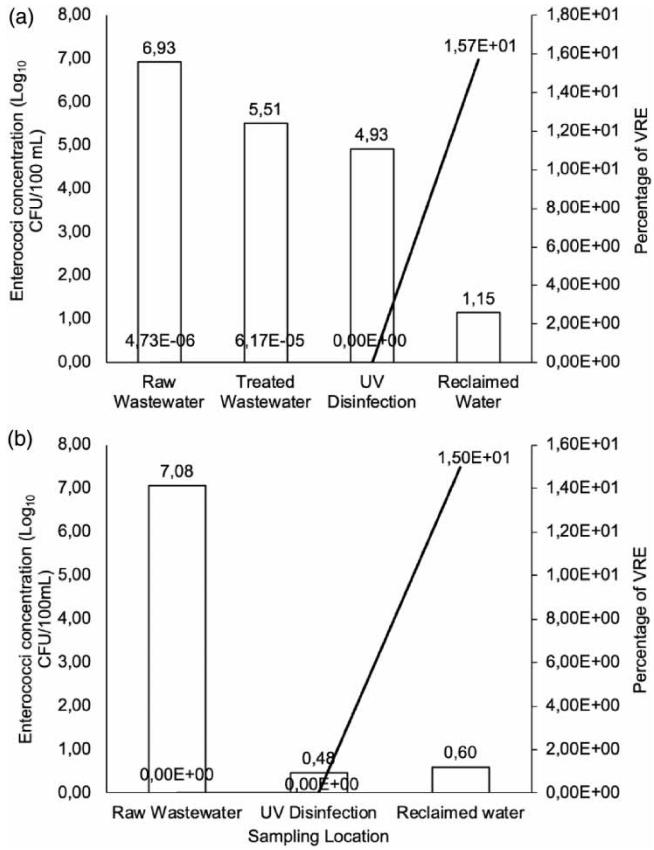

Figure 5 | Breakdown of enterococci mean concentration and VRE persistence during WWTP (a) WWTP2 and (b) WWTP3. Bars indicate the mean concentration of enterococci and line represents the percentage of VRE in each sampling location and WWTP.

DISCUSSION

In the early stages of antibiotic resistance, the main problem was considered to be the medical settings, particularly within hospitals. However, the problem has grown beyond these settings and has crossed into the environment. In the present study, we evaluated the presence of multiple-resistant enterococci and VRE in different stages of three WWTPs and in two environmental waters. Culturable enterococci were isolated from all the tested stages of the WWTPs chosen, including following UV disinfection and

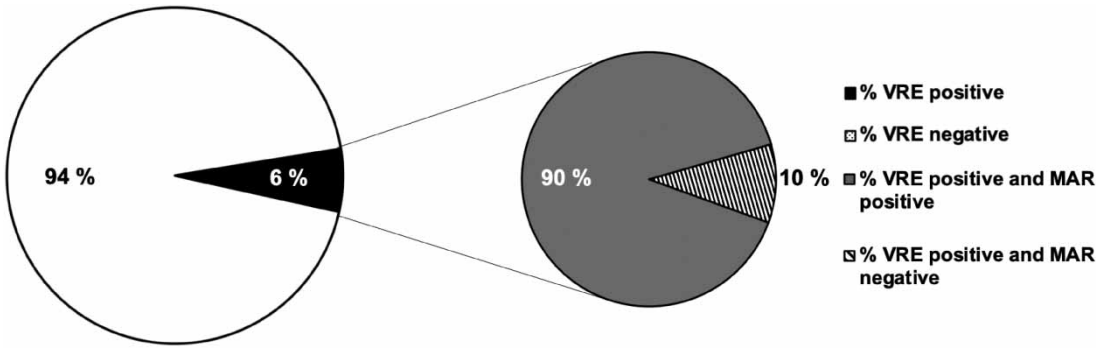

Figure 4 | Proportion of vancomycin resistant and MAR enterococci in all WWTP effluents.

in reclaimed waters at the point of use. We found that 53 and 11% of the isolated enterococci were *E. faecium* and *E. faecalis*, respectively. Such a result is in accordance with previous studies that have shown a higher prevalence rate of *E. faecium* in environmental waters, with similar percentages to those obtained in this study (Rahimi *et al*. 2007; Sadowy & Luczkiewicz 2014). *E. faecium* is often linked to human fecal contamination, even though *E. faecalis* is present in higher concentrations in humans in the community (Kühn *et al.* 2003; Thevenon *et al.* 2012). Other species such as *E. hirae* are more frequently reported in contamination from cattle and pigs (Kühn *et al.* 2003), which may justify the percentage of *Enterococcus* spp. in the receiving waters from WWTP3, situated in a region known to have cattle farms.

With respect to antibiotic resistance, half of the isolates collected during this study were resistant to two or more antibiotics. Resistance to linezolid, tetracycline, and ciprofloxacin was present in a high number of enterococci. In a study by Rahimi *et al*. (2007), enterococci were highly resistant to tetracycline and ciprofloxacin, a similar result to that obtained by Sadowy & Luczkiewicz (2014). Of particular interest was the prevalence of linezolid-resistant enterococci. Linezolid, an oxazolidinone antimicrobial agent, has been used for the treatment of infections caused by multiple-resistant Gram-positive bacteria, including VRE. According to a review by Bi *et al.* (2018), most linezolid-resistant enterococci have been documented in Europe (46% in *E. faecalis* and 64% in *E. faecium*), similar percentages to the ones obtained in our study. Additionally, the majority of the VRE also displayed resistance to linezolid (82%). MAR to three or more antibiotics was obtained for 15% of the enterococci isolates with 1% being resistance to five antibiotics, including vancomycin and linezolid. The detection of MAR enterococci, including to vancomycin, had been reported in previous studies (Luczkiewicz *et al.* 2010; Sadowy & Luczkiewicz 2014; Sanderson *et al.* 2019). Resistance to ampicillin was registered in the majority of the surveyed environmental compartments in percentages ranging from 10 to 27% (mean 16%), with the majority of the isolates being *E. faecium*. Similar results have been found in other studies performed previously (Taucer-Kapteijn *et al.* 2016; Oravcova *et al.* 2017). Molale & Bezuidenhout (2016) have found, however, a higher number of ARE with percentages above 40%. Since ampicillin is administered mainly in hospitals, ARE are considered surrogate markers for hospital-adapted lineages. VRE were detected in 6% of the isolated enterococci, with a higher prevalence rate in *E. faecalis*. A study conducted by Kühn *et al.* (2005) showed that the frequency of VRE isolated from animals, humans, and environmental waters varied between 8 and 11%. Different studies throughout Europe have described percentages of VRE in environmental waters ranging from 0 to 60% (Luczkiewicz *et al.* 2010; Morris *et al.* 2012). A report by the European Centre for Disease Prevention and Control (ECDC 2019) showed that an increase in the mean percentage of VRE*fm* occurred between 2015 (10.5%) and 2018 (17.3%) with high percentages reported throughout Europe. In the opposite direction, VRE*fm* in Portugal decreased steeply from 20.3% in 2015 to just 4.4%, which is in line with the results obtained in this study. A study from ECDC on the health burden caused by antimicrobial resistance concluded that the number of infections and deaths related to VRE practically doubled between 2007 and 2015 (Cassini *et al.* 2019), which conjugated with the steep increase observed in the latter years contributing even further to the increase in the burden.

In this study, in two of the WWTPs chosen, municipal wastewater intended for reuse in the presence of VRE and antibiotic resistance was evaluated. To our knowledge, only a few studies have addressed the potential existence of VRE and MAR in reclaimed waters that are already being used in irrigation (Goldstein *et al.* 2014). The concentration of enterococci decreased along the treatment steps at the WWTP, but the percentage of VRE out of the total enterococci actually increased for WWTP2 and WWTP3. Additionally, the vast majority of the VRE isolates were also resistant to multiple antibiotic agents, indicating a positive selective pressure from the treatments at WWTPs. A study by Goldstein *et al.* (2014) performed in WWTPs that produced reclaimed waters showed that chlorination was effective at removing VRE from the final wastewaters. The WWTPs chosen in this study had standard biological treatment (activated sludge) and disinfection by UV radiation in place. In our study, UV disinfection was not effective at removing VRE nor multidrug resistant enterococci. McKinney & Pruden (2012) looked at the disinfection efficiency of UV disinfection against several antibiotic-resistant bacteria and determined that VRE were more resistant to UV

disinfection than methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* SMS-3-5, and *Pseudomonas aeruginosa* 01. Luczkiewicz *et al.* (2011) showed similar results, with an increase in the percentage of resistant enterococci following UV disinfection compared with the results obtained for treated wastewater. The PCR of the resistant genes *vanA* and *vanB* of raw wastewater and confirmed VRE were negative. No further attempt was made to identify the isolates that did not amplify products with any of the other resistance gene primers since *vanA* and *vanB* were the genes of interest. The percentages of VRE in reclaimed waters and in the receiving waters found in this study were relatively low. However, even low percentages of VRE in these waters may represent an increased health risk, given that enterococci are not only able to replicate in environmental waters but can also transfer resistances to other bacteria in the environment, therefore increasing the risks for human health (Boehm & Sassoubre 2014). In addition, the use of VRE-positive reclaimed waters in agriculture and the consumption of fresh produce without further processing may constitute a greater risk for human health.

Nine clusters conferring resistance to glycopeptides have been previously described in enterococci: *vanA*, *vanB*, *vanC*, *vanD*, *vanE, vanG*, *vanL*, *vanM*, and *vanN* (Werner 2012). The *vanA* genotype carrying the *vanA* gene cluster involves transmissible, high-level resistance to vancomycin and teicoplanin, whereas the *vanB* genotype (with the *vanB* gene cluster) confers transmissible vancomycin resistance to diverse levels of vancomycin while retaining susceptibility to teicoplanin. *vanM* is also transferable and may be spread through conjugation to other enterococci and other Gram-positive cocci (Courvalin 2006; Xu *et al.* 2010). Another common resistance genotype is *vanC*, conferring resistance to low levels of glycopeptide, carried in the *vanC* genes which are chromosomally encoded by *vanC1* and *vanC2/3*. Resistance to low levels of vancomycin has been described in *vanC*, *vanE*, *vanL*, and *vanN* genotypes.

Our study highlights the problems and possible public health risks associated with contact with reclaimed waters. Several countries throughout the globe are already experiencing water shortage. In 2007, about 1.2 million people in less economically developed countries lived in water scarce areas, and the number is expected to increase to 1.8 million by 2025 (FAO 2007). However, more recent reports show this phenomenon to be accelerated in the past years. Over 2 billion people live in countries experiencing high water stress (United Nations 2018), whereas 4 billion people, representing nearly two-thirds of the world's population, experience severe water scarcity during at least 1 month of the year (Mekonnen & Hoekstra 2016). A third of the world's biggest groundwater systems are already in distress (Richey *et al.* 2015). Furthermore, water resources will be put under growing pressure in the next years due to growing population and climate change effects. With the existing climate change scenario, by 2030, water scarcity in some arid and semi-arid places will displace between 24 million and 700 million people. (UNESCO 2009; Global Water Institute 2013). Projections show that water demand in agriculture, industrial and domestic sectors will increase between 20 and 33% in the next decades (Burek *et al.* 2016). Industrial and domestic water demands are forecasted to grow much more rapidly than for the agricultural sector, but studies show the agriculture sector to remain the dominant water demand sector (Burek *et al.* 2016). In urban overpopulated regions, climate change and increased demand for industrial water requirements are forcing the reuse of wastewater in various fields such as agriculture, miscellaneous uses in the industry and field irrigation (Dupont 2013). Reclaimed waters are the most readily available water source not only for irrigation of urban gardens, but also in agriculture exposing the crops to antimicrobial-resistant agents and therefore further increasing the risk in the farm to fork chain. In addition to the impacts to human health, there are also severe ecological concerns regarding the receiving water of the WWTPs. Discharging treated wastewater with VRE and MAR enterococci may result in the dissemination, increasing persistence and environmental transfer of other resistant genes to environmental reservoirs other than water such as soils, sediments and bivalve mollusks. Such flow may result in a constant cycle of contamination of water and food with such organisms, posing a continued risk to human health.

CONCLUSIONS

To our knowledge, this study is one of the few studies demonstrating the presence of VRE and MAR enterococci

in reclaimed water used to irrigate urban gardens and clean the streets of a large metropolitan city. Interestingly, while the concentration of VRE decreased during the different steps of the WWTP, their proportion in relation to the total number of enterococci isolated at each stage increased, suggesting the potential for the positive selection of VRE during the common widely used wastewater treatment process. VRE were isolated from UV-disinfected wastewater and from reclaimed waters. The majority of the VRE isolated in this study also presented multidrug resistance including to linezolid and other antibiotics used to treat VRE infections. This fact implies a greater risk not only to the WWTP workers but also to the general public given the potential exposure to VRE through contact with these waters, especially if they are used for municipal irrigation and street cleaning.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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