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Antibiotic-Resistant Bacteria across a Wastewater Treatment Plant

Ofélia Godinho [1,2], Olga Maria Lage [1,2] and Sandra Quinteira [1,3,4,5,*]

[1] Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal; ofeliagodinho95@gmail.com (O.G.); olga.lage@fc.up.pt (O.M.L.)
[2] Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de 3 Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal
[3] CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO, Rede de Investigação em Biodiversidade e Biologia Evolutiva, Laboratório Associado, Universidade do Porto, 4485-661 Vairão, Portugal
[4] BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal
[5] 1H-TOXRUN-One Health Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL., Avenida Central de Gandra 1317, 4585-116 Gandra PRD, Portugal
* Correspondence: sandra.quinteira@fc.up.pt

Abstract: Antimicrobial resistance is presently one of the leading causes of death worldwide. The surveillance of different environments, namely, wastewater treatment plants (WWTPs), as hotspots of antibiotic-resistant bacteria, has become crucial under the One Health approach. This study aimed to characterize, phenotypically and genotypically, antibiotic-resistant bacteria along a WWTP receiving domestic and industrial sewage. Four sampling sites, representing distinct treatment points of the WWTP, were selected for sampling bacterial isolation in selective media supplemented, or not, with antibiotics, and subsequent antimicrobial susceptibility testing. Antibiotic resistance encoding genes were screened by molecular methods. A total of 50 bacterial isolates were obtained, 50% of which were affiliated with the genus *Enterococcus*. The antimicrobial susceptibility testing revealed antibiotic phenotypic resistance in isolates obtained from all the four treatment points of the wastewater samples, with resistance to tetracycline (32.5%) and ampicillin (25%) being the most common. Three isolates were found to be multidrug resistant and were affiliated with the genera *Citrobacter*, *Shigella* and *Klebsiella*. Molecular screening revealed the presence of *tet*(M), bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$, as well as class 1 integrons carrying *dfr*A25, ANT(3″)-IIa and *aad*A6 genes. This study highlights the relevance of bacterial isolation and their antimicrobial susceptibility evaluation in WWTP systems since antibiotic-resistant strains were found from the raw influent to the final effluent discharged into the environment, denoting the need for surveillance and containment measures.

Keywords: *Enterobacterales*; mobile genetic elements; resistance genes; integrons; sewage

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1. Introduction

One of the leading worldwide causes of death is infection caused by antimicrobial resistant pathogens. The resistance of pathogens to antimicrobial compounds leads to a lack of treatment options, resulting in increased mortality rates [1]. In fact, previous estimates have determined that 10 million deaths per year could be attributable to antimicrobial resistance by 2050, and more recent reports show that 1.27 million deaths in 2019 were due to bacterial antimicrobial resistance [2,3]. An estimated 79,000 people die each year due to infections caused by antimicrobial-resistant pathogens within 34 OECD and EU/EEA countries [4]. Apart from the loss of human lives, antimicrobial resistance in pathogens also has financial consequences since it also results in prolonged hospitalizations and increased treatment costs [5]. Within the 34 OECD and EU/EEA countries, the treatment of the complications associated with infections caused by antimicrobial-resistant pathogens was estimated to cost over USD 28.9 billion per year [4]. The main drivers of the antimicrobial

resistance burden are commonly referred as the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) and the well-known pathogen *Escherichia coli*. It is also widely known that antibiotic resistance genes can disseminate across different bacteria/ecosystems in natural environments and eventually reach human pathogens [6]. In this context, the One Health approach, which links the environment, plants, animals and humans, is highly relevant for addressing the antimicrobial resistance crisis [7] as the health of one influences the health of the others. As such, it is important to coordinate different disciplines and sectors to share information and policies in order to help in the prevention and control of different health threats [8–12]. In fact, recently, the World Health Organization (WHO), the Food and Agriculture Organization (FAO), the World Organisation for Animal Health (WOAH) and the United Nations Environment Programme (UNEP) made a quadripartite collaboration based on the One Health approach to tackle the antimicrobial resistance crisis [12].

Wastewater treatment plants (WWTPs) have long been considered hotspots for the transmission and selection of antimicrobial resistance genes [13]. Studies have shown the high presence of antibiotic resistance genes in downstream water environments from WWTP effluents [14]. This is the result of two factors: first, the urban WWTPs are source of antibiotic residues [13], and secondly, they also promote the selection of antibiotic-resistant bacteria [15]. The link between the resistance genes found in clinical isolates and the nearby wastewater treatment plants has also been established [16]. The presence of antibiotic resistance genes/antibiotic-resistant strains in downstream water environments from WWTPs constitutes a risk for public health, and consequently, their surveillance in wastewaters is crucial for tackling the antibiotic resistance crisis under the One Health approach [17]. This surveillance was historically based on culture-based methods but has recently been relying more on culture-independent approaches such as quantitative PCR (qPCR) or metagenomics [17].

With this study, our aim was to evaluate the presence of antibiotic-resistant bacteria from the influent to the final effluent of a WWTP and in the affected environment. To do so, we performed the isolation of bacteria from samples of different sites of a WWTP for which we assessed their phenotypic antimicrobial susceptibility profiles, and we performed a molecular search for the different antibiotic resistance genetic determinants.

2. Material and Methods

2.1. Sampling and Processing

Wastewater samples were collected in May 2021 and in July 2021 from four different points within a wastewater treatment plant located in the northern region of Portugal (the WWTP features are detailed in reference [18]). The four sampling points represented: A—raw influent of untreated wastewater; B—preliminary effluent already treated for the removal of coarse solids, sands, oils and fats; C—final effluent after membrane bioreactor (MBR) treatment with an ultrafiltration process but before the discharge in the river; and D—the stream receiving the WWTP effluent [18]. Samples were collected in 100 mL sterile flasks and kept in cold conditions until they were processed. An aliquot of 1mL of each sample was retrieved for serial decimal dilutions, and 100 µL of each dilution were plated on different culture media and spread with glass beads before incubation at 30 °C. Additionally, a volume of 90 mL was filtered using several 0.22 µm Whatman sterile membrane filters which were placed in flasks containing 225 mL of Buffered Peptone Water and incubated at 37 °C for 24 h without shacking. After this enrichment step, the samples were serial decimal diluted, and 100 µL of the dilutions were plated and incubated as referred to before. The following three culture media (supplemented, or not, with antibiotics) were used for the isolation of the clinically relevant bacterial strains: MacConkey Agar (MCA), Mannitol Salt Agar (MSA) and Slanetz Bartley Agar (SBA), and these were used for the isolation of *Enterobacteriaceae*, *Staphylococcus* spp. and *Enterococcus* spp., respectively. The MCA was supplemented with imipenem [2 µg/mL] or ciprofloxacin [2 µg/mL], while the

MSA and SBA were supplemented with vancomycin [4 μg/mL]. Grown colonies were selected based on different morphotypes for isolation in Luria agar (LA), stored at −80 °C in Luria broth (LB) supplemented with 20% (*v/v*) glycerol and further used for antibiotic susceptibility assays.

2.2. Antibiotic Susceptibility Assays

Antibiotic susceptibility profiles were determined based on the Kirby–Bauer method according to the CLSI standards [19,20]. When the CLSI interpretative standards were not available for an isolate, EUCAST breakpoints, if available, were used for interpretation [21]. In brief, bacterial suspensions of each isolate in phosphate buffer saline (PBS) were prepared to the equivalent of 0.5 McFarland standard. The suspensions were spread uniformly on Mueller Hinton Agar II with a sterile swab. Antibiotic discs were then placed on the surface of the inoculated media before incubation at 37 °C for 18 h. Afterwards, inhibition zone diameters were measured and interpreted as susceptible (S), intermediary (I) or resistant (R), based on the zone diameter breakpoints provided by the CLSI standards for the different tested bacterial groups [21]. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality controls in each assay. Antibiotics targeting cell wall biosynthesis, namely, β-lactams, such as ampicillin (AMP, 10 μg), cefotaxime (CTX, 30 μg) and imipenem (IMP, 10 μg), as well as the glycopeptide vancomycin (VA, 30 μg) were tested. Additionally, the protein synthesis inhibitors gentamicin (CN, 10 μg) (an aminoglycoside) and tetracycline (TE, 30 μg) (representing the tetracyclines class), as well as ciprofloxacin (CIP, 5 μg) (a fluoroquinolone, which inhibit DNA replication), were also included.

2.3. Phylogenetic Determination with 16S rRNA Sequencing

The DNA extraction of axenic cultures was performed using an E.Z.N.A. Bacterial DNA Isolation Kit (Omega BioTek, Norcross, USA) according to the manufacturer's instructions. The extracted genomic DNA was used for the PCR amplification of the 16S rRNA gene using the universal primers 27F and 1492R [22] (Table 1). The PCR mixtures and conditions were prepared as previously described [23] (Table 1). The PCR products were then visualized after electrophoresis in a 0.8% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer stained with GreenSafe Premium (NZYTech, Lisboa, Portugal). All amplicons were then purified with a GFX PCR DNA and Gel Band Purification Kit (Cytiva, Oeiras, Portugal) before the samples were sent for sequencing at Eurofins Genomics. The obtained sequences were cleaned and analyzed using Geneious Prime 2021, and the consensus sequence was compared with the NCBI Genbank database [24] using NCBI's Standard Nucleotide BLAST search. The sequences were then deposited in Genbank with the accession numbers OR872259-OR872308. The obtained 16S rRNA gene sequences were aligned, together with the closest type strains from the NCBI's Genbank database, with Molecular Evolutionary Genetics Analysis (MEGA) (version 7.0) [25] using the Clustal W algorithm [26]. This multiple-sequence alignment was then used to construct a phylogenetic dendrogram using the Maximum Likelihood method based on the General Time Reversible model and the gamma distributed with the invariant sites (G+I) [27] and bootstrap method (1000 replicates).

2.4. Detection of Antibiotic Resistance Genes and Class 1 Integrons

Screening for the antibiotic resistance genes was carried out by PCR. The respective primers for each target, as well as the reaction conditions, are presented in Table 1. A total of five targets were amplified, as follows: Class 1 integrons, *tet*(M), bla_{SHV}, bla_{TEM} and $bla_{\text{CTX-M}}$. The sequences were then deposited in Genbank with the accession numbers OR879256-OR879278.

Table 1. List of the targets, primers and amplification conditions used in this study.

Target	Primer Sequence	Amplicon Expected Size (bp)	Annealing Temperature	Program	Reference
16S rRNA	27F—AGA GTT TGA TCM TGG CTC AG 1492R—TAC GGY TAC CTT GTT ACG ACT T	~1400	56 °C	95 °C—5 min (1×) 95 °C—1 min, 56 °C—1 min, 72 °C—1.5 min, (30×) 72 °C—10 min (1×)	[22]
Class 1 integron	Int 5′CS—GGC ATC CAA GCA GCA AG Int 3′CS—AAG CAG ACT TGA CCT GA	Variable	55 °C	94 °C—5 min (1×) 94 °C—30 s, 55 °C—30 s, 72 °C—6 min, (35×) 72 °C—16 min (1×)	[28]
Tet(M)	tetMF—GTG GAC AAA GGT ACA ACG AG tetMR—CGG TAA AGT TCG TCA CAC AC	406	55 °C	95 °C—3 min (1×) 95 °C—30 s, 55 °C—30 s, 72 °C—30 s, (35×) 72 °C—10 min (1×)	[29]
bla_{SHV}	SHV-F—GGG TTA TTC TTA TTT GTC GC SHV-R—TTA GCG TTG CCA GTG CTC	930	56 °C	94 °C—10 min (1×) 94 °C—30 s, 56 °C—30 s, 72 °C—30 s, (35×) 72 °C—10 min (1×)	[30,31]
bla_{TEM}	TEM-F—ATG AGT ATT CAA CAT TTC CG TEM-R—CTG ACA GTT ACC AAT GCT TA	847	58 °C	94 °C—3 min (1×) 94 °C—30 s, 58 °C—30 s, 72 °C—30 s, (35×) 72 °C—10 min (1×)	[32]
bla_{CTX-M}	CTX-M-F′—TTT GCG ATG TGC AGT ACC AGT AA CTX-M-R′—CGA TAT CGT TGG TGG TGC CAT A	590	51 °C	94 °C—10 min (1×) 94 °C—30 s, 51 °C—30 s, 72 °C—30 s, (35×) 72 °C—10 min (1×)	[33]

3. Results and Discussion

3.1. Isolation Results and Identification

A total of 50 bacterial isolates were retrieved from the two sampling events, as follows: 16 from sample site A, 16 from sample site B, 8 from sample site C and 10 from sample site D (Supplementary Table S1). Twenty-five isolates were affiliated with the genus *Enterococcus*, eleven with the family *Enterobacteriaceae*, three with the genus *Aeromonas* and eight with the genus *Pseudomonas*, and only one was isolated with the genera *Comamonas*, *Brevundimons* and *Stenotrophomonas* (Supplementary Figure S1). Different bacteria were found across all the sampling sites, including sampling site C, which corresponded to the effluent after the MBR treatment with an ultrafiltration process. The presence of antibiotic-resistant isolates from the entrance to the exit of the WWTP was in agreement with previous reports, which indicated that the MBR treatment applied was insufficient to reduce the microbial load [18]. This reinforces the need to apply additional strategies at this WWTP to ensure the proper removal of microorganisms, which may require the development of novel technologies and procedures [34]. The presence of potentially pathogenic organisms in all sampling points also reinforces the need for protective measures for WWTP operators, such as personal protective equipment, to ensure their safety since it has been shown that microorganisms can be spread by aerosols, and thus, they present a health risk for workers [35,36].

3.2. Antibiotic Susceptibility Testing

Three of the isolated bacteria (*Brevundimonas* sp. E112, *Comomonas* sp. E11 and *Stenotrophomonas* sp. E126) belonged to taxonomic groups that were not considered in the tables from the CLSI interpretative criteria (nor in EUCAST) for the antimicrobial susceptibility test, which impaired the analyses of the obtained results for these bacteria. For the remaining 47 bacteria, only 8 isolates (17%) were pan-susceptible, and 17 (36%) presented resistance to only one antibiotic (Supplementary Table S2). The numbers of isolates resistant to each antibiotic, as well as those found to be susceptible or with intermediate phenotypes, are presented in Table 2, and an example of the different phenotypes is shown in Supplementary Figure S2. Among the tested antibiotics, tetracycline and ampicillin were the ones for which more resistant phenotypes were observed (Table 2). Three isolates presented resistance to two antibiotics, and three isolates, *Citrobacter* sp. E73, *Shigella* sp. E113 and *Klebsiella* sp. E142, presented resistant to three tested antibiotics of three distinct classes of antibiotics, as follows: *Citrobacter* sp. E73 and *Klebsiella* sp. E142 were resistant to ampicillin, tetracycline and ciprofloxacin, while *Shigella* sp. E113 was resistant to ampicillin, gentamicin and ciprofloxacin. These isolates could, thus, be classified as multidrug-resistant [37] and were affiliated with the clinically relevant pathogens (Supplementary Table S1). The genus *Citrobacter* includes different species which have been found to cause infections in humans, such as *Citrobacter braakii*, with which E73 is affiliated [38,39]. Isolate E113 was found to be affiliated with *Shigella sonnei*, an emerging pathogen that causes bloody diarrhoea, with high morbidity and mortality [40]. The genus *Klebsiella* includes the critical human pathogen *Klebsiella pneumoniae* [41] but also other clinically relevant species such as *Klebsiella variicola* and *Klebsiella quasipneumoniae* [42]. Our NCBI analysis showed that the closest-described species to isolate E142 was *Klebsiella quasivariicola* KPN1705^{T}, a recent species described from an isolate retrieved from a wound infection [43,44].

Table 2. Number of isolates showing either resistant, intermediate or susceptible phenotypes to the tested antibiotics.

	Phenotype (n.)		
Antibiotic	Resistant	Intermediate	Susceptible
Ampicillin	9	2	25
Cefotaxime	0	3	11
Imipenem	3	0	19
Tetracycline	13	0	27
Vancomycin	0	6	19
Gentamicin	2	9	6
Ciprofloxacin	5	15	27

In general, the bacteria retrieved from sampling site B showed more susceptible profiles than the bacteria from the other three sampling sites, with only three isolates (*Enterococcus* sp. E179, *Providencia* sp. E130 and *Pseudomonas* sp. E9) showing resistance phenotypes. The multidrug-resistant isolates were retrieved from sampling sites A, C and D, revealing the discharge and presence of multidrug-resistant strains into the environment.

A total of 13 isolates out of the 40, to which the interpretative criteria standards could be applied, were classified as resistant to tetracycline, of which 9 were affiliated with *Enterococcus* sp. (Supplementary Table S2). Tetracycline resistance in *Enterococcus* sp. is common and may be rising [45–48]. The isolates *Citrobacter* sp. E73, *Providencia* sp. E130, *Klebsiella* sp. E142 and *Raoultella* sp. E148 also showed resistance to tetracycline. The resistance of these strains to tetracycline has been previously reported [49–52]. The PCR screening revealed the presence of *tet*(M) in 15 isolates, of which 11 were confirmed by sequencing (Table 3). Most of the isolates (n = 13) were affiliated with *Enterococcus* spp., for which various reports of *tet*(M) exist [53,54]. Additionally, *tet*(M) was found in the isolate *Stenotrophomonas* sp. E126 and in the isolate *Brevudimonas* sp. E112, both of

which were confirmed by sequencing. The *tet*(M) gene has been previously found in a *Stenotrophomonas* isolate [55]. Although there are no available antimicrobial susceptibility interpretative breakpoints for this species, the isolate *Stenotrophomonas* sp. E126 presented an inhibition zone diameter of 9 millimetres, which we may consider as an indication of phenotypic resistance to tetracycline. The sequencing confirmed the presence of *tet*(M) in the isolate *Brevundimonas* sp. E112. *Brevundimonas* is a genus of the family *Caulobacteraceae* that has been increasingly studied due to reports of its role as an opportunistic pathogen [56]. The tetracycline resistance gene *tet39* has been detected in at least one isolate from this genus; however, most reports show the susceptibility of *Brevundimonas* isolates to tetracycline [56,57]. Our results from the phenotypic testing showed an inhibition zone of 12 mm, which we considered as indicative of susceptibility; nevertheless, the lack of CLSI or EUCAST breakpoints [21], in addition to very few reports of susceptibility testing being available, made the determination of the susceptibility/resistance profile subjective. Among the *Enterococcus* spp. isolates with *tet*(M), three were considered susceptible to tetracycline in the phenotypic susceptibility testing and one was classified as intermediate. This result reinforces the need to complement environmental molecular approaches with culture-based methods when studying antimicrobial resistance to properly correlate the presence of genes with their functionality.

Table 3. Results of the PCR screening for the antibiotic resistance genes and class 1 integrons.

Isolate ID	Resistance Phenotype	Intermediate Phenotype	Affiliation	Antibiotic Resistance Genes	Integron Content
E73	AMP; TE; CIP	CTX; CN	*Citrobacter* sp.	*bla*TEM *	ANT(3″)-IIa *
E79	AMP	CN; CIP	*Kluyvera* sp.	*bla*CTX-M *	
E82	TE	VA	*Enterococcus* sp.	*tet*(M) *	ANT(3″)-IIa *
E84	TE		*Enterococcus* sp.	*tet*(M)	
E112			*Brevudimonas* sp.	*tet*(M) *	
E113	AMP; CN; CIP		*Shigella* sp.	*bla*TEM *	
E126			*Stenotrophomonas* sp.	*tet*(M) *	
E127	IMP	CIP	*Pseudomonas* sp.	ND	*dfr*A25 *
E142	AMP; TE; CIP	CN	*Klebsiella* sp.	*bla*SHV *	
E145		CN	*Pseudomonas* sp.	ND	*aad*A6 *
E148	AMP; TE	CIP	*Raoultella* sp.	*bla*SHV *	*dfr*A25 *
E154		TE	*Enterococcus* sp.	*tet*(M) *	
E159		TE; CIP	*Enterococcus* sp.	*bla*SHV	*dfr*A25 *
E160	TE	CIP	*Enterococcus* sp.	*tet*(M) *	
E161	TE		*Enterococcus* sp.	*tet*(M)	
E164	TE	CIP	*Enterococcus* sp.	*tet*(M) *	
E170	TE		*Enterococcus* sp.	*tet*(M) *	
E171	TE	VA	*Enterococcus* sp.	*tet*(M)	
E179	TE	VA	*Enterococcus* sp.	*tet*(M) *	
E183			*Enterococcus* sp.	*tet*(M) *	
E188			*Enterococcus* sp.	*tet*(M)	
E195			*Enterococcus* sp.	*tet*(M) *	
E208		TE	*Enterococcus* sp.	*tet*(M) *	
E209			*Enterococcus* sp.	*bla*SHV *	

*, confirmed by sequencing; AMP, ampicillin; TE, tetracycline; CTX, cefotaxime; CN, gentamycin; CIP, ciprofloxacin; VA, vancomycin; ND—not determined.

Regarding ciprofloxacin, there were 47 isolates with interpretative standards available, of which 5 isolates were considered resistant and 15 presented an intermediate phenotype (Supplementary Table S2). The resistant isolates were the *Pseudomonas* sp. isolate E9, *Aeromonas* sp. isolate E115, *Citrobacter* sp. isolate E73, *Klebsiella* sp. isolate E142 and *Shigella* sp. isolate E113. Resistance to ciprofloxacin in isolates from the genera *Pseudomonas* [58], *Aeromonas* [59], *Citrobacter* [39], *Klebsiella* [60,61] and *Shigella* [62] have been reported. Our results are of concern since these ciprofloxacin-resistant bacteria (some with pathogenic behavior) were isolated from all sampling sites, including in the effluent waters released directly into the environment.

In respect to gentamicin, it was only possible to interpret results for 17 out of the 50 bacterial isolates due to the lack of interpretative standards available (Supplementary Table S2). Of these, only two isolates were considered resistant to gentamicin and nine showed intermediate phenotypes. The resistant isolates were *Pseudomonas* sp. E150 and *Shigella* sp. E113. The resistance of *Pseudomonas* to aminoglycosides, in particular, to gentamicin, has been extensively reported [63]. Some reports consider gentamicin resistance in *Shigella* spp. less common [64,65]; however, opposing statistics, showing high percentages of resistance in clinical isolates, have also been reported [66,67].

A total of 25 of our isolates were Gram-positive, of which none were found to be resistant to vancomycin (Supplementary Table S2). However, six isolates affiliated with *Enterococcus* spp. were considered to have intermediate phenotypes. Vancomycin-resistant *Enterococcus* spp. is one of the major threats to human health, being considered a high priority target by the WHO for the research and development of new antibiotics [68–70].

Resistance to β-lactams was evaluated against the following three antibiotics covering three classes: ampicillin, which belongs to penicillin; cefotaxime, which belongs to cephem; and imipenem, which belongs to carbapenem. Regarding ampicillin, 9 isolates (out of 36 isolates) presented resistance phenotypes, with, additionally, 2 presenting intermediate phenotypes (Supplementary Table S2). The resistant isolates were *Citrobacter* sp. E73, *Klebsiella* sp. E142 and E167, *Kluyvera* sp. E79, *Providencia* sp. E130, *Raoultella* sp. E148, E74 and E8, and *Shigella* sp. E113. Ampicillin resistance in the genera *Citrobacter* [39], *Klebsiella* [61], *Kluyvera* [71], *Providencia* [50], *Raoultella* [72] and *Shigella* [62] has been reported. No resistance to cefotaxime was observed in any of the 14 isolates for which interpretative standards existed; however, the following three isolates showed intermediate phenotypes: *Citrobacter* sp. E116 and E73 and *Klebsiella* sp. E167. Three isolates showed resistance to imipenem (out of twenty-two isolates), and they were *Klebsiella* sp. E167 and *Pseudomonas* sp. E127 and E144. Isolate E167 had as its closest hit *Klebsiella aerogenes* KCTC 2190, which was previously known as *Enterobacter aerogenes* [73]. Resistance to imipenem in *K. aerogenes* has been demonstrated to be due to changes in porin expression but also to efflux systems [74–77]. For both E127 and E144, the closest-described species is *Pseudomonas otitidis*, for which the constitutive expression of metallo-β-lactamase has been shown to potentially confer resistance to carbapenems, such as imipenem [78].

The PCR screening revealed the presence of bla_{SHV} in *Klebsiella* sp. E142, *Raoultella* sp. E148, and *Enterococcus* sp. E209; bla_{TEM} in *Citrobacter* sp. E73 and *Shigella* sp. E113; and $bla_{\text{CTX-M}}$ in the isolate *Kluyvera* sp. E79. Both *Klebsiella* sp. E142 and *Raoultella* sp. E148 were classified as resistant to ampicillin, and thus, it is possible that the identified bla_{SHV} was the underlying resistance determinant. Similarly, the isolates *Citrobacter* sp. E73 and *Shigella* sp. E113, which were considered resistant to ampicillin, possessed the bla_{TEM} gene.

Although present and confirmed by sequencing, the bla_{SHV} in *Enterococcus* sp. E209 did not seem to confer resistance since this isolate was considered susceptible to ampicillin (according to the CLSI breakpoints). Furthermore, $bla_{\text{CTX-M}}$ was present in the isolate *Kluyvera* sp. E79, which was considered susceptible to cefotaxime. These, in addition to the *tet*(M)-positive but phenotypically susceptible to tetracycline isolates previously mentioned, reinforce the importance of conducting culture-based methods in antimicrobial resistance surveillance. The presence of a gene does not necessarily indicate the presence of resistance, which has been shown, for instance, for bla_{TEM}, where, by the presence of weak promotors or the production of inactive mutants, no resistance was conferred [79].

Although interpretative standards were available for most of the isolates, this was not the case for the following three isolates: the *Comamonas* sp. Isolate E11, the *Brevundimonas* sp. isolate E112 and the *Stenotrophomonas* sp. isolate E126. Even without interpretative standards, some resistance phenotypes were possible to be deduced. These three isolates were resistant to ampicillin since no inhibition zones were observed. The *Brevundimonas* sp. isolate E112 showed inhibition zones of 11 and 10 millimetres for cefotaxime and imipenem, respectively, and the *Stenotrophomonas* sp. isolate E126 showed inhibition zones of 8 and 0 millimetres for the same antibiotics, respectively, which also suggested decreased

susceptibility to these β-lactams. Regarding tetracycline, the *Stenotrophomonas* sp. Isolate E126 showed an inhibition zone of 9 millimetres, indicating decreased susceptibility.

3.3. Presence of Mobile Genetic Elements

The PCR screening for integrons revealed the presence of such mobile genetic elements in multiple isolates, some of which were confirmed by sequencing (Table 3 and Supplementary Figure S3).

The amplified band for the isolates *Citrobacter* sp. E73 and *Enterococcus* sp. E82 was revealed by sequencing to possess ANT(3″)-IIa, an aminoglycoside nucleotidyltransferase which has been associated with resistance to spectinomycin and streptomycin [80]. The isolate *Citrobacter* sp. E73 was one of the multidrug-resistant strains identified in the antibiotic susceptibility testing, showing resistance to ampicillin, tetracycline and ciprofloxacin, and it was retrieved from sampling site C. Its multidrug resistance classification, the presence of a class 1 integron carrying a gene cassette that might confer resistance to aminoglycosides such as streptomycin, and the fact that it was isolated from the final effluent that was discharged into the environment established this isolate as a critical example of the potential dissemination of multidrug resistance not only within the WWTP but also, and worryingly, into the environment.

By sequencing, it was possible to detect the class 1 integron *In*51 in the isolate *Pseudomonas* sp. E145, which has been previously described in *P. aeruginosa*. This integron carries an aminoglycoside nucleotidyltransferase gene, *aad*A6, which encodes resistance to spectinomycin and streptomycin [81].

The sequencing of amplicons from the isolates *Pseudomonas* sp. E127, *Enterococcus* sp. E159 and *Raoultella* sp. E148 showed the presence of the trimethoprim resistance gene *dfr*A25, which encodes a dihydrofolate reductase that was previously identified in a class 1 integron in *Salmonella* Agona [82].

The isolates *Pseudomonas* sp. E127 and *Enterococcus* sp. E82 were retrieved from sampling site A, *Enterococcus* sp. E159 from sampling site B, *Citrobacter* sp. E73 and *Raoultella* sp. E148 from sampling site C, and *Pseudomonas* sp. E145 from sampling site D (Supplementary Table S1). The presence of bacteria harboring mobile genetic elements, in particular, integrons carrying antibiotic resistance genes, in all the sampling points, including the final effluent, further supports the importance of improvements in control measures to prevent the spread of antibiotic resistance genes in WWTPs and into the receiving environments.

4. Conclusions

Our work revealed the presence of antibiotic-resistant bacteria across a WWTP, including in the final effluent discharged into the aquatic environment.

A majority of the isolates showed resistance to tetracycline and ampicillin. Of note, three isolates belonging to clinically relevant genera, *Citrobacter* sp. E73, *Shigella* sp. E113 and *Klebsiella* sp. E142, revealed multidrug resistance phenotypes, and they were detected in three of the WWTP sampling sites, including in the river downstream. Isolated from the discharged effluent into the environment, *Citrobacter* sp. E73 is of particular interest since, apart from its multidrug-resistant phenotype, it was also shown to possess a class 1 integron carrying an aminoglycoside resistance gene.

By demonstrating the presence of antibiotic-resistant bacteria/antibiotic resistance genes and their association with mobile genetic elements throughout an WWTP, as well as in the surrounding aquatic environment, the present work reinforces the importance of surveillance/monitoring studies within these systems, as these bacteria might enter into contact with humans and animals, representing a public health risk. Thus, our data contribute to the awareness of the importance of tackling antimicrobial resistance in the framework of the One Health concept by producing actionable information for public health agencies and, ultimately, policymakers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol4010025/s1, Figure S1: Phylogenetic 16rRNA gene sequence-based dendrogram of isolates from the WWTP and closest type strains; Figure S2: Examples of the results of the antibiotic susceptibility assays; Figure S3: Example of representative gel electrophoresis for PCR amplification of class 1 integron. Table S1: Isolation conditions and NCBI closest described species for each isolate; Table S2: Results of the antimicrobial susceptibility testing.

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