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Antibiotic Resistance in Seawater Samples from East Coast of Spain

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Abstract: Seawater has been proposed as a reservoir for antibiotic-resistant bacteria and antibiotic resistance genes, thus representing a risk to public health. In this study, we evaluated the presence of antibiotic resistance determinants (bacteria and genes) in 77 seawater samples collected at different points along the coast of the Gulf of Valencia (Spain). Specifically, indicators of fecal contamination bacteria, *Escherichia coli* and *Enterococcus* sp., were isolated, and their antibiotic resistance profiles were analyzed through the use of the Sensititre® system, followed by the detection of the main antibiotic resistance genes (*bla*TEM, *qnr*S, *tet*W, *sul*I, and *erm*B). The highest frequencies of resistance in the *E. coli* isolates were detected for ampicillin (35.1%) and ciprofloxacin (17.5%), followed by sulfamethoxazole and trimethoprim (15.7%), while 23% of enterococci isolates showed resistance to a single antibiotic, 20% against tetracycline and 3% against daptomycin. Through PCR analysis, 93% of the *E. coli* strains showed the *bla*TEM and *sul*I resistance genes. Among the enterococci, the presence of the *bla*TEM gene was detected in 40% of the isolates, while the rest of the genes were present at very low rates. Among the water samples, 57% were positive for at least one of the tested genes, italic format with *bla*TEM being the most commonly found gene (47%), followed by the *qnr*S (33%) and *sul*I (23%) genes. These results show that seawater, in addition to being subjected to a high rate of fecal contamination, can contribute to the spread of antibiotic resistance.

Keywords: antibiotic resistance; fecal indicators; seawater; Mediterranean

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1. Introduction

Antimicrobial resistance (AMR) is one of the world's greatest public health challenges [1,2]. The spread of antibiotic-resistant bacteria (ARB) is directly linked to the overuse and inappropriate use of antibiotics in human and veterinary medicine, aquaculture, and agriculture, as well as inadequate disposal in several regions of the world [3]. Although most studies have focused on resistance in clinical settings, the increase in infections caused by ARB has stimulated interest in understanding the occurrence of antibiotic resistance in natural settings. The environment plays a crucial role as a potential reservoir of antibiotic resistance genes (ARGs) [4,5], contributing to their spread among environmental bacteria and human pathogens via horizontal gene transfer (conjugative plasmids and integrons) [6–8].

AMR is one of the central points of the "One Health" approach, and any strategy aimed at reducing the risk to human health posed by AMR must consider not only the health context but also all of the complex interrelationships that occur between all of the ecological niches that act as reservoirs and sources of dispersion of these resistance determinants [9,10]. Several studies have reported the presence of antibiotic-resistant bacteria and/or resistance genes in different environments such as soils [11], vegetables [12,13], livestock farms [14],

and aquatic environments such as wastewater [15,16], drinking water [17–20], surface waters [21–23], irrigation ditches [24,25], and, to a lesser extent, the marine environment. However, it is well known that run-off from land-based sources and wastewater discharge can reach the sea, introducing resistant bacteria and their genes to this environment in turn [26]. Another important source of antimicrobial resistance is the use of antibiotics in activities related to marine aquaculture [27,28]. Therefore, seawater can diffuse ARGs not only through the fluidity of water bodies but also through transfer between bacterial species [29]. Furthermore, urban coastal beaches, which are widely used by humans for recreational purposes, may provide an ideal environment for resistance spread [30,31].

Although ARB and ARG have been recognized as emerging contaminants [32,33], insufficient information is available on their prevalence and abundance in the environment [34]. Therefore, information on the environmental occurrence of ARB and ARGs will help to fill existing gaps and support national and international action plans to reduce the spread of antimicrobial resistance [35].

Although, as mentioned above, ARGs could be transferred to the sea, reports on ARG pollution in coastal areas are very few. Hence, this study aimed to evaluate the antibiotic resistance profile of fecal indicator bacteria isolated from seawater from different coastal locations in Comunitat Valenciana (Spain) by combining phenotypic and molecular analyses. For the present study, we selected a series of ARGs that confer resistance to the most widely used antibiotics in the clinical practice: *bla*TEM (β-lactams), *erm*B (macrolides), *qnr*S (fluoroquinolones), *sul*I (sulfonamides), and *tet*W (tetracyclines). These genes are involved in different resistance mechanisms, such as antibiotic efflux, antibiotic inactivation, antibiotic target substitution, antibiotic target modification, and antibiotic target protection and have been shown to be significant in estuarine and marine environments [36].

2. Materials and Methods

2.1. Samples

A total of 77 seawater samples were taken along 23 coastal locations of the Comunitat Valenciana (eastern Spain). Twenty-one seawater samples were collected from eight municipalities of the North Valencia region, thirty samples were collected from nine municipalities of the South Valencia region, and twenty-six samples were collected from six municipalities of the Alicante region. Figure 1 shows the sampling locations along the coast. All of the samples were collected aseptically, placed in a temperature-controlled box at 4 °C, transported to the laboratory, and processed without further delay.

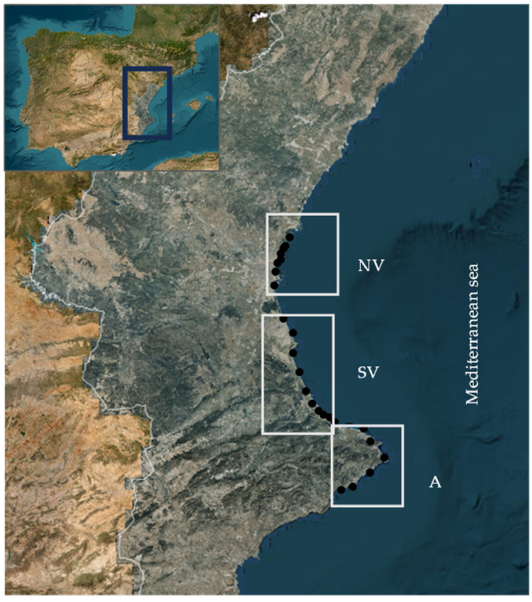

Figure 1. Coastal water sampling sites. NV: North Valencia; SV: South Valencia; A: Alicante.

2.2. Bacterial Analysis

Bacterial analyses were performed using Colilert and Enterolert test kits to detect *Escherichia coli* and *Enterococcus* sp., respectively, according to the manufacturer's instructions. Both methods have been certified as alternative methods by the standards [37,38]. The density units were expressed as the most probable number (MPN) per 100 mL, according to the Quantry-Tray/2000 and Quanty-Tray systems, respectively (IDEXX Laboratories, Hoofddorp, The Netherlands). *E. coli* and *Enterococcus* sp. colonies were isolated from positive wells and subcultured onto Tryptone Bile X-Glucuronide Agar (Oxoid, Ltd., Hampshire, UK) and Bile Esculine Azide Agar (Scharlau, Barcelona, Spain), respectively. Tentative colonies were then confirmed by the API phenotypic identification system (API20E strips, BioMèriux, Marcy-l'Étoile, France) and stored in refrigeration until their use.

2.3. Determination of Antibiotic Resistance

The antibiotic resistance of *E. coli* and *Enterococcus* sp. isolates was determined using the Sensititre System (Thermo Fischer Scientific, Madrid, Spain), an automatic system that uses a microplate format with a panel of different antimicrobial compounds that are accurately dosed at appropriate dilutions resembling the classical broth dilution method, according to the methods described by the Clinical and Laboratory Standards Institute [39], and accepted as a method of international reference [40]. EUVSEC plates were used for *E. coli* isolates, with the following antimicrobials to be tested: ampicillin (AMP), azithromycin (AZI), cefotaxime (FOT), ceftazidime (TAZ), ciprofloxacin (CIP), chloramphenicol (CHL), colistin (COL), gentamicin (GEN), meropenem (MER), nalidixic acid (NAL), sulfamethoxazole (SMX), tetracycline (TET), tigecycline (TGC), and trimethoprim (TMP). The enterococci isolates were tested against ampicillin (AMP), ciprofloxacin (CIP), chloramphenicol (CHL), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LIN), quinupristin/dalfopristin (QUIN/DAL), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC), and vancomycin (VAN), using the EUVNEC plate. The test was performed following the manufacturer's instructions. The results were interpreted by means of a digital visualization system, Sensititre Vizion (Thermo Scientific); for wells that showed bacterial growth, a sedimentation button was observed, indicating the resistance of the microorganism to the concentration of the antibiotic contained in the well. The well containing the lowest antimicrobial concentration where no growth was observed was the MIC (minimum inhibitory concentration). *E. coli* ATCC 25922 and *E. fecalis* ATCC 29212 were used as internal controls.

2.4. Detection of ARG

The analysis for the presence of ARG was carried out both in direct water samples and in bacteria isolated from these waters. For the DNA extraction from both sample types, the GenE-lute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Madrid, Spain) ref. NA2110) was used according to the manufacturer's instructions. For the water samples, a volume of 250 mL was filtered through a 0.45 µm pore size membrane (Millipore) to retain the bacterial cells. The membranes were aseptically cut and suspended in 500 µL of lysis buffer included in the kit. Next, 80 mg of glass beads was added, and the mixture was shaken for 20 min at 3000 rpm (Disruptor Gene, USA Scientific, Ocala, FL, USA). For ARG detection from *E. coli* and enterococci isolates, bacterial cells from an overnight culture were harvested and suspended in 500 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8), and then, DNA was extracted.

The ARGs chosen for this study were *bla*TEM, *erm*B, *qnr*S, *sul*I, and *tet*W, which confer resistance to β-lactam antibiotics, macrolides, quinolones, sulfonamides, and tetracyclines, respectively. These genes are involved in a variety of resistance mechanisms. These include antibiotic efflux, antibiotic inactivation, antibiotic target replacement, antibiotic target modification and antibiotic target protection.

The PCR mixture was prepared in a total volume of 50 µL containing 5 µL of the DNA template and 45 µL of the reaction mixture including 1X PCR buffer (Sigma-Aldrich), 1.5 mM of $MgCl_2$ (Sigma, Aldrich), 0.2 mM of dNTPs, 0.25 µM of each primer (TIB MOLBIOL, Madrid, Spain), and 2.5 U of Taq polymerase (Sigma, Aldrich). The primer sequences and cycling conditions for five PCRs are shown in Table 1. MiliQ water was used as a negative control; as positive controls for each gene, *E. coli* isolates from our own collection tested in previous work were used. The PCR products were analyzed using gel electrophoresis on 1.2% agarose gel in 1xTAE buffer (40 mM of Tris, 20 mM of acetic acid, 1 mM of EDTA) plus 5 µL of RedSAfe (iNtRON Biotechnology, Ecogen, Madrid, Spain) per 100 mL. Ten µL of PCR product, previously mixed with 6x loading buffer, was used. Two molecular weight markers were included in each gel (GeneRuler 100 bp DNA Ladder, 0.5 µg/µL, Thermo Fischer Scientific, Madrid, Spain). Finally, the fragments were visualized in a transilluminator under UV light.

Table 1. PCR primer sequences, targets, and conditions of the reactions.

Target Gene	Sequence	Conditions	Product (bp)	Reference
*bla*TEM	5′-GCKGCCAACTTACTTCTGACAACG-3′ 5′-CTTTATCCGCCTCCATCCAGTCTA-3′	95 °C 3 min (1 cycle); 95 °C 15 s and 60 °C 20 s (40 cycles); 72 °C 1 min	247	[41]
*erm*B	5′-GATACCGTTTACGAAATTGG-3′ 5′-GAATCGAGACTTGAGTGTGC-3′	95 °C 3 min (1 cycle); 95 °C 15 s and 58 °C 20 s (40 cycles); 72 °C 1 min	364	[42]
*qnr*S	5′-GACGTGCTAACTTGCGTGAT-3′ 5′-TGGCATTGTTGGAAACTTG-3′	95 °C 3 min (1 cycle); 95 °C 15 s and 62 °C 20 s (40 cycles); 72 °C 1 min	240	[22]
*sul*I	5′-CGCACCGGAAACATCGCTGCAC-3′ 5′-TGAAGTTCCGCCGCAAGGCTCG-3′	95 °C 3 min (1 cycle); 95 °C 15 s and 65 °C 20 s (40 cycles); 72 °C 1 min	163	[43]
*tet*W	5′-GAGAGCCTGCTATATGCCAGC-3′ 5′-CTTTATCCGCCTCCATCCAGTCTA-3′	95 °C 3 min (1 cycle); 95 °C 15 s and 60 °C 20 s (40 cycles); 72 °C 1 min	168	[44]

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2.5. Statistical Analysis

Statistical analysis was performed by using Statgraphics (Centurion XVII). A multifactor analysis of variance was carried out to compare the results obtained in *E. coli* and *Enterococcus* sp. counts for each sample, followed by a post hoc analysis using the Fisher's least significance difference (LSD) method. Resistance gene detection was analyzed by a χ^2 test, using contingency tables, in order to establish any possible dependence with location of sampling.

3. Results

3.1. Microbiological Determination of E. coli and Enterococcus sp. in Marine Water Samples

Among the 77 water samples analyzed, 74% showed contamination by *E. coli*, and 39% showed contamination by *Enterococcus* sp. The NMP of CFU/100 mL varied from 1 to 3.8 $\log_{10}$ and 1 to 2.9 $\log_{10}$ for *E. coli* and *Enterococcus* sp., respectively. The statistical analysis did not show significant differences in the contamination level among the three sampling zones. In accordance with the limits established in Spanish regulations (R.D. 1341/2007), 87% of the analyzed waters presented excellent quality (87%) as bathing waters, while the rest of them were classified as being in the good quality category.

3.2. Antibiotic Resistance Determination of Bacterial Isolates

A total of 87 strains were isolated from the seawater samples (57 *E. coli* strains, and 30 *Enterococcus* sp. strains) and subjected to the antibiotic sensitivity test. The resistance of the bacteria to antibiotics was determined according to the CLSI standards [39] for antimicrobial susceptibility testing.

Among the 57 isolates of *E. coli* (Table 2), 34 isolates (59.6%) were resistant to at least one antibiotic. Resistance to ampicillin was the most frequent observation (20 isolates) followed by ciprofloxacin (10 isolates), sulfamethoxazole, and trimethoprim with 9 isolates for each and tetracycline and nalidixic acid with 8 isolates (Supplementary Materials Table S1). The MIC values for these antibiotics were 32 - > 64 mg/L, 0.125–0.5 mg/L, >1024 mg/L, >32 mg/L, 32 - ≥ 64 mg/L, and >128 mg/L, respectively. There was no correlation ($p > 0.05$) between the number of strains resistant to any antibiotic and the area from which they were isolated. Multiple resistance (≥3 antibiotic classes) [45] was observed in 5 out of 34 resistant *E. coli* strains, (14.7%). Each of them presented a unique multi-resistance pattern. β-lactam resistance was present in all profiles, followed by sulfonamide and tetracycline resistance, present in four profiles, and quinolone resistance, observed in three profiles. Resistance to polymyxins (colistin) and aminoglycosides (gentamicin) was only present in one multi-resistance profile for each. Finally, none of the isolates showed resistance to chloramphenicol and tigecycline, with MIC ranges of ≤8–16 mg/L and ≤0.25–1 mg/L, respectively.

Table 2. Incidence resistant *E. coli* isolates for each antimicrobial compound separately and that of multi-resistance in three different zones of Comunitat Valenciana Coast.

Zone	No. Tested Isolates	Number of *E. coli* Resistant Isolates														
		SMX	TMP	CIP	TET	MER	AZI	NAL	FOT	CHL	TGC	TAZ	COL	AMP	GEN	MDR [a]
NV	14	2	3	2	2	0	1	3	0	0	0	1	0	4	1	2
SV	23	4	4	2	2	1	3	4	2	0	0	4	1	12	0	2
A	20	3	2	4	4	0	1	1	0	0	0	0	0	4	0	1
Total	57	9	9	10	8	1	5	8	2	0	0	5	1	20	1	5
% of resistant isolates		15.8	15.8	17.5	10.4	1.8	8.8	14	3.5	0	0	8.8	1.8	35.1	1.8	14.7

SMX: sulfamethoxazole; TMP: trimethoprim; CIP: ciprofloxacin; TET: tetracycline; MER: meropenem; AZI: azithromycin; NAL: nalidixic acid; FOT: cefotaxime; CHL: chloramphenicol; TGC: tigecycline; TAZ: ceftazidime; COL: colistin; AMP: ampicillin; GEN: gentamicin. NV: North Valencia; SV: South Valencia; A: Alicante. [a]: MDR: resistance to ≥3 antibiotic classes.

Only seven *Enterococcus* sp. isolates showed resistance to the tested antibiotics, specifically, six isolates against tetracycline and one against daptomycin (Table 3) (Supplementary Materials Table S4). The MIC ranges for these antibiotics were 64–128 mg/L and 16 mg/L. The statistical analysis showed no correlation ($p > 0.05$) between the resistance of the isolates to any antibiotic and the area from which they were isolated.

Table 3. Incidence of resistant *Enterococcus* sp. isolates for each antimicrobial compound in three different zones of Comunitat Valenciana Coast.

Zone	No. Tested Isolates	Number of *Enterococcus* Resistant Isolates											
		AMP	CHL	CIP	DAP	ERY	GEN	LIN	QUIN/DAL	TEI	TET	TGC	VAN
NV	9	0	0	0	0	0	0	0	0	0	0	0	0
SV	12	0	0	0	0	1	0	0	0	0	4	0	0
A	9	0	0	0	0	0	0	0	0	0	2	0	0
Total	30	0	0	0	0	1	0	0	0	0	6	0	0
% of resistant isolates		0	0	0	0	3.3	0	0	0	0	6.6	0	0

AMP: ampicillin; CHL: chloramphenicol; CIP: ciprofloxacin; DAP: daptomycin; ERY: erythromycin; GEN: gentamicin; LIN: linezolid; QUIN/DAL: quinupristin/dalfopristin; TEI: teicoplanin; TET: tetracycline; TGC: tigecycline; VAN: vancomycin. NV: North Valencia; SV: South Valencia; A: Alicante.

3.3. Antibiotic-Resistant Gene Determination in Water Samples and Bacterial Isolates

The presence of five antibiotic resistance genes in the 77 seawater samples was analyzed as described in the Section 2. Forty-four of the samples (57%) were positive for at least one of the genes. As shown in Table 4, the *bla*TEM gene was the most frequently detected gene (46.7%), followed by *qnr*S (32.5%) and *sul*I (23.4%); the *tet*W gene was detected in 17% of the samples, while *erm*B was only found to be present in one sample (Supplementary Materials Table S3). The statistical analysis only showed a significant relationship between the *tet*W gene and the region of Alicante ($\chi^2 = 10.384$, $p < 0.05$), and it was not detected in any of the samples from South Valencia. Combinations of two genes were detected in 24 seawater samples (31%), with the most abundant combinations being those containing the *bla*TEM gene. Three gene combinations were detected in nine samples (ca. 12%). Finally, four and five gene combinations were present in only one sample. A statistically significant dependence relationship was only detected between the *tet*W gene and the sampling zones of North Valencia and Alicante ($\chi^2 = 10{,}384$, $p < 0.05$) as this gene was not detected in any of the samples from South Valencia.

Table 4. Presence of antibiotic resistance genes in water samples according to sampling zone.

Zone	No. Samples	Number of Positive Samples				
		***bla*TEM**	***qnr*S**	***erm*B**	***sul*I**	***tet*W**
NV	21	6	5	0	3	5
SV	30	15	9	0	5	0
A	26	15	11	1	10	8
Total (%)	77	36 (46.7)	25 (32.5)	1 (1.2)	18 (23.4)	13 (16.9)

NV: North Valencia; SV: South Valencia; A: Alicante.

The *bla*TEM gene, together with the *sul*I gene, was the most frequently detected gene, with each of them being found in 53 *E. coli* strains (92.9%) (Table 5). They were followed in frequency by the *tet*W and *qnr*S genes, present in 40% and 36.8% of the samples, respectively. In contrast, the *erm*B gene was detected in only three isolates (5%) (Supplementary Materials Table S2). The statistical analysis showed no dependence relationship between the sampling area and the frequency of gene detection in *E. coli* isolates ($p > 0.05$).

Table 5. Presence of antibiotic resistance genes in *E. coli* isolates according to sampling zone.

Zone	No. Isolates	Number of Positive Isolates				
		***bla*TEM**	***qnr*S**	***erm*B**	***sul*I**	***tet*W**
NV	14	13	4	0	13	2
SV	23	21	11	1	21	11
A	20	19	6	2	19	10
Total (%)	57	53 (92.9)	21 (36.8)	3 (5.2)	53 (92.9)	23 (45.1)

NV: North Valencia; SV: South Valencia; A: Alicante.

The analysis of genes in enterococci (Table 6) again showed that the *bla*TEM gene was the most abundant, with it being detected in 40% of the isolates. The percentages of the other genes ranged from 3.3% for *qnr*S and *tet*W to 6.6% for *erm*B. It should be noted that *sul*I was not detected (Supplementary Materials Table S5). The statistical analysis showed no relationship between the sampling area and the occurrence of genes in the isolates ($p > 0.05$).

Table 6. Presence of antibiotic resistance genes in *Enterococcus* sp. isolates according to sampling zone.

Zone	No. Isolates	Number of Positive Isolates				
		***bla*TEM**	***qnr*S**	***erm*B**	***sul*I**	***tet*W**
NV	9	3	1	1	0	0
SV	12	1	0	1	0	0
A	9	8	0	0	0	1
Total (%)	30	12 (40.0)	1 (3.3)	2 (6.6)	0	1 (3.3)

NV: North Valencia; SV: South Valencia; A: Alicante.

For most of the genes investigated, their presence was greater in the *E. coli* isolates than in the directly analyzed waters (Figure 2). In North Valencia, the *bla*TEM and *sul*I genes were detected at significantly higher levels in the *E. coli* isolates than in the seawater samples (χ^2 = 13.988, p = 0.0002 and χ^2 = 20.896, p = 0.000, respectively). In South Valencia, besides the *bla*TEM and *sul*I genes (χ^2 = 10.194, p = 0.0014 and χ^2 = 29.020, p = 0.000, respectively), significant differences were found in the *tet*W gene, which was not detected in any of the seawater samples (χ^2 = 18.106, p = 0.0000). In Alicante, significant differences were found in the *bla*TEM and *sul*I genes, which were detected to a greater extent in the isolates (χ^2 = 8.160, p = 0.0043 and χ^2 = 15.120, p = 0.0001, respectively).

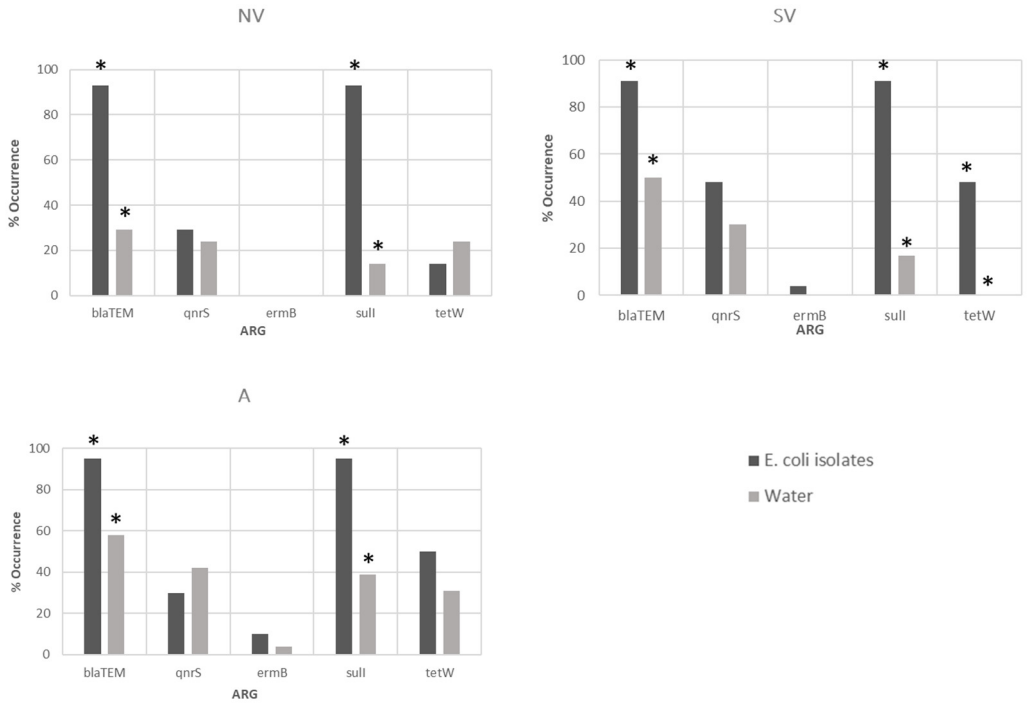

Figure 2. Comparison of ARG occurrence among the *E. coli* isolates and water from the different locations (NV: North Valencia; SV: South Valencia; A: Alicante). *: significant differences ($p < 0.05$) between gene occurrence in water samples and in the *E. coli* isolates.

When comparing the presence of ARGs in the enterococci and water samples (Figure 3), the *bla*TEM and *qnr*S genes were detected at significantly higher levels in the seawater samples than in enterococci isolates from South Valencia (χ^2 = 6.310, p = 0.0120 and χ^2 = 4.582, p = 0.0323, respectively). In addition to the *qnr*S gene (χ^2 = 5.553, p = 0.0184), significant differences were found in the *sul*I gene in the Alicante area (χ^2 = 4.846, p = 0.0277), only detected in the water samples.

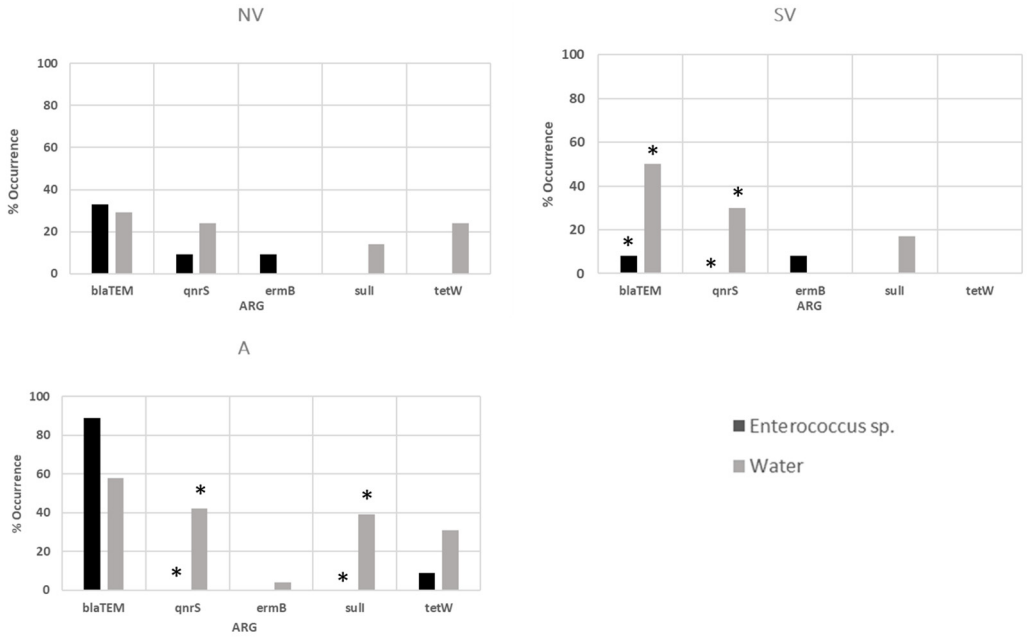

Figure 3. Comparison of ARG occurrence among the *Enterococcus* sp. isolates and water from the different locations (NV: North Valencia; SV: South Valencia; A: Alicante). *: significant differences ($p < 0.05$) between gene occurrence in water samples and in the *Enterococcus* sp. isolates.

4. Discussion

In recent decades, humanity has been confronted with the growing problem of the spread of antibiotic-resistant bacteria (ARB) and the difficulty of treating associated infections. The presence of these bacteria in coastal environments, as well as the presence of ARGs circulating freely in the water, represents a serious problem, especially for individuals who visit such areas for leisure purposes [46]. However, data on their presence and importance in the marine environment are still limited.

To this end, 77 water samples from the coastal waters of Comunitat Valencia (eastern Spain) were analyzed using microbial and molecular methods, to determine the presence of ARB and ARGs. The main species studied included *E. coli* and *Enterococcus* sp., which were tested for resistance to 12–14 antibiotics. The detected levels of both microorganisms were within the range found in the compilation of several studies in marine waters worldwide encompassing four decades of research [47], ranging from 0.59 to 3.51 $\log_{10}$ CFU/100 mL for *E. coli* and 0.63 to 3.21 $\log_{10}$ CFU/100 mL for *Enterococcus* sp. In a macro study conducted in Spain between 2012 and 2015, covering 1392 beaches throughout the country, a mean of 1.4 $\log_{10}$ CFU/100 mL for *E. coli* and 1.32 $\log_{10}$ CFU/100 mL for *Enterococcus* sp. was found for the southern Mediterranean, where our samples were collected, with a national mean of 1.93 and 1.65 $\log_{10}$ CFU/100 mL for each of the microorganisms [48]. Our values were within these averages, although we found some samples with *E. coli* values of 4.4 $\log_{10}$ CFU/100 mL and some samples of *Enterococcus* sp. with values of 4.2 $\log_{10}$ CFU/100 mL; both results were above acceptable levels and could have been due to the spillage of fecal matter due to problems with the sewerage system at the location or run-off from rainfall. It should also be noted that these exceptionally polluted samples were taken in the summer, on beaches close to the city of Valencia, where not only members of the local population (Valencia and its metropolitan area comprise a population of around one and a half million inhabitants) but also many tourists bathed during this period. Some authors have already stated that beaches in urban environments, as in our case, are of lower quality than those in semi-urban or natural environments [49–52], which is related to the number of people using the beaches, as well as the number of pets and birds, especially seagulls, that have access to them [53–55]. It is, therefore, difficult to determine the origin of this contamination.

The search for ARB was directed toward the detection of *E. coli* and enterococci of fecal origin. Both microorganisms have been recognized as important actors in the spread of antibiotic resistance [56,57]. The percentage of *E. coli* strains resistant to at least one antibiotic (59.6%) was similar to that obtained in a study conducted on beaches in Brazil [58] and lower than that obtained in other studies in Portugal [56] or China [59], with percentages of up to 80% being observed. Ampicillin resistance was the most frequent observation, confirming that β-lactam resistance was the most prevalent form in the aquatic environment [60,61]. Other antibiotics, such as ciprofloxacin, nalidixic acid, tetracycline, and sulfamethoxazole, were found to have resistance rates ranging from 14% to 18%. Similar findings were made by Hernández et al. [62] in a study conducted in Antarctica, an extreme location where the human presence is due to the establishment of scientific bases and where the removal of fecal contamination is very strictly controlled. This reflects the general resistance to β-lactams worldwide, with varying rates of resistance to other antibiotics depending on various factors such as geographical location. This fact also demonstrates the ability of antimicrobial resistance to distribute homogeneously in seawater.

Nine percent of the *E. coli* strains showed multidrug resistance, and again, the β-lactam family was present in all of the resistance profiles. Comparing these data with those of other studies, we see very different percentages of multidrug resistance. In a study carried out on the coast of Sicily, [63] found levels of multidrug resistance in their isolates ranging from 7% (against five antibiotics) to 14% (against four antibiotics). Similar results were obtained in isolates from Brazil beaches with multi-resistance levels ranging from 0 to 8.3% [64]. This supports the idea that the majority of multidrug-resistant ARBs are not of environmental origin but rather originate in places of high selective pressure, such as hospitals or highly contaminated ecosystems. The levels found in each geographical area are very consistent with the antibiotic use policies in these areas [65].

The enterococci in our study showed a low level of resistance (23%) compared with other studies conducted on Mediterranean beaches, for example, in Italy, with 38% of resistant strains [66], in Morocco (ca. 65%) [57], or in Tunisia, where this resistance is as high as 75% [67]. In our study, only strains resistant to tetracycline (20%) and daptomycin (3%) were obtained, with the latter being a rarely used drug. However, it is important to note that daptomycin is used as a last line of therapy, especially in vancomycin-resistant enterococcal infections [68]; therefore, the emergence of daptomycin-resistant enterococci is of concern in healthcare settings. No multidrug-resistant *Enterococcus* spp. isolates were found. These data are encouraging as antibiotic-resistant enterococci found in coastal marine waters are often closely related to human clinical isolates, probably originating from urban wastewater [69,70].

PCR assays revealed that *bla*TEM was the most frequently detected gene in the analyzed seawater samples. These results were in line with other studies in the Mediterranean, where this gene was detected in almost all samples [56,63,71], confirming that β-lactam antibiotic resistance genes were widely distributed in seawater samples, detecting it in fishes and wild marine species [72]. In a macro study carried out on water samples from oceans around the world, the authors found a high abundance of ARGs encoding resistance to β-lactam antibiotics, probably due to the high use of these antibiotics, as stated by the WHO [73,74]. The presence of the *qnr*S, *sul*I, and *tet*W genes was also comparable with the results of some other works that attested to their prevalence in coastal environments [75,76], but this contrasts with the absence confirmed in other coastal waters, such as Sicily [63]. This observed variability in the presence and abundance of ARGs was highly dependent on antibiotic usage patterns, which was a key factor for the generation and propagation of ARGs [77].

Once again, the *bla*TEM gene was the most frequently detected gene in both *E. coli* (92.9%) and *Enterococcus* sp. isolates (40%). The remaining genes showed more variable percentages, with the exception of *sul*I in *E. coli* isolates, which also stood out with identical percentages to *bla*TEM. Other studies have demonstrated that *bla*TEM, *tet*, and *sul* genes are ubiquitous in *E. coli* isolates from estuarine and marine environments [32,36,78,79].

In the present study, the frequency with which ARGs were detected was higher in *E. coli* isolates than in direct water samples. These results contrast with those found in other types of water, such as the study carried out by Amato et al. [24] in water from ditches that flow into the sea, specifically in the region of Valencia Norte. This, in turn, would be consistent with some studies suggesting that culture-dependent antimicrobial resistance approaches could underestimate the frequency and diversity of genetic determinants of antimicrobial resistance in environmental samples [80,81]. However, in our case, this could be due to the fact that the marine environment is a diluting medium, so the filtrate could provide little genetic material for amplification, while for the detection of *E. coli* ARGs, pure colonies with a high DNA load are used as a starting point.

Antibiotic resistance is no longer merely a clinical problem [82]. The demonstration of the presence of antibiotic resistance (bacteria and genes) in this work highlights the role of the sea in the spread of AR genes and the need to carefully consider the risks to human health associated with fecal contamination. Coastal areas, where human recreation, in addition to commercial activities, is important, are ideal environments for the transmission of resistance to the human population.

5. Conclusions

This study provides a clearer perspective on the circulation of ARG in the marine environment and its relationship with fecal pollution in seawater. This could facilitate future interventions to reduce antimicrobial resistance in the environment as part of a holistic One Health approach.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14051965/s1. Table S1: Incidence of resistant *E. coli* isolates for each antimicrobial compound according to sampling zone. Table S2: Presence of antibiotic resistance genes in *E. coli* isolates according to sampling zone. Table S3: Presence of antibiotic resistance genes in water samples according to sampling zone. Table S4: Incidence of resistant *Enterococcus* sp. isolates for each antimicrobial compound according to sampling zone. Table S5: Presence of antibiotic resistance genes in *Enterococcus* sp. isolates according to sampling zone.

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