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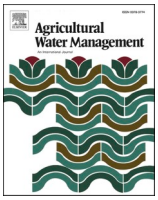

Occurrence of antibiotic resistant bacteria and resistance genes in agricultural irrigation waters from Valencia city (Spain)

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ABSTRACT

The increasing use of antibiotics has become one of the main environmental and health problems today, worldwide. In addition to causing toxic effects on non-targeted organisms, their presence in the environment promotes the horizontal transfer of resistance genes, thus increasing the presence of resistant bacteria in the environment and, consequently, the risk of super-resistant bacterial infections. In the present work, we have investigated the occurrence of antibiotic-resistant *E. coli* and genes (bla_{TEM}, *qnr*S, *tet*W, *sul*I and *erm*B) in surface water samples from different agricultural irrigation ditches located around Valencia city (Spain). Results of the research showed the presence of antibiotic resistance genes in all the analysed samples with mean percentages of 100% for *tet*W, 96% for bla_{TEM}, 80% for *sul*I, 72% for *qnr*S and *erm*B in 60% of samples. The higher antibiotic resistance rates were detected in the samples whose waters had more contact with human or animal activities. Our study detected a high proportion (79.2%) of multi-resistant *E. coli* isolates, which presented a total of 26 multi-resistance patterns. The high levels of antibiotic resistances in irrigation waters observed in this work could help to both, better understanding and management, of the contamination caused by antibiotics and related resistance genes in agricultural environments, in order to implement appropriate measures for control. Moreover, appropriate surveillance of the quality of these waters as well as developing sanitation techniques are necessary to decrease antibiotic resistance rates in irrigation water in our geographical area.

1. Introduction

Fresh water is an essential resource for domestic, agricultural and industrial purposes. Due to its high demand in agriculture, the increase of the global population and changes in consumption patterns, the availability and quality of fresh water are decreasing, causing problems in environmental sustainability and food security (Ercin and Hoekstra, 2014).

Antibiotics are some of the chemical pollutants most frequently found in the environment (Ternes et al., 2004). They can reach fresh water from urbanisation, agriculture, wastewater treatment plants (WWTPs) and pharmaceutical industry waste. Presence of antibiotics is the main factor for appearance and spread of antibiotic-resistant bacteria (ARB) in environment, posing a very serious risk for human and environmental health.

The World Health Organisation (WHO) estimates that ARB cause approximately 700,000 deaths per year worldwide (WHO, 2018). In Spain, according to the Spanish Ministry of Health, almost 3000 people die every year due to infections caused by antibiotic-resistant bacteria (PRAN, 2019).

Antibiotic contamination is not only relevant because of the presence of resistant bacteria, but also for the spreading of resistance genes (Martínez, 2003). These genes can persist in the environment or in the bacterial population even after the disappearance of the selection pressure (Pruden et al., 2006); hence, they are considered environmental pollutants.

Antibiotic resistance genes (ARG) are easily transmissible between bacteria of the same or different species (Huerta et al., 2013), due to selection pressure. They can be found in genetically transmissible elements, together with other antibiotic-resistance genes or genes that confer resistance to other pollutants (Aminov and Mackie, 2007). The mechanism that allows for the spread of resistance genes is horizontal transfer, what can happen between very different bacteria, pathogenic or non-pathogenic, Gram-negative or Gram-positive (Pruden et al., 2006). The conjunction of antibiotic residues, stress environmental conditions and the presence of microplastics (a new reactive surface for biofilm formation) in aquatic ecosystems increases the horizontal gene transfer and, consequently, the presence of resistance genes in the

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environment (Arias-Andres et al., 2018; Zhu et al., 2017). Spread of these ARG causes cross-resistance among pathogenic bacteria and increases the emergence of super-resistant microorganisms (O'Neill, 2015; Wright, 2005).

This emergence of antibiotic resistances in microorganisms of clinical concern has led to investigate their presence in environment, from where they can reach humans. Many authors have studied aquatic environments, including wastewaters, sea water, surface (rivers and lakes), recreational or drinking water (Christou et al., 2017; Niu et al., 2016; Proia et al., 2016; Hatosy and Martiny, 2015; Huerta et al., 2013; Stoll et al., 2012; Xi et al., 2009), detecting numerous antibiotics, diverse families of ARG, and ARB belonging to different populations and communities. This confirms the wide dispersion of resistances in different aquatic systems as well as the importance of the problem worldwide.

Among European countries, Spain presents the highest concentrations of antibiotics in surface waters (Danner et al., 2019; Ginebreda et al., 2010). According to Martínez (2003), 90% of the bacteria present in water are resistant to one antibiotic, and 20% are resistant to at least five antibiotics, being *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* some of the most relevant species (WHO, 2018).

Although a great number of recent investigations focus on the occurrence of antibiotics and ARB in aquatic environments, many gaps still remain (Larsson et al., 2018), especially in those related to agriculture (Franklin et al., 2016; Williams-Nguyen et al., 2016). Not only human health, but also the health of the agroecosystems may be in danger. Some studies have demonstrated that the presence of antibiotics in the environment affects the soil microbial communities, altering some important microbial reactions as respiration, iron reduction or nitrogen transformations (Franklin et al., 2016; Liu et al., 2011). A consequence, no less serious, is the possibility of contamination of vegetables, which can carry resistance genes, as well as new strains of resistant pathogens, to the food chain (Bürgmann et al., 2018; Hölzel et al., 2018).

ARB can reach vegetables through irrigation water (Araújo et al., 2017; Gekenidis et al., 2018). Recently, Dungan and Bjorneberg (2020) suggested that irrigation return flows can be a point source of ARGs that ultimately discharge into surface waters. Animals waste can also contaminate wastewater or may be used as manure, bringing antibiotics residues and resistant bacteria into crops (Grote et al., 2007). In this way, antibiotics can enter into human food chain through consumption of raw vegetables, which can also carry antibiotic-resistant food-borne pathogenic bacteria (Araújo et al., 2017; Hölzel et al., 2018). Several studies have demonstrated a correlation between the presence of resistance genes in vegetables, fruits, soil and irrigation water (Cerqueira et al., 2019a, 2019b; Hölzel et al., 2018; Araújo et al., 2017; Jongman and Korsten, 2016,). This is a main issue in the development of resistant bacterial strains, which can infect humans, increasing the difficulty to treat severe infections (Conde-Cid et al., 2018).

In this sense, it is essential to develop studies that can help to explain the importance of natural water bodies in the spread of antibiotic resistance on agricultural environments and to clarify the implicated pathways.

The "Huerta Valenciana" is a historical region around the city of Valencia (Spain) that has a huge productive, cultural and environmental value. It is an important part of the local agricultural heritage. Irrigation is achieved by drawing water from the Turia River to the surrounding fields via a complex network of irrigation ditches, which have served effectively for the irrigation of this area for centuries. Vegetables from this area are consumed, not only in this geographical area, but all around the country. Thus, the presence of antibiotics and ARB in this water not only threatens the health of the environment, but mainly human health. In this work, we aimed to study the existence of antibiotic resistance determinants, bacteria and genes, in the Turia River and its irrigation ditches network.

2. Material and methods

2.1. Water sample origin and collection

Five points were sampled monthly between January and May 2018: Turia River (TR), Royal Moncada Irrigation Ditch (MID), Rascanya Irrigation Ditch (RID), Vera Irrigation Ditch (VID) and Carraixet Ravine (CR) (Fig. 1). River water was collected in a recreational shallow area, when part of its water has already been collected to form the Royal Moncada Irrigation Ditch (MID). After this point, most of the water is distributed among several irrigation ditches, including RID and VID. The water of all these ditches network is used for crop irrigation, reaching most of agricultural fields around the urban area of Valencia. Carraixet Ravine collects the drainage waters of the surrounding fields, and the sampling point was located immediately before its outlet in the Mediterranean Sea. All the sampling sites are open channels, which receive occasional environmental contamination inputs from rainwater, soil, human and animal activity.

A total of 25 samples belonging to 5 sites and 5 different sampling times were analysed Samples were collected in 2 L refrigerated sterile containers, which were quickly transported within 2 h at 4 ºC to the laboratory and processed without further delay.

2.2. Water sample processing

One-hundred millilitres or, if necessary, decimal dilutions until 1:100 of each water sample were filtered through 0.45 μm pore diameter nitrocellulose membranes (Millipore), which were aseptically transferred to Microinstant^{R} Chromogenic Coliform Agar Base (Scharlau, Barcelona), a selective medium for *E. coli* and coliforms bacteria. In order to isolate presumptive ARB, the culture medium plates were was finally prepared adding one specific antibiotic to each one. The antibiotics used were: ampicillin, oxytetracycline, erythromycin, sulfamethoxazole and ciprofloxacin at 32, 45.5, 8, 16, and 4 μg/mL, respectively. The concentration of each antibiotic was from their minimal inhibitory concentration (MIC) for enterobacteria. Plates of the same medium without antibiotic served as positive control and for determining total viable coliforms and *E. coli* counts. All analyses were made in quadruple.

After 24 h incubation at 37 °C, rose-red coliforms and blue tentative *E. coli* colonies on the plates with each type of antibiotic were counted. One representative colony was selected from each antibiotic supplemented plate, and each sampling site and time. Presumptive antibiotic resistant *E. coli* were subcultured on the same medium and identified by API 20E system (BioMérieux España). *E. coli* cultures were re-suspended in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8) for further DNA extraction. Cultures were also preserved at − 80 °C in MicrobankTM-Blue criovials (Pro-Lab Diagnostics).

In addition, 300 mL of each water sample was filtered through a 0.45 μm pore diameter nitrocellulose membrane for direct ARG detection, without culturing. The membrane with the trapped biomass was aseptically fragmented and stored into 2 mL Eppendorf tubes for subsequent DNA extraction.

Fig. 1. Location of sampling sites in irrigation ditches network.

2.3. Antibiotic susceptibility testing of E. coli isolates

Antibiotic resistance of *E. coli* isolates was investigated by using the Sensititre System (Thermo Fisher), according to the manufacturer's instructions. This is an automatic system based on broth dilutions method, which uses a multi-well plate containing selected antimicrobials, which is filled automatically with appropriate dilutions of the bacteria suspension. After 24 h incubation, growth is read by using a digital display system (Sensititre Vizion, Thermo Fisher) and measured by VIZION-software SWIN (Thermo Fisher). The determination of the MIC (Minimum Inhibitory Concentration) corresponds to the lowest concentration with no growth. Results are interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org). *E. coli* ATCC 25922 was used as an internal control for all the assays.

Sensititre EUVSEC plates were used for testing 125 *E. coli* isolates susceptibility to fourteen antimicrobial compounds: sulfamethoxazole, trimethoprim, ciprofloxacin, tetracycline, meropenem, azithromycin, nalidixic acid, cefotaxime, chloramphenicol, tigecycline, ceftazidime, colistin, ampicillin and gentamicin.

2.4. Antibiotic resistance gene detection

Prior to DNA extraction, 80 mg of Glass Beads (Sigma-Aldrich) and 500 μL of T-lysis buffer (Sigma-Aldrich) were added to the Eppendorf tubes containing the fragmented filtration membranes and left for 20 min in agitation at 3000 rpm (Disruption Gene, USA Scientific). At the end of the bead beating, the membrane fragments were removed.

DNA was extracted from all the samples by using GenEluteTM Bacterial Genomic DNA Kit (Sigma-Aldrich), according to the manufacturer's instructions.

Specific primers (TIB MOLBIOL) for five ARG, related to five different families of antibiotics, were selected (Table 1): bla_{TEM} (β-lactams), *tet*W (tetracyclines), *erm*B (macrolides), *sul*I (sulfonamides) and *qnrS* (quinolones). The selection was performed attending to their usual presence in aquatic environments (Stange et al., 2019; Lee et al., 2017; Rodríguez-Mozaz et al., 2015; Martí et al., 2013).

The PCR reaction included 2.5 μL template DNA, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each primer, 2.5 U of Taq polymerase, 2.5 μL of extracted DNA and 1X PCR buffer, reaching a final reaction volume of 25 μL. All the PCR reagents were provided by Ecogen (Barcelona, Spain). Positive (DNA with each resistance gene previously checked in our laboratory) and negative controls (MilliQ water) were added. The amplification was developed in a Mastercycler®Pro (Eppendorf) thermal cycler. PCR products (10 μL) were detected by electrophoresis on 1.3% (w/v) agarose gel in TAE 1X (Tris 40 mM, acetic acid 20 mM, EDTA 1 mM) buffer with RedsafeTM (iNtRON Biotechonolgy) at 90 V for about 60 min and visualized by UV transillumination. A 100-bp DNA ladder (GeneRuler 100 bp DNA Ladder, Thermo Fisher) was used as molecular weight marker.

2.5. Statistical analysis

Statistical analysis was performed by using Statgraphics (Centurion XVII). A Multi-factor Analysis of Variance was carried out to compare the results obtained in *E. coli* counts for each sample, followed by a *post-hoc* analysis using the Fisher's least significance difference (LSD) method. Resistance genes detection was analysed by χ^2 test, using contingency tables, in order to establish any possible dependence with location and/or moment of sampling.

3. Results and discussion

3.1. Antibiotic-resistant faecal contamination in water samples

All water samples presented presumptive antibiotic resistant faecal coliforms at levels that were impossible to quantify, due to their elevated concentration, even when decimal dilutions were performed. This result was found in all the plates which contained antibiotics, except for samples incubated with oxytetracycline and ciprofloxacin, which could be counted, registering mean values from 1.28 to 1.96 logarithm of cfu/100 mL for ciprofloxacin, and from 1.42 to 1.79 for oxytetracycline.

E. coli counting was possible in most of the samples, except for the Vera Irrigation Ditch (VID) and some samples from Rascanya Irrigation Ditch (RID) and the Carraixet Ravine (CR), whose waters were overly contaminated to allow colony counting (Table 2).

No guidelines exist in Europe for regulating quality of irrigation waters, regarding antibiotic resistance bacteria. However, the presence of antibiotic resistant *E. coli* has been proposed as an indicator of the presence of ARB and associated clinically relevant genes (Gekenidis et al., 2018; EFSA, 2008). The limit for irrigation surface waters advised by Spanish guideline (R.D. 1620/2007) is 100 *E. coli* per 100 mL of water for water that comes into contact with edible products that will not be cooked prior to its consumption, without any harvest-time-dependent guideline. This value has been highly exceeded by *E. coli* in all the tested samples. The excessive concentration of *E. coli* (independently antibiotic resistant or not) detected, determines that the water could not be used for irrigation of raw-eaten vegetables in any case. Therefore, a surveillance of the quality of these waters, as well as developing sanitation techniques are necessary aiming to avoid microbiological risks and decrease antibiotic resistance rates in irrigation water.

The presence of the antibiotic in the culture medium had a statistically significant effect on *E. coli* growth compared with the control plates (without antibiotic) by sampling site (Table 2). Overall, counts were significantly lower in plates supplemented with ciprofloxacin

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

Target gene	Sequence	Conditions	Refs.
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	Xi et al. (2009)
*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	Chen et al. (2007)
qnrS	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	Martí and Balcázar (2013)
*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	Pei et al. (2006)
*tet*W	5' -GAGAGCCTGCTATATGCCAGC- 3' 5' -CTTTATCCGCCTCCATCCAGTCTA- 3'	95 °C 3 min (1 cycle); 95 °C 15 s and 60 °C 20 s (40 cyces); 72 °C 1 min	Aminov et al. (2001)

Table 2
E. coli counts in the water samples.

	Sampling time*	*E. coli* (cfu /100 mL ± SD) x 10^3				
		TR	MID	RID	VID	CR
Control	1	2.00 ± 0.15	1.29 ± 0.09	0.79 ± 0.19	-**	3.63 ± 1.02
	2	2.88 ± 0.09	3.60 ± 0.10	–	–	–
	3	4.57 ± 1.57	2.10 ± 0.11	–	–	–
	4	2.45 ± 0.38	1.18 ± 0.56	9.12 ± 0.70	–	–
	5	2.05 ± 0.06	1.67 ± 0.45	–	–	7.48 ± 2.80
	Mean	2.79 ± 0.45[a]	1.97 ± 0.98[a]	4.95 ± 0.44[a]	–	5.56 ± 1.92[a]
AMP	1	0.98 ± 0.02	0.45 ± 0.07	1.07 ± 0.22	–	1.05 ± 0.40
	2	1.02 ± 0.08	2.26 ± 0.34	–	–	–
	3	1.47 ± 0.08	0.39 ± 0.03	–	–	–
	4	1.02 ± 0.09	0.22 ± 0.07	–	–	2.04 ± 0.94
	5	0.86 ± 0.01	0.52 ± 0.38	6.28 ± 0.95	–	4.29 ± 0.97
	Mean	1.07 ± 0.06[c]	0.77 ± 0.18[b]	3.67 ± 0.59[a]	–	2.46 ± 0.77[b]
OXY	1	0.34 ± 0.04	0.16 ± 0.11	0.16 ± 0.04	1.12 ± 0.23	0.5 ± 0.09
	2	0.26 ± 0.11	0.98 ± 0.12	–	3.87 ± 0.36	–
	3	0.47 ± 0.06	0.27 ± 0.03	0.83 ± 0.10	–	1.73 ± 0.28
	4	0.18 ± 0.05	0.07 ± 0.08	2.80 ± 0.33	2.84 ± 0.25	0.37 ± 0.06
	5	0.17 ± 0.04	0.10 ± 0.05	6.02 ± 1.96	–	1.41 ± 0.17
	Mean	0.28 ± 0.06[e]	0.32 ± 0.80[c]	2.45 ± 0.61[a]	2.61 ± 0.28	1.00 ± 0.15[c]
SUL	1	1.61 ± 0.23	0.58 ± 0.13	0.83 ± 0.15	–	1.95 ± 0.48
	2	1.83 ± 0.15	4.90 ± 0.54	–	–	–
	3	2.63 ± 0.35	1.43 ± 0.09	–	–	–
	4	1.29 ± 0.35	0.82 ± 0.11	6.60 ± 0.42	–	3.47 ± 2.00
	5	1.34 ± 0.43	0.83 ± 0.63	9.80 ± 0.00	–	6.76 ± 2.61
	Mean	1.74 ± 0.22[b]	1.71 ± 0.30[a]	5.74 ± 0.19[a]	–	4.06 ± 1.70[ab]
ERY	1	2.19 ± 0.28	1.05 ± 0.01	1.25 ± 0.12	–	3.98 ± 0.62
	2	2.90 ± 0.15	8.32 ± 0.57	–	–	–
	3	3.95 ± 0.67	1.86 ± 0.23	–	–	–
	4	2.45 ± 0.16	1.11 ± 0.10	6.76 ± 0.97	–	5.12 ± 2.23
	5	2.46 ± 0.10	1.48 ± 1.07	6.74 ± 0.73	–	9.00 ± 0.06
	Mean	2.79 ± 0.22[a]	2.77 ± 0.40[a]	4.92 ± 0.61[a]		6.03 ± 0.97[a]
CIP	1	0.36 ± 0.01	0.06 ± 0.01	0.01 ± 0.00	–	0.29 ± 0.11
	2	0.43 ± 0.03	0.77 ± 0.10	–	–	–
	3	0.50 ± 0.06	0.11 ± 0.03	–	–	2.15 ± 0.21
	4	0.30 ± 0.03	0.14 ± 0.03	2.19 ± 0.31	–	0.76 ± 0.13
	5	0.31 ± 0.03	0.18 ± 0.16	–	–	1.05 ± 0.18
	Mean	0.38 ± 0.03[d]	0.25 ± 0.07[c]	1.10 ± 0.16[b]	–	1.06 ± 0.16[c]

Values are means ± standard deviation of cfu/100 mL in four plates for each sampling time and antibiotic. * : 1: January 22th; 2: February 19th; 3: March 12th; 4: April 16th; 5:May 14th.
* *: uncountable results.
AMP: ampicillin; OXY: oxytetracycline; ERY: erythromicin; SUL: sulfamethoxazole; CIP: ciprofloxacin. TR: Turia River; MID: Royal Moncada Irrigation Ditch; RID: Rascanya Irrigation Ditch; VID: Vera Irrigation Ditch; CR: Carraixet Ravine.
Within each column, mean values with the same superscripts are not significantly different ($p < 0.05$).

(0.57×10^3 cfu/100 mL), which showed the maximum inhibitory effect followed by oxytetracycline (2.05×10^3 cfu/100 mL), and ampicillin (1.21×10^3 cfu/100 mL), thus indicating that most *E. coli* in these water sources are not resistant to these two antibiotics. On the contrary, erythromycin (3.49×10^3 cfu/100 mL) was the less selective agent, as occurred for total coliform counts.

Statistical analysis (Multi-factor Analysis of Variance) showed that, in addition to the presence of antibiotics in the media, the sampling site and moment of sampling exerted a significant effect ($p < 0.05$) on presumptive resistant *E. coli* counts. MID and TR samples were the sites with the lowest concentration of bacteria, showing no significant differences between them.

VID (uncountable data), RID and CR samples were the sites with significant highest *E. coli* counts with mean values of 3.81×10^3 and 3.36×10^3 cfu/100 mL, respectively. VID and RID are irrigation ditches, whose waters have crossed the city of Valencia and the surrounding fields, carrying with them bacteria from urban, including hospitals, wastewaters and manure fertilised soil. Furthermore, they may also be contaminated by human or animal faecal material, present in the vicinity of the irrigation channels. The levels of *E. coli* contamination were in the same order than those obtained in previous studies (Brooks et al., 2014; Udikovic-Kolic et al., 2014; Czekalski et al., 2012), where the highest concentration of ARB was found in surface or groundwater that have been subjected to contamination by sewage, animal manure and agricultural activity.

The statistical study also showed that the moment of the sampling had a significant effect, denoting that the highest *E. coli* counts corresponded to the second sampling time (2.96×10^3 cfu/100 mL). It is probably due to heavy rain in the days prior to this sampling, which would have moved components from the agricultural soils, as well as removed microorganisms from the sediment in the irrigation ditches. Several studies argue that sediments can act as a reservoir of bacteria (Zhang et al., 2016; Sidrach-Cardona et al., 2014; De Oliveira and Pinhata, 2008; Alm et al., 2003) providing nutrients and protection against light or predation.

Table 3
Prevalence of resistance to each antimicrobial in *E. coli* isolated from each sampling site.

		Number of resistant *E. coli* isolates													
Sampling sites	Number of *E. coli* tested	SMX	TMP	CIP	TET	MER	AZI	NAL	FOT	CHL	TGC	TAZ	COL	AMP	GEN	Multi-resistance[a]
TR	25	25	16	20	16	0	14	18	4	5	0	0	0	20	0	20 (80)
MID	25	23	12	10	15	0	3	8	5	5	0	5	0	15	0	12 (48)
RID	25	25	18	11	18	0	4	11	4	7	0	4	0	18	7	18 (72)
VID	25	23	5	16	14	0	2	14	5	7	0	5	0	16	5	16 (64)
CR	25	25	14	18	18	0	7	18	7	10	0	7	0	21	4	22 (88)
TOTAL[b]	125	121 (96.8)	65 (52)	75 (60)	81 (64.8)	0	30 (24)	69 (55.2)	25 (20)	34 (27.2)	0	21 (16.8)	0	90 (72)	16 (12.8)	88 (70.4)

SMX: sulfamethoxazole; TMP: trimethroprim; 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.
TR: Turia River; MID: Royal Moncada Irrigation Ditch; RID: Rascanya Irrigation Ditch; VID: Vera Irrigation Ditch; CR: Carraixet Ravine
[a]: resistance to $\geq$ 3 antibiotic classes. [b]: number in parentheses represent percentages

3.2. Antibiotic susceptibility testing of *E. coli* isolates

A total of 125 *E. coli* strains were isolated: 25 from each sampling site, corresponding to each one of the plates supplemented with the five antibiotics. All isolates were subjected to automated antibiotic susceptibility test Sensititre. All of them showed resistance to at least one antibiotic. Resistance to sulfamethoxazole was the most frequent (96.8% of the isolates) followed by ampicillin (72%), tetracycline (64.8%) and ciprofloxacin (60%) (Table 3). Antibiotics of more recent use, like new generation cephalosporins (cefotaxime and ceftazidime) were less prevalent. Less used antibiotics, such as meropenem, tigecycline or colistin, were found to be active against all the isolates.

Overall, a higher number of antibiotic resistant *E. coli* isolates were detected in sulfamethoxazole supplemented media, while the lower value corresponded to plates containing erythromycin (Fig. 2). Statistical analysis revealed that the addition of a specific antibiotic to the media did not significantly increase the number of *E. coli* resistant to azithromycin, chloramphenicol, cefotaxime, gentamicin and ceftazidime isolated from the plates. However, a significant influence of the presence of some antibiotics in the media was observed: ampicillin resistance was favored in ampicillin-, ciprofloxacin-, oxytetracycline- and sulfamethoxazole-supplemented media. Resistances to ciprofloxacin and nalidixic acid were favored in ampicillin-, ciprofloxacin- and sulfamethoxazole-supplemented media. Resistance to trimethoprim was favored by oxytetracycline and sulfamethoxazole presence in culture media. Finally, sulfamethoxazole and tetracycline resistances were favored in all antibiotic supplemented media.

Multiple resistance, considered as resistance to $\geq$ 3 antibiotic classes (Egervärn et al., 2017), was observed in 70.4% (88 out of 125 *E. coli* isolates). A total of 15 multi-resistance patterns were detected. β-lactam resistance was present in all resistance patterns, followed by sulfonamide, quinolone and tetracycline resistance (14 (93.3%), 11 (73.3%), and 10 (66.7%) resistance patterns, respectively). The most frequent number of multi-resistance among *E. coli* isolates was against 4 and 5 antibiotic classes, with 30 isolates each one, followed by 3 with 16 isolates and 6 with 12 isolates (Table 4).

In a similar work, Wang et al. (2013) observed 31 different multi-resistance patterns out of 114 *E. coli* isolates from lake waters. Although a lower frequency of multi-resistance has been detected in other studies carried out in rivers (Hu et al., 2008; Ram et al., 2007), all of them showed a wide variety of multi-resistance patterns, which is usually attributed to the different origins of isolates (Osińska et al., 2017).

Considering the sampling site, Carraixet Ravine showed the highest rate of multi-resistance (88% of their *E. coli* isolates), presenting four different phenotypes. Moreover, three of these *E. coli* isolates were resistant to 9 out of the 14 antibiotics tested. This is a relevant result, as Carraixet ravine flows into the sea and it has been suggested that coastal waters can act as new reservoir for antibiotic resistance determinants, contributing to the dissemination of antibiotic resistant microorganisms and genes (Bennani et al., 2012).

Vera Irrigation Ditch showed a rate of 64% of multi-resistance among its *E. coli* isolates. Multi-resistance is primarily associated with the proximity of hospitals (Czekalski et al., 2012). In the present case, an increase in resistance has been observed as the analysed water had made a longer path through the subsoil of the Valencia city, which raises the possibility of contact with other human activities, such as hospitals or domestic wastewaters (Fig. 3). The high multi-resistance rate found in the river samples (80%) does not disagree with the previous statement, as these waters were collected in a recreational and shallow area and therefore, it is expected that contact with humans and animals is high.

When statistical analysis was performed, no significant difference among the site or the moment of sampling and percentage of multi-resistant bacteria was observed. This highlights the high frequency of resistances to antibiotics and the high level of dispersion through all the aquatic agricultural environments.

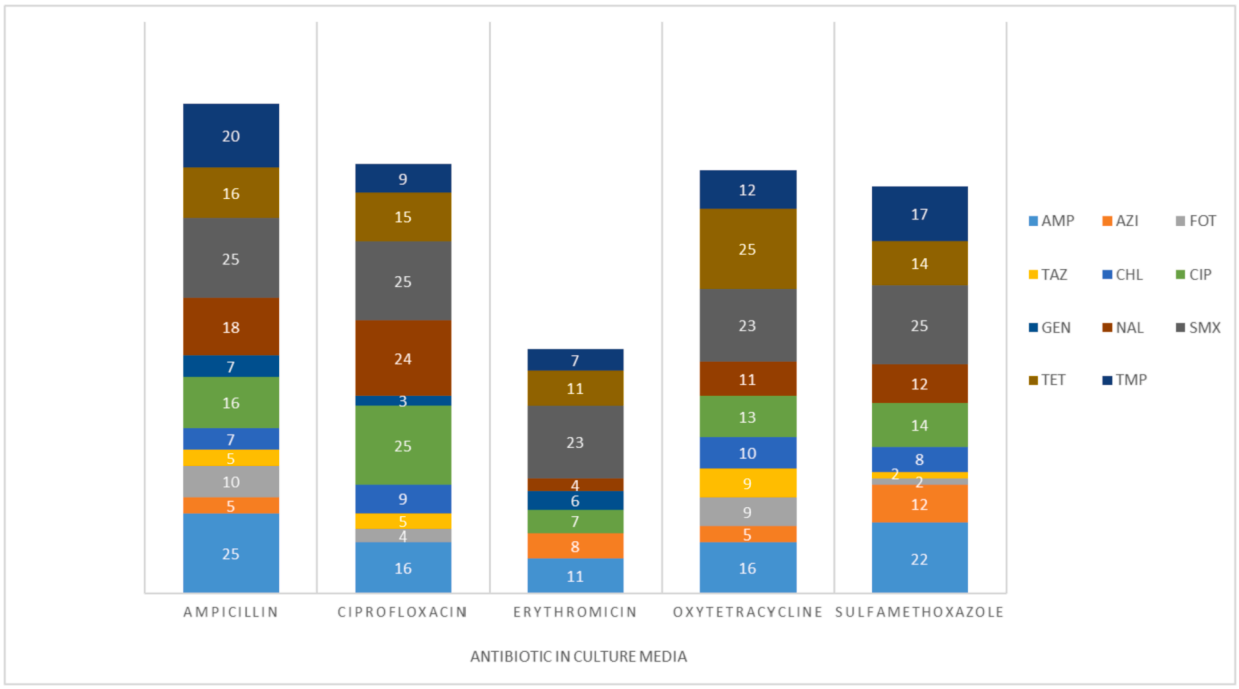

Fig. 2. Distribution of antibiotic resistance (AR) in *E. coli* isolates according to the antibiotic present in the isolation media. (SMX: sulfamethoxazole; TMP: trimethroprim; CIP: ciprofloxacin; TET: tetracycline; AZI: azithromycin; NAL: nalidixic acid; FOT: cefotaxime; TAZ: ceftazidime; AMP: ampicillin; CHL: chloramphenicol; GEN: gentamicin. Numbers in figure boxes represent the number of resistant *E. coli* isolates.

Table 4
Distribution of multi-resistance patterns and number of *E. coli* isolates that presents each pattern.

Multi resistance (no. classes of antibiotics)	Number of different patterns	Number *E. coli* isolates (%)
3	3	16 (18.2)
4	6	30 (34.1)
5	4	30 (34.1)
6	2	12 (13.6)

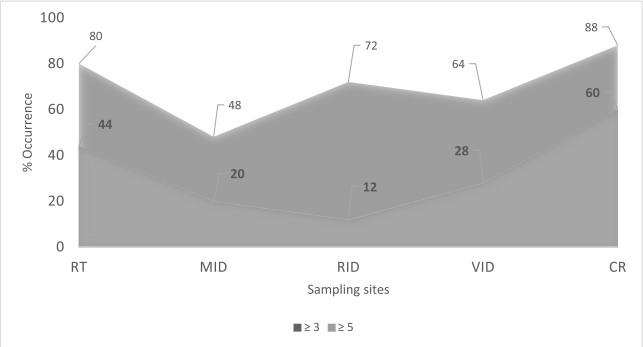

Fig. 3. Comparison of percentages of multi-resistant bacteria with three, and five antibiotic classes at the five sampling sites. (TR: Turia River; MID: Royal Moncada Irrigation Ditch; RID: Rascanya Irrigation Ditch; VID: Vera Irrigation Ditch; CR: Carraixet Ravine).

3.3. Antibiotic-resistance genes detection

Aquatic ecosystems are some of the environments of most concern in ARG research. It must be taken into account that it is impossible to isolate and analyse all the cultured microorganisms which are present in a sample. Moreover, some antibiotic-resistant bacteria could be non-culturable. Therefore, by analysing only the isolates obtained after culturing the samples it is possible to lose relevant information. Culture independent methods allow for the detection of those ARG carried by non-culturable bacteria (Henriques et al., 2006a, 2006b). For this reason, in this work, ARG direct detection from water samples was also carried out.

It has been reported that sulfonamide, tetracycline, β-lactams, macrolide, and, to a lesser extent, fluoroquinolones resistance genes are the most abundant ARG in aquatic environments (Christou et al., 2017; Dang et al., 2017; Zhai et al., 2016). Thus, a representative gene of each family was selected for this study. ARG detection was performed by PCR, amplifying specific fragments of each one of the five tested genes.

The presence of these ARG on the 125 *E. coli* isolates was studied (Table 5). Ninety-six percent (120) of the isolates presented bla_{TEM} gene, whereas *erm*B gene was only detected in one isolate (0.8%). The rest of the genes presented a frequency no higher than 50%. These results suggest that at least bla_{TEM} gene is largely widespread in aquatic environments from our geographical area. With the aim of studying the existence of a significant correspondence between presence of a determined gene in a specific *E. coli* isolate and the sampling site, frequency data was further analysed by χ^2 test. No significant correlation was found between sampling site for bla_{TEM}, *erm*B, *sul*I, and *tet*W genes, but for *qnr*S gene, occurrence was significantly lower for *E. coli* isolates from RID samples.

As mentioned above, occurrence of genes was also studied directly in the water samples (n = 25 samples). All five ARG were detected in all locations. Although some variations in the frequency of detection could be observed between locations, these were not significant. *Tet*W gene was detected in the totality of the water samples, followed in frequency by bla_{TEM} gene (96%), which was not detected in 20% of the RID samples. *Sul*I gene (80% in total) was less frequent in TR waters (60%), whereas 100% of VID samples were positive for this gene. For *qnr*S and *erm*B genes, a major variability between samples was observed (from 40% to 100%), with a percentage average of 72% for *qnr*S and 60% for *erm*B (Fig. 4).

With the aim of studying a possible relationship between the same

Table 5
Frequency of ARG (%) detection in the totality of *E. coli* isolates and water samples.

	bla$_{TEM}$	*ermB*	*sulI*	*qnrS*	*tetW*
E. coli isolates[a]	96% (120)	0.8% (1)	48.8% (61)	21.6% (27)	17.6% (22)
Water samples[b]	96% (24)	60% (15)	80% (20)	72% (18)	100% (25)

[a] n = 125
[b] n = 25.

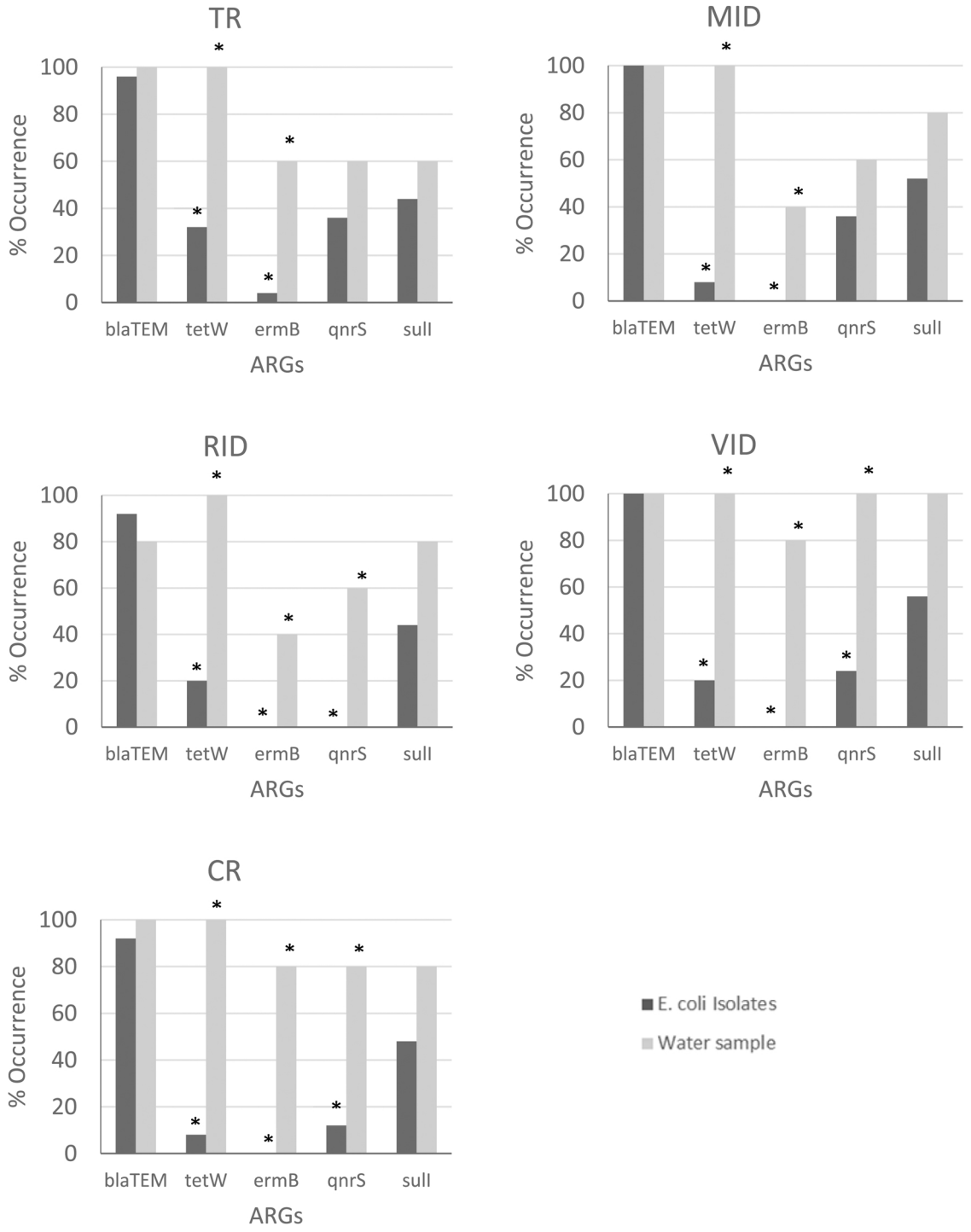

Fig. 4. Comparison of ARG occurrence among the isolates and water of the different emplacements (TR: Turia River; MID: Royal Moncada Irrigation Ditch; RID: Rascanya Irrigation Ditch; VID: Vera Irrigation Ditch; CR: Carraixet Ravine). * : Significant differences ($p < 0.05$) between gene occurrence in water samples and in *E. coli* isolates.

gene occurrence in water samples and in *E. coli* isolates for each sample point, a χ^2 test was conducted. Significant differences ($p < 0.05$) were found for *tet*W and *erm*B genes for all sampling sites, as well as for *qnr*S gene for RID, VID and CR sites. While 100% of water samples were positive for the *tet*W gene, different levels of this gene were detected in *E. coli* isolates from each point. The presence of the *qnr*S gene was higher than 70% in water samples, and below 25% in the *E. coli* isolates, with 0% in RID isolates. For *sul*I gene, the lesser percentage detected in the isolates (48.8%) compared to water samples (80%) was not significant. Finally, the gene that stands out is *erm*B, being present in 60% of water samples and only in one *E. coli* isolate (0.8%) (Fig. 4).

The present study corroborates the hypothesis that cultivation-dependent studies may underestimate the prevalence of resistance genes that are present in environmental samples (Henriques et al., 2006a), as the presence of ARG detected directly in water samples was higher than in the isolates, except for bla_{TEM}. This may be due to several reasons: ARGs can be present in non-culturable bacteria (Heritage et al., 2001), naked DNA can be present in the medium (Gaze et al., 2013; Nielsen et al., 2007) or resistance may occur in bacteria from different families or genera than the ones usually analysed (Bengtsson-Palme et al., 2018; Zhang et al., 2009). It must be considered also that in the present study, only *E. coli* isolates have been investigated. EFSA (2008) proposes to use *E. coli* and other non-pathogenic microorganisms as indicators of the occurrence of antibiotic resistances to the main antibiotics use in a given zone or country. *E. coli* has also been shown to be an important reservoir of antimicrobial resistance genes, which can transfer to pathogenic bacteria, contributing this way to spread the resistances (Katakweba et al., 2018; Odonkor and Addo, 2018). Therefore, the search and detection of resistant *E. coli* would constitute a good prediction for presence of clinically relevant resistant bacteria and associated genes (Gekenidis et al., 2018). However, the antibiotic resistance levels or the frequency of AR-genes detected in *E. coli* from a sample cannot be assumed to be the resistance levels in this same sample.

A high proportion of tetracycline, β-lactam and sulfonamide antibiotic resistance genes were detected in the present research. These antibiotics are among the oldest and most widely used in human and animal medicine on account of their broad spectrum of activity and low cost (ECDC, 2015; Harnisz et al., 2011). Results obtained here are in accordance with those reported by a variety of studies on surface waters, rivers and lakes. Stoll et al. (2012) reported *sul*I as the most detected gene in superficial waters from Germany and Australia. In other research carried out by Henriques et al. (2006a) in estuary water from Portugal, around 80% of the Enterobacteria isolates presented the bla_{TEM} gene, and more than 50% carried tetracycline resistance genes. In a pool of studies on rivers and lakes of China, sulfonamide related genes were the most prevalent (Huang et al., 2019; Stange et al., 2019; Zhang et al., 2016). Regarding studies performed in Spain, AR bacteria and genes have been detected in rivers impacted by discharges of wastewater treatment plants (Rodríguez-Mozaz et al., 2015; Sidrach-Cardona et al., 2014; Martí et al., 2013), demonstrating an incomplete removal of antibiotics and antibiotic resistance bacteria in the plants, what affects severely the receiving river.

Comparing Sensititre and PCR results, we can observe that the first method detected more frequency of resistances than what the presence of the selected genes in the strains could lead to expect. This result is not surprising, as there is a great variety of genes that encode for resistances to the same group of antibiotics beyond here selected. Likewise, if a specific gene is not detected by PCR, this does not imply the absence of resistance to a specific family of antibiotics in the isolates.

Nevertheless, bla_{TEM} gene was detected in 96.6% of 90 AMP-resistant *E. coli* isolates, in 100% of the 25 FOT-resistant and 21 TAZ-resistant *E. coli* isolates. Several studies have demonstrated that the most frequently detected gene was bla_{TEM} in AMP-resistant isolates from estuarine, river and lake water (Henriques et al., 2006a; Wang et al., 2013; Hu et al., 2008). Resistance to β-lactam antibiotics is mediated mainly by β-lactamases, which are frequently detected in *E. coli* and other Gram-negative bacteria. These enzymes, in addition to conferring resistance to ampicillin and penicillin, are associated with resistance to 3rd generation cephalosporins as shown herein by the high detection percentages in FOT- and TAZ- resistant isolates. Moreover, high percentages of bla_{TEM} gene were also detected in TET- (98.8%), SMX- (95.9%), CIP- (94.6%), NAL- (94.2%) and TMP- (93.8%) resistant *E. coli*. The simultaneous occurrence of resistance to a variety of antibiotics is corroborated by the simultaneous presence of the genes that encode them in *E. coli* isolates. This could be explained by the presence of various genes in the same plasmid. Regarding the prevalence of the *sul*I gene, it was around 50% among isolates SMX- (60 of 121) and TMP-resistant (34 of 65) *E. coli* isolates. A similar prevalence was found in SMX-resistant E. coli isolated from rivers and lakes (Wang et al., 2013; Su et al., 2012; Hu et al., 2008). A low frequency was detected for *qnrS* gene between quinolone-resistant isolates: 28% and 26% for CIP- and NAL-resistant isolates, respectively. Wang et al. (2013) reported a much lower frequency of *qnr*S regarding another quinolone resistant genes such as *qnr*A, or *qnr*B, suggesting that CIP and NAL resistance among our *E. coli* isolates is due to genes other than *qnr*S. This great variability observed in the results of the present study highlights the complexity of the factors involved in the acquisition of resistance among bacterial isolates.

4. Conclusions

Environmental impact of fecal contamination, as well as spread of antibiotic resistance genes has been extensively studied in wastewater destined for irrigation of agricultural soils, but lesser in surface irrigation channels. The ditches network in Huerta Valenciana is an ideal system to investigate the presence of ARG and ARB, as a strong interaction exists with human ecosystem and their activities (domestic, agricultural and industrial). Thus, this type of surface waters could be a dominant route by which ARGs are disseminated.

In the present study we have evaluated the occurrence of antibiotic resistant bacteria and genes in a network of irrigation ditches in the Huerta Valenciana (Valencia, Spain), by studying both, *E. coli* isolates and water samples, in order to relate antibiotic resistance levels to viable indicator bacteria present in the samples.

Results of this study showed that all the water samples presented very high contamination levels of resistant fecal coliforms. Moreover, a notable prevalence of multi-resistant *E. coli* strains (70.4% of the isolates) was detected in the analyzed irrigation waters.

ARGs for the main families of antibiotics, sulfonamide, tetracycline, β-lactams, macrolide, and fluoroquinolones, were present in all irrigation waters. The main prevalence rates corresponded to bla_{TEM} gene, which was detected in 96% of the *E. coli* isolates, while the rest of genes presented frequencies no higher than 50%.

The use of these contaminated waters for irrigation increases the potential transfer of ARB and ARG to food crops. Thus, vegetables irrigated with contaminated water can act as a potential vector for dissemination of antibiotic resistances among environment and human populations.

The high levels of antibiotic resistances in irrigation waters observed in this work could help to both, better understanding and management, of the contamination caused by antibiotics and related resistance genes in our geographical area, in order to implement appropriate measures for control.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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