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Monitoring of indicator and multidrug resistant bacteria in agricultural soils under different irrigation patterns

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ABSTRACT

The presence of multidrug-resistant bacteria has been proposed as an environmental impact indicator of wastewater use in agricultural soils. Nevertheless, the effect of untreated wastewater discharge on occurrence and persistence of these type of bacteria in soils is unclear. In this study, multidrug-resistant bacteria and microbial indicators of fecal contamination (coliforms and *Salmonella*) were determined at different depths (0–15, 15–30 and 30–50 cm), in active agricultural soils under different untreated wastewater irrigation pattern (a zone with past and present wastewater irrigation; a zone with wastewater irrigation until 2003; and a zone that had never been irrigated with wastewater) in Chihuahua, Mexico. A total of 94 multidrug-resistant strains were recovered from all tested soil samples, even from those that have never been irrigated with wastewater. Higher presence ($P < 0.05$) of multidrug-resistant bacteria was found in soils currently irrigated with untreated wastewater than in soils that has not been irrigated with wastewater for more than 10 years; the later had presence of multidrug-resistant bacteria that was statistically higher than soils that had never been irrigated with wastewater. Wastewater irrigation showed an impact on proliferation of antibiotic-resistant Gram negative bacteria in soils actually irrigated but not in Gram positive bacteria. No statistical difference was found for coliforms and *Salmonella* between the three soil groups. The effect of depth in multidrug-resistance bacteria presence was negligible. Although multidrug-resistant bacteria was also isolate from soils without wastewater irrigation, their persistence in a highest number in soils irrigated with wastewater suggest that use of untreated wastewater for irrigation enhance the proliferation of multidrug-resistant microorganisms which can be monitoring in soils even after a long period of time after the last irrigation.

1. Introduction

The increasing water demand due to human population located in arid and semi-arid zones has converted domestic wastewater in a precious resource. Treated wastewater can be re-used in urban areas, in agriculture or in industrial processes. Nevertheless, the increasing population generates a huge volume of domestic wastewater and the cost of treatment is too high for several developing countries which opt for sewage wastewater for irrigation (Singh et al., 2012). Low-quality water, including untreated wastewater will be increasingly used for irrigation in agriculture (Lonigro et al., 2016). The effect of untreated wastewater use to irrigate agricultural soils in places such as Australia and Mediterranean countries has been extensively studied (Aydin et al., 2015; Becerra-Castro et al., 2015; Jechalke et al., 2015; Lonigro et al., 2016). In Mexico wastewater irrigation to crop production for human consumption has been prohibited. Nevertheless, due to the scarcity of water with good quality in rural areas this resource is still used for crop and forage production (Carrillo et al., 2016; Montero-Aguirre et al., 2016).

Wastewater irrigation can cause several effects in soils as changes in the relations of Na^{+}/Ca^{+2}, heavy metal accumulation and translocation to cultivated vegetables or crops, heavy metal biosorption in autochthonous bacteria, salinity increase, and acid-

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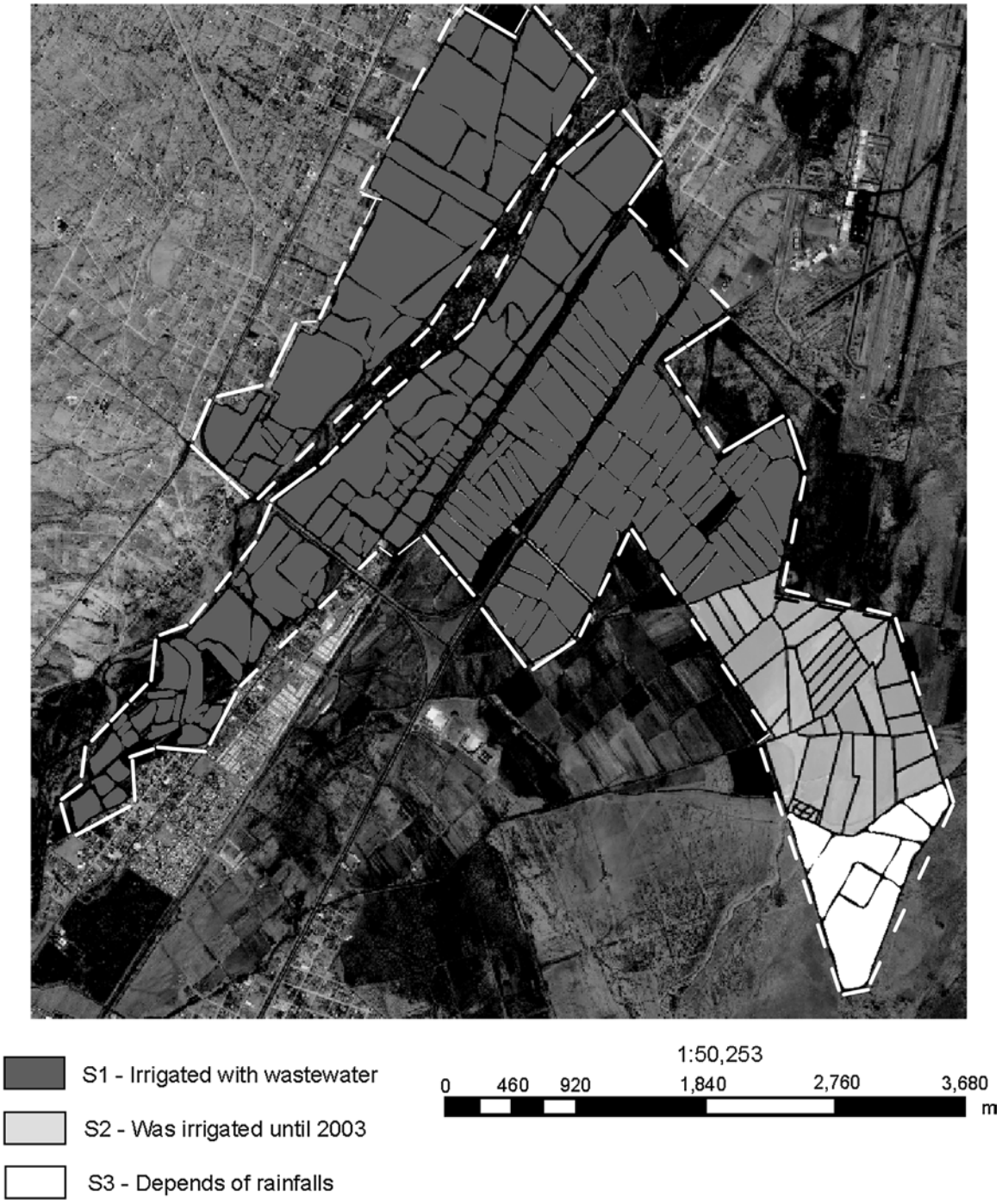

Fig. 1. Sampling site. Tabalaopa, Chihuahua, Chihuahua Mexico, site sampled with three different irrigation patterns. S1 with a stream contaminated with wastewater; S2 irrigated until 2003 and currently irrigated but with uncontaminated water; S3 has no irrigation and depends only on rainfalls.

ification (Ansari and Malik, 2007; Christou et al., 2014; Singh et al., 2010). In addition, several changes in soil properties may occur, including alterations in pH, increment of organic matter, increment of Na and P among others (Bedbabis et al., 2014; Singh et al., 2012; Xu et al., 2010).

The presence of coliforms, *Salmonella* and enterococci has been proposed as indicator of fecal contamination (Gemmell and Schmidt, 2012; Nabeela et al., 2014; Yamahara et al., 2012). Mexican government criteria recommend the use of fecal coliforms as surface water quality, as well as quantification of fecal coliforms, *Salmonella*, and helminth eggs as quality indicators of biosolids used for agricultural soil amendment (DOF, 2002). Antibiotic and antibiotic resistance genes (ARGs) present in both soil and water have been recently considered as emergent contaminants (Czekalski et al., 2015; Milić et al., 2013; Munir and Xagoraraki, 2011). In the last decades, search for antibiotic resistant patterns in faecal bacteria as indicators of human contamination has been proposed as an alternative to the commonly used methods (Niemi et al., 1983), and more recently the presence of multidrug-resistant microorganisms have been used as pollution indicators in higher organisms such as turtles (Al-Bahry et al., 2011).

Municipal wastewaters present antibiotic resistant bacteria and a large amount of antibiotics from their incomplete metabolism in humans or due to disposal of unused antibiotics (Nagulapally

Table 1
Frequency of multidrug-resistant bacteria isolated from agricultural soils with different irrigation patterns and at different sampling depth.

	Irrigated with wastewater	More than 10 years without wastewater irrigation	Rainfall	TOTAL
Gram Negative				
0–15	14 ± 2.9	1 ± 0.7	3 ± 1.2	18
15–30	9 ± 2.6	0	2 ± 1.1	11
30–50	12 ± 2.8	1 ± 0.7	1 ± 0.7	14
TOTAL	35	2	6	43
Gram Positive				
0–15	7 ± 2.2	4 ± 1.8	3 ± 1.3	14
15–30	13 ± 2.5	9 ± 2.2	2 ± 1.6	24
30–50	5 ± 2	6 ± 2	2 ± 1.19	13
Total	25	19	7	51
	60[a]	21	13	

[a] Statistical difference.

et al., 2009). Although many antibiotic resistant bacteria are not pathogenic, resistance genes can be transferred to pathogens, and as such, represents a public health risk (O'Brien, 2002). The possible contamination of groundwater by leaching of wastewater through agricultural soils is another risk since bacteria can contaminate food, and resistance genes can be further dispersed (Brennan et al., 2010; Li, 2014; Monaghan and Hutchison, 2012). Thus, physical factors that can facilitate or limit the leaching of microorganisms and contaminants present in wastewater through soil have been studied (Hentati et al., 2014; Kay et al., 2005; Langdon et al., 2015). Although the long-time effect of agricultural soil wastewater irrigation on heavy metals accumulation has already been reported (Mapanda et al., 2005), the long-time effect on prevalence of microbial indicators in different soil depths has been less reported. Studies on the prevalence of contaminants and microorganisms in soils irrigated with untreated wastewater are necessary to know the real risk in using this valuable resource. We have specially tested the hypothesis that soils irrigated for long-time with untreated wastewater show higher prevalence of fecal microbial indicators and multidrug-resistant bacteria than soils without this pattern of irrigation that served as control. The aim of this work was to determine the presence of microbial indicators of fecal contamination including coliforms, *Salmonella*, and multidrug-resistant bacteria at different depths of agricultural soils with different untreated wastewater irrigation patterns.

2. Materials and methods

2.1. Study area and sampling

A suburban site of 1094 ha located in southeastern Chihuahua City, in Chihuahua State, Mexico, namely Ejido Tabalaopa (Commonland) was used for this study. This arid area was traditionally used since 1953 to grow different crops until 1996 using untreated wastewater (water from Chuviscar river which is an tributary of the Bravo river and that crosses Chihuahua City and receives discharges of domestic wastewater from the entire city) for irrigation (DOF, 2015). However, wastewater irrigation to crop production for human consumption is regulated in Mexico by the NOM-001-SEMARNAT-1996 (DOF, 1996). For this reason, part of this area was discontinued but other part is currently being used only for forage crops for cattle production and surface water contaminated with untreated wastewater is still used for irrigation. For this study, three zones from Ejido Tabalaopa were analyzed: (S1) including soils with past and present irrigation using surface water contaminated with wastewater; (S2) a zone with wastewater irrigation until 2003 and until now is not irrigated with wastewater; and (S3) a zone that had never been irrigated with wastewater and represent the non-affected soil by wastewater irrigation (Fig. 1 samplings zones delimited by white discontinuous lines). The soil is an Orthid Aridisol with well-developed pedogenic horizons, low organic matter in virgin soil, and dry more than six months a year (Maldonado et al., 2008).

During a spring season, a total of 150 soil samples were taken from 50 different sampling points covering an area of 1059 ha. At each point, samples were taken at three different soil depths (0–15 cm, 15–30 cm and 30–50 cm), which were dried at and sieved through a 100 mesh (0.15 mm) according to Mexican Official Norm NOM-021-SEMARNAT-2000 and the samples were storage at 4 °C for less than seven days until analysis (DOF, 2001).

2.2. Determination of multidrug-resistant bacteria

As ampicillin is one of the world's most widely prescribed antibiotic, it was chosen as first step for multidrug-resistant bacterial strain isolation. The presence of ampicillin resistant bacteria was determined with the adaptation of the method described by Ash et al. (2002) as follows: dried soil was re-suspended in saline buffer (1:10 and 1:100 dilutions) and 0.1 mL were inoculated by standard spread plating techniques into the plate surface in Tryptic Soy Agar (TSA Bioxon, Mexico City, Mexico) supplemented with 250 mg/L ampicillin. Plates were incubated at 36 ± 0.2 °C for 24 h. Ampicillin-resistant pure cultures were obtained by re-streaking isolated colonies into TSA plates. Gram stain, catalase, and oxidase tests were done for preliminary identification. A total of 24 antibiotics (6 for Gram-negative: amikacin-30 μg, carbenicillin-100 μg, ceftriaxone-30 μg, chloramphenicol-30 μg, netilmicin-30 μg, nitrofurantoin-300 μg; 6 for Gram-positive bacteria: ceftazidime-30 μg, cefuroxime-30 μg, dicloxacillin-1 μg, erythromycine-15 μg, tetracycline-30 μg, penicillin-1 IU; and 6 for both: cephalotin-30 μg, cefotaxime-30 μg, gentamicin-10 μg, pefloxacin-5 μg, trimethoprim/sulfamethoxazole-25 μg) were tested to determine antibiotic resistance by the Bauer-Kirby method, which consisted in inoculations of each pure culture in TSA plates; then, a disc impregnated with a specific antibiotic (Multisensidiscs, Bio-Rad, Philadelphia, USA) was placed on top of the inoculated media, and cultures were incubated at 36 ± 0.2 °C for 24 h. Resistance profile was determined by CLSI (Clinical & Laboratory Standards Institute) criteria according to the instructions given by the supplier of the test kit. According to CLSI criteria the type of resistance profile (resistant, intermediate and sensitive bacteria) is determined by the halo of growth inhibition showed by the bacterial strain inoculated in the plate. Resistant profile are bacteria that shows total resistance to clinical concentration of antibiotic; intermediate resistance profile are bacteria that can growth in presence of below clinical concentrations of the antibiotic tested; and the sensitive profile are bacteria that can not growth in presence of the antibiotic tested. Taking into account that multidrug-resistant

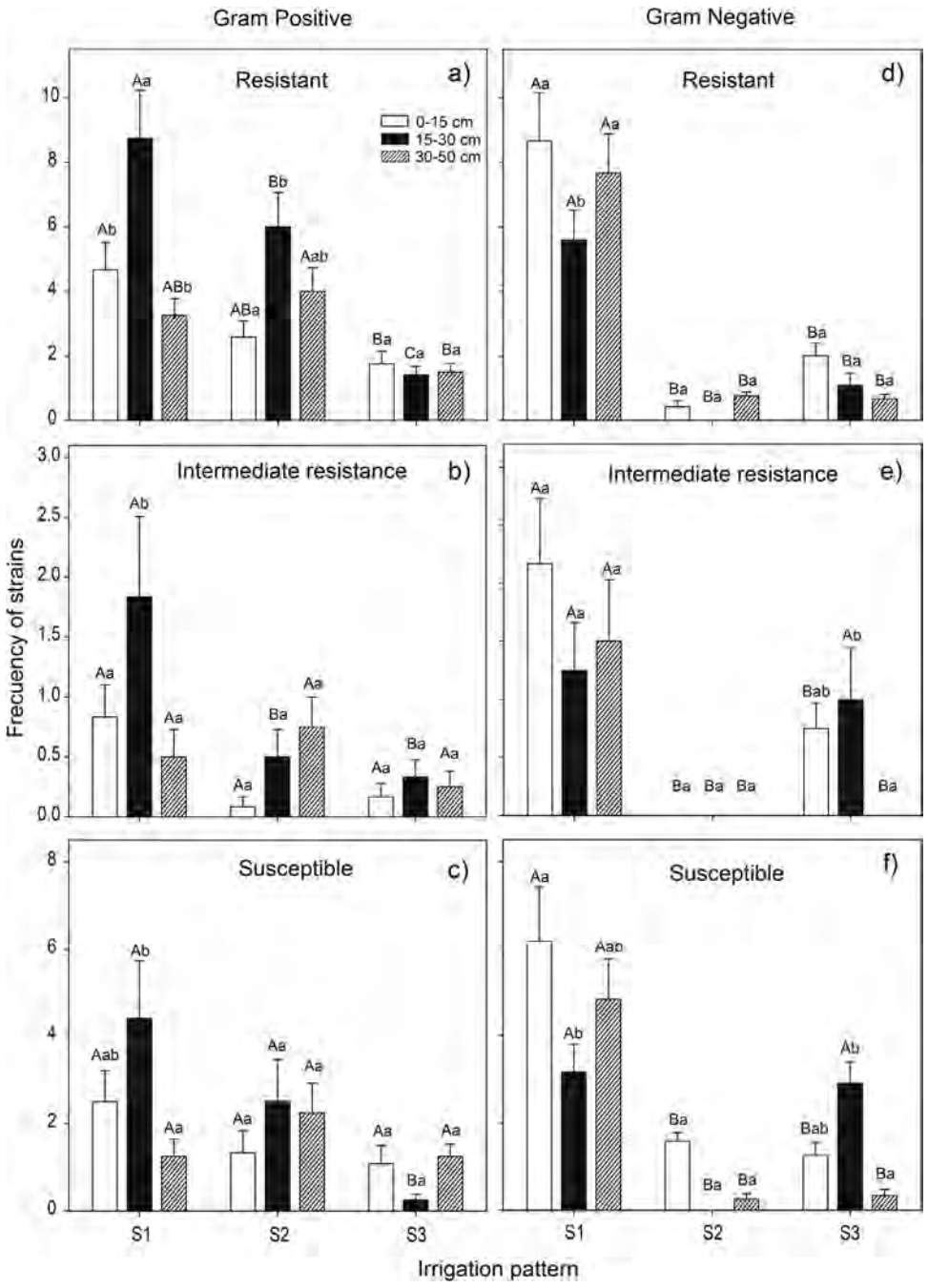

Fig. 2. Distribution of resistance phenotypes of Gram-positive bacteria (a–c) and Gram-negative bacteria (d–f) at different depth of soils with different irrigation pattern. Columns denoted by different lower letters differ significantly at different depth in a same sampling zone, using ANOVA for the nested model and lsmeans at $P < 0.05$, where values at each depth denoted by different capital letter differ between zones significantly, using ANOVA for nested model and lsmeans at $P < 0.05$. Whisker lines represent SE.

bacteria is defined as acquired non-susceptibility to three or more antimicrobial categories (Magiorakos et al., 2012), we considered all bacterial strains that showed resistance to three or more antibiotics as multidrug-resistant bacteria.

2.3. Determination of total and fecal coliforms

For total and fecal coliform analyses, MPN (Most Probable Number) technique was used, according to the Mexican Official Norm NOM-004-SEMARNAT-2002 (DOF, 2002). Eight grams of soil were mixed with 72 mL of saline buffer and mixed manually. Soil was let to settle down and a series of three tubes of Lactose Broth (Bioxon, Mexico City, Mexico) were inoculated with 10, 1, and 0.1 mL of supernatant, respectively for MPN determination. Tubes were incubated at $34.5 \pm 0.2\,^{\circ}C$ for 24 h. For fecal coliforms after incubation, positive tubes (presence of gas) were transferred to *Escherichia coli* broth (E.C. broth) (Bioxon, Mexico City, Mexico), and tubes were further incubated at $44.5 \pm 0.2\,^{\circ}C$ for 24 h. Tubes with gas trapped in the Durham tube were considered as positive. Results of bacterial cells/g of soil were obtained from MPN standardized tables.

2.4. Determination of Salmonella

For *Salmonella* determination the method described by Mexican Official Norm NOM-004-SEMARNAT-2002 (DOF, 2002) was used. Eight gram of soil were mixed with 72 mL of tetrathionate broth

(Difco, Sparks, MD, USA) and were mixed manually. Broth was incubated at 37 ± 0.2 °C for 24 h, and after incubation, serial dilutions (1:10) were done in saline buffer. From the dilutions, 1 mL was transferred to three tubes with 9 mL Selenite Cystine Broth (Difco, Sparks, MD, USA), and incubated at 41 °C for 24 h. A change of color to an intense orange was considered as positive, and a loop of those tubes was transferred to SS Agar (Bioxon, Mexico City, Mexico). Plates were incubated at 35 ± 0.2 °C for 24 h, and colonies suspected of belonging to genus *Salmonella* were confirmed by microscopy; colonial morphology and standard biochemical tests including Triple sugar iron agar, Lysine iron agar, Motility indole ornithine, Sulfite indole motility, Methyl red and Voges-Proskauer test and Urea broth (All from Difco, Sparks, MD, USA) were performed. Results of bacterial cells/g of soil were obtained from MPN standardized tables.

2.5. Statistical analysis

All determinations were done in triplicate. The difference in fecal and total coliforms in soil samples were determined with Chi-square test with a significance set at $P < 0.05$, using Minitab® statistical software (Minitab 17, Minitab Inc. State College, PA, USA).

Frequency of different resistance phenotypes and antibiotic resistant bacteria at different depths and sites of sampling were analyzed by GLM using SAS 9.3 (SAS Inst., Cary, NC, 2004) considering the follow model:

$$y_{ijk} = \mu + S_i + D_{j(i)} + \varepsilon_{ijk}$$

where y_{ijk} is the number of isolates;
S_i is the i-th sampling zone (S1–S3);
$D_{j(i)}$ is the j-th depth nested into the soil type;
ε_{ijk} is the experimental error.

Differences observed were estimated using least-square means (lsmeans) test for $\alpha = 0.05$.

3. Results and discussion

3.1. Effect of untreated wastewater irrigation pattern in multidrug-resistant bacteria presence in soil

Wastewater use for agricultural purposes is increasing due to a constant intensification in water demand for food production. Environmental impact of wastewater use has focused mainly on fecal contamination and pathogenic microorganism dispersion, but the spread of antibiotics and other emergent contaminants, as well as the dispersion of antibiotic resistance genes (ARGs) is also to be considered when using wastewater for irrigation of agricultural soils (Li et al., 2015; Malik and Aleem, 2011; Travis et al., 2010). The persistence of antibiotics in the environment, including soil (Pan and Chu, 2016) and water (Brown et al., 2014) has been documented, but the effect of untreated wastewater in the generation and persistence of antibiotic resistant strains and the distribution of these microorganisms in agricultural soil have been less reported.

From 110 pure culture ampicillin-resistant bacteria, 94 showed resistance to 3 or more antibiotics. Sampling zone currently irrigated with surface water contaminated with wastewater (S1) showed a higher presence of multidrug-resistant bacteria ($P < 0.0012$) than in the other two studied zones; a total of 60 strains were isolated from this zone while 21 were found in S2, and 13were detected in S3 control (Table 1). Several previous studies found a positive correlation between wastewater irrigation and presence of antibiotic resistant bacteria in soil (Malik and Aleem, 2011; Negreanu et al., 2012; Pignato et al., 2009), which was also found in our work. The analysis of resistance phenotypes of the isolates showed a major presence ($P < 0.05$) of bacteria resistant to antibiotics (Fig. 2a and d) and bacteria with intermediate resistance (Fig. 2b and e) in soils from S1 than in the other two studied zones. Only for Gram positive bacteria was found a statistical difference between the presence of resistant bacteria in S2 compared to S3 site (Fig. 2a); however, this difference was not found for the phenotype of intermediate resistance (Fig. 2b). Nevertheless, contrary to the resistance and intermediate resistance phenotypes, the phenotype of susceptible bacteria was only different for Gram negative bacteria from S2 soils than from the other two zones (Fig. 2c and f).

Table 2
Number of antibiotic resistance at which multidrug-resistant bacteria isolates from agricultural soils with different irrigation patterns.

Irrigation pattern	Number of antibiotic resistance	Gram Negative	Gram Positive
S1	3–6	6	4
	7–9	21	14
	10–12	8	7
S2	3–6	0	11
	7–9	1	5
	10–12	1	3
S3	3–6	4	2
	7–9	1	4
	10–12	1	1

Degradation of antibiotics is governed by their molecular composition (Thiele-Bruhn, 2003). Although it has been reported that half-life in soil of erythromycin, roxithromycin, oleandomycin, tylosin, salinomycin and tiamulin is less than 30 d (Schlüsener and Bester, 2006), several molecules with antimicrobial activity can persist more than 100 d (Thiele-Bruhn, 2003). Persistence of antibiotics has been reported to be related to their initial concentration in soil (Pan and Chu, 2016). The excessive use of water containing antibiotics leads to spreading antibiotic resistance to soil bacteria, which then serves as persistent reservoir of antibiotic resistance (Gosh and LaPara, 2007). Similar presence ($P > 0.05$) of multidrug-resistant bacteria in soils from S2 and S3 control could be explained by natural processes related with the gain of antibiotic resistance as horizontal (incorporation in the genome of whole genes from an outside sources or organism) and vertical (transfer of genetic material from parent directly to the progeny) gene transfer mechanisms or by intrinsical resistance that some bacteria can have to antimicrobial agents (Tenover, 2006).

Based on bacterial classification using Gram staining, no statistical difference was found between the number of multidrug-resistant Gram-negative and Gram-positive bacteria (35 and 25, respectively) in soils irrigated with surface water contaminated with untreated wastewater (Table 1) ($P = 0.321$). Because Gram-negative bacteria represent a major group of pathogens causing disease (Ulevitch and Tobias, 1999), it can be assumed that antibiotic compounds applied in therapy are mainly directed to Gram-negative bacteria. Nevertheless, the presence of β-lactams and erythromycin which are used as treatment against Gram-positive bacteria as *Streptococcus*, *Gonococcus*, and *Staphylococcusk*, has been reported in wastewater (Le-Minh et al., 2010; Senta et al., 2013), which can explain the similar presence between multidrug-resistant Gram-positive and Gram-negative bacteria in soils irrigated with untreated wastewater.

It has been proposed that wastewater irrigation does not have an impact on antibiotic resistance in soil microbiome (Gatica and Cytryn, 2013). Nevertheless, in our study, soil from S1 and S2 showed a high number of bacteria resistant to more than 6 antibiotics (Table 2). Moreover, the phenotype of resistant bacteria was predominant in soil from S1 compared with the other two sites (Fig. 2a and d), which suggests that a high gene transfer rate of antibiotic resistance occurs in soils irrigated with untreated wastewater.

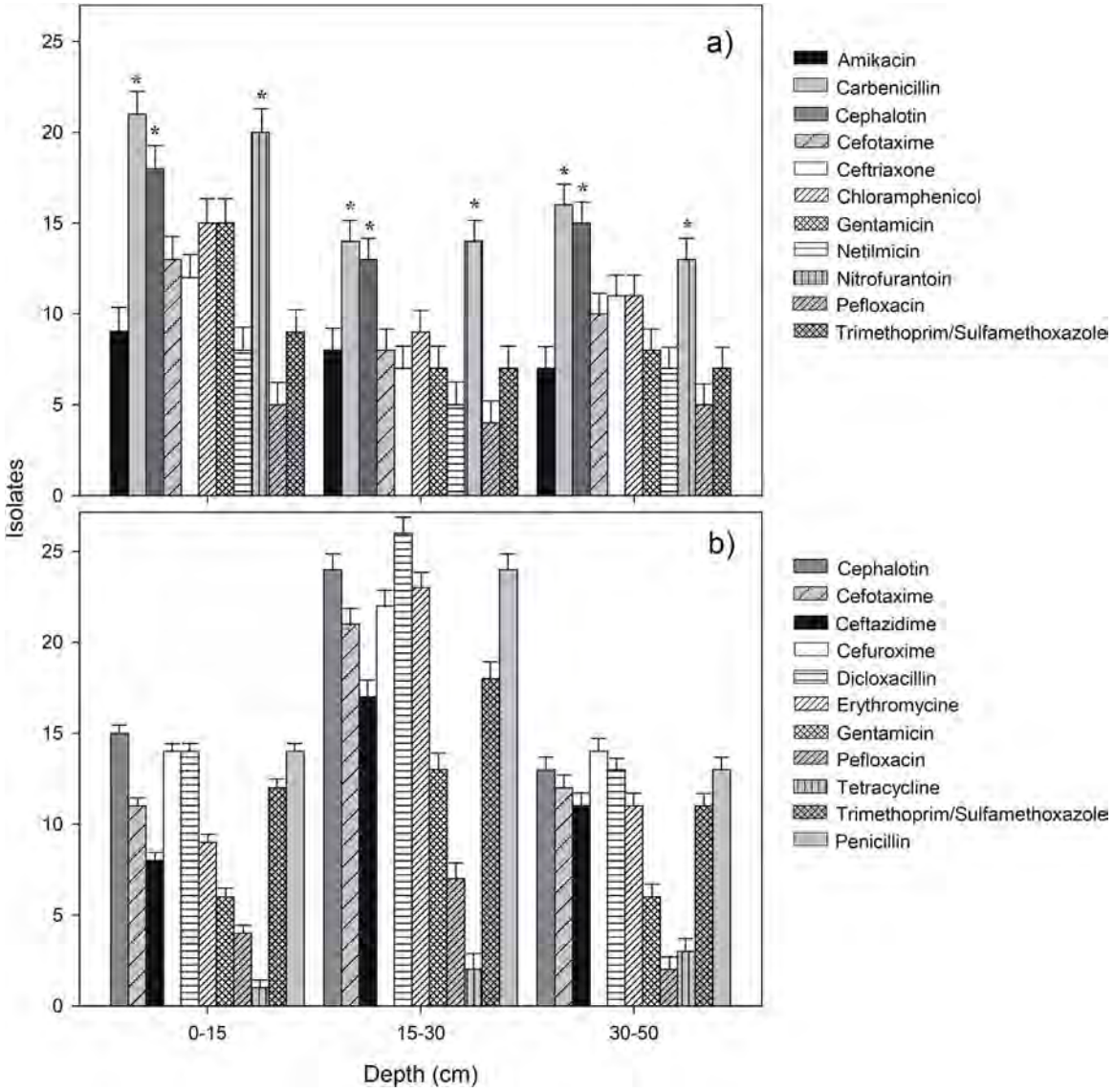

Fig. 3. Antibiotic resistant pattern of Gram-negative (a) and Gram-positive (b) bacteria at different depth of soil. Whisker lines represent SE. Asterisks indicate statistical difference.

Interestingly, a higher number of multidrug-resistant Gram-positive strains were found in S2 (Fig. 2a). Some of the strains were identified as belonging to the *Bacillus* genus that includes a diverse array of Gram-positive endospore-forming rods found in a wide range of microhabitats (McKillip, 2000). Endospore-forming ability is a long-term environmental survival strategy that is activated under stressful conditions (Titball et al., 1991) and allows dispersion through soil as a function of water movement at different soil depths (Jiang et al., 2006). On the other hand, presence of 6 isolates of multidrug-resistant Gram-negative bacteria in soils that never have never been irrigated with wastewater can be explained by the presence of farm animals since their intestine is considered the most important reservoir of multidrug-resistant Gram-negative bacteria (Wellington et al., 2013).

3.2. *Effect of soil depth in presence of multidrug resistant bacteria*

The number of multidrug-resistant bacteria at three different soil depths was not statistically different ($P = 0.91$) (Table 1). Nevertheless, the resistance phenotype in soils from S1 and S2 showed a predominant presence of resistant Gram-positive bacteria in depth from 15 to 30 cm (Fig. 2a), and this behavior is similar for the phenotype of intermediate resistance in soils from S1 (Fig. 2b). On the other hand, for Gram-negative bacteria the phenotype of resistant bacteria was predominant in soils from S1 from 0 to 15 and 30–50 cm in depth (Fig. 2d). The prevalence of a resistance phenotype bacterium in a specified depth could be due to the different patterns of antibiotic distribution in soils. Thiele-Bruhn (2003) described that distribution and prevalence of antibiotics in soil depend on physical and chemical characteristics of both the antimicrobial compounds and particles of soil.

A higher number of Gram-negative microorganisms showed resistance to carbenicillin, cephalotin and nitrofurantoin ($P < 0.001$) (Table 3), and this pattern was constant at the three different depths ($P > 0.05$) (columns with asterisks-Fig. 3a). In Gram-positive bacteria antibiotic resistance was variable (Fig. 3b). Bacterial strains were mainly resistant to cephalotin and penicillin in superficial and medium depths ($P < 0.05$). Resistance to more than one kind of antibiotic is usual in medium depth at more stable environmental conditions (Aga et al., 2005; Wu and Fassihi, 2005). However, in this study a high number of Gram-positive bacteria showed resistance to only cefuroxime ($P = 0.016$) at 30–50 cm.

Table 3
Profile of antibiotic resistance of strains isolated from agricultural soils with different irrigation patterns.

	Antibiotics tested											
Gram-positive	AM	CF	CTX	CAZ	CXM	DC	E	GE	PEF	TE	SXT	PE
Resistant	53 ± 1.6	51 ± 2.1	33 ± 3.9	34 ± 3.7	45 ± 3.0	53 ± 1.6	37 ± 3.6	6 ± 1.6	3 ± 3.0	0	41 ± 3.3	51 ± 2.1
Intermediate	2 ± 1.4	0	13 ± 3.2	2 ± 1.4	5 ± 2.1	0	11 ± 3.0	16 ± 3.5	8 ± 2.6	6 ± 2.1	0	0
Sensitive	1 ± 0.7	5 ± 2.1	10 ± 2.9	20 ± 3.6	6 ± 2.3	3 ± 0.7	8 ± 2.6	34 ± 4.2	45 ± 3.7	50 ± 3.1	15 ± 2.2	5 ± 1.7
	Antibiotics tested											
Gram-negative	AK	AM	CB	CF	CTX	CRO	CL	GE	NET	NF	PEF	SXT
Resistant	12 ± 3.9	51 ± 1.7	44 ± 2.9	45 ± 2.7	18 ± 4.1	17 ± 4.1	25 ± 4.0	22 ± 3.9	19 ± 3.6	44 ± 2.9	4 ± 1.9	21 ± 3.7
Intermediate	12 ± 3.1	0	7 ± 2.5	0	13 ± 3.2	13 ± 3.2	11 ± 3.0	8 ± 2.6	1 ± 0.7	3 ± 1.7	10 ± 2.9	2 ± 1.4
Sensitive	30 ± 4.1	3 ± 0.68	3 ± 1.7	9 ± 1.3	23 ± 3.9	24 ± 3.9	18 ± 3.6	24 ± 3.8	34 ± 3.6	7 ± 2.5	40 ± 3.8	31 ± 3.8

Note: AM = Ampicillin, CF = Cephalotin, CTX = Cefotaxim, CAZ = Ceftazidime, CXM = Cefuroxim, DC = Dicloxacillin, E = Erytromycin, GE = Gentamicin, PEF = Pefloxacin, TE = Tetracycline, SXT = Trimethoprim/Sulfamethoxazole, PE = Penicillin, AK = Amikacin, CB = Carbenicillin, CRO = Ceftriaxone, CL = Chloranfenicol, NET = Netilmicine, NF = Nitrofurantoin.

Table 4
Number of positive samples for fecal coliform determination in agricultural soils with different irrigation patterns and at different sampling depth.

	Irrigated with wastewater	Was irrigated with wastewater	Rainfall	Total
0–15	1 ± 0.9	1 ± 0.7	1 ± 0.6	3
15–30	4 ± 1.5			4
30–50	4 ± 1.4	1 ± 0.5	1 ± 0.7	6
Total	9	2	2	13

Antibiotic resistance genes in bacterial strains isolated from soil can be attributed to external factors such as irrigation with wastewater or it could be due to naturally present compounds in soil (Marti et al., 2013). For example, cephalotin belongs to the cephalosporin group originally produced by the fungus *Cephalosporium acremonium*, but it is also widely used for human and veterinary treatments worldwide (Jiang et al., 2010). This can be the explanation of similar pattern of antibiotic resistance found in strains isolated from the different zones tested, so it can be suggested that the presence of multidrug-resistant microorganisms in agricultural soil can be independent of the irrigation pattern used.

Regardless of the source of antibiotic contamination in soil, its physical-chemical properties, such as molecular structure, size, shape, solubility, hydrophobicity, electronegative charge, and organic matter affinity can influence its distribution in the soil (Kemper, 2008; Pan and Chu, 2016; Tamtam et al., 2011), with the consequent differential exposure to soil microorganisms (Kemper, 2008). Among bacterial strains isolated in our study, Gram-negative and positive multidrug-resistant bacteria were differently distributed in the soil depending on antibiotic resistance. Gram-negative bacteria isolates resistant to nitrofurantoin were predominant in superficial soil while bacterial isolates from deeper soil samples were predominantly resistant to cephalotin. The prevalence of the antibiotic resistance gene in superficial soils may be due to nitrofuran compounds, which are of lipophilic nature (Jia et al., 2014) and cannot be decreased by leaching into soil due to the irrigation process. On the other hand, cephalotin can be distributed by leaching through soil and sediments (Jiang et al., 2010; Mitchell et al., 2014), and so it could be present in sub-superficial soil samples.

An interesting fact was that 11 isolates that were initially selected based on their resistance to ampicillin, later showed sensitivity to this antibiotic (Table 3). This loss in resistance has been reported before; reversibility of antibiotic resistance in bacteria is determined by essentially two factors, dilution (effect produced by the continuous change in the microbial population which generates that the specific factor studied – in this case antibiotic resistance – is diluted by the high rate of replacement of the community) and fitness cost (any effect the resistance mechanism has on reducing the ability of the bacterium to reproduce and spread in the environment will be replacement or avoided to allow the low fitness cost). The first factor is mainly present in open systems, and the second is more prevalent in close systems (Andersson and Hughes, 2011). A bacterial mechanism to reduce biological fitness cost in a close environment would be the suppression of the resistance mechanism.

3.3. *Distribution of fecal indicators and* Salmonella *in soil*

No statistical difference was found in the presence of fecal coliforms and *Salmonella* in the three different tested zones and at different depth (Table 4). In S1, a total of nine samples were positive for fecal coliforms and only one of them was obtained from superficial soil. In S2 and S3, two soil samples were positive for fecal coliforms. Although several reports describe a positive correlation between wastewater irrigation and high presence of coliforms (Gemmel and Schmidt, 2012; Monaghan and Hutchison, 2012; Negahban-Azar et al., 2012), the existence of autochthonous *E. coli* populations that can move through soil has also been reported, compromising the use of coliforms as fecal contamination indicator (Brennan et al., 2010; VanderZaag et al., 2010). Nevertheless, the low values of coliforms detected in our study could have been due to a bias in the real concentration likely generated by the fact of letting the soil settle down and later re-suspending it in distilled water using only the supernatant. Bacteria could have also settled down with soil particles causing to underestimate coliform detection.

Regarding *Salmonella* determination, only two samples were positive, one from S2 and one from S3 medium soil. Persistence of *Salmonella* strains in soil can be affected by indigenous soil microbes (Jacobsen and Bech, 2012; You et al., 2006) and the source of those microorganisms can also be related to domestic or wild animals.

In conclusion, the presence of microbial indicators of fecal contamination in soils could be due to diverse abiotic and biotic factors, and the relationship to only one factor, such as surface water contaminated with untreated wastewater irrigation, is difficult to confirm. Our results demonstrated the relationship between the irrigation pattern in agricultural soil and the distribution and persistence of multidrug-resistant bacteria; even more, persistence of those microorganisms in soil can be traced to sites irrigated with untreated wastewater more than 10 years before. Even in agricul-

tural soils with rainfall pattern, multidrug-resistant bacteria were present, suggesting the presence of antibiotics or antibiotic-like substances from natural sources which exert a natural selection for microorganisms carrying antibiotic resistance genes. Further investigation on the interactions among antibiotics, microorganisms, and physical-chemical properties of soil is necessary to understand and predict the presence and persistence of different indicators of untreated wastewater contamination. Nevertheless, although multidrug-resistant bacteria was also isolate from soils without wastewater irrigation, their persistence o in a highest number in soils irrigated with wastewater suggest that these microorganisms can be uses as a better indicator of a long-time wastewater soil contamination than coliforms and *Salmonella*.

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