Science of the Total Environment 635 (2018) 1477–1483

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Tracking antibiotic resistance genes in soil irrigated with dairy wastewater

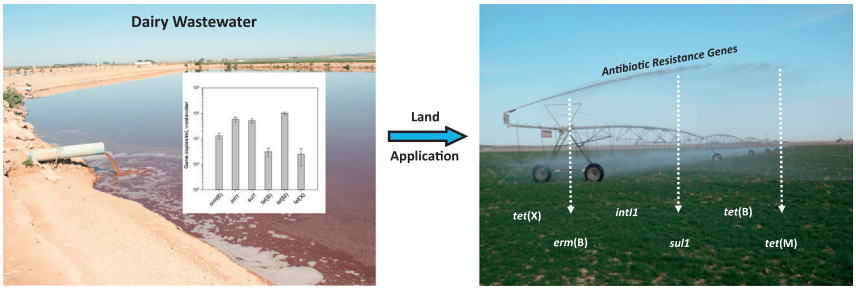
Robert S. Dungan *, Chad W. McKinney, April B. Leytem

USDA-Agricultural Research Service, Northwest Irrigation & Soils Research Laboratory, 3793 North 3600 East, Kimberly, ID 83341, United States

HIGHLIGHTS

- Dairy wastewaters contain antibiotic resistance and integron-integrase genes.
- Antibiotic resistance gene levels in soil increase after wastewater irrigation.
- Repeated monthly applications of dairy wastewater sustain the gene levels.
- Wastewater irrigation enlarges the reservoir of antibiotic resistance genes in soil.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 March 2018
Received in revised form 31 March 2018
Accepted 1 April 2018
Available online xxxx

Editor: D. Barcelo

Keywords:
Antibiotic resistance gene
Dairy
Integron-integrase
Manure
Wastewater irrigation

ABSTRACT

The application of dairy wastewater to agricultural soils is a widely used practice to irrigate crops and recycle nutrients. In this study, small-scale field plots were irrigated monthly (6 times) with dairy wastewater (100%), wastewater diluted to 50% with irrigation (canal) water, and diluted wastewater spiked with copper sulfate (50 mg Cu L^{-1}), while control plots were irrigated with canal water. In addition, half of all plots were either planted with wheat or were left as bare soil. Biweekly soil samples were collected during this period and processed to determine the occurrence and abundance of antibiotic resistance genes [$bla_{CTX\text{-}M\text{-}1}$, *erm*(B), *sul1*, *tet*(B), *tet*(M), and *tet*(X)] and a class 1 integron-integrase gene (*intI1*) via quantitative real-time PCR (qPCR). Only *sul1* and *tet*(X) were detected in soil (3 out of 32 samples) before the wastewater treatments were applied. However, the occurrence and relative abundance (normalized to 16S rRNA gene copies) of most genes [*erm*(B), *intI1*, *sul1*, and *tet*(M)] increased dramatically after wastewater irrigation and levels were maintained during the entire study period. $bla_{CTX\text{-}M\text{-}1}$ was the only gene not detected in wastewater-treated soils, which is likely related to its absence in the dairy wastewater. Relative gene levels in soil were found to be statistically similar among the treatments in most cases, regardless of the wastewater percentage applied and presence or absence of plants. The key result from this study is that dairy wastewater irrigation significantly enlarges the reservoir of ARGs and *intI1* in soils, while detection of these genes rarely occurred in soil irrigated only with canal water. In addition, elevated levels of Cu in the wastewater and treated soil did not produce a concomitant increase of the ARG levels.

Published by Elsevier B.V.

1. Introduction

Approximately 57 million kg of antibiotics were used in animal agriculture globally in 2010 (Van Boeckel et al., 2015). While antibiotics are a valuable tool to prevent and treat disease in food-producing animals, overuse of antibiotics can produce negative outcomes (Marshall and

* Corresponding author.
E-mail address: robert.dungan@ars.usda.gov (R.S. Dungan).

https://doi.org/10.1016/j.scitotenv.2018.04.020
0048-9697/Published by Elsevier B.V.

Levy, 2011). Antibiotics can select for antibiotic resistant bacteria (ARB) in the animal gut, which are then released into the farm environment via excreted feces. Antibiotic resistant bacteria can make their way from the farm environment to humans through occupational exposures on farms and at meat processing facilities, as well as by foodborne exposures among consumers, use of animal manures as crop fertilizers, and contamination of surface water and groundwater at animal production facilities (Koch et al., 2017). Antibiotic resistance genes (ARGs) are the genetic code ARB use to produce proteins that allow them to resist the effects of antibiotics. Antibiotic resistance genes can be distributed to similar, distantly related, and pathogenic bacteria through horizontal gene transfer mechanisms (Alekshun and Levy, 2007) and are considered to be emerging contaminants (Pruden et al., 2006).

There are nearly 9.2 million dairy cattle in the United States (FAO, 2014), with each animal producing about 50 to 70 kg of manure per day (Dungan and Leytem, 2014). At dairies, liquid manure (combined feces and urine) is generally subjected to a solid separation process, where the solid fraction is stockpiled, composted, or reused as bedding and the liquid fraction is sent to a wastewater storage pond. Due to limited capacity, the storage ponds require removal of the wastewater on a regular basis to accommodate new wastewater influxes. The application of dairy wastewaters to agricultural soils is a common method to irrigate and fertilize the soil to support growth of forages, and reuse the wastewater. Like other animal wastewaters, it is well known that dairy wastewaters contain ARB and ARGs (McKinney et al., 2010), as well as antibiotic residues and copper (Ippolito and Moore, 2013; Wei et al., 2011). In addition to applied nutrients, any contaminants or residues in the wastewater will accumulate in the soil during irrigation events (Chee-Sanford et al., 2009).

Most studies exploring the occurrence and fate of ARB and ARGs in soil agroecosystems focus on land application of animal manure solids or reclaimed wastewater from domestic wastewater treatment plants (Franklin et al., 2016). As a result, there are significant knowledge gaps regarding the impacts of animal wastewaters on antibiotic resistance in treated soils. The primary objective of this field study was to determine the effect of repeated applications of straight and diluted (50%) dairy wastewater on the occurrence and abundance of several ARGs [$bla_{\text{CTX-M-1}}$, *erm*(B), *sul1*, *tet*(B), *tet*(M), and *tet*(X)] and a class 1 integron-integrase (*intI1*) in receiving soil (bare or with wheat) over one growing season. We hypothesized that the long-term application of dairy wastewater would significantly expand the soil resistome when compared to soil that received regular irrigation water.

2. Materials and methods

2.1. Field site and treatments

The field site was located at the USDA–ARS Northwest Irrigation and Soils Research Laboratory in Kimberly, Idaho. Soil at the site is a Portneuf silt loam (coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcids). This field site received manure applications (type of manure was not recorded, but most likely beef cattle) in 1977 and 1981 at an amount of 112 and 45 Mg ha^{-1}, respectively. Thirty-two aluminum borders were installed (extending 7.6 cm below and 2.5 cm above ground) to create isolated mini-plots (76 cm × 38 cm) placed at least 60 cm apart.

The experimental design was a randomized complete block with 8 treatments in a 4 × 2 factorial structure. The main plot treatments consisted of 100% canal water (CW100); dairy wastewater blended to 50% with canal water (WW50); dairy wastewater blended to 50% with canal water and spiked with $CuSO_4$ to a final concentration of approximately 50 mg Cu L^{-1} (WW50Cu); and 100% dairy wastewater (WW100). In addition, half of all plots were planted with soft white spring wheat (*Triticum aestivum*) or were left as bare soil, with both treatments randomized among the plots.

On 13 April 2016, the wheat seeds were planted (105 seeds $plot^{-1}$) by hand in 3 rows with 15 cm spacing. On the same day, both bare and wheat plots received a surface application (non incorporated) of urea fertilizer at a rate of 47 kg N ha^{-1} (i.e., 1.3 g N $plot^{-1}$). The wheat biomass was cut on 27 June to a stubble height of about 3 cm, which was then allowed to grow again, followed by a second cutting on 5 Aug. The stubble and roots were pulled from the plots on 11 Oct.

A dairy that manages a mixed herd of about 1000 Holstein and Jersey cows supplied the wastewater. The wastewater was retrieved from the second storage pond in a series of four (with a settling basin preceding the entire system) and it was applied to the plots on the same day it was collected. Treatments were applied by hand using flood irrigation at 3.8 L per plot every four weeks (17 May, 14 June, 13 July, 9 Aug, 7 Sept, and 6 Oct). Due to the semi-arid climate and lack of rainfall, canal water was used to irrigate all plots between treatments as needed. Only 168 mm of precipitation were recorded during the study and additional climate data can be found on the Twin Falls (Kimberly) Agrimet web site (https://www.usbr.gov/pn/agrimet/agrimetmap/twfida.html). The Agrimet weather station was located next to the field site.

2.2. Soil sampling

Approximately 30 g of soil per plot was collected from the top 2 cm using a disposable soil core sampler (Lock N' Load, Environmental Sampling Supply, San Leandro, CA) and placed in a clean sealable plastic bag. The bags were manipulated to mix the soil within, then they were placed in a cooler with ice packs for the duration of the sampling (2−3 h). Upon arrival at the laboratory the soils were frozen at −75 °C until processed. Soil sampling was performed on a biweekly to monthly basis from May to Nov 2016 (16 [before treatment] and 31 May, 13 and 27 June, 12 and 27 July, 8 and 22 Aug, 6 and 19 Sept, 4 Oct, and 2 Nov) for a total of 12 sampling events.

2.3. Detection and quantitation of genes

DNA was extracted from approximately 500 mg of soil (wet wt.) or 0.5 mL of dairy wastewater using the FastDNA Spin Kit for Soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA) following the manufacturer's protocol. DNA was stored in DNase/pyrogen-free water at −20 °C until analyzed by quantitative real-time PCR (qPCR) on a iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each individual reaction consisted of 12.5 μL of 2× SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 250 nM of forward and reverse primers and probes, 2 μL of DNA template (10-fold diluted in molecular biology grade water), and sterile DNase/pyrogen free water to create a final volume of 25 μL. The gene targets were $bla_{\text{CTX-M-1}}$, *erm*(B), *intI1*, *sul1*, *tet*(B), *tet*(M), *tet*(X), and 16S rRNA. Primers, probes, annealing temperatures, amplicon lengths, and sequences can be found in Table 1. The thermocycler conditions consisted of 1 cycle at 95 °C for 3 min, 40 cycles at 95 °C for 15 s, and annealing temperature for 30 s. The qPCR runs included a standard curve covering seven orders of magnitude, and each sample was analyzed in duplicate. Standards were created using gBlocks Gene Fragments (Integrated DNA Technologies). All standard curve r^2 values were consistently ≥ 0.98 and amplification efficiencies ranged from 61 to 97%. The ARG and *intI1* gene copy numbers were normalized to 16S rRNA gene copies to produce relative gene abundances.

2.4. Chemical analyses

Antibiotic analysis was performed on the dairy wastewater samples that were used to irrigate the plots. In brief, one wastewater sample per sampling event was placed in a precleaned 250-mL amber jar (Environmental Sampling Supply, San Leandro, CA) and stored frozen at −20 °C. Frozen samples were shipped to Dr. Daniel Snow at the Water Sciences Laboratory in Lincoln, NE, for processing and analysis. Thawed portions

Table 1
Primer and probe sequences and annealing conditions used in the study.

Gene	Primer/probe	Sequence (5′ to 3′)	Amplicon size (bp)	T_A (°C)	Reference
bla$_{CTX-M-1}$	CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	336	58	Birkett et al. (2007)
	CTX-M-R	ATCACKCGGRTCGCCNGGRAT			
	CTX-M-1-P	CCCGACAGCTGGGAGACGAAACGT			
erm(B)	ermB-F	GGATTCTACAAGCGTACCTTGGA	92	60	Bockelmann et al. (2009)
	ermB-R	GCTGGCAGCTTAAGCAATTGCT			
	ermB-P	CACTAGGGTTGCTCTTGCACACTCAAGTC			
intI1	intI1-F	GCCTTGATGTTACCCGAGAG	196	60	Barraud et al. (2010)
	intI1-R	GATCGGTCGAATGCGTGT			
	intI1-P	ATTCCTGGCCGTGGTTCTGGGTTTT			
sul1	sul1-F	CCGTTGGCCTTCCTGTAAAG	67	60	Heuer and Smalla (2007)
	sul1-R	TTGCCGATCGCGTGAAGT			
	sul1-P	CAGCGAGCCTTGCGGCGG			
tet(B)	tetB-F	ACACTCAGTATTCCAAGCCTTTG	205	60	Peak et al. (2007)
	tetB-R	GATAGACATCACTCCCTGTAATGC			
	tetB-P	AAAGCGATCCCACCACCAGCCAAT			
tet(M)	tetM-F	GGTTTCTCTTGGATACTTAAATCAATCR	88	60	Peak et al. (2007)
	tetM-R	CCAACCATAYAATCCTTGTTCRC			
	tetM-P	ATGCAGTTATGGARGGGATACGCTATGGY			
tet(X)	tetX-F	GCAAGCGCCCATTACCCATAA	97	60	McKinney et al. (2018)
	tetX-R	AAGGCATCCACCAACCCACT			
	tetX-P	CATTTGATGCCGCCTTTTGCAGGGC			
16S rRNA	BACT1369-F	CGGTGAATACGTTCYCGG	~123	56	Suzuki et al. (2000)
	PROK1492-R	GGWTACCTTGTTACGACTT			
	TM1389F-P	CTTGTACACACCGCCCGTC			

of samples were weighed, mixed with 0.1 g of sodium EDTA and 100 ng of surrogate. After equilibration, the sample was passed through a glass microfiber filter and preconditioned 200 mg Oasis HLB (Waters Corporation, Milford, MA) solid phase extraction cartridge for enhancement of individual compounds. Group 1 analytes (ampicillin, anhydroerythromycin, ceftiofur, erythromycin A, novobiocin, penicillin G, penillic acid, tiamulin, tylosin, and virginiamycin M1) were eluted with 6 mL of an 80:20 mixture of dichloromethane/acetone. Group 2 analytes (chlortetracycline, lincomycin, monensin A, oxytetracycline, sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfathiazole, tetracycline, and trimethoprim) were eluted with 6 mL of 0.1% formic acid in methanol. All cartridge eluates were spiked with internal standards and evaporated under nitrogen gas to about 100 μL. Concentrated extracts were analyzed by liquid chromatography-tandem mass spectrometry by two different methods depending on chemical properties of analytes (Dungan et al., 2017). Analytes in each group were detected using multiple reaction monitoring mode with positive electrospray ionization. Method detection limits were determined by extraction and analysis of 8 to 10 replicates of a low-level fortified blank. All the antibiotics had a detection limit of 0.010 μg L^{-1} except for ampicillin, monensin, novobiocin, penicillin G, tiamulin, and tylosin 0.005 μg L^{-1}, virginiamycin M1 at 0.020 μg L^{-1}, and ceftiofur at 0.090 μg L^{-1}.

Copper levels in the wastewater and soils were determined by inductively coupled plasma-atomic emission spectrometry after microwave digestion according to U.S. EPA method 3051A (USEPA, 2007).

2.5. Statistical analysis

All statistical tests were performed using SAS 9.4 (SAS Institute Inc., Cary, NC). The relative genes abundances over the study period were analyzed in a linear mixed model (PROC MIXED) with repeated measures (date). The model considered date, treatment (CW100, WW50, WW50Cu, and WW100) and type (bare soil and wheat) and their interactions as fixed effects, and block as a random effect. A first-order, autoregressive, moving average covariance structure was used to account for the correlations among the repeated measures. One-way analysis of variance was performed using the general linear model procedure (PROC GLM). Least square means were calculated and pairwise comparisons were determined using a Tukey-Kramer adjustment. Paired *t*-tests for the means were performed using PROC TTEST. Correlation analysis (PROC CORR) was used to calculate Pearson correlation coefficients (r) and *P*-values between the ARGs. Highly correlated variables were defined as $r \geq 0.70$. Gene abundance data was $\log_{10}$ transformed before analysis to meet assumptions of normality and homogeneity of variance. Statements of statistical significance were declared at $P < 0.05$.

3. Results and discussion

Antibiotics from eight classes were targeted for analysis in the wastewater samples, with 15 out of 23 detected (Table 2). The most frequently detected antibiotics were lincomycin, chlortetracycline, monensin, oxytetracycline, and sulfadimethoxine (6 out of 6 wastewater samples), followed by sulfamethazine and sulfamethoxazole (5 out of 6 samples). Antibiotics or their metabolites that were detected less frequently were erythromycin, anhydroerythromycin (metabolite of erythromycin), penillic acid, sulfamethizole, tetracycline, tiamulin, trimethoprim, and tylosin. Chlortetracycline and monensin had the highest mean concentrations (9.4 and 13.3 μg L^{-1}, respectively), as well as, the highest concentrations in a single sampling event (37.3 and 42.8 μg L^{-1}, respectively).

Regarding the presence of genes in the dairy wastewater, *erm*(B), *intI1*, *sul1*, *tet*(B), *tet*(M), and *tet*(X) were detected in all six monthly samples (Fig. 1). *tet*(M), which encodes for resistance to tetracyclines through ribosomal protection, was the most abundant ARG on average. The second most abundant genes were *sul1* and *intI1*, followed by *erm* (B) at the third highest level. *sul1* encodes for resistance to sulfonamide antibiotics, while *intI1* is a class 1 integron-integrase gene which can carry any number of ARGs and can move between species by horizontal gene transfer (Gillings et al., 2015). *erm*(B) genes encode resistance to macrolide, lincosamide, streptogramin, ketolide, and oxazolidinone antibiotics, and are found in a wide host range (Roberts, 2008). *tet*(B) and *tet*(X) were found in the wastewater at levels nearly an order of magnitude lower than the other genes and encode for resistance through efflux and enzymatic modification of tetracyclines, respectively. The *bla*$_{CTX-M-1}$ gene, which encodes resistance to β-lactam antibiotics, was not detected in the dairy wastewater.

The occurrence of ARGs and *intI1* was found to be negligible in the soils before treatment (i.e., baseline soils) and in plots irrigated with canal water for the duration of the study (Table 3). Only *sul1* and *tet* (X) were detected in background soils, while *intI1*, *sul1*, and *tet*

Table 2
The occurrence and concentrations of antibiotics and antibiotic metabolites in the dairy wastewaters that were applied to the plots.

Antibiotic class	Antibiotic	Occurrence (out of 6)	Concentration (μg L^{-1})		
			Mean	Min	Max
β-Lactam	Ampicillin	0			
	Ceftiofur	0			
	Penicillin G	0			
	Penillic acid[a]	2	1.53	1.46	1.60
Macrolide-lincosamide-streptogramin	Lincomycin	6	0.13	0.07	0.18
	Erythromycin	4	0.08	0.04	0.18
	Anhydroerythromycin[b]	3	0.06	0.03	0.11
	Tylosin	1	0.05		
	Virginiamycin M1	0			
Sulfonamide	Sulfadiazine	0			
	Sulfadimethoxine	6	2.56	0.89	3.99
	Sulfamerazine	0			
	Sulfamethazine	5	0.73	0.17	2.40
	Sulfamethizole	1	0.03		
	Sulfamethoxazole	5	0.89	0.27	1.94
	Sulfathiazole	0			
Tetracycline	Chlortetracycline	6	9.36	1.67	37.3
	Oxytetracycline	6	2.57	1.73	4.20
	Tetracycline	1	0.10		
Aminocoumarin	Novobiocin	0			
Anisoles	Trimethoprim	4	1.08	0.06	3.79
Ionophore	Monensin	6	13.3	5.81	42.8
Pleuromutilin	Tiamulin	1	0.002		

[a] Metabolite of penicillin G.
[b] Metabolite of erythromycin.

(X) were detected in canal water treated soils (i.e., CW100). The lack of ARG detection is likely due to the fact that the soils and canal water contain naturally low levels of these genes or they are completely absent. In addition, sources that contain abundant ARGs, such as animal manure, have not been applied to these soils since 1981. The results from the present study, however, clearly demonstrate that dairy wastewater applications dramatically increase the occurrence of all ARGs and *intI1* in treated soils (Table 3). Not surprisingly, because these genes were present in the dairy wastewater, one would expect to find them in soils treated with 50% or 100% wastewater. The only gene that was not detected in wastewater-treated soil was $bla_{CTX\text{-}M\text{-}1}$, which is almost certainly related to the absence of this gene in the dairy wastewater. Another interesting observation related to gene occurrence was that *tet*(B) and *tet*(X) were detected less frequently in wastewater-treated soils when compared to *erm*(B), *intI1*, *sul1*, and *tet*(M) (Table 3). The difference in gene detection frequencies appears to be related to their abundance in wastewater, as both *tet*(B) and *tet*(X) were found at the lowest levels (Fig. 1). However, it should be noted that other factors, such as presence of antibiotic residues or other non-antibiotic xenobiotic compounds (e.g., heavy metals, antimicrobial disinfectants) in wastewaters, could be influencing the resistome in the treated soils (Cytryn, 2013). Because of the low number of detections for *tet*(B) and *tet*(X) and inability to properly interpret trends, the discussion from this point forward will solely focus on *erm*(B), *intI1*, *sul1*, and *tet*(M).

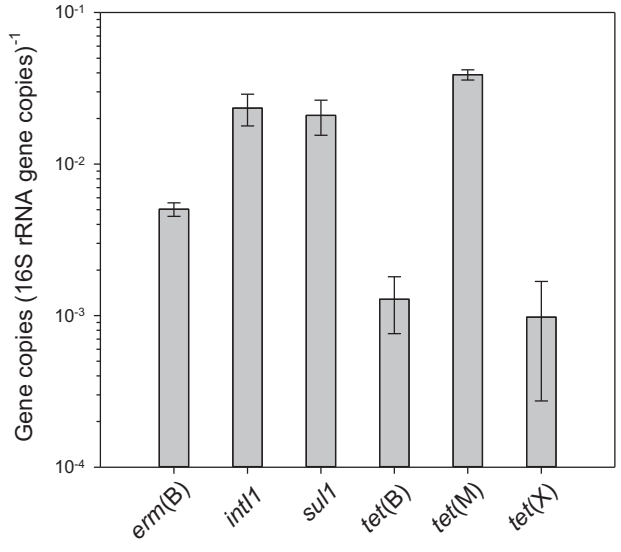


Fig. 1. The average abundance of antibiotic resistance and *intI1* gene copies (per 16S rRNA gene copies) in the dairy wastewater. Error bars are 95% confidence intervals of 6 sampling events.

When considering the wastewater gene abundance data in Fig. 1, it might be expected that gene levels in treated soil could produce the following trend: *tet*(M) > *intI1* = *sul1* > *erm*(B). Fig. 2 presents the average gene levels (normalized to 16S rRNA genes) over the study period by treatment, which shows a general trend of *tet*(M) > *sul1* > *intI1* > *erm*(B) for most of the wastewater treatments. Furthermore, while abundances of *intI1*, *sul1*, and *tet*(M) in bare soil treated with 100% wastewater had the highest average values, a pairwise comparison revealed that the levels were statistically similar to those in wheat plots treated with 100% wastewater and in bare soil treated with 50% wastewater without and with added Cu (except *intI1*). In the case of *erm*(B), the gene level in bare soil with 100% wastewater was only statistically similar to the same treatment with wheat. After reviewing the scientific literature, we could not find other studies that have investigated the effect of

Table 3
The occurrence of antibiotic resistance genes and a class 1 integron-integrase gene (*intI1*) in soil as influenced by treatment.

Treatment	Type	*n*	$bla_{CTX\text{-}M\text{-}1}$	*erm*(B)	*intI1*	*sul1*	*tet*(B)	*tet*(M)	*tet*(X)
BT		32	0	0	0	1	0	0	2
CW100	BS	44	0	0	4	2	0	0	0
	W	44	0	0	4	4	0	0	1
WW50	BS	44	0	36	43	44	7	44	4
	W	44	0	31	40	44	0	44	4
WW50Cu	BS	44	0	36	36	41	7	44	5
	W	44	0	35	35	38	4	44	2
WW100	BS	44	0	43	44	44	13	44	17
	W	44	0	41	44	44	9	44	12

BT = before treatment; CW100 = 100% canal water; WW50 = 50% dairy wastewater; WW50Cu = 50% dairy wastewater with 50 mg Cu L^{-1}; WW100 = 100% dairy wastewater; BS = bare soil; W = wheat.

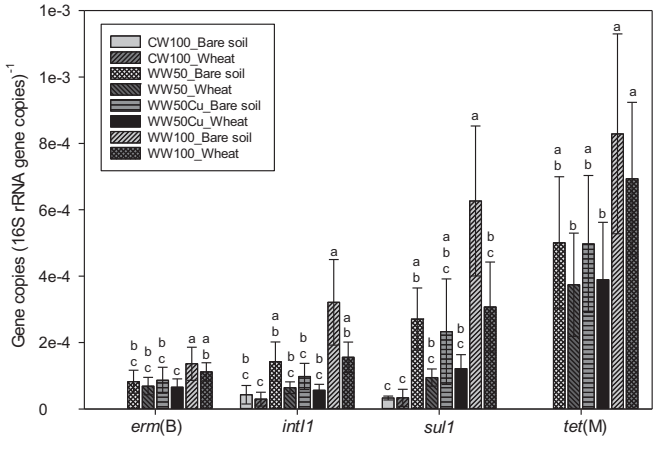


Fig. 2. The average abundance of antibiotic resistance and *intI1* gene copies (per 16S rRNA gene copies) in the treated soils. Error bars are 95% confidence intervals based on the occurrences (sample size) in Table 2. Lowercase letters above the bars indicate significant differences between the treatments at a 0.05 probability level. CW100 = 100% canal water; WW50 = 50% dairy wastewater; WW50Cu = 50% dairy wastewater with 50 mg Cu L^{-1}; WW100 = 100% dairy wastewater.

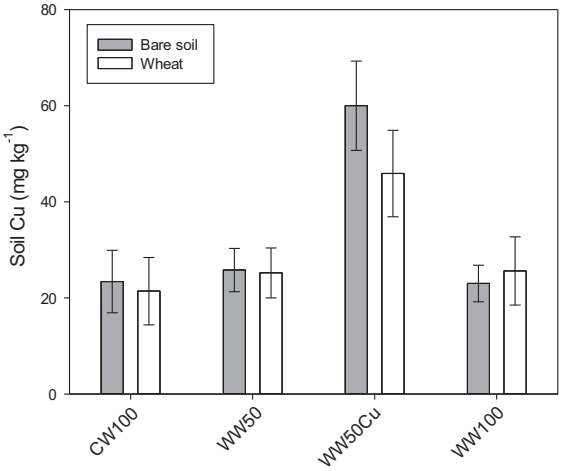


Fig. 3. The average soil copper concentrations for each treatment in bare and wheat plots. Error bars are 95% confidence intervals based on 11 sampling events.

dairy wastewater applications on ARG levels in soil. Most studies have focused on use of untreated and treated municipal wastewater in agricultural settings (Broszat et al., 2014; Chen et al., 2014; Dalkmann et al., 2012). The results from these studies demonstrate that municipal wastewater irrigation increases the level of ARGs in soil, which supports the results from our study. In contrast, Negreanu et al. (2012) found that ARG abundances in soil irrigated with freshwater to be similar or higher than in soil irrigated with treated municipal wastewater.

Elevated levels of Cu (5 to 90 mg L^{-1}) can be found in many dairy wastewaters due to the flushing or dumping of footbath waste into storage ponds (Ippolito and Moore, 2013). When the wastewater is land applied, the Cu in the soil can exceed background levels. Copper is of particular interest in contaminated soils, since it has been found to select for Cu-resistant bacteria, which in turn also indirectly selects for AR in the Cu-resistant bacteria (Berg et al., 2010; Berg et al., 2005). In this study Cu was detected in the dairy wastewater at 4.8 mg L^{-1} and in the before treatment soils at an average concentration of 14.2 mg kg^{-1} (data not shown). Upon addition of the treatments, the Cu levels in the soils increased, but the largest increases occurred in the soil receiving 50% wastewater that was amended with $CuSO_4$. Under this treatment, the bare and wheat plots had average Cu concentrations of 60.0 and 45.9 mg kg^{-1}, respectively, while the average concentration in soil under all other treatments ranged from 21.4 to 25.8 mg Cu kg^{-1} (Fig. 3). When just comparing the gene abundance data (via a paired *t*-test) in soils treated with 50% wastewater (with and without added $CuSO_4$), only *intI1* and *sul1* were found to be significantly different in bare soil with *P*-values of 0.004 and 0.002, respectively. In fact, these genes levels were found to be slightly lower on average in the plots receiving Cu amended wastewater (see Fig. 2). Gene levels for *erm*(B) and *tet*(M) in bare and wheat plots and *intI1* and *sul1* in wheat plots were determined to be similar ($P > 0.31$).

Fig. 4 shows the relative abundances of *erm*(B), *intI1*, *sul1*, and *tet*(M) in the treated soils over the course of the experiment from late May to early Nov. The data for *tet*(B) and *tet*(X) are not presented since the infrequent number of detections made it impossible to interpret the trends. As a result, the discussion from this point forward is only focused on *erm*(B), *intI1*, *sul1*, and *tet*(M). Gene data from before the first wastewater treatment (occurring on 16 May) is absent since most of the genes were not detected in the soils (Fig. 4). Irrespective of the gene, once the wastewater was applied they were then found at detectable levels in the soil. It is evident from the results that gene levels were maintained throughout the study period due to repeated applications of the wastewater. The abundance of *erm*(B) was highest on 31 May after the first application of wastewater, but subsequent applications of wastewater did not cause the gene levels to fluctuate (Fig. 4a). In contrast, *intI1* (Fig. 4b) and *sul1* (Fig. 4c) abundances appear to be associated with some wastewater applications; however, the levels decreased within two to four weeks. In the case of *tet*(M), gene abundances were highest for all wastewater treatments on 31 May, then they decreased to consistently lower levels thereafter (Fig. 4d). Overall, relative gene abundances tended to be the highest, on average, in bare soil treated with 100% dairy wastewater (black squares in Fig. 4), but overlapping error bars suggest little difference among the various wastewater treatments. Results from the statistical analysis (Table 4) revealed that there were no significant Date × Treatment × Type interactions on gene levels ($P > 0.36$), although there were significant Date × Type interactions for *intI1*, *sul1*, and *tet*(M) ($P < 0.003$).

Correlation analyses were performed between the relative abundances of *erm*(B), *intI1*, *sul1* and *tet*(M). Statistically significant correlations ($P < 0.0001$) were found between *erm*(B) and *tet*(M) ($r = 0.76$), as well as, between *intI1* and *sul1* ($r = 0.80$). Although statistically significant, all other correlations had $r \leq 0.46$ (data not shown) and will not be discussed further. The high correlation coefficients between *tet*(M) and *erm*(B) genes makes sense because they are often found together on the Tn*916*–Tn*1545* family of conjugative transposons which often reside in Gram-positive bacteria, especially streptococci, staphylococci, and enterococci (Roberts and Schwarz, 2016). Typically, the *sul1* gene is located adjacent to class 1 integrons and the *intI1* gene encodes the integrase of the class 1 integron (Gillings et al., 2015; Mazel, 2006) which could be why there is such a high correlation between these two genes.

4. Conclusions

The application of dairy wastewater to agricultural soils is a practice that will surely continue to be used as a method to irrigate crops, recycle nutrients, and reuse wastewater. What is clear from the results of this study, is that dairy wastewater irrigation dramatically increases the occurrence and abundance of ARGs and *intI1* in the treated soil. The gene levels in soil were found to be statistically similar in most cases among the treatments, regardless of the wastewater percentage applied (50% vs. 100%), presence or absence of plant growth (bare soil vs. wheat), and level of Cu in the wastewater. On average, ARGs and *intI1* made up about 0.1 to 4% of the microbial community in the wastewater when normalized to 16S rRNA gene copies, but <0.1% in soil, which indicates that ARB enrichment did not occur. The appearance of ARGs in soil simply occurs by addition of intracellular and extracellular genes that are present in the wastewater; however, it is not known if the

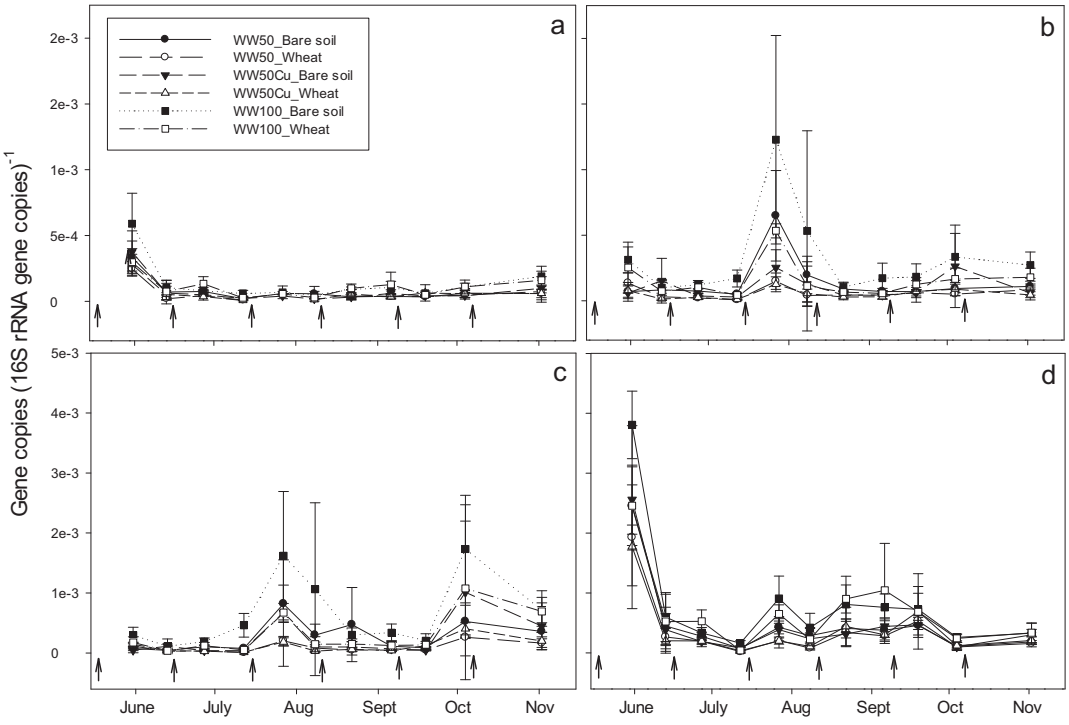


Fig. 4. The abundance of (a) *erm*(B), (b) *intI1*, (c) *sul1*, and (d) *tet*(M) gene copies (per 16S rRNA gene copies) in the treated soils during the study period. Error bars represent the standard deviation of measurements from four replicate plots. The arrows above the x-axis indicate the time at which the wastewater was applied. WW50 = 50% dairy wastewater; WW50Cu = 50% dairy wastewater with 50 mg Cu L^{-1}; WW100 = 100% dairy wastewater.

presence of antibiotic residues and other xenobiotic compounds may have affected gene selection and persistence in this study. The fact that elevated Cu levels did not cause associated increases in ARGs, suggests that ARB did not possess a co-located Cu resistance gene. Conversely, it is possible that soil Cu was not at a level high enough (and for suitable period of time) to cause enrichment of ARB. In addition, since we investigated repeated wastewater applications, there is little indication as to how quickly the genes might dissipate after wastewater application is terminated. Nevertheless, since wastewater irrigation at dairies generally occurs several months out of the year and on a more frequent basis than in this study, it can be expected that high gene levels will be maintained in affected soils. Future research efforts should determine how long-term irrigation of soils with dairy wastewater might be contributing to the growing threat of drug-resistant bacteria.

Table 4
Linear mixed model results (*F*-statistic) describing the effects of dairy wastewater application (Treat) on normalized gene levels in soil plots with and without wheat (Type).

Parameter	*erm*(B)	*intI1*	*sul1*	*tet*(M)
Date	**32.0 (<0.0001)**	**15.7 (<0.0001)**	**40.6 (<0.0001)**	**69.8 (<0.0001)**
Treat	**18.7 (<0.0001)**	**52.4 (<0.0001)**	**41.3 (<0.0001)**	**32.3 (<0.0001)**
Date × Treat	1.32 (0.1774)	0.91 (0.5931)	**1.71 (0.0334)**	1.02 (0.4455)
Type	3.79 (0.0678)	**11.7 (0.0009)**	**16.5 (0.0002)**	**23.6 (0.0002)**
Date × Type	1.37 (0.2032)	**2.82 (0.0034)**	**6.06 (<0.0001)**	**4.31 (<0.0001)**
Treat × Type	0.06 (0.9428)	1.16 (0.3413)	2.24 (0.1057)	0.56 (0.5830)
Date × Treat × Type	0.91 (0.5594)	0.63 (0.8932)	1.09 (0.3612)	0.63 (0.8843)

Significances at α = 0.05 are in bold; *P*-values are in parentheses under the *F*-statistic.

References

Alekshun, M.N., Levy, S.B., 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell 128, 1037–1050.

Barraud, O., Baclet, M.C., Denis, F., Ploy, M.C., 2010. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J. Antimicrob. Chemother. 65, 1642–1645.

Berg, J., Tom-Petersen, A., Nybroe, O., 2005. Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. Lett. Appl. Microbiol. 40, 146–151.

Berg, J., Thorsen, M.K., Holm, P.E., Jensen, J., Nybroe, O., Brandt, K.K., 2010. Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. Environ. Sci. Technol. 44, 8724–8728.

Birkett, C.I., Ludlam, H.A., Woodford, N., Brown, D.F., Brown, N.M., Roberts, M.T., et al., 2007. Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum β-lactamases. J. Med. Microbiol. 56, 52–55.

Bockelmann, U., Dorries, H.H., Ayuso-Gabella, M.N., Salgot de Marcay, M., Tandoi, V., Levantesi, C., et al., 2009. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. Appl. Environ. Microbiol. 75, 154–163.

Broszat, M., Nacke, H., Blasi, R., Siebe, C., Huebner, J., Daniel, R., et al., 2014. Wastewater irrigation increases the abundance of potentially harmful gammaproteobacteria in soils in Mezquital Valley, Mexico. Appl. Environ. Microbiol. 80, 5282–5291.

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.F., Yannarell, A.C., et al., 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J. Environ. Qual. 38, 1086–1108.

Chen, C.Q., Li, J., Chen, P.P., Ding, R., Zhang, P.F., Li, X.Q., 2014. Occurrence of antibiotics and antibiotic resistances in soils from wastewater irrigation areas in Beijing and Tianjin, China. Environ. Pollut. 193, 94–101.

Cytryn, E., 2013. The soil resistome: the anthropogenic, the native, and the unknown. Soil Biol. Biochem. 63, 18–23.

Dalkmann, P., Broszat, M., Siebe, C., Willaschek, E., Sakinc, T., Huebner, J., et al., 2012. Accumulation of pharmaceuticals, *Enterococcus*, and resistance genes in soils irrigated with wastewater for zero to 100 years in central Mexico. PLoS One 7, e45397.

Dungan, R.S., Leytem, A.B., 2014. The characterization of microorganisms in dairy wastewater storage ponds. J. Environ. Qual. 42, 1583–1588.

Dungan, R.S., Snow, D.D., Bjorneberg, D.L., 2017. Occurrence of antibiotics in an agricultural watershed in south-central Idaho. J. Environ. Qual. 46, 1455–1461.

FAO, 2014. FAOSTAT. Food and Agriculture Organization of the United Nations Statistics http://www.fao.org/faostat/en/#data (accessed 16 April 2018).

Franklin, A.M., Aga, D.S., Cytryn, E., Durso, L.M., McLain, J.E., Pruden, A., et al., 2016. Antibiotics in agroecosystems: introduction to the special section. J. Environ. Qual. 45, 377–393.

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279.

Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9, 657–666.
Ippolito, J., Moore, A., 2013. Copper Sulfate Foot Baths on Dairies and Crop Toxicities. eXtension.org http://articles.extension.org/pages/22177/copper-sulfate-foot-baths-on-dairies-and-crop-toxicities (accessed 16 April 2018).
Koch, B.J., Hungate, B.A., Price, L.B., 2017. Food-animal production and the spread of antibiotic resistance: the role of ecology. Front. Ecol. Environ. 15, 309–318.
Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24, 718–733.
Mazel, D., 2006. Integrons: agents of bacterial evolution. Nat. Rev. Microbiol. 4, 608–620.
McKinney, C.W., Loftin, K.A., Meyer, M.T., Davis, J.G., Pruden, A., 2010. tet and sul antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. Environ. Sci. Technol. 44, 6102–6109.
McKinney, C.W., Dungan, R.S., Moore, A., Leytem, A.B., 2018. Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. FEMS Microbiol. Ecol. 94, fiy010.
Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E., 2012. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. Environ. Sci. Technol. 46, 4800–4808.
Peak, N., Knapp, C.W., Yang, R.K., Hanfelt, M.M., Smith, M.S., Aga, D.S., et al., 2007. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. Environ. Microbiol. 9, 143–151.
Pruden, A., Pei, R.T., Storteboom, H., Carlson, K.H., 2006. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ. Sci. Technol. 40, 7445–7450.
Roberts, M.C., 2008. Update on macrolide–lincosamide–streptogramin, ketolide, and oxazolidinone resistance genes. FEMS Microbiol. Lett. 282, 147–159.
Roberts, M.C., Schwarz, S., 2016. Tetracycline and phenicol resistance genes and mechanisms: importance for agriculture, the environment, and humans. J. Environ. Qual. 45, 576–592.
Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl. Environ. Microbiol. 66, 4605–4614.
USEPA, 2007. Test Methods for Evaluating Soilid Wastes: Physical/Chemical Methods, SW-846. Method 3051A: Microwave Assisted Acid Digestion of Aqueous Samples and Extracts. https://www.epa.gov/hw-sw846/sw-846-compendium (accessed 16 April 2018).
Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., et al., 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. U. S. A. 112, 5649–5654.
Wei, R., Ge, F., Huang, S., Chen, M., Wang, R., 2011. Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Jiangsu Province, China. Chemosphere 82, 1408–1414.