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RESEARCH ARTICLE

Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure

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One sentence summary: This study demonstrated that dairy manure applications to soil significantly increase the abundance of clinically relevant ARGs when compared to control and inorganic fertilized plots.

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ABSTRACT

Animal manures are commonly used to enhance soil fertility, but there are growing concerns over the impact of this practice on the development and dissemination of antibiotic resistance. The aim of this field study was to determine the effect of annual dairy manure applications on the occurrence and abundance of antibiotic resistance genes (ARGs) in an agricultural soil during four years of crop production. Treatments included (i) control (no fertilizer or manure), (ii) inorganic fertilizer and (iii) dairy manure at three application rates. Quantitative PCR was used to determine absolute (per g dry soil) and relative (per 16S rRNA gene) abundances of ARGs in DNA extracted from soils. Six ARGs and one class 1 integron were targeted. This study found that (i) manure application increases ARG abundances above background soil levels; (ii) the higher the manure application rate, the higher the ARG abundance in soil; (iii) the amount of manure applied is more important than reoccurring annual applications of the same amount of manure; (iv) absolute abundance and occurrence of ARGs decreases with increasing soil depth, but relative abundances remained constant. This study demonstrated that dairy manure applications to soil significantly increase the abundance of clinically relevant ARGs when compared to control and inorganic fertilized plots.

Keywords: antibiotic resistance genes; dairy; manure; soil; class 1 integron; qPCR

INTRODUCTION

Antibiotics are commonly given to food-producing animals at therapeutic levels for treatment of infection and at subtherapeutic levels for prevention of disease and growth promotion. Enhanced selection for antibiotic-resistant bacteria (ARB) can occur in animal guts (Looft *et al.* 2012) which are excreted through defecation (Inglis *et al.* 2005; Alexander *et al.* 2008). In addition, up to 80% of antibiotic administered to animals can be excreted as the parent compound or active metabolites in urine and feces (Elmund *et al.* 1971; Winckler and Grafe 2001; Arikan *et al.* 2007). While animal manures are regularly used as a soil conditioner and fertilizer for crop production, the land application of manure is a common route for the introduction of antibiotics, ARB and antibiotic resistance genes (ARGs) to enter the environment (Chee-Sanford *et al.* 2009). Excreted antibiotics can continue exerting selection pressure in manure, soil, water/wastewater and sediment/sludge depending on their rate of degradation, hydrophobicity and sorption potential. Manure-amended soils have been documented to increase both ARB and ARGs, even in the absence of antibiotics (Heuer, Schmitt and

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Smalla 2011; Zhou *et al.* 2013; Udikovic-Kolic *et al.* 2014), but inconsistent results and limited data highlight the need for additional research on this topic (Franklin *et al.* 2016). For example, Munir and Xagoraraki (2011) found that soil ARGs significantly increased in abundance above background levels after land application of manure and biosolids to one site, but did not significantly increase after land application of biosolids to another site. This study, like most determining the abundance of ARGs in soil, utilized quantitative real-time PCR (qPCR) in which ARGs of interest must be determined a priori and only a limited number of genes are generally targeted due to time and cost constraints (Luby *et al.* 2016).

ARGs have a tendency to be shared among bacteria (related and unrelated species) through horizontal gene transfer (HGT), which is of great concern and incredibly difficult to track in the environment (Luby *et al.* 2016). The ARGs are often associated with mobile genetic elements (MGEs) such as plasmids, transposons and integrons that can be transferred via HGT mechanisms including conjugation (gene transfer through cell-to-cell contact), transduction (gene transfer mediated by bacteriophages) and transformation (uptake of extracellular DNA) (Levy and Marshall 2004). The reservoir of ARGs in soil has been termed the soil resistome (D'Costa *et al.* 2006) and it has been determined that the antibiotic resistome is shared between soil bacteria and human pathogens (Forsberg *et al.* 2012). The spread of ARGs in the environment is of significant interest, as disease-causing bacteria that acquire these genes can become resistant to medical treatment. Once in the environment, ARB and ARGs can be transmitted to humans through soil, water, air/dust, fresh produce, domesticated animals and wildlife (Huijbers *et al.* 2015). Because of these reasons, it has been suggested that ARGs themselves are emerging contaminants for which mitigation strategies are needed to prevent their widespread dissemination (Pruden *et al.* 2006).

A thorough search of the literature indicated that studies investigating the long-term influence of dairy manure application on ARGs in soils are rare (Musovic *et al.* 2014; Fang *et al.* 2015; Peng *et al.* 2015; Tang *et al.* 2015; Graham *et al.* 2016). The purpose of this study was to determine the occurrence and abundance of six ARGs [$bla_{\text{CTX-M-1}}$, *erm*(B), *sul1*, *tet*(A), *tet*(W) and *tet*(X)] and a class 1 integron-integrase gene (*intI1*) in an agricultural soil that had received dairy manure at three rates on an annual basis for 4 years. These ARGs were chosen because they include resistance to a variety of antibiotics, utilize different resistance mechanisms and cover a wide range of bacterial hosts (Skold 2000; Canton and Coque 2006; Birkett *et al.* 2007; Roberts 2008; Roberts and Schwarz 2016). Even though *intI1* is not an ARG, it can be used as a proxy for ARG contamination because it is linked to antibiotic, disinfectant and metal resistance genes, resides in a diverse number of commensal and pathogenic bacteria, often located on MGEs, and many common forms are xenogenetic (i.e. assembled relatively recently under selection pressures brought upon by human activities) (Gillings *et al.* 2015). Thus, throughout this paper, *intI1* will be included in the group called ARGs. The comparisons in this study include treatment type (control, inorganic fertilizer and three manure application rates), soil depth (0 to 120 cm), and year (2012–2016).

MATERIALS AND METHODS

Field site and treatments

The field site was located at the USDA-ARS Northwest Irrigation and Soils Research Laboratory in Kimberly, Idaho. This region has a semi-arid climate and consists of hot dry summers and cool wet winters, with a mean annual temperature of 8.9°C and precipitation of 229 mm (NRCS 2017). Soil at the site is a Portneuf silt loam (coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcids) that was surface-irrigated with Snake River water. The field was 1 ha and contained plots that were 12.2 m × 18.3 m. The experimental design was a randomized complete block with four replications. The treatments consisted of a control (Ctrl, no fertilizer or manure application), inorganic fertilizer (Fert) and manure at three application rates (17.2, 34.5 and 52.0 Mg [dry wt.] ha^{-1}). The highest manure rate (i.e. 52 Mg ha^{-1}) is representative of what is typically applied in this region. Stockpiled manure was obtained from anonymous dairies using a local manure hauler. The manure was analyzed for chlortetracycline, oxytetracycline, monensin, penicillin G, sulfamethazine, sulfadimethoxine, sulfamethoxazole, sulfathiazole and tetracycline, but only monensin and oxytetracycline were detected (concentration data is being withheld by collaborator until published). It was applied once per year (after crop harvest and soil sampling) to the same designated plots in the fall of 2012, 2013, 2014 and 2015. Inorganic fertilizer was applied using University of Idaho Extension recommendations based on spring soil test data (Brown *et al.* 2010). If necessary, individual nutrients (N, P, K, S, Mn) were added to meet the requirements for the specific crop being grown. After broadcasting the fertilizer and manure treatments, they were incorporated into the soil using a tandem disk within 24 h (Ctrl plots were also disked) to a depth of ~15 cm. The crops consisted of a wheat–potato–barley–sugar beet rotation from 2013 to 2016. Prior to this study, the crops at the field site were oats (2004), alfalfa (2005–2007), barley (2008–2009), corn (2011) and beans (2010, 2012).

Soil collection

Before treatment (BT), soils (i.e. background soils) were collected from the top 30 cm in the fall of 2012, prior to the first manure application. Pre-plant (spring) and post-harvest (fall) soil samples were collected in March and September, respectively. The pre-plant soil samples were collected every year from 2013 to 2016, but only from the top 30 cm of soil since they were also used to make fertilizer recommendations. Post-harvest samples were collected in 2014 and 2015 prior to manure addition (2013 samples collected, but were lost) and consisted of 120 cm long cores that were sectioned into five layers (0–15, 15–30, 30–60, 60–90 and 90–120 cm). Ten (0–30 cm) and two (0–120 cm) soil cores were collected per plot during the pre-plant and post-harvest sampling, respectively. The contents of each soil layer were composited and thoroughly mixed before a subsample was placed in clean sealable plastic bag and then frozen at −75°C.

Soil DNA extraction

Immediately after thawing at room temperature, one DNA extraction was performed per composited soil sample. The DNA was extracted from about 500 mg of soil (wet wt.) 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 qPCR was performed.

Primers and probes

Primers, probes, annealing temperatures, amplicon lengths, sequences and references can be found in Table 1. Due to the short sequences and low melting temperatures (T_M), the $bla_{\text{CTX-M-1}}$ and

Table 1. Primers and probes used in this study.

Gene	Type	Primer/probe name	Sequence 5′ to 3′	T_A (°C)	Amplicon size (bp)	Reference
16S rRNA	Forward	BACT1369F	CGGTGAATACGTTCYCGG	56	~123	Suzuki, Taylor and DeLong (2000)
	Reverse	PROK1492R	GGWTACCTTGTTACGACTT			
	Probe	TM1389P	6-FAM-CTTGTACACACCGCCCGTC-BHQ-1			
intI1	Forward	intI1-F	GCCTTGATGTTACCCGAGAG	60	196	Barraud *et al.* (2010)
	Reverse	intI1-R	GATCGGTCGAATGCGTGT			
	Probe	intI1-P	6-FAM-ATTCCTGGCCGTGGTTCTGGGTTTT-BHQ-1			
***bla*$_{CTX-M-1}$**	Forward	CTXM1-F	ACCAACGATATCGCGGTGAT	60	101	Colomer-Lluch, Jofre and Muniesa (2011)
	Reverse	CTXM1-R	ACATCGCGACGGCTTTCT			
	Probe (LNA)	CTXM1-P	6-FAM-TC + GTGCGC + CG + CTG-BHQ-1			
***erm*(B)**	Forward	ermB-F	GGATTCTACAAGCGTACCTTGGA	60	92	Bockelmann *et al.* (2009)
	Reverse	ermB-R	GCTGGCAGCTTAAGCAATTGCT			
	Probe	ermB-P	6-FAM-CACTAGGGTTGCTCTTGCACACTCAAGTC-BHQ-1			
sul1	Forward	sul1-F	CCGTTGGCCTTCCTGTAAAG	60	67	Heuer and Smalla (2007)
	Reverse	sul1-R	TTGCCGATCGCGTGAAGT			
	Probe	sul1-P	6-FAM-CAGCGAGCCTTGCGGCGG-BHQ-1			
***tet*(A)**	Forward	tetA-F	CCGCGCTTTGGGTCATT	60	51	Guarddon *et al.* (2011)
	Reverse	tetA-R	TGGTCGCGTCCCAGTGA			
	Probe (LNA)	tetA-P	6-FAM-TCG + GCG + AG + G + ATCG-BHQ-1			
***tet*(W)**	Forward	tetW-F	GCAGAGCGTGGTTCAGTCT	60	65	Smith *et al.* (2004)
	Reverse	tetW-R	GACACCGTCTGCTTGATGATAAT			
	Probe	tetW-P	6-FAM-TTCGGGATAAGCTCTCCGCCGA-BHQ-1			
***tet*(X)**	Forward	tetX-F	GCAAGCGCCCATTACCCATAA	60	97	This study
	Reverse	tetX-R	AAGGCATCCACCAACCCACT			
	Probe	tetX-P	6-FAM-CATTTGATGCCGCCTTTTGCAGGGC-BHQ-1			

6-FAM = fluorescein; BHQ-1 = black hole quencher 1 (Suzuki, Taylor and DeLong 2000; Smith *et al.* 2004; Heuer and Smalla 2007; Bockelmann *et al.* 2009; Barraud *et al.* 2010; Colomer-Lluch, Jofre and Muniesa 2011; Guarddon *et al.* 2011).

tet(A) probes were converted to locked nucleic acid (LNA) probes (Integrated DNA Technologies [IDT], Coralville, IA) to increase the T_M of the probes to about 68°C–70°C. A search of the literature did not reveal a primer/probe combination for *tet*(X). The following GenBank accession numbers were used to design the *tet*(X) primers and probe: M37699, JQ990987, JQ990988 and EU864422. Only four sequences were used because these were the only verified *tet*(X) gene sequences in the Gene Database within GenBank at the time of primers/probe design. ClustalX 2.1 (Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland) was used to align the sequences to find the most conserved regions, followed by use of Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA) to select the best primer pair (18–30 bp, 60°C–64°C melting temperature and 35%–65% GC content) and probe (20–30 bp, melting temperature 6°C–8°C higher than the primers and 35%–65% GC content) combinations (amplicon length between 70 and 150 bp). The primers were then checked against the NCBI BLAST database to ensure that they would not amplify non-specific products.

Quantitative real-time PCR

qPCR was performed on an 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 (IDT), 2 μL of DNA template and sterile DNase/pyrogen-free water to create a final volume of 25 μL. To help alleviate PCR inhibition, each DNA template was diluted by one order of magnitude. The temperature profile consisted of one cycle at 95°C for 3 min, 40 cycles at 95°C for 15 s and annealing temperature (T_A, Table 1) for 30 s. All qPCR runs included a standard curve covering seven orders of magnitude, and each sample was analyzed in triplicate. Standards were created using gBlocks Gene Fragments (IDT). The standard curve r^2 values were consistently >0.98, with an instrument limit of quantification (LOQ) of 10^2 gene copies μL^{-1}. Since the DNA templates were diluted by an order of magnitude to prevent qPCR inhibition, the corrected LOQ was 10^3 gene copies μL^{-1}. All ARG and *intI1* qPCR results were normalized per g of dry soil (absolute abundance) and per 16S rRNA gene copies (relative abundance). To normalize the gene data per gram of dry soil, the moisture contents of the soils were determined gravimetrically. A spreadsheet of all the absolute and relative ARG and absolute 16S rRNA gene abundance data can be found in the Supporting Information.

Statistical analysis

All statistical tests were performed using SAS 9.4 (SAS Institute Inc., Cary, NC). The data were log (base 10) transformed to achieve homogeneity of variance, which was determined from visual analysis of predicted versus residual and quantile-quantile plots. Statements of statistical significance were

Table 2. ARG occurrence by treatment.

Treatment	n	*intI1*	$bla_{\text{CTX-M-1}}$	*erm*(B)	*sul1*	*tet*(A)	*tet*(W)	*tet*(X)
BT	20	11	0	0	17	0	19	0
Ctrl	56	33	1	0	42	0	19	2
Fert	56	33	0	0	37	0	18	1
17.2M	16	16	1	4	16	0	16	13
34.5M	16	16	0	5	16	0	16	16
52.0M	56	37	2	6	48	0	40	27
Total	220	146	4	15	176	0	128	59

BT = before treatment; Ctrl = control; Fert = fertilizer; 17.2M = 17.2 Mg manure ha^{-1} yr^{-1}; 34.5M = 34.5 Mg manure ha^{-1} yr^{-1}; and 52.0M = 52.0 Mg manure ha^{-1} yr^{-1}.

defined as $P \leq 0.05$. One-way analysis of variance (ANOVA, GLM procedure) model was used to generate population means and 95% confidence limits (CL). The least significant difference (LSD) test within the GLM procedure was used for comparison of population means for categorizing significance groups. Least squares means (lsmeans) was used to generate *P*-values to determine statistical significance for comparison of individual population means. Since standard deviations have no meaning for n equal to 1 or 2, 95% CL are not included. Pooled data refer to the systematic grouping of qPCR data to strengthen the ANOVA model due to similar trends; it do not refer to the mixing of unique soil samples or DNA extracts prior to analysis. Correlation analysis (CORR procedure) was used to calculate the Pearson correlation coefficient (r) and *P*-values between the various genes. Correlations were considered significant if the Pearson correlation coefficient (r) ≥0.60 and the $P \leq 0.05$. Data were not pooled for the correlation analysis.

RESULTS

Gene occurrence

Out of a total of 220 unique soil samples (all spring, fall and depth data included) analyzed, the *intI1*, $bla_{\text{CTX-M-1}}$, *erm*(B), *sul1*, *tet*(A), *tet*(W) and *tet*(X) genes were detected 146, 4, 15, 176, 0, 128 and 59 times, respectively (Table 2). Due to limited detection of $bla_{\text{CTX-M-1}}$, *erm*(B) and *tet*(A), these genes were not included in any quantitative or statistical analysis. $bla_{\text{CTX-M-1}}$ was detected once in the Ctrl plots and three times in manured plots; *erm*(B) was only detected in soils with manure application; and *tet*(X) was not detected before treatment, but was detected twice in the Ctrl plots, once in the Fert plots and 56 times in the manured plots. All other ARGs, except for *tet*(A), were detected in BT, Ctrl, Fert and manured plots. Table 2 contains occurrence data for all ARGs broken down by treatment.

Treatment

The general treatment effects indicate that manure application increases both ARG absolute and relative abundances (i.e. per gram of dry soil and per 16S rRNA gene copies, respectively). Also, manure application rate was positively correlated with ARG abundances, although not always significantly. Figure 1 presents the absolute (A) and relative (B) abundance results from fall 2012 and spring 2013–2016 (data pooled to strengthen the ANOVA model since similar trends occurred each year). Since the fall 2012 samples were obtained prior to any fertilizer or manure addition, they were not included in the pooled data analysis, but were used as a comparison for BT gene abundances.

According to Fig. 1A, *sul1* and *tet*(W) show the following statistically significant trends: 52.0M > 34.5M > 17.2M > Ctrl ≈ Fert ≈ BT. *intI1* had a similar trend but the 17.2M was not statistically different from the Ctrl ($P = 0.60$) or Fert plots ($P = 0.24$). With *tet*(X), 34.5M and 52.0M were not significantly different according to LSD, but 52.0M was significantly greater than 34.5M using lsmeans ($P = 0.0013$). Even though 17.2M and 34.5M were not significantly different from each other or the Ctrl and Fert plots, *tet*(X) was only detected twice in the Ctrl and once in the Fert compared to 13 detections for 17.2M and 16 detections for 34.5M. The same trends occurred when normalizing to the 16S rRNA gene (Fig. 1B), except the 34.5M and 52.0M treatments were not significantly different for *sul1* ($P = 0.32$) and *tet*(W) ($P = 0.19$).

Soil depth

Unlike the treatment analysis, ARG absolute abundances show a different trend than the relative abundances for the 52.0M treatments. According to Fig. 2A, the ARG absolute abundances show a general trend of decreasing with depth (though not always significant), except for *tet*(X). The ARG relative abundances show no general trend with very few significant differences between depths (Fig. 2B). One thing that is clear with both graphs is that the detection frequency of ARGs decreases with depth, including *tet*(X). A conclusion that can be drawn from this is that total ARG copy numbers decrease with depth, but the fraction of bacteria carrying the ARGs does not change significantly.

Year

For the 52.0M (Fig. 3A and B) manure treatments, the ARG abundances for the years 2013–2016 are significantly greater than 2012, for both normalizations, except for 2012 and 2015 relative *intI1* abundances ($P = 0.28$). This makes sense because manure was applied after the fall 2012 sampling event. Both ARG abundances were quite variable when comparing individual years from 2013 to 2016.

Correlation analysis

Correlation analysis was performed to determine the relationship between ARGs and ARGs versus 16S rRNA genes (Tables 3 and 4). It should be noted that a significant correlation between two variables does not imply causation. All the ARGs [*intI1*, *sul1*, *tet*(W) and *tet*(X)] had a significant positive correlation with each other for both relative and absolute abundances. The strongest correlations were between *tet*(X) and *sul1* ($r = 0.88$, $P < 0.0001$) for both abundances and between *tet*(X) and *intI1* [$r = 0.81$ (absolute) and 0.85 (relative), $P < 0.0001$].

DISCUSSION

Occurrence of ARGs in manure-amended soil

This study was designed to determine the changes in absolute and relative abundances of various ARGs and a class 1 integron (*intI1*) in soil that had received inorganic fertilizer or dairy manure under a crop rotation that is typical for southern Idaho. While qPCR was used to track the ARGs, it should be noted that this technique cannot decipher between intracellular and extracellular DNA (Corinaldesi, Danovaro and Dell'Anno 2005), whether the DNA came from live or dead bacteria (Nocker, Cheung and Camper 2006) nor determine which bacteria carried these ARGs (Yang *et al.* 2002) and if these genes were expressed (Smith and Osborn 2009). Of the six ARGs that were targeted in

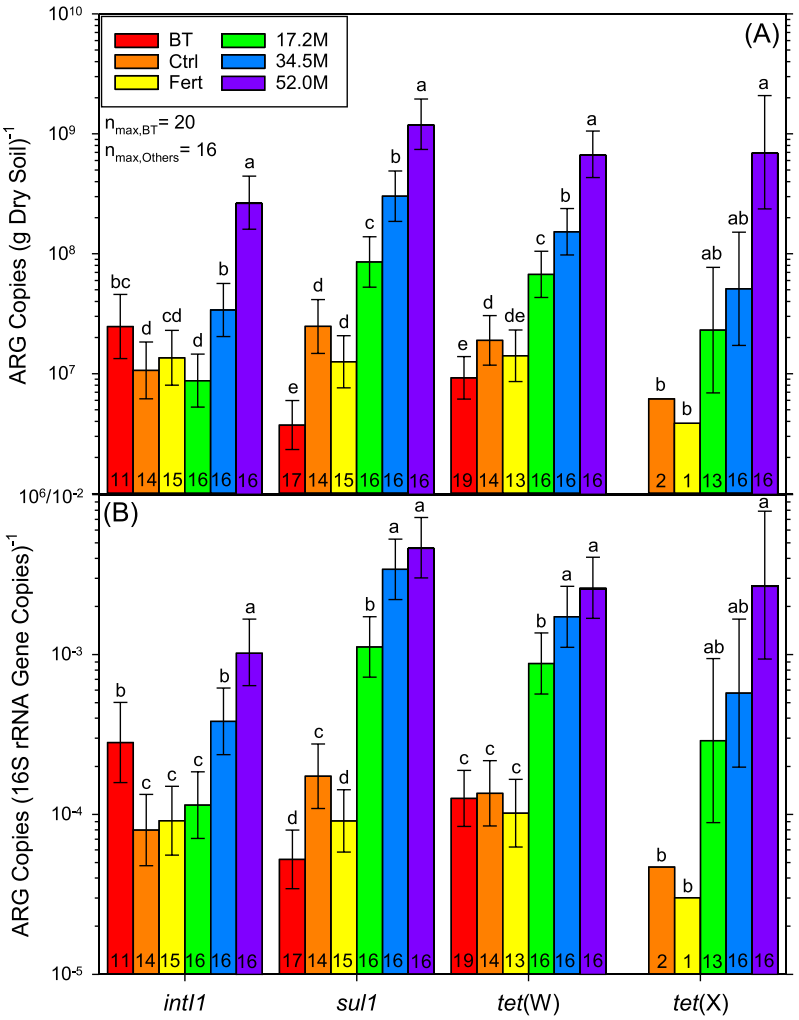

Figure 1. ARG absolute (**A**) and relative (**B**) abundances by treatment, fall 2012 (BT), spring 2013–2016 (pooled data) and soil depth = 0–30 cm. The letters above the bars are the least significant difference (LSD) category. The numbers at the base of the bars are the n detected out of the n_{max} displayed on the graph. BT = Before Treatment; Ctrl = Control; Fert = Fertilizer; 17.2M = 17.2 Mg manure ha^{-1} yr^{-1}; 34.5M = 34.5 Mg manure ha^{-1} yr^{-1}; and 52.0M = 52.0 Mg manure ha^{-1} yr^{-1}. 95% confidence level error bars are not included when n = 1 or 2.

the soils, only three [*sul1*, *tet*(W) and *tet*(X)] and *intI1* were quantified in enough samples to perform statistics and draw conclusions. Previous studies have found bla_{CTX-M} (Marti *et al.* 2013; Hu *et al.* 2016; Nolvak *et al.* 2016), *erm*(B) (Marti *et al.* 2013, 2014; Sun *et al.* 2015; Hu *et al.* 2016), *sul1* (Munir and Xagoraraki 2011; Marti *et al.* 2013, 2014; Fahrenfeld *et al.* 2014; Ross and Topp 2015; Sun *et al.* 2015; Nolvak *et al.* 2016; Ruuskanen *et al.* 2016), *tet*(A) (Marti *et al.* 2013; Hu *et al.* 2016; Nolvak *et al.* 2016; Sandberg and LaPara 2016), *tet*(W) (Munir and Xagoraraki 2011; Kyselkova *et al.* 2013; Fahrenfeld *et al.* 2014; Kyselkova *et al.* 2015; Sandberg and LaPara 2016), *tet*(X) (Sandberg and LaPara 2016) and *intI1* (Marti *et al.* 2013, 2014; Kyselkova *et al.* 2015; Sun *et al.* 2015; Hu *et al.* 2016; Nolvak *et al.* 2016; Sandberg and LaPara 2016) in soil amended with dairy or beef cattle manure. All of these ARGs encode resistance to commonly used antibiotics in animal agriculture (Chee-Sanford *et al.* 2009). Also, these ARGs are acquired from other bacteria through HGT, as opposed to intrinsically resistant. Acquired ARGs reside on MGEs such as plasmids or transposons which can be readily transferred between bacteria, even distantly related bacteria as long as they have the mechanisms to accept these MGEs (Van Hoek *et al.* 2011).

Manure application increases ARG abundances in soil

Manure contains a considerable abundance of ARB and ARGs even if the producing animal has never received antibiotics (Heuer, Schmitt and Smalla 2011). This usually translates into increased ARG abundance in the manured soil as opposed to control or background levels. However, this increased ARG abundance in the manure-treated soil may be transient without selection pressure such as high concentrations of antibiotics or metals (Heuer, Schmitt and Smalla 2011). In a study in which dairy manure was applied to agricultural soils, a 77% and 36% increase in abundance from background soil levels was reported for *tet*(W) and *sul1*, respectively (Munir and Xagoraraki 2011). In a microcosm study where dairy cow feces [from an oxytetracycline-treated cow (OE) and non-treated cow (NE)] were applied to soils from three different farms, the *tet*(W) gene was not detected in the control soils, but it was detected in similar quantities in both OE- and NE-treated soils from all three farms (Kyselkova *et al.* 2013). Fahrenfeld *et al.* (2014) conducted a field study in which dairy manure slurry was applied to a cornfield. There were significant increases in *sul1* and *tet*(W)

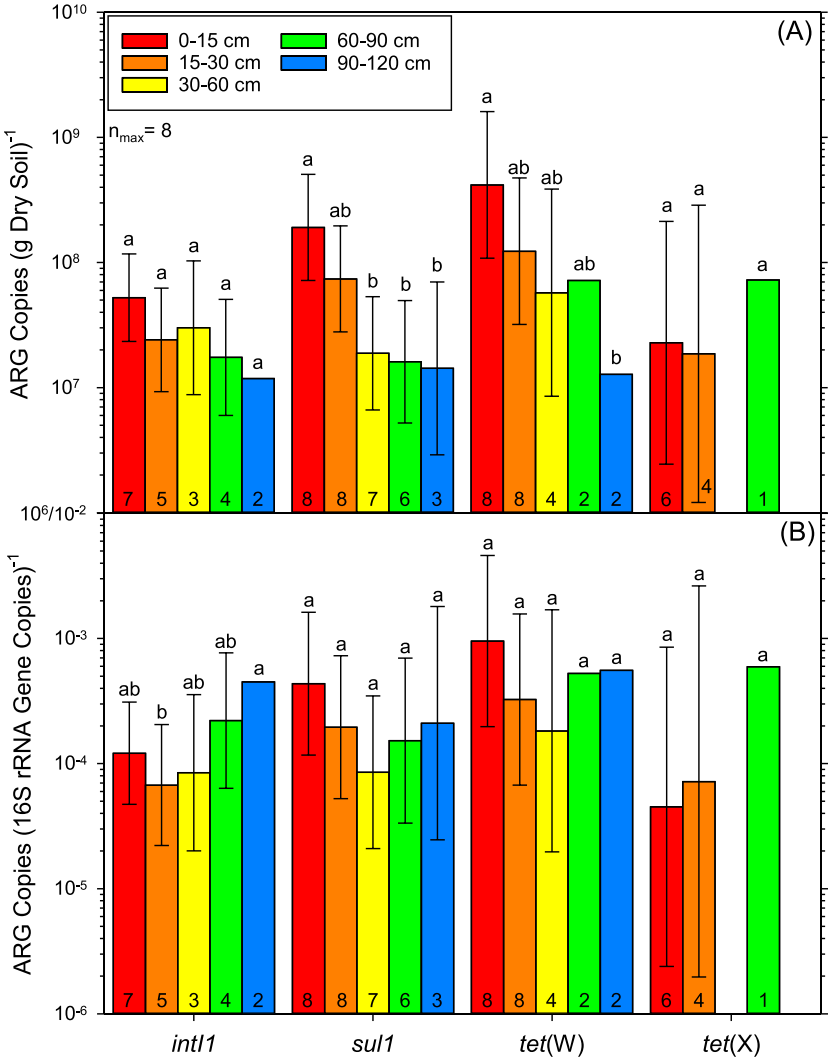

Figure 2. ARG absolute (**A**) and relative (**B**) abundances by soil depth, fall 2014–2015 (pooled data) and 52.0 Mg manure ha^{-1} yr^{-1}. The letters above the bars are the least significant difference (LSD) category. The numbers at the base of the bars are the n detected out of the n_{max} displayed on the graph. 95% confidence limit error bars are not included when n = 1 or 2.

relative abundances in post- versus pre-manured soils, as well as post-manured versus downgradient non-manure treated soils. According to another study where dairy manure was applied to soil, *sul1* and *int1* relative abundances were significantly greater in manured soils than in soil that received inorganic fertilizer (Marti *et al.* 2014). The *sul1* genes significantly increased from ~10^1 gene copies ng^{-1} DNA in control samples to ~10^5 gene copies ng^{-1} DNA in soil amended with raw manure, according to a study by Ross and Topp (2015). In a microcosm study by Sandberg and LaPara (2016), *tet*(W) increased, and *intI1* and *tet*(X) were similar in dairy manure-amended soils compared to control soils. In this study, *intI1* and *tet*(X) were not significantly different between Ctrl and 17.2M manure treatment, but were significantly different between the Ctrl and both 34.5M and 52.0M manure treatments. Nolvak *et al.* (2016) conducted a field study to determine the fate of various ARGs in soil amended with no fertilizer (control), mineral fertilizer and cattle slurry. For *intI1* and *sul1*, cattle slurry-amended soil was significantly greater than the control and mineral fertilized plots, while there were no significant differences between the control and inorganic fertilizer plots. These referenced studies, including our study, have found that applying dairy manure to soil increases the abundance of ARGs above background levels.

The higher the manure application rate, the higher the ARG level

To the knowledge of the authors, no previous paper sought to determine the effect of dairy manure application rate on the abundance of ARGs. There are many studies comparing different animal manures (i.e. dairy, beef, swine, chicken, etc.) (Chen *et al.* 2007; Ghosh and LaPara 2007; Marti *et al.* 2013, 2014; Hu *et al.* 2016; Ruuskanen *et al.* 2016; Sandberg and LaPara 2016), different manure types (composted, stockpiled, slurry, lagoon, etc.) (Munir and Xagoraraki 2011; Fahrenfeld *et al.* 2014; Ross and Topp 2015; Nolvak *et al.* 2016; Ruuskanen *et al.* 2016) or application to different types of agricultural soils (Munir and Xagoraraki 2011; Kyselkova *et al.* 2013, 2015; Sun *et al.* 2015; Hu *et al.* 2016; Ruuskanen *et al.* 2016), but none exploring the difference between application rates for the same manure source and same soil type.

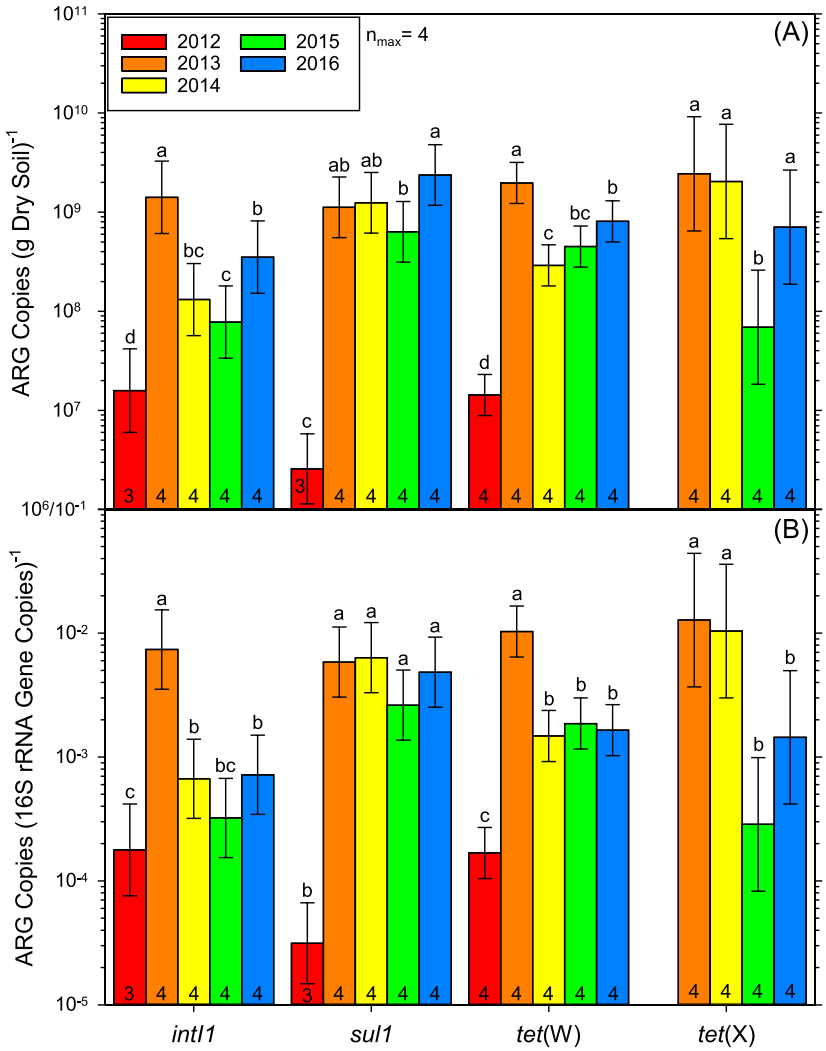

Figure 3. ARG absolute (**A**) and relative (**B**) abundances by year, fall 2012 (before treatment), spring 2013–16, 52.0 Mg manure ha^{-1} yr^{-1} and soil depth = 0–30 cm. The letters above the bars are the least significant difference (LSD) category. The numbers at the base of the bars are the n detected out of the n_{max} displayed on the graph. Error bars are 95% confidence limits.

When manure is applied to soil, it is either left on the surface of the soil or it is incorporated to a fixed depth (due to the limited penetration depth of the tillage equipment) no matter how much manure is applied. In this study, the manure was incorporated with a tandem disk to a fixed depth of ~15 cm. Therefore, soil samples from the top 15 cm of the profile had a high ratio of manure to soil mass. Since manure usually has a higher ARG abundance relative to the soil (Munir and Xagoraraki 2011; Fahrenfeld *et al.* 2014; Nolvak *et al.* 2016; Ruuskanen *et al.* 2016; Sandberg and LaPara 2016), the abundance of ARGs in the soil collected post-manure application will increase with each step increase of the manure application rate (see Fig. 1A and B).

Manure application rate is more important than repeated annual applications of manure

When comparing Figs 1A and 3A and Figs 1B and 3B, the trends clearly demonstrate that manure application rate increases ARG abundances while repeated annual applications of the same manure rate had a variable effect. The factors affecting the persistence of ARGs in manure-amended soils are numerous, including fitness effects to bacteria carrying ARGs, ability to transfer ARGs between closely and distantly related bacteria, half-lives and sorption potential of antibiotics to soil particles which can induce selection pressure, and co-selection of ARGs that reside on MGEs along with metal- and biocide-resistance genes in metal or biocide contaminated environments, to name a few (Chee-Sanford *et al.* 2009). Other studies have investigated the persistence of ARGs in dairy manure-amended soil (Fahrenfeld *et al.* 2014; Marti *et al.* 2014; Kyselkova *et al.* 2015; Nolvak *et al.* 2016), but most studies collected soil samples and applied dairy manure much more frequently, with time frames much shorter than this study. Due to these reasons, it is hard to draw a conclusion as to why ARG abundances vary among the yearly spring sampling events.

Absolute abundance and occurrence of ARGs decrease with increasing soil depth

A search of the literature did not reveal any studies that assessed the difference in ARG abundances with soil depth after receiving dairy manure application, but there are studies that

Table 3. Pearson correlation analysis of absolute ARG and 16S rRNA gene (16S) abundances.

	intI1 gDS^{-1}	*sul1* gDS^{-1}	*tet*(W) gDS^{-1}	*tet*(X) gDS^{-1}	
16S gDS^{-1}	0.3835	0.1845	0.3918	0.1383	r
	<.0001	0.0143	<.0001	0.2964	P-value
	146	176	128	59	n
***intI1* gDS^{-1}**		0.6338	0.7065	0.8141	r
		<.0001	<.0001	<.0001	P-value
		127	106	59	n
***sul1* gDS^{-1}**			0.7701	0.8788	r
			<.0001	<.0001	P-value
			121	59	n
***tet*(W) gDS^{-1}**				0.6707	r
				<.0001	P-value
				59	n

gDS = gram of dry soil; r = Pearson correlation coefficient; n = number of observations; yellow boxes: 0.8000 ≤ r ≤ 0.8999; green boxes: 0.7000 ≤ r ≤ 0.7999; blue boxes: 0.6000 ≤ r ≤ 0.6999.

Table 4. Pearson correlation analysis of relative ARG abundances.

	sul1 16S^{-1}	*tet*(W) 16S^{-1}	*tet*(X) 16S^{-1}	
***intI1* 16S^{-1}**	0.6617	0.6696	0.8464	r
	<.0001	<.0001	<.0001	P-value
	127	106	59	n
***sul1* 16S^{-1}**		0.7378	0.8824	r
		<.0001	<.0001	P-value
		121	59	n
***tet*(W) 16S^{-1}**			0.6870	r
			<.0001	P-value
			59	n

16S = 16S rRNA gene; r = Pearson correlation coefficient; n = number of observations; yellow boxes: 0.8000 ≤ r ≤ 0.8999; green boxes: 0.7000 ≤ r ≤ 0.7999; blue boxes: 0.6000 ≤ r ≤ 0.6999.

address swine manure applications (Huang *et al.* 2013; Peng *et al.* 2015; Tang *et al.* 2015). The results of this study are in agreement with Huang *et al.* (2013) in which absolute abundances of *tet*(W) and *tet*(X) were higher in the surface soils, then generally decreased with increasing soil depth, while relative abundances stayed constant throughout the soil profile. Peng *et al.* (2015) determined the absolute and relative abundances of *tet*(W) at three soil depths after fresh swine manure application. A visual analysis of the graphs in Peng *et al.* (2015) indicates that the *tet*(W) abundances did not change with depth for both normalizations, which is not in agreement with the absolute abundances determined in our study. Tang *et al.* (2015) only provided data for relative abundances of *tet*(W) and *sul1* in soil where swine manure was applied. The data from Tang *et al.* (2015) suggest that the relative abundances decreased with increasing soil depth, except for one of the sites where *tet*(W) increased with increasing soils depth, which is not consistent with our study as stated above. None of the referenced soil-depth studies performed statistical analyses to determine significant differences between different depths for a specific ARG, so all conclusions were drawn from visual inspection of graphs (Peng *et al.* 2015) or tables (Huang *et al.* 2013; Tang *et al.* 2015). Also, none of these referenced studies included occurrence data of ARGs throughout the soil profile, as was determined in the present study.

ARGs correlate with each other but not with 16S rRNA genes

In two recent studies, a significant correlation was determined between the relative (Sun *et al.* 2015; Nolvak *et al.* 2016) and absolute (Nolvak *et al.* 2016) abundance of *sul1* and *intI1*. Both studies showed stronger correlations between these two genes than in our study (Tables 3 and 4). Nolvak *et al.* (2016) also found a significant correlation between the absolute abundances of 16S rRNA gene and *sul1* (r = 0.65) in dairy cattle slurry-amended soil, as opposed to this study where no significant correlation was found (r = 0.18, Table 3).

In conclusion, the results from this field study advance knowledge that dairy manure is an ARG source and applications to soil can enlarge the reservoir of clinically relevant ARGs when compared to soil that received inorganic fertilizer or no fertilizer/manure. While the ARGs may have been associated with dead bacteria or extracellular, an increase of the ARG reservoir is a potential cause for concern, as it could facilitate acquired resistance in bacteria that are pathogenic to humans and food-producing animals. However, our results cannot be used to verify the transfer of ARGs to pathogens, nor determine the level of resistance of those specific genes. Regardless of this limitation, dairy manure applications were shown to increase the absolute abundance of ARGs in soil. Although not investigated in this study, antibiotic residues and other constituents are also present in dairy manure; thus, their influence on the enrichment of ARGs in manure-amended soil should not be ruled out and should be a subject for continued investigation.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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