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Safely Coupling Livestock and Crop Production Systems: How Rapidly Do Antibiotic Resistance Genes Dissipate in Soil following a Commercial Application of Swine or Dairy Manure?

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Animal manures recycled onto crop production land carry antibiotic-resistant bacteria. The present study evaluated the fate in soil of selected genes associated with antibiotic resistance or genetic mobility in field plots cropped to vegetables and managed according to normal farming practice. Referenced to unmanured soil, fertilization with swine or dairy manure increased the relative abundance of the gene targets *sul1*, *erm*(B), *str*(B), *int1*, and IncW *repA*. Following manure application in the spring of 2012, gene copy number decayed exponentially, reaching background levels by the fall of 2012. In contrast, gene copy number following manure application in the fall of 2012 or spring of 2013 increased significantly in the weeks following application and then declined. In both cases, the relative abundance of gene copy numbers had not returned to background levels by the fall of 2013. Overall, these results suggest that under conditions characteristic of agriculture in a humid continental climate, a 1-year period following a commercial application of raw manure is sufficient to ensure that an additional soil burden of antibiotic resistance genes approaches background. The relative abundance of several gene targets exceeded background during the growing season following a spring application or an application done the previous fall. Results from the present study reinforce the advisability of treating manure prior to use in crop production systems.

The World Health Organization, Ministers of Science from the G8 countries (United Kingdom, Russian Federation, Germany, Japan, Italy, France, Canada, United States), and the European Commission recently acknowledged that antimicrobial resistance is a seminal public health issue (1, 2). Amid concerns that the loss of antibiotic efficacy will have dire consequences for human morbidity and mortality, there is an urgent need for a comprehensive and global strategy to forestall the development of resistance to antibiotics by bacterial pathogens (3, 4). Action must include steps to promote the judicious use of antibiotics in human medicine and in animal production and to mitigate terrestrial and aquatic exposure to antibiotic residues and antibiotic resistance genes carried in agricultural wastes, effluents from municipal wastewater treatment, and effluents from antibiotic manufacturing factories (5–9).

It is important that in mixed agriculture, livestock and crop production systems be tightly coupled with respect to nutrient flow. Recycling manure appropriately to meet crop nutrient needs captures the economic value of excreted nitrogen and phosphorus for the farmer, minimizing the need to purchase costly mineral fertilizers. Efficient uptake of nitrogen and phosphorus into crops reduces the wasteful loss of nutrients in aqueous runoff or leaching, protecting surface and subsurface water quality. Efficient uptake of nitrogen into crops also reduces the availability of inorganic soil residues for nitrification and denitrification, mitigating gaseous emissions of nitric oxide and nitrous oxide to the detriment of air quality. The use of manure for production of food for human consumption is typically undertaken using mandated management practices designed to reduce the risk of food contamination with microbial pathogens (10). Typically, these can consist of treating manure prior to application to reduce the abundance of pathogens entrained into soil. Alternatively, a period of time during which entrained viable pathogen populations decline to levels that represent an acceptable risk of crop contamination must lapse between application of untreated raw manure and crop harvest. Manures typically carry antibiotic-resistant bacteria, and numerous genes associated with antibiotic resistance determinants have been detected in molecular inventories of manure microbial populations and in the environment in proximity to land fertilized with manure (11–13). We previously reported that vegetable crops grown in the ground without manure fertilization carried various antibiotic resistance genes detectable by PCR at harvest and that a number of additional antibiotic resistance genes were detected only on vegetables grown in ground that had been fertilized with swine or dairy manure (14). Antibiotic resistance genes entrained in manure therefore represent an increased risk of crop contamination with these genes and, therefore, presumably, an increased risk of human consumption of these genes. These data were obtained from field experiments in which manure was applied in the spring and vegetables were harvested in the fall, within the same growing season.

In the present study, the fate of a number of genes in soils following the application of swine or dairy manure was evaluated by quantitative PCR. The specific objective was to elucidate the dynamics of genes following an in-season application, an application the previous fall, and an application the previous spring. These three time periods encompass the range of offset times (in

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TABLE 1 Primers and probes for quantitative PCR used in the present study

Name	Sequence (5′→3′)[a]	Product size (bp)	Annealing temp (°C)	Final primer concn (nM)	Target	Reference
Universal bacteria						
BACT1369F	CGGTGAATACGTTCYCGG	123	59	300	*rrnS* gene	29
PROK1492R	GGWTACCTTGTTACGACTT					
TM1389F	HEX-CTTGTACACACCGCCCGTC-BHQ1					
erm(B)						
ermB-F	AAAACTTACCCGCCATACCA	139	65	400	Erythromycin resistance gene locus B	30
ermB-R	TTTGGCGTGTTTCATTGCTT					
str(B)						
strB-F	ATCGCTTTGCAGCTTTGTTT	143	61	300	Streptomycin phosphotransferase B	31
strB-R	ATGATGCAGATCGCCATGTA					
strB-P	HEX-ATGCCTCGGAACTGCGT-BHQ1					
sul1						
sul1-F	GACTGCAGGCTGGTGGTTAT	105	64	200	Sulfamethazine resistance gene 1	This study
sul1-R	GAAGAACCGCACAATCTCGT					
int1						
Int1F2	TCGTGCGTCGCCATCACA	67	62	400	Integrase class 1	23
Int1R2	GCTTGTTCTACGGCACGTTTGA					
IncW *repA*						
IncW-F	GGCCATCGTATCAACGAGAT	153	61	300	*repA* gene from plasmid incompatibility group W	This study
IncW-R	ATTGGTGCGCTCAAAGTAGC					
IncW-P	HEX-AGCTGGCTTAGTCGGCTACA-BHQ1					

[a] HEX, 2′,4′,5′,7′-tetrachloro-6-carboxy-4,7-dichlorofluorescein succinimidyl ester; BHQ1, black hole quencher 1.

season, previous fall, and previous spring application) between manure application and crop harvest typical of commercial farming in the Great Lakes Basin. The information should therefore be helpful with respect to validating off-set times required to ensure that antibiotic resistance gene abundance in manured soils falls to levels comparable to those in the absence of manure prior to crop harvest. Gene targets were selected that were previously detected in manured soils on the basis of PCR (14) and that represented a range of different antibiotic resistance classes and mobile genetic elements.

MATERIALS AND METHODS

Field operations. Experiments were undertaken during the 2012 and 2013 growing seasons on the Agriculture and Agri-Food Canada research farm in London, Ontario, Canada (42.984°N, 81.248°W). The field installations and methods were described in detail by Marti et al. (14). Briefly, the soil is a silt loam (gray-brown Luvisol) with the following properties: pH of 7.5, cation exchange capacity of 13.2, sand-silt-clay composition (%) of 18:67:15, and organic matter content of 3.4%. Climate conditions (temperature and precipitation) during the experimental period are available in Fig. S1 in the supplemental material.

Manure for applications in the spring and fall of 2012 and spring of 2013 was obtained from a local swine and a local dairy farm; key properties of the manures obtained for each of the three applications are summarized in Table S1 in the supplemental material. The dairy herd consisted of 180 Holstein cows. Dairy manure was stored in an open pit. Penicillin and injectable oxytetracycline (Liquamycin) were used in the dairy operation in both years. The swine herd consisted of 300 farrow-to-finish pigs. Swine manure for the present study was sampled from the manure-holding pit under the barn. In both years, the swine operation used medicated feed containing Aureo SP-250 to deliver (per kg of feed) 220 mg chlortetracycline, 220 mg sulfamethazine, and 110 mg penicillin.

All application rates were based on manure content of crop-available nitrogen determined with an Agros N quick test meter (Agros, Lidköping, Sweden). For the spring 2012 application, both dairy and swine manure were applied at 8,500 U.S. gallons/acre (usg/ac) (79,475 liters/ha). Based on a soil test, inorganic fertilizer was also applied to meet crop N-P-K needs. For the fall 2012 application, both dairy and swine manure were applied at 12,000 usg/ac (112,200 liters/ha). There was no application of inorganic fertilizer in the fall of 2012. For the spring 2013 application, both dairy and swine manure were applied at 8,500 usg/ac (79,475 liters/ha). There was no application of inorganic fertilizer in the spring of 2013 in the swine plots prior to the planting of the plots. The control plots received inorganic (N-P-K) fertilizer 16:16:16 at 200 lbs/ac (224 kg/ha) and 46:0:0 at 300 lbs/ac (336 kg/ha), and the dairy plots received 16:16:16 at 200 lbs/ac (224 kg/ha) and 46:0:0 at 150 lbs/ac (168 kg/ha). In all cases, immediately following application, manures were soil incorporated to a depth of 15 cm using a disk and "S" tine cultivator. Each application was applied to a new plot area separated by 3-m borders from the surrounding plots. Vegetable varieties planted were radish (*Raphanus sativus* variety Sora; 600 seeds per row spaced at 75 cm), carrots (*Daucus carota* variety Ibiza hybrid; 30-cm rows thinned at emergence), and lettuce (*Lactuca sativa* variety Summertime; 100 seeds per row spaced at 75 cm). Dates for planting and harvest are specified in Table S2 in the supplemental material.

Soil sampling and DNA extraction. Soil cores were taken haphazardly throughout the study period, initially on days 0, 7, and 30, and then at each crop harvest date (see Table S2 in the supplemental material). Six 2-cm-wide cores were sampled from each of 4 replicated vegetable plots to a depth of 15 cm using a T-sampler rinsed with 70% ethanol between samplings. Cores were bulked into a labeled Ziploc bag, mixed by hand until homogenous, and transported to the laboratory in a cooler with cool packs. Thus, there were four independent soil samples analyzed at each sampling time from control, dairy, and swine manure treatments.

From each bag, 50 g of soil was placed in a stomacher filtra-bag (Labplas Inc., Sainte-Julie QC, Canada; pore size of 330 μm) with 100 ml of

TABLE 2 Relative abundance of the *erm*(B) gene target in soil following the application of swine or dairy manure and in unmanured control plots

Season	Sampling (Julian) day	Relative abundance of the *erm*(B) gene target[a]					
		Control		Swine		Dairy	
		Mean	SD	Mean	SD	Mean	SD
Spring 2012	101	BLD		0.02097	0.00483	0.00012	0.00009
	108	BLQ		0.02136	0.00835	0.00009	0.00004
	131	BLD		0.00678	0.00194	0.00002	0.00002
	200	0.00059	0.00017	0.00376*	0.00302	0.00081	0.00045
	221	0.00062	0.00017	0.00241*	0.00039	0.00046	0.00022
	229	0.00027	0.00009	0.00048	0.00022	0.00048	0.00018
	254	BLD		0.00079	0.00025	BLQ	
	176	BLD		0.00051	0.00027	BLD	
	210	BLD		0.00044	0.00017	BLQ	
	303	BLD		0.00010	0.00006	BLD	
Fall 2012	275	BLD		0.000244	0.000181	0.000002	0.000002
	282	BLD		0.000112	0.000051	0.000001	0.000000
	306	BLD		0.000166	0.000047	0.000002	0.000001
	176	BLD		0.000352	0.000174	0.000013	0.000003
	210	BLD		0.000320	0.000252	BLD	
	303	BLD		0.000134	0.000023	BLQ	
Spring 2013	127	BLD		0.04122	0.02427	0.00012	0.00005
	134	0.00004	0.00006	0.01996*	0.00534	0.00019	0.00016
	158	0.00005	0.00001	0.01171*	0.00682	0.00038	0.00023
	210	BLD		0.00167	0.00061	BLQ	
	267	BLD		0.00175	0.00078	BLD	
	304	BLD		0.00201	0.00118	BLD	

[a] The relative abundance of the gene target is referenced to the total *rrnS* gene copy number. BLD, below limit of detection; BLQ, below limit of quantification; *, significant difference at P values of <0.05.

sodium metaphosphate buffer and mixed manually for at least 1 min. Filtrates were then placed in 50-ml Falcon tubes and centrifuged at 7,000 × *g* for 10 min at 4°C. Two hundred and fifty milligrams of soil pellet was used for DNA extraction using the Mobio Powersoil (MOBIO Laboratories, Medicorp, Montréal, QC, Canada) by following the manufacturer instructions. The elution volume was 100 μl.

Quantification of gene target copies. PCR amplification was performed using a Bio-Rad CFX96 real-time PCR instrument with Bio-Rad CFX Manager software, version 3.0. The primers and probes used in the present study are summarized in Table 1. Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/) was used for the design of primers and probes. The specificity of each oligonucleotide was checked with the BLAST program. All primers and probes were synthesized by Sigma (Sigma-Aldrich, Toronto, ON, Canada).

The mix reaction was performed with the Brilliant II quantitative PCR (qPCR) master mix (Agilent, Toronto, ON, Canada) for TaqMan PCR and the Brilliant II SYBR green low ROX qPCR master mix (Agilent) for SYBR green PCR. Two microliters of template DNA (corresponding to 0.1 to 10 ng of DNA) was added, and deionized water was used to reach a final volume of 25 μl. Negative controls without template DNA were run in triplicate. Each reaction was run in triplicate with the following cycle conditions: 1 cycle at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and annealing temperature for 35 s. For the SYBR green assay, a melting curve step was added in order to check the purity of the PCR product. This step consisted of a ramp temperature from 65 to 95°C, with an increment of 0.5°C and holding for 5 s for each step. The presence or absence of PCR inhibitors was verified by using an internal positive control (Applied Biosystems, Toronto, ON, Canada).

Target DNA fragments were cloned in the pSC-A-amp/kan plasmid using the StrataClone PCR cloning kit (Agilent) and transformed into *Escherichia coli* as described in reference 14. Plasmids were extracted using the Qiagen plasmid midi kit (Qiagen, Mississauga, ON, Canada). The plasmids were then linearized by NotI enzyme (New England BioLabs, Mississauga, ON, Canada) and purified with the Qiagen QIAquick PCR purification kit. Plasmid copy number was calculated using the NanoDrop ND1000 microspectrophotometer (NanoDrop Technologies, Wilmington, DE). Standard curves consisted of 10-fold serial dilution of the known concentration of plasmid solution for each marker. Plasmid insert sequences have been published previously (14). The identities of the quantified gene targets were ensured on the basis of hybridization when using TaqMan chemistry or melting behavior when using SYBR green.

Calculations and statistics. Soil gene relative abundance data are presented as the ratio of targeted gene copy number per total *rrnS* gene copy numbers in the reaction. The limit of quantification for PCRs was determined by adding known quantities of plasmid harboring the gene target insert into extracted soil DNA previously shown to be negative for the targeted gene. Serial dilution of plasmid was used in order to have a final concentration of plasmid ranging from 10^7 to 10^0 copies per microliter. Each condition was analyzed in triplicate. The limit of quantification was set at the dilution, giving 3 positive results following the linearity range. When the gene target was detected but at a copy number between 1 and 4 copies per reaction, it was determined to be below the limit of quantification. In that case, it is reported as detected but below the limit of quantification. Only soil samples with at least 3 of the 4 independent biological replicates above the limit of quantification were used to calculate and plot the average and standard deviation using SigmaPlot version 12.5 (Systat Software Inc.). Tables report data that are quantifiable and are annotated to indicate samples that were below the limit of quantification or below the limit of detection.

Gene target loading rates were estimated using gene abundance quantified in manure and are expressed on a wet weight basis (see Table S3 in the supplemental material). All estimated values and a sample calculation are available in Table S3.

Statistically significant treatment effects were determined using an un-

TABLE 3 Relative abundance of the *sul1* gene target in soil following the application of swine or dairy manure and in unmanured control plots

Season	Sampling (Julian) day	Relative abundance of the *sul1* gene target[a]					
		Control		Swine		Dairy	
		Mean	SD	Mean	SD	Mean	SD
Spring 2012	101	0.00002	0.00000	0.03661*	0.01490	0.00042*	0.00021
	108	0.00008	0.00008	0.03245*	0.01417	0.00191*	0.00070
	131	0.00003	0.00003	0.01313*	0.00676	0.00257*	0.00115
	200	0.00003	0.00003	0.00207*	0.00103	0.00050*	0.00017
	221	BLQ		0.00072	0.00014	0.00010	0.00007
	229	0.00002	0.00001	0.00028*	0.00013	0.00007*	0.00002
	254	0.00020	0.00020	0.00067*	0.00018	0.00005	0.00001
	176	0.00002	0.00002	0.00042*	0.00005	0.00003	0.00002
	210	BLQ		0.00065	0.00024	0.00004	0.00003
	303	0.00002	0.00001	0.00035*	0.00009	0.00004	0.00004
Fall 2012	275	0.000004	0.000001	0.000727*	0.000203	0.000188*	0.000005
	282	0.000005	0.000001	0.000638*	0.000289	0.000377*	0.000055
	306	0.000003	0.000000	0.000851*	0.000411	0.000440*	0.000104
	176	0.000008	0.000005	0.000139*	0.000029	0.000037*	0.000006
	210	0.000009	0.000002	0.000157*	0.000072	0.000027*	0.000010
	303	BLD		0.00027	0.000020	0.000100	0.000013
Spring 2013	127	BLQ		0.00096	0.00057	0.00034	0.00014
	134	BLD		0.00450	0.00170	0.00128	0.00030
	158	0.00002	0.00000	0.00795*	0.00244	0.00255*	0.00134
	210	BLD		0.00042	0.00016	0.00008	0.00004
	267	BLD		0.00047	0.00019	0.00008	0.00003
	304	BLQ		0.00057	0.00026	0.00008	0.00005

[a] The relative abundance of the gene target is referenced to the total *rrnS* gene copy number. BLD, below limit of detection; BLQ, below limit of quantification; *, significant difference at *P* values of <0.05.

paired *t* test without assuming equal standard deviation (Welch's correction). Data were treated using XLSTAT software version 2013.5.03 (Addinsoft). The significance level was set at *P* values of 0.05, and only cases where both control and treated samples were above the limit of quantification were used for statistical analyses.

RESULTS

In unmanured control plots, every gene target evaluated in the present study was detected at least once, with *int1* detected most frequently and with the highest relative abundance compared to the other gene targets (Tables 1, 2, 3, 4, 5, and 6). In the control plots, background gene target copy numbers were generally far less abundant than in soil that was treated with manure, and detections were generally below the limit of quantification. The abundance of gene targets in manure varied from about 10^6 to 10^9 copies per gram (wet weight) (see Table S3 in the supplemental material). Variation within gene target abundance across the three application times was within 100-fold. Taking into account the number of gene copies in the manures at application, the soil loading rates were estimated to be in the range of 10^4 to 10^7 copies per gram of soil, varying with the abundance of each gene target in manure application (see Table S4 in the supplemental material).

The dynamics of gene targets in soil, expressed as relative abundance referenced to *rrnS* gene copy number, were evaluated during the 2012 and the 2013 growing seasons (Tables 1 to 6). Various plots received manure in the spring of 2012, fall of 2012, and spring of 2013. All plots were followed through the fall of 2013. Data for *rrnS* abundance during the period of observation are available in Fig. S2 in the supplemental material.

Manuring with both swine and dairy manure increased the relative abundance of *erm*(B) relative to that of unmanured control plots (Table 2). At almost all sampling dates, the gene target was not detected in controls, whereas it was quantifiable in manured plots. In plots receiving swine manure in the spring of 2012, *erm*(B) was still quantifiable in the fall of 2013. Swine manure applications in the fall of 2012 and the spring of 2013 resulted in quantifiable *erm*(B) through the fall of 2013, whereas *erm*(B) was undetectable in control or dairy-manured plots.

The gene target *sul1* was quantifiable far more frequently in control soils than was *erm*(B) (Table 3). The relative abundance of *sul1* was frequently significantly higher in manured soils than in unmanured soils. With swine manure application in the spring of 2012, abundance was still significantly higher in the fall of 2013. In plots receiving swine manure in the fall of 2013, the abundance was higher through that fall and the following 2013 growing season.

On most sampling days, *str*(B) was not quantifiable in soil from control plots (Table 4). In contrast, the gene target was quantifiable in almost every soil sample from manured plots. The gene target was quantifiable in the 2013 growing season following application of swine manure in the spring of 2012 or swine or dairy manure in the fall of 2013.

The gene target *int1* was quantifiable on almost every sampling day in control plots (Table 5). The relative abundance of *int1* was significantly higher in manured plots throughout both growing seasons, regardless of manure application time.

The gene target IncW *repA* was never detected in control soils

TABLE 4 Relative abundance of the *str*(B) gene target in soil following the application of swine or dairy manure and in unmanured control plots

Season	Sampling (Julian) day	Relative abundance of the *str*(B) gene target[a]					
		Control		Swine		Dairy	
		Mean	SD	Mean	SD	Mean	SD
Spring 2012	101	BLD		0.02097	0.00483	0.00012	0.00009
	108	BLD		0.02136	0.00835	0.00009	0.00004
	131	BLD		0.00678	0.00194	0.00002	0.00002
	200	0.00002	0.00001	0.00376*	0.00302	0.00081*	0.00045
	221	BLD		0.00241	0.00039	0.00046	0.00022
	229	BLQ		0.00048	0.00022	BLQ	
	254	0.00002	0.00002	0.00079*	0.00025	0.00001	0.00002
	176	BLD		0.00051	0.00027	0.00002	0.00005
	210	BLQ		0.00044	0.00017	BLQ	
	303	BLD		0.00010	0.00006	BLD	
Fall 2012	275	BLD		0.00008	0.00001	0.00046	0.00010
	282	BLQ		0.00032	0.00001	0.00098	0.00006
	306	BLQ		0.00040	0.00004	0.00274	0.00039
	176	BLD		0.00002	0.00000	0.00007	0.00004
	210	BLD		0.00002	0.00001	0.00003	0.00001
	303	BLD		0.00002	0.00001	0.00006	0.00003
Spring 2013	127	BLD		0.000005	0.000005	0.000001	0.000001
	134	BLD		0.000009	0.000004	0.000003	0.000002
	158	BLD		0.000012	0.000007	0.000010	0.000002
	210	BLD		0.000013	0.000008	0.000002	0.000002
	267	BLD		BLQ		BLD	
	304	BLD		BLQ		BLD	

[a] The relative abundance of the gene target is referenced to the total *rrnS* gene copy number. BLD, below limit of detection; BLQ, below limit of quantification; *, significant difference at *P* values of <0.05.

(Table 6). It was not detected in plots receiving dairy manure in the spring of 2012, but it was transiently in plots receiving swine manure in the spring of 2012. Following an application of swine manure in the fall of 2012, the gene target was quantifiable throughout the season. Following an application of either swine or dairy manure in the spring of 2013, it was quantifiable until the fall.

DISCUSSION

The present study evaluated, over two growing seasons of normal farming practice, the persistence in soil of selected genes associated with antibiotic resistance and mobility. There were two distinct patterns to gene dynamics following manure application. In general, gene targets decayed exponentially in the 2 months following the spring 2012 application. The weather following that application was unusually warm and very dry (see Fig. S1 in the supplemental material), conditions that would disfavor bacterial growth and survival in soil. In contrast, the spring of 2013 was cool and very wet, and the fall of 2012 likewise had typically cool, wet weather (see Fig. S2 in the supplemental material). Under these conditions, essentially every gene target was found to increase in abundance in the weeks following application. We hypothesize that warmer, drier conditions reduced the persistence of bacteria carrying the gene target in soils, whereas cooler, wetter conditions promoted an increase in abundance. The location of the experimental field work in the Great Lakes region of North America has a humid continental climate (15), characteristic of much of the Northern hemisphere, including significant portions of northeastern United States and southeastern Canada, Eastern Europe, Russia, and China. The results reported in the present study would therefore be most relevant to these regions, all of which have very important areas of intensive agriculture. Clearly, the relationship between climate, soil conditions, and gene fate needs to be clarified in order to predict persistence under varying conditions.

Every gene target evaluated in the present study was detected in unmanured control soil, but other than *int1*, all remained close to the method detection limit of about 10^4 gene copies per gram of soil at every sampling. Results from the present study delineate the time required for gene copy numbers in manured soils to return to these background levels. Clearly, application in season or in the previous fall will result in gene copy numbers remaining above background throughout the crop-growing season and through harvest time. In contrast, gene copy numbers in soil receiving a spring application in the previous year fell to background levels throughout the following growing season with the exception of *int1* and *sul1* in the case of swine manure application. The present study suggests that mandated offset times greater than one season would be most protective with respect to reducing the availability of genes entrained into soil with manure and that a fall application under Ontario conditions is not protective in that respect. Previous field experiments showed more frequent detection of antibiotic resistance genes on vegetables harvested following an in-season manure application (14). Given the large variability observed in the dynamics of genes following application in the spring of 2012 and 2013, additional field data are required and observations from other areas that vary in climate conditions are called for.

Every gene target evaluated in this study was detected in soil in the absence of manure, and thus caution is required when inter-

TABLE 5 Relative abundance of the *int1* gene target in soil following the application of swine or dairy manure and in unmanured control plots

Season	Sampling (Julian) day	Relative abundance of the *int1* gene target[a]					
		Control		Swine		Dairy	
		Mean	SD	Mean	SD	Mean	SD
Spring 2012	101	0.00001	0.00001	0.01193*	0.00539	0.00016*	0.00003
	108	0.00002	0.00001	0.01225*	0.00559	0.00077*	0.00037
	131	0.00005	0.00001	0.00456*	0.00288	0.00057*	0.00020
	200	0.00010	0.00009	0.00064*	0.00035	0.00020	0.00007
	221	0.00001	0.00001	0.00062*	0.00042	0.00015*	0.00018
	229	0.00001	0.00001	0.00007*	0.00002	0.00005*	0.00002
	254	0.00014	0.00009	0.00039*	0.00017	0.00005	0.00002
	176	0.00007	0.00003	0.00028*	0.00009	0.00005	0.00001
	210	0.00007	0.00005	0.00052*	0.00022	0.00008	0.00006
	303	0.00004	0.00003	0.00042*	0.00019	0.00005	0.00001
Fall 2012	275	0.00002	0.00000	0.00034*	0.00007	0.00033*	0.00006
	282	0.00001	0.00001	0.00055*	0.00021	0.00040*	0.00005
	306	0.00001	0.00000	0.00091*	0.00053	0.00041*	0.00003
	176	0.00003	0.00002	0.00059*	0.00019	0.00015*	0.00005
	210	0.00003	0.00001	0.00030*	0.00022	0.00007*	0.00003
	303	0.00003	0.00002	0.00019*	0.00010	0.00011*	0.00006
Spring 2013	127	0.00002	0.00001	0.00017*	0.00010	0.00022*	0.00004
	134	0.00002	0.00000	0.00100*	0.00041	0.00071*	0.00017
	158	0.00005	0.00001	0.00127*	0.00100	0.00038*	0.00023
	210	BDL		0.00047	0.00043	0.00001	0.00001
	267	0.00005	0.00003	0.00025*	0.00009	0.00007	0.00002
	304	0.00002	0.00001	0.00029*	0.00008	0.00006	0.00004

[a] The relative abundance of the gene target is referenced to the total *rrnS* gene copy number. BLD, below limit of detection; BLQ, below limit of quantification; *, significant difference at P values of <0.05.

preting the contributions of manure-borne and soilborne genes following manuring (Tables 1 to 6). Exponential decay without a lag, as was generally the case for gene targets following the spring 2012 application, can reasonably be interpreted as indicating that genes entrained into soil through manure application were destroyed. On the other hand, an increase in gene copy number at any time during the period of observation could be due to an increase in the abundance of bacteria that were carried in manure or that were in the soil prior to manure application. Furthermore, there is potential for horizontal transmission of plasmid-borne genes contributing to their distribution and abundance in soil (11).

A number of previous studies have unambiguously shown that soils receiving animal manures or biosolids are enriched in antibiotic resistance genes (13, 16–20). A few studies have characterized gene abundance over time following application under field conditions. For example, an application of swine manure increased the abundance of *sul1* and *sul2* in field plots in Germany for over 4 months postapplication (21). Class 1 integrons were more abundant in field plots in the United Kingdom receiving swine manure than in control plots at least 10 months postapplication (22). Soils in the United Kingdom receiving sewage sludge contained *int1* at levels significantly above background 24 months postapplication (23). Overall, there is a body of evidence suggesting that land application of untreated fecal material can increase the abundance of some antibiotic resistance genes and mobile genetic elements for months or years. Antibiotic resistance genes amenable to horizontal transfer could still in principle represent a significant reservoir for recruitment into pathogens at abundances far lower than what can be currently measured (i.e., 10^4 copies per gram of soil for our study; see Materials and Methods), particularly in hot spots for transfer of mobile genetic elements (24, 25).

The policy-relevant significance of these results should be considered within the context of recommended or mandated manure management practices that are designed to protect produce from contamination with human pathogens. For example, the U.S. National Organic Program specifies that raw manure can be incorporated into soil not less than 120 days prior to the harvest of a product whose edible portion has direct contact with the soil surface or soil particles and that raw manure can be incorporated into soil not less than 90 days prior to the harvest of a product whose edible portion does not have direct contact with the soil surface or soil particles (32). The present study indicates this is insufficient time to reduce the abundance of antibiotic resistance genes to background levels, at least under conditions characteristic of Southwestern Ontario. This result is also consistent with the more frequent detection of some antibiotic resistance genes on vegetables grown in freshly manured soil (14). What significance this additional exposure of humans or grazing animals to soilborne antibiotic resistance genes might have for the dissemination of antibiotic resistance of human clinical concern is unknown, particularly in the context of other potential sources of exposure to antibiotic resistance genes, other human activities that enrich the environmental resistome, and the fact that these genes are naturally present in undisturbed environments (24–28). Nevertheless, our results reinforce the advisability of manure pretreatment prior to application where possible and otherwise provide new end-

TABLE 6 Relative abundance of the IncW *repA* gene target in soil following the application of swine or dairy manure and in unmanured control plots

Season	Sampling (Julian) day	Relative abundance of the IncW *repA* gene target[a]					
		Control		Swine		Dairy	
		Mean	SD	Mean	SD	Mean	SD
Spring 2012	101	BLD		0.000058	0.000003	BLD	
	108	BLD		0.000281	0.000135	BLD	
	131	BLD		0.000044	0.000019	BLD	
	200	BLD		BLD		BLD	
	221	BLD		BLD		BLD	
	229	BLD		BLD		BLD	
	254	BLD		BLD		BLD	
	176	BLD		0.000001	0.000001	BLD	
	210	BLQ		BLQ		BLQ	
	303	BLD		BLD		BLD	
Fall 2012	275	BLD		0.000002	0.000001	BLD	
	282	BLD		0.000018	0.000005	0.00001	0.00000
	306	BLD		0.000015	0.000008	0.00001	0.00001
	176	BLD		0.000011	0.000006	BLQ	
	210	BLD		0.000010	0.000004	BLD	
	303	BLD		0.000005	0.000002	BLQ	
Spring 2013	127	BLD		0.000005	0.000005	0.000001	0.000001
	134	BLD		0.000009	0.000004	0.000003	0.000002
	158	BLD		0.000012	0.000007	0.000010	0.000002
	210	BLD		0.000013	0.000008	0.000002	0.000002
	267	BLD		BLQ		BLD	
	304	BLD		BLQ		BLD	

[a] The relative abundance of the gene target is referenced to the total *rrnS* gene copy number. BLD, below limit of detection; BLQ, below limit of quantification.

points for recommending suitable offset times between the application of raw manure and crop harvest and animal grazing (5).

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