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Impact of Fertilizing with Raw or Anaerobically Digested Sewage Sludge on the Abundance of Antibiotic-Resistant Coliforms, Antibiotic Resistance Genes, and Pathogenic Bacteria in Soil and on Vegetables at Harvest

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The consumption of crops fertilized with human waste represents a potential route of exposure to antibiotic-resistant fecal bacteria. The present study evaluated the abundance of bacteria and antibiotic resistance genes by using both culture-dependent and molecular methods. Various vegetables (lettuce, carrots, radish, and tomatoes) were sown into field plots fertilized inorganically or with class B biosolids or untreated municipal sewage sludge and harvested when of marketable quality. Analysis of viable pathogenic bacteria or antibiotic-resistant coliform bacteria by plate counts did not reveal significant treatment effects of fertilization with class B biosolids or untreated sewage sludge on the vegetables. Numerous targeted genes associated with antibiotic resistance and mobile genetic elements were detected by PCR in soil and on vegetables at harvest from plots that received no organic amendment. However, in the season of application, vegetables harvested from plots treated with either material carried gene targets not detected in the absence of amendment. Several gene targets evaluated by using quantitative PCR (qPCR) were considerably more abundant on vegetables harvested from sewage sludge-treated plots than on vegetables from control plots in the season of application, whereas vegetables harvested the following year revealed no treatment effect. Overall, the results of the present study suggest that producing vegetable crops in ground fertilized with human waste without appropriate delay or pretreatment will result in an additional burden of antibiotic resistance genes on harvested crops. Managing human exposure to antibiotic resistance genes carried in human waste must be undertaken through judicious agricultural practice.

The development of bacterial resistance to antibiotics is a major public health challenge recognized by the medical and public health communities and governments (1–3). An estimated 23,000 patients in the United States die annually from bacteria that have acquired resistance to currently available antibiotics and that are therefore no longer treatable (4). Much of the scientific and regulatory discussion concerning measures to forestall resistance development has focused on prudent antibiotic use in human medicine and in food animal production (5, 6). More recently, a growing recognition of an environmental dimension to antibiotic resistance development has prompted calls for additional resistance management measures (7–9). These measures include reducing environmental exposure to antibiotic resistance genes selected for in the human gastrointestinal tract through more effective treatment of municipal sewage and reducing emissions of drug residues from antibiotic manufacturing facilities (10).

In many parts of the world, human waste is a valued source of nutrients for crop production and organic matter for improvement of soil quality (11). Waste can variously be delivered in the form of untreated raw sewage (i.e., waste handled by a municipal sewer system) or septage (i.e., waste handled by a residential septic system), untreated sewage sludge (i.e., the solid material recovered from wastewater treatment), or treated sewage sludge (also called biosolids) (12, 13). In North America, human waste used in agriculture is typically in the form of biosolids, material that has a lower abundance of pathogens than found in untreated material (14). Biosolids are produced by treating (e.g., anaerobic or aerobic digestion) raw sewage sludge to effect modest pathogen reduction (in U.S. parlance, producing class B biosolids) or, more rigorously (e.g., thermal treatment and lime stabilization), to effect significant pathogen reduction (producing class A biosolids). Both class A and class B biosolids must adhere to mandated standards of microbiological quality (15). In Ontario, Canada, about 120,000 dry tonnes of class B-equivalent biosolids are recycled onto agricultural land annually. There are limited data available on agricultural use of sewage sludge in developing countries, but anecdotally, the practice is widespread. The extent of treatment, however, is dependent on locally available technologies. Therefore, guidelines or regulations for application of sewage sludge for soil fertilization remain a challenge in many of these countries, in part due to a lack of research dedicated to evaluating the different options for sustainable and safe use of sewage sludge in agriculture, taking into consideration the local socioeconomic and climatic conditions. South Africa is an example of one of the few developing countries that has started implementing some innovative sewage

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sludge treatment options in order to reinforce restrictions and requirements for safer use of human waste in crop production (16).

The recycling of human wastes as a fertilizer in crop production represents a potentially significant source of exposure to antibiotic resistance genes and drug residues. Biosolids entrain sorbed antibiotics, including various fluoroquinolones, macrolides, and tetracycline, into soil (17). Soils fertilized with biosolids are enriched with antibiotic resistance genes and with integrons associated with genetic mobility (18, 19). There is a risk, therefore, that human consumption of crops grown in ground fertilized with human waste will increase exposure to antibiotic-resistant bacteria and genetic determinants. In the present study, by using culture-dependent and independent means, we evaluated the composition of soil and vegetable crops in plots fertilized with treated or untreated sewage sludge, referenced to plots receiving synthetic fertilizer only. We enumerated pathogenic bacteria and antibiotic-resistant coliform bacteria and evaluated the distribution of selected antibiotic resistance genes in soils and on crops at harvest. Finally, we undertook measurements during both the season of sewage sludge application and the following season to determine if a 1-year offset between fertilization and harvest was sufficient to reduce crop exposure to antibiotic resistance genes to background levels.

MATERIALS AND METHODS

Field operations. Experiments were undertaken during the 2011 and 2012 growing seasons at the Environmental Sciences Western Experimental Field Station of Western University on the outskirts of London, Ontario, Canada (latitude 43°4′47″N, longitude 81°20′24″W). 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 ratio (percent) of 18/67/15, and organic matter content of 3.4%. Climate conditions (temperature and precipitation) during the experimental period are described in Fig. S1 in the supplemental material. Prior to the present study, the field plot area was cropped to oats in 2009 and to soybeans in 2010 and had not been irrigated or received any manure or human waste amendments. In the present study, crops were periodically irrigated with well water obtained at the research farm. Periodic sanitary surveys of the irrigation water using media and methods described below indicated that all indicator and pathogenic bacteria were undetectable. Briefly, 100 ml of irrigation water (supplied as 1-liter samples) was filtered through sterile 0.45-μm-pore-size, 47-mm-diameter cellulose acetate membrane filters (Pall GN-6; VWR, Mississauga, ON, Canada). The membrane filters were aseptically placed onto all specified media and incubated at the appropriate temperatures and times (Table 1). There are no livestock farms within a radius of ~1.5 km from the research farm.

Dewatered municipal biosolids were obtained from Niagara, Ontario, Canada, in 2011, and sewage sludge was obtained from London, Ontario, Canada, in 2012. Niagara biosolids are anaerobically digested and then transferred to storage in a lagoon or in glass-lined storage tanks. They are then dewatered by using centrifugation. London adds shredded paper and dewaters the sludge with a belt press without anaerobic digestion or any other further treatment. This material is intended for incineration. Key properties of the biosolids and sewage sludge are presented in Table S1 in the supplemental material.

Based on solids content, in the spring of 2011, biosolids were applied at a rate of 10.8 wet tonnes/ha. Based on a soil test, inorganic fertilizer was also applied (banded) to the treated and control plots, 46-0-0 (NPK rating; urea) at 224 kg/ha and 6-24-24 (urea potash monoammonium phosphate blend) at 112 kg/ha. In the spring of 2012, sewage sludge was applied at 28.6 wet tonnes/ha. Based on a soil test, inorganic fertilizer was also applied to both treated and control plots, 19-16-16 at 336 kg/ha. Immediately following application, amendments were soil incorporated to a depth of 15 cm by using an "S"-tine cultivator (Kongskilde Ltd., Strathroy, ON, Canada). In the 2011 season, both control and treated blocks were subdivided into two blocks of 10 4-m by 6-m plots. Four replicates of tomatoes, radish, carrots, cucumber, and pepper were planted within the plots. Vegetable varieties planted were tomatoes (*Solanum lycopersicum* var. Bellstar) (4 transplants per row, 60 cm between rows), radish (*Raphanus sativus* var. Sora) (600 seeds per row, rows spaced at 75 cm), carrots (*Daucus carota* var. Ibiza hybrid) (30-cm rows thinned at emergence), cucumber (*Cucumis sativus* var. Straight Eight) (100 seeds per row, rows spaced at 75 cm), pepper (*Capsicum annuum* var. Early Calwonder) (4 transplants per row, rows spaced at 60 cm), and lettuce (*Lactuca sativa* var. Summertime [head-forming variety]) (100 seeds per row, rows spaced at 75 cm). Lettuce, carrots, cucumbers, and radish were planted from seed, and tomatoes and peppers were transplants. The dates when amendments were applied and vegetable planting and harvest were undertaken are presented in Table S2 in the supplemental material. Individually planted blocks were separated by borders consisting of 4 m of unplanted ground. Radishes were planted late to avoid bolting because of a wetter-than-normal spring followed by a hot summer. Plots were monitored for weeds and pest pressures on a weekly basis. Cucumbers were sprayed with chlorothalonil (Bravo; Syngenta Canada, Guelph, ON, Canada) to control mildew. Based on experience in the 2011 season, peppers and cucumbers were not included and lettuce was added in the 2012 season. Sixteen 4- by 6-m plots were delineated and planted randomly with four replicates of tomatoes, radish, carrots, and lettuce. Individually planted blocks were separated by borders consisting of 4 m of unplanted ground. Plots were monitored for weeds and pest pressures on a weekly basis. Sticky traps were laid out for flea beetles in the radish plots. Pest management, weed control, and irrigation were done manually throughout the experiments.

Water supplied to transplants while young was delivered by using a Haws plastic watering can to drench the transplants. Water for irrigation was supplied from an on-site underground well. Irrigation water was loaded into a 1,000-gal Norwesco polyethylene tank and then trucked to the field, where it was fed through a Hydro centrifugal pump (Pentair, New Brighton, MN) through a garden hose with a spray nozzle attachment. Pressure was adjusted by using the bypass system, where water was pumped back into the tank so that the water pressure at the nozzle end was adequate for irrigation but not hard enough to damage the plants. Dates for irrigation events are specified in Table S2 in the supplemental material.

Soil and vegetable sampling. Soil cores were taken randomly on days 0, 7, and 30 and at harvest, with day 0 corresponding to the day of biosolids or sludge application. Six 2-cm-wide cores were sampled from each vegetable plot to a depth of 15 cm by using a model H soil sampler (Oakfield

TABLE 1 Media and incubation conditions used for enumeration, enrichment, and primary isolation of the indicated bacteria

Medium	Incubation conditions	Bacterium(a)
Chromocult agar	37°C, 24 h	Coliforms and *E. coli*
mEnterococcus agar	37°C, 48 h	*Enterococcus* spp.
mEndo-LES agar	37°C, 24 h	Total coliforms
mFC agar	44.5°C, 24 h	Fecal coliforms
mFC-BCIG agar	44.5°C, 24 h	*E. coli*
mCP agar	44.5°C, 24 h, anaerobic	*C. perfringens*
mADA-V agar	37°C, 24 h	*Aeromonas* spp.
Campy-Line agar	42°C, 48 h, microaerophilic	*Campylobacter* spp.
XLD agar	42°C, 48 h	*Salmonella*
Salmonella chromogenic agar	42°C, 24–48 h	*Salmonella*
Chromogenic Listeria agar	37°C, 24 h	*Listeria* spp.
PALCAM agar	37°C, 24 h	*Listeria* spp.

Apparatus Co., Fond du Lac, WI) washed with 70% ethanol between samplings. Soil was taken from between vegetables and thus would not capture rhizosphere communities. Cores (12 cores on days 0 to 30 and 6 cores at harvest) were bulked into a labeled 11-lb sample bag (Alpha Poly Bag Co., Brampton, ON, Canada) that was knotted when sampling was completed to avoid spillage or cross-contamination, mixed by hand until homogenous, and transported to the laboratory in a cooler with icepacks. Samples of the edible portions of each crop were harvested when the crop was visibly ripe and ready for consumption. Four kilograms of root (carrots and radishes) and 10 individual samples (i.e., individual vegetable or fruit) of above-ground (lettuce, cucumber, tomato, and pepper) vegetables were harvested, individually bagged, and kept in a cooler on icepacks for transport to the laboratory. All implements were thoroughly washed with 70% ethanol between plots. Prior to processing for bacteriological analysis, excess soil was removed from all vegetables with a clean cloth and distilled water to achieve the cleanliness that a typical North American consumer would expect in normal food preparation. In order to avoid cross-contamination, separate cloths were used for samples from each treatment plot.

Vegetable and soil processing. Methods for preparation of vegetable and soil samples and suspensions for viable plate counts were described in detail previously by Marti et al. (20). Briefly, 50 g of soil or vegetable peel was placed into a Stomacher Filtra-Bag (pore size of 330 μm; Labplas Inc., Sainte-Julie, QC, Canada) with 100 ml of sterile sodium metaphosphate buffer (2 g/liter, pH 7.0; Thermo-Fisher Scientific, Ottawa, ON, Canada). In the case of lettuce, the head was quartered, and 50 g of material was manually removed from the center of the vegetable. Samples were homogenized for 30 s in a Smasher homogenizer (AES-Chemunex, bioMérieux, Marcy l'Etoile, France). Homogenized samples were then aseptically transferred into two 50-ml sterile conical tubes. For bacterial enumeration, 500 μl or 100 μl of sample was plated onto the specified media and incubated at the specified temperatures and times.

Chemicals and media. Viable bacteria were enumerated as described previously by Marti et al. (20), using media and conditions listed in Table 1. The following media were used: Chromocult agar (EMD Chemicals, Cedarlane Laboratories, Burlington, ON, Canada); mEnterococcus agar (Difco, Fisher Scientific, Ottawa, ON, Canada); mEndo-LES agar (Difco); mFC agar (Difco); mFC-BCIG agar, consisting of mFC basal medium (Difco) supplemented with 100 mg/liter 3-bromo-4-chloro-5-indolyl-β-D-glucuronide (BCIG) (cyclohexyl ammonium salt; Alere Canada, Ottawa, ON, Canada); mCP agar (Accumedia; Alere Canada, Stittsville, ON, Canada); ampicillin-dextrin agar (mADA-V; Hardy Diagnostics, Alere Canada); Campy-Line agar (21, 22); Salmonella chromogenic agar (Oxoid, Ottawa, ON, Canada); XLD agar (Difco); azide dextrose broth (Difco); MUG-EC broth (Sigma Chemical Co., Toronto, ON, Canada); UVM Listeria broth (Difco); Listeria enrichment broth (Difco); Listeria chromogenic agar (Oxoid); and PALCAM agar (Difco). Antibiotics were purchased from Sigma-Aldrich (Toronto, ON, Canada) and AK Scientific (Union City, CA).

Enumeration and primary isolation of bacteria. Viable bacteria were enumerated exactly as described previously by Marti et al. (20). The following bacteria were enumerated in vegetable filtrate and soil suspensions: total coliforms, fecal coliforms, *Escherichia coli*, *Enterococcus* spp., *Clostridium perfringens*, *Aeromonas* spp., *Yersinia* spp., *Campylobacter* spp., *Salmonella* spp., and *Listeria* spp. The media used and incubation conditions are presented in Table 1. Briefly, 100 μl and 500 μl of the suspension were spread plated onto agar plates containing media and incubated as specified in Table 1. Bacteria were enumerated based on colony morphology. Only plates with 20 to 200 colonies were used for enumeration. Additionally, 2-ml aliquots of each sample were inoculated into azide dextrose broth (for detection of enterococci), EC-MUG broth (for detection of *E. coli* and primary enrichment of *Salmonella*), and Listeria enrichment broth. Following an initial incubation for 48 h at 37°C, Listeria enrichment broth cultures were transferred into UVM Listeria broth and further incubated for 48 h at 37°C prior to inoculation onto Listeria chromogenic agar (Oxoid, Ottawa, ON, Canada) and PALCAM agar (Difco, Fisher Scientific, Ottawa, ON, Canada). Following an initial incubation for 24 h at 37°C, EC-MUG broth cultures were inoculated onto mFC-BCIG agar and incubated at 44.5°C and simultaneously inoculated onto XLD agar and Salmonella chromogenic agar (Oxoid), which were then incubated at 42°C for 24 h. Presumptive *Salmonella*-positive colonies were further tested by an oxidase test and a Salmonella Rapid Latex spot test (Oxoid, Ottawa, ON, Canada). Presumptive *Listeria*-positive isolates were further identified by using the API-Listeria identification system (bioMérieux, Montreal, QC, Canada). Where required, anaerobic or microaerophilic atmospheric conditions were established by using the AnaeroPack container system (Mitsubishi Gas Chemical Co., Thermo-Fisher Scientific, Ottawa, ON, Canada) in tandem with either Pack-Anaero or Pack-MicroAero gas-generating packets. Based on the sample size processed, the detection limits for the various methods were as follows: 200 CFU/g for plate counts, sludge, or biosolids; 40 CFU/g for soil; 40 CFU/g for vegetables; 100 cells/g for enrichments, EC-MUG sludge, or biosolids; 5 cells/g for soil or vegetable; 100 cells/g for azide dextrose manure; and 5 cells/g for all *Listeria* matrices.

Antibiotic-resistant bacteria in biosolids or sludge, soil, and vegetable macerates were spread plated onto Chromocult agar containing the following individual antibiotics (with breakpoint concentrations indicated in parentheses) (23) and incubated overnight at 37°C: amikacin (64 mg/liter), amoxicillin-clavulanic acid (Augmentin) (32 mg/liter–16 mg/liter), ampicillin (32 mg/liter), cefoxitin (32 mg/liter), ceftiofur (8 mg/liter), chloramphenicol (32 mg/liter), ciprofloxacin (4 mg/liter), gentamicin (16 mg/liter), nitrofurantoin (128 mg/liter), norfloxacin (16 mg/liter), sulfamethoxazole (512 mg/liter), trimethoprim-sulfamethoxazole (co-trimoxazole) (4 mg/liter–76 mg/liter), tetracycline (16 mg/liter), trimethoprim (16 mg/liter), chlortetracycline (64 mg/liter), and meropenem (4 mg/liter). Tenfold dilution series of the biosolids and soil samples were prepared in sterile sodium metaphosphate buffer (2 g/liter; Thermo-Fisher Scientific, Ottawa, ON, Canada) prior to plating 100 μl of each dilution. Coliform and *E. coli* colonies on plates were enumerated based on the manufacturer's recommendations for target colony morphology and color.

Molecular methods. DNA was obtained from samples by using Mo Bio Powersoil (Mo Bio Laboratories, Medicorp, Montréal, QC, Canada) exactly as described previously by Marti et al. (20). Briefly, DNA was extracted from 250 mg of soil in 2011 and 2012. In 2011, vegetables were placed into a bag and macerated. The macerate was then centrifuged, and the pellet was used for DNA extraction according to the same procedure as that used for soil. The method for DNA extraction from vegetables harvested in 2012 and 2013 was as follows. Rinsate (95 ml) from vegetables was filtered through a 0.45-μm-pore-size nitrocellulose membrane. Membranes were placed into 15-ml Falcon tubes containing 500 μl of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) and stored at −80°C. DNA was extracted from each filter by using the DNeasy tissue kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions, except that the proteinase K digestion step was omitted.

The frequency of detection of select gene targets was determined by PCR. Primers used to detect various antibiotic resistance genes, integrases, and plasmid incompatibility groups were obtained from the literature and are detailed in Table S3a in the supplemental material. The following targets were evaluated in the present study: plasmid incompatibility groups HI1, HI2, I1, K/B, L/M, N, P, Q, T, W, X, and Y; class 1, 2, and 3 integrases; the class A β-lactamase genes $bla_{\text{CARB-4}}$, $bla_{\text{CTX-M}}$, bla_{KPC}, bla_{SHV}, bla_{PSE}, and bla_{TEM}; the class B β-lactamase (metallo-β-lactamase) genes $bla_{\text{GIM-1}}$, bla_{IMP}, $bla_{\text{IMP-1}}$, $bla_{\text{NDM-1}}$, $bla_{\text{SIM-1}}$, $bla_{\text{SPM-1}}$, bla_{VIM}, and $bla_{\text{VIM-2}}$; the class D β-lactamase (oxacillinase) group 1 genes $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{OXA-3}}$, and $bla_{\text{OXA-5}}$; the class D β-lactamase group 2 gene $bla_{\text{OXA-20}}$; the class D β-lactamase group 4 gene $bla_{\text{LCR-1}}$; the non-affiliated class D β-lactamase genes $bla_{\text{OXA-18}}$ and $bla_{\text{OXA-48}}$; the macrolide-lincosamide-streptogramin type B resistance genes *erm*(A), *erm*(B),

TABLE 2 Abundance of viable enteric bacteria in dewatered municipal biosolids (2011) and dewatered sewage sludge (2012)[a]

Target	Log_{10} CFU g (wet wt)$^{-1}$ (no. of replicates) in 2011	Mean log_{10} CFU g (wet wt)$^{-1}$ ± SD (no. of replicates) in 2012
Total coliforms	4.88, 4.90	7.82 ± 6.57
Fecal coliforms	D (2)	6.69 ± 5.37
E. coli	D (2)	6.45 ± 6.10
Enterococcus spp.	4.29, 4.25	5.51 ± 3.81
C. perfringens	D (2)	5.45 ± 4.58
Yersinia spp.	D (2)	7.09 ± 6.36
S. enterica	BDL (2)	BDL (3)
Campylobacter spp.	D (2)	3.32 ± 2.82
Aeromonas spp.	D (2)	6.72 ± 6.41
L. monocytogenes	BDL (2)	BDL (3)

[a] Duplicate samples were taken at application in 2011, and triplicate samples were taken at application in 2012. Individual viable counts are presented for 2011, and means ± standard deviations are presented for 2012. Numbers in parentheses indicate the number of replicates that had the indicated result. BDL, below the detection limit of 1 CFU plate^{-1}; D, detected, where the viable count was below the quantification limit of 20 CFU plate^{-1}.

TABLE 3 Abundance of viable antibiotic-resistant coliform bacteria in dewatered municipal biosolids (2011) and dewatered sewage sludge (2012)[a]

Antibiotic	% resistance in 2011 (no. of replicates)	Mean % resistance ± SD in 2012 (no. of replicates)
Amikacin	BDL (2)	D (3)
Amoxicillin-clavulanic acid	0.9, 1.3	99.9 ± 11.9
Ampicillin	0.5, 0.9	96.5 ± 5.3
Cefotaxime	BDL, D	0.01 ± 0.001
Cefoxitin	D (2)	29.7 ± 1.9
Ceftiofur	BDL (2)	0.4 ± 0.2
Chloramphenicol	BDL (2)	0.1 ± 0.0
Ciprofloxacin	D (2)	0.3 ± 0.0
Gentamicin	BDL, D	0.2 ± 0.0
Nitrofurantoin	BDL (2)	BDL (3)
Norfloxacin	BDL (2)	17.4 ± 0.7
Sulfamethoxazole	D (2)	1.7 ± 0.1
Tetracycline	1.3, 1.4	1.2 ± 0.1
Trimethoprim	D, BDL	0.6 ± 0.1
Co-trimoxazole	BDL, D	1.9 ± 0.4
Chlortetracycline	D, 1.2	2.9 ± 0.2

[a] Duplicate samples were taken at application in 2011, and triplicate samples were taken at application in 2012. Individual viable counts are presented for 2011, and means ± standard deviations are shown for 2012. Numbers in parentheses indicate the number of replicates that had the indicated result. The plate counts of coliform bacteria on Chromocult agar without any antibiotic addition as a control were 4.88 and 4.90 log_{10} CFU/g (wet weight) in 2011 and 7.81 ± 6.51 log_{10} CFU/g (wet weight) in 2012. BDL, below the detection limit of 1 CFU plate^{-1}; D, detected, where the viable count was below the quantification limit of 20 CFU plate^{-1}.

erm(C), *erm*(E), and *erm*(F); the streptogramin type A resistance genes *vga* and *vat*(B); the streptogramin type B resistance gene *vgb*; the fosfomycin resistance gene *fos*(A); the fluoroquinolone resistance genes *qnr*(A), *qnr*(B), and *qnr*(S); the aminoglycoside resistance genes *aac(3)IV*, *aad*(A), *str*(A), and *str*(B); the sulfonamide resistance genes *sul1*, *sul2*, and *sul3*; the tetracycline resistance genes *tet*(A), *tet*(B), *tet*(BP), *tet*(C), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), and *tet*(T); and the vancomycin resistance genes *van*(A), *van*(B), *van*(C1), *van*(C2/C3), *mrs*(A), and *mrs*(B).

The reaction mixture consisted of deoxynucleoside triphosphates at 0.2 mM, primers at 400 nM each, 1× Green GoTaq Flexi buffer, 1.5 mM $MgCl_2$, 1 U GoTaq DNA polymerase (Promega, Thermo-Fisher Scientific, Ottawa, ON, Canada), and 2 μl of template DNA for a final volume of 25 μl. Reaction conditions were exactly as previously described (20). The detection limit of targeted genes, determined by adding known quantities of targeted genes into a 10-fold-diluted DNA template prepared from soil or vegetables, was 1 to 10 copies per μl. PCR products obtained from biosolids DNA were sequenced in order to confirm the identity of the targeted genes on the basis of the best match with sequences encoding the expected gene target. The sequences are available in Table S3b in the supplemental material. Based on sequence, putative *vat* and the bla_{SIM-1} products were unable to be confirmed and are therefore not considered here. Confirmed PCR products were cloned into plasmid pSC-A-amp/kan by using the StrataClone PCR cloning kit (Agilent) and transformed into *E. coli* according to the manufacturer's instructions. Plasmids were extracted by using the QIAprep Spin Miniprep kit (Qiagen, Mississauga, ON, Canada) and used as positive controls for PCR on soil and vegetable DNA samples. Plasmids were also used for determination of the detection limit of the PCR method. Known quantities of the cloned insert were added to DNA extracted from soil and from each vegetable type, with a final concentration ranging from 1 to 100 copies per μl of DNA. DNA extracts that were negative for each target were used for these experiments. Each gene target was analyzed in triplicate by PCR, and the plasmid quantity giving three positive results was chosen as the detection limit. Based on these results, the detection limits for the various gene targets were in the range of 10^4 to 10^5 gene copies per gram soil or per gram vegetable.

The abundance of selected gene targets was determined by quantitative PCR (qPCR) as described previously (24). Gene targets that were previously detected in biosolids-treated soil on the basis of PCR and that represented a range of different antibiotic resistance classes and mobile genetic elements were selected. Briefly, a 2-μl DNA sample (corresponding to 0.1 to 10 ng of DNA) was used as the template in a 25-μl reaction mix consisting of 12.5 μl Brilliant II qPCR master mix (Agilent, Toronto, ON, Canada) for TaqMan PCR or Brilliant II SYBR green Low ROX qPCR master mix (Agilent) for SYBR green PCR, deionized water, and selected primer pairs and probes at suitable concentrations for amplification. Each reaction was run in triplicate, including the negative controls without template DNA. Primers and probes used in the study are listed in Table S3c in the supplemental material, and the thermocycling conditions can be found in Table S3d in the supplemental material.

Data analysis and statistical methods. In order to be statistically meaningful, only viable plate counts with between 20 and 200 CFU per plate were considered quantifiable. Any plates with 1 to 19 CFU were considered to be positive but not quantifiable. On this basis, taking into account the dilutions used, the limits of quantification for viable plate counts were 400 CFU/g (wet weight) biosolids or sewage sludge and 80 CFU/g (wet weight) soil or vegetable. The detection limits were 20 CFU/g (wet weight) biosolids or sewage sludge and 4 CFU/g (wet weight) soil or vegetable. The frequency of resistance to antibiotics was calculated relative to viable plate counts on antibiotic-free Chromocult medium. The limit of quantification, expressed as percent resistance to a given antibiotic for biosolids in 2011, was 2%, and the detection limit was 0.1%. The limit of quantification for soil and vegetable samples was 2%, and the detection limit was 0.1%. The limit of quantification for sewage sludge obtained in 2012 was 0.1%, and the detection limit was 0.005%. The large difference in the limit values was due to the much higher viable counts in the sewage sludge. The limit of quantification for soil and vegetable samples was 10%, and the detection limit was 0.5%.

Means and standard deviations were imported into GraphPad Prism (version 5; GraphPad, San Diego, CA), and differences between treatments were tested by analysis of variance (ANOVA). The significance level was set at a P value of 0.05. The odds ratio for *C. perfringens* was calculated by using the VassarStats website for statistical computation (http://vassarstats.net/odds2x2.html).

Each vegetable and soil treatment was analyzed in quadruplicate.

TABLE 4 PCR detection of gene targets in DNA extracted from vegetables at harvest in the 2011, 2012, and 2013 seasons, the latter from crops grown in plots treated with sewage sludge in 2012[a]

	Gene target(s) detected					
	No amendment			+ biosolids	+ sludge	+ sludge (2012) in
Vegetable	2011	2012	2013	in 2011	in 2012	2013
Tomato	IncP *oriT*, IncF1C, *tet*(T), *str*(A), *str*(B), *sul2*, *erm*(B), *fos*(A) bla_{TEM}, *vat*(B), bla_{OXA-2}, bla_{GIM}, bla_{SPM}, *qnr*(B), *fos*(A)	IncA/C, IncP *oriT*, IncW, *int1*, *tet*(A), *str*(A), *str*(B), *sul1*, *erm*(B), bla_{OXA-1}, bla_{VIMgen}, bla_{TEM}	NA	*int3*	Nil	NA
Pepper	IncF1C, IncP *oriT*, IncP *trfA1*, IncQ *oriV*, *tet*(T), *aad*(A), *sul2*, *erm*(B), *erm*(C), *fos*(A), bla_{OXA-1}, bla_{OXA-2}, bla_{GIM}, bla_{SIM}, *qnr*(B), *fos*(A)	NA	NA	Nil	NA	NA
Cucumber	*aad*(A), *str*(A), *str*(B), *sul1*, *erm*(B)	NA	NA	Nil	NA	NA
Radish	*int1*, *str*(A), *str*(B), *fos*(A)	IncA/C, IncN, IncP *oriT*, IncW *oriV*, IncW, *int1*, *tet*(A), *tet*(S), *tet*(T), *aad*(A), *str*(A), *str*(B), *sul1*, bla_{PSE}, bla_{VIMgen}, *qnr*(S), *fos*(A), *van*(C2)	IncP *oriT*, *tet*(B), IncP *trfA2*, *tet*(T), IncQ *oriT*, *erm*(F), bla_{OXA-1}	Nil	bla_{OXA-20}	Nil
Carrot	IncP *oriT*, *qnr*(B), *fos*(A)	IncA/C, IncN, IncP *oriT*, *van*(C2), IncQ *oriV*, IncW *oriV*, IncW, *int1*, *tet*(A), *tet*(S), *aad*(A), *str*(A), *str*(B), *sul1*, *erm*(C), bla_{TEM}, bla_{VIMgen}, *qnr*(B), *qnr*(S), *fos*(A)	IncA/C, IncP *oriT*, IncQ *oriV*, IncQ *oriT*, IncW *oriV*, *aad*(A), *str*(A), *sul1*, *erm*(B), *erm*(C), *erm*(F), bla_{TEM}, *int3*, bla_{OXA-1}, bla_{PSE}, bla_{VIMgen}, *fos*(A)	Nil	bla_{OXA-20}	Nil
Lettuce	NA	IncP *oriT*, IncW, *tet*(A), *tet*(S), *aad*(A), *sul1*, *fos*(A), *erm*(B), bla_{TEM},[b] *fos*(A), bla_{CTX-M},[b] bla_{OXA-1}	IncP *oriT*, IncQ *oriT*, *tet*(B), *tet*(T), *erm*(F), *van*(C2)	Nil	bla_{OXA-20}	Nil

[a] Shown are all gene targets that were detected on at least one vegetable sample grown in soil without biosolids treatment (− biosolids) and those targets that were also detected on at least one vegetable grown with but not without biosolids (+ biosolids). All of the data are available in Table S9 in the supplemental material. NA, not applicable because the vegetable was not planted in that year. Nil indicates that no genes in addition to those detected in the unamended treatment were detected.
[b] Primers for bla_{CTX-M} and bla_{TEM} were used only in 2012 and 2013, whereas all others were used in 2011 as well.

When a bacteriological target was detected but at a concentration below the quantification limit of 20 CFU/plate, it was determined to be detected but not quantifiable. In that case, it was reported as detected but below the limit of quantification. Only soil and vegetable samples with at least 3 of the 4 independent replicates above the limit of quantification were used to calculate the averages and standard deviations using MS Excel (Microsoft Corp.). Tables report data that are quantifiable and are annotated to indicate samples with concentrations of bacteriological targets that were below the limit of quantification or below the limit of detection. Statistically significant treatment effects were determined by using an unpaired ANOVA followed by Bonferroni posttests. Data were treated by using GraphPad Prism software version 5 (GraphPad, San Diego, CA). The significance level was set at a P value of 0.05, and only cases where concentrations of targets were above the limit of quantification in both control and treated samples were used for statistical analyses. Odds ratio determinations for *C. perfringens* were calculated by using the VassarStats website for statistical computation (http://vassarstats.net/odds2x2.html).

The relative abundance of gene targets is presented as the ratio of the targeted gene copy number to the total number of *rrnS* gene copies in the reaction (24). The limit of quantification for PCRs was determined by adding known quantities of plasmid harboring the gene target insert into extracted 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 100 copies per μl. Each condition was analyzed in triplicate. The limit of quantification was set as the dilution giving three positive results following the linearity range. When the gene target was detected but with only between 1 and 4 copies per reaction, it was determined to be below the limit of quantification. In that case, it was 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 averages and standard deviations using SigmaPlot version 12.5 (Systat Software Inc.). Tables report data that are quantifiable and are annotated to indicate samples with concentrations of bacteriological targets that were below the limit of quantification or below the limit of detection. Statistically significant treatment effects were determined by using an unpaired t test without assuming an equal standard deviation (Welch's correction). Data were treated by using XLSTAT software version 2013.5.03 (Addinsoft). The significance level was set at a P value of 0.05, and only cases where concentrations of targets were above the limit of quantification in both control and treated samples were used for statistical analyses.

TABLE 5 Relative abundance of the *int1* gene target in soil and on vegetables at harvest following application of sewage sludge[a]

Season	Plot	Mean relative abundance of the *int1* gene target (SD)			
		Control soil	Treated soil	Control vegetables	Treated vegetables
2012	Lettuce	BLQ	0.000010 (0.000064)	BLD	0.000003 (0.000001)
	Carrot	BLQ	BLQ	BLD	BLD
	Radish	BLQ	BLQ	BLD	BLD
	Tomato	BLQ	0.000101 (0.000051)	BLD	0.000013 (0.000001)
2013	Lettuce	BLQ	0.000288 (0.000294)	0.000004 (0.000002)	0.000010 (0.000044)
	Carrot	0.001047 (0.00031)	0.001455 (0.000430)	0.000027 (0.000025)	0.000063 (0.000041)
	Radish	0.000048 (0.000021)	0.000137 (0.000117)	0.000098 (0.000082)	0.000080 (0.000074)

[a] In Tables 5 to 11, BLQ indicates values below the limit of quantification, and BDL indicates values below the detection limit, as defined in Materials and Methods.

RESULTS

Bacterial composition of amendments. The biosolids used in the 2011 experiment carried far fewer viable bacteria than the sewage sludge used in 2012 (Table 2). Total coliform bacteria had a 1,000-fold-lower abundance and enterococci had a >10-fold-lower abundance in the biosolids. Levels of *E. coli* and all pathogens were below the limit of detection or quantification in 2011. In the sewage sludge used in 2012, all pathogens were quantifiable, with the exception of *Salmonella* and *Listeria monocytogenes*, which were not detected in either year.

The amendments were also strikingly different with respect to the antibiotic resistance profiles of coliform bacteria enumerated on Chromocult medium (Table 3). The fraction of total viable coliform bacteria recovered from sewage sludge on Chromocult plates containing breakpoint concentrations of amoxicillin-clavulanic acid, ampicillin, cefoxitin, and norfloxacin was much larger than that recovered from biosolids in 2011. The fraction of coliforms resistant to most of the other antibiotics was small but quantifiable in the sewage sludge, whereas resistance was below the detection limit or limit of quantification in the biosolids sample.

Pathogenic and coliform bacteria in soil and on vegetables at harvest. The impact of soil amendment on the abundance of indicator and pathogenic bacteria in soil at crop harvest was evaluated (see Table S4 in the supplemental material). There were no differences in the abundance of total coliforms, fecal coliforms, *E. coli*, enterococci, or *Yersinia* spp. enumerated in amended and untreated control plots. *Campylobacter* spp., *Salmonella enterica*, and *L. monocytogenes* were never detected in any sample. The only effect of sewage sludge fertilization on soil bacteria was an increase in the frequency of detection of *C. perfringens* (see Table S4 in the supplemental material). *C. perfringens* was not enumerated in the 2011 experiment but was enumerated in 2012 and 2013. *C. perfringens* was detected significantly more frequently in amended soil than in unamended control soil, with an odds ratio of 48:1 (chi square = 38.96; $P < 0.0001$).

There was no impact of biosolids or sewage sludge application on the abundance of any indicator or pathogenic bacteria on vegetables at harvest (see Table S5 in the supplemental material). *Campylobacter* spp., *L. monocytogenes*, and *S. enterica* were never detected on any vegetable samples.

Abundance of antibiotic-resistant coliform bacteria in soil and on vegetables at harvest. The frequency of resistance of total coliform bacteria was assessed by plate counts on Chromocult medium containing breakpoint concentrations of antibiotics. There was no coherent effect of biosolids application on the frequency of resistance to any antibiotics in soil coliform bacterial samples at crop harvest (see Table S6 in the supplemental material). Resistance to ciprofloxacin and norfloxacin was never detected, and resistance to nitrofurantoin was detected in only one sample. Resistance to cefotaxime, ceftiofur, chloramphenicol, sulfamethoxazole, tetracycline, trimethoprim, and co-trimoxazole was detected in only one or two soil samples per season and was not quantifiable. Resistance to amikacin, ampicillin, cefoxitin, gentamicin, and chlortetracycline was frequently detected, at frequencies that were very variable within replicates for a given treatment or year. In one instance, there was a significant effect of treatment: 79.2% ± 15.0% of coliforms from soil cropped to carrots in soil that had received sewage sludge were resistant to ampicillin, whereas 46.4% ± 16.3% of coliforms from the untreated soil were resistant (see Table S6 in the supplemental material).

The only significant effect of soil amendment on the frequency of antibiotic resistance in coliform bacterial cultures from vegetable samples was on cefoxitin resistance in carrot samples from the

TABLE 6 Relative abundance of the *sul1* gene target in soil and vegetables at harvest following application of sewage sludge

Season	Plot	Mean relative abundance of the *sul1* gene target (SD)			
		Control soil	Treated soil	Control vegetables	Treated vegetables
2012	Lettuce	BLD	BLD	BLD	BLD
	Carrot	BLQ	0.000085 (0.000027)	BLD	BLD
	Radish	BLD	BLD	BLD	BLD
	Tomato	BLQ	0.000094 (0.000079)	BLD	0.000054 (0.000021)
2013	Lettuce	0.000247 (0.000123)	0.000330 (0.000378)	0.000004 (0.000002)	0.000009 (0.000012)
	Carrot	BLQ	0.001117 (0.000486)	BLQ	BLQ
	Radish	0.000425 (0.000073)	0.000926 (0.000553)	0.000150 (0.000108)	0.000169 (0.000095)

TABLE 7 Relative abundance of the IncW *repA* gene target in soil and vegetables at harvest following application of sewage sludge[a]

2012 season plot	Mean relative abundance of the IncW *repA* gene target (SD)			
	Control soil	Treated soil	Control vegetables	Treated vegetables
Lettuce	BLQ	0.000005 (0.000003)	BLQ	0.000002 (0.000001)
Carrot	0.000006 (0.000002)	0.000011 (0.000002)	0.000001 (0.000000)	0.000078* (0.000027)
Radish	BLQ	0.000261 (0.000201)	BLQ	0.000002 (0.000001)
Tomato	BLQ	0.000007 (0.000001)	0.000003 (0.000001)	0.000004 (0.000001)

[a] The gene target was not detected in 2013. The asterisk indicates a significant treatment effect ($P < 0.05$); only cases where the levels of gene targets were above the limit of quantification in both control and treated samples were used for statistical analyses.

2012 season (see Table S7 in the supplemental material). In the absence of sewage sludge amendment, 48.8% ± 16.2% of culturable coliforms were resistant to cefoxitin, whereas in the amended plots, 85.7% ± 7.6% were resistant. Resistance to cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin, norfloxacin, sulfamethoxazole, trimethoprim, and co-trimoxazole was never detected. Resistance to amikacin, amoxicillin-clavulanic acid, ampicillin, and cefoxitin was widespread but very variable within replicates of the same treatment. Tetracycline resistance was detected in only one tomato sample in 2012, norfloxacin resistance was detected in only one pepper sample from a control plot in 2013, and resistance to chlortetracycline was detected in carrot, radish, and lettuce samples in 2012.

Distribution of gene targets in amendments, in soil, and on vegetables at harvest. A number of gene targets associated with plasmid incompatibility groups, integrons, and antibiotic resistance were detected in biosolids and sludge by PCR (see Table S8 in the supplemental material). The following gene targets were detected in biosolids used in the 2011 season: IncP *oriT*, IncP *trfA1*, IncQ *oriT*, *int1*, *int3*, *tet*(Q), *tet*(S), *tet*(T), *aad*(A), *str*(A), *str*(B), *sul1*, *sul2*, *erm*(A), *erm*(B), *erm*(C), *erm*(F), bla_{OXA-20}, bla_{OXA-5}, bla_{OXA-1}, bla_{OXA-2}, bla_{LCR-1}, *qnr*(B), and *fos*(A2). The following gene targets were detected in sewage sludge used in the 2012 season: IncA/C, IncFIB, IncN, IncP *korA*, IncP *oriT*, IncP *trfA2*, IncQ *repB*, IncQ *oriT*, IncQ *oriV*, IncW, IncW *oriV*, IncY, *int1*, *int2*, *int3*, *tet*(A), *tet*(B/P), *tet* (M), *tet*(Q), *tet*(S), *tet*(T), *aad*(A), *str*(A), *str*(B), *sul1*, *sul3*, *erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), bla_{CTX-M}, bla_{OXA-20}, bla_{OXA-1}, bla_{LCR-1}, bla_{PSE}, bla_{TEM}, $bla_{VIMgen-2}$, *qnr*(A), *qnr*(B), *qnr*(S), *fos*(A2), and *van*(C2/C3). The possible enrichment of these gene targets in amended soils was evaluated. In a number of instances, a gene target carried in biosolids or sludge was not detected in amended soils: IncFIB, IncP, *korA*, IncP *trfA2*, IncQ *repB*, IncQ *oriT*, IncY, *int2*, *tet*(B/P), *tet*(M), *tet*(Q), *sul3*, bla_{CTX-M}, bla_{OXA-2}, bla_{LCR-1}, bla_{SPM-1}, and *qnr*(A). Many genes carried in biosolids or sludge were also detected in unamended control soil: IncA/C, IncN, IncP *oriT*, IncP *trfA1*, IncQ *oriV*, IncW, IncW *oriV*, *int1*, *int3*, *tet*(A), *tet*(S), *tet*(T), *aad*(A), *str*(A), *str*(B), *sul1*, *sul2*, *erm*(A), *erm*(B), *erm*(C), bla_{CTX-M}, bla_{OXA-20}, bla_{OXA-1}, bla_{LCR-1}, bla_{PSE}, bla_{TEM}, $bla_{VIMgen-2}$, *qnr*(S), *fos*(A2), and *van*(C2/C3). In the following instances, a gene target carried in an amendment was detected in amended soil and not in unamended control soil in the indicated sampling year: IncW in 2013, *int3* in 2012, *erm*(C) in 2013, bla_{OXA-1} in 2013, *fos*(A2) in 2013, and *van*(C2/C3) in 2013. The following genes were detected in amended plots in the indicated year and were never detected in unamended control plots: IncP *trfA2* in 2013, bla_{OXA-5} in 2011, and *qnr*(B) in 2012. There were two instances where genes were detected in control plots and were never detected in biosolids-treated plots: bla_{CTX-M} (2012; not evaluated in 2011) and bla_{LCR-1} (2012).

The possible transfer of genes carried in amendments onto vegetables at harvest was investigated (Table 4; see also Table S9 in the supplemental material). Many gene targets were detected on vegetables grown in the absence of any soil amendment by PCR (Table 4). In addition to these genes, *int3* was also detected in 2011 on vegetables grown in amended soil but not in unamended control soil. However, *int3* was detected on carrots grown in unamended soil in 2013. The gene target bla_{OXA-20} was detected on radish, carrot, and lettuce grown in sludge-amended soil in 2012 but not in 2013. The gene was never detected in unamended control soil.

The impact of sewage sludge amendment on the abundances of selected gene targets in soils and on vegetables at harvest was evaluated by qPCR (Tables 5 to 11). Soil and vegetable samples were taken at harvest time in both the year of sludge application (2012) and the following year (2013). Every gene target [*int1*, *sul1*, IncW *repA*, *erm*(B), *erm*(F), *str*(A), and *str*(B)] was detected at least once both in soil and on a vegetable during the period of observation. In the year of application, there were two instances (lettuce and tomato) where *int1* was quantifiable in soil or on vegetable in treated but not control soil (Table 5). Likewise, in 2012, *sul1* was detectable in treated soil from carrot and tomato plots and on tomato but not in the corresponding control samples (Table 6). The gene targets *int1* and *sul1* were quantifiable in both treated soil and control soil in 2013, with no significant difference (Tables 5 and 6). The gene target IncW *repA* was detected in 2012 but not 2013

TABLE 8 Relative abundance of the *erm*(B) gene target in soil and vegetables at harvest following application of sewage sludge[a]

2012 season plot	Mean relative abundance of the *erm*(B) gene target (SD)			
	Control soil	Treated soil	Control vegetables	Treated vegetables
Lettuce	0.000192 (0.000025)	0.000254 (0.000110)	0.000019 (0.000007)	0.000039 (0.000026)
Carrot	BLD	BLD	BLD	BLD
Radish	BLQ	BLQ	BLD	BLD
Tomato	BLQ	0.000002 (0.000001)	BLQ	0.000003 (0.000002)

[a] The gene target was not detected in 2013.

TABLE 9 Relative abundance of the *erm*(F) gene target in soil and vegetables at harvest following application of sewage sludge[a]

2013 season plot	Mean relative abundance of the *erm*(F) gene target			
	Control soil	Treated soil	Control vegetables	Treated vegetables
Lettuce	0.000341 (0.000182)	0.001936 (0.003676)	0.000048 (0.000392)	0.000110 (0.000174)
Carrot	0.003924 (0.002079)	0.003047 (0.001649)	0.000085 (0.000142)	0.000203 (0.000188)
Radish	0.000157 (0.000140)	0.000696 (0.000654)	0.000207 (0.000215)	0.001411 (0.001166)

[a] The gene target was not detected in 2012.

(Table 7). The gene target was often quantifiable in soil or on vegetables from treated plots but was below the level of quantification in the control plots. It was significantly more abundant on carrots from treated than from control plots at harvest (Table 7). The gene target *erm*(B) was detected in 2012 but not in 2013, whereas *erm*(F) was detected in 2013 but not in 2012 (Tables 8 and 9). There was no treatment effect on the abundance of *erm*(F), whereas in one instance (tomato), *erm*(B) was quantifiable in treated soil and vegetables but was below the limit of quantification in samples from control plots. The gene target *str*(A) was detected in both 2012 and 2013, whereas *str*(B) was detected only in 2012 (Tables 10 and 11). In 2012, *str*(A) was quantifiable in soil and on vegetables from treated plots, whereas it was below the limit of quantification or detection in samples from control plots (Table 10). In contrast, there was no treatment effect on the distribution of *str*(A) in 2013. In 2012, when it was detectable, there were six instances where *str*(B) was detectable or quantifiable in samples from treated plots and only one instance where it was detected in samples from control plots (Table 11).

DISCUSSION

Residual solid materials recovered from municipal wastewater treatment systems are rich in microorganisms and a diversity of antibiotic resistance genes (18, 21). No more viable enteric bacteria were detected in soil or on vegetables at harvest from plots receiving either biosolids or untreated sewage sludge than in control plots that were fertilized inorganically only (see Tables S4 and S5 in the supplemental material). Clearly, in both the 2011 and 2012 field seasons, conditions were thus that microorganisms entrained in the organic amendments (Table 2) lost viability in the intervening weeks between application and crop harvest. Furthermore, the absence of a treatment effect on the abundance of viable enteric bacteria in the 2013 season indicates that there was no regrowth of these organisms a year following the application of sewage sludge in 2012 (see Table S4 in the supplemental material). These field results are in agreement with previous assessments concluding that a 1-year delay prior to consumption of produce will ensure that there is very little risk of illness from biosolids-borne viable bacterial pathogens (22). As revealed by viable plate counts on Chromocult agar, there was no consistently significant effect of biosolids or sewage sludge utilization on the abundance of total coliform bacteria in soil or on vegetables that were resistant to a variety of antibiotics (see Tables S6 and S7 in the supplemental material). In a field study in Arizona, there was no impact of biosolids treatment on bacterial cultures on R2A agar containing antibiotics 15 months after treatment (23). Overall, as an endpoint, viable plate counts do not reveal significant treatment effects that last over at least two growing seasons with respect to the distribution of pathogenic or antibiotic-resistant bacteria.

Many gene targets associated with antibiotic resistance or mobility were detected on vegetables harvested from soil fertilized inorganically only (Table 4). In addition to these genes, *int3* was detected on tomatoes grown in the presence of biosolids, and $bla_{\text{OXA-20}}$ was detected on carrots, lettuce, and radish grown in the presence of sewage sludge in 2012. On the other hand, in 2013, no additional genes associated with the use of sewage sludge from the previous season were detected on any vegetable. Quantitative PCR revealed several instances where a gene target was more abundant in soils or on vegetables harvested in 2012 from plots treated with sewage sludge than on the same vegetables grown without sludge treatment (Tables 5 to 11). In contrast, gene targets evaluated on vegetables harvested in 2013 did not generally vary in abundance between sludge-treated and control plots. Taken together, these data suggest that both biosolids and sewage sludge have the potential to increase the variety and abundance of antibiotic resistance genes recovered on vegetables at harvest in the season of application but that this treatment effect is not detected in the subsequent season. Thus, under conditions in South Western Ontario, a 15-month delay between application of sewage sludge and crop harvest appears to be sufficient to attenuate exposure to sludge-borne antibiotic resistance genes. The diversity and abundance of antibiotic-resistant bacteria in biosolids will vary according to treatment, and thus, the results reported here are not rep-

TABLE 10 Relative abundance of the *str*(A) gene target in soil and vegetables at harvest following application of sewage sludge

Season	Plot	Mean relative abundance of the *str*(A) gene target (SD)			
		Control soil	Treated soil	Control vegetables	Treated vegetables
2012	Lettuce	0.000121 (0.000081)	0.000193 (0.000077)	BLD	0.000015 (0.000007)
	Carrot	BLQ	0.001462 (0.000900)	BLQ	0.000009 (0.000004)
	Radish	BLQ	BLQ	BLD	BLQ
	Tomato	BLQ	0.000109 (0.000071)	BLQ	0.000103 (0.000041)
2013	Lettuce	BLD	BLD	BLD	BLD
	Carrot	0.001844 (0.000958)	0.007378 (0.007717)	0.000044 (0.000038)	0.000146 (0.000151)
	Radish	BLD	BLD	BLD	BLD

TABLE 11 Relative abundance of the *str*(B) gene target in soil and vegetables at harvest following application of sewage sludge[a]

2012 season plot	Mean relative abundance of the *str*(B) gene target (SD)			
	Control soil	Treated soil	Control vegetables	Treated vegetables
Lettuce	BLD	0.000105 (0.000024)	BLD	BLQ
Carrot	BLD	BLQ	BLD	BLD
Radish	BLD	BLQ	BLD	BLQ
Tomato	BLQ	0.000115 (0.000027)	BLD	BLD

[a] The gene target was not detected in 2013.

resentative of all biosolids. Future work will evaluate the field behavior of microorganisms and antibiotic resistance genes following the application of biosolids obtained from municipalities that anaerobically digest, compost, or heat treat their sewage sludge.

Viable plate counts (see the supplemental material) and gene target copy numbers (Tables 5 to 11) in soils were generally very variable within treatments at each sampling time. This is presumably at least partly due to the aggregate structure of the dewatered material used in the present study. Distribution of amendment even following incorporation is relatively heterogeneous in soil, certainly compared to an application of slurry, where water entrains particulate materials into the soil far more uniformly. Physical considerations, including amendment moisture content and the rigor and depth of soil incorporation, will have an important influence on the distribution, fate, and exposure potential of microbial, and chemical, contaminants.

Taken together, these data indicate that under conditions characteristic of the Great Lakes Basin, crops harvested in the same season as the application of human waste are at risk of contamination with antibiotic resistance determinants that are either not detected or detected in lower abundance in soil that is fertilized only inorganically. The risk of crop exposure to sewage sludge-borne genes is greatly attenuated by a 15-month delay. We emphasize that the Province of Ontario does not permit the in-season harvest of vegetables from ground that was fertilized with biosolids or untreated sewage sludge, and thus, the results reported here are not representative of normal farming practice in this region. In the United States, the EPA Part 503 Biosolids Rule specifies a 14-month delay between the application of class B biosolids and the harvest of above-ground (e.g., tomato) crops for human consumption and a 20-month delay before the harvest of below-ground (e.g., carrot) crops destined for human consumption (25). The World Health Organization recommends several measures for mitigating risk from human fecal material, including preapplication treatment, crop harvest delays that permit pathogen die-off, and restricting the type of crop grown in amended ground (12). Results from the present study indicate that producing vegetable crops in ground fertilized with human waste without appropriate delay or pretreatment will result in an additional burden of antibiotic resistance genes on the harvested crops. Clearly, fertilizing ground with human waste for production of crops for human consumption represents a potential indirect "fecal-oral" route of exposure to antibiotic resistance genes. In the context of other sources of environmental or food-borne exposure to antibiotic resistance genes, the significance of this practice with respect to the global development of antibiotic resistance is unknown (8). Nevertheless, managing human exposure to antibiotic resistance genes carried in human waste through judicious agricultural practice, as commonly employed in Canada and the United States and as recommended by the WHO, is prudent and should be undertaken in all jurisdictions (10).

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