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The impact of municipal sewage sludge stabilization processes on the abundance, field persistence, and transmission of antibiotic resistant bacteria and antibiotic resistance genes to vegetables at harvest

Roger Murray [a], Yuan-Ching Tien [a], Andrew Scott [a], Edward Topp [a,b,*]

[a] Agriculture and Agri-Food Canada, London, Ontario, Canada
[b] Department of Biology, University of Western Ontario, London, Ontario, Canada

HIGHLIGHTS

- Effect of biosolids stabilization on ARGs distribution in soils and on vegetables was evaluated.
- Class A biosolids had fewer and less abundant ARGs than Class B.
- ARGs typically associated with gene cassettes persist through the growing season.
- Root vegetables carry more abundant ARGs when grown in soil with biosolids.

GRAPHICAL ABSTRACT

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ABSTRACT

Biosolids were obtained from four Ontario municipalities that vary in how the sewage sludge is treated. These included a Class B biosolids that was anaerobically digested, a Class A biosolids that were heat treated and pelletized (Propell), and two Class A biosolids that were stabilized using either the N-Viro (N-Rich) or Lystek (LysteGro) processes. Viable enteric indicator or pathogenic bacteria in the biosolids were enumerated by plate count, gene targets associated with antibiotic resistance or horizontal gene transfer were detected by PCR, and a subset of these gene targets were quantified by qPCR. Following application at commercial rates to field plots, the persistence of enteric bacteria and gene targets in soil was followed during the growing season. Carrots, radishes and lettuce were sown into the amended and unamended control plots, and the diversity and abundance of gene targets they carried at harvest determined. All three Class A biosolids carried fewer and less abundant antibiotic resistance genes than did the Class B biosolids, in particular the very alkaline N-Viro product (N-Rich). Following application, some gene targets (e.g. *int*1, *sul*1, *str*A/B, *aad*A) that are typically associated with mobile gene cassettes remained detectable throughout the growing season, whereas others (e.g. *erm*B, *erm*F, bla_{OXA20}) that are not associated with cassettes became undetectable within three weeks or less. At harvest a larger number of gene targets were detected on the carrots and radishes than in the lettuce. Overall, land application of Class A biosolids will entrain fewer viable bacteria and genes associated with antibiotic resistance into crop ground than will amendment with Class B biosolids.

1. Introduction

The development of antibiotic resistance is a seminally important public health challenge, potentially leading to a complete loss of efficacy of all medicines used to treat bacterial infections (Carlet et al., 2011;

* Corresponding author at: Agriculture and Agri-Food Canada, London, Ontario, Canada.
E-mail address: ed.topp@agr.gc.ca (E. Topp).

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Piddock, 2016). Antimicrobial resistance arises from the use of antibiotics in commercial agriculture and in human medicine, the release of antibiotics into the environment, and the transmission of resistant bacteria and resistance determinants locally and globally between these sectors. The close linkages between humans, animals and the environment is emphasized in what has been coined the One Health Framework (McEwen and Collignon, 2018). One potential route of transmission to humans from the environment is through the consumption of plant-based foods, particularly those consumed raw (http://www.fao.org/3/CA0963EN/ca0963en.pdf accessed October 1, 2018; Freitag et al., 2018; Huijbers et al., 2015). The risk of transmission may be particularly significant if the crop production system is exposed to human or animal fecal material through fertilization with organic amendments or irrigation with reclaimed wastewater (Christou et al., 2017; Pruden et al., 2013)

Many jurisdictions permit the amendment of agricultural soil with biosolids as a valued source of organic matter and crop nutrients (European Union, https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:31986L0278, Accessed April 24, 2018; US Environmental Protection Agency, http://water.epa.gov/scitech/wastetech/biosolids/503pe_index.cfm, Accessed August 5, 2014; O'Connor et al., 2005; Zerzghi et al., 2010). Biosolids can contain a variety of heavy metals, various classes of bioactive organic chemicals, and viral, bacterial, protozoal and helminthic pathogens (Clarke and Cummins, 2015; McClellan and Halden, 2010; Meng et al., 2016; Pepper et al., 2006; Smith, 2009). Thus, there are inevitably public and regulatory concerns about risks to human and environmental health associated with the practice of using biosolids in plant-based food production systems. The risk of transmission of these contaminants to crops is typically managed by mandating a delay between biosolids application and the harvest of crops destined for human consumption, limits on sewer discharges by commercial enterprises of inorganic and organic chemicals, and treating the sewage sludge prior to land application to abate the number of potentially pathogenic microorganisms, a process called stabilization (Ontario Provincial Government, http://www.e-laws.gov.on.ca/html/regs/english/elaws_regs_030267_e.htm#BK194, accessed August 21, 2018). Following appropriate treatment, sewage sludge is commonly given the designation "biosolids". Treatment options can include aerobic digestion, anaerobic digestion, heating, composting, or the addition of an alkali agent to "lime stabilize" the sewage sludge. In Ontario Canada, if the treatment is rigorous enough to reduce the abundance of *Escherichia coli* in the biosolids to a maximum of 1000 colony forming units per 100 ml, Salmonella to a maximum of three colony forming units or most probable number per 100 ml, viable Helminth ova to an undetectable level in 100 ml, and total culturable enteric viruses to an undetectable level in 100 ml, that material is designated CP1. It is equivalent to Class A biosolids in US parlance (US Environmental Protection Agency, http://water.epa.gov/scitech/wastetech/biosolids/503pe_index.cfm, Accessed August 5, 2014). If the biosolids have been insufficiently treated to achieve these microbial targets, it is designated CP2, equivalent to Class B biosolids in US parlance. Sewage biosolids categorized as CP2 have to meet the *E. coli* $<2 \times 10^6$ CFU/g of total solids dry weight standard.

Biosolids contain residues of multiple classes of antibiotics, and are rich in antibiotic-resistant bacteria excreted by the population within the sewer shed (Ju et al., 2016; Magee et al., 2018; Manaia et al., 2018; Rahube et al., 2014; Sabourin et al., 2012). There is a concern that either antibiotic residues or antibiotic-resistant bacteria will be transmitted to crops when these are grown in ground fertilized with biosolids, and that this would represent a human or animal health risk when the crops are consumed (Ashbolt et al., 2013; Lau et al., 2017; Pepper et al., 2018; Pruden et al., 2013; Rahube et al., 2014; Yang et al., 2018). The method used to treat sewage sludge has a profound impact on its microbial composition (Goberna et al., 2018).

In previous work, we systematically evaluated the microbial composition and fate of antibiotic resistance genes following application of biosolids sourced from regional municipalities that treat the biosolids using various methods (Lau et al., 2017). This, to obtain evidence under realistic field conditions that will inform best practice for pretreating biosolids to minimize the risk of environmental transmission of antibiotic resistance. In field experiments undertaken in 2014 on the University of Western Ontario research farm in London Ontario, we evaluated the fate following land application of antibiotic resistance genes carried in raw municipal sewage sludge, and in biosolids that were variously anaerobically digested, aerobically digested, or heat-treated and pelletized. In addition, the aerobically- and anaerobically-digested biosolids were applied either as a slurry, or as a dewatered cake (Lau et al., 2017). Each of the five types of materials was applied at commercial rates, following which lettuce, carrots and radishes were planted. The estimated antibiotic gene loading rates were comparable with each of the five biosolids. In the present study, we repeated the field application of the heat-treated pelletized biosolids and the anaerobic cake. We also included two municipal biosolids that were lime-stabilized using two different processes. The microbial composition of each biosolids was determined, as was the abundance of selected antibiotic resistance genes in the biosolids and in soil following application, and the potential transmission of antibiotic resistance to carrots, radishes or lettuce at harvest was evaluated. The present work broadens the suite of treatment options that have been evaluated for the ability to abate antibiotic resistance in biosolids prior to land application, notably evaluating three Class A biosolids that were stabilized using different means, and contrasting with one anaerobically digested Class B biosolid.

2. Materials and methods

2.1. Field operations

Experiments were undertaken during the 2017 growing season on the Environmental Science Western Field Station located near London, Ontario, Canada (43°4′47″N, 81°20′24″W). The field site characteristics and general methods for amendment application, cropping and sampling have been described in (Lau et al., 2017). The air temperature and precipitation events during the experimental period are presented in supplementary materials (Fig. S1), as are dates of key farm operations (Table S1). The present study compared four different types of biosolids in field experiments; one Class B dewatered anaerobically digested biosolids, and two alkali-stabilized and one heat stabilized Class A biosolids. The key physical and chemical properties of these biosolids are available in the supplementary materials (Table S2). Dewatered anaerobically digested biosolids was obtained from the municipality of Niagara Ontario. The material was field applied at 7 dry mt/ha. The product generated using the N-Viro process (termed N-Rich) was obtained from Thorold, Ontario. The material is prepared by mixing sewage sludge with cement and/or lime kiln dust. The mixing, drying and exergonic reaction produces a granular material. N-Rich material was applied at 5 dry mt/ha. The product generated using the Lystec process (termed LysteGro) was obtained from the municipality of Saint-Marys, Ontario. The proprietary Lystek process involves a combination of heat, alkali, and high shear mixing to yield a slurry material. The LysteGro slurry was applied at 33,690 l/ha. Propell biosolids pellets were obtained from the city of Windsor, Ontario. The material is produced with dewatered sewage sludge that is heat-treated and pelletized. Heat treated pellets were applied at 3.5 dry mt/ha. Amendments were incorporated to a soil depth of 15 cm with two passes in opposite directions of an "S tine" cultivator immediately following application.

Based on antecedent cropping history and soil nutrient testing, plots received a uniform application of inorganic fertilizers broadcasted using a Herd Sure Feed Broadcaster (Kasco Manufacturing of Shelbyville, IN), before application of organic amendments. These were (% volume N-P-K) 16-16-16 at 558lbs/ac (625 kg/ha), and 46-0-0 at 268lbs/ac (312 kg/ha) to supply a total of 212lbs/ac N (237 kg/ha), 89lbs/ac P and K (100 kg/ha). For each amendment 12 m × 20 m plots were staked