AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2017 – 2019

TITLE: Fate of Pathogenic and Antibiotic-Resistant Bacteria in Soil and on Vegetable Crops after Biosolids Land Application

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EXECUTIVE SUMMARY: Land application of biosolids (treated sewage sludge) is practiced around the world not only as a disposal method, but also as an alternative source of nutrients for soils to improve agricultural productivity. In the US, over 6.5 million dry tonnes of biosolids are produced annually, 55% of which are land applied. One of the main human health risks associated with biosolids use in agriculture is the potential contamination of food crops with infectious bacteria. Insufficient treatment of biosolids applied to field crops that are consumed raw represents a potentially significant transmission pathway for pathogens. To mitigate this risk, the US Environmental Protection Agency (EPA) categorizes biosolids based on microbiological quality. Class A biosolids have been treated to reduce microbial levels to achieve a standard of <1000 generic *Escherichia coli* and may be applied to land with no restrictions. Whereas, for Class B biosolids with high microbial concentrations, there are restrictions on land application, including extended application-to-harvest timeframes (30 days to up 20 months).

In addition to their infectivity, bacterial pathogens often show resistance to different environmental factors and treatment processes. Antibiotic resistance is of particular concern as a result of their use in human and veterinary medicine as well as general agricultural use. The development of resistant bacterial strains presents human health risks due to the potential failure of treatments and infection control. Each year in the US, there are two million infections with enteric bacteria resistant to antibiotics, and there are 23,000 deaths as a direct result of these infections. Municipal wastewater and biosolids are major sources of enteric antibiotic-resistant bacteria and determinant genes via incomplete metabolism in humans and the disposal of unused antibiotics. While there is some evidence on the potential impact of treatment processes on the prevalence of enteric antibiotic-resistant bacteria in biosolids, their fate (growth and persistence) in the agricultural soil environment as well as on edible crops is virtually unknown. Furthermore, the human health impacts of the presence of the enteric antibiotic-resistant bacteria in the environment and their transmission pathways are not well-defined. Consequently, there are no regulations on the presence of enteric antibiotic-resistant bacteria in biosolids used for soil amendments. There is, therefore, a critical need to identify the impact of biosolids land application on antibiotic resistance in agricultural soils and food crops. In the absence of such knowledge, the promise of empirically derived strategies to slowing or reversing the rise of illness, disability, and death due to infections with enteric antibiotic-resistant bacteria will likely remain unfulfilled.

The overall objective of this study is to investigate the prevalence and persistence of antibiotic-resistant *E. coli* in biosolids-amended soil and on harvested crops. Resistance to tetracycline and ampicillin are measured as they are often observed in the US wastewater systems. Rifampicin-resistant *E. coli* inoculum are added to the soil to distinguish between the indigenous and added resistant genes. Biosolids are obtained from local wastewater treatment plants to study the impact of treatment on the fate of antibiotic resistance in a controlled greenhouse system. At the completion of this study, we expect to have determined the impact of biosolids land application on the prevalence and persistence of enteric antibiotic-resistant bacteria in agricultural soils and on food crops. The outcomes will constitute an important early step towards the development of evidence-based policy strategies to decrease the emergence and spread of illness, disability, and death attributable to enteric antibiotic-resistant infections.

OBJECTIVES:

This study aims to achieve the following objectives using biosolids amendment to soils growing vegetable crops at an OSU greenhouse:

1. ***Investigate the prevalence, growth, and persistence of indigenous antibiotic-resistant total bacteria and inoculated (i.e. non-indigenous) antibiotic-resistant E. coli in biosolids amended soils, with respect to soil amendment and irrigation.***
   Our working hypothesis is that application of biosolids to soils promotes the prevalence of antibiotic resistance in soils and food crops.

2. ***Determine the environmental hotspots for the emergence of antibiotic resistance in soils and food crops after biosolids application.***
   Our working hypothesis is that environmental stressors such as neutral pH and certain soil types impact their persistence and dissemination.

PROCEDURES:

Lab protocols were developed and tested over winter 2017 in a greenhouse at Oregon State University. The greenhouse is constructed of solid concrete floors (700 sq-ft) with steel mesh grid tables (5′ × 15′). 4′ × 4′ growth trays are placed on mesh grid tables to collect and contain contaminated runoff. Polyvinyl chloride pipe cages with mosquito netting were constructed around each tray to prevent flying insects from accessing contaminated plants and soil. In the greenhouse, the ambient temperature is maintained by thermostatic control. We obtained soil from commercial agricultural fields with no history of biosolids/manure amendment from the Willamette Valley area in Oregon. Soil is hand ground and distributed in 300-mL growth cups or 2-gallon growth pots.

Starting spring 2017, a preliminary study was performed in an OSU greenhouse. Soil cups were prepared in different treatment categories in growth cups: no treatment vs. inoculation with rifampicin-resistant *E. coli* vs. biosolids amendment, no irrigation vs. irrigation every 3-4 days. Soil samples were collected before and after irrigation every 3-4 days. *E. coli*, rifampicin-resistant *E. coli*, aerobic plate counts (APCs, i.e., total bacteria), and tetracycline- and ampicillin-resistant

APCs were enumerated over 10 sampling days. Collected data was used to design and perform a further study in summer.

In summer 2017, to evaluate the impact of soil contamination through biosolids application, we investigated the persistence of enteric antibiotic-resistant bacteria in soils amended with biosolids. Soil samples were distributed in growth cups in a greenhouse study. A subset of cups was amended with biosolids obtained from the Corvallis wastewater treatment facility and a subset was inoculated with rifampicin-resistant *E. coli*. Growth cups were irrigated every 3-4 days with autoclaved distilled water. We tested triplicates of each treatment on each sampling day. *E. coli*, rifampicin-resistant *E. coli*, APCs, and tetracycline- and ampicillin-resistant APCs were quantified.

In summer 2018, we investigated the impact of biosolids soil amendment and wastewater irrigation on antibiotic-resistant bacteria in soil and on food crops in a greenhouse study. Soil was hand ground and distributed in growth pots. For treatment combinations with biosolids amendment, biosolids were added and mixed. Carrot seeds were planted in growth pots, and irrigated as needed with autoclaved water or wastewater effluent. Pots were prepared in triplicates for each treatment. Total and antibiotic-resistant (tetracycline, ciprofloxacin, and sulfamethoxazole) *E. coli* and *Enterococci* were quantified weekly in soil cores and at harvest on carrots.

SIGNIFICANT ACCOMPLISHMENTS:

Summer 2017

The concentrations of *E. coli*, rifampicin-resistant *E. coli*, APCs, or tetracycline-resistant APCs did not vary significantly during the 23 days of the study (*Figure 1*). Results demonstrate the persistence of the target bacteria. Biosolids amendment and inoculation with antibiotic-resistant *E. coli* increased all target bacteria levels by at least 2 log units. The increased concentrations remained relatively constant throughout the study period. This suggests higher contamination in the soil environment via amendment or irrigation can promote the persistence of enteric antibiotic-resistant bacteria. Further studies, as proposed under *procedures*, will identify how biosolids amendment in agricultural practices impacts the abundance, diversity, and dissemination of antibiotic-resistant bacteria in soils and on food crops. Collectively, these preliminary data confirm that antibiotic resistance persists in soil in high concentrations through the application of biosolids.

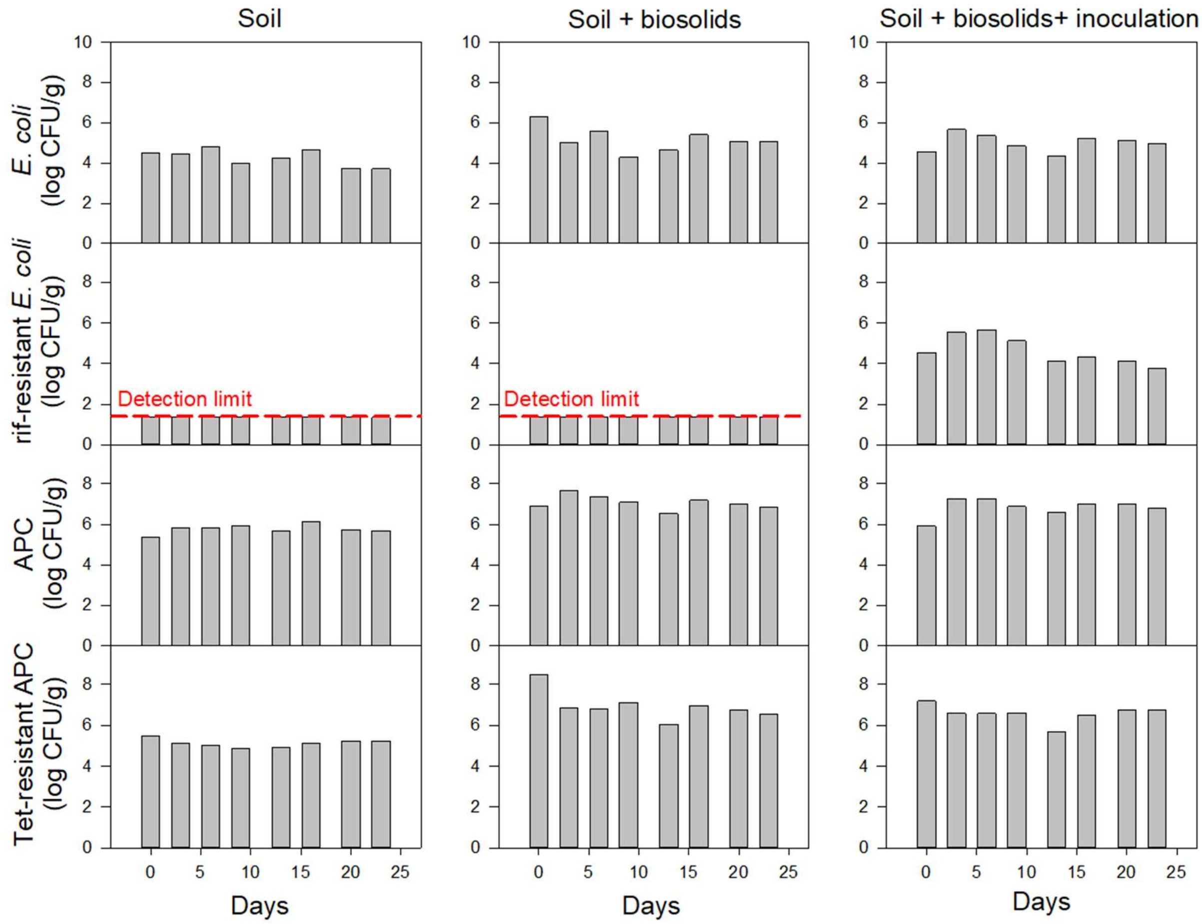

Figure 1. Prevalence and persistence of *E. coli*, rifampicin-resistant *E. coli*, aerobic plate counts (APCs; total bacteria), and tetracycline-resistant APCs in soil, soil + biosolids, and soil + biosolids with rifampicin-resistant *E. coli* inoculum monitored over 23 days. Counts are in logs of colony forming units (CFU) per gram of dry solids, and are averages of three replicates (standard errors not shown).

Summer 2018

Biosolids soil amendment increased total and resistant *E. coli* (*Figure* 2) and *Enterococci* (data not shown) concentrations by 3-5 log CFU per gram solids, whereas wastewater irrigation did not significantly impact the levels. Concentrations declined slowly to 2-3 log CFU per gram solids for *E. coli* and to the detection limit for *Enterococci*. *E. coli* was present on harvested carrots at 1.6 ± 1.2 log CFU per gram, independent of soil amendment or irrigation water. These results suggest a decline – but not cessation – of antibiotic-resistant enteric bacteria in soils irrigated or amended with wastewater and biosolids. The vegetable crops grown under the tested conditions can still harbor these resistant bacteria and genes, making them a reservoir and source of antibiotic resistance and a potential threat to public health.

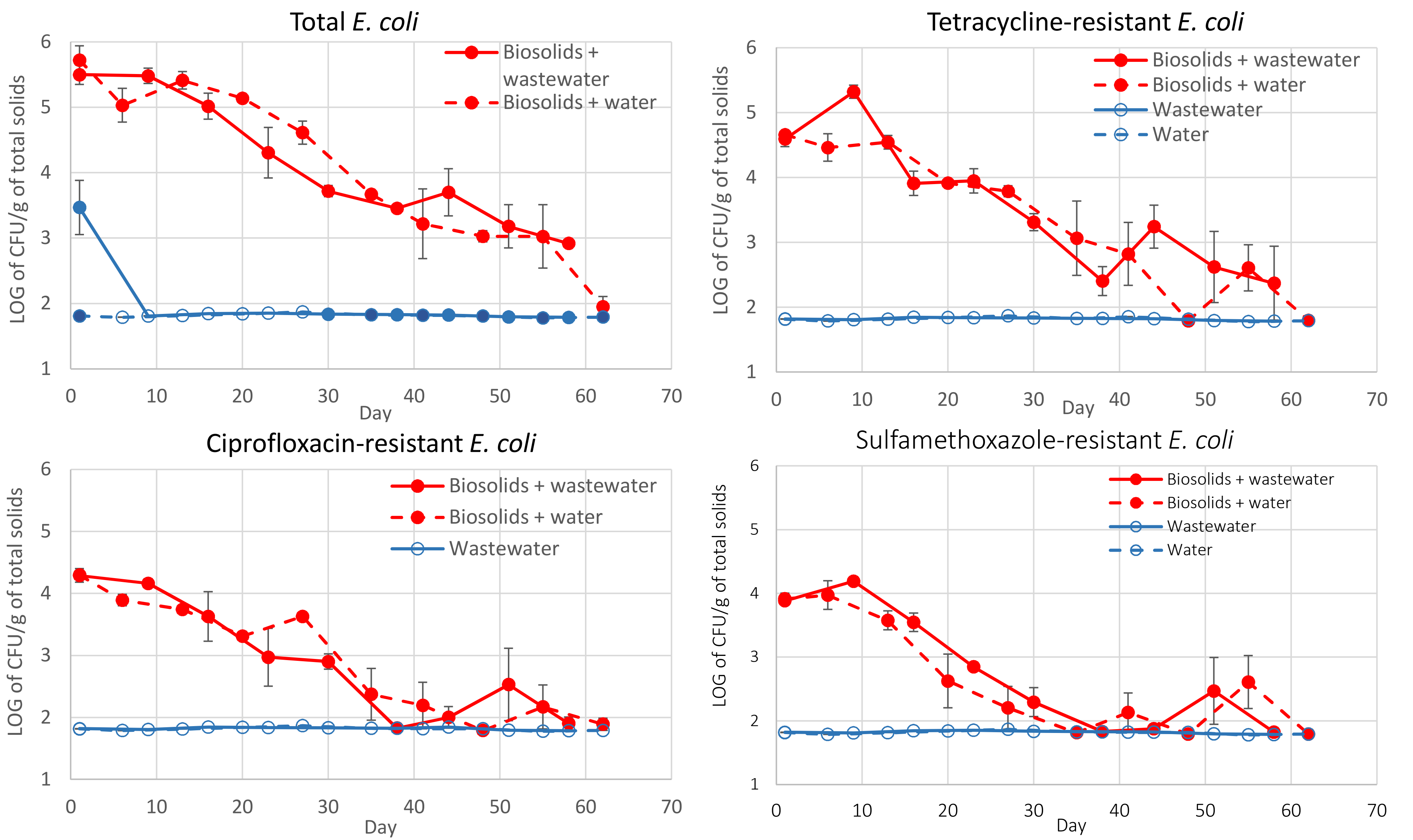

Figure 2. Concentration of *E. coli*, tetracycline-resistant *E. coli*, ciprofloxacin-resistant *E. coli*, and sulfamethoxazole-resistant *E. coli* in soil amended with biosolids and/or irrigated with wastewater or water.

BENEFITS & IMPACT:
The increased disease burden of the emergence of infections with enteric antibiotic-resistant bacteria is of global concern. In the U.S., annually more than two million people are infected by antibiotic-resistant infections resulting in over 23,000 deaths (CDC 2013). The economic cost of antibiotic resistance to the U.S. economy is an estimated $20 billion in direct healthcare costs, with an additional $35 billion indirect costs (CDC 2013). Such reports are of particular public concern due to their high morbidity and mortality, and global dissemination. Municipal wastewater is one of the major sources of antibiotic resistance, with reported resistant residues in effluent and biosolids as well as the receiving environments including surface waters, agricultural soils, and food crops. This study provides insights in the gap on the impact of wastewater and biosolids application in agriculture on antibiotic resistance in soils and food crops. We have identified the persistence of antibiotic-resistant enteric bacteria in soils irrigated or amended with wastewater and biosolids. The vegetable crops grown under the tested conditions can still harbor these resistant bacteria and genes, making them a reservoir and source of antibiotic resistance and a potential threat to public health.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:
Findings obtained from this study were the foundation for my successful proposal “Antibiotic resistance from wastewater to agricultural soils and food crops” to the U.S. Department of Agriculture, Agriculture and Food Research Initiative ($455,000) in 2017.

FUTURE FUNDING POSSIBILITIES:
None at this time.