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# Assessing uptake of antimicrobials by *Zea mays* L. and prevalence of antimicrobial resistance genes in manure-fertilized soil

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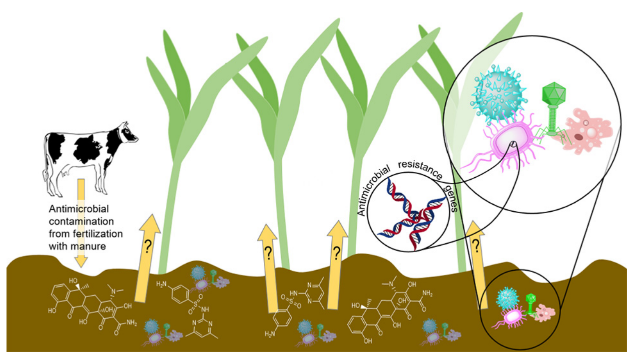
## HIGHLIGHTS

- Sulfonamides and tetracyclines in manure-ammended soil are taken up by plants, and bioaccumulated in plant shoots.
- Plant uptake factors of sulfonamides are over 10-fold higher than tetracyclines.
- No antimicrobial resistance genes were detected in *Zea mays*.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Manure-borne antimicrobials and antimicrobial resistance genes (ARGs) are of environmental concern due to their potential to be transferred into the food-web via plant-uptake. In this study, *Zea mays* L. seeds were grown in three different soil conditions: soil without dairy manure, dairy manure-amended soil, and antimicrobial spiked dairy manure-amended soil, to investigate the potential uptake of antimicrobials and ARGs present in manure. The antimicrobial spiked manure consisted of dairy manure fortified with 1 mg/Kg of each individual antimicrobial compounds belonging to the sulfonamide and tetracycline classes. Samples of the *Zea mays* L. plants were harvested over the course of three weeks to determine potential uptake of antimicrobials from soil to plant shoots, and to compare prevalence of ARGs in manure amended soils and plant tissue. Antimicrobial analysis was performed using liquid chromatography with tandem mass spectrometry (LC-MS/MS), and ARGs (*sul1*, *tetO*, and *OXA-1*) were analyzed using quantitative polymerase chain reaction (qPCR). The study found that both tetracycline and sulfamerazine antimicrobials bioaccumulated in the *Zea mays* L., reaching concentrations of nearly 3000 ng/g and 1260 ng/g, respectively. Tetracycline residues predominated in the soil, while sulfonamides had mainly bioaccumulated in *Zea mays* L. tissue. The greatest average uptake factor within the *Zea mays* L. tissue was 8 for tetracyclines and 110 for sulfonamides indicating larger bioaccumulation of sulfonamides. Additionally, three ARGs (*sul1*, *tetO*, and *OXA-1*) were detected in the soil, only after manure application. However, ARGs were not detected in any of the plant samples.



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## 1. Introduction

Both human and veterinary pharmaceuticals are introduced to the environment through a variety of pathways. Wastewater treatment plants can provide a significant source of pharmaceuticals into the environment, for these treatment systems were not originally designed to remove organic contaminants (Kostich et al., 2014). Other important routes of pharmaceutical release include: wastewater irrigation, biosolid fertilization, or land application of animal manure (Ding et al., 2011; Grossberger et al., 2014; Chiffre et al., 2016). Pharmaceutical compounds have been widely detected in aquatic environments and have been shown to accumulate in plants, where their environmental presence can be a cause for concern as some pharmaceuticals have been found to have adverse effects on aquatic or plant life (Wu et al., 2015; Carter et al., 2015; Carter et al., 2014; Cortés et al., 2013; Wu et al., 2014; Paltiel et al., 2016; Arnnok et al., 2017; Niemuth and Klaper, 2015).

Due to its high nutrient content and availability, dairy manure is often applied in agricultural fields as fertilizer. However, with the large amounts of antimicrobial use in food-producing animals, land application of dairy manure can be a significant point source for antimicrobial release into the environment. The animal antimicrobial drug market reached $3.3 billion globally in 2013 and is expected to exceed $4.1 billion in 2018 (marketsandmarkets.com). It has been estimated that 50–80% of tetracyclines and 20–50% of sulfonamides are excreted unmetabolized by livestock (Merck & Co. Inc, 1955–2018). The growth of the antimicrobial industry is widely attributed to the transition from traditional farms to concentrated animal feeding operations (CAFOs) to meet growing food demands. Since the 1950s, farm operations have become fewer in number but larger in size (FDA, 2016). The production of livestock and poultry in the United States (U.S.) has more than doubled in the past decade; however, the number of operations has decreased by 80% (EPA, 2013). Thus, the use of antimicrobials to prevent the spread of diseases among animals raised in closed confinements is inevitable. Tetracyclines and sulfonamides are both widely used antimicrobials for livestock in the U.S., with nearly 5.9 million, and 0.4 million kg being sold for livestock use in 2016, respectively (FDA, 2016). The increase in antimicrobial administration and land-application of manure can contribute to the proliferation of antimicrobials and antimicrobial resistance in the environment (Singer et al., 2016).

The impact of antimicrobial contamination on the prevalence of antimicrobial resistance genes (ARGs) in the environment is not fully understood. However, continued release of antimicrobials into the ecosystem is generally considered to be closely linked to reported increases in antimicrobial resistance in the environment (Vikesland et al., 2017). At trace levels, residual antimicrobials present in the environment can apply selective pressure on microbial communities to initiate resistance expression behavior (Heuer et al., 2011). Conferred genetic resistance can be propagated across bacterial species by mobile genetic elements such as plasmids and insertion sequences, increasing the likelihood of bacterial species harboring multiple genetic resistance mechanisms (Davies and Davies, 2010; Wellington et al., 2013).

Antimicrobials present in dairy manure can be taken up by plants. *Zea mays* L. is of particular interest, because the U.S. is the largest producer of *Zea mays* L. in the world. In the 2016–2017 crop marketing year, the U.S. grew over 385 million metric tons of *Zea mays* L. (Keller, 2017/2018). The USDA reported in 2009 that 15.8 million acres of cropland, or about 5% of all U.S. cropland is fertilized with livestock manure (James et al., 2009). Previous studies have been performed on plant-soil systems with both sulfonamides and tetracyclines. Sulfadimethoxine has been found in barley roots and leaves after growing in pig manure fertilized soil (Kumar et al., 2005). Uptake of sulfadiazine and its effects in common hazel was investigated after being grown in sulfadiazine-fortified soil at concentrations ranging from 0.01–100 mg/kg. Sulfadiazine was detected mostly in the plant roots, and the roots were shorter and dark at concentrations above 10 mg/kg. Detection was also confirmed in the leaves of plants exposed to sulfadiazine at a concentration of 100 mg/kg soil (Michelini et al., 2015). The accumulation of tetracycline in pea seedlings grown in soil with concentrations up to 150 mg/kg was examined, and up to 20 μg/g was detected in the pea seedling stem and leaves (Margas et al., 2016). Another study investigated the accumulation and distribution of tetracyclines and sulfonamides in cucumber, tomato, and lettuce grown in 5–20 mg/kg spiked soil and found that the nonedible components were higher in concentration than the fruit (Ahmed et al., 2015). Dolliver et al. reported that the highest amount of sulfamethazine was detected in lettuce followed by potato and corn after growing in manure amended soil (Dolliver et al., 2007). Increasing levels of antimicrobials in the environment are of concern for overall plant health: a study evaluating the effects of sulfadiazine on willow and *Zea mays* L. plants found that growing the plants in 200 mg/kg sulfadiazine-fortified soil caused serious stress in willow (e.g., reduced C/N ratio and total chlorophyll content) and the death of *Zea mays* L., demonstrating that sulfonamides can potentially impair plant growth and biomass at high concentrations (Michelini et al., 2012).

Previous research indicated that ARG prevalence increased in soil after fertilization with either dairy or chicken manure, most likely due to an increase in selective pressure near the minimum inhibitory concentrations for bacteria (Li, 2017). Investigative studies have shown presence of class 1 and 2 integrons as well as $bla_{CTX\text{-}M\text{-}15}$ in *Pseudomonas* spp. isolated from the surfaces of lettuce and packaged spinach, suggesting the presence of antimicrobial resistant bacteria (ARB) on the surface of vegetables (Bezanson et al., 2008; Raphael et al., 2011). The detection of ARGs, including *tetX*, $bla_{CTX\text{-}M}$, *sul1*, and *sul2*, were previously reported in endophytic DNA extracted from pakchoi (Chinese cabbage) that had been exposed to 50% of the MIC of tetracycline, cephalexin, and sulfamethoxazole under hydroponic growth conditions. The ratio of antimicrobial-resistant endophytic bacteria to total cultivable endophytic bacteria significantly increased as the antimicrobials accumulated in the plants (Zhang et al., 2017). These recent studies describe the appearance of ARGs from bacteria found in the endophyte portion of plants or from surface of crops.

This study aims to investigate the occurrence of antimicrobials and ARGs in young *Zea mays* L. plants by investigating both antimicrobial uptake and prevalence of ARGs in *Zea mays* L. that have been grown in manure-amended soil. Previous studies investigating young crops (6–8 weeks) have only investigated the uptake of antimicrobials, but did not investigate the occurrence of ARGs contributed by manure-amended soil (Kumar et al., 2005; Dolliver et al., 2007). Specifically, this study investigates *Zea mays* L. grown under three different treatment conditions: (1) *Zea mays* L. grown in a treatment plot of only soil (ST), (2) *Zea mays* L. grown in soil and unspiked manure treated plot (SUMT), and (3) *Zea mays* L. grown in soil and spiked manure treated plot (SSMT). This study provides information regarding the time-dependent fate of antimicrobials and ARGs in crops that have been fertilized using dairy manure containing veterinary antimicrobials.

## 2. Materials and methods

### 2.1. Materials

The following standards were obtained from Sigma Aldrich: sulfadiazine (SPD), sulfamethoxazole (SMX), sulfameter (SMT), sulfamerazine (SMR), sulfamethizole (SMI), sulfadimethoxine (SDM), sulfachloropyridazine (SCP), sulfamethazine (SMZ), and tetracycline (TC). Oxytetracycline (OTC), Chlortetracycline (CTC), 4-epichlorotetracycline (ECTC), anhydrochlorotetracycline (ACTC), and anhydrotetracycline (ATC) were purchased from Acros Organics. Sulfathiazole (STZ) was purchased from ICN Biomedicals, Inc. Epitetracycline (ETC) was purchased from Spectrum Chemical Mfg. Corp. Isotopically labeled compounds of $^{13}C_6$-sulfamethazine,