IMPACT OF MANURE MANAGEMENT PRACTICES ON THE ENVIRONMENTAL FATE OF ANTIBIOTICS IN MANURE-APPLIED FIELDS

Hanh Thi Van Le

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Kang Xia, Committee Chair

Rory O. Maguire

Chao Shang

Amy Pruden

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ACADEMIC ABSTRACT

Antibiotics and antibiotic resistance genes from animal manure applied to soil as fertilizer are now among the most concerned contaminants in soil. The widespread use of antibiotics in livestock might amplify the risk of developing antibiotic resistance, causing once treatable diseases to turn deadly. The World Health Organization declared antibiotic resistance as "one of the biggest threats to global health, food security, and development". The goal of this dissertation was to develop best manure management practices by understanding the behavior of manure-associated antibiotics in manure, water, and soil. In particular, my research focused on the effects of manure application methods, on-site manure treatment methods, manure application seasons, and manure-rainfall time gaps on antibiotic surface runoff losses, antibiotic distribution and movement in soil, antibiotic dissipation in soil, and development of antibiotic resistance. Rainfall simulation field-scale and soil incubation lab studies were combined to find the best manure management practices. My research has shown for the first time that using the manure soil subsurface injection method, especially during spring application season due to moist soil, applying manure at least 3 days before a subsequent rainfall, and using composted manure, can significantly reduce the quantity of antibiotic loss with runoff from manure-applied fields to the surrounding environment. The majority of applied antibiotics remained in soil. All antibiotics showed a similar dissipation pattern with fastest kinetics during the first 14 d before slowing down. The effect of two manure application methods on antibiotic dissipation kinetics varied with different antibiotics. Although the half-life of tested antibiotics in soil was short (<21 days),

some remained detectable even at 6 months after a single manure application. Results also showed that compared to the surface application, the subsurface injection slits acted as a “hot zone” with a higher amount of antibiotics, manure microbes, and antibiotic resistance. The results provide information for policy makers, manure managers, and farmers to develop better manure management practices that can use manure as fertilizer while minimizing the spread of antibiotics to surrounding water, soil, and plants.

Impact of Manure Management Practices on the Environmental Fate of Antibiotics in Manure-Applied Fields

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GENERAL AUDIENCE ABSTRACT

There is growing concern about antibiotic resistance as a serious human health threat because a resistant infection may kill, can spread, and increases health costs. Every year in the United States, there are 2 million people infected with antibiotic resistant bacteria, 23,000 people die as a direct result of these infections, and $55 billion is lost due to increased hospital stay and lost work days. Although bacteria naturally develop the ability to resist antibiotics, the problem is the length between antibiotic introduction and resistance development is shortening because of the widespread and overuse of antibiotics, especially in the livestock industry.

The goal of this study was to develop the best manure management practices balancing the benefits of antibiotics in livestock and animal manure and their impact on the environment. In particular, we monitored, using field-scale and laboratory studies, the effects of manure application methods, on-site manure treatment methods, manure application seasons, and manure-rainfall time gaps on antibiotic loss through surface runoff, antibiotic distribution and movement in soil, antibiotic dissipation in soil, and development of antibiotic resistance.

In order to reduce the amount of antibiotic loss with surface runoff from manure-applied fields to the surrounding environment, farmers are recommended to 1) compost manure before application, 2) watch the forecast to apply manure at least 3 days before a subsequent rainfall, and 3) use the subsurface injection method, especially when the soil is wet (spring season). The majority of applied antibiotics remained in soil. All tested antibiotics showed a similar dissipation pattern with the fastest rate during the first two weeks after manure application, then

slowing down. Although the half-life of tested antibiotics in soil was short (<21 days), some remained detectable even at 6 months after a single manure application. Besides, the subsurface injection slits acted as a hot zone with a concentrated amount of antibiotics, manure microbes, and antibiotic resistance. The results provide recommendations for policy makers, manure managers, and farmers to maximize benefits of manure as fertilizer while minimizing the spread of manure-associated antibiotics to surrounding water, soil, and plants.

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Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Background

In the United States, it is estimated that 70-80 % or more of total antibiotic products are used to raise animals (FDA, 2011, 2012). In the livestock industry, antibiotics have been used at therapeutic levels to control diseases and sub-therapeutic levels to help animals absorb food better and prevent negative effects from crowded and unsanitary living conditions. The most widely-used veterinary antibiotics are ionophores, tetracyclines, macrolides, lincosamides, polypeptides, penicillins, sulfonamides, aminoglycosides, and flouroquinolones (Chee-Sanford et al., 2009). Four antibiotics studied in this dissertation included chlortetracycline (a tetracycline), sulfamerazine (a sulfonamide), tylosin (a macrolide), and pirlimycin (a lincosamide), because of their common use in livestock industry. Some characteristics of these antibiotics are summarized in Table 1.1.

Animals do not absorb all administered dose, 40-95 % of antibiotics are excreted in feces and urine, depending on types of antibiotics, used dosage, treated animals, routes of administration, excreting species, and time after administration (Boxall, 2008; Jechalke et al., 2014) (Fig. 1.1). For example, tetracyclines and aminoglycosides are generally not significantly metabolized in the body and primarily eliminated from animals in unchanged forms (Boxall, 2008; Noreddin, 2012). For pirlimycin, when it was administered to dairy cows, about 24 % and 10 % of the initial dose were excreted in feces and urine, respectively, as an unchanged compound and 80 % and 45 % of pirlimycin in urine and feces, respectively, was excreted as compound conjugates (Hornish et al., 1992). In fact, a wide range of antibiotics has been detected in manure at a level as high as tens to hundreds mg kg^{-1}, for example, 20 mg kg^{-1} of

sulfonamides in liquid manure (Haller et al., 2002), 23 mg kg^{-1} of chlortetracycline in poultry manure, 972 mg kg^{-1} of oxytetracycline, and 116 mg kg^{-1} of tylosin in fresh cattle manure (He and Zhang, 2014).

Antibiotics can remain unchanged, partially degraded, or increase due to deconjugation of metabolites back to parent compounds during storage period in stockpiles or lagoons before being land-applied. The half-life of antibiotics varied from 30 d or less to many months (Table 1.2), depending on physicochemical properties of antibiotics as well as manure/slurry types (Boxall, 2008). Since lagoons are usually emptied every 6 months, antibiotics can be presented at land application time. **One approach to reduce the spread of antibiotics from manure to the surrounding environment is composting raw manure**, a method driven by activities of microbes. Composting has shown a varied effect on antibiotic removal efficiency, from a complete removal to no removal (Dolliver and Gupta, 2008; Ray et al., 2017; Zhang et al., 2019). Temperature, moisture, pH, and the C:N of manure during composting are important factors influencing transformation of antibiotics during composting. For antibiotics with an incomplete removal, they are released to soil following application of composted manure. **Research comparing the effect of raw manure versus composted manure from animals with antibiotic administration on antibiotic behaviors in soil is lacking.** For recalcitrant antibiotics with no removal during composting, their levels in raw manure and composted manure are similar. However, due to different physicochemical and biological properties of two amendment types (Miller et al., 2008; Pankow, 2017), the environmental fate of antibiotics in soil receiving raw manure and composted manure with the same antibiotic initial mass might not be similar. Generally, compared to fresh manure, composting decreases the C:N ratio, total N, NH_4-N, and total C, but increases NO_3-N (Miller et al., 2008).

Table 1.1 Physicochemical of studied antibiotics.

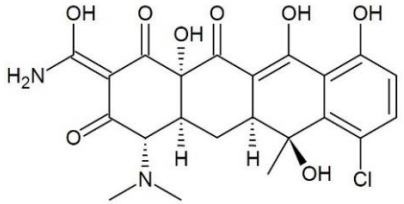
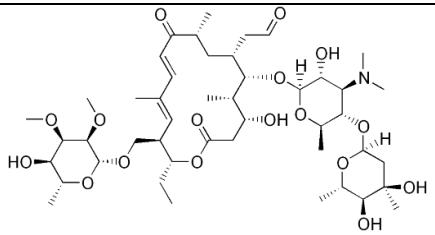
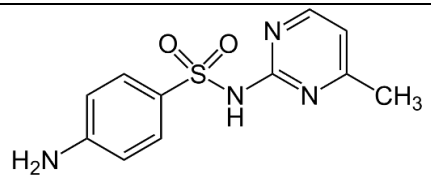
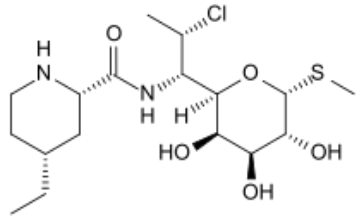
Antibiotic	Structure	Water solubility (mg L^{-1})	Log K_{ow}	pK_a	K_d (L kg^{-1})	Characteristic	Reference
Chlortetracycline		500-600	-0.62	3.3/ 7.6/ 9.3	1280 (sandy loam) 2386 (clay loam)	Naturally found, broad-spectrum, protein synthesis inhibitor, and strongly-sorbed to soil.	(Chee-Sanford et al., 2009; Dolliver and Gupta, 2008)
Tylosin		5,000	1.63-2.5	3.3/ 7.5	8.3 (loamy sand) 62.3 (sandy loam) 66 (sandy loam) 128 (sandy loam) 92 (clay loam)	Naturally found, feed additive, broad-spectrum, bacteriostatic, protein synthesis inhibitor, and highly lipid soluble.	(Dolliver and Gupta, 2008; Noreddin, 2012)
Sulfamerazine		202				Bacteriostatic and bacterial synthesis of dihydrofolic acid inhibitor.	(NCBI, 2017)
Pirlimycin		70,000 (pH 4.5) 3,000 (pH 13)		7.6	0.8 (sandy soil) 2.2 (clay soil)	Semi-synthetic, active against most gram-positive bacteria, bacteriostatic, bacterial protein synthesis inhibitor, and mastitis treatment.	(Le et al., 2018; Noreddin, 2012; USFDA, 1993)

Table 1.2 Half-life of some antibiotics in animal excretion (Boxall, 2008)

Antibiotic group	Compound	Matrix	Half-life (d)
Macrolides	Tylosin	Pig slurry	<2
	Erythromycin	Liquid manure	41
	Roxithromycin	Liquid manure	130
Sulfonamides	Sulfachloropyradizine	Broiler feces	>8
	Sulfachloropyradizine	Laying hen feces	>90
	Sulfachloropyradizine	Pig slurry	>8
Tetracyclines	Chlortetracycline	Chicken manure	>30
	Oxytetracycline	Cattle manure	<30

Manure application methods have been improved to fertilize soil and crops as well as to reduce negative impacts of manure on the environment. Broadcasting manure onto a field is a traditional method with a low cost and fast operation; however, it can cause nutrient losses through volatilization and surface runoff. Tillage following manure broadcasting can reduce these losses by moving manure under the soil surface. However, it can increase soil structure disturbance, leading to increased soil erosion and carbon loss, and can increase soil compaction, making it difficult for rainfall to infiltrate. According to an estimate in 2012, agriculture was the single largest source of nitrogen (42 %), phosphorous (58 %), and sediment (58 %) entering the Chesapeake Bay watershed (Chesapeake Bay Program, 2016). To avoid agricultural runoff into the Chesapeake Bay, more than 80 % of crop areas are in no till or reduced tillage (USDA-NRCS, 2011). The need to apply manure into soil and leave crop residue and forage on the surface to protect soil from erosion led to a new manure application method, manure soil subsurface injection. This method can significantly reduce nutrient losses via ammonia volatilization and runoff, minimize soil disturbance, and substantially increase nutrient and water use efficiency (Maguire et al., 2013; Maguire et al., 2011). However, **it is less known if this**

method can also reduce output of emerging contaminants associated with animal manure, such as antibiotics, from manure-applied fields.

Soil receiving animal manure can be a critical medium for spreading manure-associated antibiotics to the surrounding environment. A wide range of antibiotics has been detected in manure at a concentration as high as tens to hundreds mg kg^{-1} (Haller et al., 2002; He and Zhang, 2014). The amount of antibiotics entering agricultural soils from animal excrements as fertilizer has been up to kilograms per hectare (Kemper, 2008). From soil, antibiotics can find ways to surface water through surface runoff and groundwater through leaching. Carbadox, florfenicol, and chlortetracycline were detected at the highest values of 1,577 ng L^{-1}, 666 ng L^{-1}, and 570 ng L^{-1}, respectively in Kyungahn stream in South Korea, which received runoff from agricultural areas and livestock farms in the upper stream area (Kim et al., 2016). Fourteen antibiotics in 125 stream samples of an agricultural watershed in Southern Ontario, Canada were detected with the prevalence of lincomycin, monensin, carbamazepine, and sulfamethazine at a median concentration of 44 ng L^{-1} (Lissemore et al., 2006). Amprolium and monensin appeared in half of 109 water samples from 11 farm drainage tile channels and surface ditches in Lansing (Michigan, US) with an average concentration of 288 and 189 ng L^{-1}, respectively (Song et al., 2010).

The majority of manure-associated antibiotics remained in soil after manure application (Le et al., 2018; Pan and Chu, 2017; Spielmeyer et al., 2017). Because of low vapor pressure, antibiotic volatilization is insignificant. Antibiotic losses via surface runoff or leaching account for a small percentage of the initial amount. Dolliver and Gupta (2008) found that less than 5 % of applied antibiotics were lost in 3 years via surface runoff and antibiotic losses via leaching were even lower. Studies reported that surface runoff of antibiotics mostly occurred during the

first rainfall after manure application, resulting in 0.45 to 2.62 % of applied antibiotics leaving the field (Kulesza et al., 2016; Le et al., 2018). In addition, compared to the traditional manure surface application, the manure subsurface injection method is recommended to reduce surface losses of manure-associated antibiotics, probably due to the nature of the methods (Kulesza et al., 2016; Le et al., 2018). While manure is spread evenly on soil following the surface application method, manure is buried below the soil surface and concentrated in injection slits of the subsurface injection. Therefore, compared to soil where manure has been surface-applied, soil in the injection slits may have an elevated level of nutrients, organic matter, water, manure microbes, and antibiotics, which all can affect the environmental fate of antibiotics in soil. However, **effects of these two manure application methods on antibiotics in soil are unknown**.

There are various and complex soil processes determining the environmental fate of antibiotics including persistence, mobility, and impact. Mineralization to CO_2 accounts for less than 2 % of the initial amount for sulfadiazine (Schmidt et al., 2008). Photodegradation of antibiotics in soil is also limited, especially in sub soil due to poor light penetration (Ozaki et al., 2011). Transformation of antibiotics in soil has been reported to be mainly microbial driven (Accinelli et al., 2007; Pan and Chu, 2016). Sorption of antibiotics to soil particles can reduce their mobility, reactivity, and bioavailability for biodegradation (Jechalke et al., 2014). The sorption/desorption of antibiotics to soil particles varies widely depending on the properties of the antibiotics, soil, and other environmental conditions. For antibiotics, it depends on the molecular structure, size, shape, solubility, and hydrophobicity (Kemper, 2008). For example, for sulfonamides, adsorption to soil increases with aromaticity and electronegativity of functional groups (Thiele-Bruhn et al., 2004). For soil, the most important factors include soil texture,

quantity and quality of soil organic matter (SOM), composition of soil minerals, soil pH, soil CEC, and ionic strength (Boxall, 2008). Positively-charged antibiotics might bind to soil minerals (especially montmorillonite with high surface areas and swelling capacity) and SOM through electrostatic attraction or cation exchange (Gao and Pedersen, 2005). Meanwhile, negatively-charged antibiotics can form complexes with cations that are adsorbed on negatively-charged soil constituents; the cation bridging enables the pharmaceuticals to be retained in soils (Tolls, 2001). Also, anion antibiotics can strongly bind to iron and aluminum oxides/hydroxides, which are common in highly-weathered and acidic soils in the Southeast United States.

Following land application, antibiotics in soil degrade over time. Biotransformation by the microorganisms is an efficient mechanism to reduce chemical persistence (Accinelli et al., 2007; Pan and Chu, 2016). Other degradation routes are via photodegradation and hydrolysis. Since complete mineralization accounts only for a small portion of the total initial amount (Schmidt et al., 2008), concerns about antibiotics always go together with their transformation products. Most studies reported either no difference or even a decrease in toxicity between the parent compounds and their transformation products (Haddad et al., 2015). Nevertheless, some transformation products can be more persistent and harmful than the parent compounds (Majewsky et al., 2014; Yuan et al., 2011). While there are numerous studies on the environmental fate of antibiotics, **identification and quantification of their transformation products in the environment system are difficult**. First and foremost, formation of transformation products is a complex process, which is regulated by various environmental conditions and a number of transformation products are unknown. For instance, De Laurentiis et al. (2014) reported different transformation products of acetaminophen from photodegradation compared to human metabolism or microbial biodegradation. Chromatographic and mass

spectrometric instruments are commonly used to tentatively identify transformation products. Advances in high-resolution mass spectrometry such as QTOF, Orbitrap and FT-ICR have made the process easier; however, interpretation of mass spectra can be challenging (Lopez et al., 2014). Besides, the proposed structures can only be confirmed by comparing with reference standards, which are often unavailable. Finally, the majority of studies were conducted in a simplified condition and used a much higher concentration level of parent compounds than their environmental level, which to some extent hinders what happens in the real environment. Detecting transformation products at trace levels in complex matrices still needs further studies.

The presence of antibiotics and especially antibiotic resistant bacteria, antibiotic resistance genes, and associated mobile genetic elements could enhance the resistant levels of soil microorganisms due to vertical and horizontal gene transfer (Gullberg et al., 2011; Heuer and Smalla, 2007; Munir and Xagoraraki, 2011). Soil is known as a natural reservoir of diverse antibiotic resistant bacteria and antibiotic resistance genes, but the antibiotic resistance levels in background soil would be significantly lower than in antibiotic-treated manure (Munir and Xagoraraki, 2011; Thiele-Bruhn, 2003). Under selective pressure due to an elevated level of antibiotics, soil native resistance populations might survive and increase. Another mechanism is transferring resistance via mobile genetic elements such as bacteria IncQ plasmids, which were detected in pig manure as well as environmental habitats (Kemper, 2008). Several studies suggested that following manure amendment, levels of antibiotic resistance in soil first increased and after a period of time the abundance of antibiotic resistant bacteria/genes decreased over time (Heuer and Smalla, 2007; Sengeløv et al., 2003). In the study of Heuer and Smalla (2007) applying manure spiked with sulfadiazine increased the abundance of *sul*1 and *sul*2 resistant genes in soil for at least 2 months compared to soil that received manure without sulfadiazine.

Also, there was an increase in frequency of sulfadiazine-resistant-plasmid capture in *E. coli* in manure-amended soil. Higher occurrence of resistance in soil appeared following a higher manure application rate (Lin et al., 2016; Sengeløv et al., 2003). The decrease of antibiotic resistant level could be because manure-borne bacteria cannot adapt well in the soil environment. The long or short period varied depending on properties of antibiotics as well as the environment condition. About one-year period was enough for antibiotic resistance genes and associated genetic mobile elements to approach background levels (Marti et al., 2014).

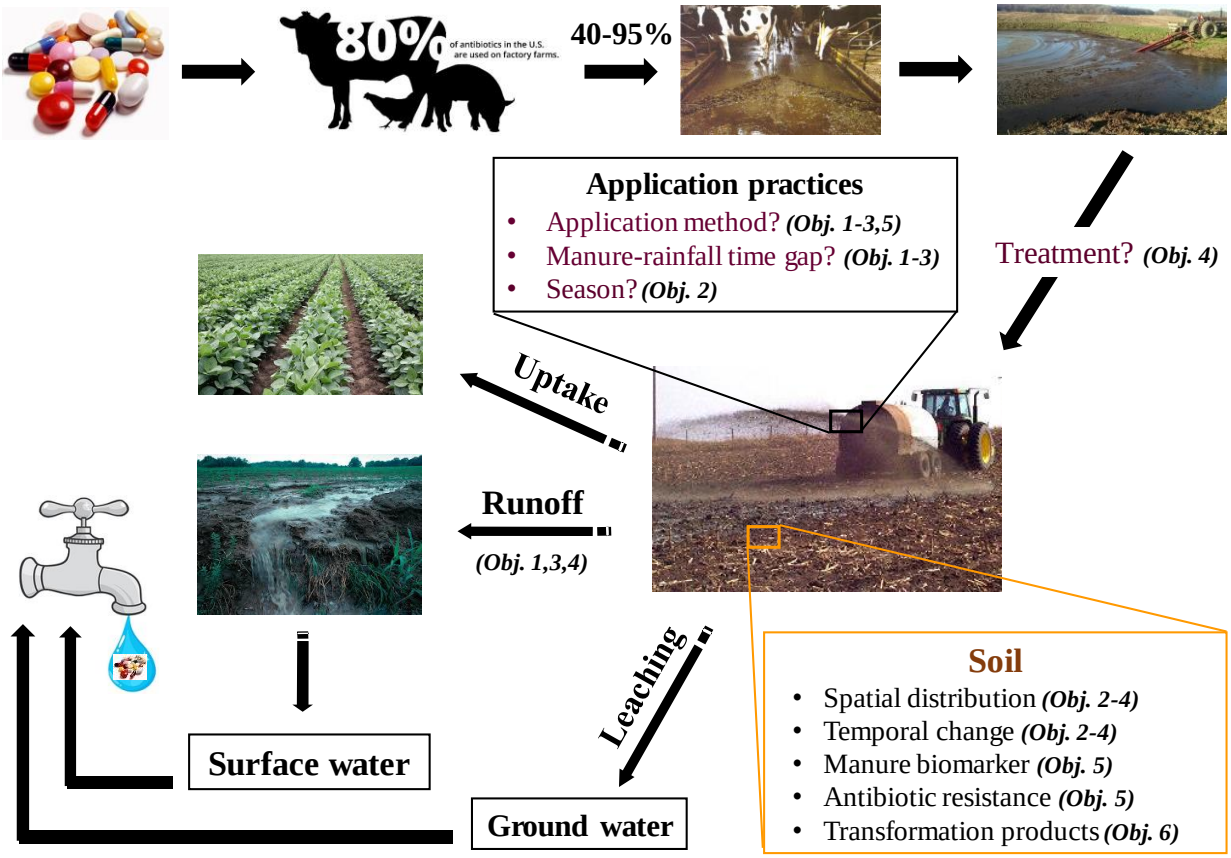


Figure 1.1. Overall concept diagram showing six major objectives of this dissertation.

1.2 Objectives

The overall objective of this dissertation was to understand the effect of different manure management practices on the environmental fate of veterinary antibiotics associated with animal manure. Based on the acquired results, the best manure management practices would be recommended to balance manure benefits as fertilizer and environmental impact from manure-associated contaminants. Field-scale and lab studies were combined to accomplish the following specific objectives with their corresponding chapters (Fig. 1.1).

Objective 1: To investigate (1) how manure soil subsurface injection influences the losses of pirlimycin, chlortetracycline, tylosin, and sulfamerazine in surface runoff water and sediment compared to the traditional surface manure application method; and (2) how the time gap between manure application and a subsequent rain event affect antibiotic losses through surface runoff (Chapter 2).

Objective 2: To monitor how manure application methods and manure-rainfall time gaps affect spatial distribution and temporal change of pirlimycin, chlortetracycline, tylosin, and sulfamerazine in soil in the field conditions (Chapter 3).

Objective 3: To compare the seasonal impact (dry and warm fall versus wet and cold spring) on the environment fate of pirlimycin in fields amended with liquid manure using surface application and subsurface injection (Chapter 4).

Objective 4: To determine the environmental fate of pirlimycin in fields that were subsurface injected with pirlimycin-spiked dairy manure/compost and manure/compost from pirlimycin treated cows (Chapter 5).

Objective 5: To determine co-distribution/change between manure-associated antibiotics and manure biomarker (*Rum-2-bac*) and a mobile genetic element (*IntI*1) in soil (Chapter 6).

Objective 6: To study transformation products of pirlimycin by coupling a computer metabolites prediction software (Eawag-BBD/PPS) with mass spectrometry (Chapter 7).

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Chapter 2: Method of dairy manure application and time before rainfall affect antibiotics in surface runoff

Le, H.T.V., R.O. Maguire, and K. Xia. 2018. Method of dairy manure application and time before rainfall affect antibiotics in surface runoff. Journal of Environmental Quality 47: 1310-1317. DOI: 10.2134/jeq2018.02.0086.

Abstract

Although research has shown that manure soil subsurface injection reduces nutrient input to the aquatic environment, it is less known if it also reduces antibiotic surface runoff from manure-applied fields. Surface runoff of four dairy production antibiotics was monitored comparing a) surface application and subsurface injection of manure and b) time gaps between manure application and a subsequent rain event. Liquid dairy manure spiked with pirlimycin, tylosin, chlortetracycline, and sulfamerazine was applied to 1.5x2 m test-plots at an agronomic N rate via surface application and subsurface injection. On the day of (day 0), and 3 and 7 days after manure application, a simulated rainfall (70 mm h^{-1}) was conducted to collect 30 min runoff. Target antibiotics in runoff water and sediment were quantified using UPLC/MS/MS. Results demonstrated that runoff was a significant route for transporting antibiotics off manure-applied fields, amounting to 0.45-2.62 % of their initial input with manure. However, compared to manure surface application, subsurface injection reduced sulfamerazine, chlortetracycline, pirlimycin, and tylosin losses in runoff by at least 47, 50, 57, and 88 %, respectively. Antibiotic distribution between aqueous and solid phases of runoff was largely determined by water solubility and partition capacity of antibiotics to soil particles. Masses in the aqueous phase were 99±0.5 %, 94±4 %, 91±7 %, and 22±15 % of pirlimycin, sulfamerazine, tylosin, and chlortetracycline, respectively. Manure application three days or longer before a subsequent rain event reduced antibiotic runoff by 9-45 times. Therefore, using subsurface injection and avoiding manure application less than 3 days before rain would be a recommended manure land management best practice.

Highlights

- Compared to surface application, subsurface injection reduced antibiotic runoff.
- ~3 % of antibiotics was lost in 30-min runoff from a manure surface-applied field.
- ~1 % of antibiotics was lost in 30-min runoff from manure subsurface-injected fields.
- Antibiotic distribution in runoff water and sediment was compound dependent.
- Manure application at least 3 days before rain reduced antibiotics in surface runoff.

2.1 Introduction

Manure application methods have been improved to fertilize soil and crops as well as to reduce negative impacts of manure on the environment. Broadcasting manure onto a field is a traditional method with a low cost and fast operation; however, it can cause nutrient losses through volatilization and surface runoff. Tillage following manure broadcasting can reduce these losses by moving manure under the soil surface. However, it can increase soil structure disturbance, leading to increased soil erosion and carbon loss, and can increase soil compaction, making it difficult for rainfall to infiltrate. According to an estimate in 2012, agriculture was the single largest source of nitrogen (42 %), phosphorous (58 %), and sediment (58 %) entering the Chesapeake Bay watershed (Chesapeake Bay Program, 2016). To avoid agricultural runoff into the Chesapeake Bay, more than 80 % of crop areas are in no till or reduced tillage (USDA-NRCS, 2011). The need to apply manure into soil and leave crop residue and forage on the surface to protect soil from erosion led to a new manure application method, manure soil subsurface injection. This method can significantly reduce nutrient losses via ammonia volatilization and runoff, minimize soil disturbance, and substantially increase nutrient and water use efficiency (Maguire et al., 2013; Maguire et al., 2011). However, it is less known if this method can also reduce output of emerging contaminants associated with animal manure, such as antibiotics, from manure-applied fields.

Soil receiving animal manure can be a critical medium for spreading manure-associated antibiotics to the surrounding environment. A wide range of antibiotics has been detected in manure at a concentration as high as tens to hundreds mg kg^{-1} (Haller et al., 2002; He and Zhang, 2014). The amount of antibiotics entering agricultural soils from animal excrements as fertilizer has been up to kilograms per hectare (Kemper, 2008). From soil, antibiotics can find ways to

surface water through surface runoff and groundwater through leaching. Carbadox, florfenicol, and chlortetracycline were detected at the highest values of 1,577 ng L^{-1}, 666 ng L^{-1}, and 570 ng L^{-1}, respectively in Kyungahn stream in South Korea, which received runoff from agricultural areas and livestock farms in the upper stream area (Kim et al., 2016). Fourteen antibiotics in 125 stream samples of an agricultural watershed in Southern Ontario, Canada were detected with the prevalence of lincomycin, monensin, carbamazepine, and sulfamethazine at a median concentration of 44 ng L^{-1} (Lissemore et al., 2006). Amprolium and monensin appeared in half of 109 water samples from 11 farm drainage tile channels and surface ditches in Lansing (Michigan, US) with an average concentration of 288 and 189 ng L^{-1}, respectively (Song et al., 2010). Research has shown toxicity of antibiotics on aquatic organisms such as algae and daphnia at concentrations between 5 and 100 µg L^{-1} (Halling-Sørensen, 2000; Wollenberger et al., 2000). In addition, dissemination of antibiotics into the environment can enhance selection, development, and spread of single, cross-, and even multiple resistance in pathogens either directly or indirectly (Kemper, 2008). Because of a growing demand for animal products of a growing population, antibiotic residues in animal manure are expected to be on an upward trend.

Current manure application methods are designed for capturing nitrogen and phosphate for crops and decreasing their input to water bodies. Little research with inconsistent results has been published about the effects of these application methods on input of manure-borne antibiotics off the field. According to Isensee et al. (1990), organic contaminant losses with leachate under no till were often greater than other conservation or conventional tillage methods due to macroporous persistence. However, Kreuzig et al. (2005) observed that runoff volume and mass loss of sulfonamide with runoff from manure-incorporated arable land were much less than from manure surface-applied grassland. In a 3-yr field study by Dolliver and Gupta (2008), an

average annual loss of tylosin with leachate from the no-tillage treatment was significantly higher than from the chisel tillage treatment for the field which received liquid hog manure, but there was no difference between two manure application methods for the field which received beef manure. For antibiotic losses with runoff, effects of tillage showed no consistent pattern. In one year out of three years, monensin and tylosin were detected in runoff from the no-tillage treatment much more frequently than from the chisel plow treatment. A study by Joy et al. (2013) showed no significant effects of manure land application methods (broadcast, incorporation, and injection) on the concentration of antibiotics (chlortetracycline and tylosin) in runoff water. However, broadcast treatments had the highest total antibiotic mass loss in runoff water compared to incorporated and injected treatments. Kulesza et al. (2016) reported that manure subsurface injection treatments reduced pirlimycin concentrations six and three times in runoff water and sediment, respectively, compared to surface application treatments.

There is a need to reduce antibiotic transport from manure-amended fields. The objectives of this study were to investigate (1) how manure soil subsurface injection, a new application method, influenced losses of pirlimycin, chlortetracycline, tylosin, and sulfamerazine in surface runoff water and sediment compared to the traditional surface application and (2) how the time gap between manure application and a subsequent rain event affected antibiotic losses through surface runoff. Collected results can provide some suggestions for a better manure land management practice in an effort to minimize manure-borne antibiotic transport from manure-applied fields to the surrounding environment through agricultural surface runoff.

2.2 Materials and Methods

2.2.1 Field test-plot setup and manure application

A test-plot rainfall simulation study was conducted in fall 2015 after corn harvesting and before the next planting on a no-till corn field with Braddock Loam (46 % sand, 44 % silt, and 10 % clay) in Blacksburg, Virginia (Kulesza et al., 2016). Each test plot (1.5 x 2.0 m) was framed using 20-cm wide metal sheets, which were hammered approximately 10 cm into the soil. Each plot was installed with the 1.5-m sides perpendicularly to a slope of 9 to 11 %. Distance between two test-plots was approximately 4.5 m to avoid cross contamination. A metal pan was installed at the down slope edge of each plot to transport surface runoff through a hose to a collecting container at a lower slope further down. All connection between the metal pan and the metal frame was sealed carefully with waterproof caulk to prevent any water leakage and tested with water before conducting simulated rainfall. A total of 21 plots were established, including seven treatments, each with three replicates. The seven treatments included two manure application methods (surface application and subsurface injection) × 3 time-gaps [0 (2 hours), 3, and 7 days] between manure application and a subsequent simulated rainfall event + no-manure control. A randomized complete block design was used to generate an overall plot layout with one replicate of each treatment in each block.

Fresh dairy manure from a barn floor of the Virginia Tech Dairy Farm was collected five days before the first application day and stored at -20^{o}C. Two days before the application day, frozen manure was taken out to thaw at room temperature. Two subsamples of wet manure were collected to measure manure moisture content by drying at 110^{o}C to a constant weight. Immediately before application, wet manure was weighed and mixed with water from a nearby

well to make a slurry with 5 % dry matter content to mimic typical lagoon dairy manure in Virginia (VADCR, 2014). For each plot, 42 mL of a stock solution containing pirlimycin (lincosamide class), chlortetracycline (tetracycline class), sulfamerazine (sulfonamide class), and tylosin (macrolide class) at 100, 380, 380, and 114 mg L^{-1}, respectively, was evenly mixed with 31.96 L of liquid manure to achieve target concentrations of 131.25 µg kg^{-1}, 498.75 µg kg^{-1}, 498.75 µg kg^{-1}, and 149.63 µg kg^{-1}, respectively in liquid manure. These target concentrations were based on an excretion study of antibiotic residues in manure from antibiotic-treated cows (Chen et al., 2018). After being thoroughly mixed, two sub liquid manure samples were collected in 400 mL mason jars and placed on ice before being stored at -20^{o}C in the lab for later analysis for the initial concentration of four target compounds. The four antibiotic compounds, pirlimycin (PIRSUE; Zoetis, Florham Park, NJ), chlortetracycline (Sigma Aldrich, ≥75 %), sulfamerazine (Sigma Aldrich, ≥99.0 %), and tylosin (Fluka, ≥95.5 %) were commercially available.

The antibiotic-spiked liquid manure was applied at a recommended agronomic N application rate of 56 Mg ha^{-1} (wet weight), which is typical in Virginia. For the surface application treatment, liquid manure was manually poured on a metal tray to spread it evenly on the surface of a plot. For the subsurface application treatment, each plot had three pre-cut injection slits (5 cm wide × 10 cm deep), which were created perpendicular to the slope using a Yetter injection disc mounted on a three-point hitch. Spiked liquid manure was manually poured into the injection slits and hand covered with soil on top to simulate manure subsurface injection equipment (Appendix A).

2.2.2 Rainfall simulation and runoff collection

Rainfall was simulated by a rainfall simulator (2.44 m x 3.05 m), which is an aluminum frame covered with tarps to act as a windscreen. Plot establishment and rainfall simulation setup followed the established protocol of the national research project for simulated rainfall-surface runoff studies (SERA-17, 2008) to allow for comparison between rainfall simulations conducted in different areas. The SERA-17 protocol was specifically developed to standardize methods when comparing between studies with the selected nozzle type and height above plots to standardize droplet size and velocity. At the center of the rainfall simulator, there was a TeeJet nozzle, which was placed 305 cm above the soil surface. There is a pressure regulator connected the water source and the TeeJet nozzle. The water flow rate was established at 210 mL s^{-1}. Water from a nearby well was used, and it was analyzed to be sure it is free of target antibiotics. Rainfall simulation was conducted at an intensity of 70 mm h^{-1}, which is a standard rate used in the SERA-17 protocol. On top of the runoff-collection metal tray, there was a plastic sheet covering the tray to avoid rainwater getting into the tray and only collect surface runoff. Plot borders directed surface runoff into the metal tray at the bottom edge of the plot, which transferred the runoff through a hose to a receiving container. When surface runoff started to flow into the receiving container, 30 min of collection started, and the time to runoff was recorded (Appendix I). Runoff from the receiving container was then pumped to a pre-weighed large plastic tub, which was placed on a scale to measure the weight of collected runoff from each plot (Appendix I). Immediately after finishing runoff collection, 1.5 to 1.8 L of surface runoff was collected using pre-cleaned mason jars after a vigorous stir to make a homogeneous mixture of runoff water and sediment. These subsamples were immediately placed on ice before being stored at -20^{o}C until analysis.

2.2.3 Sample extraction and cleanup

2.2.3.1 Liquid manure

See Appendices B & C.

2.2.3.2 Surface runoff water

See Appendix D.

2.2.3.3 Surface runoff sediment

See Appendices C & E.

2.2.4 Statistical analysis

All data were analyzed using JMP Pro 13 with a significant level of α at 0.05. A Wilcoxon / Kruskal-Wallis test (rank sums) was applied to compare surface runoff loss of antibiotics (relative to total initial input with manure) among different treatments. Because of the small sample size, the chi-square approximation was used in the Wilcoxon / Kruskal-Wallis test (JMP Pro 13). Individual treatment factor effect and interaction effect between two treatment factors were tested using a generalized regression model with a Beta distribution and a maximum likelihood estimation method. The Beta distribution was used since the percent mass loss of antibiotics with surface runoff was continuous and ranged from 0 to 100 %.

2.3 Results and Discussion

2.3.1 Surface runoff as a route of antibiotic output from manure-applied fields

Concentrations of four tested antibiotics were below detection limits in the well water used for rainfall simulation and in surface runoff of no-manure control treatments. Up to 2.62±0.31 %, 1.77±0.68 % 1.64±0.53 %, and 0.45±0.14 % of pirlimycin, tylosin, sulfamerazine, and chlortetracycline, respectively, that were initially introduced to the field via manure application, left the field with surface runoff during the 30-min runoff event (Fig. 2.1 and Appendix K). These maximum surface runoff losses were from the plots that received manure via surface application, which could be at least twice as much as those from the manure soil subsurface-injected plots. All four antibiotics were detectable in both surface runoff water and sediment from the manure-applied plots. Surface runoff of pirlimycin, tylosin, and sulfamerazine occurred mostly in the runoff water, while that of chlortetracycline mostly occurred in the runoff sediment (Fig. 2.1).

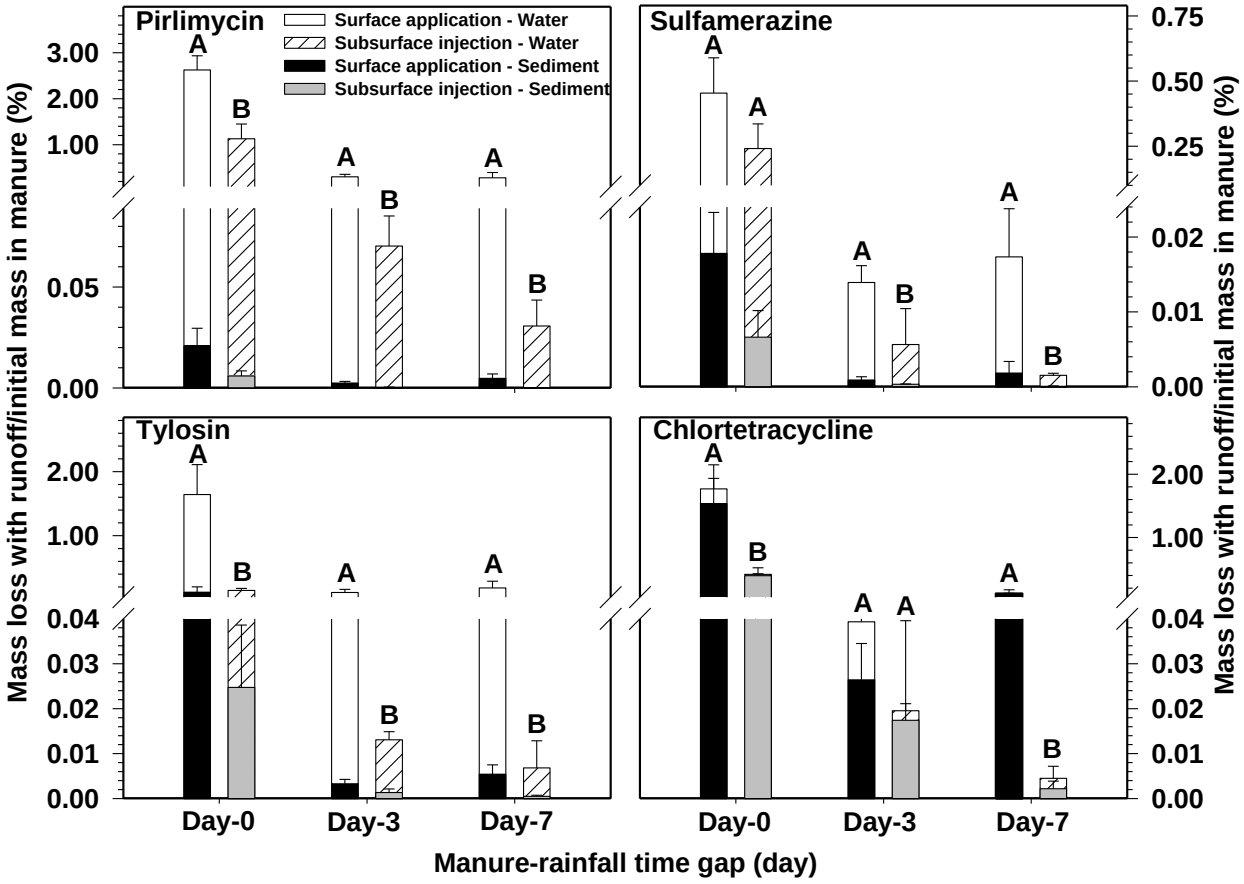

Figure 2.1 Surface runoff loss of pirlimycin, tylosin, sulfamerazine, and chlortetracycline that were initially applied with the manure to the surface applied and subsurface injected test plots. For each antibiotic, two stacked bars illustrated the loss with surface runoff water and surface runoff sediment. For each antibiotic, its mass loss in surface runoff water or sediment was calculated based on its concentration in the runoff water or sediment and the volume of runoff water and mass of runoff sediment collected during 30 min of runoff (Appendix I). Different letters within the same manure-rainfall time gap treatment indicate statistical significance at $p < 0.05$ (Appendix L).

Studies on various soils and runoff collections have confirmed our observation that surface runoff is a route of antibiotic output from manure-applied fields and have reported a range of antibiotic losses that are comparable with our findings. In a study by Davis et al. (2006),

less than 0.1 % of antibiotics (tetracycline, chlortetracycline, sulfathiazole, sulfamethazine, erythromycin, tylosin, and monensin), which were sprayed directly on a cultivated bare soil 1 h before a 1-h rainfall event, were lost with surface runoff water and sediment. The loss of sulfonamides from arable land ranged from 0.1 to 2.5 % in the aqueous phase and 0.004 % in the suspended-matter phase after 2 h of sprinkler irrigation with an intensity of 50 mm h^{-1} (Kreuzig et al., 2005). According to Burkhardt et al. (2005) after 1-day contact, the average loss with runoff from manure surface-applied plots on grassland irrigated at a rate of 20 mm h^{-1} and the total amount of 30 mm was 0.3 % for sulfadiazine, 0.8 % for sulfathiazole, and 1.4% for sulfadimidine. Kay et al. (2004) also reported a single maximum loss of 0.48 % and 0.015 % for sulfachloropyridazine and oxytetracycline, respectively, in drain flow in two consecutive years after pig slurry was surface applied. Although leaching is another way for antibiotics to move from soil, a study by Dolliver and Gupta (2008) showed more antibiotics were detected in surface runoff than in leachate. (Dolliver and Gupta, 2008) measured losses of chlortetracycline, tylosin, and monensin with leaching and runoff water in a 3-yr field study on silt loam soils, but antibiotic losses with sediment were not measured. The highest amount of antibiotic loss with runoff was 5 % for tylosin, 2 % for monensin, and 0.2 % for chlortetracycline. For all three antibiotics, leaching losses were <0.005%.

In summary, antibiotics from manure-applied fields can be disseminated to the surrounding environment via surface runoff. During the 30-min runoff, the mass loss of antibiotic with runoff ranged from 0.45 % (chlortetracycline) to 2.62 % (pirlimycin) of those initially applied to the field. Because majority of antibiotics remained in the soil, the dissipation rates of antibiotics remained in soil would affect the losses of antibiotics with subsequent surface runoff.

2.3.2 Impact of manure application methods on antibiotic losses in surface runoff

For all four antibiotics, the manure soil subsurface injection method significantly reduced the amount of antibiotic loss in surface runoff water compared to the traditional surface application method for all manure-rainfall time gap treatments (Fig. 2.1). The only exception was the Day-0 treatment for sulfamerazine, in which although the sulfamerazine loss with runoff water from the subsurface injection treatment was lower than that from the surface application treatment, the difference was not significant. For Day-0 treatments, the reduction of antibiotic loss with runoff water due to the new manure application method was 2, 12, and 13 times for pirlimycin, chlortetracycline, and tylosin, respectively. The reduction was 4, 2, 6, and 9 times for pirlimycin, sulfamerazine, chlortetracycline, and tylosin, respectively for Day-3 treatments, and 9, 11, 7, and 28 times for pirlimycin, sulfamerazine, chlortetracycline, and tylosin, respectively for Day-7 treatments.

Similarly, compared to the surface application method, the manure soil subsurface injection method significantly decreased losses of four antibiotics with surface runoff sediment in most cases (Fig. 2.1). The exception belonged to the Day-3 treatment for chlortetracycline with no significant difference in the chlortetracycline loss with runoff sediment between surface application and subsurface injection treatments. The significant reduction of pirlimycin loss associated with runoff sediment due to the manure soil subsurface injection method was 3, 6, and 41 times for the Day-0, Day-3, and Day-7 treatments, respectively. It was 3, 3, and 26 times for sulfamerazine and 5, 2, and 46 times for tylosin.

Therefore, the manure soil subsurface injection method can significantly reduce antibiotic losses with both surface runoff water and sediment for all time gaps between manure application and a subsequent rain event (Table 2.1). The reason could be that antibiotics associated with

liquid manure in surface application treatments were more available to interact with rainfall over a much larger area of 2×1.5 m^2. Meanwhile, for subsurface injection treatments, antibiotics with manure were buried in three injection slits ($3 \times 0.05 \times 1.5$ m^2) and covered with soil, resulting in much less interaction with rainfall.

Table 2.1 Effects of manure application methods, manure-rainfall time gaps, and the interaction effect of the two on the percent mass loss of antibiotics with surface runoff relative to the initial input to each treatment plot

Factor	Pirlimycin	Sulfamerazine	Chlortetracycline	Tylosin
Manure application method	<0.0001	<0.0001	<0.0001	<0.0001
Manure-rainfall time gap (Day-0 & Day-3)*	<0.0001	<0.0001	<0.0001	<0.0001
Manure application method x manure-rainfall time gap (Day-0 & Day-3)*	0.2051	0.9659	0.3570	0.1873

*Day-0 and Day-3 were compared because the result from the Day-3 and Day-7 treatments is statistically much more similar than that of Day-0 and Day-3 treatments (Appendix L).

2.3.3 Distribution of antibiotics in surface runoff water and sediment

Up to 2.62±0.31 %, 1.77±0.68 % 1.64±0.53 %, and 0.45±0.14 % of initial field-applied pirlimycin, tylosin, sulfamerazine, and chlortetracycline, respectively, were lost with runoff from the Day-0 surface application treatment. These numbers were 1.13±0.32 %, 0.14±0.04 % 0.24±0.1 %, and 0.42±0.12 % for pirlimycin, tylosin, sulfamerazine, and chlortetracycline, respectively, for the Day-0 subsurface injection treatment. For all treatments, the majority of pirlimycin (99±0.5 %), sulfamerazine (94±4 %), and tylosin (91±7 %) left the manure-amended fields with surface runoff water, while chlortetracycline was primarily lost with surface runoff sediment (78±15 %) (Fig. 2.2). The mass distribution of an antibiotic between the aqueous and solid phases of surface runoff from both manure surface-applied and subsurface-injected fields was largely determined by its physiochemical properties.

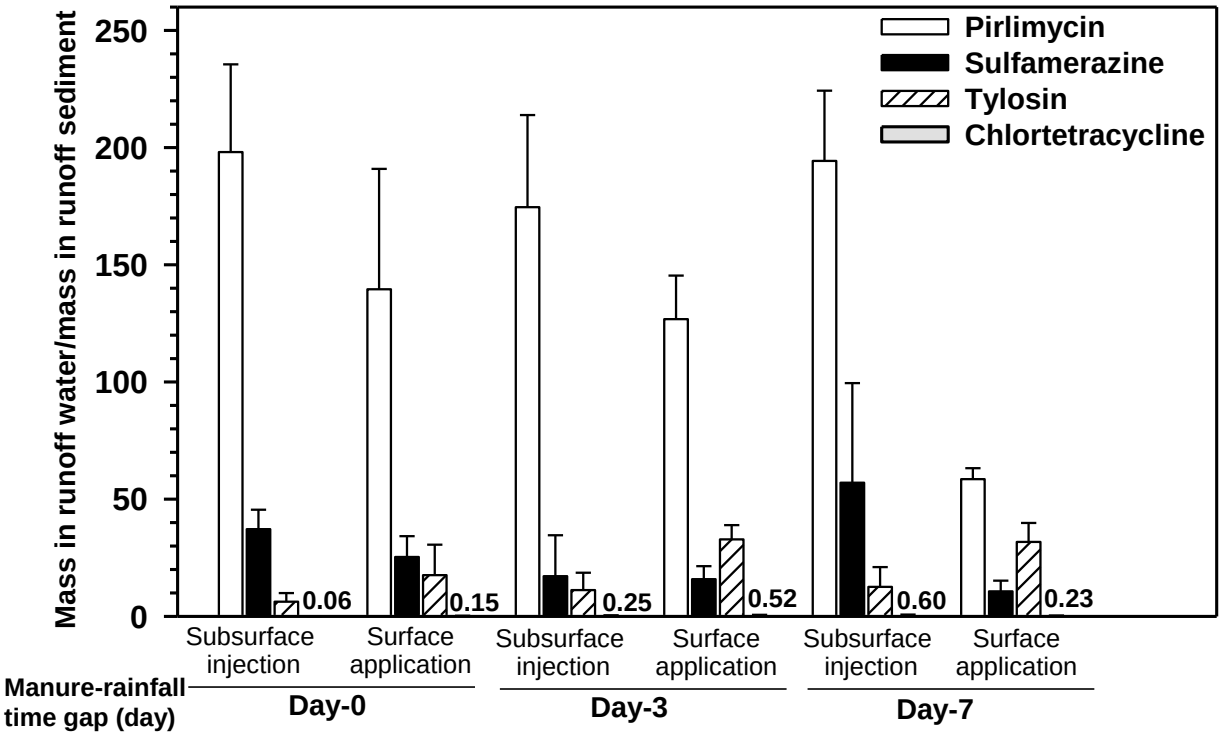

Figure 2.2 Mass distribution of pirlimycin, tylosin, sulfamerazine, and chlortetracycline between the aqueous and solid phases of surface runoff from different treatment plots.

Similar results were observed in other studies. Kreuzig et al. (2005) observed that sulfonamide loss with runoff was predominant in the aqueous phase for both manure-amended arable and grassland. Sulfonamides were present in surface water and groundwater samples collected throughout the U.S. (Lindsey et al., 2001). In a 3-yr study about antibiotic losses through leaching and runoff from a manure-applied field, chlortetracycline was only detected in runoff, while tylosin was detected in both leachate and runoff (Dolliver and Gupta, 2008). In a study by Davis et al. (2006), among seven compounds, including tetracycline, chlortetracycline, sulfathiazole, sulfamethazine, erythromycin, tylosin, and monensin, which were sprayed directly to soil 1 h before rainfall, monensin had the highest concentration and tetracyclines and tylosin had the lowest concentrations in the aqueous part. In the sediment part, the highest concentration

belonged to erythromycin, followed by monensin and then tylosin, while sulfamethazine concentration was below the limit of quantification.

Clearly, the mobility and transport of antibiotics are influenced by their physicochemical properties. In particular, differences in molecular structure, size, shape, solubility, and hydrophobicity of antibiotics result in their differences in sorption and fixation to soil particles (Kemper, 2008). Sorption can occur through hydrophobic partitioning to soil organic matter, hydrogen bonding, and electrostatic interactions (Verlicchi and Zambello, 2015; Wegst-Uhrich et al., 2014). Our results have shown that among the tested antibiotics, chlortetracycline had the strongest interaction with soil particles. In fact, tetracyclines can strongly bind to soil organic matter via cation exchange, surface complexation, and cation bridging sorption (Wegst-Uhrich et al., 2014). Tetracyclines have the ability to form complexes with divalent charged cations, which are readily available in soil in high concentrations (Kemper, 2008). In addition, tetracyclines strongly bind to proteins and silanol groups (Thiele-Bruhn, 2003).

A common indicator to predict behavior of antibiotics in soil is their affinity to the solid phase, K_d value (solid liquid partition coefficient) (Tolls, 2001). K_d values range from 0.6 to 4.9 L kg^{-1} for sulfonamides, 8.3 to 128 L kg^{-1} for macrolides, and 420 to 1030 L kg^{-1} for tetracyclines (He and Zhang, 2014). Since there was no published K_d value for pirlimycin, a sorption batch experiment was conducted and determined that its K_d were 0.8 (sandy soil) to 2.2 L kg^{-1} (clay soil). In general, an antibiotic with a higher K_d value tends to bind more strongly to soil particles and tends to be lost with the solid phase of surface runoff. Therefore, antibiotic sorption to sediment tends to increase in the following order: pirlimycin ~ sulfamerazine < tylosin << chlortetracycline. Indication from K_d values matched with our results in terms of the antibiotic mass loss in surface runoff sediment.

The mass loss of the four tested antibiotics increased in the following order: chlortetracycline < sulfamerazine < tylosin < pirlimycin with the aqueous runoff phase and pirlimycin ~ sulfamerazine < tylosin << chlortetracycline with the solid runoff phase. Therefore, weakly bound antibiotics tend to be transported from manure-applied fields in surface runoff water, while the strongly bound antibiotics tend to be off the field in associated particles eroded by runoff water.

2.3.4 Effect of manure-rainfall time gaps on surface runoff of antibiotics

Another crucial finding of this study was that the time gap between manure application and a subsequent simulated rainfall could significantly affect the amount of antibiotic loss with surface runoff (Fig. 2.3). In particular, compared to treatments of simulated rainfall events immediately after manure application (Day-0 treatments), applying manure three days before the rain event (Day-3 treatments) significantly reduced the mass loss of all four antibiotics in surface runoff for both manure application methods (Table 2.1). These reductions for pirlimycin, tylosin, chlortetracycline, and sulfamerazine were 9, 14, 45, and 33 times, respectively, for surface application treatments and 16, 11, 21, and 43 times, respectively, for subsurface injection treatments. However, there was no significant difference between Day-3 and Day-7 treatments in terms of antibiotic losses with surface runoff for all four antibiotics from surface application treatments. Meanwhile, for subsurface injection treatments, Day-7 treatments significantly lowered the mass loss of pirlimycin (by 2 times) and sulfamerazine (by 5 times) with surface runoff compared to Day-3 treatments, but there was no significant difference between Day-7 and Day-3 treatments for chlortetracycline and tylosin. The interaction of manure application

methods and manure-rainfall time gaps (Day-0 and Day-3) had no significant effect on the mass loss of antibiotics (Table 2.1).

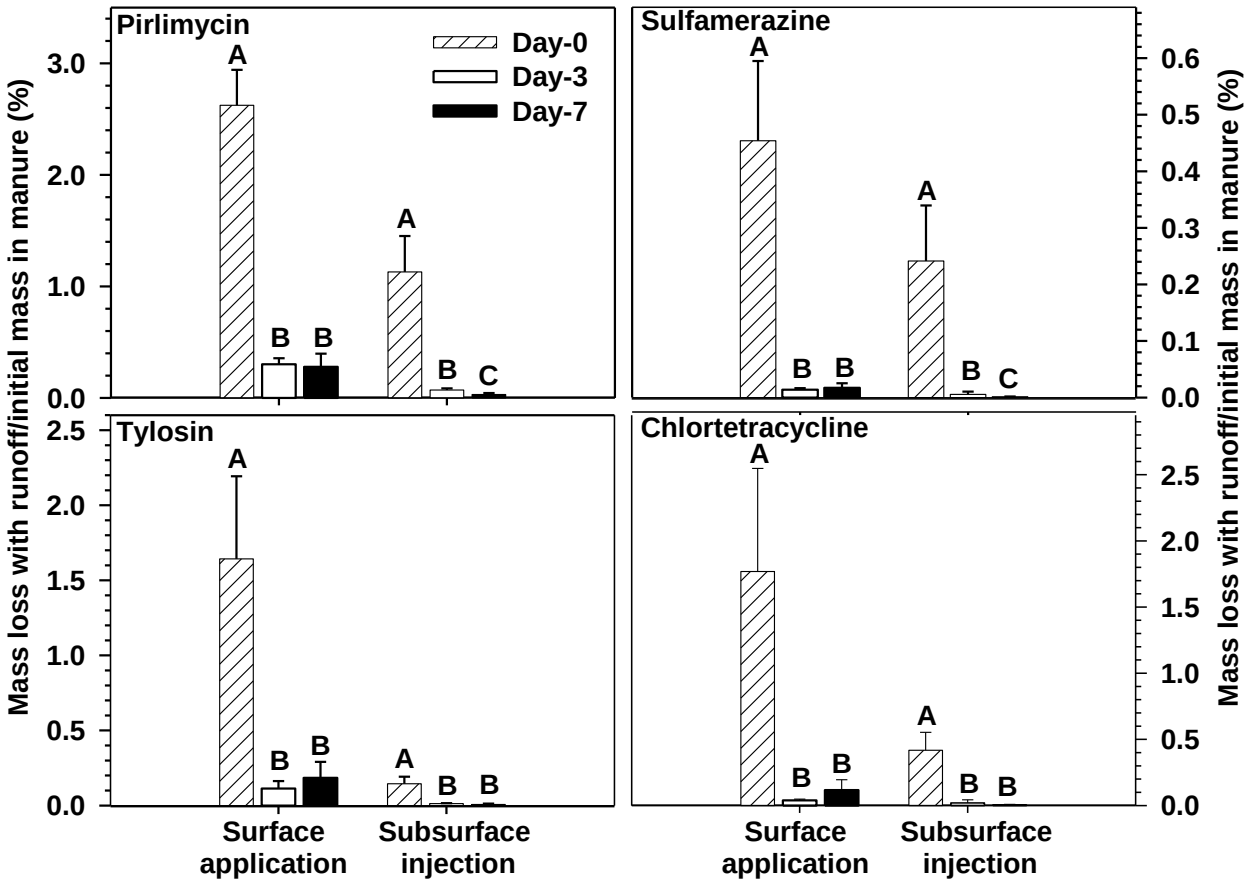

Figure 2.3 Surface runoff loss of pirlimycin, tylosin, sulfamerazine, and chlortetracycline that were initially added with manure to the test plots, which received simulated rainfall immediately (Day-0), 3 days (Day-3), and 7 days (Day-7) after manure surface application and subsurface injection. Different letters within the same manure application method indicate statistical significance at $p < 0.05$ (Appendix L).

As a result, applying manure onto the field at least three days before a subsequent rainfall would dramatically decrease the amount of antibiotics that left the field in both surface runoff water and sediment. Lertpaitoonpan (2008) also observed that total mass loss of sulfamethazine

in leachate from a soil column which received rainfall 1 day after manure amendment was significantly higher than in leachate from a column which received rainfall 4 days after manure amendment. Also, the sulfamethazine masses leached from soil columns which received rainfall on 4 or 7 days after manure addition showed the same magnitude. A similar trend was shown for herbicide transport from soils to leachate (Isensee and Sadeghi, 1995; Neurath et al., 2004). In contrast, Burkhardt et al. (2005) reported an increased total loss of sulfonamides with runoff water on manured grassland after 3-day contact (0.6, 1.1, and 2.1 % for sulfadiazine, sulfathiazole, and sulfadimidine, respectively) compared to 1-day contact (0.2, 0.4, and 0.8 % for sulfadiazine, sulfathiazole, and sulfadimidine, respectively) after 90 min of irrigation at 30 mm h^{-1} intensity. However, for control plots (antibiotics spiked in aqueous solution), the authors reported a reverse trend that sulfadiazine and sulfathiazole were detected in runoff water after 1-day contact, but both were below the limit of quantification after 3-day contact.

The decreased antibiotic loss with surface runoff with an increased time gap between manure application and a subsequent rain event could be explained by three mechanisms. First, sorption of antibiotics to soil was time dependent. For Day-0 treatments, there was an approximately 2-h time gap between manure application and the simulated rainfall, while the time gap increased to 3 and 7 days for Day-3 and Day-7 treatments, respectively. Sorption of most antibiotics onto soil happens fast (Thiele-Bruhn, 2003). For example, sulfonamides reach sorption equilibrium after several hours, while tetracyclines first quickly bind to outer surfaces, then penetrate into interlayers of clay minerals and micro-pores (Thiele-Bruhn, 2003). Second, several antibiotics are degradable in manure-amended soil with a half-life less than 30 days by biotic and abiotic mechanisms (He and Zhang, 2014). The half-life of sulfonamides in soil ranged from 14 – 64 days (Lertpaitoonpan, 2008). The half-life of tylosin A in soil was 7 days

(Boxall, 2008). The degradation half-life in sandy loam was 21 – 24 days for chlortetracycline and 5 – 6 days for tylosin (Carlson and Mabury, 2006). Tetracyclines are sensitive to photolysis, while sulfonamides are sensitive to hydrolysis (He and Zhang, 2014). High temperatures during summer can accelerate antibiotic transformation and/or degradation processes. Finally, antibiotics can transport deeper into the soil, reducing their interaction with surface runoff. After liquid manure was applied onto the surface or into the injection slits, it took some time for the manure containing antibiotics to completely infiltrate into the soil. Compared to the Day-0 treatments, Day-3 and Day-7 treatments had a longer time before the beginning of simulated rain for antibiotics to travel to deeper soil.

2.4 Conclusions

The crucial finding of this study was that the manure soil subsurface injection method, which was designed for nutrient loss reduction, can also significantly reduce the mass loss of antibiotics associated with surface runoff compared to the traditional manure surface application method. Therefore, the manure soil subsurface injection method is a promising manure management practice to reduce input of nutrients as well as manure-borne emerging chemicals of concerns such as antibiotics from manure-applied fields to water bodies. Also, this study reported for the first time that the time gap between manure application and a subsequent rainfall can decrease the antibiotic mass loss associated with surface runoff from manure-applied fields. For all tested compounds, applying manure three days or longer before a rain event can reduce the antibiotic mass loss in both surface runoff water and sediment by at least nine times with both manure application methods. Therefore, as a better manure land management practice, using the manure soil subsurface injection method and avoiding manure application less than three days before a rainfall are recommended to prevent the spread of antibiotics from manure-applied fields to surrounding waters.

The results from this study bring up new questions related to the recommended manure soil subsurface injection method. First, whether reduction of antibiotic surface runoff due to the subsurface injection would be observed when manure is applied in spring with moist soil or on various soil types. Second, compared to surface application, whether the subsurface injection would also reduce surface runoff of other emerging contaminants associated with manure such as antibiotic resistance genes and antibiotic resistant bacteria. Furthermore, unlike the surface application, the subsurface injection creates injection slits rich in animal manure, resulting in localized hotspots rich in nutrients, organic matter, water, and an altered microbial community.

The dissipation of antibiotics and antibiotic resistant genes in these hotspots over time would be different from that in surface-applied soils. Finally, further testing to determine if subsurface injection would facilitate antibiotic downward movement in soil through leachate compared to surface application is needed.

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Chapter 3: Spatial distribution and temporal change of antibiotics in soils amended with manure using two field application methods

Hanh Thi Van Le, Rory O. Maguire, and Kang Xia

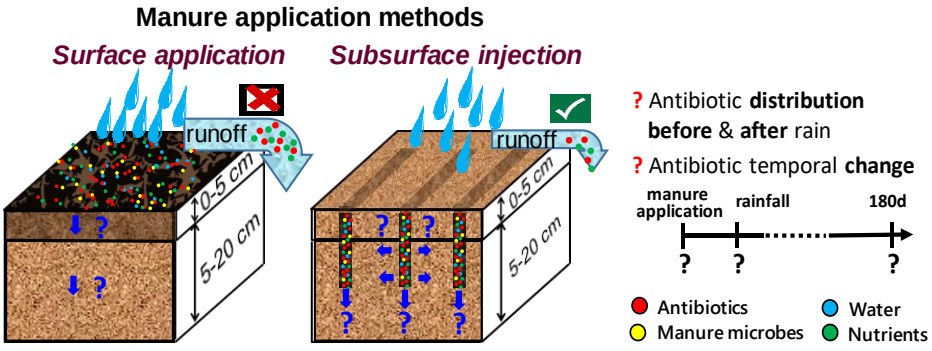
(To be submitted to Science of Total Environment)

Abstract

Compared to surface application, manure subsurface injection significantly reduces transport of manure-associated antibiotics via surface runoff; however, the environmental fate of antibiotics in injection slits is unknown. A field investigation was conducted to monitor distribution and dissipation of pirlimycin, tylosin, chlortetracycline, and sulfamerazine in soil following either surface application or subsurface injection of dairy manure. Simulated rainfall was conducted on days 0, 3, or 7 after manure application. Soil samples were collected before, on the day of, and 5, 14, 60, and 180 days after the simulated rainfall. Target antibiotics in soil were quantified using ultra performance liquid chromatography-tandem mass spectrometry. Antibiotic concentrations in the subsurface injection slits were 4-26 times higher than those in the surface applied plots. Antibiotics were concentrated in the injection slits for an extended period of time with limited horizontal and vertical movement from the slits. Thus, the microbial community inside the slits was exposed to elevated levels of antibiotics, possibly resulting in a "hot zone" for antibiotic resistance development in manure subsurface injected fields. Effects of manure application-rainfall time gaps on antibiotic distribution were strongest immediately after manure application and decreased with increased time gaps after manure application. During 180 d after the 1st rainfall, antibiotic transformation rates were fastest during the first 14 d before slowing down, and the effect of two manure application methods on antibiotic transformation kinetics varied with different antibiotics. The half-life of antibiotics ranged from 3 to 11 d for pirlimycin, 3 to 10 d for sulfamerazine, 5 to 12 d for tylosin, and 3 to 21 day for chlortetracycline in the fall. However, pirlimycin, sulfamerazine, and tylosin remained detectable in soil even at 6 months after a single manure application, indicating that antibiotics from the previous manure

application can be carried over to the next manure application season. The long-term accumulation of antibiotics in the injection slits compared to the surface application treatment would not be a concern, therefore, using the subsurface injection method and applying manure at least three days before rain is recommended.

Graphical abstract



Highlights

- Impact of manure subsurface injection on antibiotic fate in soil is unknown.
- A field study with spatial distribution and temporal change of antibiotics in soil.
- Antibiotics concentrated in the injection slits (the hot zone) with limited movement.
- Half-life of antibiotics in soil ranged from 3 to 21 days.
- Some antibiotics remained detectable at 180 d after a single manure application.

3.1 Introduction

Following manure application, the majority of manure-associated antibiotics remained in soil (Le et al., 2018; Pan and Chu, 2017; Spielmeyer et al., 2017). Because of low vapor pressure, antibiotic volatilization is insignificant. Antibiotic losses via surface runoff or leaching account for a small percentage of the initial amount. Dolliver and Gupta (2008) found that less than 5 % of applied antibiotics were lost in 3 years via surface runoff and antibiotic losses via leaching were even lower. Studies reported that surface runoff of antibiotics mostly occurred during the first rainfall after manure application, resulting in 0.45 to 2.62 % of applied antibiotics leaving the field (Kulesza et al., 2016; Le et al., 2018). In addition, compared to the traditional manure surface application, the manure subsurface injection method is recommended to reduce surface losses of manure-associated antibiotics, probably due to the nature of the methods (Kulesza et al., 2016; Le et al., 2018). While manure is spread evenly on soil following the surface application method, manure is buried below soil surface and concentrated in injection slits of the subsurface injection. Therefore, compared to soil where manure has been surface-applied, soil in the injection slits may have an elevated level of nutrients, organic matter, water, manure microbes, and antibiotics, which all can affect the environmental fate of antibiotics in soil. However, effects of these two manure application methods on antibiotics in soil are unknown.

There are various and complex soil processes determining the environmental fate of antibiotics including persistence, mobility, and impact. Mineralization to CO_2 accounts for less than 2 % of the initial amount for sulfadiazine (Schmidt et al., 2008). Photodegradation of antibiotics in soil is also limited, especially in sub soil due to poor light penetration (Ozaki et al.,

2011). Transformation of antibiotics in soil has been reported to be mainly microbial-driven (Accinelli et al., 2007; Pan and Chu, 2016). Sequestration of antibiotics within soil particles can reduce their mobility, reactivity, and bioavailability for biodegradation (Jechalke et al., 2014).

Following manure application, compared to soil from the surface application, soil in the injection slits of the subsurface probably has a much higher organic matter content. On the one hand, presence of a large amount of manure organic matter can increase the sequestration of antibiotics in soil by acting as an effective sorbent, reducing their mobility, reactivity, as well as availability for biodegradation (Li et al., 2010; Wang et al., 2015). On the other hand, C sources and nutrients from manure can promote activities of microbes, which can enhance biotransformation of antibiotics. Following manure amendment, several studies reported higher microbial biomass carbon and nitrogen, and higher soil enzymes such as dehydrogenase, alkaline phosphatases, β-glucosidasen, and urease (Lin et al., 2016; Tejada et al., 2006). Previous investigations have demonstrated that manure application could increase dissipation rates for tylosin, sulfadiazine, sulfamethoxazole, sulfamethazine, and sulfachloropyridine (Accinelli et al., 2007; Carlson and Mabury, 2006; Zhang et al., 2017), decrease dissipation rates for sulfonamides (Albero et al., 2018; Carlson and Mabury, 2006), or leave dissipation rates for chlortetracycline, monensin, and lincomycin unchanged (Albero et al., 2018; Carlson and Mabury, 2006). Differences in antibiotic physicochemical properties such as structure, size, shape, solubility, and hydrophobicity can definitely be a part of varied results in their persistence and mobility. For antibiotics that strongly bind to soil particles, it is expected that their mobility is more limited and they can persist for a longer time.

In addition to organic matter, the difference in water content after liquid manure application between soil from the subsurface injection and soil from the surface application can

alter the antibiotic fate. Due to decreased gas diffusion and rapid degradation of bioavailable organic C, the local environment in the subsurface injection slits can enhance reduction reactions and anaerobic microbes, while the top soil of the surface application facilitates oxidation reactions and aerobic microbes. These differences can affect both biotic and abiotic transformation of antibiotics in soil. In terms of abiotic transformation, hydrolysis of functional groups such as esters, amides, alkyl halides can be a major abiotic degradation pathway for antibiotics (Mitchell et al., 2014). Drier soil would enhance antibiotic dissipation processes by promoting rigidity of soil organic matter, which prevents organic compounds such as antibiotics from adhering to organic matter (Pan and Chu, 2016; Rosendahl et al., 2011). Nonetheless, Wang et al. (2006) found that the half-life of sulfadimethoxine in manure-amended soil decreased with increased soil moisture content since more sulfadimetoxine became available for degradation.

The occurrence and persistence of antibiotics in soil have raised some environmental concerns. Ding and He (2010) reviewed the effect of antibiotics on soil microbial composition and functions. Changes in microbial community structure in soil upon exposure to antibiotics can be as broad as a group of microbes and as narrow as a single genus or species. Ecological functions such as nitrogen transformation, methanogenesis, and sulfate reduction would also be influenced. Furthermore, according to Gullberg et al. (2011), presence of antibiotics even at a low level can effectively select and maintain resistance traits in bacteria. Studies have shown an enhanced level of antibiotic resistance genes and antibiotic resistant bacteria together with antibiotic use (Pei et al., 2006; Rodriguez-Rojas et al., 2013).

In order to gain a comprehensive picture on the environmental fate of antibiotics when manure is applied via subsurface injection, it is essential to know antibiotic behavior in soil. The

objectives of this study were to monitor how manure application methods and manure-rainfall time gaps alter spatial distribution and temporal change of antibiotics in soil under field conditions.

3.2 Materials and Methods

3.2.1 Experimental setup

3.2.1.1 Field setup

A field rainfall simulation study was conducted in Blacksburg, Virginia. A detailed experimental setup was described by Le et al. (2018). In summary, the 1.5- × 2.0-m test-plot experiment was conducted on Braddock Loam (fine, mixed, semiactive, mesic Typic Hapludults; 46 % sand, 44 % silt, and 10 % clay) with a 9 to 11 % slope (Kulesza et al., 2016). There were three treatments, dairy manure surface application, dairy manure subsurface injection, and no-manure application as control. For manure-applied plots, time to rainfall sub-treatments were based on the time gap between manure application and a subsequent simulated rain event. After manure application, the simulated rainfall was conducted at 2 h (defined as Day-0 treatment), 3 d (defined as Day-3 treatment), or 7 d (defined as Day-7 treatment). In total, seven experimental treatments with triplicate each made up 21 test-plots.

Dairy manure from a barn floor of Virginia Tech Dairy Farm was mixed with water from a nearby well to create a slurry with 5 % of dry matter content, mimicking typical liquid dairy manure commonly applied in the fields of Virginia. During this process, an appropriate amount of a mixture of antibiotic stock solution containing the most widely-used antibiotics in livestock, pirlimycin (lincosamide class), tylosin (macrolide class), sulfamerazine (sulfonamide class), and chlortetracycline (tetracycline class), was spiked to the liquid manure to achieve final concentrations of 131, 150, 500, and 500 μg kg^{-1} liquid manure, respectively. These concentrations were based on a previous excretion study measuring antibiotic residues in manure from antibiotic-treated cows (Ray et al., 2017).

The antibiotic-spiked liquid dairy manure was then applied to the test-plots at a typical application rate used in Virginia (56 Mg ha^{-1}, wet weight) (Maguire et al., 2013). As shown in Fig. S1, the liquid manure was applied to an area larger than the 1.5 × 2.0-m test-plot, as described below, to avoid disturbance inside the plot from soil collection before the simulated rainfall. For the surface application plots, manure slurry was poured on a metal tray to spread it evenly on top of soil to an area of 2.3 × 2.4 m (40 cm extension on both sides of the plot and 20 cm extension from the top and bottom edges of the plot). For the subsurface application plots, manure slurry was slowly poured into three injection slits (2.3 m long × 5 cm wide × 10 cm deep), which were created perpendicular to the slope using a Yetter injection disc mounted on a three-point hitch. The injection slits were then covered with soil by hand to simulate slit covering by closing discs.

3.2.1.2 Rainfall simulation experiment

A simulated rainfall was conducted at 2 h, 3 d, or 7 d after manure application. The simulated rainfalls were conducted at a standard rate (70 mm h^{-1}) in order to compare rainfall simulations in different areas using a rainfall simulator (2.44 m × 3.05 m) (SERA-17, 2008). A nearby well free of target antibiotics supplied water for the simulated rainfalls. After surface runoff started to flow into a receiving container, rainfall was conducted for 30 min.

3.2.1.3 Soil sample collection

There were six soil sampling times (Appendix I). The first sampling time was conducted at 1 h before the simulated rainfall for the Day-0, Day-3 and Day-7 treatments. The later five soil sampling events occurred at 1 h, 5 d, 14 d, 60 d, and 180 d after the simulated rainfall. Soils were sampled at two depths: 0-5 cm by using a stainless-steel soil probe and 5-20 cm by using a hand-held drill STIHL BT 45. For each sample, six soil cores (2-cm diameter) were collected

randomly and composited. Soil sampling scheme was illustrated in Appendix I. Soil cores were collected outside the test plot before the rainfall and inside the test plot after the rainfall. For the control and surface application plots, soil cores were collected randomly in designated areas. For the subsurface injection plots, in order to measure antibiotic movement in soil from injection slits, soil cores were sampled randomly in the injection slits, and at 5 cm, 20 cm, 25 cm, and 60 cm away from the injection slits in both up slope and down slope direction for the 1st and 2nd sampling times (1 h before and after the simulated rainfall). For the other four sampling times, soil cores were collected randomly in the injection slits and in between injection slits. All samples were placed on ice in the field and stored at -20^{o}C until analysis.

3.2.2 Sample extraction and analysis

3.2.2.1 Sample extraction

See Appendices B, C, and E.

3.2.2.2 UPLC-MS/MS analysis

See Appendix F. Method detection limit and recovery of antibiotics in manure-amended soil were listed in Appendix N.

3.2.3 Statistical analysis

Data were analyzed using JMP Pro 14 (SAS Institute, 2018) with a significant level (α) at 0.05. Effect of individual treatment factors (such as manure application methods, manure-rainfall time gaps, types of antibiotics, and soil depth) and interaction effect were analyzed using a generalized regression model with a maximum likelihood estimation method and a Gamma distribution. Because antibiotic concentrations were positive and had extreme values, the Gamma distribution was appropriate for data analyses. If the factor effect was significant, all pairwise

Comparisons-Tukey HSD were applied. In order to compare antibiotic concentrations/masses before and after the rain, a Wilcoxon / Kruskal-Wallis test (rank sums) with Chi-Square approximation (for small sample size) was applied. The effect of manure application method, manure-rainfall time gap, and soil depth on the dissipation kinetics of antibiotics over time was measured using a mixed model, in which the manure application method, the manure-rainfall time gap, and the soil depth were used as the fixed effects and the time was used as the random factor. For samples below detection limits, their concentrations were assigned a value equal half of the method detection limit for statistical analyses.

3.3 Results and Discussion

In this study, results were first focused on the vertical and horizontal distribution of antibiotics in soil for (1) after manure application and before the 1st rain event (the 1st soil sampling event) and (2) 1 h after the 1st simulated rain event (the 2nd soil sampling event). Secondly, results were focused on the temporal change of antibiotics during (1) short-term (at 1 h, 3 d, and 7 d after manure application but before the simulated rainfall) and (2) long-term (up to 180 d after the simulated rainfall, the remaining four sampling events).

3.3.1 Vertical and horizontal distribution of antibiotics in soil

3.3.1.1 Between manure application and a subsequent simulated rainfall

Following manure application (1 h, 3 d and 7 d), the distribution of manure and manure-associated antibiotics in soil were different between the surface application and subsurface injection plots (Fig. 3.1). Manure was spread evenly on soil surface for the surface application treatments, whereas manure was concentrated in subsurface injection slits for the subsurface injection treatments. Therefore, after manure application, the antibiotic concentrations in soil of the injection slits were significantly higher than those in the soil from the manure surface-applied plots ($p < 0.05$ for all antibiotics, Appendix P). In particular, compared to the manure surface-applied plots, the levels of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in 0-5 cm of the injection slits were 4-12, 7-15, 6-11, and 6-17 times higher, respectively. For 5-20-cm soil, the levels of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in the injection slits were 2-19, 10-26, 5-25, and 6-16 times higher, respectively. This result was consistent with findings by Kulesza et al. (2016). Antibiotics in soil can move to the surrounding soil by diffusion and together with water from liquid manure (95% water content) or bulk flow.

Since the injection slits were approximately 10-cm deep, it is reasonable to detect the target antibiotics in both 0-5 and 5-20 cm soil of the injection slits (Fig. 3.1). However, if the tested lower concentrations in the 5-20 cm soil in the injection slits were a result of dilution due to mixing of the 5-10-cm soil with the 10-20-cm soil, they should be one third of those in the 0-5-cm soil, assuming there was no significant downward movement and/or soil depth did not affect the dissipation rate of antibiotics. Appendix N showed that at 1 h after manure application but before the simulated rain (Day-0 treatment), the 0-5-cm/5-20-cm concentration ratio was 3.3-3.8 on average for the four antibiotics, suggesting their minimum downward movement with water from the applied liquid manure or dissipation in the manure subsurface injection slits within 1 h. For pirlimycin, this ratio increased to 8.9 (±3.5) on the 3rd day and 18.8 (±8.1) on the 7th day after manure application. For chlortetracycline, this ration increased to 9.9 (±3.7) on the 3rd day and dropped back to 5.0 (±1.4) on the 7th day. For sulfamerazine and tylosin, compared to the concentration ratio on the day of manure application, the ratio on the 3rd day did not significantly change but the ratio on the 7th day increased. This result suggested that 1) movement and dissipation rates might be different among the four compounds and 2) pirlimycin might have higher downward mobility and/or faster dissipation rate in deeper soil depth than the other three compounds.

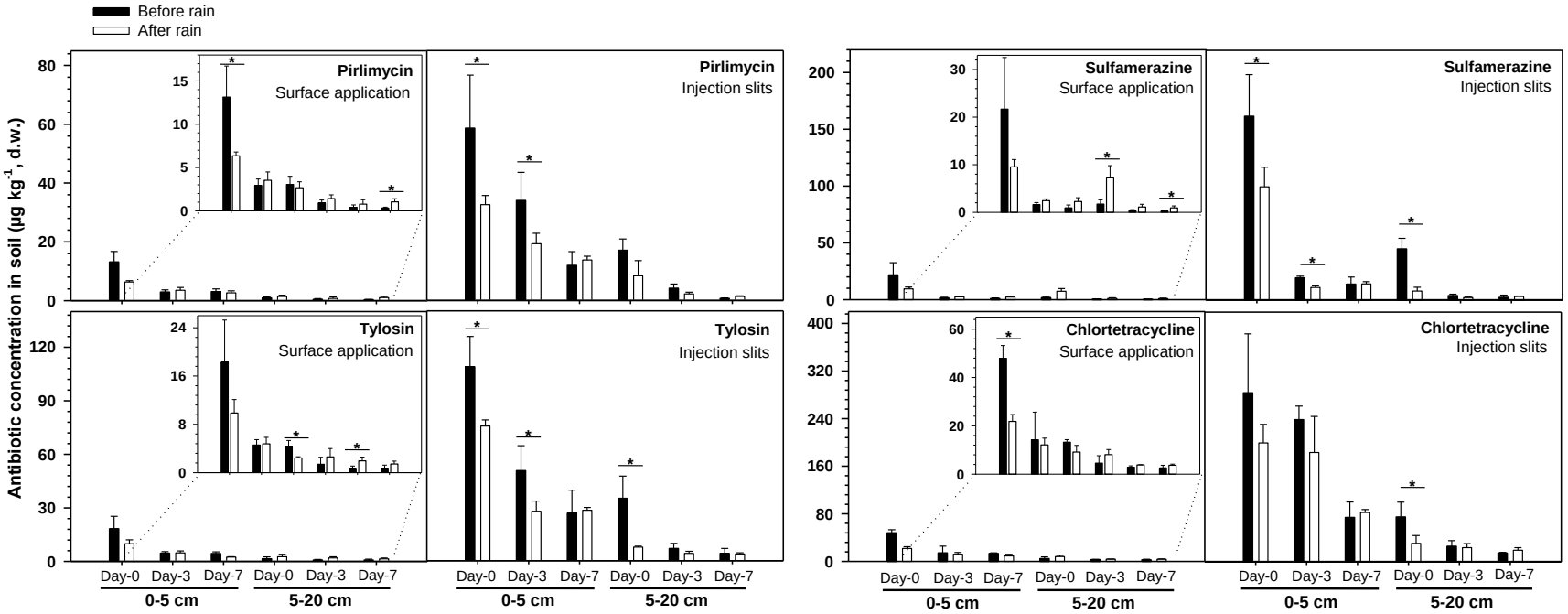


Figure 3.1 Concentration of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in 0-5- and 5-20-cm soil (µg kg^{-1}, dried weight) collected from the manure surface-applied plots and from the injection slits of the manure subsurface-injected plots at 1 h before (*black bars*) and after the simulated rainfall (*white bars*) for the three manure-rainfall time gap treatments (0, 3, and 7 days). * indicated a significant difference in antibiotic concentrations between before and after simulated rain (p<0.05, Appendix S).

For the manure surface-applied plots, although manure was only applied on the surface, all antibiotics were detected in the 5-20-cm soil (Fig. 3.1). The concentration ratios between the 0-5 cm soil and the 5-20 cm soil ranged from 5.8-12.9 for sulfamerazine, 4.9-13.5 for chlortetracycline, 9.6-14.9 for pirlimycin, and 5.0-19.8 for tylosin (Appendix O). This result suggested a similar downward movement of all four antibiotics in the surface application treatments despite their physicochemical differences. Studies have shown tetracycline (oxytetracycline and tetracycline) and macrolide (tilmicosin) antibiotics, with low water solubility, mostly partition onto the solid fraction of manure (Gros et al., 2019), whereas lincosamide (lincomycin) and sulfanomide (sulfamethazine, sulfadiazine, sulfamethoxazole) antibiotics, with high water solubility, mainly stay in the liquid fraction of manure (Bailey et al., 2016; Gros et al., 2019). Therefore, following liquid manure application, strongly-bound and weakly-bound antibiotics can be transported through soil macrospores and microspores in liquid manure fine particles and water, respectively. Also, the concentration ratio between the 0-5 cm soil and the 5-20 cm soil did not significantly change with different gaps after manure application for four antibiotics. The results suggested that effect of two manure application methods on antibiotic movement and dissipation rates would vary based on antibiotics.

Similar to our results, both previous lab and field studies have reported the accumulation of various antibiotics in 0-5-cm soil compared to deeper soil in various conditions (Biel-Maeso et al., 2018; Gros et al., 2019; Kulesza et al., 2016; Pan and Chu, 2017; Zhao et al., 2018). The results suggested that when liquid manure (5 % solid content) was surface applied to dry soil (soil water content of 16 % in 0-5 cm and 13 % in 5-20 cm; the soil water content is defined as ratios of the mass of water in soil samples to the mass of wet soil samples), although manure-associated antibiotics migrated to deeper soil profile, they remained at higher levels in 0-5-cm

soil, making them more susceptible to the surrounding environment via surface runoff. Le et al. (2018) has found antibiotics in surface runoff at all time-gaps (1 h, 3 d, and 7 d) between manure application and the sampling time points.

Results also indicated horizontal movement of antibiotics from the subsurface injection slits to surrounding soil, which has not been studied; however, antibiotics primarily concentrated within the injection slits (the black bars in Fig. 3.2). For each target antibiotic, its concentrations in the injection slits were significantly higher than in surrounding soil ($p < 0.05$ for all antibiotics, Appendix Q). In fact, the ratios of antibiotic concentrations in soil inside the injection slits to at 5 cm from the slits were 2 to 10 times for pirlimycin, 2 to 5 times for sulfamerazine, 3 to 14 times for tylosin, and 2 to 13 times for chlortetracycline. Moreover, antibiotics concentrations at 5 cm from the injection slits were significantly higher than at 25 cm from the slits ($p < 0.05$ for all antibiotics, Table S3). There were no differences in antibiotic concentrations between 20 and 25 cm from the injection slits. Additionally, target antibiotics were below detection limits at 60 cm from the injection slits for all time gaps after manure application (1 h, 3 d, or 7 d) and before the simulated rainfall. Also, when the time gaps after manure application increased from 1 h to 3 d then 7 d, differences in antibiotic concentrations between soil in the slits and at 5 cm decreased for all antibiotics at both soil depths. Although the time gaps after manure application definitely affected the antibiotic concentrations in soil, four antibiotics were detected at 20 or 25 cm from the injection slits for all time gaps. As a result, even the horizontal movement of antibiotics from injection slits occurred, it was very limited for all four tested antibiotics, antibiotics concentrated in the injection slits for all time gaps, suggesting the injection slits as a hot zone with elevated levels of antibiotics.

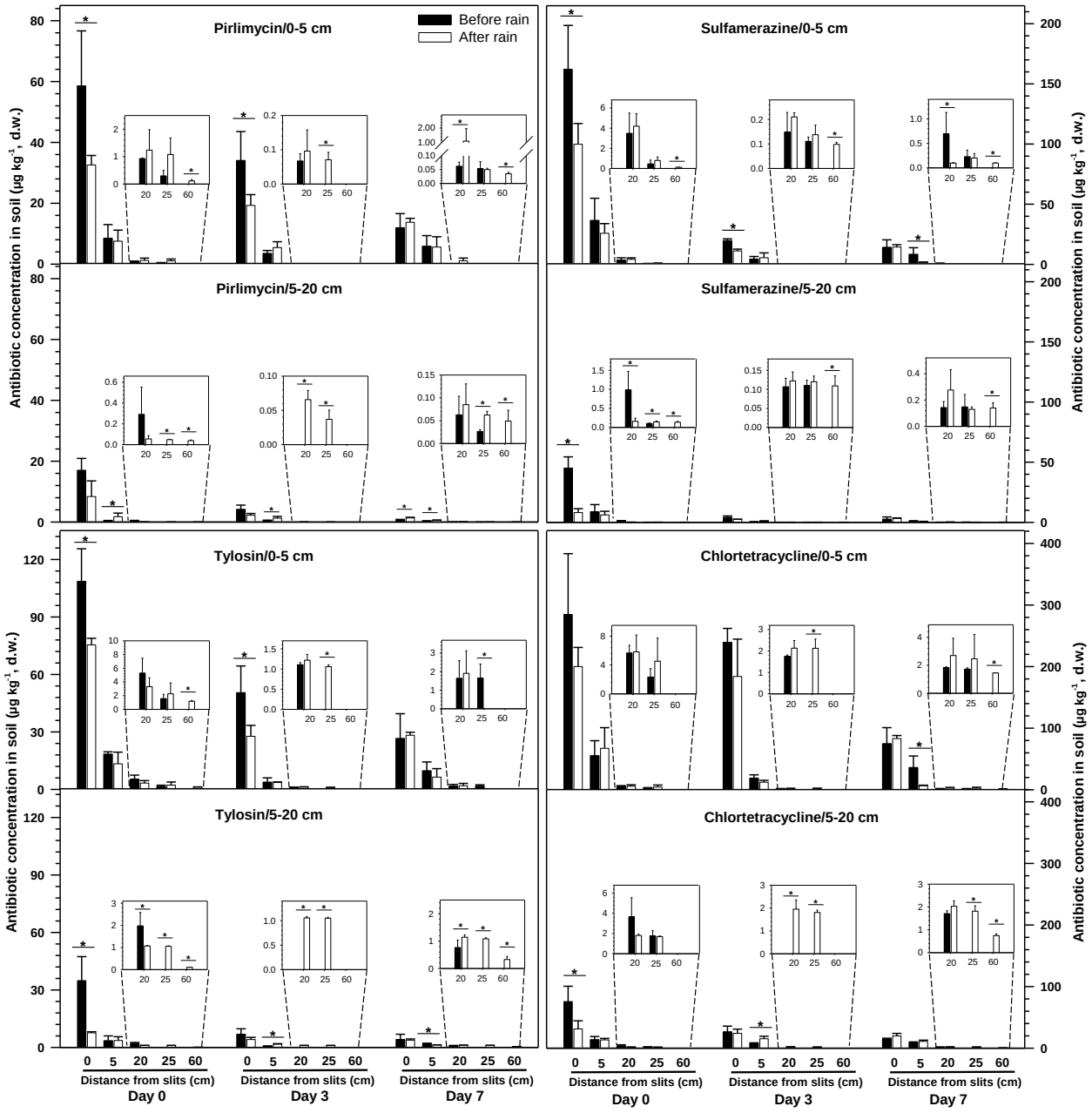


Figure 3.2 Concentration of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in 0-5- and 5-20-cm soil (µg kg^{-1}, dried weight) collected in the injection slits and 5, 20, 25, and 60 cm from the injection slits at 1 h before (*black bars*) and after simulated rainfall (*white bars*) with three manure-rainfall time gaps. * indicated a significant difference in antibiotic concentrations between before and after rain (p<0.05, Appendix T).

3.3.1.2 Effect of the subsequent simulated rainfall

Effects of simulated rainfall on the antibiotic distribution in soil depended on the manure application method, antibiotic, as well as manure-rainfall time gap (Day-0, Day-3, and Day-7 treatments). Such effects were presented as antibiotic concentrations in Fig. 3.1 and antibiotic mass in Appendix R. Effects of a rain event on antibiotic distribution in soil were strongest immediately after manure application (Day-0 treatment, the worst-case scenario). The mass and concentration of antibiotics in soil showed similar stories. Compared to before the rain, antibiotic concentrations in 0-5-cm soil after the rain generally decreased for both application methods and the decrease was significant for 5 out of 8 treatments (* in Fig. 3.1, *p* values in Appendix S). For 5-20-cm soil of the Day-0 treatment, antibiotic concentrations in the surface-applied treatment slightly increased after the rain, possibly due to downward movement of antibiotics from the 0-5-cm soil. However, the increase was only significant for sulfamerazine. In contrast, antibiotic concentrations in 5-20-cm soil in the injection slits were significantly reduced compared to before the rain, except for pirlimycin, which suggested downward movement of antibiotics to even deeper soil and/or lateral movement of antibiotics from the injection slits to surrounding soil. Several column studies and far fewer field studies have shown leachability of antibiotics in soil after a heavy rain (Biel-Maeso et al., 2018; Blackwell et al., 2009; D'Alessio et al., 2018; Halling-Sorensen et al., 2005; Park and Huwe, 2016). During a rain event, the soil water pressure increases, antibiotics can be mobilized via flows through soil structural pathways. When the manure-rainfall time gap increased to 3 d and 7 d, the simulated rainfall did not significantly change the antibiotic concentrations in soil from the surface application treatment.

The results were slightly different for the subsurface injection treatments. Simulated rainfall significantly reduced concentrations of pirlimycin, tylosin, and sulfamerazine in 0-5-cm

soil of the Day-3 treatment. However, simulated rainfall did not significantly change antibiotic concentration in 5-20-cm soil of the Day-3 treatment and in both soil depths of the Day-7 treatment. Hence, increased manure-rainfall time gaps decreased influence of a rain event on antibiotic distribution in soil possibly due to lower antibiotic concentrations in soil and increased time for antibiotics to sorb to soil particles. Among four tested antibiotics for both manure application methods, chlortetracycline showed the least mobility, lowering its risk of groundwater contamination via leaching. Sorption is considered as a major factor regulating mobility of antibiotics in soil and several studies have shown stronger sorption capacity of tetracycline groups compared to other antibiotic groups. In fact, K_d value (solid–liquid partition coefficient), a common indicator to predict affinity of antibiotics to the soil solid phase (Tolls, 2001), of tetracyclines was significantly higher than the other three antibiotic groups (0.6 to 4.9 L kg^{-1} for sulfonamides (sulfamerazine), 8.3 to 128 L kg^{-1} for macrolides (tylosin), 420 to 1030 L kg^{-1} for tetracyclines (chlortetracycline), and 0.8 to 2.2 L kg^{-1} for pirlimycin) (Le et al., 2018; Song and Guo, 2014).

Additionally, the simulated rainfall facilitated the horizontal movement of antibiotics from the injection slits to surrounding soil in both soil depths on a 9-11 % slope field. After the simulated rainfall, antibiotics were detected at further distances from the injection slits compared to before the simulated rainfall (antibiotic concentrations presented in Fig. 3.2 with p values listed in Appendix T and antibiotic mass presented in Appendix U). For sulfamerazine, it was detected up to 25 cm at both soil depths before the rain and it was detected up to 60 cm (0.10 to 0.14 µg kg^{-1}) after the rain for all manure-rainfall time gaps. After the simulated rainfall, pirlimycin and tylosin were detected at 60 cm for the Day-0 (except for tylosin at 0-5 cm) and 25 cm for the Day-3 treatment. For chlortetracycline, the simulated rainfall did not change its

detections and concentrations in both soil depths for the Day-0 treatment (the only exception was a significant decrease in 5-20 cm soil after the simulated rainfall). However, for the Day-3 and Day-7 treatments, chlortetracycline was moved further from the slits with rainwater, up to 25 cm and 60 cm for the Day-3 and Day-7 treatments, respectively. In spite of the horizontal movement, antibiotics mainly concentrated inside the injection slits and 5 cm from the slits. In fact, for all target antibiotics, their concentrations in the injection slits were significantly higher than in surrounding soils for both soil depths ($p < 0.05$ for all antibiotics, Appendix V), making the area as a hot zone compared to surrounding soil.

3.3.2 Temporal change of antibiotics in soil

3.3.2.1 Short-term change after manure application and before a subsequent simulated rainfall

Antibiotic concentrations in soils following 1 h, 3 d, and 7 d after manure application were illustrated as concentrations over time (Appendix W). Following 1 h after manure application at 0-5-cm soil, the initial concentrations of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in surface-applied soil and in the injection slits were 13.14 and 58.59 μg kg^{-1}, 21.69 and 161.94 μg kg^{-1}, 18.29 and 108.60 μg kg^{-1}, and 47.92 and 278.78 μg kg^{-1}, respectively. The initial concentrations of pirlimycin, sulfamerazine, tylosin, and chlortetracycline at 5-20 cm in surface-applied soil and in the injection slits were 0.91 and 17.03 μg kg^{-1}, 1.75 and 45.14 μg kg^{-1}, 1.41 and 34.88 μg kg^{-1}, and 4.59 and 75.66 μg kg^{-1}, respectively. These concentrations were environmentally relevant in soil (Pan and Chu, 2016; Thiele-Bruhn, 2003). In this study, the term "dissipation" is used to indicate a net decrease in antibiotic levels in soil due to biodegradation, transformation, sorption, and other processes. Dissipation of antibiotics has been tested in several

incubation studies in controlled conditions, but a few field studies have been conducted to reflect the dissipation of manure-associated antibiotics in reality (Carlson and Mabury, 2006; Halling-Sorensen et al., 2005; Heuer et al., 2008).

Antibiotic dissipation was faster during first 3 d and slowed down or even levelled off during 3 to 7 d following manure application for all target antibiotics, especially for sulfamerazine. The antibiotic dissipation rates depended on manure application methods and soil depths. At 3 d after manure application in 0-5 cm, percentages of antibiotics remaining in surface-applied soil and soil in the injection slits were 22 and 59 % for pirlimycin, 8 and 12 % for sulfamerazine, 26 and 46 % for tylosin, and 28 and 93 % for chlortetracycline. On the other hand, at 3 d after manure application in 5-20-cm, percentages of antibiotics remaining in surface-applied soil and soil in the injection slits were 42 and 24 % for pirlimycin, 18 and 9 % for sulfamerazine, 60 and 19 % for tylosin, and 77 and 35 % for chlortetracycline. During the first 3 d after manure application, antibiotic dissipation rates in the hot zone, injection slits, were slower in 0-5-cm soil but faster in 5-20-cm soil compared to the surface application treatment. The faster dissipation of antibiotics in the surface application treatment compared to in the injection slits in 0-5-cm soil could be explained by a better contact of antibiotics to soil particles, aerobic conditions at the soil surface, and photodegradation. The slower dissipation of antibiotics in the surface application treatment compared to in the injection slits in 5-20-cm soil could be attributed to the higher amount of nutrients and manure organisms in the injection slits. The Day-7 treatment showed that antibiotic concentrations during 3 to 7 d after manure application did not change as much as during the first 3 d after manure application. At 7 d after manure application in 0-5-cm soil, the concentrations of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in soil from the surface application treatment and in the injection slits were 3.05 and 11.91 μg kg^{-1},

0.95 and 14.08 µg kg^{-1}, 2.44 and 26.54 µg kg^{-1}, and 13.24 and 74.7 µg kg^{-1}, respectively. In 5-20-cm soil, the concentrations of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in surface-applied soil and in the injection slits were 0.60 and 0.67 µg kg^{-1}, 0.25 and 2.51 µg kg^{-1}, 0.75 and 4.05 µg kg^{-1}, and 2.63 and 14.77 µg kg^{-1}, respectively. Therefore, by applying manure at least 3 d before a heavy rainfall, antibiotic concentrations in soil would decrease significantly, resulting in a lower risk of antibiotic contamination via runoff (Le et al., 2018) and/or leaching.

3.3.2.2 Long-term change up to 180 days after the simulated rainfall

Antibiotic dissipation in soil from surface application and subsurface injection treatments

Longer-term changes of antibiotic concentrations in soil from immediately after the 1st simulated rainfall to 180 d after the rainfall suggested inconstant dissipation rates during 6 months (Fig. 3.3). In general, our data showed the fastest dissipation rates during the first 14 d after the simulated rainfall, slower dissipation rates between 14 to 60 d, and slowest rates after 60 d. Previous studies have also shown rapid dissipation of antibiotics in soil within first 10-15 days (Albero et al., 2018; Heuer et al., 2008; Pan and Chu, 2016; Shen et al., 2018). Within 14 d after the rain event, more than 50 % of antibiotics dissipated in soil for all antibiotics in all treatments. In an incubation study by Pan and Chu (2016), <60 % of tetracycline, <70 %, <20 % of norfloxacin, <40 % of erythromycin, and <40 % of chloramphenicol remained in soil at 14 d in aerobic and anaerobic conditions. At 60 d after the simulated rainfall, chlortetracycline concentrations in soil dropped below the detection limits, while more than 80 % of pirlimycin, sulfamerazine, and tylosin dissipated in soil. In an incubation study by Albero et al. (2018), at 60 d, ~ 20 % sulfamethazine and sulfamethoxazole (sulfonamides), <10 % lincomycin (lincosamides), and ~20 % chlortetracycline and doxycycline (tetracyclines) remained in soil. The maximum concentrations of pirlimycin, sulfamerazine, and tylosin in surface-applied soil

and soil in the injection slits at 60 d after the simulated rainfall were 0.30, 0.55, and 1.83 μg kg^{-1}, respectively. Additionally, at 6 months after the manure application, pirlimycin, sulfamerazine, and tylosin can be detected in manure-amended soil up to 0.18, 0.30, and 0.64 μg kg^{-1}, respectively. The detection of antibiotics in soil even after 6 months after a single manure application indicated that soils could be a long-term source for antibiotics to the surrounding environment, especially after repeated fertilizations (Hamscher et al., 2002).

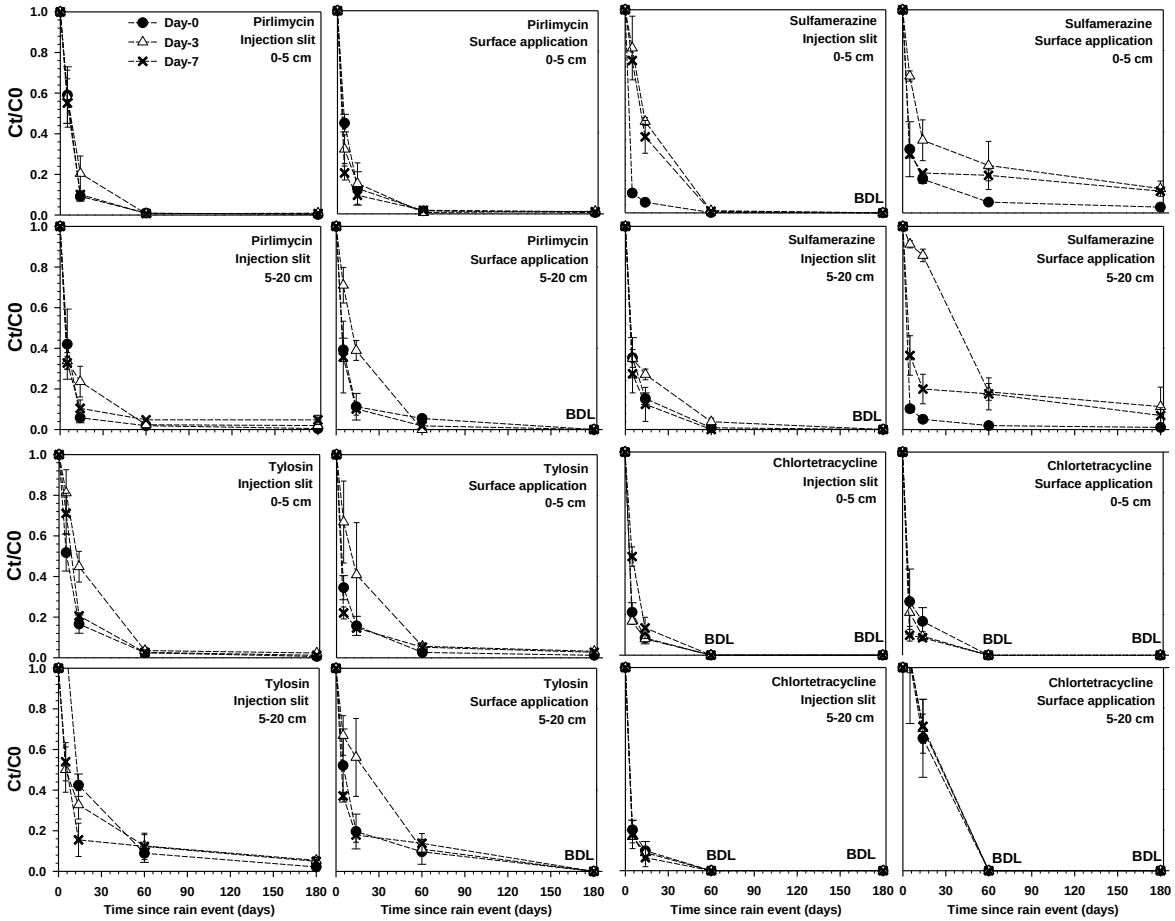


Figure 3.3 Concentration of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in 0-5- and 5-20-cm soil (µg kg^{-1}, dried weight) from the surface application treatment and the injection slits of the subsurface injection treatment during 180 d after the 1st rainfall which occurred at 2 h, 3 d, and 7 d after manure application.

Effects of the manure application method on dissipation kinetics of antibiotics in soil during 180 d varied by antibiotics, manure-rainfall time gaps, and soil depths (Appendix Y). In our previous study (Le et al., 2018), applying manure at least 3 d before a subsequent rainfall was recommended to reduce surface runoff loss of antibiotics, therefore discussion here focused on the Day-3 treatment. For chlortetracycline and tylosin, their dissipation kinetics between two application methods were not significant in 0-5-cm soil and significant in 5-20-cm soil. For pirlimycin, there were significant differences in its dissipation kinetics between two application methods for both soil depths, which contrasted with no differences between two application methods for both soil depths for sulfamerazine. Moreover, for the Day-3 treatment, dissipation kinetics between two soil depths for both application methods were different for sulfamerazine and indifferent for tylosin (Appendix Z). For chlortetracycline and pirlimycin, their dissipation kinetics between two soil depths were different for the surface application and indifferent for the injection slits. Different environmental conditions such as concentrations of antibiotics, nutrients, manure microbes, and water between two manure application methods as well as characteristics of antibiotics can all contribute to the observed results. For antibiotics, differences in sorption behavior and susceptibility to degradation can lead to different dissipation rates. Studies have also reported impact of starting concentrations (Day-0, Day-3, Day-7 treatments) on antibiotic dissipation in soil.

Due to the horizontal movement of antibiotics in soil from the injection slits, antibiotic concentrations in soil collected in between slits were measured up to 180 d after the simulated rainfall (Appendix X). In general, antibiotics were detected in in-between-slit soil at 1 h after the simulated rainfall. At 5 d after the simulated rainfall, antibiotics were detected at trace levels in these soils, <0.37 μg kg^{-1} soil for pirlimycin, <0.18 μg kg^{-1} soil for sulfamerazine, and <2.77 μg

kg^{-1} soil for chlortetracycline. Antibiotic levels dropped below detection limits from 5 d onward for tylosin, 60 d onward for pirlimycin and chlortetracycline, and 180 d onward for sulfamerazine.

Antibiotic half-life in soil from surface application and subsurface injection treatments

As mentioned above that antibiotic dissipation was fastest in the first 14 d after the simulated rainfall, slowed down during day 14 and day 60, and was slowest during day 60 and day 180. One single-phase first order kinetics did not appropriately illustrate antibiotic transformation in soil during 6 months from the surface application and subsurface injection treatments. Results showed that antibiotic half-life in soil samples from this study was within 14 d, therefore, data were fitted into a first-order kinetic ($C_t = C_0 e^{-kt}$) with three time points, 0, 5, and 14 d after the simulated rainfall for all target antibiotics, as shown in previous studies (Appendix AA) (Albero et al., 2018; Carlson and Mabury, 2006; Chen et al., 2018; Halling-Sorensen et al., 2005; Shen et al., 2018; Wind et al., 2018; Yang et al., 2018a). The half-life ($t = \ln 2/k$) of antibiotics and the coefficient of determination (R^2) of the linear regression between $\ln(C_t/C_0)$ and time were listed in Appendix AB and showed in Fig. 3.4. The half-life of antibiotics in soil ranged from 3 to 11 d for pirlimycin, 3 to 10 d for sulfamerazine, 5 to 12 d for tylosin, and 3 to 21 day for chlortetracycline. Reported half-lives of antibiotics in soil were in agreement with the half-lives obtained by other field and lab studies with 6 to 59 d for sulfonamides (Albero et al., 2018; Pan and Chu, 2017), 23 to 60 d for chlortetracycline (Albero et al., 2018; Carlson and Mabury, 2006; Chen et al., 2018; Halling-Sorensen et al., 2005; Pan and Chu, 2017), 5 to 8 d for pirlimycin (Chen et al., 2018; Wind et al., 2018), and 3 to 67 d for tylosin (Carlson and Mabury, 2006; Chen et al., 2018; Halling-Sorensen et al., 2005).

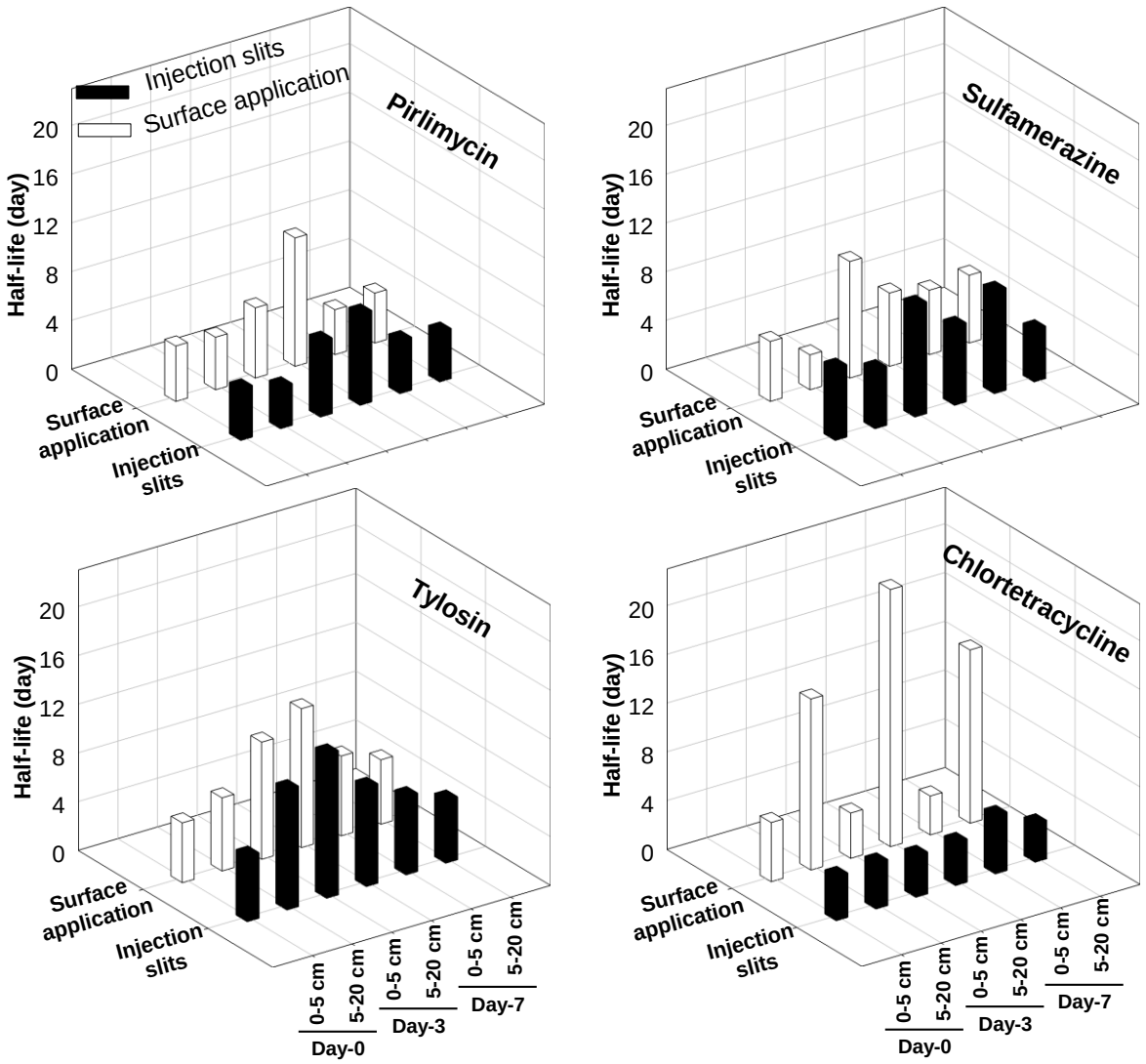


Figure 3.4 Half-life (day) of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in 0-5 and 5-20 cm soil from the surface application treatments and the subsurface injection slits with three manure-rainfall time gaps.

Results showed that there were no significant differences in terms of half-life (day) of pirlimycin, sulfamerazine, and tylosin in soil from surface application treatments and the subsurface injection slits in spite of their differences in initial concentrations. Meanwhile, for chlortetracycline, there was no difference in its half-life in 0-5 cm between two application methods; however, in 5-20-cm soil, chlortetracycline dissipated faster in soil in the injection slits

than from the surface application treatment. A study by Zhang et al. (2017) also showed no differences in half-life values of sulfadiazine and sulfamethoxazole at varying initial concentrations.

3.4 Conclusions

Following manure application, manure-associated antibiotics were spread evenly on soil surface for the surface application treatments, whereas antibiotics were concentrated in subsurface injection slits for the subsurface injection treatments. Even with vertical distribution of antibiotics to deeper soil profile, antibiotics remained at higher concentrations in 0-5-cm soil, making them more susceptible to the surrounding environment via surface runoff. Additionally, the horizontal movement of antibiotics from injection slits was very limited, suggesting the injection slits as a hot zone with elevated levels of antibiotics even after a rain event. Effects of simulated rainfall on antibiotic distribution in soil were strongest immediately after manure application and significantly decreased with increased manure-rainfall time gaps to 3 d and 7 d. Antibiotic dissipation rates were fastest during the first 14 d after the simulated rainfall with no significant difference between two manure application methods. In particular, at 3 d after manure application, 41 to 88 % of pirlimycin, 72 to 92 % of sulfamerazine, 40 to 81 % of tylosin, and 7 to 72 % of chlortetracycline dissipated in soil; therefore, applying manure at least 3 d before a heavy rainfall can lower a risk of antibiotic contamination via runoff and/or leaching. The half-life of antibiotics in surface-applied soil and soil in the injection slits was short and ranged from 3 to 11 d for pirlimycin, 3 to 10 d for sulfamerazine, 5 to 12 d for tylosin, and 3 to 21 day for chlortetracycline in the fall. However, pirlimycin, sulfamerazine, and tylosin remained detectable in soil even at 6 months after a single manure application indicating that antibiotic residues from a previous manure application season can be carried over to the next manure application season. In summary, this study provided additional evidence that farmers should implement manure subsurface injection and apply manure at least 3 d before a subsequent rain event. The governmental subsidy programs at certain states will help farmers implement the manure

subsurface injection method with a lower cost. However, the manure subsurface injection method might not be implemented on sandy soil or areas with high water table, in order to reduce groundwater contamination with manure-associated contaminants. Future studies are needed to test if the injection slit, the hot zone with elevated levels of antibiotics, would amplify antibiotic resistant genes/bacteria and if the subsurface injection slits would promote antibiotic leaching down the soil profile.

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Chapter 4: How fall and spring application seasons affect the fate of pirlimycin in manure surface applied and subsurface injected fields?

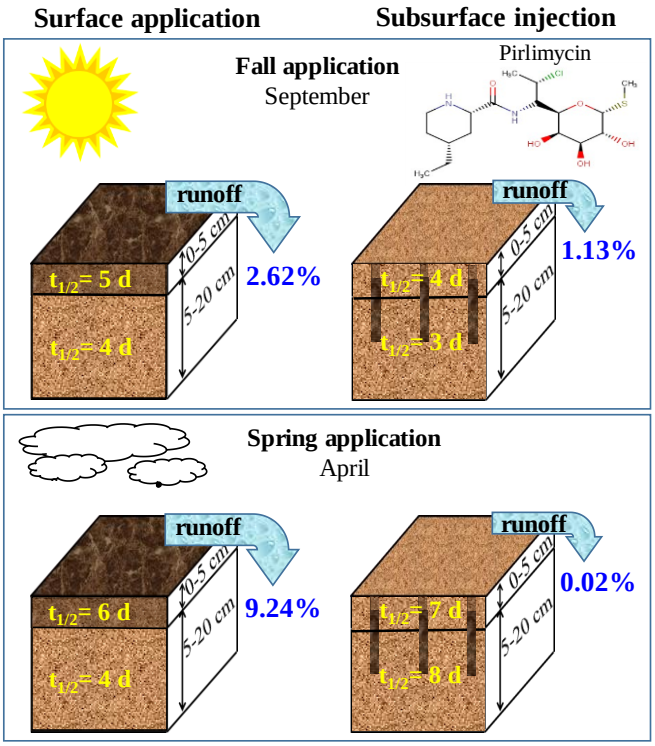
Hanh Thi Van Le, Partha Ray, Katherine Knowlton, Rory Maguire, and Kang Xia

(To be submitted to Chemosphere)

Abstract

Our previous study in the fall application season showed a significant reduction of antibiotic losses in surface runoff using manure subsurface injection compared to surface application. Another study was conducted in the spring application season to compare the effect of the two manure application methods on (1) antibiotic surface loss and (2) distribution and dissipation of antibiotics in soil in two application seasons. A lincosamide antibiotic-spiked dairy manure was applied at a typical application rate using subsurface injection and surface application methods. Rainfall simulation was conducted 1 h after amendment application to collect 30-min runoff. Following amendment application, soil samples (0-5 and 5-20 cm) were collected on day 0 (1 h before and after the rainfall), 5, 14, 60 and 150. The subsurface injection reduced the pirlimycin loss via surface runoff in both fall (>57 %) and spring (>99 %) compared to the surface application. The pirlimycin loss in runoff from the surface application was higher in the spring than that in the fall when manure was surface applied, which makes it more important to implement the subsurface injection in the spring. The manure application method did not affect the pirlimycin transformation kinetics in soil, but the manure application season did. Pirlimycin half-life in soil in fall (3-5 d) was shorter than in spring (4-8 d), which can be explained by differences in soil temperature and moisture. Using the manure subsurface injection would be the best practice to reduce the environmental risk of antibiotics in soil, especially in the spring.

Graphic abstract



Highlights

- Manure subsurface injection reduces antibiotic runoff, especially in the spring
- Application methods did not affect antibiotic dissipation in soil.
- Half-life of pirlimycin in soil in the spring is longer than in the fall
- Antibiotic movement in moist soil is limited compared to dry soil
- Soil temperature and moisture affect antibiotic transformation kinetics

4.1 Introduction

It is estimated that 40 to 95 % of the amount of antibiotics administrated to an animal is excreted in the feces and urine, depending on types of antibiotics, used dosage, species of animals, routes of administration, and time after administration (Boxall et al., 2006; Jechalke et al., 2014). A wide range of antibiotics has been detected in manure at a level as high as tens to hundreds mg kg^{-1} (Haller et al., 2002; He and Zhang, 2014). Therefore, applying animal manure onto a field as fertilizers introduces manure-associated antibiotics to soil, especially after repeated applications. The presence of antibiotics might exert selection pressure on manure and soil microbes, amplifying development and spread of antibiotic resistance genes/bacteria (Gullberg et al., 2011; Guo et al., 2018). Besides, from manure-applied fields, antibiotics can travel via surface runoff to surface water bodies, which can be used as water for irrigation and/or linked with drinking water sources. Several studies have confirmed that surface runoff is indeed a mean of spreading antibiotics to the surrounding environment. Kreuzig et al. (2005) reported that the total loss of sulfonamides via surface runoff from manure-surface applied grassland ranged from 13 to 28 % of sulfonamides applied initially after 2 h of irrigation (50 mm h^{-1}). On arable land, up to 2.5 % of applied sulfonamides was lost with surface runoff. In a 3-yr field study, Dolliver and Gupta (2008) reported that monensin, tylosin, and chlortetracycline were detected in runoff with a highest concentration of 57.5, 6.0, and 0.5 µg L^{-1}, respectively. Chlortetracycline and tylosin were also detected in surface runoff from fields receiving swine manure slurries in a rainfall simulation study by Joy et al. (2013). In manure-applied fields, after 30 min of simulated rainfall (70 mm h^{-1}), losses of chlortetracycline, tylosin, sulfamerazine, and pirlimycin via surface runoff were up to 3 – 5 % of applied antibiotics, respectively (Kulesza et al., 2016; Le et al., 2018). Carbadox, florfenicol, and chlortetracycline were detected at

concentrations of 1577, 666, and 570 ng L^{-1}, respectively, in Kyungahn stream in South Korea, which received runoff from agricultural areas and livestock farms in the upper stream area (Kim et al., 2016).

Detection of antibiotics in surface runoff leads to efforts to reduce them in surface runoff from manure-applied field. To overcome the disadvantage of increased nutrient loss following manure application using a traditional method i.e. broadcasting manure onto a field, subsurface injection has been developed as a technology to incorporate manure into soil (Maguire et al., 2013; Maguire et al., 2011). It has been reported that the subsurface injection of manure can significantly reduce the antibiotic loss via surface runoff. Kulesza et al. (2016) reported reductions of six and three times in pirlimycin concentrations in runoff water and sediment, respectively, from the manure subsurface injection treatments compared to surface application treatments. In our previous study the subsurface injection reduced sulfamerazine, chlortetracycline, pirlimycin, and tylosin losses in runoff by at least 47, 50, 57, and 88 %, respectively, compared to the surface application (Le et al., 2018). Reduction of the antibiotic loss in surface runoff following subsurface injection of manure is likely due to less exposure of manure-associated antibiotics to rain water.

Reduced antibiotic loss via surface runoff following subsurface injection of manure and limited loss of antibiotics due to volatilization, mineralization to CO_2, and photodegradation mean that manure-associated antibiotics following manure application primarily remain in soil (Ozaki et al., 2011; Pan and Chu, 2017; Schmidt et al., 2008; Spielmeyer et al., 2017). Sequestration of antibiotics within soil particles and biotic degradation of antibiotics play an important role in determining the fate of antibiotics in soil (Accinelli et al., 2007; Jechalke et al., 2014; Pan and Chu, 2016). Manure application methods might affect dissipation kinetics of

antibiotics in soil. While manure is spread evenly on soil surface via surface application, it is concentrated in injection slits of the subsurface injection. Thus, compared to soil where manure has been surface-applied, soil in the injection slits can be seen as a hot zone with an elevated level of nutrients, organic matter, water, manure microbes, and antibiotics, all of which can affect the environmental fate of antibiotics (Unpublished data). The presence of a large amount of organic matter from manure amendment can decrease the mobility of antibiotics by acting as an effective sorbent (Li et al., 2010; Wang et al., 2015). Organic matter and inorganic nutrients from manure can promote microbial activities, which can lead to increased biodegradation of antibiotics (Lupwayi et al., 2019; Urra et al., 2019). In fact, compared to the no manure treatment, addition of manure can alter dissipation rates of antibiotics (Accinelli et al., 2007; Albero et al., 2018; Carlson and Mabury, 2006; Zhang et al., 2017). Compared to the surface application, the injection slits have a higher content of water and nutrients, which are covered below soil surface. Due to decreased gas diffusion and rapid degradation of bioavailable organic C, conditions in the injection slits support reduction reactions and anaerobic microbes, while the top soil of the surface application facilitates oxidation reactions and aerobic microbes. Sulfadiazine and sulfamethoxazole persisted longer in soil under anaerobic conditions compared to aerobic conditions (Shen et al., 2018).

In addition to manure application methods, manure application seasons can affect the environmental fate of manure-associated antibiotics. Winter application is limited due to the high risk of nutrient loss via transport pathways created by frozen and water-saturated soil and the minimal nutrient uptake from crops. Crops are grown in the summer making it difficult for application. There are two big windows for manure application: spring and fall. Spring application often occurs after soil warms up for the major crop production of the year. Fall

application occurs after the crops have been harvested for the winter crops. With differences in soil conditions (such as moisture content and soil temperature) and weather conditions (such as precipitation and air temperature) between fall and spring application seasons, it is expected that the environmental fate of antibiotics in soil following manure application would be different. In a 11-yr field study, Liu et al. (2017) reported a lower P loss in the spring manure application than the fall application. The spring application also had a lower N loss in drain water than the fall manure application in a 3-yr field study by van Es et al. (2006).

The objective of the study was to compare the environment of fate of pirlimycin in liquid manure-applied field via surface application vs. subsurface injection during fall (drier and hotter) and spring (wetter and colder). In particular, we were interested in the surface runoff loss of pirlimycin and the spatial distribution and temporal change of pirlimycin in soil.

4.2 Materials and Methods

4.2.1 Experimental setup

4.2.1.1 Field setup

Two field rainfall simulation studies were conducted in fall 2015 (starting September) and spring 2016 (starting April), simulating the fall and spring application seasons, on the same field adjacent to each other at Kentland Farm, Blacksburg, VA. The field is no till growing corn and barley and soil is Braddock loam (fine, mixed, semiactive, mesic Typic Hapludults; 46 % sand, 44 % silt, and 10 % clay) with a slope of 9 to 11 % (Kulesza et al., 2016). Distance among test plots (1.5 × 2.0 m) was ~4.5 m to avoid cross contamination. A metal pan was installed at the down slope edge of each plot to direct surface runoff through a hose to a collecting container. In both fall and spring, there were three replicates of three treatments: 1) the control without manure, 2) the manure surface application, and 3) the manure subsurface injection. The experiment was set up as a randomized complete block design with one replicate of each treatment in each block. For each test plot receiving manure, right before manure application, liquid diary manure (5 % solid content) was spiked with pirlimycin (PIRSUE, Zoetis, NJ, U.S.A) to achieve the concentration of 131 μg kg^{-1} liquid manure, which was the concentration of pirlimycin in manure from cows injected with pirlimycin (Ray et al., 2017). Pirlimycin is a lincosamide antibiotic which is commonly used to treat mastitis in dairy cows on U.S. dairy farms (USDA-APHIS-VS-CEAH, 2008). The pirlimycin-spiked liquid manure was applied based on an agronomic N rate of 56 Mg wet wt. ha^{-1}, which is typical in Virginia. For the surface application, manure was poured on a metal tray to spread evenly on soil surface, meanwhile, for the subsurface injection, manure was poured into the precut injection slits (5 cm wide × 10 cm

deep) and the injection slits were close with soil afterward. The injection slits were cut perpendicular to the slope using a Yetter injection disc mounted on a three-point hitch.

4.2.1.2 Rainfall simulation experiment

Rainfall simulation was conducted following the protocol of the National Research Project for Simulated Rainfall – Surface Runoff Studies allowing result comparisons between studies (SERA-17, 2008). In particular, rainfall was simulated by a rainfall simulator (2.44 × 3.05 m) using water from a nearby well (free of pirlimycin) at an intensity of 70 mm h^{-1}. At the center of the rainfall simulator, there was a TeeJet nozzle and the water flow rate was established at 210 mL s^{-1}. To avoid rain water getting into the runoff collection pan at the bottom of the test plot, a plastic sheet was placed on top the pan. The simulated rainfall was conducted at approximately 2 h after manure application to mimic the worst case scenario since applying manure at least 3 d before a subsequent rainfall could significantly reduce the antibiotic loss in surface runoff (Le et al., 2018). For each test plot, 30 min of surface runoff collection started when the runoff started to flow into a receiving container located at a down slope. The time to start runoff, the amount of collected runoff, and the total runoff sediment load were reported in Appendix AC.

4.2.1.3 Sample collection

Subsamples of liquid manure (spiked manure) were collected before applying to each test plot to measure the initial concentration of pirlimycin in amendment. When the runoff collection finished, ~2 L of surface runoff were collected in clean mason jars after a vigorous stir to create a homogeneous mixture of runoff water and sediment. Soil samples were collected at 1 h after manure application and immediately before the simulated rainfall (referred to as before rain), 2 h

after manure application and 1 h after the simulated rainfall (referred to as 1 h after rain), and on day 5, 14, 60, and 150 after manure application. Soil samples were collected at two depths, 0-5 cm and 5-20 cm. Each soil sample was comprised of six soil cores (2 cm in diameter) collected randomly in designated areas. To avoid any disturbance inside the test-plot due to soil sampling before the simulated rainfall, liquid manure was applied to a larger area than the test plot and soil samples were collected outside the test plot in the designated area. For the sampling time points following rainfall simulation, soil samples were collected from inside the test plot. For the subsurface injection treatments, soil samples were collected from the injection slits and between injection slits at equal distance between two slits. All collected samples were immediately placed on ice before being stored at -20°C until analysis.

4.2.2 Sample extraction and analysis

4.2.2.1 Sample extraction

See Appendices B, D, and E.

4.2.2.2 UPLC-MS/MS analysis

See Appendix F.

4.2.3 Measurement and statistical analysis

Measurements of pirlimycin in this paper included the concentration of pirlimycin in different matrices, the surface runoff loss of pirlimycin, and the dissipation kinetics of pirlimycin showed as its concentration at time t over its initial concentration (Ct/C0). The surface runoff loss of pirlimycin in runoff water or sediment was calculated by multiplying its concentration in the runoff water or sediment and the volume of runoff water or the mass of runoff sediment collected during 30 min runoff (Appendix AC). Soil moisture content (%) in samples was

measured as the ratio of the amount of water in the samples to the total amount of the samples. Based on data collected from a weather station located at Kentland farm (Virginia Agricultural Experimental Station, 2019), the soil temperature (^{o}C) during the experimental period (Sep 2015 – Sep 2016) was presented using the medium in a day.

All statistical analyses were conducted using JMP Pro 14 (SAS Institute, 2016) and significant differences were declared at $p < 0.05$. The effect of the manure application method, the manure application season, or the simulated rainfall on the concentration of pirlimycin, the surface runoff loss of pirlimycin, or the half-life of pirlimycin was tested using a Wilcoxon/Kruskal–Wallis test (rank sums). Because of the small sample size, the χ^2 approximation was used in the Wilcoxon/Kruskal–Wallis test (JMP Pro 14). The effect of manure application method, manure application season, and soil depth on the dissipation kinetics of pirlimycin over time was measured using a mixed model, in which these three factors were used as the fixed effects and the time was used as the random factor. For the sample with a pirlimycin concentration below the detection limit, its value was assigned as half of the detection limit for statistical analysis.

4.3 Results and Discussion

4.3.1 Surface runoff loss of pirlimycin from raw manure-applied fields

The manure subsurface injection is recommended over the surface application to reduce the surface runoff loss of pirlimycin in both application seasons, especially in wet spring (Fig. 4.1). Compared to the surface application, the subsurface injection lowered the loss of pirlimycin with surface runoff water and surface runoff sediment by 57 % and 71 %, respectively in the fall, and >99 % in the spring. The fact that manure is buried below the soil surface in the subsurface injection, whereas manure is spread on the soil surface in the surface application. Thus, the subsurface injection can reduce interaction of rain water with manure and manure-associated antibiotics, resulting in a lower loss of antibiotics with surface runoff. Kulesza et al. (2016) also reported that manure injection treatments reduced pirlimycin concentrations six and three times in runoff water and sediment, respectively, compared to surface application treatments. Kreuzig et al. (2005) observed a lower loss of sulfonamides with runoff from manure-incorporated arable land than from manure-surface applied grassland. Joy et al. (2013) also showed that broadcast treatments had a higher total antibiotic mass loading in runoff water compared to incorporated and injected treatments.

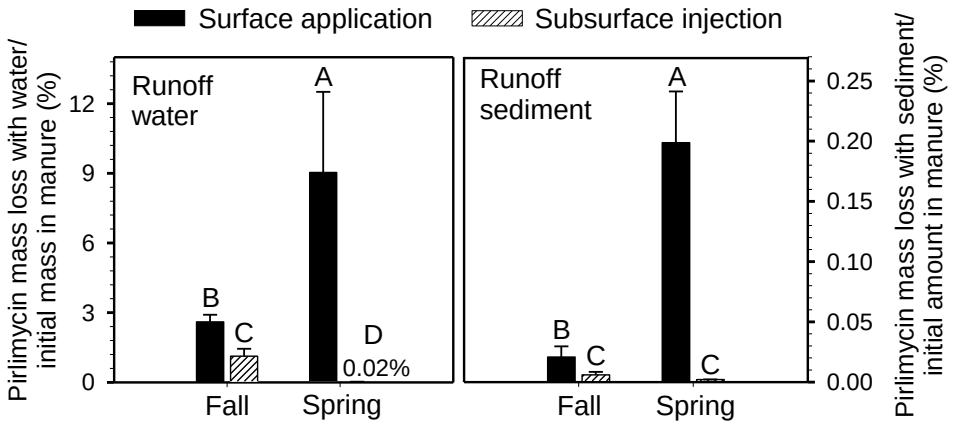


Figure 4.1. Surface runoff loss (water and sediment) of pirlimycin that was initially applied with liquid manure to the surface application and subsurface injection test plots in fall and spring. Different letters indicate a significant difference ($p < 0.05$).

Obviously, the surface runoff loss of pirlimycin from the surface application treatments in the spring was significantly higher than that in the fall, which makes it more important to use the subsurface injection in the spring. Surface runoff occurs when soil is saturated or the intensity of rainfall is higher than the capacity of soil absorbing water. Following raw manure application via surface application method, soil moisture in 0-5 cm in the fall and spring was 18 ± 2 and 26 ± 1 (%), respectively, and in 5-20 cm was 12 ± 2 and 17 ± 2 (%), respectively (Appendix AE). The moister soil from the surface application treatment in the spring compared to the fall can contribute to its higher runoff loss of pirlimycin in the spring. Studies have showed an abruptly increased runoff coefficient after a certain moisture threshold (Penna et al., 2011). In practice, since farmers usually grow corn in spring and wheat, barley, or oats in the fall, they tend to apply manure at a higher rate in the spring than in the fall due to a higher N requirement of growing corn, which adds more reasons for using the subsurface injection in the spring. Additionally, in all treatments, >90 % of pirlimycin, a hydrophilic antibiotic, in surface runoff was detected in the aqueous part. Physicochemical properties of antibiotics regulate distribution of antibiotics between aqueous and solid parts of the surface runoff. A study by Le et al. (2018) showed that in surface runoff, sulfamerazine (94 ± 4 %) and tylosin (91 ± 7 %) primarily left the manure-amended fields with runoff water, whereas chlortetracycline (78 ± 15 %) mainly travelled with runoff sediment. Therefore, weakly bound antibiotics [K_d of 0.8 to 2.2 L kg^{-1} for pirlimycin (Le et al., 2018), 0.6 to 4.9 L kg^{-1} for the sulfonamide group, 8.3 to 128 L kg^{-1} for the macrolide group (He and Zhang, 2014)] tend to be moved from manure-applied fields in surface

runoff water. Meanwhile, the strongly bound antibiotics [K_d of 420 to 1030 L kg^{-1} for the tetracycline group (He and Zhang, 2014)] tend to be associated with particles eroded by runoff water.

4.3.2 Spatial distribution of pirlimycin in soil after raw manure application and after a subsequent first rainfall

The distribution of pirlimycin in soil immediately after liquid manure application showed some similarities in both application seasons. In fact, pirlimycin was detected in 0-5- and 5-20-cm soil in both surface application and subsurface injection treatments in fall and spring (Fig. 4.2, black bars). The detection of pirlimycin in 5-20-cm soil of the surface application treatment suggested a downward movement of the antibiotic in soil, since liquid manure was spread on the soil surface. The concentration of pirlimycin in 5-20-cm soil of the surface application treatment in the fall and spring was 0.91 ± 0.33 and 0.47 ± 0.23 µg kg^{-1}, respectively. According to a study by Bailey et al. (2016), lincosamide antibiotics, the group that pirlimycin belongs to, mainly stay in the liquid fraction of a liquid manure mixture. Thus, following raw manure application, pirlimycin can move in soil with the manure liquid solution and stay in the soil aqueous phase. In another incubation study, in which soil was amended with composted manure containing antibiotics, sulfonamide and lincosamide groups had the highest level in the soil aqueous phase and tetracycline groups had very low levels in the soil aqueous phase (Albero et al., 2018). The detection of pirlimycin in 5-20-cm soil in the injection slits of the subsurface injection was expected since the injection slits containing manure was approximately 10 cm deep. The concentration of pirlimycin in 5-20-cm soil in the injection slits was 17.03 ± 3.87 and 9.35 ± 2.77 µg kg^{-1} in the fall and spring, respectively. There was no pirlimycin detected in soil

collected in between the injection slits immediately after manure application, confirming the limited movement of antibiotics in soil from the injection slits. In soil, antibiotics can spread via gradient diffusion or bulk flow with water through preferential pathways. Furthermore, regardless of the manure application method, the pirlimycin concentration in 0-5-cm soil was significantly higher than in 5-20-cm soil in both seasons (Fig. 4.2), suggesting susceptibility of pirlimycin to spread to the nearby environment via surface runoff. Both field and lab studies in various conditions have observed the accumulation of various antibiotics in 0-5-cm soil compared to deeper soil (Biel-Maeso et al., 2018; Gros et al., 2019; Kulesza et al., 2016; Pan and Chu, 2017; Zhao et al., 2018).

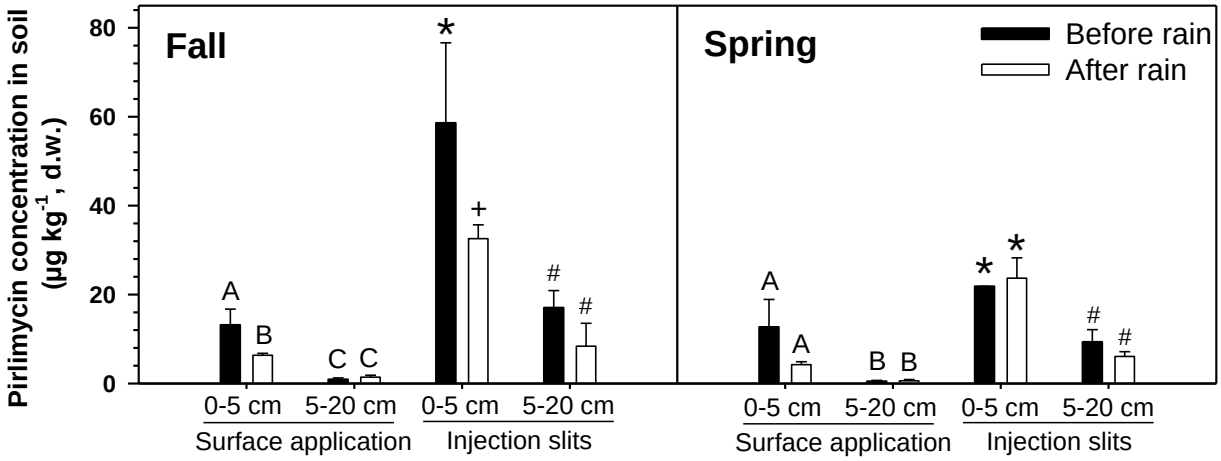


Figure 4.2. Concentration of pirlimycin in 0-5- and 5-20-cm soil (µg kg^{-1}, dried weight) collected in the manure surface application treatments and in the injection slits of the subsurface application treatments 1 h before (*black bars*) and after the first rainfall (*white bars*) following liquid manure application in fall and spring. Different letters/symbols indicate significant differences for each application method in each application season ($p < 0.05$).

Effect of a subsequent rainfall event on the distribution of pirlimycin in soil depended on the application season instead of the application method (Fig. 4.2, black and white bars). In the spring, the simulated rainfall did not significantly affect the concentration of pirlimycin in both

soil depths for both manure application methods. Meanwhile, in the fall, compared to the pirlimycin concentration before the rain, the pirlimycin concentration after the rain significantly decreased in 0-5-cm soil and did not significantly change in 5-20-cm soil for both manure application methods. Immediately after liquid manure application, the moisture content in 0-5-cm and 5-20-cm soil in the injection slits was 35 ± 7 and 25 ± 3 (%), respectively, in the spring and 25 ± 3 and 16 ± 3 (%), respectively, in the fall. For the subsurface injection test plots, since the injection slits only account for a small area, the soil moisture content in the remaining areas (background soil) of the test plot immediately after manure application can influence the effect of rainfall on antibiotic distribution in soil. The moisture content in 0-5 and 5-20 cm of the background soil was 21 ± 1 and 18 ± 1 (%), respectively in the spring and 16 ± 1 and 13 ± 0 (%), respectively in the fall. For the surface application treatment, the moisture content immediately after manure application in 0-5-cm and 5-20-cm soil was 26 ± 1 and 17 ± 2 (%), respectively, in the spring and 18 ± 2 and 12 ± 2 (%), respectively, in the fall. The moister soil in the spring compared to in the fall can contribute to reduce the amount of rain water that soil absorbs, resulting in more limited movement of pirlimycin in soil.

4.3.3 Temporal change of pirlimycin in manure-applied fields

Transformation kinetics of pirlimycin in 0-5- and 5-20-cm soil from the surface application treatment and the injection slits of the subsurface injection treatment during 150 d after liquid manure application in the fall and spring were showed in Fig. 4.3. Overall, pirlimycin transformed at unequal rates during 150 d after manure application with a faster kinetic during first 14 d before slowing down, probably because the availability of antibiotics in soil decreases exponentially with time, mainly due to sorption, protecting them from degradation (Pan and Chu,

2016; Stoob et al., 2007; Wang et al., 2006). Similar transformation patterns of antibiotics with decreasing rates were observed in various conditions such as composting processes (Ray et al., 2017), lagoon storage (Kuchta and Cessna, 2009), field manure-applied soil (Kulesza et al., 2016; Wind et al., 2018), and lab incubation soil (Albero et al., 2018; Chen et al., 2018). By 14 d after liquid manure application, 70 to 85 % of pirlimycin reduced in soil in the spring and 88 to 94 % of pirlimycin reduced in soil in the fall. For all treatments, < 6 % and 1 % of the initial amount of pirlimycin remained in soil by 60 d and 150 d, respectively after manure application.

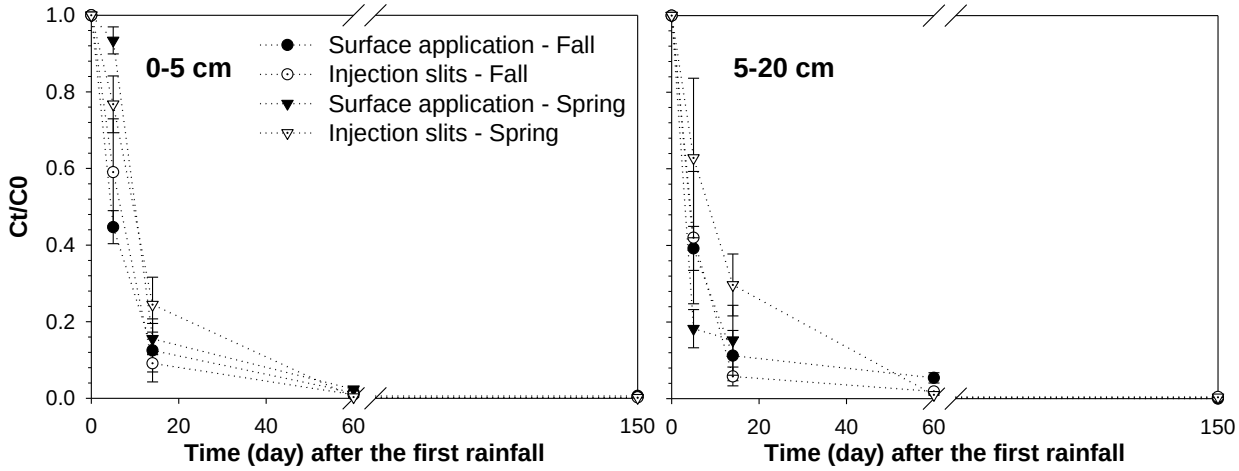


Figure 4.3 Ratios of pirlimycin concentrations (µg kg^{-1}, dried weight) at time t over its initial concentrations (Ct/C0) in 0-5- and 5-20-cm soil from the surface application treatment and the injection slits of the subsurface injection treatment during 150 d after manure application in fall and spring.

One single-phase first order kinetics did not appropriately illustrate antibiotic transformation in soil. Since results showed that pirlimycin half-life in soil samples from this study was within 14 d, therefore, pirlimycin data were fitted into a first-order kinetics ($C_t = C_0e^{-kt}$) with three time points, 0, 5, and 14 d after manure application, as shown in previous studies (Albero et al., 2018; Carlson and Mabury, 2006; Chen et al., 2018; Halling-Sorensen et al., 2005;

Shen et al., 2018). The half-life (t = ln2/k) of pirlimycin in soil ranged from 3 (±1) to 8 (±2) d (Fig. 4.4). Similar half-lives of pirlimycin in soil were reported in both field (Wind et al., 2018) and lab studies (Chen et al., 2018). The half-life of pirlimycin for compost was 4 to 5 d (Ray et al., 2017).

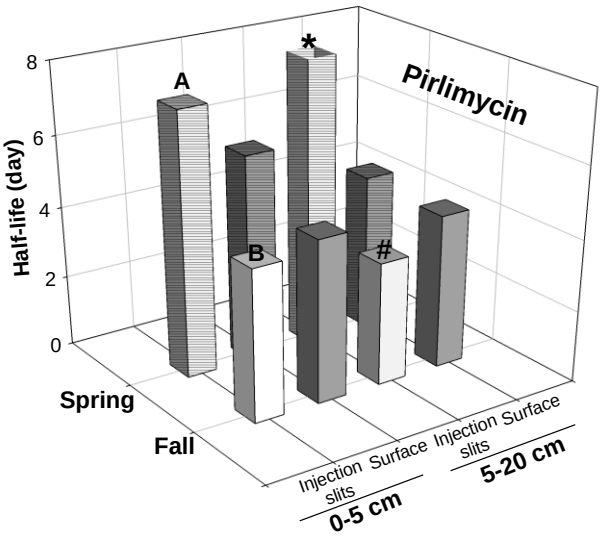


Figure 4.4. Half-life (day) of pirlimycin in 0-5 and 5-20 cm soil from the surface application treatments and the subsurface injection slits in fall and spring. The only significant difference belonged to the half-life of pirlimycin in soil in the injection slits between spring and fall.

If raw manure was applied in the fall, there were no significant differences in pirlimycin dissipation in soil between two application methods in both 0-5 and 5-20 cm (Appendix AD). However, if the raw manure was applied in the spring, no significant difference in pirlimycin dissipation between two application methods was observed in 0-5-cm soil and a significant difference was seen in 5-20-cm soil. In other words, there were similar changes of pirlimycin over time in manure-applied soil via surface application and subsurface injection in both application seasons. The only exception belongs to the 5-20-cm soil in the spring. However, the

half-life of pirlimycin in soil confirmed no significant difference between two application methods.

Overall, the application season had a significant effect on the dissipation of pirlimycin in soil and the pirlimycin half-life in soil from the injection slits (Appendix AD and Fig. 4.4). For the subsurface injection method, pirlimycin dissipation over time in both soil depths was significantly different between fall and spring. Meanwhile, for the surface application treatment, the difference was significant in 0-5-cm soil and insignificant in 5-20-cm soil. The different dissipation kinetics between two seasons happened mainly by 60 d after manure application. Furthermore, the half-life of pirlimycin in soil in the spring appeared higher than in the fall, in general. In particular, the pirlimycin half-life in soil in the injection slits was significantly higher in the spring than in the fall in both soil depths and the differences in the half-life of pirlimycin in soil between the fall and spring for the surface application were not significant in both soil depths. Therefore, although pirlimycin tends to stay longer in manure-amended soil in the spring compared to the fall, when pirlimycin concentrations dropped below certain thresholds (after 60 d), there were no differences in pirlimycin trace levels among treatments by 150 d.

Environmental conditions play an important role in dissipation rates of antibiotics in soil, especially soil temperature and moisture. While soil temperature can regulate the overall dissipation rates, soil moisture mainly alters the sequestration of antibiotics in soil (Rosendahl et al., 2011). The antibiotic degradation rate normally becomes faster under higher temperature. The higher temperature can not only enhance microbial activities but also the energy of molecules decreasing their stability. The enhanced dissipation of chlortetracycline with increased temperature was observed in manure and soil (Loftin et al., 2008; Zhang and Zhang, 2010). In terms of water content, drier soil would promote antibiotic dissipation processes since dry soil

favors oxidation reactions and aerobic soil microbes. Sulfadiazine and sulfamethoxazole persisted longer in soil under anaerobic conditions compared to aerobic conditions (Shen et al., 2018).

Generally, the soil temperature peaked at the beginning of the fall 2015 study, decreased until mid-Feb (~120 d after fall manure application) before increasing until mid-August (~120 d after spring manure application) (Appendix AE). The soil temperature during 14 d after manure application in the fall 2015 (18.1 ± 0.3 ^{o}C) was higher than in the spring 2016 (16.2 ± 0.8 ^{o}C). Based on the temperature, it is expected that pirlimycin dissipation rates during 14 d after manure application in the fall are faster than in the spring. Then between 14 and 150 d, the temperature can slow down pirlimycin dissipation rates after the fall application and speed up pirlimycin dissipation rates after the spring application, resulting in no difference in pirlimycin residues between two seasons at 150 d. Soil moisture content in the injection slits at 1 h after the manure application and 1 h after the rainfall was lower in the fall than in the spring at both soil depths, supporting the shorter half-life of pirlimycin in soil in the injection slits in the fall. There were no differences in soil moisture content in the injection slits at 5 and 14 d after manure application between two seasons. For the surface application, soil moisture content in the fall was only lower in the spring at 1 h after manure application and there were no differences at other soil samplings during the 14-d period, supporting the similar pirlimycin half-life in soil of the surface application between two seasons.

4.4 Conclusions

Two rainfall simulation studies were conducted in the fall and spring to demonstrate that compared to the surface application, the subsurface injection reduced the loss of

pirlimycin with surface runoff in both the fall (>57 %) and spring (>99 %). The surface runoff loss of pirlimycin from the surface application treatment in the spring was significantly higher than that in the fall, probably due to the moister soil in the spring compared to in the fall at the application time, which makes it more important to use the subsurface injection in the spring. Especially, manure is often applied at a higher rate in the spring application for the major crops of the year than the fall application. The moister soil in the spring compared to in the fall at manure application time can contribute to the limited movement of pirlimycin in soil in the spring compared to in the fall. Finally, pirlimycin in soil showed continuously-decreased transformation rates during 150 d after manure application with a faster kinetic during first 14 d before slowing down. The manure application method did not significantly affect the pirlimycin transformation kinetics in soil, but the manure application season did. In particular, pirlimycin levels tend to decrease faster in the fall than in the spring. Although its half-life in manure-applied soil is rather short (3 to 8 d), it remained in soil at detectable concentrations by 150 d after one single manure application in both fall and spring.

4.5 References

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Chapter 5: Environmental fate of pirlimycin in manure subsurface injected soil with pirlimycin-spiked dairy manure/compost and manure/compost from pirlimycin treated cows

Hanh Thi Van Le, Partha Ray, Katherine Knowlton, Rory Maguire, and Kang Xia

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Abstract

Several studies have been conducted to understand the environmental fate of antibiotics. Currently, it is uncertain that the fate of antibiotics in soil receiving spiked manure and manure from animals with antibiotic administration can be cross-compared. Effects of composting with different or similar antibiotic levels compared to raw manure, on antibiotic behaviors in soil are not clear. This field study was to determine how the manure types and the antibiotic sources influence the antibiotic fate in soil following manure application via subsurface injection. About 2 h after the manure application, a rainfall was simulated for 30-min runoff collection and soil samples were collected up to 150 d after manure application. Results showed that the antibiotic source did not affect the runoff loss or short-term distribution of pirlimycin in soil immediately after manure application and after the rainfall. However, the transformation rate of pirlimycin in soil from the Raw Manure – Spiked treatment was significantly faster than that from the Raw Manure – Cow Fed treatment. As a result, studies using spiked manure can be used to mimic studies using manure from animals with antibiotic administration for antibiotic runoff and antibiotic distribution in soil. However, spiked studies can underestimate the antibiotic transformation rates compared to the real conditions, especially for partly-metabolized antibiotics in animals. Composting was very effective on reducing pirlimycin (>99 %) before application. With a low initial mass of pirlimycin, the Composted Manure – Cow Fed treatment had a lower pirlimycin runoff loss than the Raw Manure – Cow Fed treatment. The Raw Manure – Spiked and Composted Manure – Spiked treatment with a similar pirlimycin level before application had a similar pirlimycin runoff loss, but the Composted Manure – Spiked treatment enhanced pirlimycin movement in soil after the rain. Despite a similar dissipation pattern,

pirlimycin dissipated faster in composted manure applied soil than in raw manure applied soil. The pirlimycin half-life of in soil followed: Composted Manure – Spiked (3.76 d) < Raw Manure – Spiked (7.72 d) < Raw Manure – Cow Fed (18.12 d). A combination of composting raw manure and using the subsurface injection would be the best practice reducing the spread of antibiotics to surrounding environment.

Highlights

- The antibiotic source significantly affected the pirlimycin dissipation in soil.
- Composting reduced pirlimycin contamination potential by lowering its initial levels.
- Pirlimycin dissipated faster in soil with composted manure than raw manure.
- $t_{1/2}$: Composted Manure – Spiked < Raw Manure – Spiked < Raw Manure – Cow Fed.

5.1 Introduction

The majority of antibiotics administered to animals are excreted in urine and feces, thus soil receiving animal manure fertilizer might be exposed to a pool of antibiotics (Jechalke et al., 2014; Munir and Xagoraraki, 2011). There is a global concern that antibiotic resistance genes from non-pathogenic antibiotic resistant bacteria can transfer to human pathogens and/or the spread of antibiotics via various pathways might amplify this potential (Suzuki et al., 2017b). Several studies have been conducted to understand the environmental fate of antibiotics in different media in order to reduce their spread into the environment. Studies using spiked manure are more common due to its economical advantage than studies using manure collected from animals with antibiotic administration. However, studies using spiked manure might not reflect antibiotic behavior in the real world since antibiotics can partly metabolize in the animal body yielding metabolites that can transform back to the original compounds after entering the environment. To our best knowledge, no study has been conducted to compare the environmental fate of antibiotics in soil between using spiked manure and using manure from animals with antibiotic administration (referred to as the *antibiotic source*).

One approach to reduce the spread of antibiotics from manure to the surrounding environment is composting raw manure, a method driven by activities of microbes. Composting has showed a varied effect on antibiotic removal efficiency, from a complete removal to no removal (Dolliver and Gupta, 2008; Ray et al., 2017; Zhang et al., 2019). For antibiotics with an incomplete removal, they are released to soil following application of composted manure. Research comparing the effect of raw manure versus composted manure from animals with antibiotic administration (referred to as the *on-site manure treatment method*) on antibiotic behaviors in soil is lacking. For recalcitrant antibiotics with no removal during composting, their

levels in raw manure and composted manure are similar. However, due to different physicochemical and biological properties of two amendment types (Miller et al., 2008; Pankow, 2017), the environmental fate of antibiotics in soil receiving raw manure and composted manure with the same antibiotic initial mass might not be similar. Generally, compared to fresh manure, composting decreases the C:N ratio, total N, NH_4-N, and total C, but increases NO_3-N (Miller et al., 2008).

The manure subsurface injection method was developed to overcome disadvantages of the traditional manure application method or surface application. Advantages of this subsurface injection method include nutrient loss reduction, odor reduction, soil compaction reduction, and increased water and nutrient use efficiency (Maguire et al., 2011). Furthermore, in our previous studies [(Le et al., 2018) and unpublished data], using manure subsurface injection can significantly decrease the antibiotic loss with surface runoff in various conditions compared to using manure surface application. Meanwhile, there were no significant differences in antibiotic dissipation in soil between the two methods. With these advantages, the manure subsurface injection method becomes more and more popular. Therefore, in this study, the manure subsurface injection was chosen to apply manure into a field. The chosen antibiotic was pirlimycin (a lincosamide antibiotic), which is used to treat about 20 % of mastitis infections in dairy cows (Pol and Ruegg, 2007).

It is reasonable to expect that the on-site manure treatment methods (raw vs. composted manure from cows receiving antibiotic administration) and the antibiotic sources (spiked antibiotics vs. antibiotics passing thought animal body) will affect the environmental fate of antibiotics in soil differently. To our best knowledge, it is currently uncertain that the environmental fate of antibiotics in soil from studies using spiked manure and studies using

manure from animals with antibiotic administration can be cross-compared. Also, a recommendation of using composted manure to reduce the spread of antibiotics needs to consider their behavior during composting as well as in soil. The objective of this study was to compare effects of the manure types and the antibiotic sources on the environmental fate of pirlimycin, including its surface loss, spatial distribution in soil, and its temporal change in soil from manure-applied field via subsurface injection method.

5.2 Materials and Methods

5.2.1 Experimental setup

5.2.1.1 Field setup

A field rainfall simulation study was conducted on Braddock loam (fine, mixed, semiactive, mesic Typic Hapludults; 46 % sand, 44 % silt, and 10 % clay) at Kentland farm, Blacksburg, Virginia (Kulesza et al., 2016). Small test plots (2 × 1.5 m) were installed on a 9 to 11 % slope with a longer side perpendicular to the slope. A metal pan was installed at the bottom edge of each test-plot metal frame to direct surface runoff through a hose to a receiving container. Raw or composted manure was applied to soil via subsurface injection method at an agronomic N rate of 56 Mg wet wt. ha^{-1}, which is typical in Virginia. In fact, 26 kg of raw manure (5 % solid content) or ~7 kg of composted manure (40 % solid content) was used per plot. To mimic the manure subsurface injection method, raw or composted manure was manually poured into pre-cut injection slits (5 cm wide × 10 cm deep) and covered with soil on top. There were six treatments in total with three replicates each, including control raw manure, raw manure from cows receiving pirlimycin, control raw manure spiked with pirlimycin, control composted manure, composted manure from cows receiving pirlimycin, and control composted manure spiked with pirlimycin. Raw manure was collected from control cows (Raw Manure – Control) and cows treated with pirlimycin (PIRSUE, Zoetis) via intramammary with two 24 h apart doses at 50 mg/cow (Raw Manure – Cow Fed). Immediately before application, control raw manure was spiked with pirlimycin to create a third treatment, Raw Manure – Spiked. There was no pirlimycin detected in control raw manure [a detection limit of 1.47 µg kg^{-1} (Ray et al., 2014)] and the total amount of pirlimycin in manure before application for the Raw Manure – Cow Fed

and Raw Manure – Spiked treatments was 3,124 ± 199 and 2,378 ± 56 (μg $plot^{-1}$), respectively. Control raw manure and raw manure collected from cows receiving pirlimycin were composted with hay and hardwood chips for 12 weeks to generate control composted manure and composted manure from cows receiving pirlimycin (Composted Manure – Cow Fed), respectively. Immediately before application, pirlimycin was spiked to control composted manure to generate the final treatment, Composted Manure – Spiked. There was no pirlimycin detected in control composted manure [a detection limit of 0.03 μg kg^{-1} (Ray et al., 2017)] and the total amount of pirlimycin in composted manure before application for the Composted Manure – Cow Fed and Composted Manure – Spiked treatments was 1.76 ± 0.73 and 2,036 ± 947 (μg $plot^{-1}$), respectively. A randomized complete block design was used to generate the plot layout with one replicate of each treatment in each block.

5.2.1.2 Rainfall simulation experiment

Rainfall simulation setup was followed the protocol of National Research Project for Simulated Rainfall – Surface Runoff Studies (SERA-17, 2008). Rainfall was simulated at an intensity of 70 mm h^{-1} using a rainfall simulator (2.44 × 3.05 m) with water from a nearby well (free of pirlimycin). The simulated rainfall was conducted at approximately 2 h after manure application to mimic the worst case scenario since increased manure-rainfall time gaps can significantly reduce the antibiotic loss in surface runoff (Le et al., 2018). For each test plot, when the surface runoff started to flow into a receiving container located at a down slope, a 30-min sample collection started. Physical properties including time to start runoff, the amount of collected runoff, and the total runoff sediment load were documented in Appendix AF.

5.2.1.3 Sample collection

All samples of raw or composted manure, surface runoff, and soil samples were stored on ice immediately after being collected in the field, then stored at -20^{o}C until analysis. Manure samples were collected immediately before being applied to soil. For surface runoff samples, ~ 2 L of samples was collected in clean mason jars after stirring the total collected surface runoff vigorously. In terms of soil, each soil sample was comprised of six soil cores (2-cm diameter), which were collected randomly in the designated areas. Soil samples were collected inside the injection slits and in between the injection slits at equal distance between the slits. There were five sampling time points, including ~1 h after the manure application and before the simulated rainfall (referred as to before rain), ~ 1 h after the simulated rainfall (referred as to 1 h after rainfall), 5 d, 14 d, 60 d, and 150 d after the manure application. To avoid any disturbance inside the test plot before the simulated rainfall, the injection slits were extended outside the plot (40 cm on each side) and manure was also applied to these extended injection slits. Soil samples before rain were collected outside the test plot, while for the other sampling times, soil samples were collected inside.

5.2.2 Sample extraction and analysis

See Appendices B, D, E, and F.

5.2.3 Measurement and statistical analysis

Measurements of pirlimycin in this paper include a mass loss of pirlimycin with surface runoff water/sediment to its initial mass in manure (%), concentrations of pirlimycin in soil (μg kg^{-1}, dry weight), transformation kinetics of pirlimycin showed as its concentration at time t over its initial concentration (C_t/C_0), and pirlimycin half-life. The mass loss of pirlimycin with surface

runoff water/sediment was calculated based on its concentrations in surface runoff water/sediment and the amount of collected runoff water/sediment (Supplemental Table S1). The half-life ($t_{1/2} = \ln 2/k$) of pirlimycin in soil was calculated by fitting data into a first-order kinetic ($C_t = C_0 e^{-kt}$), as in previous studies (Albero et al., 2018; Carlson and Mabury, 2006; Chen et al., 2018; Halling-Sorensen et al., 2005; Shen et al., 2018). Three time points of 0, 5, and 14 d after manure application were used for the Raw Manure – Spiked and Composted Manure – Spiked treatment, while four time points of 5, 14, 60, and 150 d were used for the Raw Manure – Cow Fed treatment. Transformation rate constant (k), coefficient of determination (R^2) of a linear regression and half-life of pirlimycin in soil were documented in Supplemental Table S2. Moisture content (%) in manure and soil samples was measured as the ratio of the amount of water in the samples to the total amount of the samples.

JMP Pro 14 (SAS Institute, 2016) with a significant level of α at 0.05 was used for all statistical analyses. A Wilcoxon/Kruskal–Wallis test (rank sums) was applied to test effect of the antibiotic source and manure type on the concentration of pirlimycin, its surface runoff loss, and its half-life. Due to the small sample size, the χ^2 approximation was used in the Wilcoxon/Kruskal–Wallis test (JMP Pro 14). The effect of antibiotic source and manure type on the dissipation kinetics of pirlimycin over time was measured using a mixed model, in which the antibiotic source and manure type were used as a fixed effect and sampling time was used as a random factor. For samples with a pirlimycin concentration below the detection limit, its value was assigned as half of the detection limit for statistical analysis.

5.3 Results and Discussion

5.3.1 Pirlimycin loss with surface runoff from manure-amended fields

5.3.1.1 Effect of the manure type

The pirlimycin concentrations in raw manure and composted manure from control cows receiving no pirlimycin were below the detection limits [1.47 µg kg^{-1} raw manure (Ray et al., 2014) and 0.03 µg kg^{-1} composted manure (Ray et al., 2017)]. The initial mass input of pirlimycin to each test plot receiving raw manure and composted manure from cows with pirlimycin administration was 3,453.86 ± 397.26 (µg) and 1.76 ± 0.73 (µg), respectively. Therefore, the composting process (12 weeks) significantly reduced the pirlimycin mass input per plot receiving composted manure compared to the plot receiving raw manure (>99 % reduction). Composting, a method driven by activities of microbes, can break down a wide range of contaminants such as explosives (Williams et al., 1992), aromatic and petroleum hydrocarbons (Semple et al., 2001), pesticides (Büyüksönmez et al., 1999), personal care products (Xia et al., 2005), and hormones (Hakk et al., 2005). In a 35-d composting study by Dolliver and Gupta (2008), almost all chlortetracycline content disappeared (>99 %), whereas monensin and tylosin concentrations reduced from 54 to 76 %. In contrast, sulfamethazine was very resistant with no degradation. In another 42-d composting by Ray et al. (2017), significant reductions in antibiotic concentrations were observed for pirlimycin (100 %), sulfamethazine (97–98 %), chlortetracycline (71–84 %) and tetracycline (66–72 %), while tylosin removal during composting was relatively poor. During a longer composting (171 d), removal rates were >89.7 % for lincomycin, trimethoprim and the macrolides and <63.7 % for the sulfonamides, tetracyclines and fluoroquinolones (Zhang et al., 2019). Therefore, properties of antibiotics and

manure as well as composting conditions during composting are important factors influencing transformation of antibiotics during composting.

With a much lower initial mass input, it was expected that test plots receiving composted manure (Composted Manure – Cow Fed) would have a far lower surface runoff loss of pirlimycin compared to those receiving raw manure (Raw Manure – Cow Fed). In fact, the concentrations of pirlimycin in both surface runoff water and sediment from the Composted Manure – Cow Fed treatment were below the detection limits, meanwhile, the pirlimycin loss in surface runoff water and sediment from the Raw Manure – Cow Fed was 0.06 ± 0.03 and 0.013 ± 0.009 (%), respectively (Fig. 5.1). Composted manure showed a clear advantage over raw manure on antibiotic reduction via surface runoff. Other environmental benefits from using composted manure included lower concentrations of total phosphorus, particulate phosphorus, dissolved reactive phosphorus, total nitrogen, NH_4^+, and NO_3^- in runoff from treatments receiving composted manure than treatments receiving fresh manure (Miller et al., 2006). Our other data (not presented) showed that the surface runoff loss of pirlimycin from plots receiving raw and composted manure via surface application was 9.24 ± 3.51 (%) and 0.1 ± 0.07 (%), respectively. As a result, the combined recommendations for farmers in order to reduce the surface runoff loss of pirlimycin to the surrounding environment follow this sequence: (surface application + raw manure) < (surface application + composted manure) ~ (subsurface injection + raw manure) < (subsurface injection + composted manure).

The Raw Manure – Spiked vs. Composted Manure – Spiked treatments did not affect the pirlimycin loss with surface runoff water and sediment from manure-amended fields via subsurface injection, in which a simulated rainfall was conducted at ~2 h after amendment application (Fig. 5.1). Compared to the raw manure with 5 % solid content, the composted

manure with 40 % solid content might have more sorptive areas for antibiotic partition, which can influence surface runoff loss of antibiotics (Zhang et al., 2012). However, the similar surface runoff loss of pirlimycin between Raw Manure – Spiked and Composted Manure – Spiked treatments can be explained by its low K_d values (0.8 to 2.2 L kg^{-1}) (Le et al., 2018). The fact that >80 % of pirlimycin in surface runoff stayed in the aqueous part for all treatments supports its preference in the liquid phase, which was observed in another simulated rainfall study (Le et al., 2018). Moreover, the raw and composted manure was buried below the soil surface, limiting interactions of antibiotics with rain water.

5.3.1.2 Effect of the antibiotic source

Spiked manure and manure from cows with antibiotic administration were compared to determine effect of the antibiotic source on antibiotic behaviors in soil. Because the initial mass of pirlimycin for each test plot receiving composted manure from cows with antibiotic administration was low (1.76 µg), it is hard to study its behaviors in soil. In this study, spiked raw manure and raw manure from cows with pirlimycin administration were used. The total amount of pirlimycin in manure before application for the Raw Manure – Cow Fed and Raw Manure – Spiked treatments was 3,124 ± 199 and 2,378 ± 56 (µg $plot^{-1}$), respectively.

Similar to the manure type, the antibiotic source did not affect the pirlimycin loss with surface runoff water and sediment from manure-amended fields via subsurface injection (Fig. 5.1). In spiked liquid manure (5, 10, and 15 % solid content), sulfonamide antibiotics (sulfadiazine, sulfamethazine, and sulfamethoxazole), which have similar K_d values (0.6 to 4.9 L kg^{-1}) to pirlimycin (0.8 to 2.2 L kg^{-1}), mainly stay in liquid phase (He and Zhang, 2014; Le et al., 2018). Thus, pirlimycin probably remains in the liquid manure part of both Raw Manure – Cow Fed and Raw Manure – Spiked treatments, leading to their similar surface runoff loss.

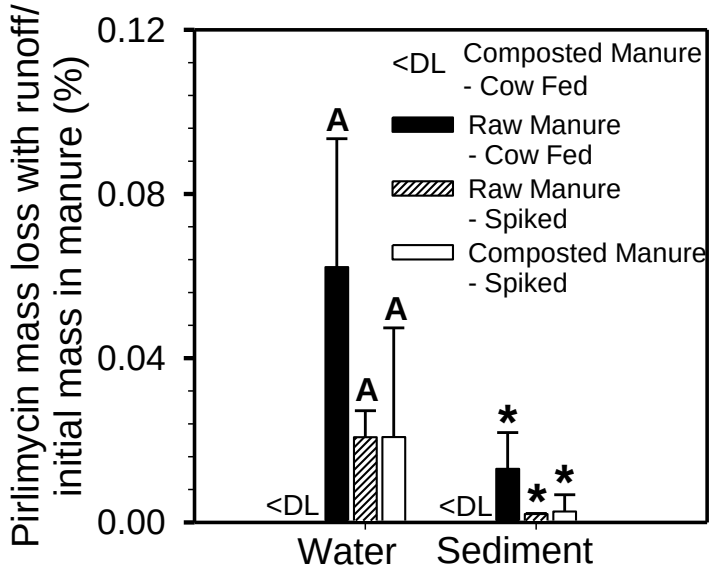

Figure 5.1 The mass loss of pirlimycin with runoff water and sediment to its initial mass in manure (%) from Composted Manure – Cow Fed, Raw Manure – Cow Fed, Raw Manure – Spiked, and Composted Manure – Spiked treatments. Pirlimycin concentrations in surface runoff water and sediment from raw manure – control and composted manure – control treatments were below the detection limit. Similar letters or symbols showed an insignificant difference among treatments ($p < 0.05$).

5.3.2 Short-term distribution of pirlimycin in soil of manure-amended fields after application and after a subsequent simulated rainfall

Due to several similarities in terms of distribution of pirlimycin in soil after application and after the simulated rainfall among all treatments, effects of the manure types and the antibiotic sources were discussed together (Fig. 5.2). Concentrations of pirlimycin in 0-5- and 5-20-cm soil collected in the injection slits and in between slits of the raw manure – control and composted manure – control treatments were below detection limits at all sampling time points.

At 1 h after manure application, pirlimycin concentrations in soil depended on its initial amount of pirlimycin in raw or composted manure before application. For example, with a low

initial amount of pirlimycin in composted manure from cows with antibiotic administration, pirlimycin was detected at 0.24 ± 0.06 and 0.02 ± 0.00 (μg kg^{-1}) in 0-5-cm and 5-20-cm soil, respectively at 1 h after application, for the Composted Manure – Cow Fed treatment. Meanwhile, pirlimycin was detected at a far higher concentration for the Raw Manure – Cow Fed treatment (30.33 ± 7.50 and 5.15 ± 1.05 μg kg^{-1} in 0-5-cm and 5-20-cm soil, respectively). Also, at 1 h after manure application, the pirlimycin concentration in 0-5-cm soil was significantly higher than in 5-20-cm soil ($p < 0.05$) for all treatments, and the rainfall, which was conducted ~ 2 h after manure application, did not change this pattern. The accumulation of pirlimycin in top soil was observed in several studies (Biel-Maeso et al., 2018; Gros et al., 2019; Kulesza et al., 2016; Pan and Chu, 2017; Zhao et al., 2018), which shows its potential contamination to spread to the surrounding environment via surface runoff rather than via leaching. In a 3-year study, Dolliver and Gupta (2008) found that antibiotic losses via surface runoff were much higher compared to via leaching.

Furthermore, despite differences in pirlimycin concentrations among treatments, the concentrations of pirlimycin in soil at 1 h before and after rain in the injection slits and in between slits were generally not influenced by the simulated rainfall for both soil depths. The only exception was that the simulated rain event significantly lowered the pirlimycin concentration in 0-5-cm soil in the injection slits of the Composted Manure-Spiked treatment, which was consistent with an increase in the pirlimycin concentration in soil in between slits from these test plots after the rainfall. Whereas, the pirlimycin concentrations in soil in between slits were below the detection limit for all the other samples, indicating that pirlimycin concentrated in the injection slits with limited movement to surrounding soil (Kulesza et al., 2016). Immediately after manure application, soil in the injection slits became moister after raw

manure application compared to after composted manure application, and the moister soil can contribute to limiting further distribution of pirlimycin in soil from the injection slits due to the simulated rainfall. In fact, following manure application, the soil moisture content of the Raw Manure – Cow Fed, Raw Manure – Spiked, Composted Manure – Spiked, and Composted Manure – Cow Fed treatments was 32 % (± 4), 35 % (± 7), 27 % (± 5), and 30 % (± 2), respectively in 0-5 cm and 22 % (± 4), 25 % (± 3), 15 % (± 3), and 16 % (± 5), respectively in 5-20 cm. For both composted manure treatments, the pirlimycin concentrations of the Composted Manure – Cow Fed treatment were far lower than those of the Composted Manure – Spiked treatment, in order to reflect the effect of the simulated rainfall.

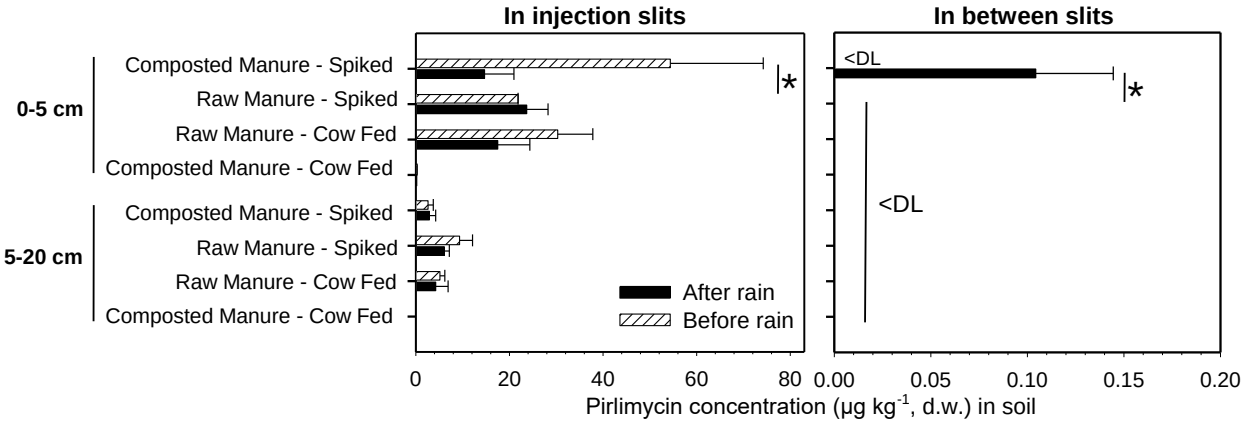

Figure 5.2 Concentration of pirlimycin in 0-5- and 5-20-cm soil (µg kg-1, dried weight) collected in the injection slits and in between slits of the subsurface application treatments 1 h before (white bars) and after rainfall (black bars) from Composted Manure – Cow Fed, Raw Manure – Cow Fed, Raw Manure – Spiked, and Composted Manure – Spiked treatments. * showed a significant difference in pirlimycin concentrations between before and after rain.

5.3.3 Long-term change of pirlimycin in soil of manure-amended fields

5.3.3.1 Effect of the manure type

On Day 0, the pirlimycin concentrations in 0-5-cm and 5-20-cm soil were 17.49 ± 6.87 and 4.25 ± 2.65 μg kg^{-1}, respectively, for the Raw Manure – Cow Fed treatment and 0.18 ± 0.01 and below the detection limit of 0.02 μg kg^{-1}, respectively for the Composted Manure – Cow Fed treatment. Despite these large differences in pirlimycin initial concentrations in both soil depths between two treatments, there were some similarities in pirlimycin dissipation patterns between two treatments (Fig. 5.3 and Table 5.1). In particular, at Day 5 after application, pirlimycin concentrations did not decrease. In particular, pirlimycin concentrations in soil of the Raw Manure – Cow Fed treatment increased sharply by 5 d after manure application (340 % in 0-5-cm soil and 175 % in 5-20-cm soil), while pirlimycin concentrations on soil of the Composted Manure – Cow Fed treatment remained stable (117 % in 0-5-cm soil). After that, pirlimycin started to decrease for both treatments. Deconjugation of conjugated pirlimycin (pirlimycin-sulfoxide, pirlimycin-sulfone, pirlimycin-adenylate, pirlimycin-uridylate, and pirlimycin sulfoxide-adenylate) probably contributes to the observed increase (Hornish et al., 1992). In addition, for both treatments, the concentrations of pirlimycin in soil collected in between slits were below the detection limit at all sampling times at both soil depths, suggesting a limited movement of pirlimycin in soil from the injection slits, which was observed in another field study by Kulesza et al. (2016).

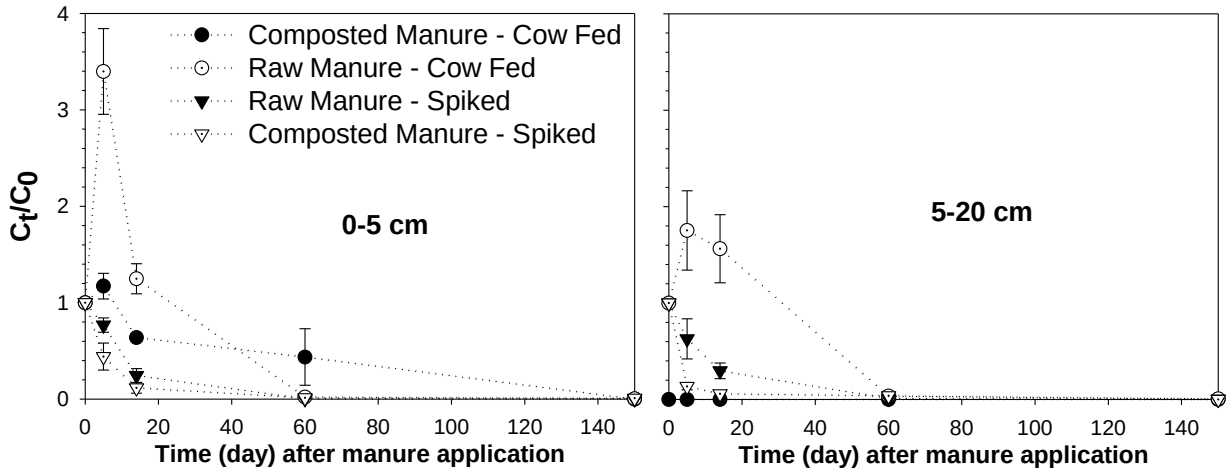

Figure 5.3 Dissipation kinetics of pirlimycin showed by its concentrations in 0-5- and 5-20-cm soil (µg kg-1, dried weight) at time t over its initial concentrations (Ct/C0) from the injection slits of the subsurface injection treatment during 150 d after manure application for Composted Manure – Cow Fed, Raw Manure – Cow Fed, Raw Manure – Spiked, and Composted Manure – Spiked treatments.

However, the pirlimycin dissipation kinetics in 0-5-cm soil between two treatments were different (Table 5.1, $p = 0.0253$). Since pirlimycin concentrations in 5-20-cm soil of the Composted Manure – Cow Fed treatment were below the detection limits at all sampling time points, it is unreasonable to compare pirlimycin dissipation kinetics between two treatments in the 5-20-cm soil. The trace amount of pirlimycin in 0-5-cm soil (<0.2 µg kg^{-1}) of the Composted Manure – Cow Fed treatment can completely sorb to soil particles, lowering the its dissipation rate compared to the Raw Manure – Cow Fed treatment. However, by Day 150, while pirlimycin concentrations in soil dropped below the detection limit for the Composted Manure – Cow Fed treatment, they were still detectable for the Raw Manure – Cow Fed treatment. Therefore, the differences in pirlimycin starting concentrations between two treatments can explain the faster but incomplete in soil from the Raw Manure – Cow Fed treatment and slower but complete in

soil from the Composted Manure – Cow Fed treatment. Because the pirlimycin concentration in 0-5-cm soil of the Composted Manure – Cow Fed treatment was below the detection limit at Day 150 and data of Day 5, 14, and 60 were not fitted well into a first-order kinetic ($R^2 = 0.63 \pm 0.31$), the pirlimycin half-life in 0-5-cm soil of the Composted Manure – Cow Fed treatment was not calculated.

In summary, compared to the Raw Manure – Cow Fed treatment, the Composted Manure – Cow Fed treatment showed a lower potential of antibiotic contamination from soil to the surrounding, indicating an advantage of composting raw manure before application. Other advantages of composted manure over fresh manure include reducing the antibiotic resistance gene load to soil and the detection frequency of resistance genes on vegetable at harvest (Tien et al., 2017). However, a field study by Wind et al. (2018) reported a significantly higher level of total ceftazidime-resistant and erythromycin-resistant fecal coliforms from plots receiving composted manure as compared to those receiving raw manure. Besides, despite benefits of lowering concentrations of manure-associated contaminants and pathogens during composting processes, a great amount of essential nutrients such as N, C, P, K, and Na, loses during composting (Tiquia et al., 2002).

Table 5.1 Dissipation kinetics of pirlimycin in soil (Ct/C0) and p values comparing effects of the manure types and the antibiotic sources on its dissipation kinetics in soil in the injection slits during 150 d after manure application.

Day	0-5 cm				5-20 cm			
	Composted Manure – Cow Fed	Raw Manure – Cow Fed	Raw Manure – Spiked	Composted Manure – Spiked	Composted Manure – Cow Fed	Raw Manure – Cow Fed	Raw Manure – Spiked	Composted Manure – Spiked
	C_t/C_0 (%)							
5	117.1 ± 13.3	339.8 ± 44.5	76.7 ± 7.4	44.1 ± 14.0	n/a	175.3 ± 41.1	62.8 ± 20.8	13.4 ± 3.4
14	63.8 ± 0.5	124.8 ± 15.6	24.4 ± 7.2	11.6 ± 5.5	n/a	156.2 ± 35.3	29.7 ± 8.1	5.8 ± 0.9
60	43.6 ± 29.3	2.1 ± 0.5	0.3 ± 0.1	1.3 ± 0.3	n/a	3.3 ± 1.3	1.0 ± 0.1	3.7 ± 2.7
150	n/a	0.4 ± 0.1	0.1 ± 0.0	0.9 ± 0.3	n/a	0.6 ± 0.3	0.3 ± 0.1	n/a
p value								
Raw Manure – Cow Fed vs. Composted Manure – Cow Fed				0-5 cm	0.0253			
				5-20 cm	n/a			
Raw Manure – Spiked vs. Composted Manure – Spiked				0-5 cm	0.0125			
				5-20 cm	0.0060			
Raw Manure – Cow Fed vs. Raw Manure – Spiked				0-5 cm	0.0019			
				5-20 cm	0.0011			

n/a: pirlimycin concentrations were below the detection limit, therefore the values were unavailable.

Although pirlimycin dissipation pattern in soil of the Raw Manure – Spiked and Composted Manure – Spiked treatments showed a similar trend (a continuous decrease with a faster rate during first 14 d), pirlimycin dissipation kinetics in soil of the Composted Manure – Spiked treatment was significantly faster than the Raw Manure – Spiked treatment (Fig. 3 and Table 1). The half-life of pirlimycin in soil from the Composted Manure – Spiked treatment (3.76 ± 1.02 d) was significantly shorter than from the Raw Manure – Spiked treatment (7.72 ± 1.67 d) (Appendix AG). A batch study by Berendsen et al. (2018) showed that dissipation of antibiotics during 24-day manure storage strongly depended on manure types (pig manure, broiler manure, and calve manure). There are many factors that can affect the dissipation of antibiotics and aerobic microbial activities can be a major route. In the injection slits filled with composted manure (61 % water content), it would be easier for oxygen movement in and out than in the injection slits filled with raw liquid manure (95 % water content). Also, raw manure would have a higher amount of readily biodegradable materials, which quickly consume available oxygen during degradation processes, than composted manure. It is because these readily biodegradable materials have been degraded during composting processes to generate composted manure. Pankow (2017) showed a significantly different microbial community composition between composted manure applied soil and raw manure applied soil. For example, at the day of dairy manure application, the relative abundance of alphaproteobacteria, deltaproteobacteria, and bacilli was higher in composted manure applied soil than in raw manure applied soil. In addition to degradation, high-lignocellulosic materials added for composting (such as wood chip and alfalfa hay) increase sorption sites for pirlimycin (Zhang et al., 2012) and strong sorption interaction can lower the extracted portion of pirlimycin. Therefore, with a

similar initial mass of pirlimycin in amendment, composted manure can reduce the pirlimycin concentration in soil faster than raw manure.

5.3.3.2 Effect of the antibiotic source

The dissipation pattern of pirlimycin in soil during 150 d after manure application was significantly different between Raw Manure – Spiked and Raw Manure – Cow Fed treatments (Fig. 5.3 and Table 5.1). In the Raw Manure – Spiked treatment, the pirlimycin concentration continuously decreased with a faster rate during first 14 d probably due to an exponential decrease of available antibiotics in soil with time (Pan and Chu, 2016; Stoob et al., 2007; Wang et al., 2006). By 14 d, ~75 % and 70 % of pirlimycin was dissipated in 0-5-cm and 5-20-cm soil, respectively. The continuous decrease and a faster dissipation rate of antibiotics in soil during the first 10-15 d after application were well-documented in other studies soil within first 10-15 days (Albero et al., 2018; Shen et al., 2018). Meanwhile, the pirlimycin concentration in soil of the Raw Manure – Cow Fed treatment increased sharply by 5 d after manure application due to deconjugation of conjugated pirlimycin, then started to decrease continuously. A similar increase (by 3 d) in incubated soil mixed with raw manure from cows receiving pirlimycin was showed by Chen et al. (2018). However, Ray et al. (2017) described a continuous reduction in pirlimycin concentrations (Day 0, 4, 7, 14, 21, 28, and 42) during composting raw manure from cows receiving pirlimycin. During composting with strong microbial activities and high temperature, the rate of breaking down pirlimycin can be faster than producing pirlimycin from its conjugates, resulting in a net decrease. Another field study by Wind et al. (2018), in which soil receiving raw manure from cows with pirlimycin administration was sampled on Day 0, 7, 28, 42, 56, 90, and 120; showed a continuous decrease. Probably, deconjugation of conjugated pirlimycin could happen immediately after manure application and by Day 7, more pirlimycin were dissipated

than generated leading to a decline compared to its level on Day 0. Results also showed that pirlimycin in soil dissipated faster in the Raw Manure – Spiked treatment than in the Raw Manure – Cow Fed treatment at all sampling time points. The half-life of pirlimycin in soil from the Raw Manure – Cow Fed treatment (18.12 ± 1.36 d) was significantly higher than from the Raw Manure – Spiked treatment (7.72 ± 1.67 d) (Appendix AG). For the Raw Manure – Cow Fed treatment, when pirlimycin passed through the animal body, it had time to establish equilibrium conditions in the manure, which can increase the persistence of pirlimycin. Another contributing factor would be a different microbial composition in manure with and without antibiotic administration (Pankow, 2017).

5.4 Conclusions

This field study compared effects of the manure types and the antibiotic sources on the surface runoff loss, short-term distribution, and long-term dissipation of pirlimycin in soil. Our data suggested that using manure spiked with antibiotics can mimic manure with antibiotics passing though animal body if the surface runoff loss of antibiotics and short-term distribution of antibiotics in soil are interested. However, studies using spiked manure might not reflect the antibiotic long-term behaviors in soil as studies using manure from animals with antibiotic administration, especially when antibiotics are partly metabolized in the animal body. In case of pirlimycin, its dissipation kinetics in the Raw Manure – Cow Fed treatment were significantly different from those in the Raw Manure – Spiked treatment with a significantly higher pirlimycin half-life in soil. Moreover, composting manure was very effective to decrease the initial concentration of pirlimycin before application (>99 %), leading to a significantly low surface runoff loss of pirlimycin as well as a trace amount of pirlimycin in compost-amended soil. In addition, the manure type (raw vs. composted manure with similar pirlimycin concentrations) can significantly affect the movement of antibiotics in soil as well as the dissipation rate of antibiotics in soil. In fact, pirlimycin in composted manure applied soil dissipated faster than in raw manure-applied soil, which added another benefit of composting raw manure before application. The combination of the subsurface injection and composted manure is recommendations for farmers in order to reduce the spread of pirlimycin to the surrounding environment via runoff as well as enhance pirlimycin dissipation in soil following manure application.

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Chapter 6: Spatial distribution and temporal change of manure biomarker and class 1 integron-integrase gene in soils following manure surface application and subsurface injection

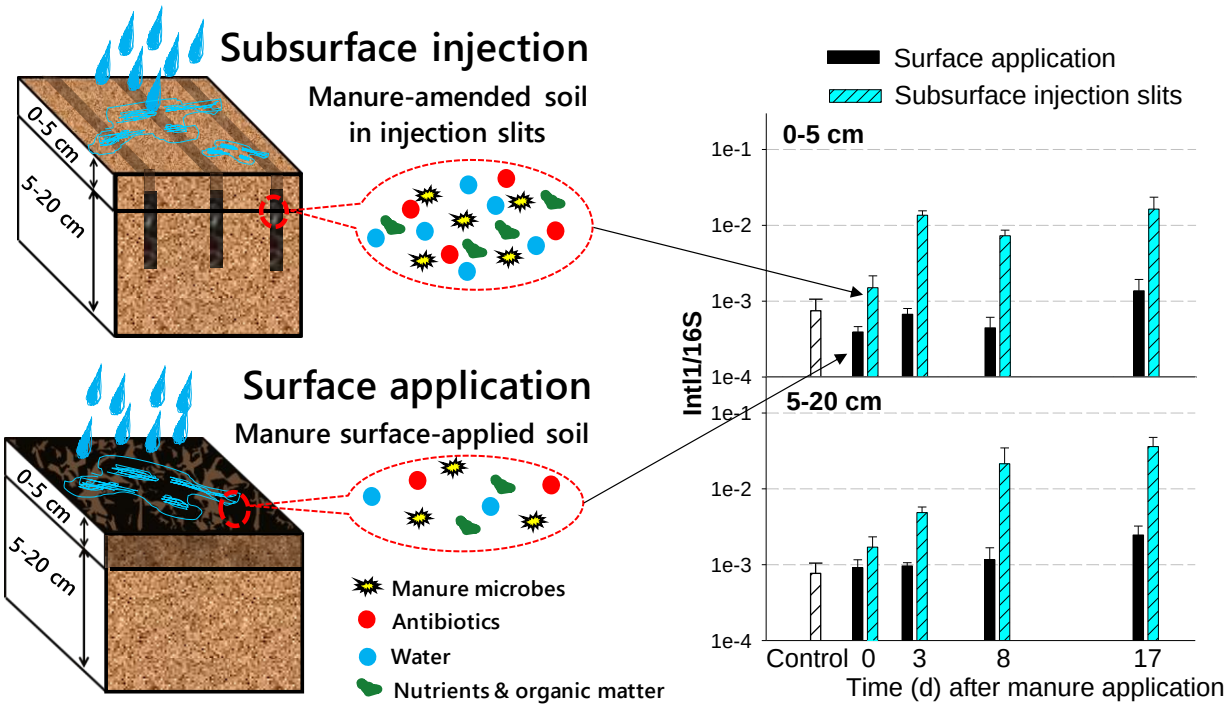
Hanh Thi Van Le, Chaoqi Chen, Rory O. Maguire, and Kang Xia

(To be submitted to Chemosphere)

Abstract

Compared with surface application, manure subsurface injection is promising to reduce nutrients and manure-borne emerging contaminants in surface runoff from manure-applied fields. The concern is that manure injection slits, which have elevated levels of nutrients, organic matter, water, manure microbes, and antibiotics, might be a “hot zone” to encourage antibiotic resistance gene transfer and growth of antibiotic resistant bacteria. A field rainfall simulation study was conducted to: 1) monitor spatial distribution and temporal change of Rum-2-bac (a ruminant biomarker) and IntI1 (a mobile genetic element marker); and 2) examine their correlation with antibiotics in soils following dairy manure surface application and subsurface injection. Pirlimycin, sulfamerazine, tylosin, and chlortetracycline-spiked dairy manure was applied at an agronomic rate, followed by a simulated rainfall (70 mmh^{-1}) conducted after manure application. The results showed that the absolute abundance of Rum-2-bac was significantly enriched in the injection slits compared to surface-applied plots. Three days after manure application, the relative abundance of intI1 significantly increased in the injection slits, where the manure was concentrated, suggesting a hot zone of potential horizontal gene transfer. The simulated rain did not significantly affect the abundance of Rum-2-bac and intI1, as well as the distribution pattern of the genes in soil, indicating that the rain has negligible effect on the fate of tested genes. The abundance of Rum-2-bac was positively correlated with antibiotics, indicating co-transport and co-dissipation of antibiotics and the manure-biomarker. Compared to Rum-2-bac, intI1 gene was much more stable in soil after manure application. It is recommended to avoid planting directly onto the subsurface injection slits, which reduces exposure of plants to antibiotics, manure microbes, and antibiotic resistance genes/bacteria.

Graphical abstract



Highlights

- The abundance of Rum-2-bac and intI1 gene was enriched in the injection slits
- Abundance of intI1 significantly increased after three days in the injection slits
- Simulated rain had a negligible effect on abundance of intI1 and Rum-2-bac
- Rum-2-bac dissipated fast in soil, while IntI1 was stable

6.1 Introduction

According to the U.S. Food and Drug Administration (2011), more than 70 % of antibiotics in the United States are administered to livestock, resulting in concentrations as high as tens to hundreds mg kg^{-1} in liquid manure (Haller et al., 2002). Besides antibiotic residues, animal manure is a pool of antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs), and associated mobile genetic elements (MGEs) (Haller et al., 2002; Jechalke et al., 2014; Munir and Xagoraraki, 2011; van Den Bogaard et al., 2000). Since ARGs are frequently associated with MGEs, which facilitate transfer of these genes between microorganisms, spreading ARGs is of great concern (Aminov and Mackie, 2007). Land application of animal manure, sewage sludge, and biosolids as fertilizers is a common practice to provide essential nutrients for crop production. Meanwhile, it introduces these emerging contaminants to soil, raising concerns about increasing antibiotic resistance levels in soil (Heuer and Smalla, 2007; Sengeløv et al., 2003). Although a wide range of ARGs was found in pristine environment (D'Costa et al., 2011), the overuse and misuse of antibiotics have raised concerns about accelerated development and spread of ARGs and/or antibiotic resistant bacteria (ARB), because antibiotics can exert selection pressure on the native microbial community (Cleary et al., 2016; Nordenholt et al., 2016) and enrich ARGs and/or ARB (Jechalke et al., 2014). Studies have showed an enhanced level of ARGs and ARB together with antibiotic use (Pei et al., 2006; Rodriguez-Rojas et al., 2013). In contrast, some other studies suggested no significant effects of antibiotics on amplification of ARGs and ARB (Hund-Rinke et al., 2004; Sengeløv et al., 2003). Therefore, the effect of antibiotics on amplification of ARGs and/or ARB is not conclusive yet.

Compared to surface application or surface broadcast (a traditional method of land manure application), manure subsurface injection has showed many advantages to the

environment. These advantages include nutrient loss reduction, soil compaction reduction, increased C and water use efficiency, odor reduction, and especially reduced manure-associated contaminant loss with surface runoff (Kulesza et al., 2016; Le et al., 2018; Maguire et al., 2013; Maguire et al., 2011). These advantages probably come from the nature of the manure subsurface injection method, in which, manure is injected below soil surface into injection slits. However, compared to the surface application, the injection slits also create a "hot zone", which has elevated levels of nutrients, manure microbes, water, and antibiotics. Higher density of microbes and substrate levels from manure slurry can increase genetic exchanges, which therefore can amplify antibiotic effects on microbial resistance (Ding and He, 2010). Besides, higher antibiotic concentrations in the injection slits can effectively select and maintain resistance traits in bacteria (Gullberg et al., 2011). However, no information is available on the effects of the subsurface injection on the potential of ARGs to spread in soil compared to surface application. Research is therefore needed to monitor the distribution and change of genes associated with transfer of ARGs in the manure subsurface-injected soil compared to manure surface-applied soil.

In order to trace sources of fecal contamination from different animal species, methods to detect the host-specific bacterial genetic markers such as *Bacteroidales* 16S rRNA gene have been developed (Mieszkin et al., 2010). *Bacteroidales* were chosen because they have some desirable characteristics such as ability to be quantified, wide distribution in target animals, and wide geographic stability (U.S. Environmental Protection Agency, 2005). Mieszkin et al. (2010) developed a reliable ruminant-specific marker, Rum-2-bac, discriminating ruminant fecal sources from other animals.

From contaminated soil, ARGs can disseminate to larger areas via multiple routes. ARGs can travel from contaminated soil to aquatic bodies through surface runoff and be disseminated

to drinking water sources (Kim et al., 2016; Song et al., 2010). They can also spread from contaminated soil onto surface of fresh produce and this is a particular concern for fruits and vegetables that are typically eaten raw. Studies have shown an increased quantity of ARGs on raw vegetables from manure-applied fields (Marti et al., 2013; Tien et al., 2017). There is a global concern that ARGs from non-pathogenic antibiotic resistant bacteria can transfer to human pathogens, causing once treatable diseases to turn deadly, and/or the spread of antibiotics and ARGs via various pathways might amplify this potential (Suzuki et al., 2017a). Compared to intrinsic antibiotic resistance due to spontaneous gene mutation, acquired antibiotic resistance under pressure is believed to be more common (Alanis, 2005). The acquisition of foreign genes or mobile genetic elements is defined as horizontal gene transfer (HGT). Common mobile genetic elements, including integrons, transposons, phages, plasmids, or chromosomal islands, play an important role in introducing resistant genes from environmental sources to clinically relevant bacteria, and the class 1 integron is the most efficient mechanism among Gram-negative bacteria (Djordjevic et al., 2013; Jain et al., 2003; Poirel et al., 2009). The *intI1* gene codes integrase, an enzyme catalyzing site-specific recombination of floating genome into the host (Stokes and Hall, 1989) and has been considered as a good indicator of ARGs in the environment (Gillings et al., 2015; Ma et al., 2017; Yang et al., 2018b).

With the above mentioned knowledge gaps, this field study addressed two major questions. Firstly, how the manure application method and timing influenced the spatial distribution and temporal change of cow manure (presented by Rum-2-bac) and horizontal gene transfer (presented by *IntI1*) in soil. Secondly, if the “hot zone” in the injection slits facilitates development of antibiotic resistance (presented by *IntI1*).

6.2 Materials and Methods

6.2.1 Experimental design

Detailed experimental design can be found at Le at al., (2018). In summary, a test-plot rainfall simulation study was conducted in 2015 in Blacksburg, VA, USA on Braddock loam. The test-plot (1.5 × 2.0 m) was installed on 9-11 % slope. There were five treatments including two manure application approaches (surface application and subsurface injection) with two manure-rainfall time gaps (0 [2 h] and 3 d) plus one no-manure control (Appendix AH). Pirlimycin, sulfamerazine, tylosin, and chlortetracycline were spiked to fresh control dairy manure (5 % solid content) to achieve target concentrations of 131, 500, 500, and 150 mg kg^{-1}, respectively. The manure slurry was then applied at an agronomic N application rate of 56 Mg wet wt. ha^{-1}. For surface application, manure was poured to a metal sheet to be spread evenly on soil surface, while, for subsurface injection, manure was injected into injection slits (5 cm wide × 10 cm deep), which were created perpendicular to the slope using a Yetter injection disc. 2 h or 3 d after manure application, a simulated rainfall (70 mmh^{-1}) was conducted for 30 min runoff collection following the established protocol of the national research project for rainfall simulation studies.

6.2.2 Soil sample collection

Soil samples were collected from 0-5 and 5-20 cm at five time points following manure application, including immediately before and after rainfall, and 3, 8, and 17 d after manure application. For each soil sample, six soil cores (2-cm diameter) were collected randomly in designated areas and composited. Before the rain, soil cores were collected in manure-applied areas outside the plot to avoid any disturbance inside, meanwhile, soil cores were collected inside the plots for the other sampling time points. For surface application, soil cores were

collected randomly in manure-applied areas. For subsurface injections, soil cores were collected inside the injection slits and 5 and 25 cm from the injection slits for immediately before and after rainfall to monitor effect of rain events and only inside the injection slits at 3, 8, and 17 d after manure application. All samples were placed on ice in the field and freezed at -20^{o}C until analysis.

6.2.3 Gene analysis: DNA extraction and qPCR assays

In order to quantify the abundance of target genes in soil, DNA from freeze-dried soil was first extracted then the target genes from extracted DNA were quantified using quantitative polymerase chain reaction (qPCR). In particular, DNA from 0.2 g freeze-dried soil was extracted using DNeasy® PowerSoil® Kit (MOBIO Laboratories INC, USA) following the manufacturer's protocol. DNA concentrations in the extract were measured using the Qubit 2.0 fluorometer (Invitrogen, USA). The absolute abundance of manure biological markers was represented by copy number of Rum-2-bac, which was calculated by comparing cycle threshold values to known standards of plasmids containing the target gene. Primers/probes, reaction mixtures, and thermal profiles used for qPCR were listed in the Appendix AI. qPCR assays were performed using Mastercycler® ep realplex (Eppendorf, USA). For quality control, positive controls and no template controls were included in each instrument run and each sample was run in triplicates. To quantify the gene copies in each sample, standard curves spanning from 10^3 to 10^8 copies for 16S rRNA, 10^1 to 10^6 copies for Rum-2-bac, and 10^1 to 10^6 copies for IntI1 were included for each instrument run. Precision of measurements for all assays was shown by the amplification efficiencies from 95 % to 105 % for all target genes and standard curve R^2 >0.99.

6.2.4 Antibiotic analysis

See Appendices C, E, and F.

6.2.5 Statistical analysis

Microbial genes were analyzed in two ways, including absolute abundance (the number of gene copies g^{-1} soil, dry weight) and relative abundance (the number of gene copies normalized to the 16S rRNA gene). Statistical analyses were conducted using JMP (JMP®, Version Pro 14, SAS Institute Inc., Cary, NC). The effect of manure application methods was examined by comparing the abundance of biomarkers in the soil collected from the injection slits to that of soil collected from plots with surface application. The effect of rainfall was examined by comparing the abundance of biomarkers in soil before and after rainfall. The horizontal and vertical distribution of biomarkers in the soil was examined at different distances from the injection slits and at different depths. The abundance of biomarkers in soil over time was examined. Pairwise comparisons were conducted using student's test for two-pair conditions or Turkey's HSD under multiple-pair conditions. Multivariate correlation analyses were conducted to obtain Spearman's coefficients to examine the correlation between the abundance of biomarkers and the concentrations of antibiotics. All significance tests were carried out at the 95 % confidence level.

6.3 Results and Discussion

In the subsequent sections, the absolute abundance of Rum-2-bac refers to the total load of manure associated bacteria (i.e. ruminant-associated *Bacteroidales*) in soil, assuming that a greater abundance of Rum-2-bac implies a higher degree of fecal contamination in soil. The absolute abundance of intI1 refers to the total load of bacteria carrying the integron-integrase gene in soil, assuming a greater abundance of intI1 implies a higher potential of antibiotic resistance to spread. The relative abundance of intI1 is the proportion of bacteria carrying intI1 in total soil bacterial community, which implies potential selective pressure affecting this proportion.

6.3.1 Effect of manure application methods on Rum-2-bac and intI1 abundance

As expected, Rum-2-bac was not detected in the control soil, indicating free of manure contamination in the testing field. Immediately after manure application, the absolute abundance of Rum-2-bac in 0-5-cm and 5-20-cm soil in the subsurface injection slits was 2.2×10^6 copies g^{-1} and 3.4×10^5 copies g^{-1}, respectively, which was 6.8 (p = 0.002, for 0-5-cm soil) and 25.7 times (p = 0.008, for 5-20-cm soil) higher than those from the surface application (Fig. 6.1, Appendix AK). For the subsurface injection, manure was buried below soil surface, up to 10 cm in the injection slits, while manure was spread on the soil surface of the surface application. With a higher load of manure, it was reasonable that the abundance of Rum-2-bac in 5-20-cm soil of the injection slits was higher than that of the surface application. However, three days after the manure application, the difference in Rum-2-bac abundance between the two application methods largely reduced (Fig. 6.1, Appendix AK). For the 0-5-cm soil, the absolute abundance of Rum-2-bac in the injection slits was only 2.3 times higher than that in the surface application (p = 0.023). For the 5-20-cm soil, there was no significant difference in Rum-2-bac absolute

abundance between the two application methods by 3 days after manure application (p = 0.667, Fig. 6.1).

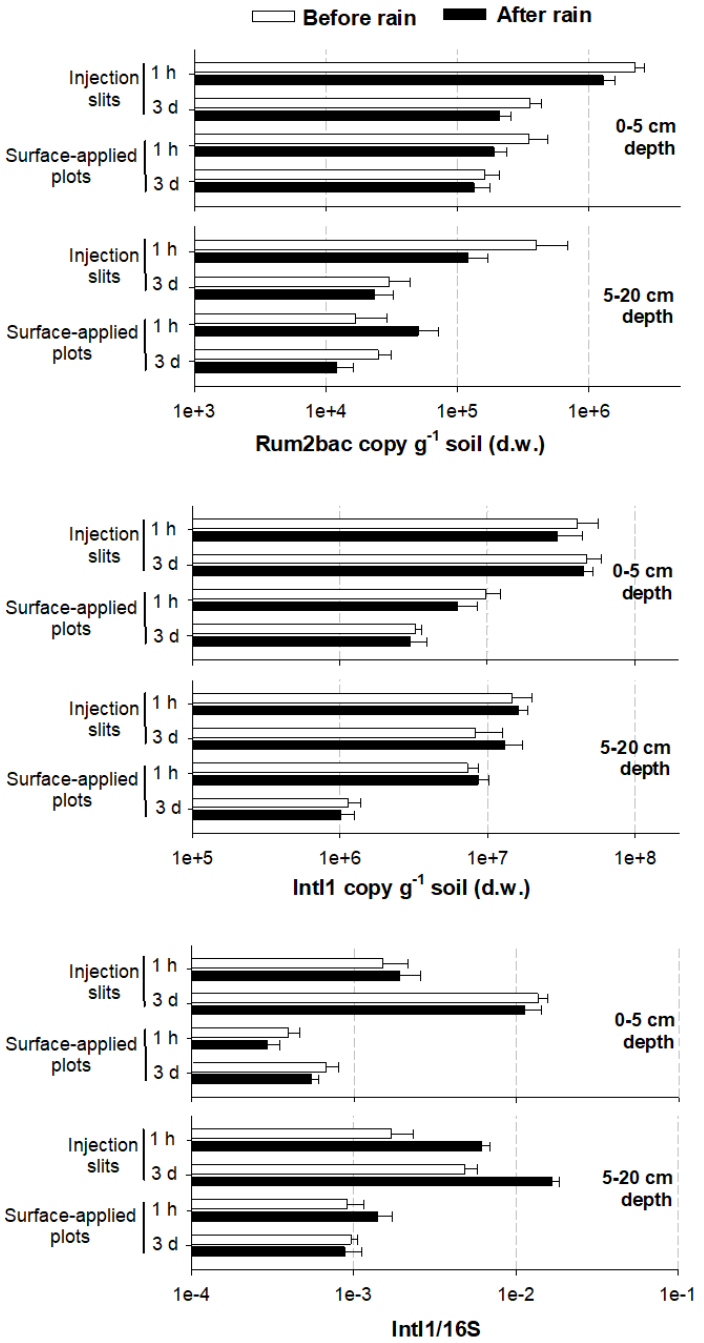


Figure 6.1 Abundance of Rum2bac and Intl1 in 0-5- and 5-20-cm soil collected in manure surface application treatments and injection slits of subsurface application treatments 1 h before and after rainfall with manure-rainfall time gaps of 1 h or 3 d.

After the 3 days, the absolute abundance of Rum-2-bac in the 0-5-cm soil reduced by 6.3 times and 2.1 times for the injection slits and surface application, respectively. The reduction of Rum-2-bac may be resulted from death of manure-associated bacteria carrying Rum-2-bac, which may be due to a competition interaction with native soil bacteria, as well as unfavorable environmental conditions for the survival of manure-associated bacteria. Competition interaction of indigenous soil bacteria plays an important role in governing the survival of manure-associated bacteria. Particularly, most *Bacteroidales* species are obligate anaerobic, indicating that they are unlikely to survive in the surface soil under aerobic conditions or compete with indigenous soil bacteria. In this study, the dissipation rate of manure-associated bacteria was higher in the soil from the injection slits than the soil from the surface application. For the surface application, the manure was evenly distributed to the entire plot. There was no gradient difference in the Rum-2-bac abundance along the field slope, except for the soil located at the edge of the plot. Comparably, for the injection slits, manure was concentrated in the slits, where manure-associated bacteria and genes were enriched compared to the soil surrounding the slits, where manure was not applied. As a result, the diffusion or downward movement (i.e. along the slope) of bacteria and genes from the injection slits to the surrounding soils may be driven by the gradient difference between the soil in the injection slits and the surrounding soil.

In the control soil, the absolute abundance of intI1 was detected at a level of 1.5×10^{7} and 6.5×10^{6} copies g^{-1}, in 0-5-cm and 5-20-cm soil, respectively. According to a field manager, the field has been using as the research field for rainfall simulation studies on and off for years and it received dry stack manure at 2.24 kg m^{-2} in the spring 2013 before this study. IntI1 is considered a proxy for anthropogenic pollution (Gillings et al., 2015; Ma et al., 2017) and plays an important role in horizontal gene transfer. It is possible that the intI1 after anthropogenic

activities (e.g. repeated manure application practices) decreases very slowly and significant decreases do not occur within a time scale of a few years. For instance, the absolute and relative abundance of the intI1 gene were stable at a range from 10^7 to 10^8 copies g^{-1} and from 10^{-4} to 10^{-3}, respectively, in a control soil with no manure application during a 200-day incubation period (Sandberg and LaPara, 2016).

Immediately after manure application, the absolute abundance of intI1 in the 0-5-cm soil in the injection slits was 3.8×10^7 copies g^{-1}, significantly higher than that in the surface application ($p = 0.007$, Fig. 6.1). However, there was no significant difference in the intI1 absolute abundance in the 5-20-cm soil between the two methods ($p = 0.067$). The high background level of intI1 may mask the difference of intI1gene induced by different application methods. Three days after manure application, the absolute abundance of intI1 gene was still significantly higher in the injection slits ($p < 0.001$ for 0-5 cm and $p = 0.004$ for 5-20 cm soil, respectively) than in the surface application.

The relative abundance of intI1 gene also showed a similar story. For instance, immediately after manure application, the relative abundance of intI1 was significantly higher in the injection slits relative to the surface application for 0-5-cm soil ($p = 0.007$), but not for 5-20-cm soil ($p = 0.104$). Strikingly, after three days, the relative abundance of intI1 in the injection slits significantly increased in both 0-5-cm and 5-20-cm soil and it was significantly higher than soil from the surface application ($p < 0.001$).

Antibiotics released into soils can remain active and have been observed to exert selection pressure on soil bacteria (Heuer et al., 2008). In addition, susceptible bacteria in soil can become resistant via horizontal gene transfer, which may be enhanced by the presence of antibiotics (Ubeda et al., 2005). As a mobile genetic element, intI1 plays a part in horizontal gene

transfer, which can enhance the spread of antibiotic resistance (Gillings et al., 2008). Further, manure provides nutrients, which can enhance the proliferation of antibiotic resistant bacteria in soil (Heuer and Smalla, 2007; Udikovic-Kolic et al., 2014). Our results revealed, for the first time, that the injection slits, where the manure is concentrated, should be considered as a "hot zone" of horizontal gene transfer. Awareness is needed regarding the extended survival time of manure-borne bacteria in the injection slits, as well as the frequency of horizontal gene transfer that may increase with high concentrations of antibiotics, high abundance of manure associated bacteria, and high amount of nutrients provided.

6.3.2 Horizontal and vertical distribution of Rum-2-bac and intI1 in soil surrounding the injection slits

On Day 0, the absolute abundance of Rum-2-bac in soil at 5 cm or 25 cm away from the injection slits was 5.8×10^5 and 1.9×10^5 copies g^{-1}, respectively, for the 0-5-cm soil and 1.2×10^5 and 1.8×10^4 copies g^{-1}, respectively, for the 5-20-cm soil (Fig. 6.2). The detection of Rum-2-bac in the soil surrounding the injection slits and at both 0-5-cm and 5-20-cm soil indicates an immediate horizontal (i.e. parallel to the slope of the tested field) and vertical transport. The field has a slope of 9-11°, therefore, the gravity can provide force for the manure to move in both directions. At three days after the manure application, the abundance of Rum-2-bac followed the order of: in the injection slits > 5 cm away from the silts > 25 cm away from the slits, for both 0-5 cm and 5-20 cm (Appendix AN). Particularly, after three days, in the soil at 25 cm away from the slits, the abundance of Rum-2-bac in the 0-5-cm and 5-20-cm soil reduced to the level close to the background. Transport of manure-associated bacteria from the injection slits to the surrounding soil may occur during the three days, however, our results suggested that the dissipation of Rum-2-bac is the main factor governing their abundance in the soil surrounding

the slits. It is possible that the manure-associated bacteria, which transported from the injection slits to the surrounding soil, cannot compete with soil bacteria and dissipate fast.

In terms of vertical distribution, the absolute abundance of Rum-2-bac was significantly higher in the 0-5-cm soil compared to the 5-20-cm soil (Appendix AM). At three days after manure application, the absolute abundance of Rum-2-bac was still significantly higher in the 0-5-cm soil for both application methods, suggesting that the vertical transport of Rum-2-bac was limited. The result was consistent with a rainfall simulation study, in which the vertical transport of ARGs was not significant during the period of study (Joy et al., 2013).

On Day 0, the absolute abundance of intI1 in soil at 5 or 25 cm away from the injection slits was 3.2×10^7 and 2.7×10^7 copies g^{-1}, respectively, for the 0-5-cm soil and 1.3×10^7 and 2.5×10^7 copies g^{-1}, respectively, for the 5-20-cm soil, respectively (Fig. 6.2). The abundance was not significantly different from that in soil from the injection slits for both 0-5-cm and 5-20-cm soil (Appendix AN). For the relative abundance, a similar trend was observed. There was no significant difference between the soil in the injection slits and the soil surrounding the injection slits. These results indicated that the immediate transport of intI1 was limited and the difference between manure-amended soil and control soil is largely masked by the high background levels of intI1 in the control soil.

At three days after manure application, the abundance of intI1 in the soil surrounding the injection slits was significantly lower than that of soil from the injection slits. With a wait period of 3 days, the antibiotics transported from the injection slits to the surrounding soil (unpublished data) may be limited to exert selection pressure favoring resistant bacteria or promote horizontal gene transfer. Thus, our results indicated that the injection slits can provide a barrier preventing the spread of antibiotic resistance in soil.

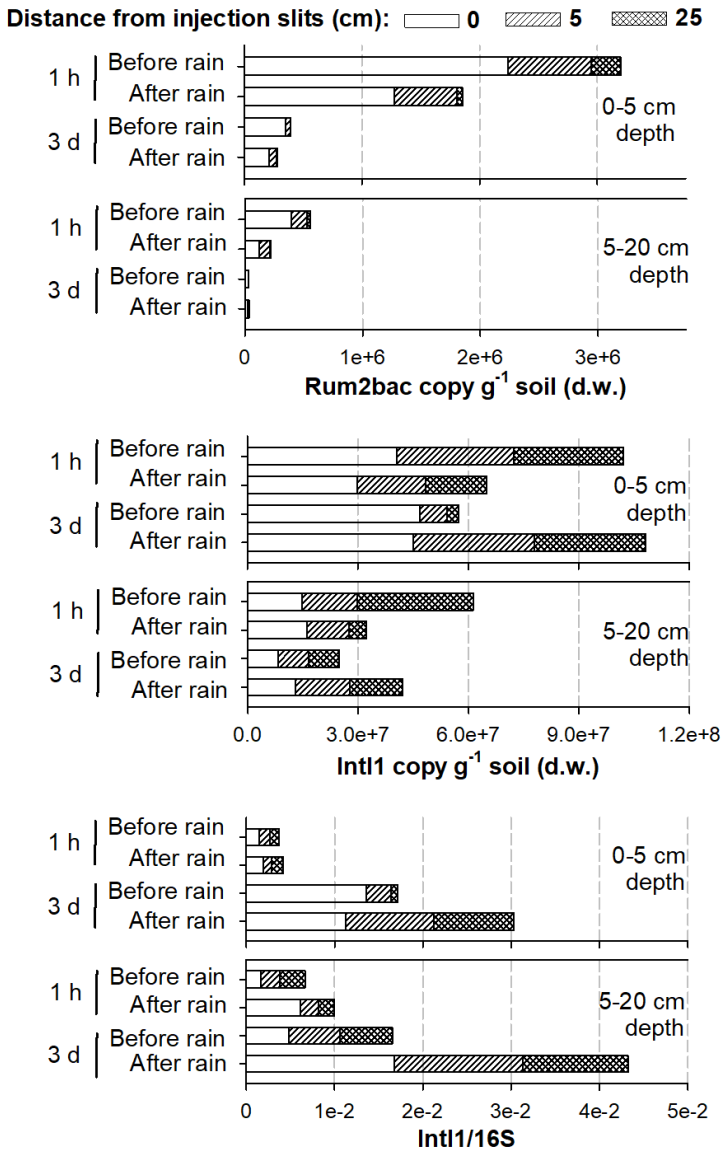


Figure 6.2 Absolute and relative abundance of Rum2bac and Intl1 in 0-5- and 5-20-cm soil collected in injection slits and 5 and 25 cm from injection slits 1 h before and after rainfall with manure-rainfall time gaps of 1 h and 3 d.

6.3.3 Effect of a simulated rainfall on rum-2-bac and intI1 abundance

Overall, the simulated rain did not significantly change the abundance of Rum-2-bac or intI1, regardless of the time gap between manure application and simulated rain (Figs. 6.1 and 6.2). There was not a consistent trend for the abundance of Rum-2-bac and intI1 before and after

the rain event. The only exception was that the relative abundance of intI1 was significantly increased in the 5-20-cm soil in the injection slits (p = 0.005 for the 2-h time gap and p = 0.001 for the 3-d time gap). However, there was no significant change in the intI1 relative abundance in the 0-5-cm soil in the injection slits after rain. The increase in the relative abundance of intI1 in the 5-20-cm soil was resulted from factors other than a higher rate of downward transport of bacteria carrying intI1 relative to bacteria not carrying intI1. Water irrigation may affect the spread of antibiotic resistance (Gatica and Cytryn, 2013; Negreanu et al., 2012) and the increased moisture content may contribute to the frequency of horizontal gene transfer (Aminov, 2011). The change of soil moisture by simulated rain may be less significant in the 0-5 cm compared to 5-20 cm soil as the 0-5-cm soil was already wet from the liquid manure application. However, increase in the intI1 relative abundance was not observed in the soil from the surface application after rain for both 0-5-cm and 5-20-cm soil. Further study is needed to confirm the effect of soil moisture on the potential of horizontal gene transfer.

The simulated rain did not significantly affect the horizontal and vertical distribution of Rum-2-bac in soil surrounding the injection slits (Figs. 6.1 and 6.2). The abundance of Rum-2-bac was maintained in the order of: in the silts > 5 cm away from the silts > 25 cm away from the slits for both the 0-5-cm and 5-20-cm soil. Similarly, the simulated rain did not significantly affect the horizontal and vertical distribution of intI1 (Figs. 6.1 and 6.2). There was no significant difference in the intI1 abundance between the soil in the injection slits and the soil surrounding the injection slits, whether the simulated rain was conducted or not. These results suggested that the rain did not significantly enhance the transport of Rum-2-bac and intI1, most likely because the soil was already wet from liquid manure application.

6.3.4 Short-term change of Rum-2-bac and intI1 in soil

For both manure application methods, Rum-2-bac was gradually decreased from the day 0 and returned to the background level of control soil by either Day 8 or Day 17. The short survival time of manure-borne bacteria was suggested by the observation that manure-borne bacteria only temporally increased after application of pig manure slurry but returned to the background level in a few days (Sengelov et al., 2003).

The intI1 gene was much more stable compared to the Rum-2-bac gene (Fig. 6.3). Up to day 17, the absolute and relative abundance of intI1 remained consistently and significantly higher in the injection slits compared to the control soil, in both 0-5-cm and 5-20-cm soil. Previous studies described various dissipations rates of intI1 gene in soil. The half-life of intI1 in soil following application of wastewater was determined to be 81 days (Burch et al., 2014), while another study showed no significant change during nearly 10 months in agricultural soil after manure slurry application (Byrne-Bailey et al., 2011).

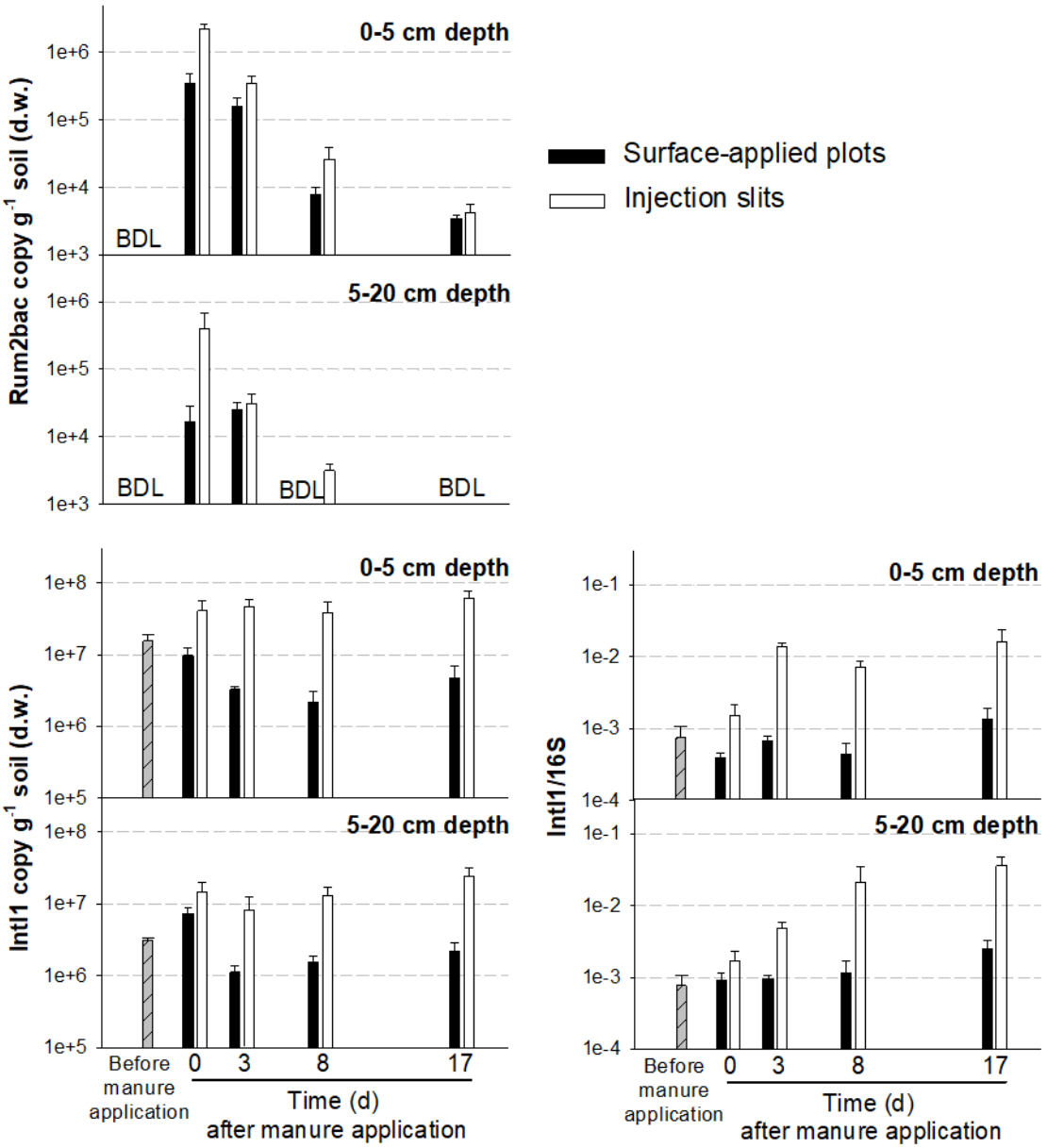


Figure 6.3 Absolute and relative abundance of Rum2bac and Intl1 in 0-5- and 5-20-cm soil collected from the surface application treatments and injection slits of the subsurface application treatments following 0 (1 h), 3, 8, and 17 d after manure application compared to control soil without manure application.

6.3.5 Correlations between antibiotic levels and gene abundance in soil

The abundance of Rum-2-bac displayed significant positive correlations with the concentrations of all four antibiotics (Table 6.1). In this study, the antibiotics were spiked into

manure before applying to soil, resulting in an elevated concentration of antibiotics in the soil where the manure was applied (Appendix AJ). As a manure biomarker, the abundance of Rum-2-bac was also elevated in the manure-amended soil compared to the control soil. Therefore, the positive correlations between Rum-2-bac and antibiotics are likely due to the fact that they come from the same source. The positive correlations between Rum-2-bac and antibiotics also suggested that the two factors have similar fate in soil after the manure application. For example, both Rum-2-bac and antibiotics dissipated fast after being released into soil and the transport of Rum-2-bac and antibiotics from the injection slits to the soil surrounding the slits was not significant (Appendix AJ).

Table 6.1 Spearman's correlation coefficient between antibiotics and abundance of Rum-2-bac and intI1 (***<0.001)

Antibiotic	Rum-2-bac (copies g^{-1})	IntI1 (copies g^{-1})	IntI1/16S
Pirlimycin (ng g^{-1})	0.786^{***}	0.423^{***}	0.047
Sulfamerazine (ng g^{-1})	0.840^{***}	0.453^{***}	-0.028
Tylosin (ng g^{-1})	0.795^{***}	0.493^{***}	0.037
Chlortetracycline (ng g^{-1})	0.820^{***}	0.468^{***}	0.130

The absolute abundance of intI1 also displayed significant positive correlations with the concentrations of all the four antibiotics. When the manure was applied to soil, the manure-associated bacteria carrying intI1 was released into soil, resulting in an increase in the total load of bacteria carrying intI1 (i.e. absolute abundance) in the soil. In contrast to the absolute abundance, the relative abundance of intI1 was not significantly correlated with the concentrations of antibiotics (Table 7.1). The antibiotics may exert selection pressure on antibiotic resistant bacteria and horizontal gene transfer. However, the antibiotics dissipated very fast in the soil (Appendix AJ), and the resistance selection may be limited within a short period

of exposure time. In addition, there may be a time interval between the antibiotic exposure and the response of intI1. For example, the relative abundance of intI1 in the injection slits significantly increased three days after the manure application, suggesting that the antibiotics posed a selection pressure on the bacteria carrying intI1. However, the antibiotics concentrations significantly decreased in the injection slits during the same period of time (Appendix AJ). More importantly, the background level of the relative abundance of intI1 in the control soil was considerably high in the test field. Although the manure application introduced a significant amount of manure-associated bacteria carrying intI1 into soil, it did not result in a significant increase in terms of relative abundance, as the manure may also introduce a significant amount of bacteria that did not carry intI1 into soil. Notably, there was no significant change in the relative abundance of intI1 during the same period of time, while a significant decrease in antibiotic concentrations was observed in the manured-amended soil. Thus, the relative abundance of intI1 generally appeared to be independent of antibiotic occurrence.

6.4 Conclusions

The manure subsurface injection method has its own advantages and disadvantages, in terms of its impact on the environment. At one hand, it can significantly reduce nutrients and manure-borne emerging contaminants such as antibiotics to the environment via surface runoff. On the other hand, this method concentrates manure microbes and antibiotics inside the manure injection slits, a “hot zone”, with limited movement of manure microbes and antibiotics to surrounding soil. In addition, the “hot zone” showed a higher level of intI1, an indicator for horizontal gene transfer, compared to surface application. Besides, the intI1 gene remained stable in the “hot zone” by 2 weeks after manure application. As a result, for farmers who are using the manure subsurface injection method, it is recommended to avoid planting directly onto the subsurface injection slits, which can reduce the exposure of plants to antibiotics, manure microbes, and antibiotic resistance genes/bacteria.

There were several limitations of our study. Firstly, we only analyzed intI1 as an indicator of the potential for horizontal gene transfer, while there are dozens of known ARGs corresponding to the resistance of the four antibiotics spiked in the manure. It would be interesting to examine corresponding antibiotic resistance genes as the genes may have correlation to the fate of antibiotics in this study. It would also be of interest if the metagenomics approach can be used to achieve a high resolution of ARGs in the soil. Secondly, the antibiotics were spiked to manure. We may have observed additional effects if we had used manure containing antibiotics which pass through animal gut. Thirdly, we only examined the concentrations of antibiotics in the soil samples. More information would be provided if the abundance of Rum-2-bac and intI1 in the runoff had been included.

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Chapter 7: Identifying transformation products of pirlimycin in soil using Eawag-BBD/PPS and mass spectrometry

Hanh Thi Van Le, Jun Xu, Sheldon Hilaire, and Kang Xia

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Abstract

A lincosamide antibiotic, pirlimycin, which is used to treat mastitis in dairy cows, can be found in raw manure at a level of 131 μg L^{-1}. Following manure land application, pirlimycin dissipates fast in soil with a reported half-life of 3 to 8 days. Since transformation products of pirlimycin have not been investigated, a pilot-scale study was conducted using Eawag-BBD/PPS and mass spectrometry to identify its transformation products from a soil incubation study. Loamy soil spiked with pirlimycin at an initial level of 1000 μg kg^{-1} soil (dried weight) was incubated at room temperature for 30 days. Pirlimycin and its transformation products predicted by Eawag-BBD/PPS were monitored at day 0, 1, 3, 5, 14, and 30 of incubation using the full-scan and selected ion monitoring functions of UPLC/MS/MS. Out of five likely transformation products predicted by Eawag-BBD/PPS, up to three were detected in soil during the 30-d incubation period. The transformation products A and/or B were detected at all sampling time points, even within hours after spiking. Their levels increased sharply within the first 5 d before levelling off for the rest of the incubation period. It indicated that these transformation products were more persistent in soil than the pirlimycin. Also, the transformation product A was predicted to be more harmful for rats than the pirlimycin. The transformation product A1 was detected in soil by day 1 and continuously increased throughout the incubation. However, its level was far lower than the levels of the transformation products A and/or B. In summary, this

study proposed a simple and economical approach to decide if studying transformation products of a chemical is needed.

7.1 Introduction

The widespread use of veterinary antibiotics in agriculture has led to their detection in various media such as animal manure, soil, wastewater treatment plants, surface water, ground water, and sediment (Karnjanapiboonwong et al., 2010; Kemper, 2008; Kim et al., 2016; Lissemore et al., 2006; Ray et al., 2017). In the United States, 13.9 million kg of antimicrobial drugs were sold for livestock and poultry in 2017 (USFDA, 2018). On one hand, the veterinary antibiotics degrade over time, reducing their impact to the environment. In soil, biotransformation by the microorganisms is an efficient mechanism to reduce chemical persistence (Accinelli et al., 2007; Pan and Chu, 2016). On the other hand, since complete mineralization accounts only for a small portion of the total initial amount (Schmidt et al., 2008), concerns about antibiotics always go together with their transformation products. Most studies reported either no difference or even a decrease in toxicity between the parent compounds and their transformation products (Haddad et al., 2015). Nevertheless, some transformation products can be more persistent and harmful than the parent compounds (Majewsky et al., 2014; Yuan et al., 2011).

While there are numerous studies on the environmental fate of antibiotics, identification and quantification of their transformation products in the environment system are difficult. First and foremost, formation of transformation products is a complex process, which is regulated by various environmental conditions and a number of transformation products are unknown. For instance, De Laurentiis et al. (2014) reported different transformation products of acetaminophen from photodegradation compared to human metabolism or microbial biodegradation. Chromatographic and mass spectrometric instruments are commonly used to tentatively identify

transformation products. Advances in high-resolution mass spectrometry such as QToF, Orbitrap and FT-ICR have made the process easier, however, interpretation of mass spectra can be challenging (Lopez et al., 2014). Besides, the proposed structures can only be confirmed by comparing with reference standards, which are often unavailable. Finally, the majority of studies were conducted in a simplified condition and used a much higher concentration level of parent compounds than their environmental level, which to some extent hindered what happened in the real environment. Detecting transformation products at trace levels in complex matrices still needs further study.

With the presence of hundreds of different pharmaceuticals in the environment, it is difficult to study transformation products for each compound. Thus, pathway prediction system tools are used to predict possible transformation pathways and corresponding transformation products. They typically rely on a database of biotransformation rules that recognize compound functional groups and transform them into transformation products such as Eawag-BBD/PPS, PathPred, and BNICE (Gao et al., 2011; Moriya et al., 2010; Wicker et al., 2016). Eawag Biodegradation / Biocatalysis Database Pathway Prediction System (Eawag-BBD/PPS), formerly known as the University of Minnesota Biodegradation/Biocatalysis Database Pathway Prediction System (UM-BBD/PPS), is considered the most extensive collection of manually curated biotransformation pathways of xenobiotics (Wicker et al., 2016). While the rule-based system can provide a fairly comprehensive list of transformation products, some transformation products can be irrelevant products (unlikely to form) under specific environmental conditions.

The aim of this paper was to study transformation products of antibiotics using Eawag-BBD/PPS and mass spectrometry, and pirlimycin was used as an example. Pirlimycin, a lincosamide antibiotic, is usually used as mastitis treatment in dairy cows. When pirlimycin was

administered to dairy cows, about 24 % and 10 % of the administered dose were excreted in feces and urine, respectively, as an unchanged compound, and 80 % and 45 % of pirlimycin in urine and feces, respectively, was excreted as compound conjugates (Hornish et al., 1992). Following manure land application, pirlimycin dissipated fast in soil with a half-life of 3 to 8 d (Le et al., 2018; Wind et al., 2018), however, its transformation products in soil are unknown.

7.2 Materials and Methods

7.2.1 Prediction of pirlimycin transformation products using Eawag-BBD/PPS

Uing instruction on the Eawag-BBD/PPS Pathway Prediction System (http://eawag-bbd.ethz.ch/predict/), with all biotransformation, the system predicted that pirlimycin was likely transformed into two products (transformation products A & B, Fig. 7.1) and neutrally transformed into several products. Similarly, the transformation products A and B were likely transformed into transformation products A1 and B1, respectively, which were then likely transformed into transformation product AB1 (Fig. 7.1). For the transformation product AB1, all further transformation pathways were neutral rather than likely. As a result, in this pilot study, only five likely transformation products were considered.

7.2.2 Toxicity prediction of pirlimycin transformation products

To estimate the toxicity of the pirlimycin transformation products predicted by Eawag-BBD/PPS, the Toxicity Estimation Software Tool (TEST) provided by the EPA based on the physical characteristics of the chemical was used (https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test#sysrequirements).

7.2.3 Soil incubation study

There were two treatments in the experiment, the control and spiked treatments. Braddock loam soil (fine, mixed, semiactive, mesic Typic Hapludults; 46 % sand, 44 % silt, and 10 % clay; free of pirlimycin) from Kentland farm, Blacksburg VA (37°11'50" N, 80°35'2"W), was collected from the 0-5 cm depth (Kulesza et al., 2016). Rock, roots, and leaves were removed before the soil was ground manually and air-dried for 2 days at room temperature. The soil was then spiked with pirlimycin stock at 50 ppm, mixed well before being added de-ionized

water to reach 60 % of water holding capacity (23 %). The starting pirlimycin concentration was 0 and 1000 µg kg^{-1} soil (dried weight) for the control and the spiked treatments, respectively. Soil samples were mixed thoroughly, then 25 g of soil mixture was transferred to a clean glass jar. All jars were slightly covered with aluminum foil and kept at room temperature. Every 2 days, each jar was weighed and the de-ionized water was added to 60 % of water holding capacity to compensate the weight loss due to water evaporation. Soil samples were destructively sampled at 0, 1, 3, 5, 14, and 30 days for the spiked treatment and at 0, 14, and 30 days for the control treatment. With 3 replicates for each treatment, there were 27 jars in total. Collected samples were freeze-dried, manually ground, and stored at -20^{o}C before extraction.

Due to differences in chemical structure of predicted pirlimycin transformation products, each soil sample experienced two extractions, the base and acid extractions. The pirlimycin compound was effectively extracted using the base extraction method. In the base extraction, 1 g of dried soil was mixed with 1 g of Na_2SO_4, 1 mL of concentrated NH_4OH, and 6 mL of CH_2CL_2, vortexed (1 min), sonicated (25 min at 10-15^{o}C), and centrifuged (1200 × g, 5 min). 5 ml of the CH_2CL_2 supernatant was then transferred to another glass tube and the sample was extracted again with 1 mL of concentrated NH_4OH and 6 mL of CH_2CL_2. The 10-mL combined CH_2CL_2 supernatant was transferred to a new tube containing 12.5 mg PSA, centrifuged again, then 6 mL of the CH_2CL_2 layer was evaporated to dryness (Labconco, 60 % vortex, 500 mbar, 30°C, 65 min). The sample was then reconstituted with 1 mL of 0.1% formic acid in 3:7 MeOH/H_2O, filtered through a 0.2 µm PTFE syringe filter (Thermo Scientific, MA, U.S.A) before being analyzed on UPLC-MS/MS. Samples were diluted if needed. For QA/QC, there were one spiked sample and one method blank every ten samples. In the acid extraction, a

similar procedure was used, however, 1 g of dried soil was extracted with 1 g of Na_2SO_4, 0.5 mL of HCl (2.42 N), and 5 mL of acetonitrile.

7.2.4 Confirmation of computer predicted pirlimycin transformation products using UPLC-MSMS

The measurements of pirlimycin concentrations and identification of pirlimycin transformation products were achieved using of an Agilent 1290 Infinity UPLC coupled with Agilent 6490 Triple Quad tandem mass spectrometry. For the purpose of identification, the full-scan (MS2 scan) and Selected Ion Monitoring (SIM) functions were used to collect ion mass spectra from 100 to 475 *m/z* and transformation product *m/z*, respectively in both electrospray ionization positive and negative modes. Pirlimycin and its transformation products were separated in a Zorbax Extend C18 analytical column (4.6 × 50 mm, 5-µm particle size, Agilent) together with a Zorbax Extend C18 guard column (4.6 × 12 mm, 5-µm particle size, Agilent). The mobile phase with a flow rate of 5 mL min^{-1} was composed of (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in methanol with the following A:B gradient at 0, 3, 3.5, 4, and 6 min: 70:30, 5:95, 5:95, 70:30, and 70:30. The *m/z* for pirlimycin, transformation product A, B, A1, B1, and AB1 was 411, 427, 427, 443, 426, and 442, respectively in the positive mode and 409, 425, 425, 441, 424, and 440, respectively in the negative mode.

7.3 Results and Discussion

7.3.1 Prediction of pirlimycin transformation products in soil using Eawag-BBD/PPS

The chemical structure of pirlimycin, a lincosamide antibiotic, includes a pyranose ring, an amide moiety, and a pyrrolidine ring (Chen et al., 2010). The predicted transformation products of pirlimycin at the first level were transformation products A and B with a molecular weight of 426 g mol^{-1} (M + 16), in which M refers to molecular weight of pirlimycin (Fig. 7.1). In the second level, the transformation product A was transformed into the transformation product A1 (441 g mol^{-1} or M + 31), which was then transformed into the transformation product AB1 (440 g mol^{-1} or M + 30). Similarly, the transformation product B was transformed into the transformation product B1 (425 g mol^{-1} or M + 15), which was then transformed into the transformation product AB1. In all reactions, it was suggested that the pyranose ring and amide group remained intact while the reactive site was located at the pyrrolidine ring, opening it up. Pathways included reduction of an alkyl group to an aldehyde group, oxidation of an aldehyde group to a carboxyl group, and deamination. Whereas, in aqueous solution, Chen et al. (2010) reported another mechanism. In particular, manganese oxides/hydroxides caused rapid and extensive decomposition of clindamycin and lincomycin (lincosamides) by breaking the C-O-C ether bond located at the pyranose ring.

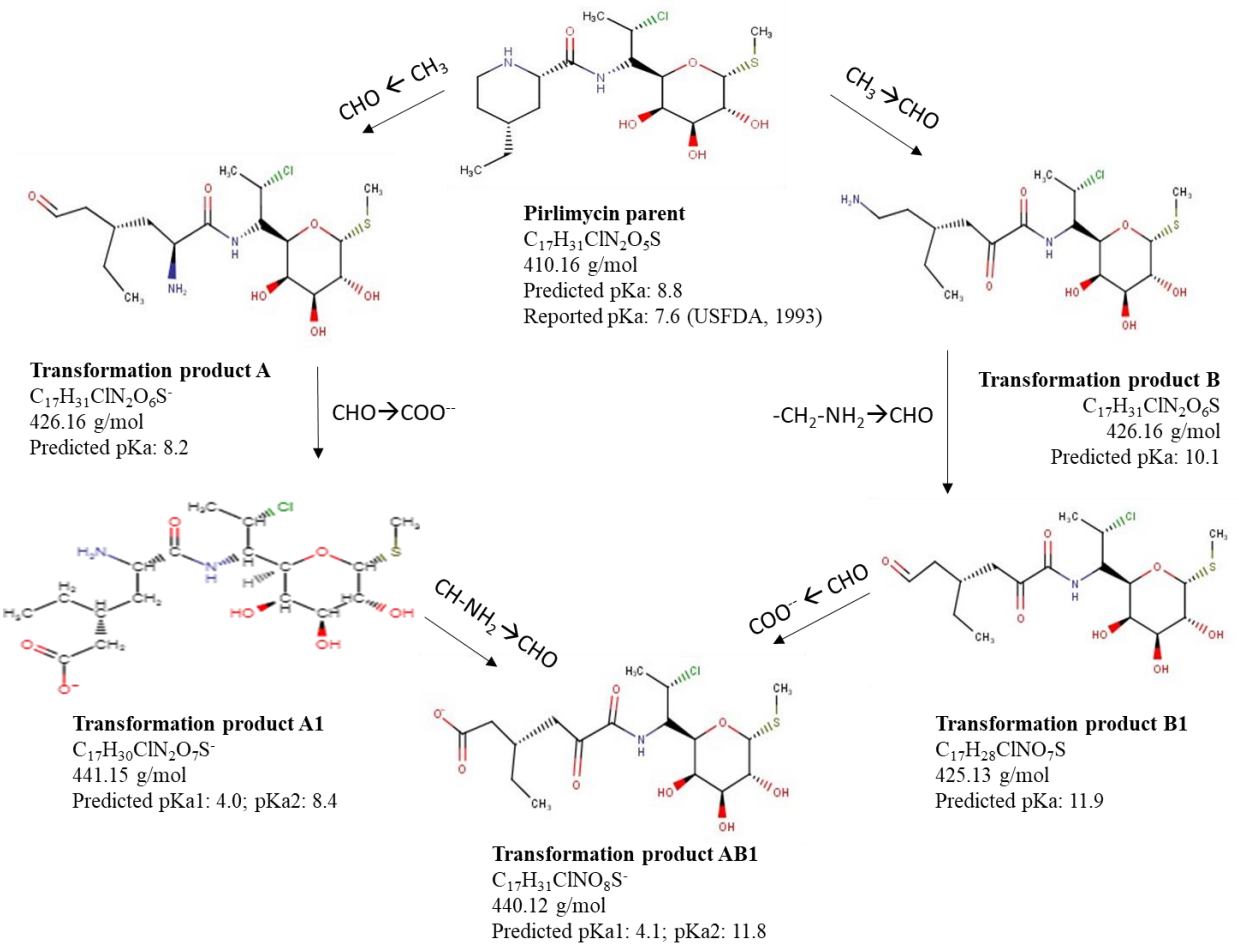


Figure 7.1 Structure, formula, molecular weight, and (predicted) pKa value of pirlimycin and its predicted transformation products using Eawag-BBD/PPS.

7.3.2 Confirmation of the computer-predicted transformation products of pirlimycin using tandem mass spectrometry

Base and acid extract of control and pirlimycin-spiked soil samples were scanned for the presence of target *m/z* during 30 d of incubation using both positive (Table 7.1) and negative ESI modes (Table 7.2). Pirlimycin and all predicted transformation products were not detected in control soil samples at 0, 14, and 30 d after incubation using base or acid extraction in both analysis modes. Therefore, the presence of pirlimycin and its predicted transformation products in spiked soil samples came from pirlimycin spiked into soil samples. In fact, pirlimycin

dissipated fast in soil (Fig. 7.2). More than 50 and 90 % of the initial added pirlimycin dissipated within 5 and 14 d of incubation, respectively. By D 30, less than 2 % of the initial added pirlimycin remained detected in soil. Even though the initial concentration of pirlimycin in this incubation study was higher than its environmental concentration, its dissipation pattern and rate were similar to other field studies using pirlimycin environmental concentrations (Unpublished data). Results also suggested that base extraction and positive analysis mode were the most sensitive for pirlimycin detection. Its fast dissipation and instrument sensitivity can explain the below detection of pirlimycin at D 30 for the acid extraction + positive mode and for the base extraction + negative mode and at from D 3 afterward for the acid extraction + negative mode.

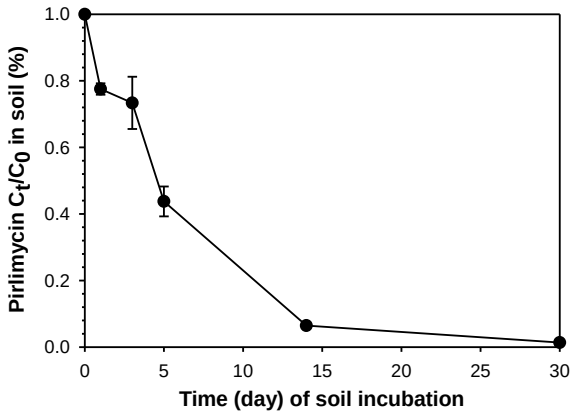


Figure 7.2 Pirlimycin dissipation in soil during a 30-d incubation period presented by its C_t/C_o (%).

In terms of transformation products of pirlimycin, out of five predicted transformation products, two (transformation products B1 and AB1) were not detected in spiked soil samples in all conditions (Tables 7.1 & 7.2). Under different conditions, different transformation products can be formed. For example, De Laurentiis et al. (2014) found several different transformation products of acetaminophen during photochemistry from microbial degradation or human

metabolism. Thus, the absence of these two transformation products can be explained by several reasons, including (1) typical environmental conditions of the study, (2) the short incubation period, and/or (3) the compound trace level. Two detected ions belonged to the transformation product A and/or B and transformation product A1 (Tables 7.1&2 and Fig. 7.3). The formation of the transformation product A1 indicated the presence of the transformation product A. The formation of the transformation products A and B followed a similar pathway, oxidation of an aldehyde group to a carboxyl group at the pyrrolidine ring, therefore, it is likely that both transformation products A and B were formed.

Table 7.1 Confirmation results of pirlimycin and its computer predicted transformation products in soil samples from a 30-d incubation study using LC-MS/MS positive ESI mode and SIM function.

Extraction type	Pirlimycin or its predicted transformation product		Sample type	Time (d) of soil incubation					
				0	1	3	5	14	30
	Name	*m/z*							
Base extraction	Pirlimycin	411	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	√	√	√	√	√	√
	Transformation production A and/or B	427	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	√	√	√	√	√	√
	Transformation production A1	443	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	√	√	√	√	√
	Transformation production B1	426	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p
	Transformation production AB1	442	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p
Acid extraction	Pirlimycin	411	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	√	√	√	√	√	n/p
		427	Control soil	n/p	n/a	n/a	n/a	n/p	n/p

	Transformation production A and/or B		Pirlimycin-Spiked soil	√	√	√	√	√	√
	Transformation production A1	443	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	√	√	√	√
	Transformation production B1	426	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p
	Transformation production AB1	442	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p

n/p: not presented; n/a: not available

Table 7.2 Confirmation results of pirlimycin and its computer predicted transformation products in soil samples from a 30-d incubation study using LC-MS/MS negative ESI mode and SIM function.

Extraction type	Pirlimycin or its predicted transformation product		Sample type	Time (d) of soil incubation					
				0	1	3	5	14	30
	Name	*m/z*							
Base extraction	Pirlimycin	409	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	√	√	√	√	√	n/p
	Transformation production A and/or B	425	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	√	√	√	√
	Transformation production A1	441	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	√	√	√	√
	Transformation production B1	424	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p
	Transformation production AB1	440	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p
Acid extraction	Pirlimycin	409	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	√	√	n/p	n/p	n/p	n/p
		425	Control soil	n/p	n/a	n/a	n/a	n/p	n/p

	Transformation production A and/or B		Pirlimycin-Spiked soil	√	√	n/p	n/p	n/p	n/p
	Transformation production A1	441	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p
	Transformation production B1	424	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p
	Transformation production AB1	440	Control soil	n/p	n/a	n/a	n/a	n/p	n/p
			Pirlimycin-Spiked soil	n/p	n/p	n/p	n/p	n/p	n/p

n/p: not presented; n/a: not available

The dissipation of pirlimycin was accompanied by the formation of transformation products A, B and A1 (Figs. 7.3 & 7.4). The combination of the base extraction and positive mode was most sensitive for detection of these transformation products. In fact, the transformation products A and B were detected at all soil sampling time points, even just within hours after spiking (D 0). Their levels increased continuously within 5 d of incubation, before levelling off during the rest of incubation period. The result suggested that the transformation products A and B were more persistent than the pirlimycin. The transformation product A1 was detected as early as by 1 d after incubation. Even its level was far lower than the transformation products A and B, its level continuously increased during the incubation period.

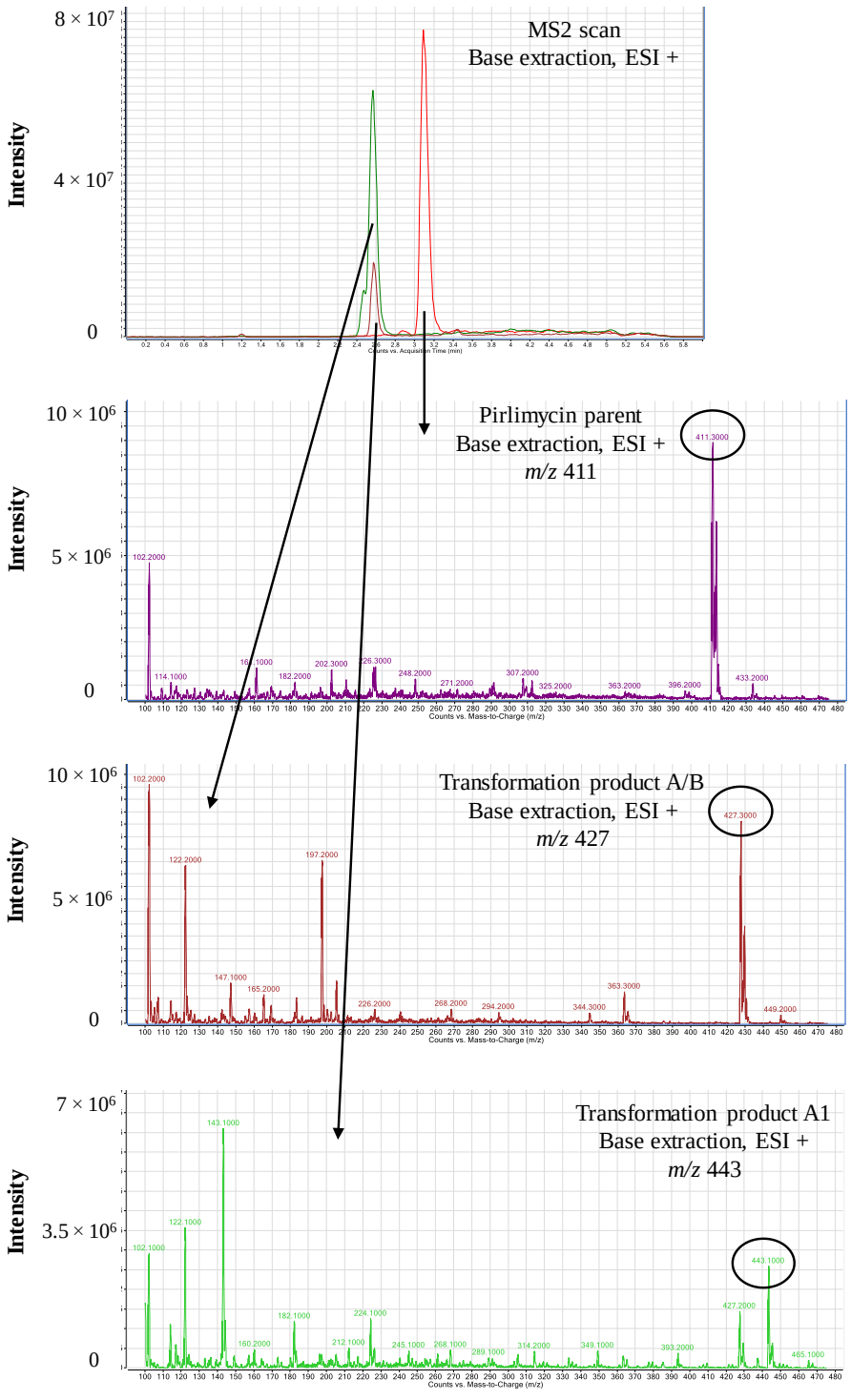


Figure 7.3 An illustration of pirlimycin and its predicted transformation products A/B and A1 in soil samples with base extraction by UPLC-MS/MS positive ESI mode and MS2 function.

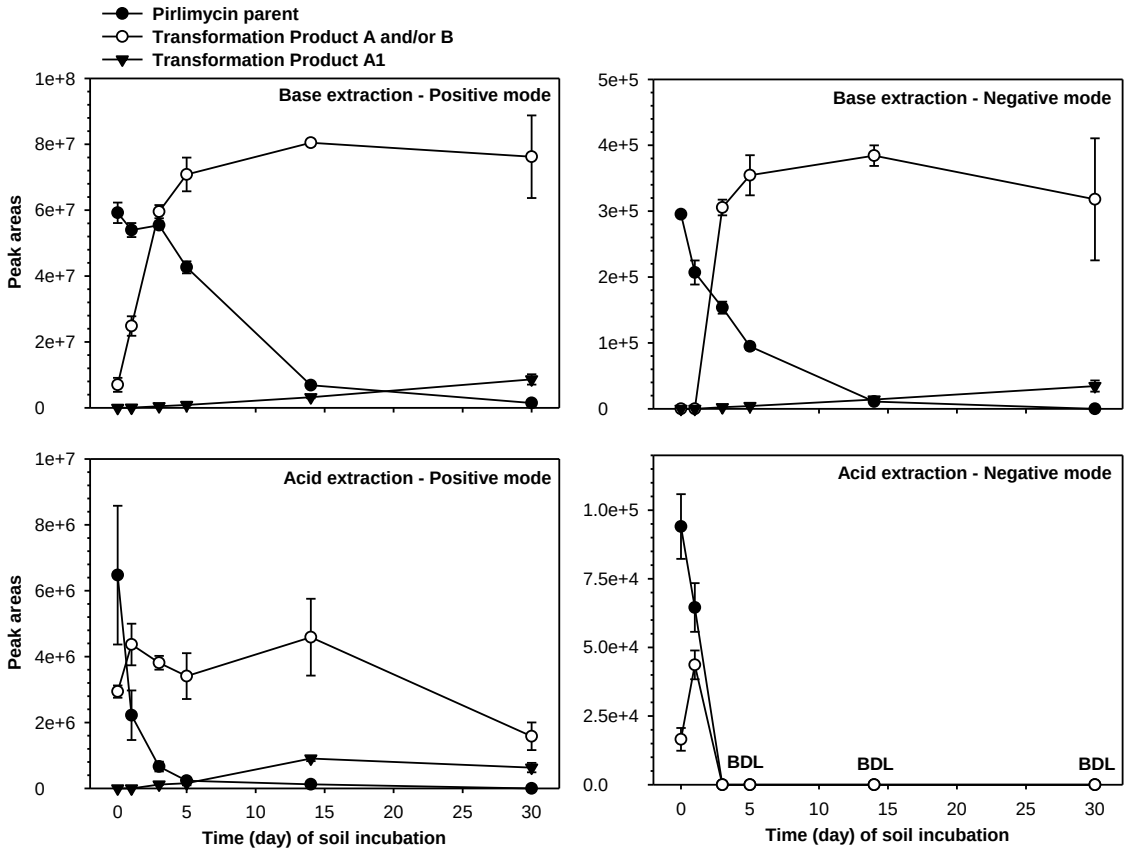


Figure 7.4 Abundance of pirlimycin, the transformation products A and/or B, and the transformation product A1 in soil measured by UPLC-MS/MS during the 30-d incubation period.

In term of toxicity, the predicted results indicated that among five transformation products, transformation products A and B1 had higher toxicities than the parent compound for rats (Table 3). The higher toxicity and persistence of the transformation product A compared to its parent might raise an environmental concern. Besides, the transformation products B1 can be potentially mutagenic (Table 7.3). The predicted toxicity reinforces the importance of studying pirlimycin transformation products. In the aquatic environment, two transformation products of sulfonamide were shown to have higher toxicity to *Vibrio fischeri* during 24 h exposure than sulfonamide (Majewsky et al., 2014). Also, photoproducts of oxytetracycline, doxycycline, and

ciprofloxacin that had the characteristic structure of the parent compounds in UV photolysis showed increased toxicity to *Vibrio fischeri* (Yuan et al., 2011).

Table 7.3 Toxicity endpoint information for pirlimycin and its predicted metabolites estimated from TEST EPA.

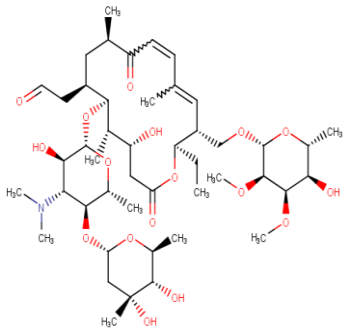
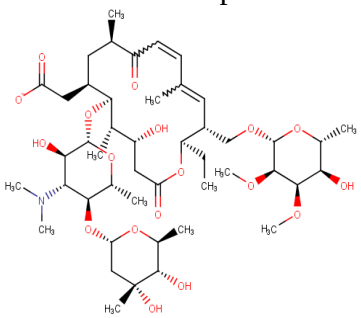
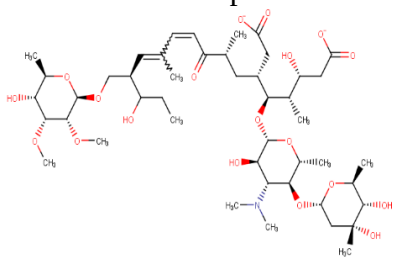
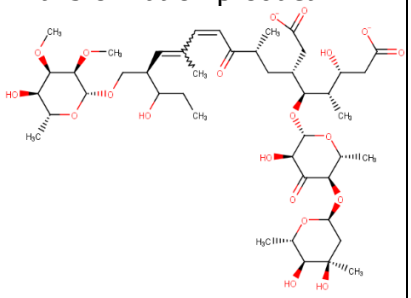
Compound	Toxicity Endpoint						
	96 hour fathead minnow LC_{50} (mg/L)	*48 hour *D. magna* LC_{50} (mg/L)	*48 hour *T. pyriformis* IGC_{50} (mg/L)	Oral rat LD_{50} (mg/kg)	*Bioaccumulation factor	Developmental toxicity	Mutagenicity
Pirlimycin	2.52	145.95	11.50	2582.87	2.09	Developmental NON-toxicant	Negative
Transformation production A	5.81	175.30	11.94	962.45	2.09	Developmental NON-toxicant	Negative
Transformation production A1	9.29	181.45	11.27	3955.15	1.44	Developmental NON-toxicant	Negative
Transformation production AB1	7.75	102.27	213.34	2038.15	1.17	Developmental NON-toxicant	Negative
Transformation production B	33.75	94.36	10.89	3195.50	1.19	Developmental NON-toxicant	Negative
Transformation production B1	12.59	54.71	10.87	789.02	1.29	Developmental NON-toxicant	Positive

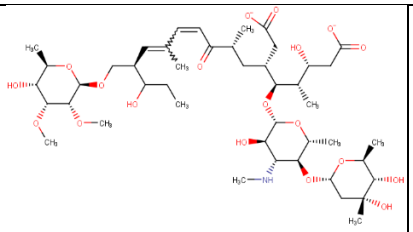
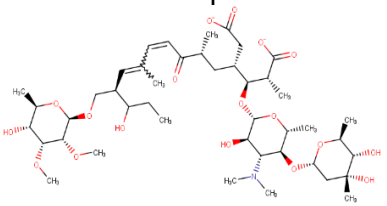
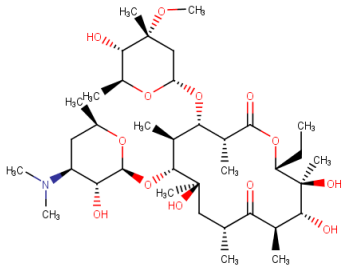
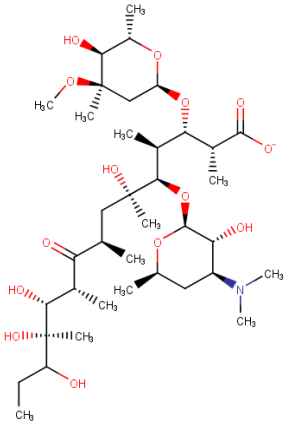
All values are presented using “consensus method” except for those denoted by * that use the “nearest neighbor method”.

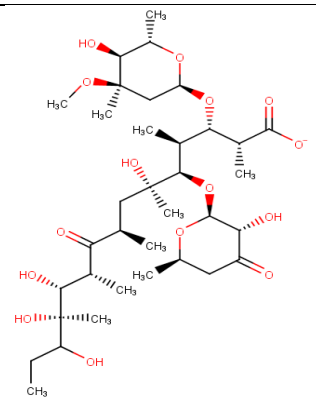
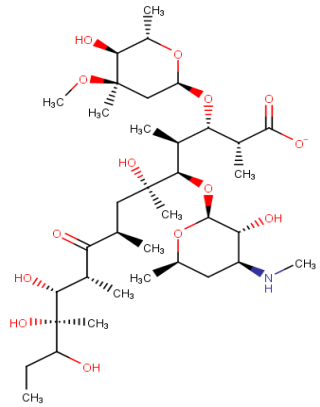
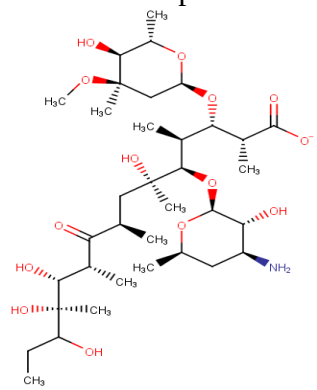
7.3.3 Computer-predicted transformation products using Eawag-BBD/PPS and literature reported transformation products

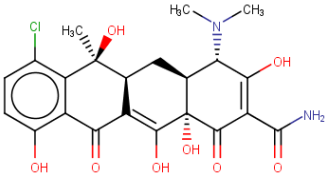
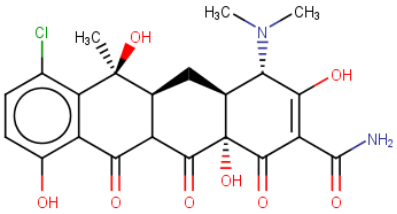
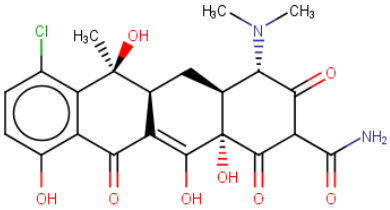
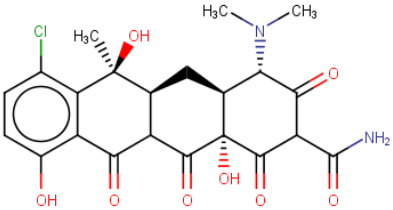
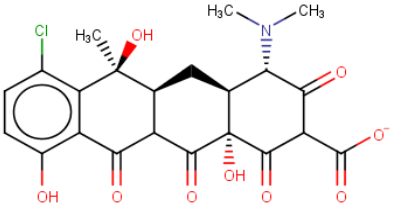
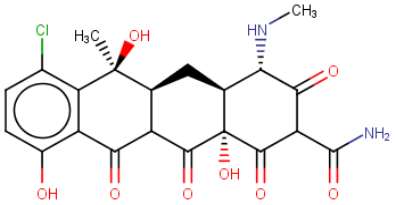
In Table 7.4, the computer predicted transformation products of antibiotics using Eawag-BBD/PPS were compared to their reported transformation products in literature. Chosen antibiotics included tylosin, erythromycin, and chlortetracycline. For tylosin A, four common transformation products are tylosin B, tylosin C, tylosin D, and dihydrodesmycosin; which were not matched with predicted transformation products using Eawag-BBD/PPS. In an incubation study of manure lagoon slurries by Kolz et al. (2005), an unknown transformation product with *m/z* of 934.5 was detected using an ion trap LC-MSMS (positive mode). This *m/z* matched with one out of five predicted transformation products. For erythromycin, four predicted transformation products were not matched with the two commonly-reported transformation products (Jia et al., 2018; Zhou et al., 2017). Similarly, two reported transformation products of chlortetracycline, 4-epichlortetracycline and 4-epi-anydrochlotetracycline, were not matched with any of the six predicted transformation products (Kwon, 2011). The mismatch between the computer-predicted transformation products and reported transformation products of some commonly-used antibiotics enhanced the need of a simple screening method for potential transformation products.

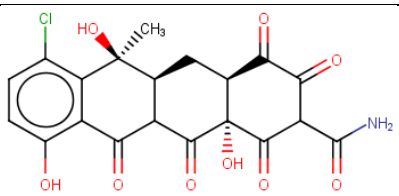
Table 7.4 Comparison of computer predicted transformation products using Eawag-BBD/PPS and reported transformation products of tylosin, erythromycin, and chlortetracycline.

Parent compound	Predicted transformation product using Eawag-BBD/PPS	Published transformation product
Tylosin  $C_{46}H_{77}NO_{17}$ 915.519 g mol^{-1}	Transformation product A  $C_{46}H_{76}NO_{17}[O^-]$ 930.50 g mol^{-1}	×
	Transformation product A1  $C_{46}H_{77}NO_{17}[O^-]_2$ 947.51 g mol^{-1}	×
	Transformation product A1-1  $C_{44}H_{70}O_{18}[O^-]_2$ 918.447 g mol^{-1}	×
	Transformation product A1-2	(Kolz et al., 2005)

	 $C_{45}H_{75}NO_{17}[O^-]_2$ 933.49 g mol^{-1}	
	Transformation product A1-3  $C_{44}H_{73}NO_{16}[O^-]_2$ 903.48 g mol^{-1}	×
	×	Tylosin B Tylosin C Tylosin D Dihydrodesmycosin (Kolz et al., 2005)
Erythromycin  $C_{37}H_{67}NO_{13}$ 733.46 g mol^{-1}	Transformation product A  $C_{37}H_{68}NO_{13}[O^-]$ 750.46 g mol^{-1}	×
	Transformation product A1	×

	 $C_{35}H_{61}O_{14}[O^-]$ 721.40 g mol^{-1}	
	Transformation product A2  $C_{36}H_{66}NO_{13}[O^-]$ 736.448 g mol^{-1}	×
	Transformation product A2-1  $C_{35}H_{64}NO_{13}[O^-]$ 722.43 g mol^{-1}	×
	×	Erythromycin A enol ether Anhydroerythromycin A (Jia et al., 2018; Zhou et al., 2017)

Chlortetracycline  $C_{22}H_{23}ClN_2O_8$ 478.882	Transformation product A  $C_{22}H_{23}ClN_2O_8$ 478.11 g mol^{-1}	×
	Transformation product B  $C_{22}H_{23}ClN_2O_8$ 478.11 g mol^{-1}	×
	Transformation product AB  $C_{22}H_{23}ClN_2O_8$ 478.11 g mol^{-1}	×
	Transformation product AB-1  $C_{22}H_{21}ClNO_8[O^-]$ 478.09 g mol^{-1}	×
	Transformation product AB-2  $C_{21}H_{21}ClN_2O_8$ 464.09 g mol^{-1}	×
	Transformation product AB-3	×

	 $C_{20}H_{16}ClNO_9$ 449.05 g mol^{-1}	
	×	4-epichlortetracycline 4-epi-anydrochlotetracycline (Kwon, 2011)

7.4 Conclusions

Lincosamide antibiotics are commonly detected in the environment, thus occurrence and potential toxicity of their transformation products need to be studied. Powerful instruments such as QToF, Orbitrap, NMR, and IR are expensive and not frequently available. Besides, due to complex components in samples, interpretation of mass spectra can be challenging. This study proposed a simple way to study transformation products of antibiotics and pirlimycin was used as a model. Potential transformation products and their toxicity were predicted using Eawag-BBD/PPS and TEST, respectively. The corresponding mass of predicted transformation products in soil samples during an incubation study spiked with pirlimycin was confirmed using UPLC-MSMS. The results indicated that up to three out of five predicted transformation products of pirlimycin were detected during the 30-d incubation period. The transformation products A and B were more persistent in soil than the pirlimycin. Also, the transformation product A was predicted to be more toxic for rats than pirlimycin. The mismatch between the computer-predicted transformation products and reported transformation products enhanced the need of a simple and cost-effective screening method to decide if studying transformation products of a compound is needed.

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Chapter 8: Conclusions and Application

Results in this dissertation demonstrated that runoff was a route for transporting antibiotics from manure-applied fields. During 30 min of runoff collection from a simulated rainfall (70 mm h^{-1}), surface losses of antibiotics amounted to 0.45-2.62 % of their initial input with manure. Antibiotic distribution between aqueous and solid phases of runoff was largely determined by the water solubility and the partition capacity of antibiotics to soil particles. For instance, masses in the aqueous phase were 99±0.5 %, 94±4 %, 91±7 %, and 22±15 % of pirlimycin, sulfamerazine, tylosin, and chlortetracycline, respectively. The good news is there are some manure management practices that can lower the antibiotic surface loss. First, compared to the traditional manure surface application, the manure subsurface injection method reduced sulfamerazine, chlortetracycline, pirlimycin, and tylosin losses in runoff by at least 47, 50, 57, and 88 %, respectively. The advantage of the subsurface injection method over the surface application in antibiotic loss reduction via surface runoff was observed in both fall and spring application seasons. Furthermore, the surface runoff loss of pirlimycin in the spring was significantly higher than that in the fall, which makes it more important to use the subsurface injection in the spring. Second, compared to plots that received simulated rainfall at 2 h after manure application, plots that received liquid manure amendment three days or longer before a subsequent rain event reduced surface runoff losses of antibiotics by 9-45 times. Third, composting manure was very effective to decrease the initial concentration of pirlimycin before application (>99 %), as a result, surface runoff loss of pirlimycin from plots receiving composted manure was significantly lower than that from plots receiving raw manure. The combined recommendations for farmers in order to reduce the surface runoff loss of pirlimycin to the

surrounding environment follow this sequence: (surface application + raw manure) < (surface application + composted manure) ~ (subsurface injection + raw manure) < (subsurface injection + composted manure). Therefore, using composted manure, the manure subsurface injection method, and avoiding manure application less than 3 days before rain would be recommended as manure land management best practices to reduce the spread of manure-associated antibiotics from the field to the surrounding environment.

The majority of antibiotics remained in soil, especially in the 0-5 cm following manure application. In terms of distribution, antibiotic concentrations in the subsurface injection slits were 4-26 times higher than those in the surface applied plots for 1 h, 3 d and 7 d after manure application. For the surface application, downward movement of antibiotics to a deeper soil profile was observed. For the subsurface injection, antibiotics concentrated in the injection slits for an extended time with limited horizontal and vertical movement from the slits. Effects of simulated rainfall on antibiotic distribution in soil depended on the antibiotic, the manure-rainfall time gap, and the application season. In general, effects of manure application-rainfall time gap on antibiotic distribution were stronger with decreased manure-rainfall time gaps and stronger in the fall than in the spring. In terms of long-term dissipation of antibiotics in soil, all antibiotics showed a similar pattern with fastest kinetics during the first 14 d before slowing down, and the effect of two manure application methods on antibiotic dissipation kinetics varied with different antibiotics. The half-life of antibiotics ranged from 3 to 11 d for pirlimycin, 3 to 10 d for sulfamerazine, 5 to 12 d for tylosin, and 3 to 21 day for chlortetracycline. These short antibiotic half-lives raise another concern about their transformation products, which are not well-documented. This dissertation proposed a simple way to screen antibiotic transformation products using Eawag-BBD/PPS for predicting potential transformation products, using TEST

EPA for predicting their toxicity, and using LC-MSMS for confirmation of these transformation products. One transformation product of pirlimycin was found to be more persistent in soil than the pirlimycin. Besides, it was predicted to be more toxic for rats than the pirlimycin. Pirlimycin, sulfamerazine, and tylosin remained detectable in soil even at 6 months after a single manure application, indicating that soils could be a long-term source for antibiotics to the surrounding environment. Their presence, especially with higher levels in the subsurface injection slits, might encourage development and growth of antibiotic resistance. Results showed a higher absolute abundance and relative abundance of rum-2-bac and intI1 in the injection slits compared to the surface application in both 0-5-cm and 5-20-cm soils during 17 d. Finally, using manure spiked with antibiotics can mimic manure with antibiotics passing though animal body if the surface runoff loss of antibiotics and distribution of antibiotics in soil are of interest. However, studies using spiked manure might not reflect the antibiotic long-term behavior in soil like studies using manure from animals with antibiotic administration, especially when antibiotics are partly metabolized in the animal body.

Appendices

Appendix A: Field test-plot installation, subsurface injection slits of the manure subsurface injection treatment, and surface runoff collection

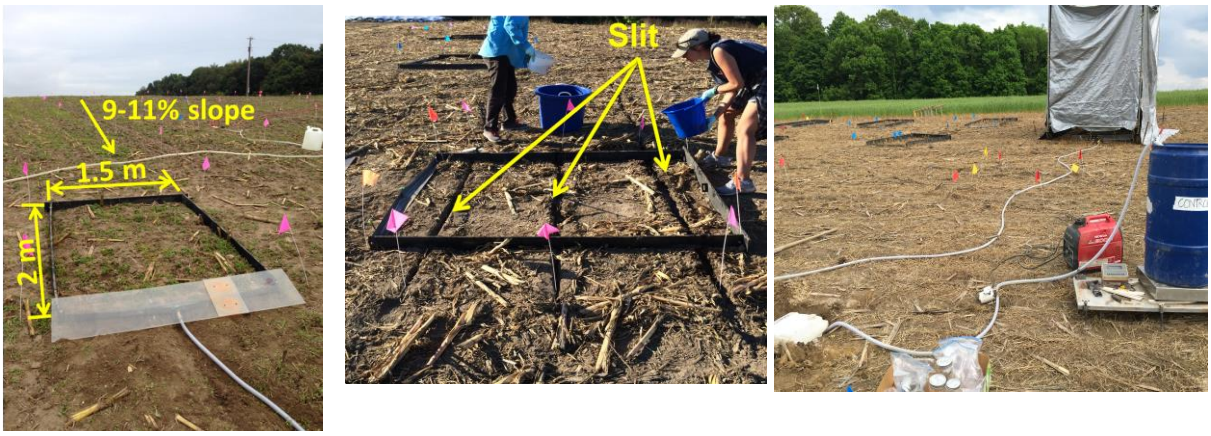


Figure A. Field test-plot (1.5 m × 2 m) installation (left), subsurface injection slits of the manure subsurface injection treatment (middle), and surface runoff collection (right).

Appendix B: Extraction and cleanup liquid raw manure or composted manure samples for pirlimycin analysis

To extract and clean up liquid raw manure or composted manure samples for pirlimycin analysis, a published method by Ray et al. (2014) was used. In summary, 1 g of liquid manure or 0.5 g of freeze-dried composted manure was mixed with 0.5 mL of 500 mM phosphate buffer (pH 8.5), 3.5 mL methanol, and 1 mL water followed by vortexing, sonicating, shaking, and centrifuging. Supernatant was transferred to another container and diluted to 50 mL using 50 mM phosphate buffer. The sample was then loaded through solid-phase extraction (SPE) and cleaned up using OASIS HLB Plus Short cartridges (250 mg sorbent, Waters). Then 1 mL of eluted extract was dried down under nitrogen gas, reconstituted in 1 mL solution of

methanol:water (3:7, v/v) with 0.1 % formic acid, and filtered through a 0.2 µm polytetrafluorethylene (PTFE, Thermo Scientific) into an amber glass high-performance liquid chromatography (HPLC) vial before being analyzed for pirlimycin on an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) (Appendix F).

Appendix C: Extraction and cleanup liquid raw manure or composted manure or runoff sediment or soil samples for chlortetracycline, sulfamerazine, and tylosin analysis

In order to extract and clean up liquid raw manure or composted manure or soil samples for chlortetracycline, sulfamerazine, and tylosin analysis, 2 mL of liquid manure or 0.5 g of freeze-dried composted manure or 2 g of freeze-dried soil samples was mixed with 10 mL extraction solution of methanol: 0.1 M ethylenediaminetetraacetic acid (EDTA): McIlvaine buffer (50:25:25, v/v/v, pH = 7). Before the extraction solution was added, all samples were spiked with internal standards that are structurally similar to the target analytes but with different molecular masses. For chlortetracycline, sulfamerazine, and tylosin, which were the antibiotics of interest for this field study, the internal standards included oxytetracycline, sulfamethazine, and roxithromycin, respectively, to correct for the loss of target analytes during sample extraction and cleanup. The mixture was then vortexed, sonicated (20 min at room temperature), and centrifuged (3500 rpm for 10 min at 10^{o}C). All of the supernatant was transferred to a 400 mL mason jar. Samples were extracted twice, and the combined supernatant was diluted to 300 mL with D.I. water and adjusted to pH of 3 with 85 % H_3PO_4 before solid-phase extraction and cleanup using OASIS HLB cartridges (60 mg sorbent, Waters). These cartridges were conditioned with 3 mL methanol followed by 3 mL water, washed with 3 mL water, and eluted

with 3 mL methanol. The collected extract was dried down using a RapidVap Vacuum (50 % Speed, 40^{o}C, 100 mbar, Labconco) and reconstituted in 1 mL solution of acetonitrile:water (3:7, v/v) with 0.1 % formic acid. The extract was then filtered through a 0.2 µm PTFE syringe filter into an amber glass HPLC vial and analyzed on an UPLC/MS/MS (see Appendix F).

Appendix D: Extraction and cleanup runoff water samples for pirlimycin, chlortetracycline, sulfamerazine, and tylosin analysis

To separate surface runoff water and sediment, the runoff samples were vacuum filtered through 0.7 µm glass microfiber filters (Whatman) using glass filter funnels and vacuum flasks. A traditional 0.45 µm nylon membrane filter was not used because it became blocked too quickly, making filtration of a relatively large volume difficult. Besides, results of spiked tests using nylon membrane and glass microfiber filters showed that samples with the nylon membrane filter had a much lower recovery than those with the glass microfiber filter. Both the traditional 0.45 µm nylon membrane filter and the 0.7 µm glass microfiber filter are operational defined. In a water-resources investigations report from the U.S. Geological Survey by Sandstrom (1995), the 0.7 µm glass microfiber filter was used to define the dissolved and solid phases of organic compounds. In fact, this filter has been used to analyze total suspended sediment (Cournane et al., 2010), polycyclic aromatic hydrocarbons (PAHs) and heavy metals associated with sediment from storm water (Brown and Peake, 2006), and insecticides in runoff (Mersie et al., 2003). Filters with sediment were freeze dried, stored at -80^{o}C, and analyzed shortly after that. For the runoff water fraction, collected runoff water was filtered twice through PTFE syringe filters of 0.45 µm and 0.2 µm immediately before analysis on an UPLC/MS/MS

using a direction injection method for pirlimycin and sulfamerazine or an Online SPE method for chlortetracycline and tylosin (see Appendix F).

Appendix E: Extraction and cleanup runoff sediment or soil samples for pirlimycin analysis

The freeze dried runoff sediment and filter were mixed with 1.00 g anhydrous sodium sulfate and 7 mL concentrated ammonium hydroxide:methylene chloride (1:6, v/v). The mixture was then vortexed, sonicated (20 min at room temperature), and centrifuged (5 min, 1200 x *g*, 10^{o}C). Exactly 5 mL of the methylene chloride layer of the supernatant was then transferred to another centrifuge tube, and the solid phase left in the first centrifuge was extracted again with the same volume of the concentrated ammonium hydroxide and methylene chloride as the first extraction step and then centrifuged (5 min, 1200 x *g*, 10^{o}C). Exactly 6 mL of the methylene chloride layer of the supernatant was combined with the 5 mL methylene chloride collected from the first extraction and centrifuged (5 min, 1200 x *g*, 10^{o}C). Exactly 5 mL of the supernatant was transferred to a clean test tube and evaporated to dryness using a RapidVap Vacuum (Labconco). The dried sample was reconstituted with 1 mL solution of methanol:water (3:7, v/v) with 0.1 % formic acid, filtered, and analyzed for pirlimycin on an UPLC/MS/MS (see Appendix F).

Appendix F: Analysis of antibiotics using UPLC/MS/MS

Concentrations of target antibiotics in filtered runoff water and sediment extract were analyzed on an Agilent 1290 Infinity UPLC coupled with Agilent 6490 Triple Quad tandem mass spectrometry in the electrospray ionization positive mode. For QA/QC, there were one method blank and one spiked sample every ten samples. To analyze pirlimycin and sulfamerazine, filtered runoff water was directly injected into the instrument, while for

chlortetracycline and tylosin analysis, 1 mL of filtered runoff water was loaded onto the Online SPE based on the 1290 Infinity Flexible Cube with Agilent ZORBAX Eclipse plus C18 cartridges. A Zorbax Extend C18 analytical column (4.6 × 50 mm, 5-µm particle size, Agilent) together with a Zorbax Extend C18 guard column (4.6 × 12 mm, 5-µm particle size, Agilent) were used for all analyses. A gradient of mobile phase with a flow rate of 0.5 mL min-1 was used for separation of all analyses, except for the Online SPE method (a flow rate of 0.3 mL min-1). Regarding the pirlimycin analysis, the mobile phase included (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in methanol with the following A:B gradient at 0, 3, 3.5, 4, and 6 min: 70:30, 5:95, 5:95, 70:30, and 70:30. For the chlortetracycline, sulfamerazine, and tylosin analysis, the mobile phase included (A) 0.1 % formic acid in water and (B) 95 % acetonitrile with the following A:B gradient at 0, 3.5, 3.6, 5, and 5.5 min: 70:30, 20:80, 0:100, 0:100, and 70:30. For the Online SPE method, the mobile phase included (A) water with 2 % acetonitrile and (B) 100 % acetonitrile with the following A:B gradient at 0, 0.1, 4.6, 8.1, 8.2, 9.6, and 10.1 min: 70:30, 70:30, 70:30, 20:80, 0:100, 0:100, and 70:30. The mass to charge ratios (*m/z*) for the parent, qualifier daughter, and quantifier daughter ions were 411.0, 363.0, and 112.0, respectively for pirlimycin; 479.1, 153.9, and 443.9, respectively for chlortetracycline; 265.0, 108.0, and 156.0, respectively for sulfamerazine; and 916.0, 772.0, and 174.0, respectively for tylosin. The concentration of all antibiotics in runoff water and the concentration of pirlimycin in runoff sediment were quantified using the matrix-matched standards. The concentrations of chlortetracycline, sulfamerazine, and tylosin in runoff sediment were quantified based on the calibration curves by plotting the ratio of the target analyte signal to each target compound's internal standard signal as a function of the target analyte standard concentrations. The method detection limit and recovery of target compounds in their matrix were listed in Appendix H.

Appendix G: Initial mass of antibiotics in liquid manure

The antibiotic amount detected in the liquid manure (Appendix J) was a product of the antibiotic concentration in the liquid manure and the amount of liquid manure applied to each test-plot (32 kg). The initial mass of pirlimycin and tylosin in liquid manure was about half of the target mass (57±4 % for pirlimycin and 52±7 % for tylosin), while the initial mass of sulfamerazine and chlortetracycline in liquid manure was more than half of the target mass (75±21 % for sulfamerazine and 69±21 % for chlortetracycline). Variation of triplicates for each treatment of sulfamerazine and chlortetracycline was smaller than that of all treatments combined. The differences between target and measured amount of antibiotics could be due to antibiotic transformation during sample storage and unextractable antibiotics strongly bound to the solid phase of liquid manure.

Appendix H: Method detection limit and recovery of target compounds in their matrix

Compound	Matrix	Method detection limit (ng/g or ng/mL)	Recovery (%)
Pirlimycin	Runoff water	0.005	91±7
	Runoff sediment	0.020	87±5
Sulfamerazine	Runoff water	0.010	95±5
	Runoff sediment	0.050	71±3
Chlortetracycline	Runoff water	0.010	93±7
	Runoff sediment	0.500	148±18
Tylosin	Runoff water	0.005	85±2
	Runoff sediment	0.100	88±33

Appendix I: Physical properties of surface runoff for all treatments

Treatment	Time to start runoff (min)	Weight of collected surface runoff (kg)	Runoff sediment concentration (g kg^{-1})	Total runoff sediment load (g)

Control	12.31 ± 2.40	16.31 ± 9.96	0.42 ± 0.24	7.58 ± 5.60
Surface application Day-0	7.33 ± 2.91	46.48 ± 6.73	0.62 ± 0.54	26.32 ± 19.17
Subsurface injection Day-0	8.74 ± 2.32	32.49 ± 9.82	1.01 ± 0.41	30.33 ± 7.45
Surface application Day-3	13.74 ± 2.97	18.16 ± 5.57	0.17 ± 0.04	3.04 ± 1.03
Subsurface injection Day-3	29.35 ± 27.14	14.21 ± 8.40	1.34 ± 0.31	10.80 ± 11.21
Surface application Day-7	4.32 ± 0.75	20.58 ± 6.67	0.54 ± 0.12	11.27 ± 5.30
Subsurface injection Day-7	18.43 ± 22.19	11.67 ± 15.87	0.70 ± 1.03	1.76 ± 1.12

Appendix J: Average initial mass of antibiotics in applied liquid manure for each treatment plot

Treatment	Initial mass of antibiotics (mg) in applied liquid manure for each treatment			
	Sulfamerazine	Tylosin	Chlortetracycline	Pirlimycin
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Surface application Day-0	12.88 ± 1.40	2.09 ± 0.17	8.31 ± 3.21	2.23 ± 0.07
Subsurface injection Day-0	10.42 ± 2.46	2.91 ± 0.18	14.82 ± 4.52	2.50 ± 0.12
Surface application Day-3	14.97 ± 2.61	2.42 ± 0.13	13.03 ± 1.87	2.29 ± 0.08
Subsurface injection Day-3	7.06 ± 0.76	2.22 ± 0.28	10.44 ± 2.77	2.40 ± 0.22
Surface application Day-7	11.62 ± 2.55	2.90 ± 0.15	9.16 ± 2.28	2.24 ± 0.06
Subsurface injection Day-7	15.35 ± 1.05	2.44 ± 0.09	10.47 ± 2.34	2.64 ± 0.04

Appendix K: Surface runoff loss of pirlimycin, tylosin, sulfamerazine, and chlortetracycline that were initially applied with the manure to the surface applied and subsurface injected test plots. (Different letters within the same manure-rainfall time gap treatment indicate statistical significance at $p < 0.05$.)

Treatment	Matrix	Mass loss of antibiotics with surface runoff /initially mass applied with the manure to the surface applied and subsurface injected test plots (%)			
		Pirlimycin	Sulfamerazine	Chlortetracycline	Tylosin
Surface application Day-0	Runoff water	2.6030 ± 0.3094	0.4362 ± 0.1353	0.2323 ± 0.1664	1.5245 ± 0.4682
	Runoff sediment	0.0208 ± 0.0088	0.0178 ± 0.0055	1.5364 ± 0.6128	0.1186 ± 0.0821
	Total runoff	2.6238 ± 0.3135A	0.4540 ± 0.1384A	1.7687 ± 0.6777A	1.6431 ± 0.5280A
Subsurface injection Day-0	Runoff water	1.1238 ± 0.3188	0.2347 ± 0.0946	0.0199 ± 0.0109	0.1202 ± 0.0331
	Runoff sediment	0.0059 ± 0.0025	0.0066 ± 0.0035	0.3974 ± 0.1256	0.0247 ± 0.0139
	Total runoff	1.1297 ± 0.3210B	0.2413 ± 0.0977A	0.4173 ± 0.1218B	0.1449 ± 0.0413B
Surface application Day-3	Runoff water	0.2989 ± 0.0535	0.0130 ± 0.0022	0.0129 ± 0.0006	0.1103 ± 0.0487
	Runoff sediment	0.0024 ± 0.0008	0.0009 ± 0.0004	0.0264 ± 0.0081	0.0033 ± 0.0010
	Total runoff	0.3014 ± 0.0543A	0.0139 ± 0.0024A	0.0393 ± 0.0087A	0.1136 ± 0.0496A
Subsurface injection Day-3	Runoff water	0.0699 ± 0.0149	0.0053 ± 0.0048	0.0021 ± 0.0016	0.0117 ± 0.0018
	Runoff sediment	0.0004 ± 0.0000	0.0003 ± 0.0000	0.0174 ± 0.0222	0.0014 ± 0.0008
	Total runoff	0.0703 ± 0.0149B	0.0056 ± 0.0047B	0.0195 ± 0.0238A	0.0131 ± 0.0023B

Surface application Day-7	Runoff water	0.2742 ± 0.1154	0.0155 ± 0.0064	0.0162 ± 0.0040	0.1792 ± 0.1041
	Runoff sediment	0.0047 ± 0.0022	0.0018 ± 0.0016	0.1004 ± 0.0748	0.0054 ± 0.0021
	Total runoff	0.2789 ± 0.1176A	0.0173 ± 0.0079A	0.1167 ± 0.0730A	0.1846 ± 0.1059A
Subsurface injection Day-7	Runoff water	0.0306 ± 0.0129	0.0010 ± 0.0009	0.0023 ± 0.0027	0.0063 ± 0.0060
	Runoff sediment	0.0001 ± 0.0001	0.0001 ± 0.0001	0.0022 ± 0.0017	0.0005 ± 0.0002
	Total runoff	0.0307 ± 0.0128B	0.0010 ± 0.0008B	0.0045 ± 0.0044B	0.0068 ± 0.0062B

Appendix L: Chi-square* and *p* values of the Wilcoxon / Kruskal-Wallis test showing the effect of manure application methods and manure-rainfall time gaps on the percent mass loss of antibiotics with surface runoff relative to initially applied to each treatment plot.

Pair comparison		Pirlimycin		Sulfamerazine		Chlortetracycline		Tylosin	
		Chi-square	p	Chi-square	p	Chi-square	p	Chi-square	p
Manure application methods									
Surface application vs. subsurface injection	Day-0	3.8571	0.0495	2.3333	0.1266	3.8571	0.0495	3.8571	0.0495
	Day-3	3.8571	0.0495	3.8571	0.0495	0.4286	0.5127	3.8571	0.0495
	Day -7	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495
Manure-rainfall time gaps									
Surface application	Day-0 vs. Day-3	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495
	Day-0 vs. Day-7	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495
	Day-3 vs. Day-7	0.4286	0.5127	0.0476	0.8273	1.1905	0.2752	1.1905	0.2752
Subsurface injection	Day-0 vs. Day-3	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495
	Day-0 vs. Day-7	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495	3.8571	0.0495
	Day-3 vs. Day-7	3.8571	0.0495	3.8571	0.0495	1.1905	0.2752	1.1905	0.2752

*Chi-square value = 3.8414 at 95 % confidence level and degree of freedom of 1. If the calculated Chi-square shown in the table is higher than this value (which means the p value is less than 0.05), it indicates a significant difference between two factors.

Appendix M: Soil sampling scheme

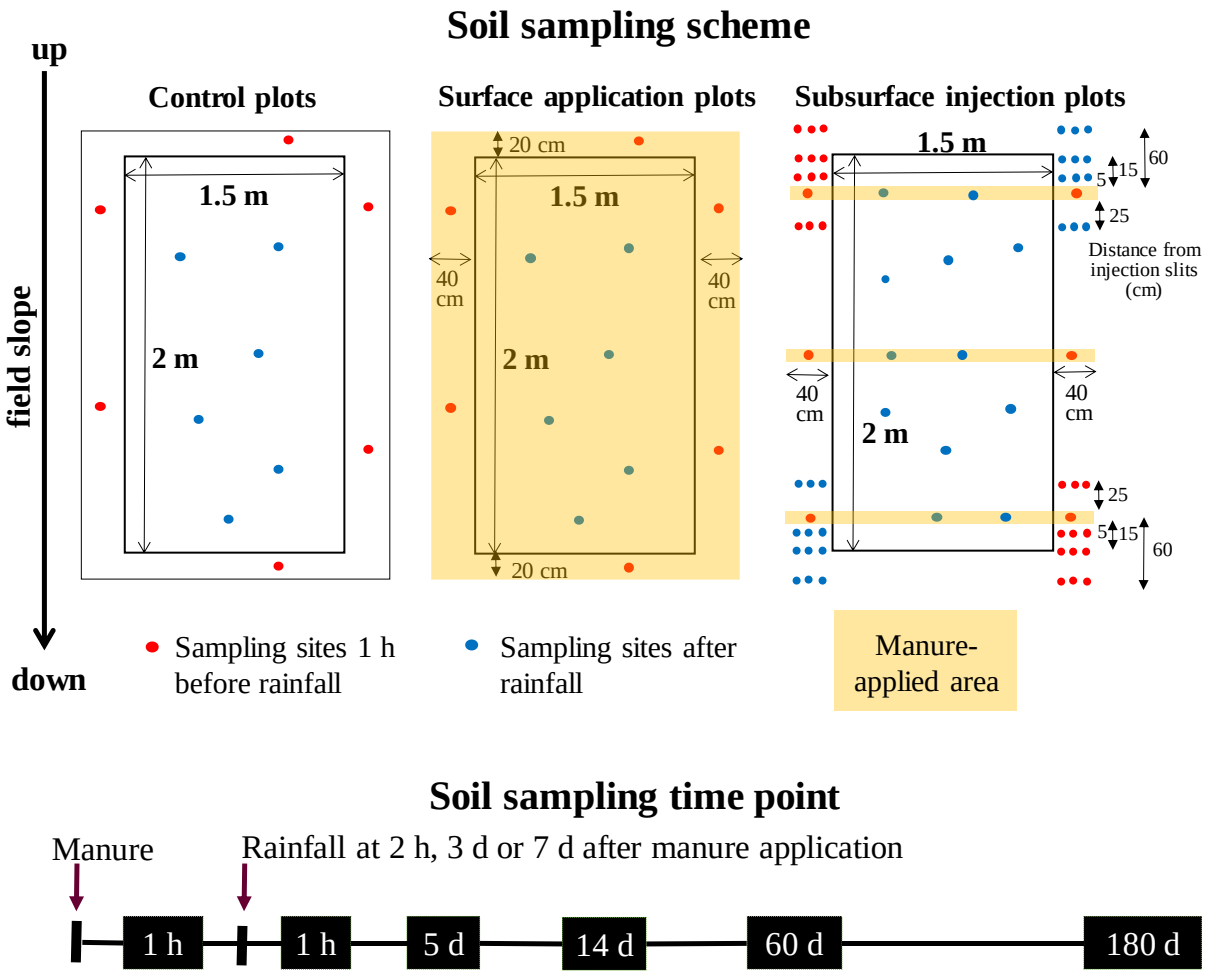


Figure M. Soil sampling scheme for control, surface application, and subsurface injection plots at 1 h after manure application and before simulated rainfall and different time points after the rain.

Appendix N: Method detection limit and recovery of antibiotics in manure-amended soil

Compound	Method detection limit (ng g^{-1} soil, dried weight)	Recovery (%)
Pirlimycin	0.02	92±7
Sulfamerazine	0.05	89±6
Tylosin	0.1	168±12
Chlortetracycline	0.5	81±10

Appendix O: Ratio between the concentration of an antibiotic in the 0-5-cm soil to that in the 5-20-cm soil from the injection slits of the manure subsurface injection plots

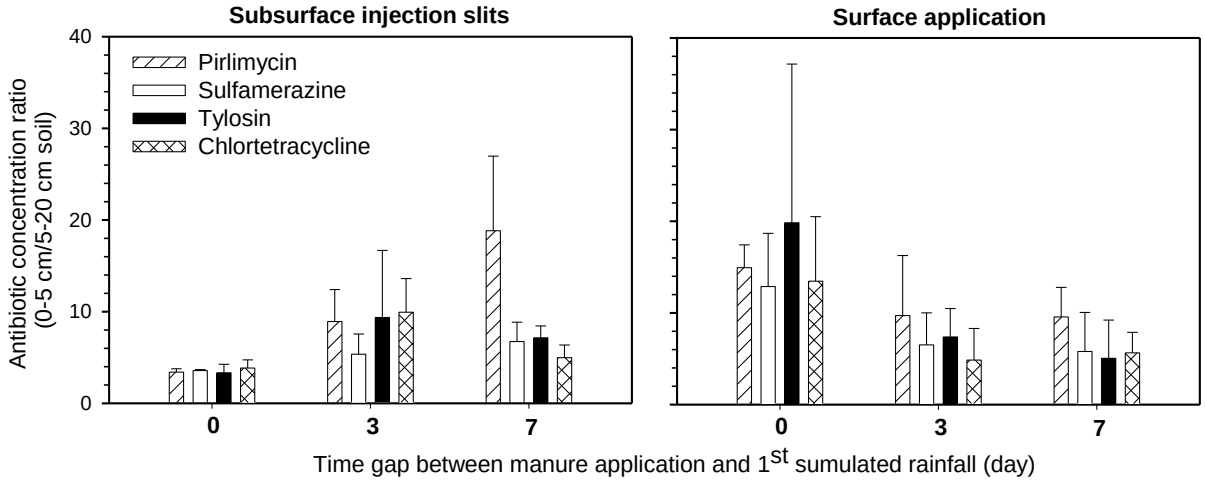


Figure O. Ratio between the concentration of an antibiotic in the 0-5-cm soil to that in the 5-20-cm soil from the injection slits of the manure subsurface injection plots on the day of manure application and the 3rd and 7th day of manure application but before the simulated rainfall.

Appendix P: *p* value comparing effect of manure application method on concentrations of antibiotics in soil from the surface application and in the injection slits following manure application and 1 h before the simulated rainfall.

Treatment	Pirlimycin	Sulfamerazine	Tylosin	Chlortetracycline
Manure application method	*p* value			
	0.0005	0.0013	0.0004	0.0003

Appendix Q: *p* value comparing effect of distance from the injection slits on concentrations of antibiotics in soil in the injection slits and 5, 20, and 60 cm from the injection slits following manure application and 1 h before the simulated rainfall.

Distance from the injection slits	Pirlimycin	Sulfamerazine	Tylosin	Chlortetracycline
	p value			
0 cm vs. 5 cm	0.0012	0.0498	0.0002	0.0004
0 cm vs. 20 cm	<0.0001	<0.0001	<0.0001	<0.0001
0 cm vs. 25 cm	<0.0001	<0.0001	<0.0001	<0.0001
0 cm vs. 60 cm	<0.0001	<0.0001	<0.0001	<0.0001
5 cm vs. 20 cm	0.1103	0.1868	0.4153	0.0092
5 cm vs. 25 cm	0.0187	0.0453	0.0186	0.0007
5 cm vs. 60 cm	0.0046	0.0028	0.0021	<0.0001
20 cm vs. 25 cm	0.9565	0.9710	0.6139	0.9286
20 cm vs. 60 cm	0.7732	0.5193	0.2199	0.5602
25 cm vs. 60 cm	0.9903	0.8766	0.9559	0.9550

Appendix R: Total mass of antibiotics in soil from the surface application treatment immediately before and after the simulated rainfall

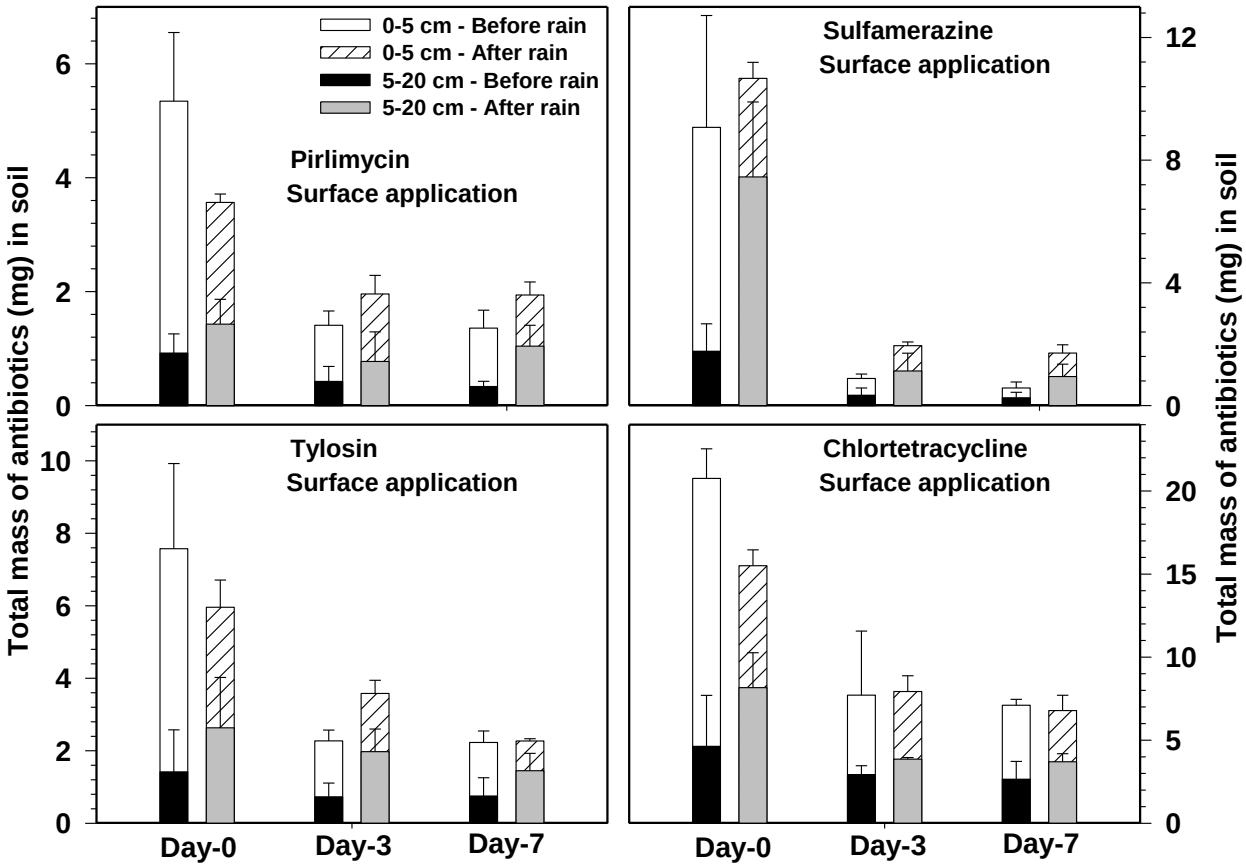


Figure R. Total mass of antibiotics (mg) in 0-5- and 5-20-cm soil from the surface application treatment immediately before and after the simulated rainfall with three manure-rainfall time gaps.

Appendix S: *p* value comparing effect of simulated rainfall on concentrations of antibiotics in soil from the surface application and in the injection slits at 1 h before and after the simulated rainfall.

Treatment	Manure application method	Manure-rainfall time gap	Pirlimycin		Sulfamerazine		Tylosin		Chlortetracycline	
			0-5 cm	5-20 cm	0-5 cm	5-20 cm	0-5 cm	5-20 cm	0-5 cm	5-20 cm
			p value							
Simulated rainfall (before vs. after)	Surface application	Day-0	0.0495	0.1266	0.1266	0.0495	0.1266	0.1266	0.0495	0.2683
		Day-3	0.2752	0.2752	0.2752	0.1266	0.8273	0.0495	0.8273	0.2752
		Day-7	0.5127	0.1266	0.1266	0.0495	0.0495	0.1266	0.0765	0.5127
	Subsurface injection	Day-0	0.0495	0.1266	0.0495	0.0495	0.0495	0.0495	0.5127	0.0495
		Day-3	0.0495	0.1266	0.0495	0.1266	0.0495	0.2752	0.2752	0.8273
		Day-7	0.5127	0.0495	0.8273	0.5127	0.5127	0.5127	0.5127	0.2752

Appendix T: *p* value comparing effect of simulated rainfall on concentrations of antibiotics in soil in the injection slits and 5, 20, and 60 cm from the injection slits at 1 h before and after the simulated rainfall.

Manure-rainfall time gap	Distance from injection slits (cm)	Pirlimycin		Sulfamerazine		Tylosin		Chlortetracycline	
		0-5 cm	5-20 cm	0-5 cm	5-20 cm	0-5 cm	5-20 cm	0-5 cm	5-20 cm
		p value							
Day-0	0	0.0495	0.1266	0.0495	0.0495	0.0495	0.0495	0.5127	0.0495
	5	0.8273	0.0495	0.5127	0.8273	0.2752	0.8273	0.2752	0.5127
	20	0.5127	0.1266	0.5127	0.0463	0.2752	0.0495	0.8273	0.5127
	25	0.1266	0.0339	0.2752	0.0495	0.8273	0.0369	0.5127	0.5066
	60	0.0369	0.0369	0.0253	0.0369	0.0369	0.0495	n/a	n/a
Day-3	0	0.0495	0.1266	0.0495	0.1266	0.0495	0.2752	0.2752	0.8273
	5	0.1266	0.0495	0.5127	0.1266	0.5127	0.0495	0.2752	0.0495
	20	0.3758	0.0369	0.5127	0.3758	0.1840	0.0369	0.1266	0.0369
	25	0.0369	0.0339	0.3458	0.8222	0.0369	0.0369	0.0369	0.0369
	60	n/a	n/a	0.0369	0.0369	n/a	n/a	n/a	n/a
Day-7	0	0.5127	0.0495	0.8273	0.5127	0.5127	0.5127	0.5127	0.2752
	5	0.8273	0.0495	0.0495	0.5127	0.5127	0.0495	0.0495	0.1266
	20	0.0495	0.2683	0.0495	0.2752	0.8273	0.0495	0.5127	0.1266
	25	0.6531	0.0463	0.8273	0.5002	0.0369	0.0369	0.5066	0.0369
	60	0.0339	0.0369	0.0339	0.0369	n/a	0.0369	0.0253	0.0369

n/a: concentrations of antibiotics were below the detection limit at both 1 h before and after the simulated rainfall.

Appendix U: Total mass of antibiotics in soil from the subsurface injection treatment, including soil inside the injection slits and at 5, 20, 25, and 60 cm from the injection slits immediately before and after the simulated rainfall

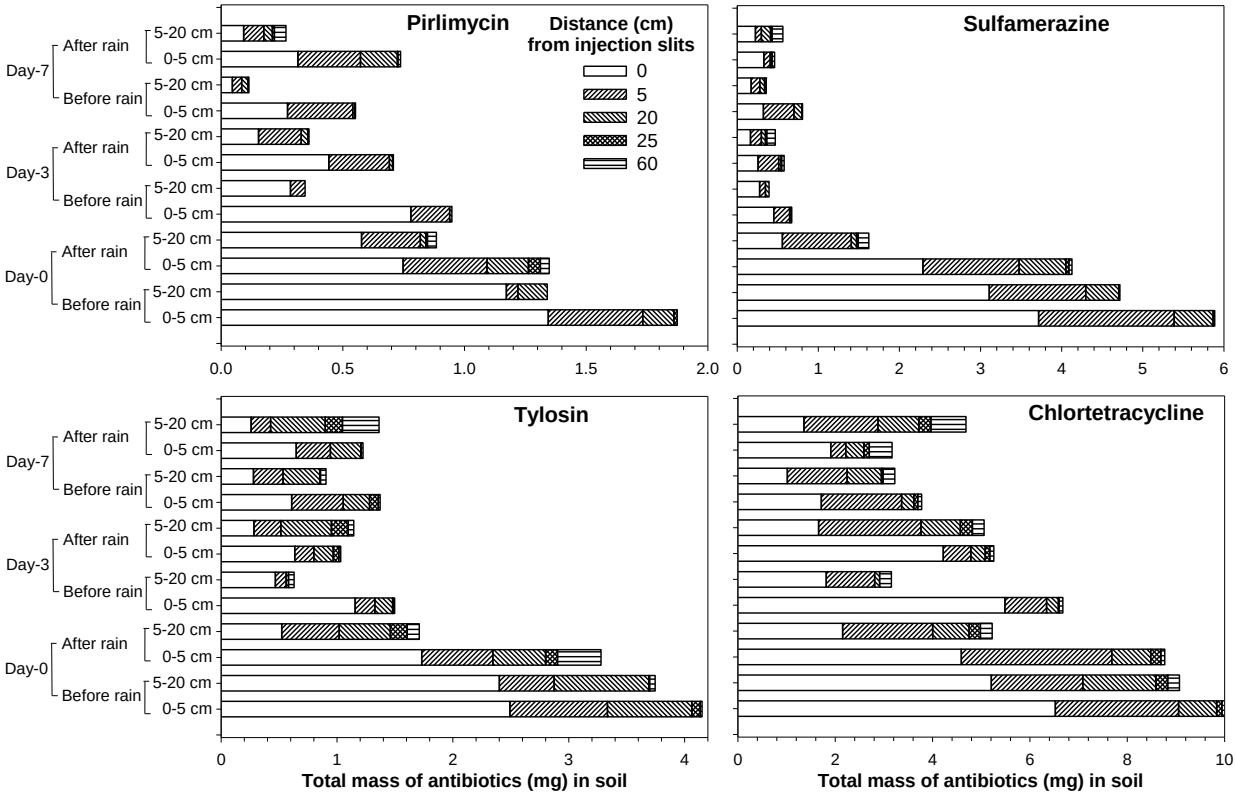


Figure U. Total mass of antibiotics (mg) in 0-5- and 5-20-cm soil from the subsurface injection treatment, including soil inside the injection slits and at 5, 20, 25, and 60 cm from the injection slits immediately before and after the simulated rainfall with three manure-rainfall time gaps.

Appendix V: *p* value comparing effect of distance from the injection slits on concentrations of antibiotics in soil in the injection slits and 5, 20, and 60 cm from the injection slits at 1 h after the simulated rainfall.

Distance from the injection slits	Pirlimycin	Sulfamerazine	Tylosin	Chlortetracycline
	p value			
0 cm vs. 5 cm	0.0077	0.0498	0.0014	<0.0001
0 cm vs. 20 cm	<0.0001	<0.0001	<0.0001	<0.0001
0 cm vs. 25 cm	<0.0001	<0.0001	<0.0001	<0.0001
0 cm vs. 60 cm	<0.0001	<0.0001	<0.0001	<0.0001

Appendix W: Concentration of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in soil from the surface application treatment and the injection slits of the subsurface injection treatment at 1 h, 3 d and 7 d after manure application

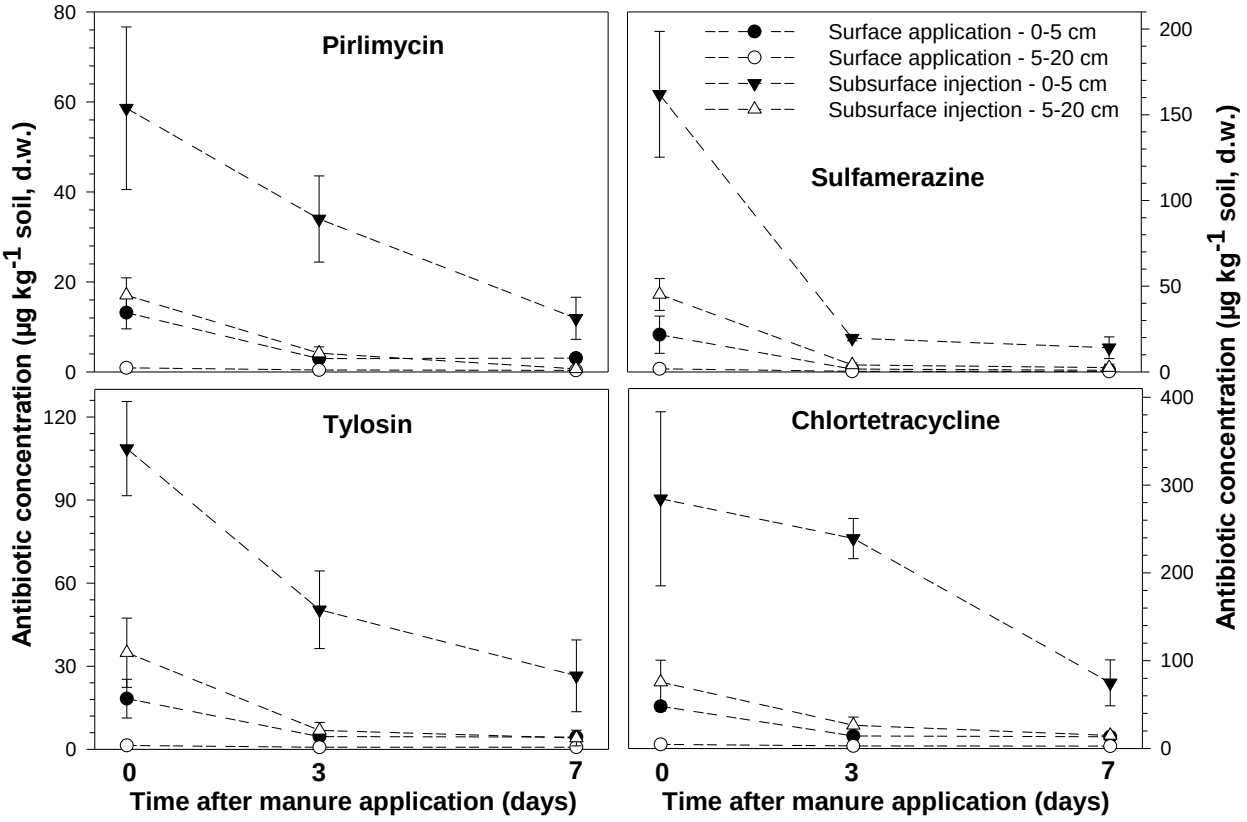


Figure W. Concentration of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in 0-5- and 5-20-cm soil (µg kg^{-1}, dried weight) from the surface application treatment and the injection slits of the subsurface injection treatment at 1 h, 3 d and 7 d after manure application.

Appendix X: Change in antibiotic concentrations in soil collected in between injection slits from 1 h to 180 d after the simulated rainfall

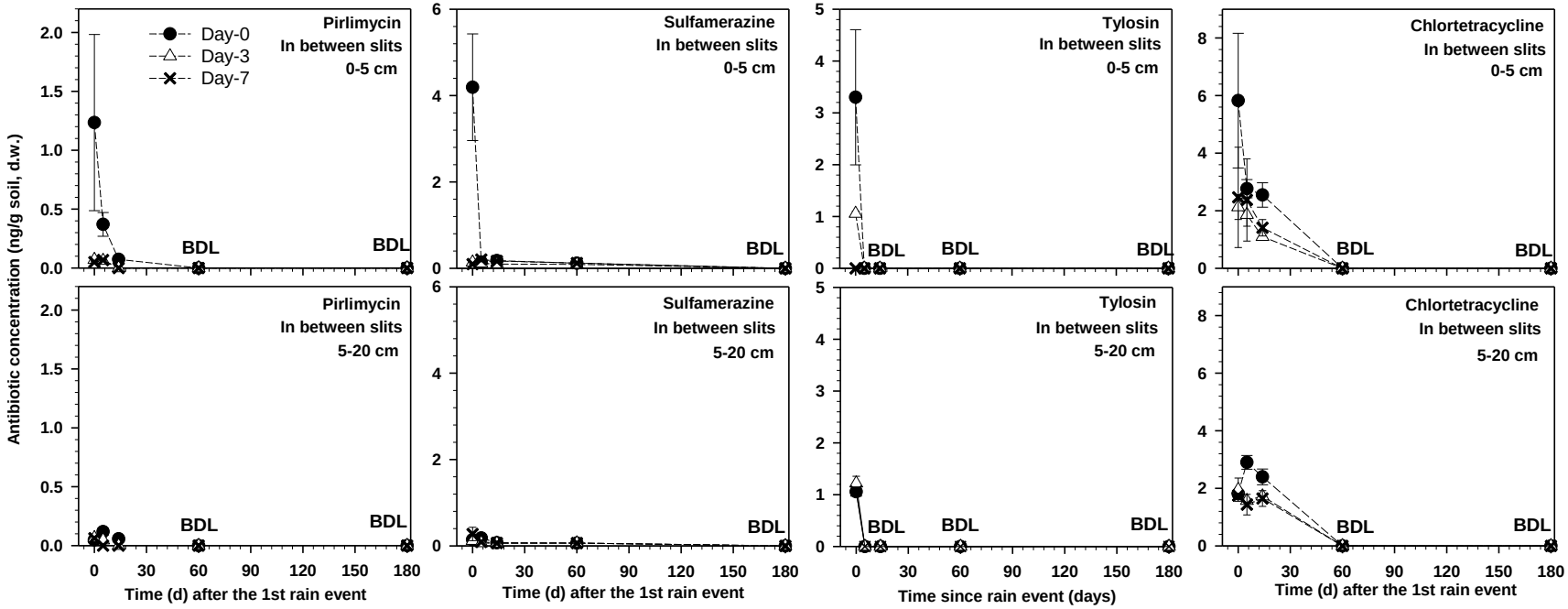


Figure X. Change in antibiotic concentrations in 0-5- and 5-20-cm soil collected in between injection slits from 1 h to 180 d after the simulated rainfall for pirlimycin, sulfamerazine, tylosin, and chlortetracycline with three manure-rainfall time gaps.

Appendix Y: *p* value comparing effect of the manure application method on change of antibiotic concentrations in soil from the surface application and in the injection slits of the subsurface injection over the 6-month period.

Treatment	Antibiotic	Manure-rainfall time gap	0-5 cm	5-20 cm
			p value	
Manure application method (surface application vs. subsurface injection)	Pirlimycin	Day-0	0.3706	0.5123
		Day-3	0.0429	0.0011
		Day-7	0.0376	0.9826
	Sulfamerazine	Day-0	0.0005	0.0022
		Day-3	0.9137	0.0920
		Day-7	0.0301	0.1290
	Tylosin	Day-0	0.0887	0.0023
		Day-3	0.4470	0.0184
		Day-7	0.0215	0.2567
	Chlortetracycline	Day-0	0.2089	<0.0001
		Day-3	0.2025	<0.0001
		Day-7	0.0057	<0.0001

Appendix Z: *p* value comparing effect of soil depth on change of antibiotic concentrations in soil from the surface application and in the subsurface injection slits over the 6-month period.

Treatment	Antibiotic	Manure application method	Day-0	Day-3	Day-7
			p value		
Soil depth (0-5 vs. 5-20 cm)	Pirlimycin	Surface application	0.7319	0.0003	0.1186
		Subsurface injection	0.1743	0.2088	0.2717
	Sulfamerazine	Surface application	0.0014	<0.0001	0.7361
		Subsurface injection	0.0026	0.0049	<0.0001
	Tylosin	Surface application	0.0252	0.2814	<0.0001
		Subsurface injection	0.0005	0.0953	0.5045
	Chlortetracycline	Surface application	0.0004	<0.0001	<0.0001
		Subsurface injection	0.9417	0.6806	0.0041

Appendix AA: Dissipation of pirlimycin, sulfamerazine, tylosin, and chlortetracycline (Ct/C0) in soil from the surface application treatment and the injection slits of the subsurface injection treatment during 180 d after the 1st rainfall conducted at 3 d after manure application.

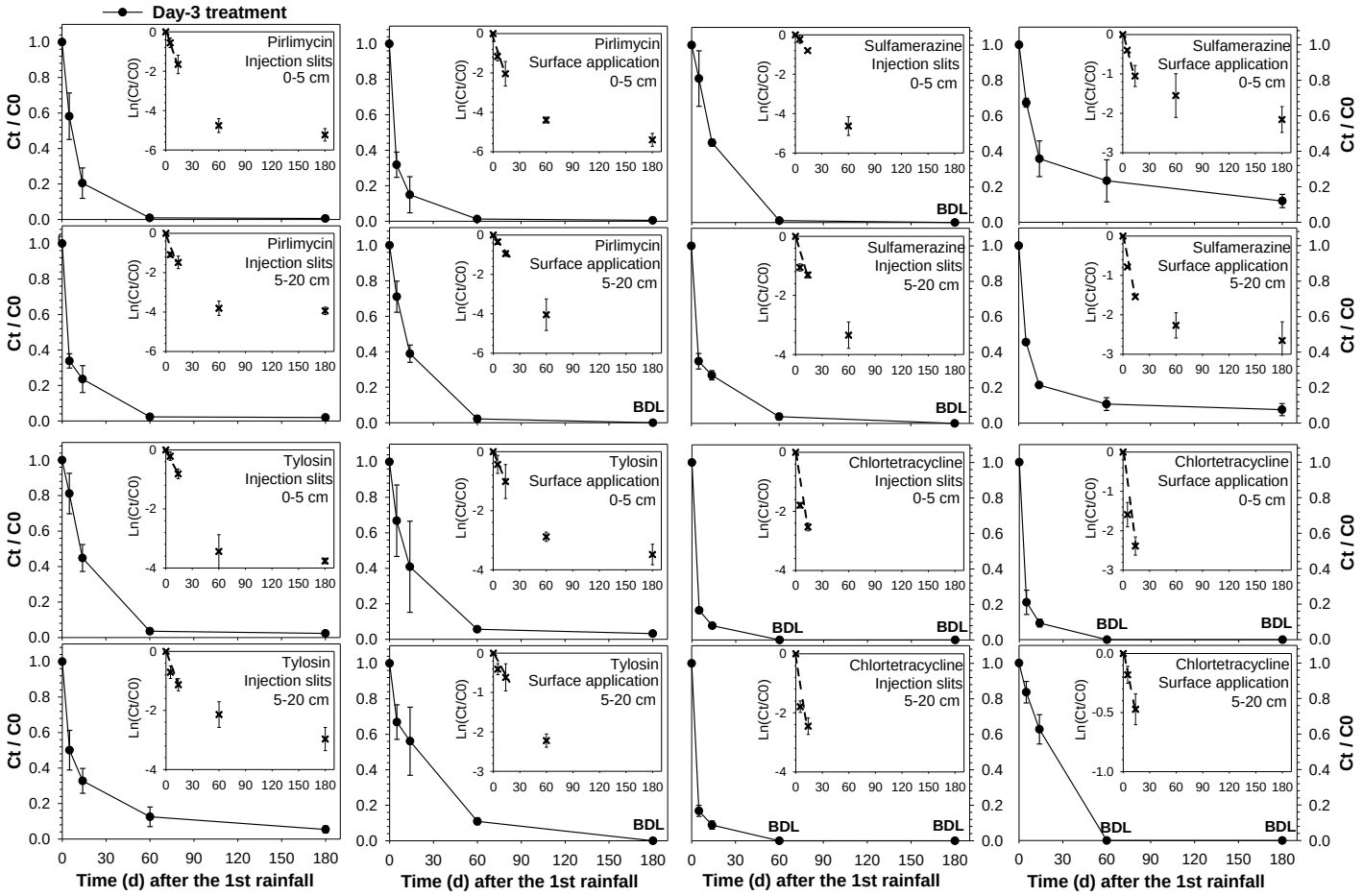


Figure AA. Concentrations of pirlimycin, sulfamerazine, tylosin, and chlortetracycline in 0-5- and 5-20-cm soil (μg kg^{-1}, dried weight) at time t over their initial concentrations (Ct/C0) from the surface application treatment and the injection slits of the subsurface injection treatment during 180 d after the 1st rainfall conducted at 3 d after manure application.

Appendix AB: Antibiotic half-life (day) in soil and coefficient of determination (R^2) of a linear regression.

Treatment			Pirlimycin		Sulfamerazine		Tylosin		Chlortetracycline	
			$t_{1/2}$ (d)	R^2	$t_{1/2}$ (d)	R^2	$t_{1/2}$ (d)	R^2	$t_{1/2}$ (d)	R^2
Injection slit	Day-0	0-5 cm	4	0.93-1.00	6	0.73-0.80	5	0.97-1.00	4	0.69-0.97
		5-20 cm	3	0.98	5	0.81-0.98	10	0.97-1.00	4	0.71-0.92
	Day-3	0-5 cm	6	0.98-1.00	9	0.99	12	0.90-1.00	3	0.77-0.80
		5-20 cm	8	0.81-0.90	6	0.55-0.86	8	0.79-0.96	4	0.55-0.87
	Day-7	0-5 cm	4	0.96-1.00	8	0.99-1.00	6	0.92-0.99	5	0.99-1.00
		5-20 cm	4	0.90-0.99	4	0.84-0.95	5	0.91-0.98	3	0.64-0.99
Surface application	Day-0	0-5 cm	5	0.90-1.00	5	0.67-0.99	5	0.83-0.95	5	0.53-0.92
		5-20 cm	4	0.94-1.00	3	0.66-0.86	6	0.98-0.99	14	0.68-0.92
	Day-3	0-5 cm	6	0.88-1.00	10	0.97-1.00	10	0.93-1.00	4	0.62-0.96
		5-20 cm	11	0.99-1.00	6	0.96	11	0.70-0.98	21	0.79-1.00
	Day-7	0-5 cm	4	0.76-0.89	5	0.70-0.77	7	0.69-0.85	3	0.57-0.80
		5-20 cm	4	0.86-0.98	6	0.63-0.98	5	0.88-0.94	14	0.75-1.00

Appendix AC: Physical properties of surface runoff for surface application and subsurface injection treatments receiving liquid raw manure spiked with pirlimycin

Season	Treatment	Time to start runoff (min)	Weight of collected surface runoff (kg)	Total runoff sediment load (g)
Fall	Surface application	7.33 ± 2.91	46.48 ± 6.73	26.32 ± 19.17
	Subsurface injection	8.74 ± 2.32	32.49 ± 9.82	30.33 ± 7.45
Spring	Surface application	4.76 ± 1.12	66.06 ± 25.78	97.76 ± 11.25
	Subsurface injection	23.15 ± 9.59	31.43 ± 15.06	53.95 ± 47.29

Appendix AD: Change in concentrations of pirlimycin in soil from the surface application and in the injection slits of the subsurface injection following manure application in fall and spring

Day	0-5 cm				5-20 cm			
	Fall		Spring		Fall		Spring	
	Surface application	Injection slits	Surface application	Injection slits	Surface application	Injection slits	Surface application	Injection slits
	% of initial concentration (Ct/C0, Mean ± SD)							
5	44.7 ± 4.3	59.0 ± 13.9	93.4 ± 3.5	76.7 ± 7.4	39.2 ± 5.7	42.0 ± 17.3	18.3 ± 5	62.8 ± 20.8
14	12.5± 8.2	9.1 ± 1.3	15.5 ± 4.0	24.4 ± 7.2	11.2 ± 6.6	5.8 ± 2.5	15.2 ± 9.1	29.7 ± 8.1
60	1.5 ± 0.2	0.9 ± 0.1	2.4 ± 0.3	0.3 ± 0.1	5.4 ± 1.3	1.9 ± 0.2	n/a	1.0 ± 0.1
150	0.6 ± 0.1	0.2 ± 0.0	n/a	0.1 ± 0.0	n/a	0.4± 0.1	n/a	0.3 ± 0.1

			p value
Application method	Fall	0-5 cm	0.3706
		5-20 cm	0.5523
	Spring	0-5 cm	0.3530
		5-20 cm	0.0177
Application season	Surface application	0-5 cm	0.0053
		5-20 cm	0.0619
	Subsurface injection	0-5 cm	0.0048
		5-20 cm	0.0124
Soil depth	Surface application	Fall	0.7276
		Spring	0.0262
	Subsurface injection	Fall	0.1743
		Spring	0.5977

n/a: the pirlimyicin concentration in soil samples was below the detection limit.

Appendix AE: Soil temperature and moisture content of soil samples from the surface application treatments and the subsurface injection slits from Sep 2015 to Oct 2016

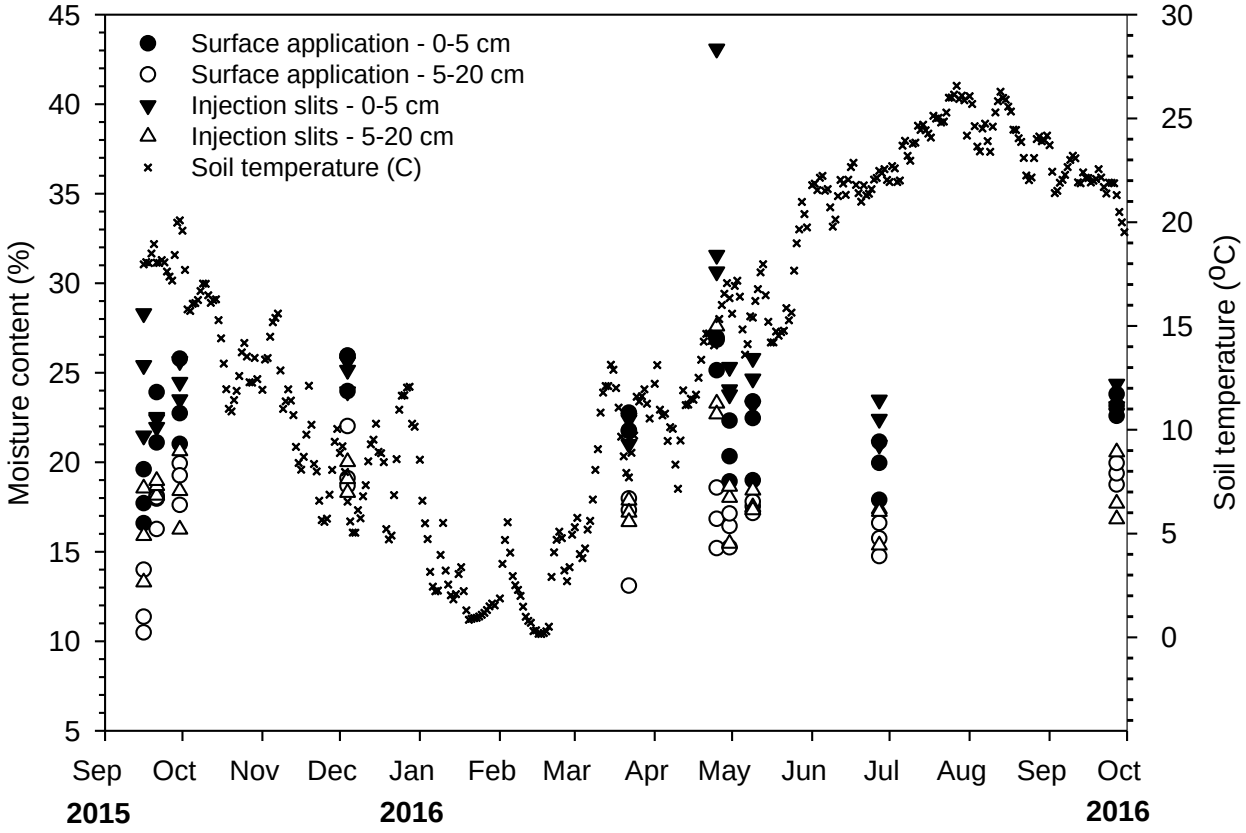


Figure AE. Soil temperature (°C) and moisture content (%) of soil samples in 0-5 and 5-20 cm from the surface application treatments and the subsurface injection slits from Sep 2015 to Oct 2016. The fall application season started at 26 September 2015 and the spring application season started at 24 April 2016.

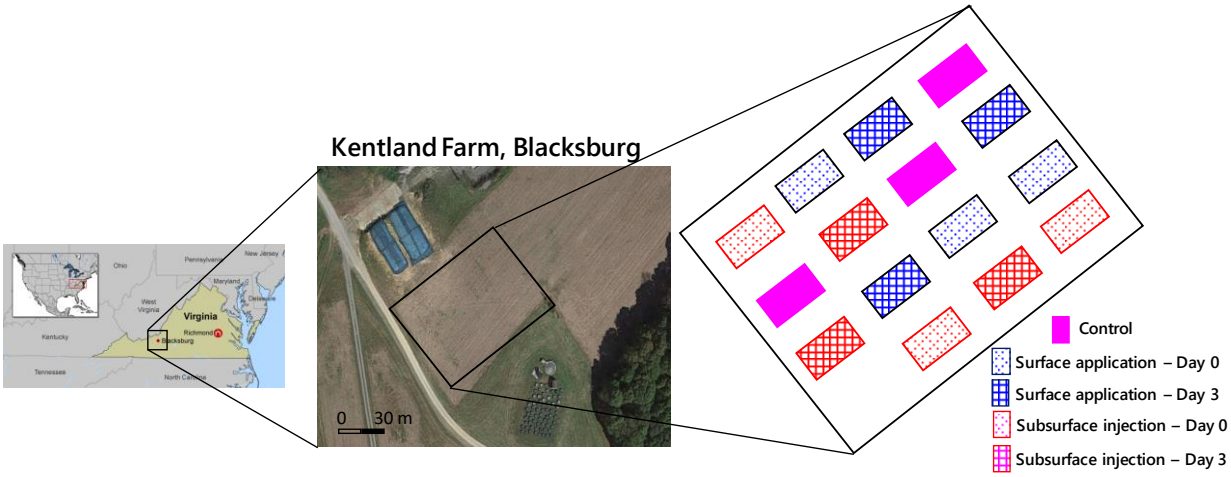
Appendix AF: Physical properties of surface runoff for all treatments receiving amendment via subsurface injection.

Treatment	Time to start runoff (min)	Weight of collected surface runoff (kg)	Total runoff sediment load (g)
Raw manure – Control	7.87 ± 4.57	45.25 ± 8.11	101.46 ± 83.65
Composted manure – Control	10.67 ± 5.12	38.21 ± 8.18	118.57 ± 53.11
Composted manure – Cow fed	14.56 ± 9.25	48.15 ± 23.18	156.66 ± 189.14
Raw manure – Cow fed	7.54 ± 3.62	43.52 ± 29.22	140.97 ± 112.48
Raw manure – Spiked	23.15 ± 9.59	31.43 ± 15.06	57.21 ± 46.06
Composted manure – Spiked	18.65 ± 11.00	25.32 ± 19.58	46.02 ± 39.90

Appendix AG: Dissipation rate constant (k), coefficient of determination (R^2) of a linear regression and half-life of pirlimycin in soil. Different letters showed a significant difference in pirlimycin half-life.

Treatment	Soil depth	k (d^{-1})	R^2	$t_{1/2}$ (day)
Raw manure – Cow fed	0-5 cm	0.04 ± 0.00	0.83 ± 0.02	17.48 ± 0.71a
	5-20 cm	0.04 ± 0.00	0.87 ± 0.06	18.76 ± 1.69a
Raw manure – Spiked	0-5 cm	0.10 ± 0.02	0.95 ± 0.01	7.37 ± 1.58b
	5-20 cm	0.09 ± 0.03	0.95 ± 0.05	8.07 ± 2.02b
Composted manure – Spiked	0-5 cm	0.16 ± 0.04	0.99 ± 0.01	4.46 ± 1.05c
	5-20 cm	0.23 ± 0.02	0.79 ± 0.05	3.07 ± 0.21c

Appendix AH: Field site and test-plot arrangement



Appendix AI: Primers/Probe

Target gene	Primer	Primer / Probe sequence (5'-3')	Annealing temperature (°C)	Size (bp)	Reference
16S rRNA	EUB338	ACTCCTACGGGAGGCAGCAG	55	180	(Fierer et al., 2005)
	EUB518	ATTACCGCGGCTGCTGG			
Rum-2-bac	BacB2 590F	ACAGCCCGCGATTGATACTGGTAA	60	99	(Mieszkin et al., 2010; Oladeinde et al., 2014)
	Bac708 Rm	CAATCGGAGTTCTTCGTGAT			
	BacB2-626P	(FAM)ATGAGGTGGATGGAATTCGTGGTGT-TAMRA			
Intl1	HS463a	CTGGATTTCGATCACGGCACG	66	473	(Hardwick et al., 2008)
	HS464	ACATGCGTGTAAATCATCGTCG			

Appendix AJ: Antibiotic concentrations in soils

Manure application method	Manure-rain time gap (day)	Soil depth (cm)		Before rain				After rain			
				PLY (ng g^{-1})	SMR (ng g^{-1})	TYL (ng g^{-1})	CTC (ng g^{-1})	PLY (ng g^{-1})	SMR (ng g^{-1})	TYL (ng g^{-1})	CTC (ng g^{-1})
Surface application	0	0-5		13.1	21.7	18.3	47.9	6.35	9.55	9.88	21.8
		5-20		0.91	1.75	1.41	4.59	1.42	7.39	2.61	6.87
	3	0-5		2.93	1.63	4.58	14.2	3.52	2.45	4.77	12.1
		5-20		0.42	0.33	0.73	2.90	0.77	1.11	1.96	3.43
Subsurface injection	0	Injection slit	0-5	58.6	161.9	108.6	278.8	32.6	99.8	75.4	205.7
			5-20	17.0	45.1	34.9	75.7	8.38	8.10	6.24	31.29
		5 cm from injection slit	0-5	8.47	36.4	18.4	55.2	7.51	25.8	13.38	67.4
			5-20	0.34	8.64	3.43	13.7	1.76	6.17	3.60	13.4
		25 cm from injection slit	0-5	0.93	3.48	5.30	5.68	1.23	4.19	3.30	5.82
			5-20	0.29	0.98	1.98	3.64	0.05	0.16	1.07	1.81
	3	Injection slit	0-5	34.0	19.7	50.4	239.1	19.3	11.2	27.7	184.0
			5-20	4.14	4.03	6.80	26.4	2.23	2.39	4.12	24.1
		5 cm from injection slit	0-5	3.47	4.27	3.72	18.7	5.43	5.55	3.58	12.33
			5-20	0.44	0.52	0.67	7.22	1.27	0.97	1.69	15.30
		25 cm from injection slit	0-5	0.07	0.15	1.11	1.75	0.10	0.21	1.22	2.13
			5-20	0.01	0.11	0.05	0.25	0.04	0.12	1.05	1.81

Appendix AK: Absolute and relative abundance of Rum-2-bac and intl1 in soils collected from the manure surface-applied plots and in the manure injection slit.

Manure application-rain time gap (day)	Soil depth (cm)	Relative to rain	Rum-2-bac – absolute abundance			Rum-2-bac/16S – relative abundance		
			Injection slit	surface application plots	p value (t-test)	Injection slit	surface application plots	p value (t-test)
0	0-5	1 h before	2.2E+06	3.3E+05	0.002	8.2E-05	1.4E-05	0.001
		1 h after	1.3E+06	1.8E+05	0.001	8.2E-05	8.9E-06	0.001
	5-20	1 h before	3.4E+05	1.3E+04	0.008	3.9E-05	1.6E-06	0.021
		1 h after	1.1E+05	4.7E+04	0.054	4.4E-05	7.6E-06	0.002
3	0-5	1 h before	3.5E+05	1.6E+05	0.023	1.0E-04	3.2E-05	0.001
		1 h after	2.1E+05	1.3E+05	0.101	5.1E-05	2.4E-05	0.004
	5-20	1 h before	2.8E+04	2.4E+04	0.667	1.8E-05	2.1E-05	0.595
		1 h after	2.2E+04	1.2E+04	0.107	2.9E-05	1.0E-05	0.014
			intI1 – absolute abundance			intI1/16S – relative abundance		
			Injection slit	surface application plots	p value (t-test)	Injection slit	surface application plots	p value (t-test)
0	0-5	1 h before	3.8E+07	9.5E+06	0.007	1.4E-03	3.9E-04	0.007
		1 h after	2.8E+07	5.9E+06	0.013	1.8E-03	2.9E-04	0.002
	5-20	1 h before	1.4E+07	7.2E+06	0.067	1.6E-03	8.9E-04	0.104
		1 h after	1.6E+07	8.5E+06	0.012	6.1E-03	1.4E-03	0.001
3	0-5	1 h before	4.6E+07	3.3E+06	<0.001	1.4E-02	6.6E-04	<0.001
		1 h after	4.5E+07	2.9E+06	<0.001	1.1E-02	5.5E-04	<0.001
	5-20	1 h before	7.6E+06	1.1E+06	0.004	4.8E-03	9.6E-04	<0.001
		1 h after	1.3E+07	9.9E+05	<0.001	1.7E-02	8.6E-04	<0.001

Appendix AL: Absolute and relative abundance of Rum-2-bac and intl1 in soils collected from 1 hour before rain and 1 hour after the simulated rain.

Manure application methods	Manure application-rain time gap (day)	Soil depth (cm)		Rum-2-bac – absolute abundance			Rum-2-bac/16S – relative abundance		
				1 h before rain	1 h after rain	p value (t-test)	1 h before rain	1 h after rain	p value (t-test)
Surface application	0	0-5		3.3E+05	1.8E+05	0.092	1.4E-05	8.9E-06	0.063
		5-20		1.3E+04	4.7E+04	0.088	1.6E-06	7.6E-06	0.047
	3	0-5		1.6E+05	1.3E+05	0.466	3.2E-05	2.4E-05	0.157
		5-20		2.4E+04	1.2E+04	0.036	2.1E-05	1.0E-05	0.102
Subsurface injection	0	Injection slit	0-5	2.2E+06	1.3E+06	0.033	8.2E-05	8.2E-05	0.982
			5-20	3.4E+05	1.1E+05	0.076	3.9E-05	4.4E-05	0.878
		5 cm from injection slit	0-5	5.8E+05	5.3E+05	0.852	2.1E-05	2.6E-05	0.617
			5-20	1.2E+05	9.2E+04	0.461	1.9E-05	1.6E-05	0.744
		25 cm from injection the slit	0-5	1.9E+05	2.4E+04	0.121	6.9E-06	1.6E-06	0.186
			5-20	1.8E+04	4.7E+03	0.148	2.0E-06	1.4E-06	0.665
	3	Injection slit	0-5	3.5E+05	2.1E+05	0.052	1.0E-04	5.1E-05	0.002
			5-20	2.8E+04	2.2E+04	0.535	1.8E-05	2.9E-05	0.062
		5 cm from injection slit	0-5	3.3E+04	6.6E+04	0.206	1.2E-05	2.1E-05	0.376
			5-20	4.1E+03	1.1E+04	0.081	2.9E-06	1.1E-05	0.013
		25 cm from injection the slit	0-5	2.8E+03	4.3E+03	0.360	6.1E-07	1.3E-06	0.088
			5-20	4.1E+03	2.5E+03	<0.001	3.0E-06	2.0E-06	0.034
				intI1 – absolute abundance			intI1/16S – relative abundance		
				1 h before rain	1 h after rain	p value (t-test)	1 h before rain	1 h after rain	p value (t-test)
Surface application	0	0-5		9.5E+06	5.9E+06	0.179	3.9E-04	2.9E-04	0.138
		5-20		7.2E+06	8.5E+06	0.336	8.9E-04	1.4E-03	0.118

	3	0-5		3.3E+06	2.9E+06	0.562	6.6E-04	5.5E-04	0.206
		5-20		1.1E+06	9.9E+05	0.595	9.6E-04	8.6E-04	0.557
Subsurface injection	0	Injection slit	0-5	3.8E+07	2.8E+07	0.405	1.4E-03	1.8E-03	0.482
			5-20	1.4E+07	1.6E+07	0.622	1.6E-03	6.1E-03	0.005
		5 cm from injection slit	0-5	3.2E+07	1.7E+07	0.098	1.1E-03	8.5E-04	0.537
			5-20	1.3E+07	1.1E+07	0.733	2.0E-03	2.0E-03	0.888
		25 cm from injection the slit	0-5	2.7E+07	1.4E+07	0.295	1.0E-03	9.2E-04	0.894
			5-20	2.5E+07	3.5E+06	0.078	2.7E-03	1.1E-03	0.353
	3	Injection slit	0-5	4.6E+07	4.5E+07	0.906	1.4E-02	1.1E-02	0.331
			5-20	7.6E+06	1.3E+07	0.196	4.8E-03	1.7E-02	0.001
		5 cm from injection slit	0-5	7.1E+06	3.2E+07	0.004	2.6E-03	9.9E-03	0.014
			5-20	8.2E+06	1.4E+07	0.148	5.8E-03	1.4E-02	0.009
		25 cm from injection the slit	0-5	3.0E+06	3.0E+07	0.001	6.6E-04	8.7E-03	0.001
			5-20	7.7E+06	1.4E+07	0.123	5.5E-03	1.2E-02	0.065

Appendix AM: Absolute and relative abundance of Rum-2-bac and intl1 in soils collected from 0-5 cm and 5-20 cm.

Manure application methods	Manure application-rain time gap (day)	Relative to rain		Rum-2-bac – absolute abundance			Rum-2-bac/16S – relative abundance		
				0-5 cm	5-20 cm	p value (t-test)	0-5 cm	5-20 cm	p value (t-test)
Surface application	0	1 h before		3.3E+05	1.3E+04	0.005	1.4E-05	1.6E-06	0.015
		1 h after		1.8E+05	4.7E+04	0.008	8.9E-06	7.6E-06	0.535
	3	1 h before		1.6E+05	2.4E+04	0.001	3.2E-05	2.1E-05	0.267
		1 h after		1.3E+05	1.2E+04	0.001	2.4E-05	1.0E-05	0.014
Subsurface injection	0	1 h before	Injection slit	2.2E+06	3.4E+05	0.010	8.2E-05	3.9E-05	0.354
			5 cm from the slit	5.8E+05	1.2E+05	0.052	2.1E-05	1.9E-05	0.870
			25 cm from injection slit	1.9E+05	1.8E+04	0.061	6.9E-06	2.0E-06	0.223
		1 h after	Injection slit	1.3E+06	1.1E+05	0.001	8.2E-05	4.4E-05	0.047
			5 cm from the slit	5.3E+05	9.2E+04	<0.001	2.6E-05	1.6E-05	0.110
			25 cm from injection slit	2.4E+04	4.7E+03	0.156	1.6E-06	1.4E-06	0.880
	3	1 h before	Injection slit	3.5E+05	2.8E+04	0.001	1.0E-04	1.8E-05	<0.001
			5 cm from the slit	3.3E+04	4.1E+03	0.008	1.2E-05	2.9E-06	0.058
			25 cm from injection slit	2.8E+03	4.1E+03	0.220	6.1E-07	3.0E-06	0.001
		1 h after	Injection slit	2.1E+05	2.2E+04	0.001	5.1E-05	2.9E-05	0.039
			5 cm from the slit	6.6E+04	1.1E+04	0.018	2.1E-05	1.1E-05	0.127
			25 cm from injection slit	4.3E+03	2.5E+03	0.142	1.3E-06	2.0E-06	0.189
				intI1 – absolute abundance			intI1/16S – relative abundance		
				0-5 cm	5-20 cm	p value (t-test)	0-5 cm	5-20 cm	p value (t-test)
Surface application	0	Before rain		9.5E+06	7.2E+06	0.241	3.9E-04	8.9E-04	0.014
		After rain		5.9E+06	8.5E+06	0.233	2.9E-04	1.4E-03	0.001

	3	Before rain		3.3E+06	1.1E+06	0.002	6.6E-04	9.6E-04	0.044
		After rain		2.9E+06	9.9E+05	0.008	5.5E-04	8.6E-04	0.07
Subsurface injection	0	1 h before	In the slit	3.8E+07	1.4E+07	0.037	1.4E-03	1.6E-03	0.705
			5 cm from the slit	3.2E+07	1.3E+07	0.081	1.1E-03	2.0E-03	0.081
			25 cm from the slit	2.7E+07	2.5E+07	0.890	1.0E-03	2.7E-03	0.041
		1 h after	In the slit	2.8E+07	1.6E+07	0.134	1.8E-03	6.1E-03	0.006
			5 cm from the slit	1.7E+07	1.1E+07	0.233	8.5E-04	2.0E-03	0.133
			25 cm from the slit	1.4E+07	3.5E+06	0.168	9.2E-04	1.1E-03	0.903
	3	1 h before	In the slit	4.6E+07	7.6E+06	0.005	1.4E-02	4.8E-03	0.002
			5 cm from the slit	7.1E+06	8.2E+06	0.517	2.6E-03	5.8E-03	0.063
			25 cm from the slit	3.0E+06	7.7E+06	0.062	6.6E-04	5.5E-03	0.004
		1 h after	In the slit	4.5E+07	1.3E+07	0.003	1.1E-02	1.7E-02	0.071
			5 cm from the slit	3.2E+07	1.4E+07	0.059	9.9E-03	1.4E-02	0.147
			25 cm from the slit	3.0E+07	1.4E+07	0.020	8.7E-03	1.2E-02	0.323

Appendix AN: Abundance of Rum-2-bac and inIt1 in soils at different distances away from the slit. Top values are absolute abundance and bottom values are relative abundance. Significant letters are labelled according to multiple student's t-test with means in the order of A>B>C>D and levels not connected by same letter are significantly different.

Rum-2-bac [Rum-2-bac/16S]						
Time gap	Rain	Depth	Mean [in the slit	Mean [5 cm away from the slit]	Mean [25 cm away from the slit]	Mean [Control soil]
Day 0	Before rain	0-5 cm	2.2E06 A [8.2E-05 A]	5.8E05 B [2.1E-05 B]	1.9E05 B [6.9E-06 C]	2.5E03 C [1.2E-07 D]
		5-20 cm	3.4E05 A [3.9E-05 A]	1.2E05 A [1.9E-05 A]	1.8E04 B [2.0E-06 B]	2.5E03 C [5.8E-07 B]
	After rain	0-5 cm	1.3E06 A [8.2E-05 A]	5.3E05 A [2.6E-05 A]	2.4E04 B [1.6E-06 B]	2.5E03 C [1.2E-07 C]
		5-20 cm	1.1E05 A [4.4E-05 A]	9.2E04 A [1.6E-05 B]	4.7E03 B [1.4E-06 C]	2.5E03 C [5.8E-07 D]
Day 3	Before rain	0-5 cm	3.5E05 A [1.0E-04 A]	3.3E04 B [1.2E-05 B]	2.8E03 C [6.1E-07 C]	2.5E03 C [1.2E-07 D]
		5-20 cm	2.8E04 A [1.8E-05 A]	4.1E03 B [2.9E-06 B]	4.1E03 B [3.0E-06 B]	2.5E03 C [5.8E-07 C]
	After rain	0-5 cm	2.1E05 A [5.1E-05 A]	6.6E04 B [2.1E-05 B]	4.3E03 C [1.3E-06 C]	2.5E03 C [1.2E-07 D]
		5-20 cm	2.2E04 A [2.9E-05 A]	1.1E04 A [1.1E-05 B]	2.5E03 B [2.0E-06 C]	2.5E03 B [5.8E-07 D]
intI1[intI1/16S]						
Time gap	Rain	Depth	Mean [in the slit	Mean [5 cm away from the slit]	Mean [25 cm away from the slit]	Mean [Control soil]

Day 0	Before rain	0-5 cm	3.8E07 A 1.4E-03 A]	3.2E07 A 1.1E-03[A]	2.7E07 A B 1.0E-03[A]	1.5E07 B 7.1E-04[A]
		5-20 cm	1.4E07 A [1.6E-03 A]	1.3E07 A [2.0E-03 A]	2.5E07 A [2.7E-03 A]	3.1E06 B [7.3E-04 B]
	After rain	0-5 cm	2.8E07 A [1.8E-03 A]	1.7E07 A [8.5E-04 A]	1.4E07 A [9.2E-04 A]	1.5E07 A [7.1E-04 A]
		5-20 cm	1.6E07 A [6.1E-03 A]	1.1E07 A [2.0E-03 AB]	3.5E06 B [1.1E-03 B]	3.1E06 B [7.3E-04 B]
Day 3	Before rain	0-5 cm	4.6E07 A [1.4E-02 A]	7.1E06 C [2.6E-03 B]	3.0E06 D [6.6E-04 C]	1.5E07 B [7.1E-04 C]
		5-20 cm	7.6E06 A [4.8E-03 A]	8.2E06 A [5.8E-03 A]	7.7E06 A [5.5E-03 A]	3.1E06 B [7.3E-04 B]
	After rain	0-5 cm	4.5E07 A [1.1E-02 A]	3.2E07 A [9.9E-03 A]	3.0E07 A [8.7E-03 A]	1.5E07 B [7.1E-04 B]
		5-20 cm	1.3E07 A [1.7E-02 A]	1.4E07 A [1.4E-02 A]	1.4E07 A [1.2E-02 A]	3.1E06 B [7.3E-04 B]

Appendix AO: Abundance of Rum-2-bac and inIt1 in soil collected from different days after rainfall. Top values are absolute abundance and bottom values are relative abundance. Significant letters are labelled according to multiple student's t-test with means in the order of A>B>C>D>E and levels not connected by same letter are significantly different.

Rum-2-bac [Rum-2-bac/16S]						
Application Method	Depth	Mean [Day0]	Mean [Day3]	Mean [Day8]	Mean [Day17]	Mean [Control soil]
Subsurface injection	0-5 cm	2.2 E+06 A [8.2 E-05 A]	3.5 E+05 B [1.0 E-04 A]	2.4 E+04 C [4.8 E-06 B]	4.1 E+03 D [1.0 E-06 C]	2.5 E+03 D [1.2 E-07 D]
	5-20 cm	3.4 E+05 A [3.9 E-05 A]	2.8 E+04 B [1.8 E-05 A]	3.1 E+03 C [4.7 E-06 B]	8.3 E+02 D [1.2 E-06 C]	2.5 E+03 C [5.8 E-07 C]
Surface application	0-5 cm	3.3 E+05 A [1.4 E-05 B]	1.6 E+05 B [3.2 E-05 A]	7.7 E+03 C [1.6 E-06 C]	3.5 E+03 D [1.0 E-06 D]	2.5 E+03 D [1.2 E-07 E]
	5-20 cm	1.3 E+04 A [1.6 E-06 B]	2.4 E+04 A [2.1 E-05 A]	2.5 E+03 B [1.7 E-06 B]	2.5 E+03 B [2.8 E-06 B]	2.5 E+03 B [5.8 E-07 C]
intI1[intI1/16S]						
Application Method	Depth	Mean [Day0]	Mean [Day3]	Mean [Day8]	Mean [Day17]	Mean [Control soil]
Subsurface injection	0-5 cm	3.8 E+07 A [1.4 E-03 C]	4.6 E+07 A [1.4 E-02 AB]	3.6 E+07 A [7.2 E-03 B]	5.9 E+07 A [1.5 E-02 A]	1.5 E+07 B [7.1 E-04 D]
	5-20 cm	1.4 E+07 AB [1.6 E-03 C]	7.6 E+06 B [4.8 E-03 B]	1.3 E+07 AB [1.9 E-02 A]	2.4 E+07 A [3.5 E-02 A]	3.1 E+06 C [7.3 E-04 D]
Surface application	0-5 cm	9.5 E+06 A [3.9 E-04 B]	3.3 E+06 BC [6.6 E-04 AB]	2.1 E+06 C [4.2 E-04 B]	4.4 E+06 B [1.3 E-03 A]	1.5 E+07 A [7.1 E-04 AB]
	5-20 cm	7.2 E+06 A [8.9 E-04 B]	1.1 E+06 D [9.6 E-04 B]	1.5 E+06 CD [1.1 E-03 B]	2.1 E+06 BC [2.4 E-03 A]	3.1 E+06 B [7.3 E-04 B]