

Effects of Manure Injection on Transport and Transformation of Nutrients and Antibiotics

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Abstract

Overapplication of manure in sensitive watersheds is an issue of increasing environmental concern due to increased nutrient loading and antibiotic release into aquatic environments. Manure is typically surface applied, leaving nutrients and antibiotics vulnerable to loss at the soil surface. Elevated nutrient and antibiotic loading into water bodies can increase the rate of eutrophication and occurrence of antibiotic resistance genes in areas of high animal agriculture production, such as the Chesapeake Bay watershed. Manure injection is a new technology that incorporates manure into the soil with minimal disturbance, and management strategies that reduce manure loss from agricultural fields could prevent the transport of nutrients and antibiotics to sensitive waterways. However, little is known about the efficacy of dry litter injection to decrease nitrogen (N) loss when compared to surface application. Also, there are no studies that determine the effects of injection on antibiotic transport and transformation after manure application. Therefore, this project focused on changes in N cycling, orchardgrass hay yield and quality, and transport and transformation of pirlimycin and cephalixin, two common antibiotics in dairy production, when manure is injected. Subsurface injection eliminated ammonia volatilization and N loss in runoff and increased soil inorganic N when compared to surface application after volatilization, incubation, and rainfall simulation studies. Although these benefits did not translate to higher yields in orchardgrass hay, protein increased when poultry litter was injected, indicating greater N uptake. Injection of dairy manure decreased losses of pirlimycin to levels of the control when compared to surface application. Although, pirlimycin had a slower degradation rate within the injection slit compared to surface application,

potentially increasing the amount of time soil microbes are exposed to antibiotics. In an incubation study, pirlimycin concentrations decreased after 7 days, but concentrations increased sharply after 14 days. This indicates that conjugates formed in the liver or digestive tract of dairy cows may revert back to the parent compound after manure application. With increased retention of nutrients and antibiotics, injection could be a best management practice used to reduce the loss of these compounds to the environment while increasing the quality of crops produced.

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Chapter 1: Introduction

Manure produced from large scale animal agriculture operations has increased dramatically since the 1950's due to consolidation of animal farms and increased stocking rates on relatively small land areas (USEPA, 2013). In 2007, 2.2 billion animal units (e.g. 1 beef cow, 0.75 dairy cows, 9 market swine, or 455 broiler chickens) produced one billion Mg of manure (Kellogg et al., 2000; Gollehon et al., 2001; USDA, 2009). Of that, 173 million Mg of dairy manure and 48 million Mg of broiler litter are produced annually in the U.S. (USDA, 2009). Considering most manure is land applied near the farm where it is generated, there is growing concern regarding the overapplication of manure in sensitive watersheds. Nitrogen (N) and phosphorus (P) movement into waterways has been the primary concern in the past, with efforts to reduce the rate of eutrophication and frequency of algal bloom occurrence (USEPA, 2013). However, there is also concern of emerging contaminants, such as antibiotics, found in these manures. Through manure application, antibiotics are applied to agricultural fields where they may leach, sorb to soil particles, volatilize, dissolve in surface runoff, or degrade (Thiele-Bruhn, 2003). The transport and transformation of these compounds is very dependent on the compound's chemical properties and the farmer's management of the manure and fields where the manure is applied. For example, surface application is a very common application method because it is quick and cost-effective. However, surface application leaves manure at the soil surface where nutrients and other constituents are vulnerable to loss through volatilization or in surface runoff (Maguire et al., 2011a). Tillage is used by many farmers as part of their typical management to decrease N volatilization. However, tillage increases soil erosion and loss of chemicals and nutrients attached to this eroded soil. Also, tillage is not an option for fields under no-till or forage management. Manure injection is an application method that places manure

below the soil surface with little soil disturbance (Maguire et al., 2011a). Manure injection has been demonstrated to decrease runoff losses of nutrients contained in manures (Pote et al., 2003; Pote et al., 2009; Watts et al., 2011). The Subsurfer, a poultry litter injector, is a new technology that incorporates poultry litter similarly to a no-till planter (Pote et al., 2011). A cutting disk cuts through plant residues while a disk opener creates a slit for injection to a depth of 2-4 in, depending on manure type and application rate. Then, the manure drops into the opening and a presswheel closes the injection slit. While there have been studies on the effectiveness of dairy manure injection for reducing nutrient losses, there has been little research on poultry litter injection using the newest prototype, the Subsurfer. While research has shown that litter injection decreases nutrient losses in runoff, there has been no research determining the effects of injection on the fate and transport of antibiotics in manure.

For this study, incubation, volatilization, rainfall simulation, and field experiments were conducted to determine the effects of poultry litter injection on N cycling and orchardgrass hay yields and quality. Also, a rainfall simulation experiment was conducted to determine the effects of injection on the fate and transport of pirlimycin from dairy manure application when compared to surface application, and an incubation study was conducted to determine the rate of transformation of pirlimycin and cephalixin in soil when manure from treated dairy cows is incorporated into two soil types.

Objectives

The overall objective of this study was to determine the effects of manure injection on nutrient and antibiotic transport and transformations when compared to traditional surface application.

The individual study objectives were to determine:

1. Effects of poultry litter injection on N and P dynamics
2. Effects of poultry litter injection on orchardgrass hay yields and quality
3. Effects of dairy manure injection on antibiotic movement
4. Effects of pH and temperature on antibiotic transformation in soil after dairy manure incorporation

Chapter 2: Literature Review

Water Quality Regulation in the U.S.

In 1972, the Federal Water Pollution Control Act (Clean Water Act) (33 U.S.C. §1251) was signed into law, establishing the backbone for creating water quality standards in the country. The goal of the Clean Water Act was to “restore and maintain the chemical, physical, and biological integrity of the Nation’s waters” (Clean Water Act of 1972, p3). Since passage of this law, many regulations have been put in place to protect or improve water quality in the U.S., with major focus on point source pollution from industry or waste water treatment plants and nonpoint source pollution from agriculture or urban runoff. The Clean Water Act is partially implemented through the use of total maximum daily loads, which set numerical limits on the concentration of certain pollutants in water bodies and discharged through point sources. When a river or stream is impaired or threatened, it is added to the 303(d) list or list of impaired and threatened waters within the U.S., and a TMDL is created to address the areas of concern (40C.F.R.§130.7(b)(4)). By setting limits for total maximum daily loads in waterways, various stakeholders in watersheds may be required to limit the release of sediment, metals, toxic chemicals, fecal coliform bacteria, pH, nutrients, or other pollutants from their land or facilities to maintain water quality necessary for supporting the beneficial uses of that waterway, which are set by individual states (USEPA, 2012). In 1988, the Chesapeake Bay was added to the 303(d) list for Virginia, Maryland, and the District of Columbia (Chesapeake Bay Program, 2008). The Chesapeake Bay watershed covers 64,000 square miles, with 150 major rivers, and consists of seven watershed jurisdictions to implement and report on water quality improvement measures (Chesapeake Bay Program, 2015). Nutrients and sediment flowing from these rivers and streams cause major water quality issues in the bay, including the formation of algal blooms and increased turbidity. Therefore, there have been many efforts to reduce the amount of

nutrients flowing through these waterways from point and nonpoint sources. While this is a complex problem that cannot be solved by reducing nutrient inputs from one source, agriculture is the major contributor of nutrients and sediment in the bay (Chesapeake Bay Program, 2015)

Eutrophication

Eutrophication is a result of excessive nutrient enrichment, creating conditions favorable for algal growth in aquatic systems. Nitrogen (N) and phosphorus (P) are the limiting nutrients for primary production in aquatic systems. Nitrogen is needed for protein synthesis, and P is a component of DNA, RNA, and ATP. The increase of N in saltwater systems and P in freshwater systems has led to increased occurrence of harmful algal blooms in areas such as the Chesapeake Bay (Boesch et al., 2001). Therefore, it is clear that a nutrient management strategy for N and P is necessary to reduce the frequency of eutrophication in these water systems (Conley et al., 2009). The demand for dissolved oxygen increases as the algae decomposes, creating anoxic conditions. Anoxic “dead zones” are formed in areas of high nutrient induced primary productivity, and these areas have reduced biodiversity due to fish kills and increased turbidity. It was estimated that 40% of the N applied to land within the Chesapeake Bay watershed comes from manure application, and manure application on agricultural lands contributes an estimated 18% of N and 27% of P flowing into the Chesapeake Bay (Chesapeake Bay Program, 2004; Chesapeake Bay Program, 2010). Due partly to these sources of excess N, eutrophication has significantly decreased water quality within the Chesapeake Bay (Jaworski et al., 1997).

Manure Production and Management

Since the 1950's, consolidation of animal agriculture and increased stocking rates have significantly increased the amount of manure generated in a relatively small geographic area (USEPA, 2013). In that time, the amount of livestock and poultry produced in the U.S. has more

than doubled while the number of operations has decreased by 80% (Graham and Nachman, 2010). In 2007, 2.2 billion animal units produced one billion tons of manure in the U.S. (USDA, 2009). Considering most manure is applied near where it is generated, new manure management strategies are necessary to prevent overapplication of manure and movement of nutrients or other contaminants from agricultural fields into sensitive waterways.

In Virginia, manure is typically surface applied to agricultural fields to provide nutrients for crop production (Maguire et al., 2011b). However, surface application can leave the manure vulnerable to losses at the soil surface through ammonia volatilization and in runoff (Pote et al., 2009; Maguire et al., 2011a). Incorporation of manure through tillage can decrease volatilization. However, tillage is not compatible with no-till or forage systems. Therefore, new application technologies, such as poultry litter injection, are needed to incorporate poultry litter with minimal soil disturbance to prevent destroying the benefits provided through no-till (Maguire et al., 2011a).

Nitrogen Chemistry

Nitrogen is the most common limiting nutrient in agricultural production and can exist in many forms in the environment. When applied through manure application, N is present in organic (proteins and amino acids) and inorganic (NH_4 and NO_3^-) forms. The amount of N in each fraction depends on the manure type and conditions of manure storage. For example, the majority of N present in poultry litters from broiler production in Virginia is in organic forms ($27 \text{ g}^{-1} \text{ kg}$) with a small portion of total nitrogen existing as inorganic ammonium ($6 \text{ g}^{-1} \text{ kg}$) (VADCR, 2005). After application to land, organic nitrogen can undergo mineralization to inorganic nitrogen through microbial degradation of organic matter. Heterotrophic bacteria break down protein molecules into amines, amino acids, and urea, and these are further broken down to produce ammonium. Uric acid and undigested proteins, two forms of organic N, accounted for

50-90% of the total N in poultry litter, depending on the storage method, and 60-70% of total N was excreted as uric acid (Nahm, 2003). Uric acid undergoes transformation to glyoxylate and urea (Lee et al., 2013). Urea produced through uric acid metabolism can be converted to carbon dioxide and ammonia by urease enzymes (Kim et al. 2009). A small portion of the ammonium can be assimilated by plants, but the majority of ammonium undergoes nitrification or volatilizes as ammonia gas.

Nitrification is the oxidation of ammonium by obligate aerobic bacteria (commonly nitrosomonas in equation 2.1 and nitrobacter in equation 2.2) to nitrite and finally to nitrate (Havlin et al., 2005).

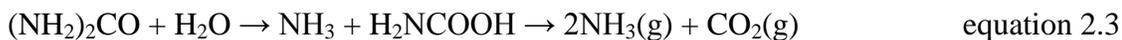


Nitrification can only occur in aerobic environments due to the oxygen demand of these chemical reactions. Nitrate can then be taken up by plants, leached from the system, or transformed through denitrification.

Nitrate is an anion preferentially taken up by plants as a nitrogen source for amino acid formation and eventually protein synthesis. Because nitrate is an anion, it can be easily leached from the soil into groundwater. Most soils have a net negative charge due to isomorphic substitution of Mg^{2+} or Fe^{2+} for Al^{3+} in the octahedral sheet or Al^{3+} for Si^{4+} in the tetrahedral sheet of phyllosilicate clays. While cations are retained on the surfaces of clays, nitrate will move with the flow of water through the soil. Therefore, it is important to apply the appropriate amount of N at the right time and place to prevent N loss.

Ammonia volatilization is a major loss mechanism from application of manure and inorganic fertilizers, such as urea. In manure such as poultry litter, the main form of organic

nitrogen is uric acid, which is broken down by bacteria to form urea. Urea undergoes hydrolysis to ammonia (equation 2.3) through urease catalysis (Kim et al. 2009). The resulting ammonia can then form ammonium, remain as ammonia in solution, or volatilize as ammonia gas.



Ammonia volatilization is dependent upon the pH of the substrate, humidity of air, temperature, wind speed, and exposure to the atmosphere (Nathan and Malzer, 1994). Higher temperatures and soil water evaporation increase the rate of ammonia volatilization due to equilibrium reactions and elevated biological activity. The acid dissociation constant (pKa) of the ammonium ion is 9.25. At pH below 9.25, nitrogen will mainly exist as solid ammonium, and at pH above 9.25, the majority of nitrogen will be in the form of ammonia gas (equation 2.4). Soil pH typically ranges from 5-7 with some exceptions occurring in calcareous (high pH) or acidic (low pH) soils. However, the pH of poultry litter is typically around 8.5, which can leave a large amount of nitrogen present as ammonia. Keeping pH well below the pKa is crucial to preventing loss of nitrogen through ammonia volatilization. Acidifiers, such as sodium bisulfate, are commonly added to poultry litter while in the poultry house to reduce microbial activity and pH of the litter, but the pH eventually rises over time (Hunolt, 2015). Therefore, limiting exposure to the atmosphere is an important practice used to prevent ammonia volatilization.



When ammonia is applied to the soil surface, ammonia will continuously volatilize due to the low partial pressure of ammonia in the atmosphere, and higher wind speeds increase the intensity of ammonia volatilization (Meisinger and Jokela, 2000). When ammonia is incorporated into the soil through injection, the slow diffusion of oxygen between the soil and atmosphere can eliminate ammonia volatilization.

Nitrogen Benefits with Manure Injection

Few studies have been conducted comparing yield and quality of crops when comparing injection and surface application, which show poultry litter injection maintains or increases crop yields in forage systems. Forage yield and quality were increased when using subsurface banding compared to surface application, and poultry litter injection showed a strong tendency to increase yield of bermudagrass (Pote et al., 2011; Pote et al., 2003). Warren et al. (2008) found that yields were similar when injecting or surface applying poultry litter into tall fescue and bermudagrass fields. While differences for individual cuttings were not always significant, total yields were increased by 40% when poultry litter was injected into bermudagrass and mixed grass pastures when compared to surface application (Pote et al., 2009). Increased yield and quality of crops could be an indicator of increased capture of nutrients through reducing N loss as ammonia volatilization and in runoff. Nitrogen is the most common limiting nutrient in agricultural systems, and additional N can increase crop yield and quality (Eckert, 2007). There are several indicators used to determine feedstock quality: e.g. crude protein, acid detergent fiber, neutral detergent fiber, and total digestible nutrient content (Ball et al., 2001). Protein and total digestible nutrients are common indicators of feedstock quality and nutritive value (Adesogan et al., 2009). Although not always significant, dairy manure injection increased N content of wheat, barley, and canola compared to surface application (Mooleki et al., 2001). In bermudagrass and mixed grass pasture, the mean protein and total digestible nutrient contents were higher when poultry litter was injected, but differences were not always significant for individual cuttings (Pote et al., 2009).

Manure incorporation through tillage has been shown to decrease N losses through ammonia volatilization and in runoff, but tillage is not compatible with no-till or forage systems (Thompson and Meisinger, 2002; Maguire et al., 2011a). Poultry litter injection can reduce

nutrient losses through runoff or ammonia volatilization when compared to surface application (Maguire et al., 2001a; Moore et al., 2011; Pote et al., 2003; Pote et al., 2009; Pote et al., 2011). Pote et al. (2009), Pote et al. (2003), and Watts et al. (2011) found that N and P concentrations in runoff were reduced to levels similar to a no manure control when poultry litter was injected versus surface applied. Also, poultry litter injection eliminated ammonia loss through volatilization (Moore et al., 2011). Manure injection can decrease ammonia volatilization by limiting the exposure of manure to the atmosphere, which increases the immobilization of ammonium (Dell et al., 2011). When manure rates are based on N requirements, there is a tendency to overapply P because poultry litter has an average N:P ratio of 3 and major grain and hay crops require an N:P ratio of 8 (Moore et al., 1995; Fertilizer Handbook, 1982). Through retention of nutrients, manure injection increases the N use efficiency of manure applied through this method and more closely mimics plant requirements (Maguire et al., 2008). While there is little information on the cost-benefit of poultry litter injection, profits from grass production were increased by \$340 yr⁻¹ using shallow disk injection of dairy and swine manure, and the greatest environmental benefits at the lowest cost or greatest profit for the producer were obtained using shallow disk injection (Rotz et al., 2011). While liquid injection can increase nitrogen loss through nitrous oxide production, these losses do not offset the benefits of increased ammonia capture and reduction of nutrients in runoff provided when liquid manure is injected (Dell et al., 2011). Because injection can reduce the movement of nutrients in agricultural fields, injection could change the behavior of other contaminants, such as antibiotics, that enter the environment through manure application.

Antibiotic use in Agriculture

Antibiotics are commonly used in animal agriculture for treatment of disease and as growth promoters (Chee-Sanford et al., 2009). It is believed that antibiotics added to water or

feed for growth promotion alter the intestinal microbial population, creating conditions favorable for weight gain by reducing competition for nutrients, and the majority of antibiotics used in livestock or poultry are administered to promote animal growth (MacDonald and McBride, 2009; Mellon et al. 2001). However, there is increasing concern over emerging contaminants, such as antibiotics, and their impact on environmental quality (USEPA, 2013). In the U.S., it was estimated that 13.2 million kg of antimicrobials were sold for use in livestock and poultry production in 2010, which is four times the amount used for human health (USFDA, 2011; Loglisci, 2010). Intensive indoor facilities produce large amounts of manure that can be stored until land application using a variety of storage methods (e.g. lagoon or dry stacked) (Boxall, 2004). The composition of manure is dependent on the storage type, animal species and life stage, diet, and waste management plan (Campagnolo et al., 2002). A large portion of antibiotics administered to animals for therapeutic or subtherapeutic purposes are excreted through urine and/or feces unchanged or as metabolites. However, antibiotic dose and excretion are dependent on animal type and life stage, making it difficult to estimate livestock excretion of antibiotics in animal manure (Boxall et al., 2002; USEPA, 2013). Application of antibiotics to soil through manure application appears to be the main pathway for releasing these compounds into the environment, which may increase the occurrence of antibiotic resistance (Bager et al., 2000; Boxall et al., 2002; Kumar et al., 2005).

Antibiotic Resistance

Microbes may gain resistance through selection of resistant microbes in the animal gut and/or horizontal gene transfer (e.g. conjugative transfer, transduction, or transformation) (Chee-Sanford et al., 2009). Antibiotic resistance is an issue of growing concern as the evidence of close association between use of antibiotics in animal agriculture and emergence of antibiotic resistance in human pathogens are increasing (Kumar et al., 2005). In Denmark, a region where

fewer antibiotics are used in agriculture, there were lower frequencies of antibiotic resistance genes in human feces (Osterblad et al., 1995; Chee-Sanford et al., 2009). Also, very low antibiotic concentrations can impart resistance, making it important to determine the potential for manure application to introduce these compounds into the environment (Tello et al., 2012).

Antibiotics in the Environment

Antibiotics can enter the environment through human wastewater streams or land application of manure or biosolids, with the potential for subsequent transport dissolved in runoff water, sorbed to eroded sediment, or leached into groundwater (Boxall, 2008). Antibiotics may degrade during storage, but treated pasture animals can deposit antibiotics in high concentrations to small areas during urination or defecation (Boxall, 2012; Halling-Sorensen et al., 1998). The fate of antibiotics in the environment is dependent on initial antibiotic concentration, soil properties (e.g. texture, mineralogy, pH, organic matter), antibiotic properties/chemistry (e.g. photostability, binding to soil solids, biodegradation, and water solubility), and abiotic factors (e.g. temperature and rainfall) (Kumar et al., 2005).

With antibiotic pKa's in the pH range commonly found in soils, antibiotics could exist in ionized or unionized forms, controlling their sorption to soil solids (Boxall, 2012). Temperature and pH are two major factors impacting the transformation of antibiotics in soil (Kumar et al., 2002; Boxall, 2012). Temperature is important for regulating microbial activity and pH controls antibiotic sorption and degradation (Kemper et al., 2007; Boxall, 2012). For example, lower temperatures increase half-lives of antibiotics added to soil through manure application (Gavalchin and Katz, 1994). Ceftiofur, a third generation cephalosporin, degradation was affected by soil pH, and cefepime, a fourth generation antibiotic, stability was affected by pH when dissolved in aqueous solution (Gilbertson et al., 1990; Fubara and Notari, 1998). However, the effect of temperature and pH is dependent on the specific antibiotic and properties of the soil,

and there is little information on several antibiotics commonly used in animal agriculture. Also, movement of antibiotics applied through manure application will depend on the soil properties, manure management, and field management.

There have been few studies conducted to determine the movement of antibiotics in surface runoff after manure application to agricultural fields, and these studies show that a small portion (typically <5%) are lost through this process (Dolliver and Gupta, 2008; Burkhardt et al., 2005; Kreuzig et al., 2005). After surface application of swine manure, Burkhardt et al. (2005) found 0.3% to 1.4% of sulfadioazine, suladimidine, and sulfathiazole antibiotics in runoff after 1 day and 1.6% to 6.3% after 3 days. After incorporation of bovine manure by tillage, mass loss of sulfonamides ranged from 0.1% to 2.5% of the total applied (Kreuzig et al., 2005).

Chlortetracycline, tylosin, and monensin had mass losses less than 5% when swine and beef manure were applied to agricultural fields, and no-till plots generally had higher mass loss when compared to plots that were chisel plowed (Dolliver and Gupta, 2008). Since plowing is not compatible with no-till or forage production, injection could reduce the losses of antibiotics to aquatic systems where manure is typically surface applied.

Pirlimycin Chemistry

Mastitis is an infection of the mammary gland and can be caused by many bacterial pathogens (Gehring and Smith, 2006; du Preez, 2000). However, staphylococci, streptococci, coliforms, and *arcanobacterium pyogenes* bacteria are the source of the majority of mastitis cases (du Preez, 2000). Mastitis is a very common problem for U.S. dairy operations, with 85% of farms reporting at least one or more cows affected annually, and pirlimycin and cephalosporins are two of the most common antibiotics used for treatment of mastitis in dairy cows (Pol and Ruegg, 2007; USDA, 2005). Pirlimycin, a lincosamide antibiotic, is effective against gram-positive bacteria and binds to the 23s portion of the 50S subunit of the bacterial ribosome, inhibiting

protein synthesis (Kulczycka-Mierzejewska et al., 2012). The structure of pirlimycin is pictured below (Image 2.1).

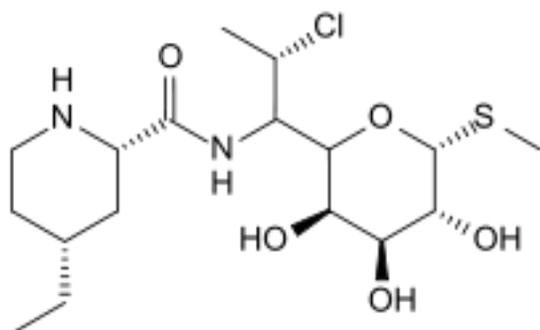


Image 2.1

After radiolabeled pirlimycin was administered, Hornish et al. (1992) found that 68% of the initial dose was excreted in milk, urine, and feces as unchanged parent pirlimycin when administered to dairy cows, and 24% and 10% of the administered dose was excreted in feces and urine, respectively (Hornish et al., 1992). Of that, 80% and 45% of the pirlimycin in urine and feces, respectively, was excreted as the parent compound, with other portions existing as conjugates. In the liver, pirlimycin can undergo oxidation to pirlimycin sulfoxide or pirlimycin sulfoxone, and approximately 8% of the pirlimycin excreted in urine was present in the sulfoxide form. When in the intestinal tract, pirlimycin or pirlimycin sulfoxide can be altered by microflora to ribonucleotide adducts. Two of these adducts accounted for 50% of the pirlimycin excreted in feces, and these adducts may revert back to parent pirlimycin through oxidation in excreted feces or after application to soil (Hornish et al., 1992). The pKa of pirlimycin is 8.38, and with soils commonly in the pH range of 5-7, pirlimycin will remain protonated, which could limit mobility due to retention on permanently charged clay surfaces (USFDA, 1993).

Cephapirin Chemistry

Cephapirin belongs to the group of cephalosporin antibiotics, and cephalosporins are beta-lactam antibiotics, which gain their biocidal activity through the use of a four-membered beta-

lactam ring (Hornish and Kotarski, 2002). Once the beta-lactam ring is cleaved, cephalosporins lose their biological activity. Cephalosporins have the same central structure (beta-lactam ring fused with a dihydrothiazine ring) but differ in their side chain constituents, and are generally polar, hydrophilic, non-volatile, and thermolabile (Hornish and Kotarski, 2002; Pehoureq and Jarry, 1998). Cephapirin (pictured below in image 2.2) is effective against many gram-positive and gram-negative aerobic bacteria, and according to a national survey, cephapirin was the most commonly used drug on dairy farms, administered to 42% of cows raised in the U.S. in 2002 (Donowitz and Mandell, 1988; USDA, 2005).

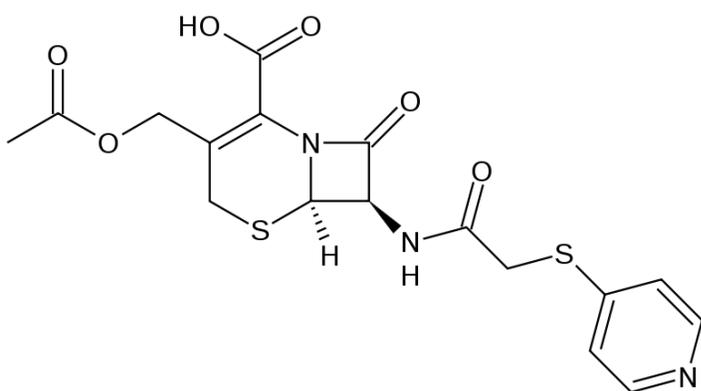


Image 2.2

Once administered, cephapirin undergoes rapid transformation to the deacetylated metabolite desacetylcephapirin, which has 54% of the biological activity of cephapirin when assayed on *S. lutea* plates (Cabana et al., 1976). The pKa's of cephapirin sodium are 2.15 and 7.3, and cephapirin is a weak acid (Gennaro, 1990; Pehourcq and Jarry, 1998). In a sandy soil collected from a sand dune, cephapirin from cephapirin sodium has a K_d range between 0.94 to 3.45 L kg⁻¹, which is similar to other antibiotics in sandy soils (Miropolskiy, 2009; Peterson and O'Mears, 2008). With a low K_d (<5 L kg⁻¹) cephapirin sodium would likely have a low affinity to soil and high bioavailability (Tolls, 2001; Lawrence et al., 2000). However, little is known about cephapirin benzathine. The pKa's of cephapirin benzathine are 2.67 and 4.49 (Willing, 2013).

Cephapirin benzathine has very low solubility when compared to cephapirin sodium because the goal of cephapirin benzathine is to treat localized infections of mastitis in the intramammary tissue. Due to low solubility, cephapirin benzathine maintains a higher concentration within the udder for longer periods of time, increasing the duration of therapy during the dry period (Ray et al., 2014). Longer residence time within the cow allows for a higher effectiveness of treatment using this method. However, with increased residence time within the cow, cephapirin is excreted over a longer period of time, potentially impacting the formation of antibiotic resistance (Ray et al., 2014).

Summary

1. Excess nutrients can negatively impact water quality, and poultry litter injection could reduce nutrient runoff and N volatilization enough to increase crop yield, improve crop quality, and improve water quality.
2. Injection reduces nutrients in runoff and may increase forage yield and quality, but little is known about the effects of injection on N cycling when compared to surface application. Injection could increase ammonia retention and N mineralization due to increased contact with soil solids.
3. Antibiotics are commonly used for disease treatment and growth promotion in animal agriculture. However, little is known about the transport and transformation of two of the most common antibiotics used in dairy production, cephalosporin and tetracycline, after manure is applied to soil.
4. Many factors control the transformation of antibiotics applied to soil through manure application (e.g. antibiotic chemical properties, manure properties and handling, soil properties, and climate), and there is little information on the effects of pH and temperature on the transformation of cephalosporin and tetracycline.

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Chapter 2: Effects of poultry litter injection on ammonia volatilization, nitrogen availability, and nutrient losses in runoff

Abstract

Poultry litter is a common organic amendment in agricultural production, but nutrient losses can reduce its effectiveness as a fertilizer. Three experiments were conducted to evaluate ammonia-N ($\text{NH}_3\text{-N}$) volatilization, nitrogen (N) availability, and runoff losses of nutrients by conducting a closed chamber volatilization study, soil incubation, and rainfall simulation. In all studies, poultry litter was applied at a rate of 6.7 Mg ha^{-1} either on the surface or injected and compared to an unamended control. In the volatilization and soil incubation studies, treatments were applied to Braddock Loam and Bojac Sandy Loam surface soils. Of the ammonium-N ($\text{NH}_4^+\text{-N}$) added, cumulative loss of $\text{NH}_3\text{-N}$ by volatilization was 3% from injected and 121% from surface applied poultry litter after 7 days in the Loam. In the Sandy Loam, cumulative loss of $\text{NH}_3\text{-N}$ was 9% from injected and 153% from surface applied poultry litter after 7 days. After a 40 day soil incubation, injection increased total inorganic N by 52% and 99% for the Loam and Sandy Loam soils, respectively, when compared to surface application. Compared to surface application, injection reduced Total Kjeldahl N (TKN) by 59%, Total Kjeldahl P (TKP) by 53%, dissolved reactive P (DRP) by 96%, dissolved $\text{NO}_3^-\text{-N}$ by 73%, and dissolved $\text{NH}_3\text{-N}$ in runoff by 99%. Injection reduced $\text{NH}_3\text{-N}$ volatilization and nutrients in runoff to levels of the control. These studies show that injection increases plant available N while decreasing losses through volatilization and runoff.

Introduction

Manures contain valuable nutrients for crop production, but those nutrients may be vulnerable to losses through surface runoff or gaseous emissions (Maguire et al., 2011a). Manure is typically surface applied to agricultural fields as this is quick and cost effective.

Sometimes manure is incorporated by tillage to decrease ammonia-N ($\text{NH}_3\text{-N}$) volatilization, and many farmers use tillage as part of their typical management (Maguire et al., 2011a). However, tillage is not compatible with no-till and forage systems. Manure injection offers the opportunity to incorporate manure into the soil with minimal soil disturbance (Pote et al., 2011).

Soil disturbance is minimal with application methods such as disk injection, chisel injection, or high pressure injection, potentially making them compatible with no-till and forage systems (Maguire et al., 2011b). Technologies are commercially available for injection of liquid manures, but not for injection of dry poultry litter. The Subsurfer is a new technology that injects poultry litter into no-till soils or grasslands with minimal disturbance to the soil (Pote et al., 2011). The Subsurfer is a tractor-drawn applicator that uses an internal auger system to grind and provide a constant flow of poultry litter to injection slits. Poultry litter is typically injected to a 5 cm depth. However, adjustments can be made to accommodate slightly different depths. Disturbance created from subsurface injection of poultry litter is comparable to that of no-till planter operation (Pote et al., 2011).

Injection of poultry litter increased mean yield and quality in bermudagrass (*Cynodon dactylon*) hay, although not always significant for individual cuttings (Pote et al., 2009). Corn yields were increased by 36% and 20% in 2008 and 2009, respectively, when comparing injection to surface application of poultry litter (Pote et al., 2011). However, it is unclear whether yields were increased due to improved mineralization of organic nitrogen (N) or through increased utilization of manure ammonium-N ($\text{NH}_4^+\text{-N}$) due to decreased volatilization. Nitrogen mineralization is the conversion of organic N to inorganic forms of N, $\text{NH}_4^+\text{-N}$ and nitrate-N ($\text{NO}_3^-\text{-N}$). Organic N in poultry litter applied in spring or early fall in Virginia has a calculated mineralization rate of 60% for all application methods (VADCR, 2005). Poultry litter incubated

for 180 days had a mineralization rate of 38, 68, 73, 83, and 80% for sampling intervals of 0, 45, 90, 135, and 180 days, respectively (Hanselman et al., 2004). Cabrera et al. (1994) reported that there were no differences in mineralization of poultry litter after 14 days when comparing surface application and incorporation.

Injection of manure has been shown to decrease $\text{NH}_3\text{-N}$ volatilization and increase NH_4^+ -N immobilization by limiting the exposure of manure to the atmosphere (Dell et al., 2011). In a 4-year study by Powell et al. (2011), liquid manure injection reduced annual $\text{NH}_3\text{-N}$ volatilization by 40-95% when compared to traditional surface broadcast application. However, the majority of research on $\text{NH}_3\text{-N}$ volatilization from manure injection deals with liquid manures. In a study by Moore et al. (2011), ammonia volatilization was reduced to zero using simulated poultry litter injection in bermudagrass pasture.

Manure injection can not only reduce losses of $\text{NH}_3\text{-N}$ to the atmosphere, but it can reduce losses of nutrients in runoff water. Nitrogen and phosphorus (P) are the key limiting nutrients in aquatic systems, and the reduction of nutrients in runoff water has the potential to reduce eutrophication of surface waters. The increase of N in saltwater and brackish estuarine systems and P in freshwater systems has led to increased occurrence of eutrophication (Boesch et al., 2001). It is clear that a dual-nutrient-reduction strategy of N and P is needed to reduce the effects of eutrophication (Conley et al., 2009).

Nutrients in runoff can be significantly reduced when manure is injected into the soil (Maguire, 2008). Total N, NO_3^- -N, dissolved reactive P (DRP), and total P concentrations in runoff were reduced by 35, 25, 61, and 64% respectively when poultry litter was incorporated with a rotary tiller compared to surface application (Adeli et al., 2011). Injection of poultry litter has been shown to reduce losses of N and P in runoff to levels of the control. Compared to

surface broadcast, there was a reduction greater than 85% in inorganic N (91%), total N (90%), DRP (86%), and total P (86%) when poultry litter was injected (Watts et al., 2011). In a study by Pote et al. (2003), nutrient losses of N and P in runoff were reduced by 90% using simulated injection compared to broadcast application. Using the Adjustable-Band prototype, P loss in runoff was reduced by 75% in the first runoff event in a rainfall simulation on soil columns and a three year watershed study (Pote et al., 2011). Cumulative P losses in runoff were reduced by 55% over three years using injection compared to broadcast application (Pote et al., 2011). The poultry litter injector used in this study was an improved design from that used in previous studies (Pote et al., 2003; Pote et al., 2011; Watts et al., 2011), so the current manuscript is the first to report data from the latest dry litter injection technology. The new injector design differs from the old prototype in several respects, but only those that most directly affect litter placement and soil disturbance are given here. Spacing between soil openers (and resulting parallel subsurface litter bands) was decreased from 30 cm to 24 cm, so the new injector can apply more litter per hectare with a single pass. To accommodate the narrower band spacing, no depth-gauge wheels were included on soil openers of the new injector, and the mechanism for closing each soil trench filled with litter was changed from a pair of angled press wheels to a single convex press wheel.

Most research on manure injection has been done with liquid manure injection, older prototypes of the poultry litter injector, or on pasture, and there is a need to evaluate dry poultry litter injection using the Subsurfer in other cropping systems. More information is needed to determine what effects poultry litter injection has on nutrient cycling and losses relative to surface application. Therefore the objective of this study was to determine if injection of poultry litter would decrease NH_3 volatilization, change cycling of N calculated by mass balance,

increase soil inorganic N, and decrease nutrients in runoff when compared to traditional surface application.

Materials and Methods

Soil and Poultry Litter Collection and Analysis

Surface soil collected from a Braddock (fine, mixed, semiactive, mesic typic hapludult) soil series and Bojac (coarse-Loamy, mixed, semiactive, thermic typic hapludult) soil series were used for the volatilization and incubation studies (Table 1). The Bojac surface soil consisted of 71.2% sand, 21.5% silt, and 7.3% clay and the Braddock surface soil consisted of 46.2% sand, 43.9% silt, and 10.0% clay and will be referred to as the Sandy Loam and Loam soil, respectively, throughout. These soils were collected from the Eastern Shore AREC near Painter, VA and Kentland farms near McCoy, VA. Soil was sampled from a 0 to 10 cm depth range, sieved in the field through a 1.3 cm sieve, and air dried. Soil particle size analysis was conducted using the pipette method in combination with wet sieving (USDA-NRCS, 2004). Organic matter (OM) was determined by loss on ignition on a weight basis adapted from AOAC method 2.7.08 (Cunniff, 1996). The Loam soil had higher OM than the Sandy Loam soil (Table 1). Water pH was determined using a 1:1 volume to volume ratio of soil to distilled water. Soil solutions were stirred for 10 minutes to allow equilibration and analyzed with an automated pH analyzer (Kalra, 1995). The pH in the Loam and Sandy Loam soils used in the N mineralization, NH₃-N volatilization, and rainfall simulation studies were within the agronomic range for crop production (Maguire and Heckendorn, 2011). Mehlich-I P was extracted at a solution: soil ratio of 1:1 and filtered through a 0.45 µm filter (Mehlich, 1953). Extracts were analyzed on ICP-OES to determine P content. Mehlich-1 P levels were in the “low” category for VADCR (2005) soil P fertility ratings in the Loam (6 mgkg⁻¹) and Sandy Loam (3 mg kg⁻¹) soils used in the soil incubation and NH₃-N volatilization studies. For the rainfall simulations, soil Mehlich-1 P was in

the “high” category for soil P fertility ratings with 69 mgkg^{-1} . To determine initial soil NO_3^- -N and NH_4^+ -N, a representative soil sample was collected, dried in an oven at 57°C , and sieved through a 2mm sieve. A three gram subsample was extracted with 30 mL of 2M KCl and shaken for one hour (Bremner and Keeney, 1966). Samples were then filtered through a $0.45 \mu\text{m}$ filter connected to a vacuum pump. Filtered samples were refrigerated for up to one week until analysis on a Lachat flow injected colorimeter to determine NH_3 -N and NO_3^- -N concentrations with QuickChem sodium salicylate method 12-107-06-2-A (Hofer, 2001) and QuickChem 12-107-04-1-B using Cd reduction (Knepel, 2001), respectively.

Fresh poultry litter samples were collected for each experiment. Litter for the volatilization study was collected after cleanout from the top 2.5 cm of the litter pack, and litter for the incubation and rainfall simulation studies was a composite sample collected at time of cleanout. No inhibitor was used on any of the litters used in these experiments. Total N was measured by Kjeldahl (1883) digestion modified by Peters (2003) using concentrated sulfuric acid, and organic N was determined by subtracting inorganic N from total N. Ammonium-N was measured by distillation using a 1:10 manure to KCl extraction and the boric acid indicator method adapted from AOAC method 973.49 and EPA method 350.2 (Peters, 2003). Total P was quantified using wet ashing adapted from AOAC 985.01 (Peters, 2003). Moisture was calculated by drying a representative 10-20 g sample at 110°C until a constant weight was achieved (Peters, 2003). Average values in poultry litters tested in Virginia are 26.7 gkg^{-1} organic-N, 5.7 gkg^{-1} NH_4^+ -N, 11.4 gkg^{-1} total P, and 27.8% moisture content (VADCR, 2005). The P and organic N in poultry litter for the N mineralization, NH_3 -N volatilization, and rainfall simulation studies (Table 2) were similar to these average values in Virginia. Poultry litter used for the rainfall simulations had higher NH_4^+ -N than the Virginia averages and the poultry litter used in

the other two studies. In all studies, percent moisture of poultry litter was lower than average values in Virginia (VADCR, 2005).

Ammonia Volatilization Study

Setup and procedures for the ammonia volatilization chambers described by Woodward et al. (2011) were followed. Soil was added to 100 x 150 mm (diameter x depth) enclosed glass chambers, brought to 70% field capacity and placed in temperature controlled boxes. Field capacity was determined by saturating 300 g of soil in cups with holes at the bottom. The cups were reweighed after 2 days of free draining, and field capacity was calculated on a weight by weight basis (Cassel and Nielsen, 1986). Chambers with 500 g dry soil were brought to 70% field capacity and kept in the boxes overnight to bring the soil to temperature (26°C). After initial setup, soil moisture was not adjusted. Three boxes containing six volatilization chambers were used in a randomized complete block design. Three treatments (surface applied poultry litter, injected poultry litter, and no poultry litter control) were combined factorially with two soils (Loam and Sandy Loam) to generate six treatments, which were arranged in a randomized complete block design with three replications. Each replication of a treatment was placed inside a temperature box. Poultry litter was collected fresh from a chicken house raising broilers (*Gallus gallus domesticus*) from the top 10 cm of the litter pack. There were three replicates of three treatments using the two soils totaling 18 chambers. Poultry litter was applied at a rate of 6.7 Mgha⁻¹ or 4.30 g pot⁻¹ to surface and injection treatments and injected using cardboard pieces to create an injection slit to a depth of 5 cm from the soil surface. The rate of 6.7 Mgha⁻¹ was based on the common application rate of poultry litter for pasture in Virginia. Injection slits were opened in the shape of a triangular prism along the diameter of the chamber. After injection, the cardboard pieces were removed, and the slit was closed with soil covering the bottom and top of the added poultry litter. The soil covering the litter was placed loosely to reflect conditions

similar to those created by the Subsurfer, where individual press wheels move soil over injection slits. The glass chambers were sealed and placed into the temperature controlled boxes which were kept at 26°C for the extent of the experiment. Air from the chambers was pumped through 100 mL of 0.02 M phosphoric acid scrubbing solution at a rate of 1 L min⁻¹, which is equivalent to 1.56 and 1.93 headspace exchanges per minute for Loam and Sandy Loam soils, respectively. Acid scrubber bottles were changed at 1, 3, 6, 9, 12, 18, 24, 36, 48, 60, 72, 96, 120, 144, and 168 hours. At time of sampling, bottles were weighed and refrigerated immediately. Samples were run on a Lachat flow injected colorimeter to determine NH₃-N concentrations using QuickChem phenol Method 10-107-06-1-G (Prokopy, 1993). Weight and concentration of the acid solution were used to calculate mass loss of ammonia. A three gram subsample of soil from the chambers was dried, ground, 2 mm sieved, and analyzed for NO₃⁻-N and NH₄⁺-N using the methods cited above.

Soil Incubation Study

The same two soils were used in this soil incubation, the Loam and Sandy Loam. Dry soil (100 g) of each series was added to 5.7 x 5.7 cm planter cups lined with coffee filters to prevent soil loss through drainage holes. There were three treatments: surface applied poultry litter, injected poultry litter, and no poultry litter control. Poultry litter was collected fresh from a chicken house raising broilers. There were four replicates × three treatments × five sampling intervals × two soils totaling 120 soil cups set up in a randomized complete block design. All soil cups were placed in an incubator at 21°C after amendment with litter and kept there until time of sampling. Sampling times were 0, 10, 20, 30, and 40 days. Poultry litter was applied at a rate of 6.7 Mgha⁻¹ or 2.20 g pot⁻¹ to surface and injection treatments based on the cup surface area. The soils were kept at 70% field capacity by watering every three days to maintain weight. Surface replicates were brought to 70% field capacity prior to addition of poultry litter to prevent

movement of ammonia into the soil. Poultry litter injected into the soil was simulated by adding a layer of dry soil to the bottom of each lined planter cup, and cardboard pieces were used to keep a slit in the shape of a triangular prism open for injection of poultry litter. Soil was added around the cardboard pieces, and poultry litter was injected to 5 cm depth to the bottom of the poultry litter band from the soil surface. After injection, the cardboard pieces were removed, and the slit was closed with soil covering the bottom and top of the added poultry litter. Then, the soil was brought to 70% field capacity. Sampling time 0 replicates were dried immediately after all sampling cups were prepped and placed in the incubator. At the appropriate sampling time, the entire 100 g soil for each replicate treatment was transferred from the planter cup. The filter paper and thin layer of soil in contact with the filter were removed to reduce any influence of the filter paper on soil N. The sample was then dried at 57°C, 2mm sieved, subsampled, and extracted for NO_3^- -N and NH_4^+ -N using the methods detailed above.

Rainfall Simulation Study

Rainfall simulation plots were installed as described by the National Research Project for Simulated Rainfall - Surface Runoff Studies (SERA-17, 2008) with slopes ranging from 9% to 11%. Runoff plots were 1.5 m × 2 m, rainfall intensity was 70 mm per hour and 30 mins of runoff was collected. Runoff collection plots were installed in September and October 2012 on a Braddock Loam (fine, mixed, semiactive, mesic typic hapludult) soil that was previously under corn production. The surface soil texture of the Braddock soil consisted of 46% sand, 44% silt, and 10% clay. A metal pan was installed at the downhill edge of each plot to drain runoff through a hose to a collection container. Borders of injection plots were installed after poultry litter was injected using the newest prototype of the Subsurfer produced by BBI in 2011 into the soil at a rate of 6.7 Mgha⁻¹ and depth of 5 cm to the bottom of the poultry litter band using the poultry litter Subsurfer (Pote et al., 2009). The Subsurfer injects the poultry litter by opening a

slit to 5 cm depth, dropping in poultry litter at the given rate, and closing the injection slit using press wheels. Therefore, the poultry litter was covered by soil after injection. Rainfall simulations were conducted on the same day as injection. For the surface application treatment, poultry litter was applied evenly over the plot by hand immediately prior to rainfall simulation. The time to runoff was recorded, and the total runoff was weighed to determine a volume. A 1-L subsample was collected after vigorous mixing of the total volume of runoff to ensure representative sampling of the sediment. Subsamples were immediately stored on ice and refrigerated at 4°C until filtering and/or analysis.

Unfiltered runoff water was analyzed for total Kjeldahl N (TKN) and P (TKP) using QuickChem Methods 10-107-06-2-D (Wendt, 1995a) and 10-115-01-1-C (Liao, 1998), respectively. A subsample of runoff water was filtered once samples reached the lab, no more than 6 hours after collection, through a 0.45 µm filter connected to a vacuum pump. Filtered runoff was analyzed for dissolved reactive P (DRP) colorimetrically using phosphomolybdate reduction outlined in QuickChem Method 10-115-01-1-A (Diamond, 1995). Ammonia-N and NO₃⁻-N were determined colorimetrically using QuickChem phenol Method 10-107-06-1-G (Prokopy, 1993) and QuickChem sulfanilamide Method 10-107-04-1-A (Wendt, 1995b), respectively. Sediment was determined by weight by taking 50 mL of runoff and drying at 110 °C until a constant weight was attained.

Statistical Analysis

Data were analyzed with ANOVA in JMP Pro 10 statistical package (SAS Institute, 2012). Means were separated using the Student's t-test. All statements of significance were declared at $\alpha < 0.05$. Error bars demonstrate one standard deviation of the mean.

Results and Discussion

Ammonia Volatilization

Relative to surface application, injection of poultry litter reduced $\text{NH}_3\text{-N}$ volatilization to that observed in the control throughout the 7 d volatilization study (Fig. 1). This is similar to results of Moore et al. (2011) where $\text{NH}_3\text{-N}$ losses were reduced to zero when simulating poultry litter injection into a pasture. Moore et al. (2011) used mobile wind tunnels to measure $\text{NH}_3\text{-N}$ loss in bermudagrass pasture for 14 days after poultry litter was applied to the surface or using simulated injection. This study is the first to investigate $\text{NH}_3\text{-N}$ loss from injection of poultry litter using a closed system, offering the opportunity to calculate a mass balance. Poultry litter applied to both treatments added 23.65 mg of $\text{NH}_4^+\text{-N}$ to each soil chamber. Cumulative losses of $\text{NH}_3\text{-N}$ from the Loam soil resulted in 3% of the $\text{NH}_4^+\text{-N}$ applied for injected and 121% for surface applied poultry litter. In the Sandy Loam soil, cumulative losses of $\text{NH}_3\text{-N}$ from injected and surface applied were 9% and 153%, respectively. Surface applied treatments probably had losses greater than 100% of $\text{NH}_4^+\text{-N}$ added through poultry litter application due to volatilization of $\text{NH}_3\text{-N}$ after ammonification of poultry litter and soil organic N. Injection of poultry litter decreased $\text{NH}_3\text{-N}$ volatilization by 98% and 94% for Loam and Sandy Loam soils, respectively, when compared to surface application. Reducing $\text{NH}_3\text{-N}$ volatilization is important from agricultural and environmental perspectives. Increasing $\text{NH}_3\text{-N}$ captured through injection could increase crop yields while reducing N released to the environment (Pote et al., 2011). Also, ammonia-N loss reduces the N:P of the poultry litter nutrients applied to soil, which can increase soil P levels over time when manures are applied to meet crop N needs (Pote et al., 2011). For surface application, the highest rate of $\text{NH}_3\text{-N}$ volatilization occurred between 12 and 24 hours with 1.00 mg h⁻¹ and 0.86 mg h⁻¹ for Loam and Sandy Loam soils, respectively. In both soils, neither injected nor control treatments exceeded 0.03 mg h⁻¹ of $\text{NH}_3\text{-N}$ volatilization. At the end

of the 7 day study, ammonia-N volatilization from surface application was still occurring at a rate of 0.09 mg h⁻¹ from the Loam and 0.11 mg h⁻¹ from the Sandy Loam. At 7 days, ammonia-N volatilization in control and injected treatments had ceased, and this agrees with the study by Moore et al. (2011) who observed negligible NH₃-N loss following poultry litter injected into pasture.

Soil Inorganic Nitrogen after 7-Day Ammonia Volatilization

Using inorganic N measured in the soil and NH₃-N loss, a mass balance of inorganic N was calculated (Table 3). For both soils, the injected treatment always had the greatest, and the control always had the lowest, soil NO₃⁻-N and NH₄⁺-N (Table 3). The NO₃⁻-N and NH₄⁺-N for surface applied litter was always numerically intermediate to injected and control treatments, but this difference was not significant for NO₃⁻-N in the Sandy Loam. Nitrate-N concentrations were likely higher in the Loam soil compared to the Sandy Loam soil due to increased nitrification from higher microbial activity in the Loam soil. Microbial activity would be elevated in the Loam soil due to higher soil OM content and larger specific surface area of the Loam soil, which correlates to higher water holding capacity (Brady and Weil, 2002). The control having the lowest total inorganic N was not surprising, as the other two treatments had NH₄⁺-N and organic N added with the poultry litter while the control did not. Differences between soil inorganic N (NO₃⁻-N + NH₄⁺-N), NO₃⁻-N, and NH₄⁺-N were determined using equation 1.

$$\text{Percent increase (\%)} = \left(\frac{|\text{Injected-Surface}|}{(\text{Surface-Control})} \right) (100\%) \quad \text{equation 2.1}$$

In the Loam soil, injection increased soil inorganic N by 71%, NO₃⁻-N by 47%, and NH₄⁺-N by 92%, when compared to surface application. In the Sandy Loam soil, the average soil NO₃⁻-N was 58.6% higher when poultry litter was injected versus surface applied but not significantly different. Injection increased soil inorganic N by 105% and NH₄⁺-N by 110%, compared to

surface application in the Sandy Loam soil. However, surface application increased total inorganic N in the system by 9.2% in the Loam soil and 10.6% in the Sandy Loam soil. This could indicate a difference in organic N mineralization rates between the two application methods.

The total inorganic N in the system was higher in surface treatments of both soil types relative to litter injection. Mineralization of litter organic N by 7 d for both surface and injected litter can be calculated using equation 2 for each treatment, where PL represents poultry litter and soil total inorganic N is the sum of soil inorganic N and NH₃-N volatilized.

$$\text{Org N min (\%)} = \frac{(\text{Soil total inorganic N} - \text{Control total inorganic N}) - (\text{PL NH}_4^+ - \text{N})}{\text{PL Organic N Added}} \times 100\%$$

equation 2.2

In both soil types, organic N mineralization was numerically higher in the surface treatment when compared to the injected treatment, however, these were not significant at the $\alpha=0.05$ level. In the Loam soil, organic N mineralization was 26.9% for injected and 30.8% for surface applied litter. Organic N mineralization was 22.2% for injected and 26.6% for the surface applied litter in the Sandy Loam soil. Higher organic N mineralization in the surface treatment was probably a result of increased aerobic decomposition due to constant supply of oxygen at the soil surface. Although there are no organic N mineralization rate comparisons between surface applied and injected poultry litter in the literature, these mineralization rates are similar to those found by Agehara and Warncke (2005) and Hanselman et al. (2004) for poultry litter mixed with soil. Agehara and Warncke (2005) found that organic N mineralization was 29.5% for poultry litter mixed with a sandy clay loam soil after 1 week of incubation at 25/20 °C (14/10 h). Hanselman et al. (2004) found slightly higher organic N mineralization of 38%, 3 days after incorporation of poultry litter with soil at 30 °C. Higher temperatures are likely the reason for higher organic N

mineralization found by Hanselman et al. (2004) when compared to this study. Further research is needed to evaluate how mineralization of organic N would proceed over the course of a full growing season for injected versus surface applied poultry litter. While organic N mineralization was higher for surface treatments, total soil inorganic N was higher in injected treatments due to increased $\text{NH}_3\text{-N}$ capture.

Soil Incubation

Unlike the volatilization study, there was no forced air flow for the soil incubation and ammonia loss was not measured. Therefore a mass balance was not possible, but this enabled changes in forms of soil inorganic N to be seen with time with destructive sampling (Table 4). Injected and surface applied litter treatments always had greater $\text{NO}_3^- \text{-N}$ and $\text{NH}_4^+ \text{-N}$ compared to the unamended control, due to the N added with the litter. As reported earlier, ammonia-N volatilization was greatly reduced by injecting litter, as compared to surface application. This captured ammonia shows up as increased $\text{NH}_4^+ \text{-N}$ in the injected versus surface treatments at 10 d for both soils. Injection limits the interaction of poultry litter with the atmosphere by reducing the surface area exposed at the soil surface and increasing contact with the soil (Dell et al., 2011). The increase of soil $\text{NH}_4^+ \text{-N}$ (41% and 38% for Loam and Sandy Loam, respectively) from using injection was not as dramatic in the soil incubation as in the volatilization study (71% and 110% for Loam and Sandy Loam, respectively). This could be attributed to a lack of $\text{NH}_3\text{-N}$ volatilization from the surface treatment due to little air movement in the incubation study. Higher wind speed increases the intensity of $\text{NH}_3\text{-N}$ volatilization by increasing the mass transfer and air exchange between the surface of the manure and the atmosphere (Meisinger and Jokela, 2000). However, it is still clear that injection increased soil $\text{NH}_4^+ \text{-N}$ when compared to traditional surface application, even with minimal airflow. After 10 d, the $\text{NH}_4^+ \text{-N}$ decreased in both soils in injected and surface applied litter. During this decrease in $\text{NH}_4^+ \text{-N}$ there was a

concomitant increase in NO_3^- -N, presumably due to nitrification. To determine differences in NH_4^+ -N and NO_3^- -N between surface applied and injected treatments, the control was subtracted from each treatment, and the percent increase was calculated (equation 1). Injection of poultry litter increased total inorganic N by 52% and 99% for Loam and Sandy Loam soils, respectively, when compared to surface application. As discussed in the previous study, increased capture of NH_3 -N is the main reason for increased inorganic N at 40 days. These results agree with those of the volatilization study where total inorganic N in the soil was increased by 71.0% in the Loam and 105% in the Sandy Loam soil.

Nitrate-N in all treatments in both soil types increased throughout the experiment from nitrification of NH_4^+ -N and mineralization of organic N (Table 4). Nitrate-N is used as a measure for plant available N (PAN), and injection has the potential to significantly increase it (Maguire, 2008). Elevated NO_3^- -N concentrations are beneficial for crop production because plants take up NO_3^- -N more than other forms of N (Eckert, 2007). Therefore, higher NO_3^- -N production could lead to increased crop yields. In the Loam soil, the injected treatment increased NO_3^- -N concentrations by 81, 58, and 57% at 20, 30, and 40 day sampling times, respectively, when compared to surface application. In the Sandy Loam soil, the injected treatment increased NO_3^- -N concentrations by 53, 56, and 60% at 20, 30, and 40 day sampling times, respectively.

Rainfall Simulation

The time between start of rainfall to initial runoff did not vary between treatments, and the mean time before initial runoff was 19 minutes. There was no difference in mass of runoff collected between treatments, and the mean runoff mass collected was 18.04 kg. Sediment loss did not vary between treatments, and the mean sediment concentration was 2.3 gkg^{-1} in runoff water (data not shown). Injection of poultry litter reduced TKN, TKP, DRP, dissolved NO_3^- -N, and dissolved NH_3 -N in runoff water to concentrations similar to those of the control (Table 5).

When comparing concentrations of nutrients in runoff, equation 3 was used to determine differences between injected and surface applied.

$$\text{Change in runoff nutrient content (\%)} = \left(\frac{\text{Surface} - \text{Injected}}{\text{Surface}} \right) (100\%) \quad \text{equation 2.3}$$

Injection of poultry litter decreased concentrations of TKN by 59%, TKP by 53%, DRP by 96%, dissolved NO_3^- -N by 73%, and dissolved NH_3 -N by 99% in runoff water, when compared to surface application. The TKN and TKP differences are smaller than those found in similar research by Watts et al. (2011), who reported reductions of 86, 86, 91, and 90% in DRP, TKP, total inorganic N, and TKN, respectively, when using injection compared to surface application. In the study by Watts et al. (2011), rainfall simulations were conducted on established bermudagrass pasture. Intense rainfall events on an established pasture or forage setting will generate less soil disturbance when compared to fields used for row crops, such as the one used in this study, from increased root density and surface cover (Power and Dick, 2000).

Concentrations of TKN, TKP, DRP, NO_3^- -N and NH_3 -N were not statistically different for injected litter and the unamended control. This is similar to results of Adeli et al. (2011), Watts et al. (2011), Pote et al. (2009), Pote et al. (2003), Kibet et al. (2011), and Sistani et al. (2009) where losses of N and P in runoff were reduced to that of the control when poultry litter was incorporated or injected. This confirms the effectiveness of the new prototype to reduce nutrients lost in runoff after injection into corn residue.

Conclusions

Poultry litter injection is a new technology that can place nutrients below the soil surface, and is compatible with no-till and forage systems. Results presented here show that injection increases soil NO_3^- -N concentrations relative to surface application, mostly by decreasing volatilization of NH_3 -N. With increased retention of N in soil, injection could reduce the need for

inorganic N fertilizer. This could decrease fertilizer costs and/ or increase crop yield for farmers. Injection also protects water quality by reducing N and P in runoff, relative to surface application. When poultry litter was injected, nutrients were reduced to levels of the control, where no manure was applied. Therefore, injection could be used as a best management practice for manure application, allowing farmers to capture more valuable N from poultry litter, while decreasing environmental impacts.

Table 2.1. Soil physical and chemical properties for Bojac and Braddock soils.

Experiment	Soil series	OM g kg ⁻¹	pH	Surface Soil texture	Mehlich-1 P mg kg ⁻¹
Volatilization and Incubation	Braddock	52	6.48	Loam	6
Volatilization and Incubation	Bojac	17	6.51	Sandy Loam	3
Rainfall Simulation	Braddock	47	6.29	Loam	69

Table 2.2. Poultry litter chemical properties for three experiments determined on “as is” basis.

Experiment	Organic N	Ammonium-N	P	Moisture
Soil Incubation	29.1	5.9	10.4	20.4
Ammonia Volatilization	34.0	5.5	10.2	19.2
Rainfall Simulation	39.1	8.4	15.0	18.9

Table 2.3. Nitrogen mass balance using soil Nitrate-N, soil ammonium-N, and ammonia-N volatilization loss after surface, injected, and control treatments for a 7 day volatilization study using two soil types with Sandy Loam and Loam surface soil textures.

Soil Type	Treatment	Ammonia-N		Soil Nitrate-N		Soil Ammonium-N		Total inorganic N	
		Loss							
		mg							
Loam	Control	0.1	b†	53.2	c	2.0	c	55.4	b
	Injected	0.7	b	78.8	a	38.8	a	118.3	a
	Surface	32.3	a	70.5	b	21.2	b	124.1	a
Sandy Loam	Control	0.1	b	7.9	b	6.1	c	14.0	c
	Injected	2.3	b	12.5	a	55.3	a	70.1	b
	Surface	36.3	a	10.8	ab	29.5	b	76.6	a

† Means within the same column for each soil type followed by different letters are significantly different at a 0.05 probability level

Table 2.4. Soil Nitrate-N and Ammonium-N in 10 day increments over a 40 day soil incubation study using soil types with Sandy loam and Loam surface soil textures.

Soil Type	Treatment	Nitrate-N										Ammonium-N									
		days																			
		0†	10	20	30	40	0†	10	20	30	40	0†	10	20	30	40	0†	10	20	30	40
		mg kg ⁻¹																			
Loam	Control‡	45.9	Dc	91.1	Cb	96.5	BCc	104.3	Bc	115.3	Ac	20.6	Ab	7.5	Bc	3.4	Cb	5.6	BCb	3.3	Cc
	Injected	52.3	Db	121.8	Ca	284.8	Ba	298.6	Ba	324.1	Aa	122.1	Aa	126.2	Aa	22	Ba	7.8	Cb	5.2	Cb
	Surface	55.3	Da	113.7	Ca	200.5	Bb	226.9	Ab	248.3	Ab	125.7	Aa	91.5	Bb	27.8	Ca	14.7	Da	8.8	Da
Sandy Loam	Control	2	Db	12.2	Cb	17.1	Abc	19.2	Ac	15.9	Bc	5.9	Bb	6.7	Ac	2.3	Dc	4.2	Cc	2.1	Db
	Injected	9.5	Da	13.6	Dab	60.1	Ca	124.9	Ba	170.6	Aa	97.7	Ba	124.2	Aa	103	Ba	85.4	Ca	57	Da
	Surface	9.5	Da	14.8	Da	45.2	Cb	86.8	Bb	112.3	Ab	96.9	Aa	91.6	Ab	51.9	Bb	25.7	Cb	11.3	Db

† Means within the same column for each soil type followed by the same lower case letter are not significantly different at a 0.05 probability level

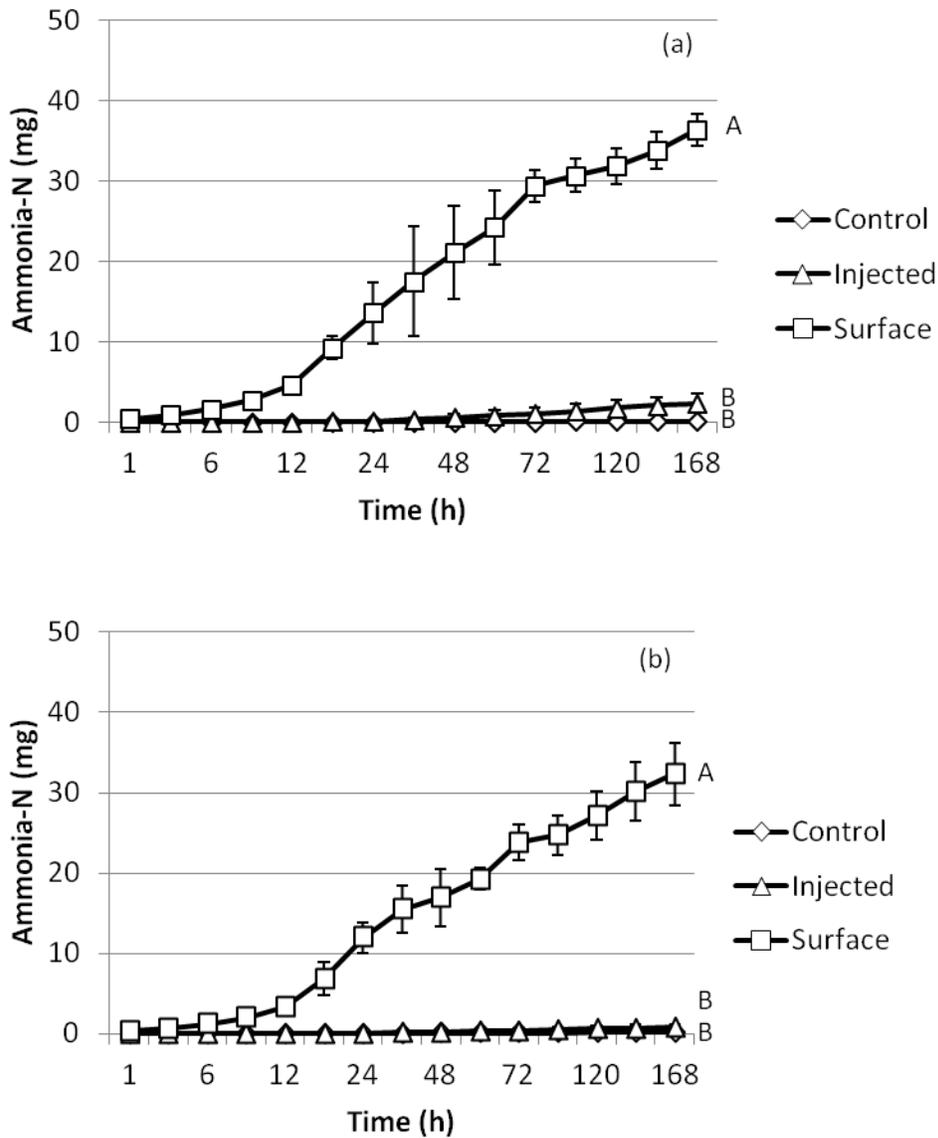
‡ Means within the same row followed by a the same upper case letter are not significantly different at 0.05 probability level

Table 2.5. Total Kjeldahl N (TKN), Total Kjeldahl P (TKP), dissolved reactive P (DRP), dissolved nitrate-N, and dissolved ammonia-N concentrations (mg L^{-1}) in runoff water collected from a single rainfall simulation immediately after surface, injection, and control treatments were applied to an agricultural soil.

Treatment	TKN	TKP	DRP	Water Nitrate-N	Water Ammonia- N
				mg L^{-1}	
Control	14.3b†	3.1b	0.2b	1.3ab	0.1b
Injected	23.7b	4.4b	0.2b	0.6b	0.1b
Surface	57.7a	9.3a	3.8a	2.1ab	8.5a

† Means within the same column followed by the same letter are not significantly different at a 0.05 probability level

Figure 2.1. Cumulative ammonia-N loss from surface, injected, and control treatments throughout a 7 day volatilization study using soil types with a) Sandy Loam and b) Loam surface soil textures. Different letters indicate significant differences between treatments from hour 1 through the extent of the 7 day volatilization study. Error bars represents one standard deviation.



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Chapter 3: Effects of poultry litter injection on orchardgrass hay yield and quality

Abstract

Traditional surface application of poultry litter leaves nutrients vulnerable to loss through volatilization and runoff. However, injection can increase capture of these nutrients in agricultural fields. Therefore, a field experiment was conducted to determine the effects of poultry litter injection on orchardgrass (*Dactylis glomerata* L.) hay yield and quality. Poultry litter was injected or surface applied using the Subsurfer poultry litter injector at the recommended agronomic rate (high) and half that rate (low) in 2012 and 2013 in an established field of orchardgrass. Soil was sampled to 15 cm and analyzed for soil nitrate. No significant differences in soil nitrate were detected between treatments. Though not always statistically significant, first cutting orchardgrass yields tended to be greater with surface litter application. Injected treatments had greater protein concentrations than their respective surface treatment, showing greater nitrogen uptake, when protein was weighted by yield. Protein was the same for high surface and low injected treatments showing that similar N uptake was achieved.

Introduction

Manures are typically surface applied to agricultural fields and sometimes incorporated through tillage to provide nutrients that are valuable for crop production. While tillage is fast and cost efficient, tillage is not compatible with no-till or forage systems and can destroy benefits provided by no-till farming, which means surface application of manure is common in these settings (Maguire et al., 2011a). However, traditional surface application leaves manure on the soil surface vulnerable to loss through runoff and volatilization of ammonia-N ($\text{NH}_3\text{-N}$). Recent technical advances mean that manure can be incorporated into the soil without tillage, using manure injection techniques (Maguire et al., 2011b).

Manure injection systems create minimal soil disturbance while partially burying surface residues, which could make them compatible with no-till and forage systems (Dell et al., 2011). However, commercially available equipment for injecting manure only accommodates liquid manure, and much of the research available on manure injection excludes dry poultry litter. Poultry litter injection using the Subsurfer places poultry litter into the soil without tillage, reducing soil disturbance (Pote et al., 2011). Injection of manure reduces N and P losses from agricultural fields in runoff and $\text{NH}_3\text{-N}$ volatilization relative to surface application (Maguire et al., 2008; Maguire et al., 2011a; Maguire et al., 2011b; Moore et al., 2011; Pote et al., 2003; Pote et al., 2009; Pote et al., 2011; Watts et al., 2011).

Watts et al. (2011) reported that losses of N and P in runoff were reduced to those of a no poultry litter control when poultry litter was injected. Manure injection not only reduces nutrient losses but also increases the N use efficiency of manures (Maguire et al., 2008). Ammonia-N volatilization was reduced to zero when poultry litter was injected (Moore et al., 2011). Reductions in $\text{NH}_3\text{-N}$ volatilization are due to increased contact of manure with soil solids and decreased contact of manure with the atmosphere, which increases immobilization of ammonium-N ($\text{NH}_4^+\text{-N}$) (Dell et al., 2011). Although there is only limited research available, this decrease in ammonia volatilization by poultry litter injection should increase the amount of N in the soil available for crop uptake. For example, a laboratory study by Kulesza et al. (2014) simulated poultry litter injection and reported increased soil total inorganic N when compared to surface application. Increased N availability has the potential to increase yields because N is the most common limiting nutrient in agricultural production and can increase the quality of crops produced (Eckert, 2007). A major concern for forage production is maintaining forage quality that supports the desired level of weight gain or milk production, which is partially dependent on

N. Crude protein, acid detergent fiber (ADF), neutral detergent fiber (NDF), and total digestible nutrient (TDN) concentrations can be used as indicators of feedstock quality (Ball et al., 2001). Although not always statistically significant, poultry litter injection showed a tendency to increase yields and crude protein of bermudagrass hay (Pote et al., 2009). When poultry litter was injected rather than surface applied, corn yields increased by 36% and 20% in 2008 and 2009, respectively (Pote et al., 2011). With increased nutrient capture, crop yield, and crop quality, poultry litter injection could be a valuable alternative to traditional surface application, providing agricultural and environmental benefits.

While much research has been done on liquid manure injection, more research is needed to evaluate injection of dry poultry litter. Information is needed to determine the effects of poultry litter injection on yield and quality of crops when compared to traditional surface application. Therefore, the objective of this study was to determine the effect of poultry litter injection on soil inorganic N, yield, and quality of orchardgrass hay when compared to traditional surface application.

Materials and Methods

Site and Soil Description and Poultry Litter Analysis

This study was conducted on a private farm near Mauzy, VA in the Shenandoah Valley. Prior to this study, the orchardgrass plots had been under established orchardgrass hay production. Field soils were fairly uniform ranging from the Frederick (fine, mixed, semiactive, mesic typic paleudult) to the Lodi (fine, mixed, subactive, mesic typic hapludult) soil series. Initial soil samples (n=5 cores) were combined from 0-15 cm depth, dried, and sieved (2mm) prior to initiation of these field studies in 2012. Organic matter concentration was determined on a weight basis using loss on ignition adapted from AOAC method 2.7.08 (Cunniff, 1996). Soil organic matter concentration in the orchardgrass field was 3.1 g kg⁻¹ prior to starting the

experiment in 2012. Water pH was determined using a 1:1 soil to distilled water volume to volume ratio stirred for 10 minutes to allow equilibration. Water pH was then measured using an automated pH analyzer (Kalra, 1995). The pH was 6.61 and within agronomic range for crop production (Maguire and Heckendorn, 2011). Mehlich I P was determined using a method adapted from Mehlich (1953) as described by Maguire and Heckendorn (2011) by extraction of a 1:1 solution:soil ratio, filtered (Whatman #2), and analyzed on an ICP-OES (Mehlich, 1953). Mehlich-I P was in the “very high” category at 100 mg kg^{-1} in the orchardgrass field.

Average values of organic N, NH_4^+ -N, total P, and moisture in poultry litters tested in Virginia are 26.7 g kg^{-1} , 5.7 g kg^{-1} , 11.4 g kg^{-1} , and 27.8%, respectively (VADCR, 2005). Fresh poultry litter samples were collected at the time of application each year. Organic N was determined by Kjeldahl (1883) digestion using the concentrated sulfuric acid method as described by Peters (2003). In 2012, poultry litter had 28.7 g kg^{-1} organic N and 33.6 g kg^{-1} in 2013. Also, litter from both years had higher organic N concentrations than the average organic N of poultry litters tested in Virginia (VADCR, 2005). To determine NH_4^+ -N, poultry litter was extracted with KCl at a 1:10 litter to KCl ratio, and ammonium-N was determined using the boric acid indicator method adapted from AOAC method 973.49 and EPA method 350.2 (Peters, 2003). Ammonium-N was 8.1 g kg^{-1} in 2012 and 7.4 g kg^{-1} in 2013, and both litters had higher NH_4^+ -N than the average of litters tested in Virginia (VADCR, 2005). Total P was determined by digestion with concentrated nitric acid using the wet ashing method as described by Peters (2003) adapted from AOAC 985.01 and analyzed on ICP-OES. The concentration of P in poultry litter was 8.0 g kg^{-1} in 2012 and 12.3 g kg^{-1} in 2013. A representative 10-20 g subsample of litter was dried at 110°C until a constant weight was achieved to determine moisture (Peters, 2003). Poultry litter from both years had lower moisture than averages in Virginia (VADCR, 2005).

Orchardgrass Hay Trials

Orchardgrass hay plots 2.5 m x 6.1 m were laid out in a split-plot design with application rate being the main plot and application method the subplot. Four treatments were used with four replications: high rate injection (HI), high rate surface (HS), low rate injection (LI) and low rate surface (LS) applications totaling 16 plots. All plots were treated with poultry litter collected on site from houses for broiler (*Gallus gallus domesticus*) production. Poultry litter was applied using the Subsurfer poultry litter injector (Pote et al., 2009) to ensure consistent application rate, with the coulters being lifted above ground for the surface application. Poultry litter was injected to a depth of 5 cm as described by Pote et al. (2009). High rate application was determined using a nitrogen based application rate, poultry litter analysis, soil productivity grouping, and state nutrient management regulations (VADCR, 2005). Plant available N from the poultry litter application was calculated using 60% organic N mineralization in a growing season for all treatments and 95% NH_4^+ -N availability for the injected treatment (VADCR, 2005). There is no book value for NH_4^+ -N availability when injecting poultry litter, therefore the values for liquid manure injection (95%) were used (VADCR, 2005). In 2012, the high rate (10.80 Mg ha^{-1}) application was determined assuming 95% NH_4^+ -N capture and 60% organic N mineralization for the injected treatment (Table 1). As NH_4^+ -N capture for surface application is only 50%, the calculated plant available N (PAN) for HS was slightly below the recommended PAN (VADCR, 2005). Low rate application was half of the high rate application at 5.40 Mg ha^{-1} because an N response from injection would be more easily detected with a suboptimal N rate. Poultry litter was injected to a depth of 5 cm as described by Pote et al. (2009) on March 28, 2012 and April 23, 2013 into a field of orchardgrass established using the Shilo cultivar, and treatments were applied to the same plots in both years. According to VADCR (2005), poultry litter can be applied at 90 kg N ha^{-1} per expected cutting not to exceed 280 kg N ha^{-1} annually. In 2012,

poultry litter was applied at 269 kg N ha⁻¹ for the HI treatment in one spring application with anticipation of three hay cuttings. As only two cuttings were achieved in 2012, high rate and low rate poultry litter treatments were applied at 179 kg N ha⁻¹ (7.20 Mg ha⁻¹) and 90 kg N ha⁻¹ (3.60 Mg ha⁻¹), respectively, with anticipation of two hay cuttings in 2013. However, three cuttings were achieved in 2013 due to good growing conditions. Reference plots (3.0 m x 6.1 m) were laid out in a randomized complete block design with three replicates of four urea rates. Reference plots were fertilized using split applications of urea. For reference plots, five urea rates were used with three replications: 0, 22, 45, 67, and 90 kg N ha⁻¹ for each cutting totaling 15 plots. These N rates were applied once in spring and after each cutting except the last one in fall. Potash was applied each spring to reference plots at a rate of 151 kg K ha⁻¹ based on soil test recommendations (VADCR, 2005). Forage samples were collected by harvesting 0.76 m width at 7.5 cm cutting height from the middle of the plot after plot edges had been mowed using a walk behind harvester. Forage yields were determined by dividing the weight of sample by the area of cutting. At time of cutting, a subsample was collected, weighed, dried to a constant weight at 57°C, and ground using a Wiley mill fitted with a 1mm screen. Samples were then analyzed using a FOSS XDS NIR rapid content analyzer (FOSS NIR Systems, Laurel, MD, USA) to determine protein, ADF, NDF, and TDN. Using equation 1, TDN was calculated using the western formula (Adams, 1980).

$$TDN\% = 80.4 - (0.481 * ADF\%) \quad \text{equation 3.1}$$

Protein was weighted by yield to determine the protein content of the cumulative forage collected annually. Weighted protein (WP) was determined using the protein and yield of each cutting (equation 2).

$$WP = \frac{\left(cut\ 1\ protein\ \left(\frac{Mg}{Mg}\right) * yield\left(\frac{Mg}{ha}\right) \right) + \left(cut\ 2\ protein\ \left(\frac{Mg}{Mg}\right) * yield\left(\frac{Mg}{ha}\right) \right) + \dots}{total\ yield}$$

equation 3.2

Milk equivalence was then determined using the University of Wisconsin MILK2006 equation for alfalfa (Undersander et al., 2006). Fertilizer N equivalence was determined by inputting each treatment yield into the respective inorganic N yield response curve equation of each cutting or cumulative yield found in figure 1.

Soil samples were collected from 0-15 cm to determine soil inorganic N approximately 3 months after litter application in 2012 and 2 months after application in 2013. Soil samples within the litter treatment plots and reference plots were collected, dried at 57 °C, ground, sieved (2mm), and analyzed for NO₃⁻-N and NH₄⁺-N. For NO₃⁻-N and NH₃-N determination, a three gram subsample was extracted with 30 mL of 2M KCl by shaking for one hour (Bremner and Keeney, 1966). Samples were filtered through a 0.45 µm filter connected to a vacuum pump and refrigerated until analysis. Nitrate-N and NH₃-N were determined colorimetrically using QuickChem 12-107-04-1-B (Knepel, 2001) by means of Cd reduction and QuickChem sodium salicylate method 12-107-06-02-A (Hofer, 2001) on a Lachat flow injected colorimeter.

Statistical Analysis

Data were analyzed using analysis of variance, and mean separation was achieved using the Student's T test in JMP Pro 10 (SAS Institute, 2012). Statements of significance were declared at $\alpha < 0.1$ for main treatment factors. Analysis of variance was used to determine significant linear or quadratic regression fits when conducting a simple linear regression. Error bars in all figures represent the standard deviation of the mean.

Results and Discussion

Orchardgrass Hay Trials

Soil Nitrate

In 2012, all treatments were similar with 6.44, 3.67, 3.08, and 3.38 mg kg⁻¹ NO₃⁻-N for HI, HS, LI, and LS treatments, respectively. In 2013, it is suggested that soil NO₃⁻-N was much greater than in 2012 due to more rainfall, warmer temperatures, and less time for plant uptake of N between application and soil sampling. Between application of manure and sampling for soil NO₃⁻-N, the average temperature for 2012 was 23.2°C with 17.5 cm of precipitation. In 2013, the average temperature was 24.3°C with 20.0 cm precipitation between application of manure and sampling for soil NO₃⁻-N. There were no significant differences in soil NO₃⁻-N in 2013. However, the average soil NO₃⁻-N was numerically greatest for the HI treatment with 34.13 mg kg⁻¹, but this was not significantly greater than the HS treatment which had 27.71 mg kg⁻¹. There were no significant differences between low injected and low surface treatments for soil NO₃⁻-N in either 2012 or 2013.

Yield

In 2012, average yield of the first cut was similar across treatments (Table 2). Nitrogen would not have been a limiting factor for the first cut of hay, as sufficient N was applied at the start of the season for 3 cuts over the growing season. Therefore, any extra N availability would be expected to show up later in the growing season. For the second cut of 2012 and for the cumulative yield, there were no significant differences between injection and surface application for either high or low poultry litter rates. Due to more rain and better orchardgrass regrowth, an extra hay cutting was harvested in 2013 than in 2012. In 2013, there was a similar trend for hay growth between the treatments. There was no significant difference between HI and HS treatments throughout the growing season. However, yield of LS was greater than LI in the first

cut of 2013 with 3.0 Mg ha^{-1} and 2.5 Mg ha^{-1} , respectively, indicating surface application may provide more N initially due to rapid aerobic microbial activity at the soil surface. However, looking at the cumulative yields from all 3 cuts in 2013, there was no effect of injection on yield, with $HI = HS \geq LI = LS$. These results were similar to those found by Warren et al. (2008) but different to those found by Pote et al. (2003) and Pote et al. (2009). Warren et al. (2008) found that yield was the same when injecting or surface applying poultry litter into tall fescue and bermudagrass fields. This study further supports literature showing that injection has no detrimental impact on yield in warm or cool season perennial grass production due to root damage during application in the spring. Pote et al. (2003) found that cumulative forage yields were 25% higher when injection was simulated; however, these differences were not always significant. Pote et al. (2003) injected by hand and stepped on the injection slits to ensure closure which could have caused less soil disturbance and increased closure of injection slits when compared to this study, which used mechanized injection. Pote et al. (2009) found that the total yield was increased by 40% when poultry litter was injected compared to surface broadcast, but these differences were not always significant.

Yield response curves showed quadratic relationships for cut one yield and cumulative yield in 2012 and cut two yield, cut three yield, and cumulative yield in 2013 in response to increased inorganic fertilizer application (Fig. 1). Linear relationships were found for cut two in 2012 and cut one in 2013 in response to increasing inorganic fertilizer applications. Using the first derivative of the quadratic equations, the optimum N application rate was 43 kg N ha^{-1} cutting⁻¹ for cut one in 2012, and 69 and 59 kg N ha^{-1} cutting⁻¹ for cut two and cut three,, respectively, in 2013. For cumulative yields, optimum N application rate was 154 and 128 kg N ha^{-1} for 2012 and 2013, respectively. Optimum N rate could not be calculated for cut two in 2012

or cut one in 2013 because the optimum N rate was exceeded by the application rates used in this experiment. In the first cut of 2012, the N equivalence was greatest in HS treatment with 12.3 kg N ha⁻¹ and lowest in the LI treatment with 2.3 kg N ha⁻¹. LS and HI treatments were intermediate with 10.6 kg N ha⁻¹ and 6.7 kg N ha⁻¹, respectively, and similar to the HS and LI treatments (data not shown). In the second cut of 2012, the N equivalence was 51.9, 22.7, 17.3, and 11.5 kg N ha⁻¹ for HI, HS, LS, and LI, respectively. However, the HS treatment was not significantly different from any other treatment, and the LS treatment was similar to the LI treatment. Using cumulative yield of 2012, HI had the greatest and LI had the lowest N equivalence provided by poultry litter application. HS and LS were intermediate but were similar to HI and LI treatments. In all cuts of 2013, there were no differences in N equivalence between any treatments.

Forage Quality

No observable trends were found between treatments for ADF, NDF, and TDN in 2012 or 2013 (data not shown). The protein concentration in the first cut of hay from 2012 was greatest in the HI treatment with 17.1% and lowest in the LS treatment with 12.7% (Fig. 2a). The HS and LI treatments had similar protein concentrations and yields in cut one and two in 2012. Therefore, N uptake was similar for HS and LI treatments, which indicates that injection increased NH₄⁺-N capture which provided more N for crop production when using injection. Protein is directly related to N uptake by forages (Malzer and Schoper, 1984; Johnson et al. 2001). Johnson et al. (2001) found that increasing inorganic N fertilization rates elevated crude protein in bermudagrass. Malzer and Schoper (1984) showed that crude protein in orchardgrass, reed canary grass (*Phalaris arundinacea*), and quackgrass (*Elytrigia repens*) increased with increasing N rates using inorganic fertilizer. Increased protein in injected treatments can be related to soil NO₃⁻-N values discussed earlier, where soil NO₃⁻-N concentrations were similar for HS and LI treatments, therefore the amount of N available for plant uptake and protein

synthesis was the same. Also, protein concentrations for both injected treatments were greater than their corresponding surface treatments in cut one of 2012 which correlates to higher N use efficiency of poultry litter N when using injection. In the second cut of 2012, all treatment combinations had similar protein concentrations. In the first cut of 2013, HI had the greatest protein concentration with 13.9% and the LS treatment had the lowest with 10.8% (Fig. 2b). HS and LI treatments were not significantly different in the first cut of 2013 and intermediate to the other two treatments with 12.9% and 12.6%, respectively. In the second and third cut of 2013, there were no significant treatment effects on protein concentration. When protein concentration was weighted by yield in 2012, it followed the order $HI > HS = LI > LS$. Therefore, for both the high and low rates, injection increased nitrogen use efficiency of the manure applied in 2012 (Fig 2c). The trend was similar in 2013, although HI was not significantly greater than HS. On average, protein was 1.2% and 2.0% greater for high and low rate injection treatments, respectively, relative to the equivalent surface application rate in 2012, and protein was 0.5% and 1.0% greater than the respective surface application rate for high and low rate, respectively, in 2013. Similar results were found by Mooleki et al. (2001) and Pote et al. (2009). Mooleki et al. (2001) found that N concentration was higher in wheat, barley, and canola when dairy manure was injected, however this was not always significant. Pote et al. (2009) found that mean protein and digestibility of bermudagrass was higher for injected poultry litter when compared to surface broadcast, although not always significant. Pote et al. (2009) found a range of 13% to 21% protein concentration for forages when poultry litter was surface applied or injected. Protein averages reported by Pote et al. (2009) are greater than averages found in this study. However, Pote et al. (2009) injected into bermudagrass, a warm season grass, pasture while this study involved orchardgrass, a cool season grass, pasture. Therefore, this study shows that injection

can increase protein content when compared to surface application in a cool season grass. Protein and TDN are common indicators of nutritive value of feedstocks (Adesogan et al., 2009). Greater protein and TDN would correlate to higher quality feed. Using the MILK2006 equation, calculated milk equivalence showed a general trend of HI > HS > LI > LS (data not shown). However, there were no significant differences between treatments.

Conclusions

Poultry litter injection using the Subsurfer had a tendency to increase average soil NO_3^- -N compared to surface application, but cumulative orchardgrass yields showed no differences between treatments, which could indicate over application of poultry litter. However, there was frequently increased protein when injecting poultry litter, versus surface applying at the same rate. With previously established environmental benefits and increased quality of orchardgrass hay, injection could be a valuable alternative to traditional surface application in agricultural production systems.

Table 3.1. Poultry litter and plant available N (PAN) from litter applied to orchardgrass plots.

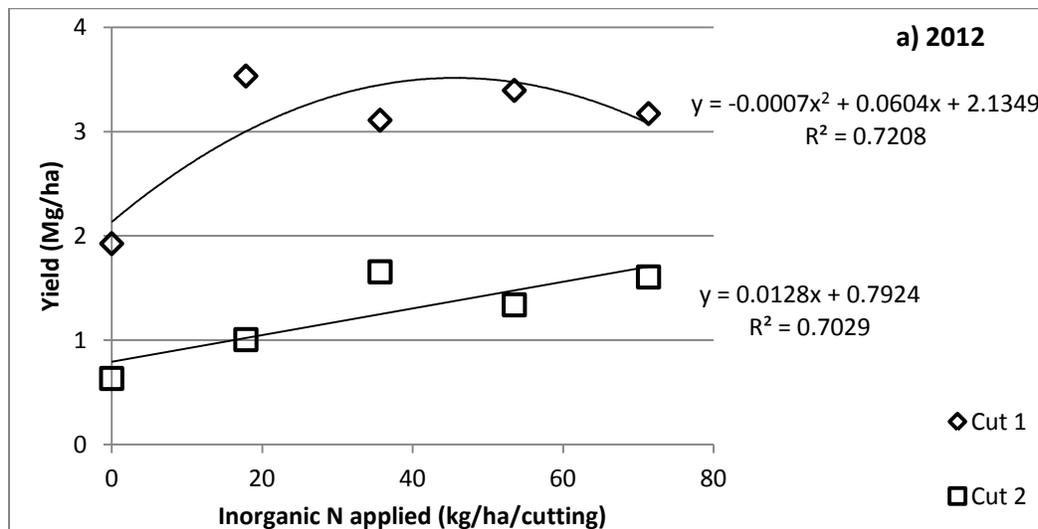
		2012		2013	
Rate	Application method	Litter rate	PAN	Litter rate	PAN
		Mg ha ⁻¹	kg ha ⁻¹	Mg ha ⁻¹	kg ha ⁻¹
High	Injected	10.8	269	7.3	179
	Surface	10.8	230	7.3	153
Low	Injected	5.4	135	3.7	90
	Surface	5.4	115	3.7	77

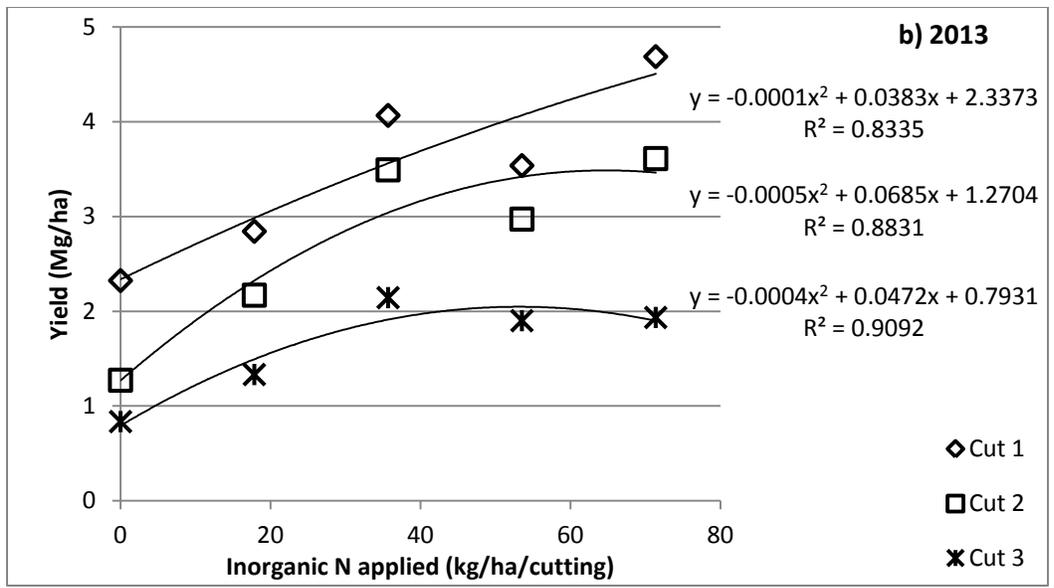
Table 3.2. Orchardgrass yield for 2012 and 2013 after poultry litter was surface applied or injected at high and low rates.

Treatment	2012			2013			
	cut 1	cut 2	cumulative	cut 1	cut 2	cut 3	cumulative
	Mg ha^{-1}						
High Injection	2.5 ns	1.5 ns	4.0 ns	3.1 A†	2.5 ns	1.6 ns	7.2 A
High Surface	2.7 ns	1.1 ns	3.8 ns	3.0 A	2.2 ns	1.3 ns	6.6 AB
Low Injection	2.3 ns	0.9 ns	3.2 ns	2.5 B	1.8 ns	1.3 ns	5.6 B
Low Surface	2.7 ns	1.0 ns	3.7 ns	3.0 A	1.8 ns	1.2 ns	6.1 B

† Means within the same column followed by different letters are significantly different at the 0.05 probability level.

Figure 3.1. Yield response to varying application rates of urea fertilizer in orchardgrass hay for a) two cuts in 2012, b) three cuts in 2013, and c) cumulative yields for 2012 and 2013.





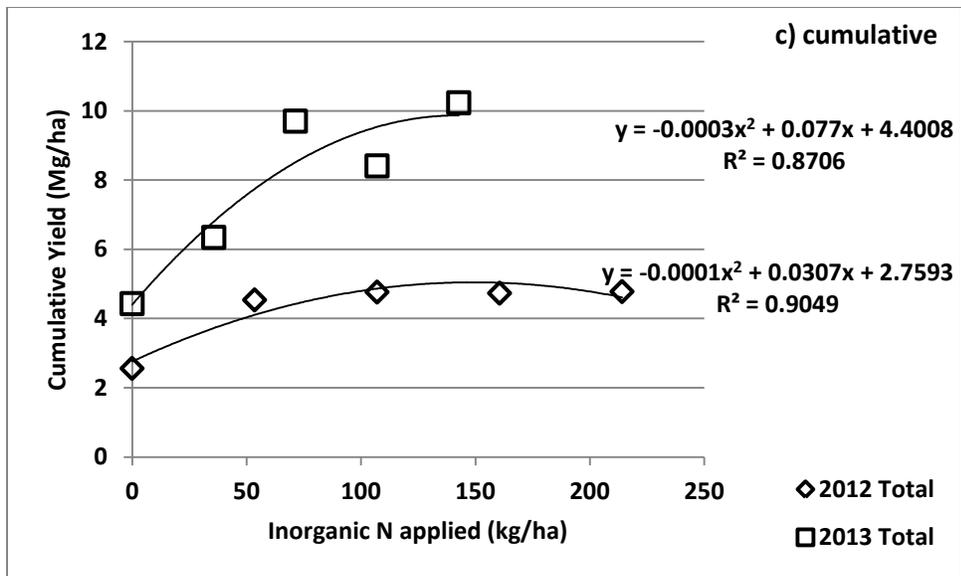
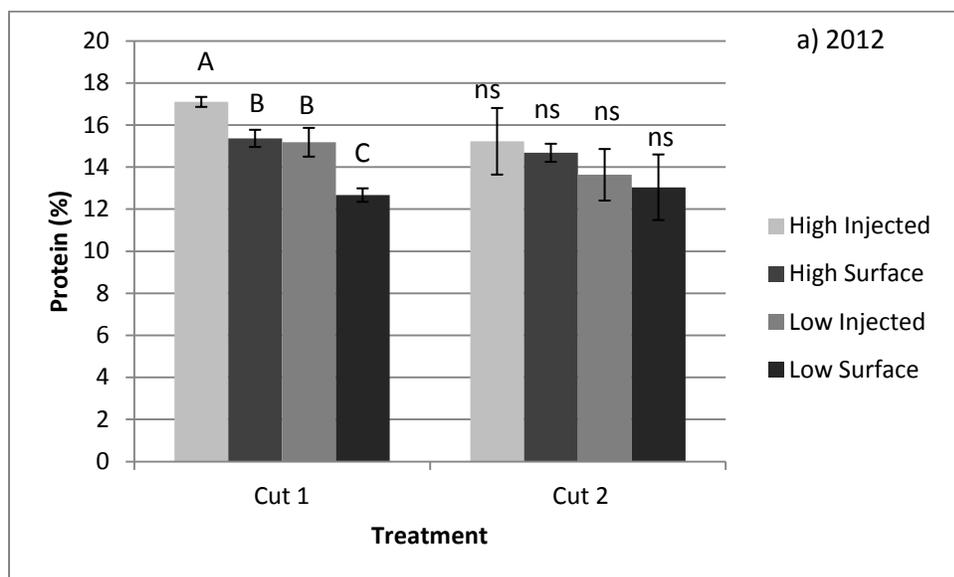
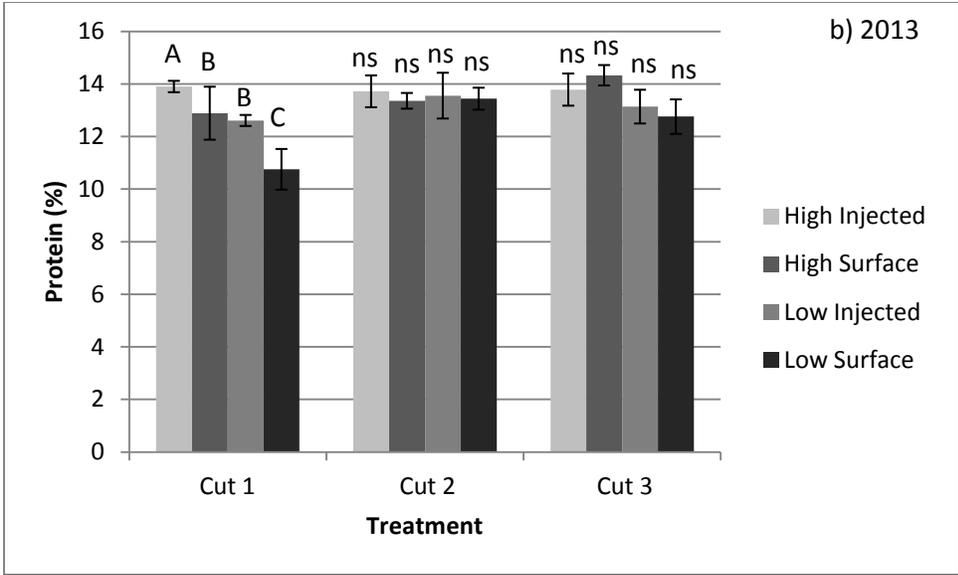
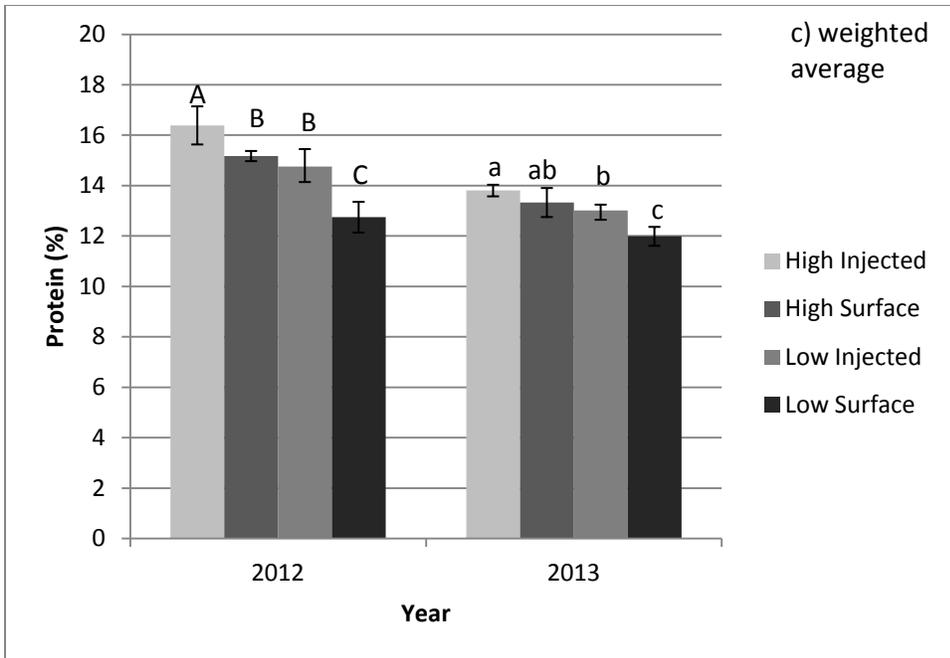


Figure 3.2. Percent protein in orchardgrass hay for a) two cuts in 2012, b) three cuts in 2013, and c) weighted average of all cuts in 2012 and 2013 for high injected, high surface, low injected, and low surface treatments.







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Chapter 4: Manure Injection Impacts Fate of Pirlimycin in Surface Runoff and Soil

Abstract

Antibiotics used in animal agriculture are of increasing environmental concern due to the potential for increased antibiotic resistance following land application of manure. Yet, little is known about impacts of different manure application technologies on the environmental behavior of these antibiotics. Therefore, rainfall simulations were conducted on plots receiving three manure treatments (surface application, subsurface injection, and no manure control) to determine the fate and transport of pirlimycin, an antibiotic commonly used in dairy production. Rainfall simulations were conducted immediately and seven days after application of dairy manure spiked with pirlimycin at 128 ng g⁻¹ (wet weight). Soil samples were collected from all plots at two depths (0-5 cm and 5-20 cm). For injection plots, soil was collected from injection slits and between slits. Over seven days, pirlimycin concentrations were higher in soil of injection slits compared to plots receiving surface application. Pirlimycin concentrations in the surface soil decreased by 30, 55, and 87% in injection slit, between injection slit, and surface applied plots over seven days. Mass loss of pirlimycin in sediment and water from surface application was 21 and 29 times that of injection. After seven days, pirlimycin levels in runoff sediment and water decreased 80-98%, and surface application had 7 and 3 times higher pirlimycin concentrations in water and sediment than subsurface injection. This indicates that pirlimycin is most susceptible to loss in runoff immediately after manure application. Thus, subsurface injection could be used as a best management practice to prevent loss of pirlimycin in surface runoff.

Introduction

Manure from large scale animal operations in the U.S. is typically land applied to provide nutrients for crop production. In 2007, 2.2 billion food animals raised in the U.S. produced one

billion Mg of manure (USDA 2009). Of that, approximately 173 million Mg of manure was produced from dairy operations. Considering most manure is land applied near where it is generated, these manures can have significant adverse impacts on the surrounding environment (USEPA, 2013). Most research on animal manure has centered on phosphorus and nitrogen cycling due to their value to crops and negative impacts on water quality. However, there is increasing concern over emerging contaminants such as antibiotics in manures. Antibiotics are widely used in animal agriculture, with 13.2 million kg sold in the U.S. in 2010 for livestock and poultry use (USFDA 2011). A large portion (60-80%) of livestock and poultry are administered antibiotics regularly with 90% of dairy operations administering intramammary antibiotics during non-lactating periods and 80% of those operations administering to all cows at the facility in 2007 (Carmosini and Lee 2008; USDA, 2008). A recent study conducted by the EPA revealed that there are many gaps in knowledge regarding how much of these antibiotics are administered to livestock and their behavior in the environment (USEPA, 2013).

Pirlimycin is a common lincosamide antibiotic used for treatment of mastitis in dairy cows. According to a pharmacokinetic study conducted by Hornish et al. (1992), 68% of the administered dose is excreted in the milk, urine, and feces as unchanged pirlimycin. The same study found that 80% of the pirlimycin in urine and 45% of pirlimycin in feces is present as unchanged parent compound. The parent pirlimycin excreted by dairy cows via feces and urine maintains its biological activity and might contribute to the development of antibiotic resistance in the environment (Hornish et al., 1992; USEPA, 2013). Antibiotic resistance is an issue of growing concern in the U.S. as the evidence of close association between use of antibiotics in animal agriculture and emergence of antibiotic resistance in human pathogens are increasing (Kumar et al., 2005). Following administration, antibiotics can be excreted in feces and urine.

Excreted antibiotics, even at very low concentrations, can impart antibiotic resistance (Tello et al., 2012). When antibiotics are used in animal agriculture and released continuously at low concentrations through land application of manure, microbes might gain resistance through several possible mechanisms compiled by Kumar et al. (2005): selection of resistant microbes in the animal gut, transfer of resistance genes in manure to native microbes, accumulation of antibiotic residues in plant and animal tissues, etc. Therefore, it is important to reduce the spread of these compounds, and associated resistance, to prevent the occurrence of antibiotic resistance in the environment.

Traditional animal manure surface application leaves manure at the soil surface, vulnerable to losses through runoff (Maguire et al. 2011). Nitrogen and phosphorus are transported from agricultural fields through ammonia volatilization, leaching, dissolution in runoff water, and bound to particulates in runoff. Therefore, it is beneficial to prevent manure/atmosphere contact and reduce soil erosion to increase the capture of these nutrients for crop production and to reduce nutrient pollution. However, incorporation of manure through tillage reduces the benefits that no-till agriculture provides (increased soil structure, increased organic matter, reduced erosion, increased soil moisture, etc). Manure injection increases the contact of manure with soil solids using minimal soil disturbance, which decreases ammonia volatilization and nutrient losses in runoff (Dell et al., 2011). Several studies have shown the benefits of injection for nutrient cycling and preventing nutrient losses in runoff (Maguire et al., 2011). However, there are no studies comparing transport and transformation processes of manure-borne antibiotics when injecting or surface applying dairy manure. Therefore, a field rainfall simulation experiment was conducted to compare the effect of manure surface application and subsurface injection on the fate and transport of pirlimycin in soil amended with

dairy manure containing pirlimycin. The findings from this study on pirlimycin would apply to other similar antibiotics.

Materials and Methods

Field Site

Rainfall simulations were conducted on a Braddock Loam (fine, mixed, semiactive, mesic typic hapludult) soil near McCoy, Virginia. Prior to the rainfall simulation study, soil was sampled from 0-10 cm, sieved, and air dried. Then, soil particle analysis was conducted using the pipette method in conjunction with wet sieving (USDA-NRCS, 2004). The surface soil consisted of 46% sand, 44% silt, and 10% clay, making it a loam soil texture. The field site was previously under no-till corn production and seeded with barley prior to installation of plot borders. Rainfall simulation plots were installed in September 2014 according to the National Research Project for Simulated Rainfall-Surface Runoff Studies protocol (SERA-17, 2008). Runoff plots measured 1.5 x 2 m, and a metal pan was installed on the downhill edge to transport runoff through a hose to a collection container. This experiment was set up in a randomized complete block design with three replicates of three treatments: dairy manure surface application, dairy manure subsurface injection, and a no manure control. Each block contained one replicate of each treatment, and blocks were arranged perpendicular to the slope. Runoff plots were installed on a 9% to 11% slope. Rainfall intensity for the simulations was 70 mm hr^{-1} , which is a standard rate to allow for comparison between rainfall simulations conducted in different areas (SERA-17, 2008). Rainfall simulations were conducted on the day of and 7 days after manure application. Thirty minutes of runoff was collected from each plot during each simulated rainfall. Borders of injection plots were installed after creation of injection slits to 15 cm for dairy manure injection in the injection plots. Injection slits were created using a Yetter injection disc with 75 cm disc spacing mounted on a three-point hitch.

Manure Collection and Application

Fresh dairy manure was collected from the Virginia Tech Dairy Farm in Blacksburg, VA. Manure was collected three days before the start of rainfall simulations. Manure moisture content was calculated by drying a 10 g sample at 110°C until a constant weight was achieved (Peters, 2003). Immediately before application, dairy manure was diluted to 95% moisture to mimic average lagoon dairy manure in Virginia (VADCR, 2005) and spiked with pirlimycin to achieve the final concentration of 128 ng g⁻¹. The pirlimycin target concentration was determined using a previous study that recorded the average excretion for five days following administration of pirlimycin to dairy cows (unpublished data). A urine to feces ratio of 2:1 and dry matter (DM) content of 13% were used in this calculation. Once manure was mixed to reach the appropriate antibiotic concentration and DM content, it was applied to the soil at 56.12 Mg ha⁻¹ (wet weight) by subsurface injection or surface application. This manure application rate is a typical application rate in Virginia (Maguire et al., 2013) for crop production. Manure was applied to a 2 x 2 m area immediately prior to rainfall initiation via surface spreading by hand or manual subsurface injection using strips of metal to hold open the previously created injection slits as described above. Runoff collection plots were 1.5 x 2 m and manure was applied to a 2x2 m area. Manure was applied to a larger area than the runoff collection plot to allow soil sampling after the first rainfall simulation without disturbing the soil within the runoff collection plot prior to the second rainfall simulation. After manure was injected, slits were closed by hand.

Sample Collection and Extraction

Approximately 100 mL of manure mixture was collected prior to application to determine the initial spiked pirlimycin concentrations in manure. Time to runoff and total runoff weight were recorded, and a 1-L subsample of runoff was collected after vigorous mixing to ensure representative sampling of the water and sediment. Seven drops of 6 M HCl were added to each

jar containing approximately 500 mL of runoff water to decrease the pH to < 2 in order to reduce microbial activity. Soil samples were collected from 0-5 cm and 5-20 cm depths outside the plot border after the first rainfall simulation and inside the plot border after the second rainfall simulation to prevent soil disturbance within the plot before the second simulation. In injection plots, soil cores were collected from the injection slits (15 cm surrounding slit) and between injection slits (60 cm between injection slit sampling) at both depths to determine spatial variability within the plots receiving subsurface injection treatment. Approximately 15 and 7 soil cores were collected from 0-5 and 5-20 cm depths from each plot and composited. The surface runoff and soil samples were stored on ice in the field for no more than 8 hours before being placed in a -20°C freezer in the lab until ready for analysis.

For extraction, cleanup, and analysis of pirlimycin in soil samples, 6 mL of methylene chloride (CH_2Cl_2) and 1 mL of ammonium hydroxide (NH_4OH) were mixed with 1 g of freeze dried soil. Then, the mixture was sonicated for 20 min, centrifuged for 5 min at 15°C and 1200 xg, and the supernatant was transferred to a clean glass centrifuge tube. The remaining soil sediment was extracted again with 6 mL CH_2Cl_2 and 1 mL NH_4OH , and the two supernatants were combined and centrifuged for five min at 15°C and 1200 xg. Then, 5 mL of the CH_2Cl_2 layer was collected and evaporated to dryness using the RapidVap Vacuum evaporation system from Labconco (Kansas City, MO). The dried sample was reconstituted in one mL of methanol (MeOH): water (3:7, v/v) with 0.1% FA before filtering through a $0.2\ \mu\text{m}$ PVDF syringe filter (Fisher, Pittsburg, PA) into amber glass HPLC vials for analysis on an ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

To analyze pirlimycin in runoff samples, the runoff samples were filtered through a Millipore $0.45\ \mu\text{m}$ nylon filter (Millipore, Billerica, MA) using glass filter funnels and vacuum

flasks attached to a vacuum pump to separate sediment and water fractions. Filters with sediment were freeze dried and stored at -80°C until extraction, cleanup, and analysis of pirlimycin in runoff sediment using the same method previously stated for soil samples. The runoff sediment content was determined by weight using the initial filtered volume and freeze dried sediment. The filtered runoff samples were analyzed for pirlimycin using the method outlined by Ray et al. (2014). Briefly, the filtered water was mixed at a 1:1 ratio with 100 mM phosphate buffer before solid phase extraction and cleanup using OASIS HLB Plus Short cartridges (250 mg sorbent; Waters, Milford, MA). After the extraction and cleanup, a total of 1 mL aliquot of the extract was dried under nitrogen gas and reconstituted in MeOH:water (3:7, v/v) before analysis on a UPLC-MS/MS. The same procedure was used for analysis of pirlimycin in the liquid manure applied to the soil.

UPLC-MS/MS Analysis for Pirlimycin

Pirlimycin in the final sample extracts were analyzed using Agilent 1290 UPLC coupled with Agilent 6490 Triple Quad tandem mass spectrometry (Agilent, Santa Clara, CA). A Zorbax Extend C_{18} guard column (4.6x12 mm, 5 μm particle size, Agilent) and a Zorbax Extend C_{18} analytical column (4.6x50 mm, 5 μm particle size, Agilent) were used in tandem for chromatographic separation (Agilent, Santa Clara, CA). A gradient mobile phase elution was used for separation with a flow rate of 0.5 mL min^{-1} . The mobile phase consisted of A: 0.1% formic acid (FA) in water and B: 0.1% FA in MeOH. The gradient elution program consisted of the following gradients at 0, 6, 7, 7.5, and 12 min: 70:30, 5:95, 5:95, 70:30, and 70:30 (A:B). Electrospray ionization with selected reaction monitoring mode was used with mass spectrometer capillary voltage, fragment voltage, and collision energies of 3000, 380, and 15 V, respectively. Ion source temperature of the mass spectrometer was 250°C . The mass to charge ratios (m/z) for the precursor, qualifier, and quantifier ions were 411, 363, and 112, respectively.

Statistical Analysis

Data were analyzed by time using analysis of variance through a PROC MIXED repeated measures analysis in SAS 9.3 (SAS Institute, 2011). Means were separated using P diff and LSMEANS in SAS. All statements of significance are based on $p < 0.5$. Error bars demonstrate one standard deviation of the mean.

Results and Discussion

Runoff and Sediment Collection

There was no difference in time to initiation of runoff between treatments at time 0, and start to runoff was significantly faster for injection on day 7 than the surface or control treatments (Table 1). The mass of runoff collected did not vary between treatments, with 27.9 kg collected on average for all treatments and simulations. Sediment concentrations were different among treatments, with sediment concentration following the trend of subsurface injection > control > surface application, during simulated rainfall on both times (Table 1). The reduction of sediment runoff over time was similar among treatments. During the simulated rainfall event on day 7, there was significantly less sediment runoff. The amount of sediment runoff during the 2nd rainfall event varied from 27-34% of that during the 1st rainfall event. These findings are similar to other studies involving dairy manure injection and surface application. For example, Little et al. (2005) found that shallow incorporation had greater sediment loss than no incorporation, when comparing surface application and incorporation using several tillage methods. Sediment loss is controlled by soil properties (compaction, drainage, etc) and the amount of soil disturbance created by the manure application process. Surface application can reduce sediment loss, but it leaves the nutrients vulnerable to other losses at the surface in runoff water or through ammonia volatilization (Maguire et al., 2011). It might have similar effect on antibiotic loss via runoff.

Concentration of Pirlimycin in the Soil

For 0-5 cm and 5-25 cm soil samples, the concentrations of pirlimycin followed the trend of injection slit > surface application for both day 0 and 7 (Fig. 1). Although pirlimycin was detected in the 0-5 cm soil of between injection slits on both days, its concentrations were not statistically higher than its detection limit. Its levels were below the detection limit for the 5-25 cm soil collected between the injection slits. Pirlimycin was below the detection limit for control treatment at both soil depths. On the day of manure application, the average pirlimycin concentrations were 21, 13, and 1.2 ng g⁻¹ (dry soil) for 0-5 cm soils collected from the injection slit, surface, and between injection slits, respectively. Seven days after manure application, the concentration of pirlimycin had dropped drastically, by 87, 55, and 30% in the 0-5 cm soil depth of surface application plots, between injections slits, and injection slits, respectively. For example, for the 0-5cm depth of injection slits, pirlimycin concentration dropped from 21 ng g⁻¹ on day 0 to 14 ng g⁻¹ on day 7.

Although there is a lack of information on pirlimycin transformation rate and mechanisms in the environment, it is speculated that the faster disappearance of pirlimycin in the surface soil of surface applied treatment compared to subsurface injection treatment could be attributed to its rapid aerobic transformation at the soil surface, which was proposed to be the main route for transformation of antibiotics in the environment (Boxall et al., 2011). Closure of the injection slit on the surface could have prevented the transfer of oxygen and slowed transformation of pirlimycin in injection slits. As reported by Petersen et al. (1995), fresh manure hot-spots caused rapid depletion of oxygen, forming anoxic soil conditions. Oxygen diffuses much more slowly into water than air, and water added to the soil through the rainfall simulation in addition to water applied through manure application reduces exchange of oxygen into the soil. This could explain why pirlimycin disappeared more slowly in the injection slit when

compared to surface application. In a study by Markfoged et al. (2011), a well defined zone of anoxic conditions was measured three hours after liquid swine manure was injected. The anoxic zone decreased in size during the first day, but soil oxygen was depleted in this zone for the extent of the three-day experiment (Markfoged et al., 2011). A better understanding of pirlimycin transformation mechanisms in the soil environmental will further validate the above speculation.

For 5-20 cm soil samples, there was a similar trend as in 0-5 cm soil samples, with the concentration of pirlimycin in the injection slit being the highest on day 0 and 7 (Figure 1). This is not surprising, as manure was injected below 5 cm, so the pirlimycin in the manure was placed in the soil to the depth where this soil sample was taken. Although pirlimycin was detected in the 5-20 cm depth of the surface application treatment, its levels were not significantly different from its detection limit. Pirlimycin was below the detection limit in the 5-20 cm soil from between the subsurface injection slits and the control treatment on both day 0 and day 7. This indicates that pirlimycin had not moved laterally from the injection slit in the injection treatment. Over seven days, pirlimycin concentrations decreased by 63% and 48% in the 5-20 cm soil from the surface application plots and injection slits, respectively. Although statistically there is no significant difference between the control and the surface applied plots at 5-20 cm depth, pirlimycin concentration was above the detection limit at this depth for the surface applied plots. This indicates there may have been some limited downward movement of pirlimycin from manure applied on the soil surface. Because of its high water solubility (64.9 mg mL^{-1}), downward movement of pirlimycin along soil profile is possible (FDA, 1993). However, because of its high pKa value (8.38), pirlimycin remains mostly protonated with positive charge on its structure at environmentally relevant pH values (FDA, 1993). Therefore, its mobility maybe limited due to its retention on permanently charged clay surfaces through electrostatic attraction.

A previous study on sulphachloropyridazine, oxytetracycline, and tylosine showed that only sulphachloropyridazine was detected in lysimeters due to its low sorption to soil, but the losses only accounted for approximately 0.00015% of that applied through manure application (Kay et al., 2005). Also, Kay et al. (2005) attributes leaching losses to movement within macropores in the soil cores, where contact of leachate with the soil is less than in situations with piston flow.

Concentration of Pirlimycin in Runoff Water and Sediment

Concentration of pirlimycin in filtered runoff water was significantly higher from the surface application treatment when compared to subsurface injection treatment, and it was not detected in runoff water from the control (Fig. 2). Statistically, injection reduced pirlimycin concentrations to levels of the control in filtered runoff water on day 0 and 7, but pirlimycin concentrations were above the detection limit in injected samples at both time points. The same trends were seen in the second rainfall simulation seven days after manure application, where surface > injection \approx control. The pirlimycin concentration in runoff water was 0.15 ng mL^{-1} for the subsurface injection plots and 4.67 ng mL^{-1} for the surface application plots at day 0 and reduced to 0.01 ng mL^{-1} (93% reduction) and 0.03 ng mL^{-1} (99% reduction) after seven days for the two treatments, respectively.

Pirlimycin concentration in runoff sediment followed a similar trend as runoff water with surface > injection \approx control (Fig. 2). Immediately after manure application, pirlimycin concentrations were 4.90 and 105.53 ng g^{-1} (dry weight basis) in runoff sediment for subsurface injection and surface application plots, respectively. During the simulated rainfall event 7 days after manure application, pirlimycin levels in the runoff sediment decreased 83% to 0.83 and 95% to 5.63 ng g^{-1} (dry weight basis) for the two treatments, respectively. Pirlimycin concentrations were 21 and 32 times higher in runoff sediment and water of surface application plots compared to subsurface injection plots during the simulated rainfall event on the day of

manure application. Even 7 days after manure application, simulated rainfall on the surface application plots resulted in 6 and 3 times higher levels of pirlimycin in the runoff sediment and water compared to those from the subsurface injection plots. Therefore, manure subsurface injection could be considered as a possible solution for reducing loss of manure-borne antibiotics to aquatic environment adjacent to manure-applied fields.

Although the overall pirlimycin concentrations in both runoff sediment and water decreased drastically 7 days after manure application, pirlimycin partitioned into runoff sediment increased over this period of time. Initially, pirlimycin sediment/water partition ratios in surface runoff were 23 and 33 L kg⁻¹ for surface application and subsurface injection treatments, respectively. After seven days, the partition ratios increased to 175 and 75 L kg⁻¹ in the surface runoff of the two treatments, respectively.

Concentration of Pirlimycin in Runoff Sediment

Pirlimycin concentration in runoff sediment followed a similar trend as runoff water with surface > injection ≈ control (Fig. 2). Immediately after manure application, pirlimycin concentrations were 4.90 and 105.53 ng g⁻¹ (dry weight basis) in runoff sediment for subsurface injection and surface application plots, respectively. During the simulated rainfall event 7 days after manure application, pirlimycin levels in the runoff sediment decreased 83% to 0.83 and 95% to 5.63 ng g⁻¹ (dry weight basis) for the two treatments, respectively. Pirlimycin concentrations were 21 and 32 times higher in runoff sediment and water of surface application plots compared to subsurface injection plots during the simulated rainfall event on the day of manure application. Even 7 days after manure application, simulated rainfall on the surface application plots resulted in 6 and 3 times higher levels of pirlimycin in the runoff sediment and water compared to those from the subsurface injection plots. Therefore, manure subsurface

injection could be considered as a possible solution for reducing loss of manure-borne antibiotics to aquatic environment adjacent to manure-applied fields.

Pirlimycin Mass Balance

The amount of pirlimycin detected in manure, soil, runoff water, and sediment were calculated using its concentration in each matrix, known volume of manure applied, standard soil bulk density ($1.33 \text{ g (cm}^3\text{)}^{-1}$), area of plots, sampling depth, volume of runoff water, and weight of runoff sediment. Also, mass lost in runoff from day 0 was taken into account when calculating values for day 7. The calculated amount of pirlimycin detected in each matrix compared to that initially added to the field through manure application are listed in Table 2 in order to demonstrate pirlimycin mass distribution in soil and surface runoff. The sum of pirlimycin detected in each matrix compared to the total amount initially added to the field ranged from 135.5% for the surface application plots to 70.12% for the subsurface injection plots. However, there was no significant difference between treatments. Detecting greater than 100% is most likely due to variability in sampling within the plots. Also, high variability within the injection treatment could be due to an accumulation of errors associated with mass balance calculations (i.e. assumed volume of injection slit and standard soil bulk density) or movement of pirlimycin out of the sampling profile. On the day of manure application, 122% and 61% of initially added pirlimycin respectively remained in the surface soil (0-5 cm) when surface applied and within injection slits (0-5 cm and 5-20 cm) when injected. Only 8.45% of initially added pirlimycin remained in the 5-20 cm soil for the surface application plots while none were detected in the same soil depth of between the subsurface injection slits, indicating there was downward movement of pirlimycin in the plots receiving manure via surface application, however, these values were not significantly different. It is clear that compared to runoff sediment, a higher percentage of added pirlimycin was lost through runoff water. For example, on the day of

manure application, 4.94% of added pirlimycin was detected in runoff water from the surface application plots, while only 0.06% of added was associated with runoff sediment. A similar trend was observed for the subsurface injection plots, but with overall lower loss via runoff sediment and water. Only 0.17% and 0.01% of added pirlimycin were detected in the runoff sediment and water, respectively, on the day of manure subsurface injection. Similar trends of loss in runoff water was observed for other antibiotics, although none of these studies include pirlimycin (Dolliver and Gupta, 2008; Burkhardt et al., 2005; Kreuzig et al., 2005; and Davis et al., 2006). Dolliver and Gupta (2008) found that chlortetracycline, tylosin, and monensin had mass losses less than 5% when liquid hog and solid beef manure were applied over 3 years, and snowmelt had a significant effect on whether the majority was lost through runoff or percolation. Also, antibiotic loss from no-till plots was generally higher than from a chisel plowed treatment, which is attributed to greater water percolation and reduced surface roughness in no-till plots (Dolliver and Gupta, 2008). Mass losses of sulfadiazine, suladimidine, and sulfathiazole in runoff after surface application of liquid swine manure accounted for 0.3 to 1.4% of the total applied to soil 1 day after application and 1.6 to 6.3% after 3 days (Burkhardt et al., 2005). Losses reported by Burkhardt et al. (2005) are below those found in this study where total loss in runoff from surface application accounted for 5% of the total pirlimycin applied to soil. Mass loss of sulfonamides from runoff collected immediately after tillage of liquid bovine manure ranged from 0.1 to 2.5% of antibiotics applied to soil, and losses of several antibiotics from the tetracycline, macrolide, sulfonamide, and ionophore classes of antibiotics had less than 0.1% loss of the total applied through application using a backpack sprayer (Kreuzig et al., 2005; Davis et al., 2006). These studies highlight the importance of investigating many classes of antibiotics, as

their chemistry and differences in soil properties play a large role in their fate and transport (Kumar et al., 2005).

After seven days, the total amount of pirlimycin detected relative to initially added decreased 38% and 85% for subsurface injection and surface application plots, respectively. The relative total amount of pirlimycin left for the subsurface injection plots was significantly higher than that for the surface applied treatment. Differences in pirlimycin detected in both 0-5 and 5-20 cm soil samples over time are likely due to decreased transformation in the subsurface injection treatment and rapid aerobic transformation in the surface application treatment as discussed earlier. Therefore, injection could increase the residence time of antibiotics applied through this method. This could have implications for the formation of localized antibiotic resistance in the environment, and more research is needed to determine if lengthening the amount of time microbes are exposed to antibiotics added through manure subsurface injection could increase the likelihood of elevated antibiotic resistant microbial population. Studies have shown that levels of antibiotic resistance genes associated with several classes of antibiotics can return to background levels within days or months after manure application to agricultural fields depending on the genes being monitored and soil/field conditions (Fahrenfeld et al., 2014). Fahrenfeld et al. (2014) found that antibiotic resistant genes returned to background levels within two months after application of dairy manure.

There are no research studies on the effect of manure injection on antibiotics in runoff. However, concentrations of pirlimycin in runoff water detected in this study follow similar trends as previous studies on nutrient transport in runoff after injection of manures. After injection of swine manure, total phosphorus and dissolved reactive phosphorus concentrations in runoff were reduced to that of a no manure control (Kovar et al., 2011). This has very important

implications for the transport of antibiotics from agricultural fields to adjacent surface water bodies. Application of manures to agricultural fields can result in the spread of antibiotic resistance to surface and groundwater sources (USEPA, 2013). Continuous application of manure with antibiotics increases the occurrence of antibiotic resistance in the environment, and water environments are an important substrate for formation of antibiotic resistant bacteria (Banquero et al., 2008). Therefore, it is very important to prevent the transport of antibiotics from agricultural fields. The total loss of pirlimycin in runoff (water and sediment) at the simulated rainfall intensity and duration and manure application rate on the day of manure subsurface injection and manure subsurface injection were equivalent to 12.49 and 354.82 mg ha⁻¹, respectively. The results of this investigation demonstrated that manure subsurface injection in agricultural fields could reduce the transport of manure-borne antibiotics to surface water bodies via surface runoff, and therefore potentially slow the development of antibiotic resistance in these water systems.

Conclusions

While most of the pirlimycin remained in the surface soil or within the injection slit, nearly five percent of added pirlimycin was lost in runoff when manure was surface applied. Subsurface injection of manure dramatically decreased pirlimycin loss in runoff compared to surface application, suggesting this could be used as a best management practice to decrease losses of manure-borne antibiotics to adjacent surface water bodies. As total pirlimycin associated with sediment in runoff was low, management practices that reduce erosion may not be adequate to prevent movement of this highly water soluble antibiotic. This study suggested that it is highly likely that 85% of added pirlimycin through surface application had transformed within 7 days after manure application, indicating that avoiding manure applications immediately before heavy rain will also help decrease its input to the adjacent aquatic environment.

Table 4.1. Rainfall simulation properties after two simulations zero and seven days after application of three treatments: surface applied dairy manure, injected dairy manure, and no manure control.

Treatment	Time to start		Water applied				Runoff collected				Sediment					
	Day 0‡		Day 7		Day 0		Day 7		Day 0		Day 7					
	minutes						kg				g kg ⁻¹					
Control†	13.0	Aa	9.5	Aa	553.5	Aa	506.7	Ab	30.0	Aa	23.6	Aa	87.4	Ba	84.2	Ba
Surface	7.0	Aa	8.7	ABa	457.5	Ba	483.6	Aa	36.3	Aa	22.7	Aa	65.9	Ca	79.57	Ca
Injection	16.4	Aa	7.7	Ba	517.3	Aa	469.9	Ab	19.6	Aa	33.4	Aa	107.3	Ab	135.16	Aa

†Means within the same column followed by the same capital letter are not significantly different at a 0.5 probability level.

‡Means within the same row, comparing Time 0 to Time 7, followed by the same lower case letter are not significantly different than those in the same observation type at a 0.5 probability level.

Table 4.2. Pirlimycin detected relative to initially added zero or seven days after surface application and injection of dairy manure.

		Detected/Initially Added (%)									
		0-5 cm Soil†		5-20 cm Soil		Water		Sediment		Sum	
Treatment		Day 0									
		%									
Surface		122.07	A	8.45	AB	4.94	A	0.06	A	135.52	A
Injection	Slit	42.75	B	18.29	A	0.17	B	0.01	B	70.12	A
	Between Slit	8.91	B	0.00	B						
		Day 7									
Surface		17.19	b	3.37	b	0.05	a	0.01	a	20.62	b
Injection	Slit	29.98	a	9.45	a	0.01	a	0.00	a	43.46	a
	Between Slit	4.01	c	0.00	b						

†Means within the same column followed by the same capital (day 0) or lowercase (day 7) letter

are not significantly different at a 0.5 probability level.

Figure 4.1. Concentration of pirlimycin in soil from control plots with no manure applied, injection slit and between injection slit of subsurface injection plots, and surface application plots zero or seven days after dairy manure application. Means with the same capital (0d) or lowercase (7d) letters are not significantly different from means within the same rainfall simulation. Error bars represent the standard deviation of the mean.

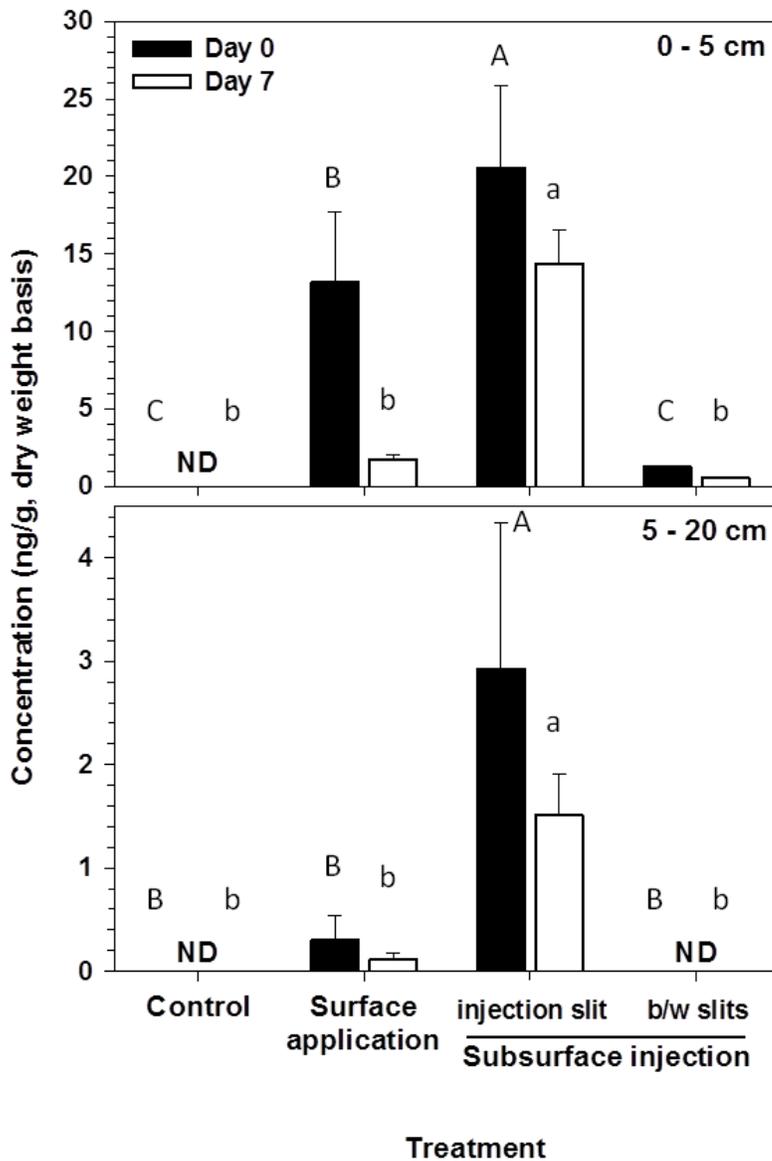
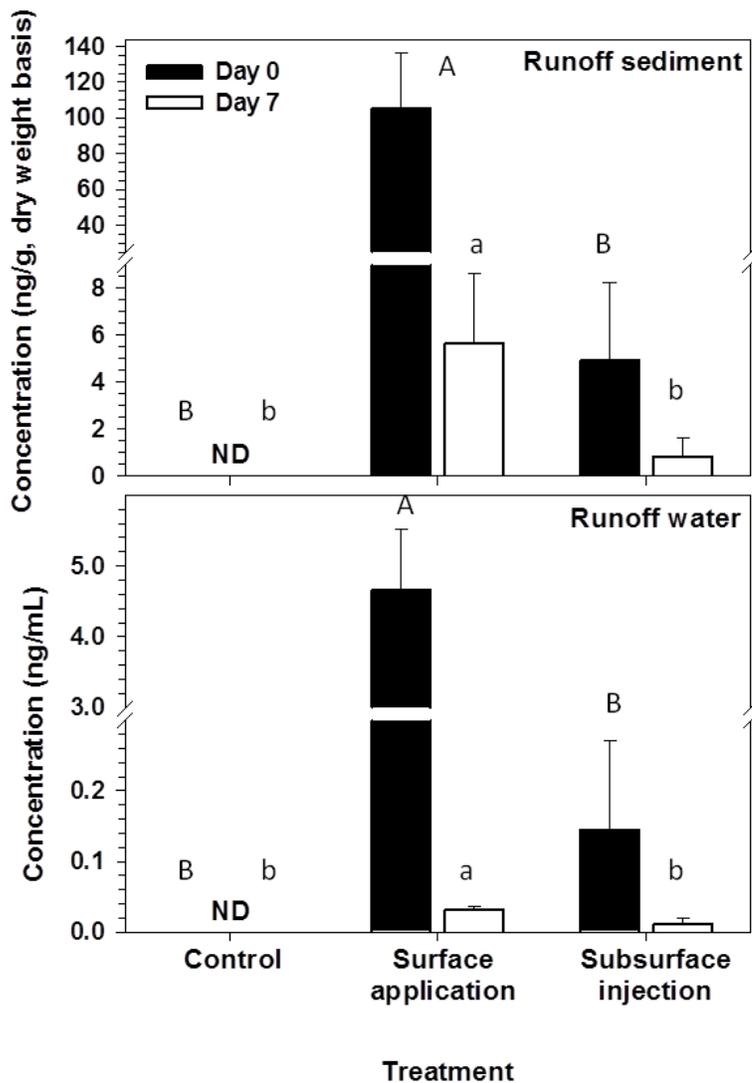


Figure 4.2. Pirlimycin concentrations in runoff sediment or runoff water collected during simulated rainfall from plots on the day and seven day of dairy manure application via surface application and subsurface injection, as well as control plots receiving no manure treatments. Means separated by the same capital (0d) or lowercase (7d) letter are not significantly different at a 0.5 probability level. Error bars represent the standard deviation of the mean.



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Chapter 5: Effect of Temperature and pH on Decrease of Pirlimycin and Cephapirin in Soil Amended with Manure

Abstract

Antibiotics applied to soil through application of manure are of increasing concern due to the formation of antibiotic resistance in the environment. Therefore, two 90-day incubation studies were conducted to determine the effects of manure temperature, pH, and soil texture on the persistence of pirlimycin and cephalosporin after manure application to soil. For the temperature experiment, manure collected from treated cows was applied to two soils and incubated at 10 or 21°C. For the pH experiment, manure pH was adjusted to 5, 7, or 9 before application to soil and incubated at 21°C. Cephalosporin was not detected in initial manure or soil extracts, indicating rapid transformation in manure. In the temperature experiment, the time to 50% decrease of initial pirlimycin was 15 and 19 days for loam 21°C and 10°C and 35 and 89 days for sandy loam 21°C and 10°C treatments, respectively. An increase in pirlimycin was detected in the sandy loam 10°C treatment, indicating possible deconjugation of pirlimycin ribonucleotide adducts added through manure. In the pH experiment, the time to 50% decrease of initial pirlimycin followed trends of sandy loam pH 9 \geq loam pH 9 \approx sandy loam pH 5 \geq loam pH 5 \approx sandy loam pH 7 \geq loam pH 7. This indicates that soil type, pH, and temperature were important factors for pirlimycin disappearance over time, which could impact development of resistance genes if soil microbes are exposed to pirlimycin for longer periods of time. The influence of pirlimycin deconjugation should be further explored.

Introduction

Land application of manure as a nutrient source for crop production is the primary use for animal manure, and most manure is applied near the farm where it is generated. While much of

the research has focused on the fate of nutrients after application of manure, there is increasing concern over the movement and impact of antibiotics applied to soil through manure application due to the potential for increased frequency of antibiotic resistance (USEPA, 2013). Land application of manure is the primary route for release of antibiotics in the environment, and there is increasing evidence that the use of antibiotics in animal agriculture and emergence of antibiotic resistant pathogens are connected (Baguer et al., 2000; Kumar et al., 2005). Antibiotics are administered to dairy cows to prevent spread of disease or treat an active infection. They are widely used, with 13.2 million kg of antibiotics sold in 2010 for use in livestock or poultry in the U.S. (USFDA, 2011). The majority (60-80%) of livestock and poultry population receives antibiotics regularly, and it is estimated that 40-80% of antibiotics are excreted in feces and/or urine as the parent compound or metabolites (Kemper, 2008). However, little is known about the transformation of these antibiotics in the environment, and different classes of antibiotics can behave very differently due to their chemistry (USEPA, 2013).

Pirlimycin, a lincosamide antibiotic, and cephalixin, a 1st generation cephalosporin antibiotic, are commonly used for treatment of mastitis and for prophylactic dry cow therapy in dairy cows. Pirlimycin and cephalixin are widely used in dairy production, but there is little information on the fate of these compounds after they are excreted in the feces and/or urine. A pharmacokinetic study on pirlimycin found that 68% of the initial dose was excreted as parent pirlimycin in milk, feces, and urine (Hornish et al., 1992). Of that, 48% and 80% of the pirlimycin excreted through feces and urine, respectively, was excreted as the unchanged parent compound (Hornish et al., 1992). The excreted unchanged compound maintains its biological activity in the manure and soil after land application. Stockler et al. (2009) found that about 60% of the initial dose of cephalixin was excreted in milk. Therefore, the remaining 40% must have

been excreted in urine or feces or degraded to metabolites (Stockler et al., 2009). Some antibiotic metabolites can maintain biological activity, with the deacetylated form of cephapirin (desacetylcephapirin) having 54% of the bioactivity compared to cephapirin when assayed on *S. lutea* plates (Cabana et al., 1976). The antibiotics and antibiotic metabolites added to soil through manure application can, even at very low concentrations, impart antibiotic resistance in the environment (Carmosini and Lee, 2008; Tello et al., 2012).

Animals raised in intensive indoor facilities produce large quantities of manure that are stored until land application (Boxall, 2004). These compounds may degrade during storage in lagoons or waste pits, but cows administered antibiotics that are allowed to graze can deposit high concentrations in localized areas at time of urination or defecation (Boxall, 2012; Halling-Sorensen et al., 1998).

The pH and temperature are very important in determining the fate of antibiotics in the environment due to the chemistry of individual antibiotics, with pKa's commonly within the pH range found in soils (Boxall, 2012). For example, the pKa's of cephapirin sodium are 2.15 and 7.3, and the pKa of pirlimycin is 8.38 (Gennaro, 1990; USFDA, 1993). The difference in pKa between these compounds could affect their sorption to mineral surfaces and availability for degradation by microbes. The degradation of ceftiofur, a third generation cephalosporin antibiotic, was affected by soil pH when comparing soils collected from Florida, Wisconsin, and California, with pH's ranging from 3.96 to 8.02 (Gilbertson et al., 1990). Varying pH also affected the stability of cefepime, a fourth generation cephalosporin antibiotic, when dissolved in an aqueous solution. However, there is no information on the effect of initial manure pH on the degradation of cephapirin and pirlimycin in soil.

Temperature is a major factor regulating microbial activity within soils, and degradation of antibiotics in soil is controlled by microbial activity (Kemper et al., 2007). Soil temperature and degradation rate are positively correlated, with longer half-lives in systems with lower temperatures (Kumar et al., 2005). Gavalchin and Katz (1994) found that lower temperatures increased the half-lives of antibiotics in soil amended with manure. At three different temperatures (4°C, 20°C, and 30°C), the degradation rate of seven antibiotics (bacitracin, chlortetracycline, erythromycin, bambarmycins, penicillin, streptomycin, and tylosin) increased with increasing temperature (Gavalchin and Katz, 1994). Almost all of the chlortetracycline, erythromycin, and bambarmycins remained in the soil after 30 days at 4°C, while 44% of the chlortetracycline and none of the erythromycin and bambarmycin remained after 30 days at 30°C. It is clear that temperature plays a major role in the degradation rate of several classes of antibiotics, but there is little information on the effects of temperature on the transformation of pirlimycin and cephalixin in soil after manure application, especially when manure is collected from cows receiving these antibiotics instead of spiking control manure. Therefore, the objective of this incubation study was to determine the effects of initial manure pH and incubation temperature on pirlimycin and cephalixin soil concentrations after manure application to soil.

Materials and Methods

Soil Collection

One soil near Painter, VA and one near McCoy, VA were collected from the top 10 cm, sieved through a 1.3 cm sieve, and air dried. These soils were a Bojac sandy loam (coarse-loamy, mixed, semiactive, thermic typic hapludult) and a Braddock loam (fine, mixed, semiactive, mesic typic hapludult). These two soils will be referred to as the Sandy Loam and Loam throughout.

These soils were chosen because they are soils representative of those found in the state of Virginia used for agricultural production, and they vary in soil properties (Table 5.1).

Manure generation for incubation study

The manure used in this study was generated from an experiment conducted with dairy cows. All procedures for this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC protocol: 13-145-DASC). Three end-of-lactation and three lactating cows were used in this study. All cows were housed in individual tie stalls (1.25 × 2.25 m), and were offered free choice water and *ad libitum* total mixed ration throughout the study. On day 1, cows were fitted with urinary catheters to allow separate collection of feces and urine. After acclimation to the stalls and catheters, cows were infused with respective antibiotic preparations.

Three end-of-lactation cows were infused with cephapirin benzathine (ToMorrow®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) into the four quarters of the udder at 300 mg cephapirin per quarter, equaling a total dose of 1,200 mg cephapirin per cow. Three lactating cows received pirlimycin hydrochloride (Pirsue®, Zoetis, Madison, NJ) twice (24 h apart). Only one quarter of the udder was infused with 50 mg of pirlimycin resulting in a total dose of 100 mg per cow over a period of 48 h.

Control fecal and urine samples were collected from dairy cows before cephapirin and pirlimycin infusions. Feces and urine were collected on day 1 following cephapirin and 2nd dose of pirlimycin infusions. This sample collection scheme was based on peak cephapirin and pirlimycin excretion in feces and urine of dairy cows following intramammary infusions (Ray et al., 2014). Feces and urine were stored at -20°C within 60 min of collection. Feces and urine

were mixed at a ratio of 2.11. This ratio was calculated using the 24-h cumulative amount of feces and urine excreted by the experimental cows for 5 consecutive days.

Incubation Setup

Dry soil (200 g) was added to 400 mL glass beakers the day before initiation of the experiment. For the temperature experiment, pirlimycin, cephalixin, or a no antibiotic control manure was mixed with two soil types, with three replications. Once mixed, beakers for each manure-soil combination were placed in each of the incubators set at 10°C or 21°C to mimic spring or winter application of manure. These experiments were set up in a completely randomized design. All soil containers were incubated in two model 815 Precision low temperature incubators (Thermo Fisher Scientific, Waltham, NJ) for the extent of the experiment. Prior to manure application, the pH of the manure was adjusted to 5, 7, or 9 with 1 M HCl or 1 M NaOH to mimic the common range of pH's found in livestock manure and soil. Pirlimycin, cephalixin, or a no antibiotic control manure was applied to three replicates of two soil types for each pH treatment (5, 7, and 9), and all beakers were placed in the same incubator at 21°C for the extent of the experiment. Manure was applied at a rate of 56,000 L ha⁻¹ (30 g cup⁻¹), and the soil and manure were mixed thoroughly. The soil-manure mixture was brought to 70% field capacity prior to mixing. Field capacity was determined by saturating 300 g of soil and reweighing after 2 days of free draining (Cassel and Nielsen, 1986). The field capacity was calculated on a weight-by-weight basis, and the moisture from added manure was subtracted from water required to bring the soil to 70% field capacity. After mixing, the initial 10 g sample was collected and stored at -20°C within 6 hours, and for both temperature and pH experiments, samples were collected on day 0, 1, 3, 7, 14, 28, 56, and 90. After sampling, the containers were covered with parafilm and placed in their respective incubators. After 1 day, the parafilm was replaced with loose fitting foil to increase air transfer because there was an odor, indicating

possible anaerobic activity. By day 3, the odor had subsided. Every three days, water was added to the containers to maintain 70% field capacity, and the soil was thoroughly mixed immediately prior to sampling at each time point.

Analytical Sample Preparation

Initial manure samples were extracted and analyzed for pirlimycin and cephalixin using the procedure as described in Ray et al. (2014). To determine pirlimycin concentrations in the soil, 1 g of freeze dried soil was mixed with 6 mL of methylene chloride (CH_2Cl_2) and 1 mL of ammonium hydroxide (NH_4OH), and the mixture was sonicated at 20-25°C for 20 min. Sonication was followed by centrifugation at $1200 \times g$ for 5 min at 15°C. The supernatant was transferred to a clean centrifuge tube, and the soil pellet was extracted again by repeating each step. The supernatants from two extractions were combined and centrifuged at $1200 \times g$ for 5 min at 15°C. The layer of CH_2Cl_2 was separated from the NH_4OH layer, and 5 mL of the CH_2Cl_2 layer was evaporated to dryness using a RapidVap Vacuum evaporation system from Labconco (Kansas City, MO). The dried samples were then reconstituted in 1 mL of mobile phase consisting of 0.1% formic acid (FA) in methanol (MeOH) and 0.1% FA in water (3:7 v/v), filtered through a 0.2 μm PTFE syringe filter (Fisher, Pittsburg, PA) into amber glass HPLC vials, and stored in a -80°C freezer for no more than 48 hours before analysis on ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

To analyze cephalixin in soil samples, 10 mL of acetonitrile (ACN): water (8:2, v/v) was mixed with 1 g freeze dried soil. The mixture was vortexed for 2 min, sonicated for 20 min at 20-25°C, and centrifuged at $1880 \times g$ for 5 min at 15°C. The supernatant was transferred to a clean centrifuge tube, and the soil was extracted again with 5 mL of ACN:water (8:2 v/v) by repeating same steps. The supernatants from two extractions were combined with 150 mg PSA, 150 mg C18 sorbent, and 900 mg anhydrous MgSO_4 . The mixture was vortexed for 1 min and

centrifuged at $1880 \times g$ for 5 min at 15°C . A 10 mL aliquot of the supernatant was evaporated to dryness as previously mentioned and reconstituted in the same mobile phase used for pirlimycin analysis.

UPLC-MS/MS Quantification of Pirlimycin and Cephapirin

Pirlimycin and cephapirin in the final extracts were analyzed with an Agilent 1290 UPLC in tandem with an Agilent 6490 Triple Quad tandem mass spectrometry (Agilent, Santa Clara, CA). Chromatographic separation was achieved using a Zorbax Extend C_{18} guard column (4.6 x 12 mm, 5 μm particle size, Agilent) in line with a Zorbax Extend C_{18} analytical column (4.6 x 50 mm, 5 μm particle size, Agilent). A gradient mobile phase elution was used for separation with a mobile phase consisting of A: 0.1% FA in water and B: 0.1% FA in MeOH. The flow rate was 0.5 mL min^{-1} with a gradient elution program consisting of the following gradients at 0, 6, 7, 7.5, and 12 min: 70:30, 5:95, 5:95, 70:30, and 70:30 (A:B). MS/MS parameters are described in Table 5.2

Statistical Analysis

Proc Mixed repeated measures analysis was used to analyze data in SAS 9.3, and all factors were considered fixed effects (SAS Institute, 2011). Mean separation was achieved using P diff and LSMEANS in SAS. Polynomial regressions were calculated for each treatment by soil type, and intercepts were set at one after the measured soil concentrations were divided by a calculated initial manure concentration. Statements of significance were declared at $P < 0.5$.

Results and Discussion

Soil Description

The Braddock loam and Bojac sandy loam soils are two common soil types found in the state of Virginia. However, they were chosen due to differences in their physical and chemical properties. The loam soil had more than twice as much organic matter as the sandy loam soil,

which could affect microbial respiration and mineralization of organic nutrient sources such as manure. Also, soil moisture and temperature are two important factors positively correlated to microbial activity in soil, and the loam soil had a higher water holding capacity compared to the sandy loam soil (Lloyd and Taylor, 1994; Orchard and Cook, 1983). Finer texture soils have higher clay content and higher specific surface area, allowing more contact with soil surfaces and greater water holding capacity (Brady and Weil, 2002).

Pirlimycin

Temperature Incubation

Time, temperature, and soil type were significant main treatment factors affecting pirlimycin reduction over 90 days, and temperature*time and soil type*time interactions were significant. At day 0, pirlimycin concentrations in the loam soil (mean = 10.49 ng g⁻¹; n = 6) were significantly greater than the sandy loam soil (mean = 4.46 ng g⁻¹; n = 6). Because all replicates were treated from the same batch of manure and spiking tests provided similar recoveries for both soil types, it is unclear why there was a difference in initial pirlimycin concentration between the two soil types. Once manure was added, soil was mixed for 5-10 minutes, so there is no clear explanation for this disparity. Therefore, the data were transformed by dividing the measured concentration at each time point by the day 0 concentration for comparison.

At day 7, there was a significant difference between soil types, with lower remaining pirlimycin in loam treatments compared to sandy loam treatments (Figure 5.1). Aerobic microbial transformation is the suggested primary mechanism of antibiotic degradation in soil, and higher organic matter and water holding capacity are positively correlated to microbial activity (Boxall et al., 2011; Brady and Weil, 2002). The loam soil had more organic matter and greater specific surface area due to a finer texture than the sandy loam soil, which could increase

microbial activity and explain the rapid reduction in the loam soil over the first 7 days when compared to the sandy loam soil. However, this trend did not continue at day 14, when temperature treatments significantly impacted pirlimycin reduction in the 10°C treatments.

At day 14, there was an increase in pirlimycin in the loam 10°C treatment. Also, the remaining pirlimycin in soil was not significantly different in the sandy loam 10°C between days 0, 7, and 14. An increase in pirlimycin concentration from 7 to 14 days indicates possible deconjugation of pirlimycin ribonucleotide adducts added to soil through manure application. It is suggested that pirlimycin was partially excreted as ribonucleotide adducts and sulfoxone and sulfoxide metabolites in manure collected from treated cows (Hornish et al., 1992). These ribonucleotide adducts and metabolites may have been added to soil through manure application within the feces and urine, respectively. There have been several studies that examine ribonucleotide adduct formation within different lincosamide antibiotics, as it can be a mechanism for resistance in *streptococci* and *staphylococci* bacteria (Neu and Gootz, 1996). Lincosamide resistance can be achieved through methylation of the binding site or deactivation of the antibiotic through enzymatic adenylation, which is the addition of adenylate at the C-3 hydroxyl group of lincosamides (Pascale and Wright, 2010). Hornish et al. (1992) found that 32% of pirlimycin excreted in feces was in the form of pirlimycin 3- (5'-adenylate) and was the major adduct excreted in feces, with the combination of uridylylate and adenylated sulfoxone metabolites making up 18% of the pirlimycin in feces. Also, ribonucleotide adducts can be formed by simply incubating pirlimycin in manure from untreated cows, and 90% of maximum conversion was achieved within 24 hours when pirlimycin, lincomycin, and clindamycin were incubated in a broth of the crude enzyme produced by *S. coelicolor*, magnesium chloride, and potassium phosphate in water. After incubation, antibiotic activity was restored through addition

of a phosphodiesterase catalyst, which indicates the adducts can be reverted back to the original parent compound (Marshall et al., 1985).

Once excreted, pirlimycin ribonucleotide adducts can revert back to parent pirlimycin through deconjugation (Hornish et al., 1992). Hornish et al. (1992) found that 50% of pirlimycin in feces was excreted as ribonucleotide adducts, while 8% of pirlimycin in urine was excreted as sulfoxide and sulfoxone metabolites. Based on results from a companion manure incubation study using the same manure as this soil incubation, pirlimycin concentrations in manure collected from treated cows increased over 14 days when incubated at 10°C and 25°C, while spiked manure collected from untreated cows showed a constant decrease of pirlimycin concentrations in manure over 14 days (Ray, unpublished data, 2015). The manure incubation study further supports the proposal that ribonucleotide adducts and metabolites influenced the rate of loss of pirlimycin over time in this soil incubation.

Pirlimycin concentrations in soil were above the detection limit (LOD = 0.01 ng g⁻¹) in all samples over the 90 day experiment. All treatments decreased from day 14 to day 90, and the sandy loam 10°C treatment had significantly greater remaining pirlimycin when compared to all other treatments, which were similar, on day 56 and day 90. This indicates that, while lower temperatures played a role in increasing persistence, the soil type also influenced pirlimycin reduction over time, with less remaining pirlimycin at day 7 in the loam soil.

With a polynomial fit, linear regressions were significant with strong correlations ($r^2 \geq 0.69$) for all treatments (Table 5.3). Using equations from the linear regression analysis, the time to 50% decrease was calculated. The time to 50% decrease from the initial pirlimycin concentration was 15, 19, 35, and 61 days, following a trend of loam 21°C \approx loam 10°C \geq sandy

loam 21°C > sandy loam 10°C, respectively, which indicates both temperature and soil type significantly affect the persistence of pirlimycin after manure application.

These results are similar to other studies on the effect of temperature on antibiotic degradation in soils. While this study did not measure or quantify the actual half life of pirlimycin due to the possible influence of ribonucleotide adduct deconjugation, temperature is an important factor controlling the transformation of antibiotics in soils (Kumar et al., 2005). Lower temperatures increased the persistence of ivermectin, an antiparasitic drug, when soil-feces mixtures were incubated under summer and winter conditions (Halley et al., 1993; Halley et al., 1989). This is important as the concentration of pirlimycin in the 10°C treatments increased to the same level or above the initial pirlimycin concentration in both soil types, which could be an important factor in controlling pirlimycin movement in the environment.

pH Incubation

Time, temperature, and soil type were the significant main treatment factors affecting pirlimycin loss over time, with significant pH*time and soil type*time interactions. Initial soil concentrations were significantly different at time 0, and initial manure concentrations were also different among the pH treatments. USFDA (1993) found that pirlimycin was rapidly hydrolyzed above pH 9 and solubility decreased with increasing pH, with maximum solubility at pH 4.5 (70 mg L⁻¹) and minimum solubility at pH 13 (3 g mg L⁻¹). It is possible that pH adjustment hydrolyzed pirlimycin, affected the solubility of pirlimycin within manure, or affected the presence of pirlimycin conjugates. Pirlimycin concentrations in manure followed a trend of pH 5 (50 ng g⁻¹) > temperature (44 ng g⁻¹) > pH 7 (37 ng g⁻¹) > pH 9 (13 ng g⁻¹). However with only one replicate of the initial manure, statistical comparison was not available. The measured pirlimycin concentration in soil was divided by the initial soil concentration for comparison.

In both soil types, the pH 5 treatment followed a similar trend over time (day 0 > day 7 > day 14 > day 56 ≈ day 90), with all other treatments showing a slower rate of pirlimycin reduction (day 0 > day 7 ≈ day 14 > day 56 ≈ day 90). While loam pH 7, loam pH 9, sandy loam pH 7, and sandy loam pH 9 numerically increased, there were no significant differences between day 7 and day 14 for these treatments. In both soils, the pH 5 treatment significantly decreased from day 7 to day 14, while other treatments remained constant or increased. This indicates that adducts may be less stable in manure with a lower pH and exist as parent compound when initially added through manure application, as the initial concentration of pirlimycin in manure and soil was greatest in the pH 5 treatment at day 0. Also, there were no significant differences between treatments after day 14. This implies that manure pH impacts pirlimycin concentration in soil over a shorter period of time than temperature.

The time to 50% reduction was calculated as previously described for temperature treatments, with $r^2 \geq 0.47$ for all treatments (Table 5.3). Time to 50% reduction followed a trend of sandy loam pH 9 \geq loam pH 9 \approx sandy loam pH 5 \geq loam pH 5 \approx sandy loam pH 7 \geq loam pH 7. It appears that pH adjustment did not affect the time to 50% reduction of pirlimycin in soil as much as it affected the initial concentration of pirlimycin in manure and day 0 soil samples. Several studies show that pH can affect the degradation of antibiotics differently. For example, cefipeme was stable in aqueous solutions ranging from pH 4 to 6, but higher pH's increased the degradability. The sandy loam pH 9 treatment had the longest time to 50% decrease in pirlimycin over time, which indicates a different trend of pirlimycin degradability.

While the initial manure pH would control whether pirlimycin exists in an ionized or neutral form, soil has a high pH buffering capacity, which means soil pH would return to the initial pH shortly after manure application. According to USFDA (1993), pirlimycin is rapidly

hydrolyzed above pH 9, which may have affected the initial pirlimycin concentration in manure. More testing is needed to determine the effects of pH on pirlimycin forms and availability in manure. Similar trends of lower initial pirlimycin concentrations in pH adjusted manure were detected in the companion manure incubation study conducted with the same manure used for this soil incubation (Ray, unpublished data, 2015). While this soil incubation study was unable to produce values for half lives of pirlimycin, regressions provide valuable information as to the trend within a soil system, where pirlimycin exists in multiple forms and can conjugate or deconjugate after application to soil. More information is needed to provide a clear picture of what happens in a soil system after manure collected from treated cows is applied to soil.

Cephapirin

Cephapirin was not detected in the manure that was stored in the freezer or day 0 soil samples, indicating rapid transformation within the manure prior to application or within the time of experiment setup. Also, freezing manure samples could have influenced the solubility of cephapirin in manure. Ceftiofur, a third generation cephalosporin, degraded rapidly in urine, with a half life of 23 hours, and when urine and feces were mixed, the half life decreased to 17 hours (Gilbertson et al., 1990). Beta lactamases are found in the digestive tract of cows. Therefore, the presence of fecal material increases the degradation rate of cephalosporins, which are susceptible to deactivation by these enzymes. While the parent compound was not detected, this does not suggest that cephapirin is not a compound of concern in reference to antibiotic resistance gene formation. Cephapirin is widely used, and desacetylcephapirin maintains 54% of the biological activity of the parent compound (Cabana et al., 1976). Therefore, analyzing samples for desacetylcephapirin could provide valuable information to confirm the degradation of cephapirin and determine the potential influence of this compound on antibiotic resistance.

Because cephalosporin was not detected in soil or manure, MS2 screening was conducted to locate evidence of cephalosporin and this major metabolite, desacetylcephalosporin. Using m/z values provided through previous studies, manure samples showed a possible peak for cephalosporin products at m/z of 226 (Figure 5.3) (Heller et al., 2000). The possible peak of cephalosporin or desacetylcephalosporin degradation products was detected at 1 min (Figure 5.3 and 5.4). Further analysis is necessary to verify the identity and quantify the compound creating the peak.

Conclusions

Results showed that pirlimycin decreased with time when manure collected from cows administered antibiotics was added to soil. However, the decrease with time was faster in a fine textured soil with greater OM than in a coarse textured soil with low OM. The decrease with time was faster with higher temperature, as would be expected due to greater microbial activity at 21°C than 10°C. The pH of the manure affected the initial concentration of pirlimycin within the manure and day 0 soil samples, which indicates the pH is important in determining the form of pirlimycin in manure. Cephalosporin was not detected in initial manure or soil samples, but there were indications that metabolites were present in the manure. This is supported by literature stating that cephalosporin rapidly degrades in the presence of fecal material.

Table 5.1. Physical and chemical properties of two soils collected in Virginia, a loam and sandy loam soil, used in a 90 day manure-soil incubation study.

Soil Series	Soil Texture	Sand	Silt %	Clay	OM g/kg	pH	Mehlich-1 P mg/kg	CEC	Base Saturation
Braddock	Loam	46	44	10	52	6.48	6	8.9	99.3
Bojac	Sandy Loam	71	22	7	17	6.51	3	4.5	98.9

Table 5.2. MS/MS operating conditions for detection and quantification of pirlimycin and cephalirin in soil and manure extracts.

Parameters	Pirlimycin	Cephapriin
Ionization	Electrospray negative ionization	
Capillary voltage (V)		3000
Fragment voltage (V)		380
Collision energy (V)		15
Ion source temperature (°C)		250
Nebulizer gas flow (L min ⁻¹)		16
Parent ion (m/z)	424	411
Qualifier ion (m/z)	181	363
Quantifier ion (m/z)	292	112

Table 5.3. Regression analysis of temperature and pH soil incubation experiments conducted with a loam and sandy loam soil collected in Virginia.

Experiment	Soil Type	Treatment	Regression Equation	r^2	Time to 50% Decrease (d)
Temperature	Loam	10	$y = 0.0002x^2 - 0.0304x + 1$	0.83	19 B
		21	$y = 0.0003x^2 - 0.0389x + 1$	0.78	15 B
	Sandy Loam	10	$y = 0.00005x^2 - 0.0163x + 1$	0.87	89 A
		21	$y = -0.0001x^2 + 0.0032x + 1$	0.69	35 AB
pH	Loam	5	$y = 0.0003x^2 - 0.038x + 1$	0.76	15 BC
		7	$y = 0.0004x^2 - 0.042x + 1$	0.48	13 C
		9	$y = 0.0003x^2 - 0.034x + 1$	0.47	17 AB
	Sandy Loam	5	$y = 0.0002x^2 - 0.033x + 1$	0.96	17 AB
		7	$y = 0.0003x^2 - 0.0376x + 1$	0.72	15 BC
		9	$y = 0.0002x^2 - 0.0308x + 1$	0.92	19 A

Figure 5.1. Pirlimycin disappearance when incubated at two temperatures (10 °C and 21 °C) over 90 days after application of dairy manure to loam and sandy loam soil types collected in Virginia.

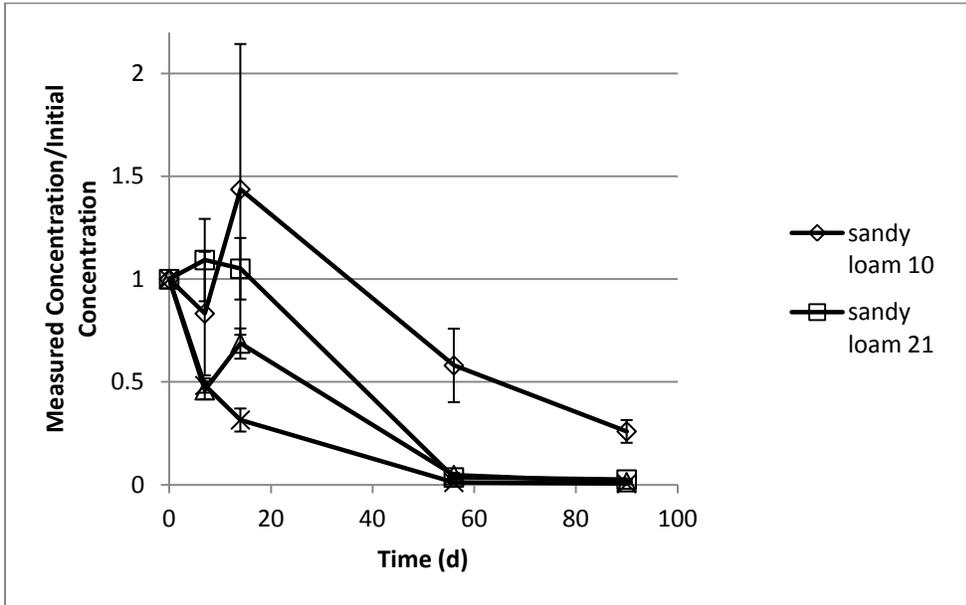
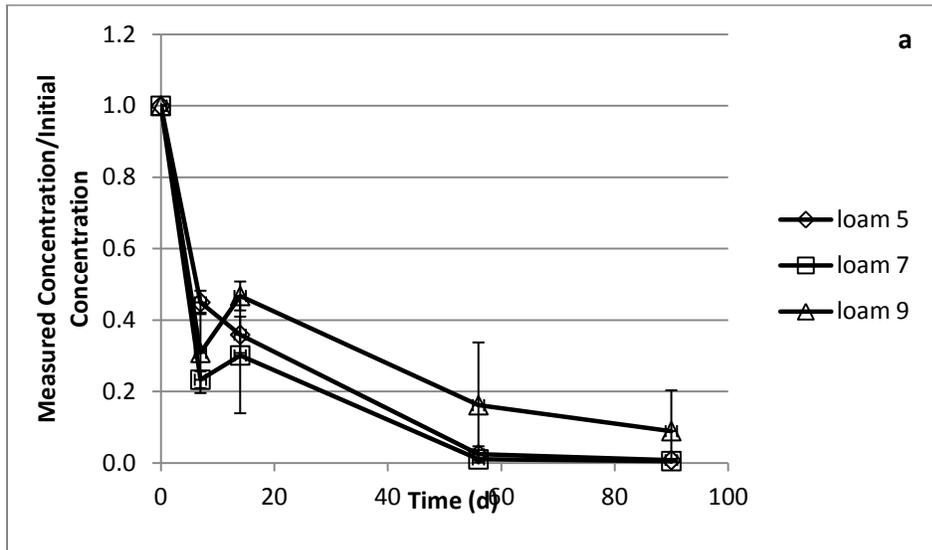


Figure 5.2. Pirlimycin disappearance over 90 days after application of dairy manure at 3 pH's to a.) loam and b.) sandy loam soil types collected in Virginia.



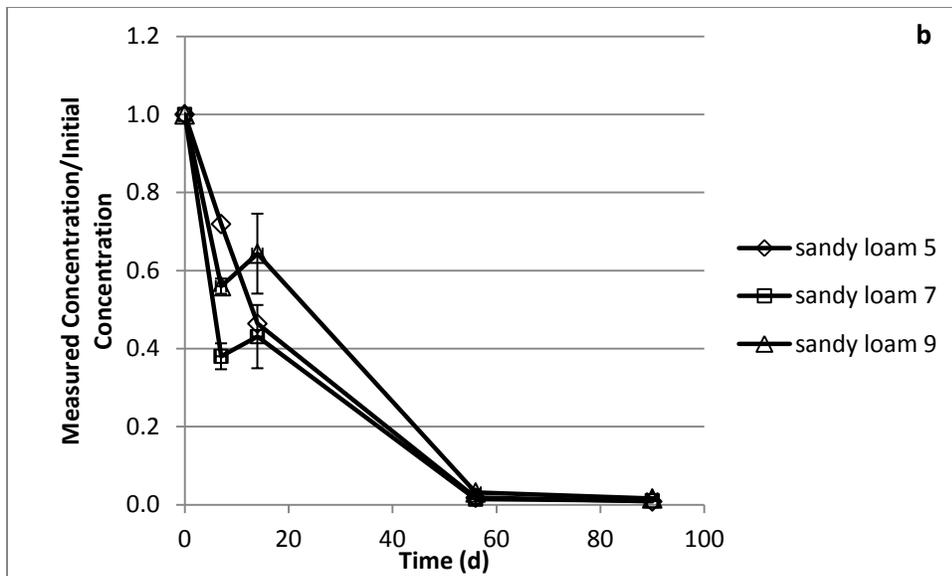
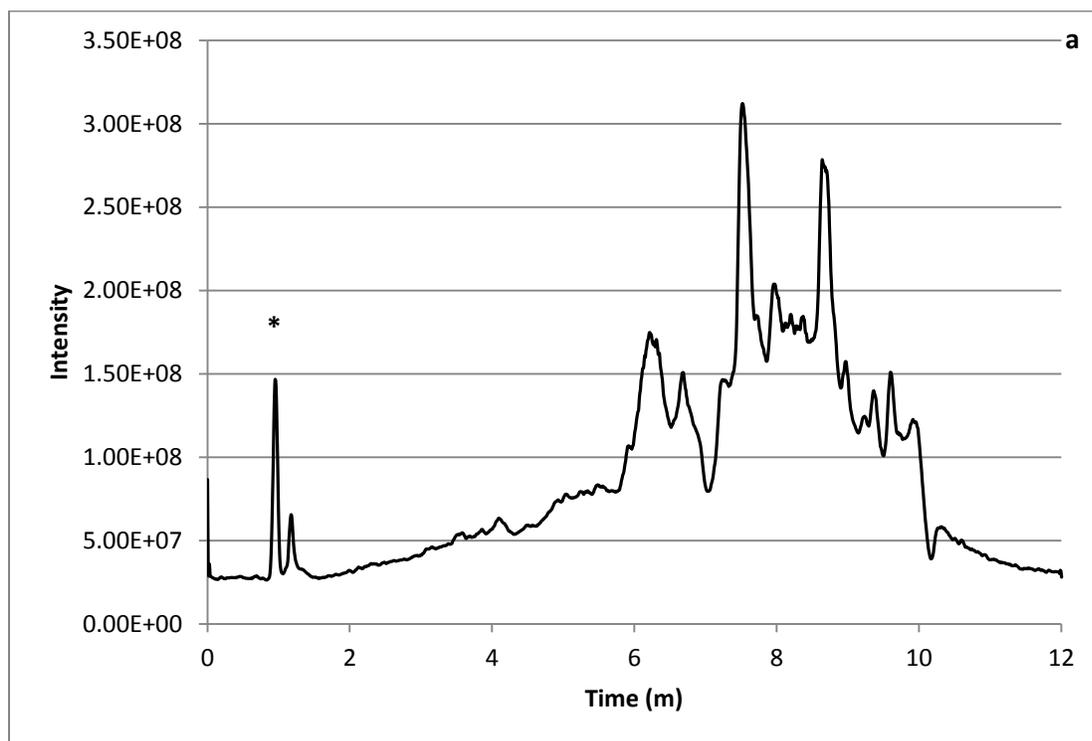
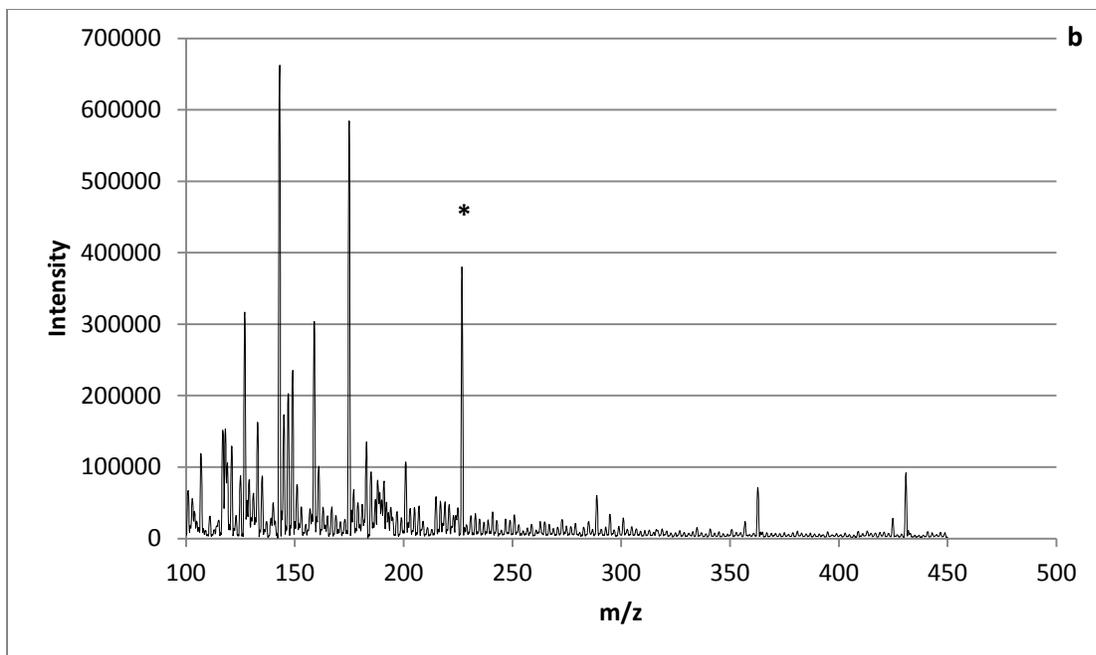


Figure 5.3. Chromatogram of MS2 screening of extracted manure sample collected from cows treated with cephalosporins and (b) mass spectrum analysis of potential degradation products (m/z 226) of cephalosporins or desacetylcephalosporins at 1 min.





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Chapter 6: Implications of Presented Research

Manure management and related environmental concerns are complex issues requiring a concerted effort of all stakeholders involved: farmers, regulators, land owners, etc. Over application of manure in sensitive watersheds can contribute nutrients and antibiotics to the environment, potentially increasing the rate and occurrence of eutrophication and antibiotic resistance gene formation. Therefore, new technologies are necessary to maintain crop production goals while preventing the release of these compounds into the environment. In the research presented, injection increased the capture of nutrients and antibiotics in agricultural fields and provided more soil nitrogen for crops.

While orchardgrass hay yields were not increased, protein content in injected treatments was greater than surface applied treatments, indicating greater N availability when poultry litter is injected. However, increased quality may not provide adequate compensation for farmers to adopt poultry litter injection, and the Subsurfer is not ready for farm use. To be farm ready, the Subsurfer must accommodate a full load of litter without failure of the auger system to provide poultry litter to injection slits. The environmental benefits of manure injection indicate that injection could be a valuable tool for manure management, and further research involving other crop types is required to fully understand the impact of poultry litter injection on crop production.

Poultry litter injection reduced ammonia volatilization and nutrients in runoff to levels of a control, and dairy manure injection reduced antibiotic loss in runoff to levels of a control. Future research should include a cost-benefit analysis of both injection technologies, and these analyses should include environmental (nutrient and antibiotic transport) and crop yield and quality benefits provided through injection. Also, injection could be implemented in or near setback areas, such as near houses or roads, due to reduced nutrient transport and increased

control of manure placement when using the Subsurfer. Increasing land area available for manure application could provide a benefit for farmers that could encourage adoption of this technology.

Antibiotic resistant gene formation is an issue of increasing concern due to the possibility of reduced antibiotic effectiveness in animal and human medicine. The potential for transport of pirlimycin to the environment through surface runoff was diminished when dairy manure was injected, and studies have shown that antibiotic resistance genes found in agricultural soils after manure application return to background levels over time. However, more information is needed to determine how time to the initial runoff event affects the transport and transformation of pirlimycin and whether pirlimycin applied through manure injection can leach downward into the soil. If manure is applied hours or days before a runoff-generating rainfall event, pirlimycin transport to the environment could be reduced due to photodegradation and soil sorption. It is suggested that more soil samples should be collected at discrete distances from the injection slit to determine pirlimycin movement within the soil, and soil should be sampled to a depth greater than 20 cm to provide further information on the potential for pirlimycin leaching.

The incubation experiment focusing on the effects of initial manure pH and incubation temperature on pirlimycin transformation left many unanswered questions. A companion incubation experiment should be conducted using spiked manure to determine the degradation kinetics of pirlimycin when applied to soil through manure application and compared to results found in this experiment. The difference in soil concentrations from incubations using spiked manure and manure collected from treated cows could provide useful information as to the magnitude of influence of pirlimycin conjugates within soil systems. If pirlimycin conjugates are

reverting back to the parent compound over time within soils, there could be an impact on antibiotic resistance gene formation over time.

Cephapirin was not detected in any manure, soil, or water samples in the rainfall simulation and incubation studies. Due to the low water solubility of cephapirin benzathine, stock solutions should be made from cephapirin sodium and any spiking tests should be conducted using cephapirin sodium. Also, there was evidence indicating the presence of desacetylcephapirin within manure samples, and desacetylcephapirin maintains 54% of the biological activity of cephapirin. Analysis of samples from these experiments should involve desacetylcephapirin due to the rapid transformation of cephapirin to this metabolite, and the potential influence of desacetylcephapirin on antibiotic resistance gene formation should be investigated.