FATE OF IONOPHORES UNDER TYPICAL BROILER LITTER MANAGEMENT FOR PASTURE PRODUCTION SYSTEM

by

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(Under the Direction of Miguel Cabrera and Aaron Thompson)

ABSTRACT

Several drugs used in livestock production belong to a major veterinary drug class known as polyether ionophores. In broiler (*Gallus gallus domesticus*) production, monensin and salinomycin are widely used ionophore anticoccidials (i.e. against intestinal coccidian parasites) that can appear in broiler litter (manure plus bedding material). Applying broiler litter as fertilizer to agricultural grasslands could introduce polyether ionophores to nearby water bodies if they are mobilized during runoff or leaching events. This dissertation aimed to evaluate the occurrence of ionophores in broiler litter and surface runoff under typical field handling procedures, and to evaluate the impact of long-term broiler litter application to soil on its capacity to retain monensin. First, a simulated rainfall experiment was conducted to examine the occurrence of ionophores in surface runoff in response to (a) addition of aluminum sulfate (alum) to broiler litter and (b) increased length of time between broiler litter application and the first simulated rainfall event. Results from this experiment showed that both methods could decrease levels of monensin and salinomycin in surface runoff from grassed soils receiving surface-applied broiler litter. Second, a broiler litter storage (or stacking) study

was conducted to assess the change in ionophore concentrations in broiler litter in response to (a) stacking and (b) alum addition. Results showed that 112 d of stacking broiler litter did not have any impact on monensin concentration but it did slowly reduce salinomycin concentration by 55%, regardless of whether broiler litter was amended with alum or not. Adding alum to broiler litter reduced monensin concentration by approximately 20% relative to unamended broiler litter, but it did not change salinomycin concentration. Such findings call for continued search for alternative strategies that potentially reduce litter ionophore concentrations prior to broiler litter application to agricultural soils. Third, sorption and desorption experiments were carried out to determine the impact of long-term surface application of broiler litter on the capacity of pasture soil to sorb and desorb monensin. These experiments showed that long-term broiler litter application would increase mobility of monensin from grassland soils, if such litter applications increase soil pH.

INDEX WORDS: Polyether ionophores, monensin, salinomycin, broiler litter, aluminum sulfate, pasture, surface application, time of rainfall, surface runoff, litter stacking, sorption, desorption

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by

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To The One, through Whom and for Whom everything exists

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CHAPTER 1

INTRODUCTION

Several antiparasitic veterinary drugs used in the US broiler (*Gallus gallus domesticus*) industry belong to the chemical family of polyether ionophores. Before ionophores were introduced in the early 1970's, a parasitic protozoan disease, known as coccidiosis, had been a major cause of weak poultry performance and productivity (Chapman 2010). Recently, the Food and Drug Administration (2014) ranked polyether ionophores as the second domestic top-selling veterinary drug with more than 4 million kg sold in 2011 and constituting 78% of marketed antimicrobials solely used for animals. These drugs are also used in swine, sheep, and cattle production, either for controlling coccidiosis or for promoting growth or both.

In poultry production, monensin and salinomycin are among the most commonly used ionophore anticoccidial drugs (Peek and Landman, 2011; Landoni and Albarellos, 2015). Residues of these compounds have been found in broiler litter, a mixture of broiler manure, feathers and bedding material (Furtula et al., 2009; Biswas et al., 2012; Sun et. al., 2013a). Application of broiler litter as fertilizer could introduce monensin and salinomycin to nearby water bodies if they are mobilized during runoff or leaching events. In the US, where poultry production is the largest in the world (USDA-ERS, 2012), up to 12.8 million Mg of broiler litter are generated per year based on an average litter production of 1.5 kg per broiler (Vervoort et al., 1998; Endale et al., 2002) and an annual US broiler production of 8.52 billion broiler as of 2013 (USDA-NASS, 2014).

Based on this estimate, as much as 64 Mg of ionophores may be applied to agricultural soils in the USA every year, assuming a median ionophore concentration (monensin and salinomycin combined) of approximately 5,000 μg kg^{-1} present in the broiler litter (Sun et al., 2013a).

Several researchers have detected polyether ionophores in natural waters mostly in areas close to agricultural activities. For instance, monensin and salinomycin (<0.01 to ~0.04 μg L^{-1}) have been found in Cache la Poudre River in Northern Colorado (Cha et al., 2005; Kim and Carlson, 2006; 2007). Monensin has also been detected (0.02 to 0.21 μg L^{-1}) in the Grand River in Ontario, Canada (Hao et al., 2006), in dairy lagoons (3.91 to 16.24 μg L^{-1}), and in shallow groundwater (0.04 to 0.39 μg L^{-1}) beneath dairy farms in the Central Valley of California (Watanabe et al., 2008). In Australian waters, monensin and salinomycin have also been detected along with 26 other antibiotics, with monensin being the most frequently detected veterinary drug in 92 out of 98 river samples (Watkinson et al., 2009).

The presence of the ionophores in the environment may cause harm to native biota. These compounds are known to be toxic to Gram-positive bacteria, protozoa, fungi, and even to higher organisms, including humans, which makes these drugs unsafe for human medicine (Rutkowski and Brzezinski, 2013; Hansen et al., 2009a; Khan et al., 2008). Based on reported ecotoxicological tests, polyether ionophores can be damaging to certain organisms living in aquatic and terrestrial environments (Žižek et al., 2011; Hillis et al., 2007; European Food Safety Authority, 2004, 2005; Hoagland, 1996). After considering the toxicological effects of ionophores and their occurrence in the

environment, Hansen et al. (2009a) reported that ionophores might pose an environmental risk, especially to sediment-dwelling organisms in streams.

Despite the potential environmental risks associated with ionophores, little research has been directed towards controlling sources of ionophore contamination to the environment, particularly from agricultural grasslands receiving surface-applied broiler litter. Previous studies examining the movement of ionophores from agricultural lands have focused on bare tilled soils (Davis et al., 2006; Kim et al., 2010). These studies, however, do not represent conditions that are typical of grasslands, which do not get tilled and have a grass cover throughout the year. Given recent findings that monensin and salinomycin were detected in natural runoff from grasslands fertilized with broiler litter (Sun et al., 2013a), the need to develop methods for mitigating ionophore mobility in surface runoff from these systems becomes more important.

One method to reduce movement of ionophores to surface runoff from grasslands may be to expose them to acidic conditions (pH 3 to 5), which allows decomposition of the ionophores through acid-catalyzed hydrolysis reactions (Sun et al., 2013b; Bohn et al., 2013). This may be achieved by decreasing litter pH with aluminum sulfate (alum), a recommended and commonly used chemical amendment for broiler litter to decrease ammonia inside broiler houses and dissolved phosphorus in surface runoff (Moore, 2011). Another potential method for controlling presence of ionophores in surface runoff is timing broiler litter applications. Applying litter during longer dry periods may allow for inonophores to degrade in the surface-applied litter prior to occurrence of rainfall.

Aside from decreasing ionophore transport during field applications, the mobility of ionophores in the environment may also be minimized if ionophores are allowed to

degrade in broiler litter over time prior to application to the field. This may be achieved by stockpiling (or stacking) broiler litter under an open storage (roofed but without walls), which many farmers currently do while waiting for the proper time to apply litter to their fields (Cunningham, et al., 2012). Moreover, stacking broiler litter in the presence of alum may further reduce ionophore concentrations in the litter through abiotic hydrolysis at low pH. To our knowledge, no study has evaluated ionophore degradation during broiler litter stacking when broiler litter is amended with alum.

The mobility of broiler litter-derived ionophores depends on their chemical properties and their interactions with the soil. However, limited information is available on sorption and desorption of polyether ionophores on soils. In particular, no information is available on the impact of repeated long-term application of animal manure such as broiler litter on the capacity of litter-amended soils to retain monensin, the most commonly used ionophore in poultry.

In terms of its chemical properties, monensin consists of a series of oxygenated heterocyclic rings and a single terminal carboxyl group. It is capable of forming lipophilic, quasi-cyclic neutral complexes with monovalent alkali ions particularly Na at pH above its pKa of 4.5 (Sun, 2014). During complexation, the molecule orients its polar groups around the cation, thereby making the inner surface of the complex hydrophilic and its outer surface hydrophobic. The lipophilic nature of monensin has also been apparent from its low solubility limits in water (5-63 mg L^{-1}) and high octanol-to-water partitioning constants (log Kow = 2.8 to 4.2) (Hansen et al., 2009b).

The suggested hydrophobic properties of monensin might indicate its preference to sorb to soil organic matter. Previous works done with monensin indicated soil organic

matter as among the factors likely controlling its sorption to soil (Sassman and Lee, 2007; Hussain and Prasher, 2011). However, several soils used in previous studies were dominated by 2:1 clay minerals (Sassman and Lee, 2007; Hussain and Prasher, 2011), which have higher cation exchange capacities than 1:1 clay minerals, and which through cation briding, could sorb greater soil organic matter. These soils are not typical in inherently nutrient-poor, 1:1 clay mineral-dominated, highly weathered soils, which underlie many grasslands along the Southern Piedmont in the United States (West et al. 2008). Sorption of monensin to soils could differ in magnitude depending on existing soil properties other than organic matter (i.e. mineral composition, pH, etc.). Hence, there is a need to evaluate sorption and desorption of monensin from grassland soils receiving long-term litter application.

Understanding the fate of the ionophores in different aspects under pasture/grassland production systems would help us design management strategies that limit ionophore transport following broiler litter applications. The overall goal of my dissertation was to evaluate the occurrence of ionophores in broiler litter under typical field handling procedures of the litter and to evaluate the impact of long-term soil broiler litter application on ionophore retention. My specific objectives were 1) to evaluate the effect of alum additions to broiler litter and the effect of applying broiler litter during longer dry periods on the occurrence of ionophores in surface runoff, 2) to evaluate the change in ionophore concentrations in broiler litter when stacked with or without alum, and 3) to evaluate the capacity of soils under long-term broiler litter application to sorb monensin as influenced by factors such as pH and temperature, and to evaluate the capacity of these soils to desorb monensin as a function of reaction time. These were

accomplished by examining the occurrence of ionophores in surface runoff from a simulated rainfall experiment, investigating in-situ degradation of ionophores under real litter stacking conditions, and evaluating the removal of monensin from solution through sorption and desorption experiments.

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CHAPTER 2

LITERATURE REVIEW

BROILER PRODUCTION AND THE ENVIRONMENT

Broiler production in the USA

The US poultry production is among the largest throughout the globe. In 2012, the USA ranked as the world's largest poultry producer and the second-largest poultry meat exporter (USDA ERS, 2012). The value of poultry products totaled $44.1 billion in 2013, with broiler (*Gallus gallus domesticus*) production accounting for 70% of it (USDA NASS, 2014).

Among other factors, the vertically integrated system of the US broiler industry is ascribed for its productivity. This system is organized through the establishment of production contracts between chicken companies (or integrators) and independent growers. To reduce transportation costs, integrators locate their hatcheries, feed mills, and processing plants close to grow-out broiler farms. This encourages growth of large facilities and renders broiler production geographically concentrated particularly in the Southeastern US (MacDonald, 2008). In 2013, the US produced up to 8.52 billion broilers in total, with 78% of this production coming from the top ten broiler-producing states in descending order as follows: Georgia, Alabama, Arkansas, North Carolina, Mississippi, Texas, Kentucky, Maryland, Missouri, and Virginia (USDA NASS, 2014).

Environmental issues associated with broiler litter

Coupled with the success of the broiler industry is the massive production of broiler litter. Broiler litter is generated when bedding materials such as sawdust, pine chips, or straw are placed on the floor inside broiler houses to absorb moisture from broiler manure. After being used, the litter is removed, stored inside stack houses and/or immediately utilized as fertilizer for agricultural fields. In the state of Georgia, most of the generated broiler litter is surface-applied (or spread) to pastures.

The production of broiler litter (i.e., manured bedding material) and its application to agricultural soil has been subject to a lot of environmental issues. Problems associated with broiler litter include elevated levels of volatilized ammonia inside broiler houses and loading of dissolved nutrients (particularly nitrogen and phosphorus), metals, and soluble organic carbon in runoff from pastures receiving surface-applied broiler litter. Currently, the US EPA has reported some 2,787 km of US streams that are impaired due to runoff from animal wastes (US EPA, 2015) such as broiler litter.

To mitigate these environmental problems, several litter amendments have been available for use. For example, among those used to control ammonia emissions include clay, organic carbon amendments, microbial inhibitors, enzyme inhibitors, acids and acid salts (Moore et al., 2001). Among these amendments, however, aluminum sulfate (or alum) has been shown and recommended for best management practice (BMP) and is estimated to be used on approximately one billion broilers grown throughout the US annually (Moore, 2011). The BMP recommendation for alum was brought about not only because of its effect on addressing the above-mentioned environmental issues associated

with unamended litter but also because of its effect on improving broiler productivity and the fertilizer value of the litter (Moore et al., 2000).

While control measures have been widely studied for ammonia emissions and excess nutrients and metals in runoff, limited research has been done on the fate of other potential pollutants from broiler liter such as veterinary drugs particularly polyether ionophores. To date, the US EPA's list of priority pollutants do not include the polyether ionophores (US EPA, 2014). Hence, discharge standards have not yet been developed for any of these veterinary pharmaceuticals, making these drugs currently unregulated in the environment.

NATURE AND PROPERTIES OF POLYETHER IONOPHORES

Ionophores (coined from the Greek words *ion* for ion and *phore* for carrier) are compounds capable of forming lipid-soluble cation complexes that serve as mediums for carrying ions across cellular membranes (Pressman and Fahim, 1982). Ionophores occur in vast diversity from those that are produced naturally by microorganisms to those that are prepared synthetically. Naturally-existing ionophores are produced mostly by the genus *Streptomyces* although other genera such as *Streptoverticillium*, *Nocardiopsis*, *Nocardia*, and *Actinomadura* are also capable of producing them (Benno et al. 1988). Based on their mode of transporting ions, ionophores are classfied as either neutral ionophores, channel-forming quasi-ionophores or carboxylic ionophores (Pressman, 1976).

Ionophores utilized for veterinary purposes constitute only a small subset of the entire class of ionophores (Butaye et al., 2003). The commonly used ionophore additives

in broiler feeds, monensin and salinomycin, belong to the carboxylic ionophores. Carboxylic ionophores consist of a series of several cyclic ether functional groups, one terminal carboxyl group on one end of the molecule, and a hydroxyl group on the other (Fig. 2.1a & 2.1b). Hence, they are also called polyether ionophores. Although they are depicted as having an open-chain structure, these compounds actually form rings through an intramolecular head-to-tail hydrogen bonding (Pressman, 1976) (Fig. 2.1c & 2.1d). In their deprotonated anionic form, monensin and salinomycin polyether ionophores complex with alkali metal ions with preference for Na^+ and K^+ in particular over other monovalent cations, respectively. During complexation, the cation loses its water of solvation and fits right into the core of the quasi-cyclic complexes (Pressman, 1976). This pseudo-cyclic conformation is created by orienting the polar groups of the molecule towards the core, thereby making the inner surfaces of the complex hydrophilic and its outer surfaces lipophilic. The carboxyl group may be involved in the cation liganding as is the case for salinomycin (Paulus et al., 1998; Miao et al., 2003) or may not be involved as is the case for monensin (Pressman, 1976; Mollenhauer et al., 1990) (Figs. 2.1c and 2.1d). The positive charge of the engulfed cation and the negative charge of the deprotonated carboxyl are internally compensated, making the cation-polyether ionophore complexes electrically neutral.

Polyether ionophores are non-volatile compounds owing to their low Henry's constant (in the range of 10^{-18} Pa L mol^{-1}; Thiele-Bruhn, 2003). They have low solubility limits in water and high-octanol-to water partitioning constants (Kow) (Table 2.1), explaining their high lipophilicity. Water solubility limits compiled by other workers (Dolliver and Gupta, 2008a; Hansen et al. 2009) showed a rather wide range. The same is

true for their pKa, a property indicating their undissociated or dissociated forms as a function of pH. This may be due to differences in experimental conditions under which these parameters were obtained such as differences in pH, ions present in solution, and organic solvent or organic solvent/water mixtures used (Sun, 2014). The use of organic solvent in determining several of the compounds' physicochemical properties (especially their pKa) is due to their low water solubility. Nevertheless, measurements of several of these properties were successfully carried out by Sun (2014) under aqueous conditions. These included the pKa of monensin (Table 2.1), as well as the water solubility of the different polyether ionophore species and their metal dissociation constants (Table 2.2).

The capacity of polyether ionophores to complex ions in their unique conformation underlies their antimicrobial properties. Their biological activity lies on their capacity to transport ions across the lipid bilayer of cellular membrane, thereby impairing the natural osmotic balance of cells. Osmotic balance is critical for proper cellular functioning and survival. As the polyether ionophores alter the cellular ionic gradient, the cell spends too much energy in trying to maintain proper ionic balance and eventually, the cell exhausts its energy source (Bergen and Bates, 1984). Additionally, increased intracellular ionic levels cause water to enter, which ultimately leads to swelling and bursting of the cell (Smith and Galloway, 1983).

Under extremely acid conditions (i.e., pH 3-4), however, polyether ionophores become unstable as their molecules break down through acid-catalyzed hydrolysis, an abiotic degradation process (European Food Safety Authority, 2004; Sun et al., 2013b; Bohn et al., 2013). As the pH of aqueous solutions is increased, hydrolytic degradation of

these compounds become slower. Table 2.3 shows their hydrolytic rate constants and half-lives under different pH conditions based on the findings of Sun et al. (2013b).

USE OF POLYETHER IONOPHORES IN LIVESTOCK PRODUCTION

The biological mechanism of action of polyether ionophores makes them effective against coccidia, a group of parasitic protozoa commonly infesting the intestines of broilers and turkeys. Hence, their use as anticoccidial drugs in poultry production. These veterinary drugs, however, are not exclusively used as feed additives for broilers and turkeys. They are also used in swine, sheep, and cattle production mainly for growth promotion.

Recently, the Food and Drug Administration (2014) ranked polyether ionophores as the second domestic top-selling veterinary drug with more than 4 million kg sold in 2011 and constituting 78% of marketed antimicrobials solely used for animals. Although no data are available on the amounts of polyether ionophores used exclusively for anticoccidial purposes in broiler production, demand for these drugs might increase as other popularly used anticoccidial drugs (i.e., organoarsenic) were voluntarily suspended by its manufacturer in 2011 in response to the Food and Drug Administration's concerns for potential carcinogenic impact on public health of increased inorganic arsenic levels in chicken livers (Sun et al., 2013b; Food and Drug Administration, 2011).

ENVIRONMENTAL CONCERNS WITH POLYETHER IONOPHORES

The interfering action of polyether ionophores to natural ion transport affect both prokaryotic and eukaryotic cells (Butaye et al. 2003). Besides its known inhibitory effects

to Gram-positive bacteria, fungi and protozoa (Rutkowski and Brzezinski, 2013), these compounds have also shown to cause mortality or deteriorate growth and development or reproduction of other organisms such as fish, zooplankton, earthworm and some plants. For instance, these drugs caused mortality to 50% of fish (*Oncorhychus mykiss*) population after a 96-h exposure to concentrations of 1.88 and 1.66 mg L^{-1} for monensin and salinomycin, respectively (European Food Safety Authority 2004, 2005). Harmful ionophore effects to other organisms include decreased zooplankton abundance and richness of specific taxonomic groups (at 500 µg monensin L^{-1}) (Hillis et al., 2007), suppressed earthworm (*Eisenia andrei*) reproduction (EC_{50} of 12.7 mg monensin kg^{-1} soil) (Žižek et al., 2011) and even injury to several plant species (at 10^{-4} mol L^{-1} monensin) (Hoagland, 1996).

Although the toxicological concentrations of ionophores are much higher than the reported occurrences of these compounds in the environment (as described in Chapter 1), more information is needed to understand what the chronic losses may be and how these losses impact native biota. Boxall et al. (2003) raised concerns regarding other unknown effects of ionophore degradation products; the effects of ionophores when present with other veterinary drugs or other chemical pollutants; and the effect of ionophore presence in the environment on development of pathogenic resistance in the long-run.

IONOPHORE TRANSPORT IN RUNOFF

The fact that polyether ionophores are solely used for animal welfare and not for human medicine renders agricultural production areas as major sources for introducing these drugs to the environment. Boxall et al. (2003) has identified runoff from fields

fertilized with manure containing polyether ionophores as a potential major pathway for transporting veterinary drugs to the environment. Indeed, Kim et al. (2010) and Davis et al. (2006) demonstrated transport of monensin in surface runoff in their simulated rainfall experiment. In their study, monensin not only showed the highest average concentrations in runoff over their 1-hr simulated rainfall but also lost the highest absolute mass and fraction of amounts applied in runoff among a suite of other veterinary drugs (tetracycline, chlortetracycline, sulfathiazole, sulfamethazine, erythromycin and tylosin). Peak concentrations for monensin reported in these studies (< 3 µg L^{-1}) occurred at the first 10 minutes of runoff collection. Cumulative absolute mass loss of 0.2 mg per 6 m^2 plot accounted for <0.1% of their total amount of monensin applied. Kim et al. (2010) recovered 34% of the applied monensin concentrated most particularly within the 10 to 20 cm depth cm of the soil after the rainfall simulation. Due to the low recovery of monensin in all phases in the runoff and in different soil depths after the experiment (34.6% of the amount applied), it is possible that monensin had moved to deeper soils beyond the sampling depth in their study.

Furthermore, surface runoff can potentially contribute ionophore loads to streams more so than leaching. In the 3-yr field study of Dolliver and Gupta (2008a) on a Rozetta silt loam soil, annual runoff losses of monensin generally accounted for <0.05% of the applied monensin, whereas annual leaching losses of monensin accounted for <0.005% from beef manure under chisel plowing and no-tillage corn production systems. In their study, the highest concentration of monensin detected in runoff was 57.5 µg L^{-1} whereas the peak concentration of monensin in the leachate was 40.9 µg L^{-1}. Both leaching and runoff detections of monensin were found to be significantly greater during the non-

growing season compared to measurements taken during the growing season. They attributed these findings to higher temperatures during the growing season periods, which presumably may have enhanced degradation and thus decreased the amounts of monensin available for transport through runoff from the fields. Furthermore, in the second year of their study, when several considerable snowmelt and rainfall events occurred, mass losses of monensin in runoff were found to be significantly greater from no-tillage compared to chisel plowed fields. The authors did not explicitly provide a clear explanation for this. However, they found greater runoff under no-tillage than under plowed soils for this particular year. The authors attributed greater runoff in no-tillage to its smooth surface which did not provide any form of obstruction to water flowing downslope during snowmelt as opposed to the rough surfaces of the soil created by plowing along contours for the plowed plots. Their findings suggest that greater losses of polyether ionophores may be larger in agricultural grasslands than in tilled soils when storms produce greater surface runoff in the former system.

To date, research examining transport of polyether ionophores in surface runoff remains limited to the above-mentioned studies. Such existing literature, however, do not represent other agricultural production systems such as those of grasslands receiving surface-applied broiler litter. For instance, previous works have focused on areas simulated for corn production systems on bare, recently tilled sandy clay loam soils (Aridic Haplustalf) (Davis et al., 2006; Kim et al., 2010) or on tilled vs no-till systems on silt loam soils (Typic Hapludalf) (Dolliver and Gupta, 2008a). These studies, however, are not representative of the conditions typical of grasslands, which do not get tilled and remain covered with grasses throughout the year. In addition, the soils used in previous

studies (Alfisols) were either alkaline or neutral (pH 7.9 or 7.0) (Davis et al., 2006; Kim et al., 2010; Dolliver and Gupta, 2008a) which are not prevalent in acidic, highly weathered soils of many grasslands along the Southern Piedmont of Georgia (West et al., 2008), a geographic area containing most of Georgia's grasslands for livestock production (Hancock et al., 2014). Depending on management systems and the type of soils, losses of monensin in runoff could be different in magnitude. Because grasslands (pastures and rangelands) and/or no-till systems receiving broiler litter applications may become more prevalent with the expansion of sustainable agricultural practices in the future (Vadas et al. 2011; Godfray et al., 2010), further research is needed on these systems to determine if these systems can be a significant source of ionophore pollution. Given recent findings that monensin and salinomycin were detected in natural runoff from grasslands fertilized with broiler litter (Sun et al., 2013a), the need to develop methods for mitigating ionophore mobility on these systems becomes more important.

At present, no published information is available on controlling runoff losses of polyether ionophores from grasslands receiving surface-applied broiler litter, particularly on how certain recommended management practice such as mixing of alum with broiler litter could influence their mobility or how time between surface application of broiler litter and occurrence of rainfall influences their presence in surface runoff. Amending broiler litter with alum might decrease losses of polyether ionophores in surface runoff by first decreasing litter pH, which is expected to enhance hydrolytic degradation of polyether ionophores as previously mentioned. Likewise, increasing the time between litter application and rainfall occurrence might reduce presence of these compounds in

surface runoff by giving time for the ionophores to degrade in the surface-applied litter prior to the occurrence of rainfall.

IONOPHORE DEGRADATION IN SOIL AND ANIMAL MANURE

Dissipation kinetics of polyether ionophores in soils occurs in a matter of days to a few weeks depending on the soils used and the experimental conditions imposed. For instance, at field capacity (-0.03 MPa), Sassman and Lee (2007) reported an estimated monensin half-life of up to 2 days, less than 4 days at 40-50% water holding capacity (Žižek et al., 2011), and about 4.2 days at 80% field capacity (Yoshida et al., 2010) although the latter also reported a half-life of 22.7 days for another Argentinian soil which had high organic carbon (4.69%). Moreover, Carlson and Mabury (2006) reported a monensin half-life of 13.5 days at 20% gravimetric soil moisture under laboratory settings. However, a much shorter half-life of 3.3 to 3.8 days was observed in the same soils under natural field conditions (Carlson and Mabury, 2006). In terms of salinomycin, a degradation half-life of 5 days was reported in soil at 20% gravimetric moisture content (Schluesener and Bester, 2006).

Unlike studies of degradation of polyether ionophores done in soils, information on their degradation in animal manure is limited. In an unprotected (unsheltered) stockpile of beef manure, a monensin half-life of 31.5 days during warmer and wetter weather and 231 days during colder and dryer weather can be estimated from the work of Dolliver and Gupta (2008b) assuming first-order decay. These workers attributed the slower degradation of monensin to lower air temperature (3°C vs 19 °C) with accompanied freeze/thaw effects on the manure. In their warmer experiment, degradation

of monensin in their unsheltered beef stockpile was estimated to account for >50% of the initial extractable monensin in the manure compared to only up to 1.9% lost to stockpile runoff from natural precipitation. These findings indicate that degrading polyether ionophores prior to manure or litter application may help limit the movement of these compounds to the environment.

In the interest of degrading polyether ionophores in turkey litter prior to litter application to the field, Dolliver et al. (2008) compared degradation of monensin in the following treatments: stockpiled litter as control (at 40% gravimetric water content of the litter), litter compost pile (managed with weekly moisture adjustments), or litter under vessel composting. Dolliver et al. (2008) observed degradation of monensin in all treatments with estimated half-lives ranging from 11 to 22 days. However, these half-lives did not differ significantly between any of the treatments. It is interesting to note the lack of significant difference in the degradation kinetics of monensin among the treatments despite significant differences in peak temperatures, and significant mass and nutrient (i.e., C and N) losses from the composted treatments in comparison to the stockpiled control. Dolliver et al. (2008) suggested stockpiling of manure after preadjustment of moisture content would be sufficient to degrade monensin.

Ramaswamy et al. (2010) also observed relatively greater differences in factors such as temperature profile, nutrient and mass losses between composted and uncomposted poultry manure (poultry type not specified) over time. However, contrary to the findings of Dolliver et al. (2008) for monensin, Ramaswamy et al. (2010) observed significantly lower concentrations of salinomycin in composted mixtures of poultry manure and hay in comparison to uncomposted open pile of poultry manure without hay.

Ramaswamy et al. (2010) estimated a salinomycin half-life of 1.3 days in the composted treatment and 4 days of salinomycin half-life for the uncomposted control. Based on their findings, Ramaswamy et al. (2010) suggested composting as a strategy to decrease concentrations of salinomycin in manure.

The quite contrasting findings of Dolliver et al. (2008) and Ramaswamy et al. (2010) impart difficulty in deducing the relative degradation behavior of different polyether ionophores due to differences in the type of manure or litter used, manure/litter composition or differences in the experimental conditions used. The work of Sun et al. (2014), however, has provided information concerning the relative degradation behavior of monensin and salinomycin in the same broiler litter material under laboratory conditions. In their work, monensin was found to persist in broiler litter microcosms across a wide range of litter moisture (from 320 to 2570 g H_2O kg^{-1} dry litter) and temperature (35, 45 or 60°C) levels (Sun et al., 2014). It is possible that the tested moisture and temperature conditions of Sun et al. (2014) were not favorable for monensin degradation or that unknown factors other than moisture and temperature likely limited the degradation of monensin in broiler litter. In contrast, salinomycin was found to degrade but only at certain combined moisture and temperature levels (Sun et al., 2014). Furthermore, Sun et al. (2014) predicted that in order for salinomycin to degrade in broiler litter during stacking, both temperature and moisture have to be maintained outside of a particular combined temperature and moisture domain wherein salinomycin is not expected to degrade based on their model prediction. However, Sun et al. (2014) cautioned that the said "no degradation" domain for salinomycin might be different for individual broiler litter samples.

Based on existing literature so far, it is apparent that degradation of polyether ionophores in one type of manure or litter cannot be assumed to hold true for other types of manure or litter. This is due to differences in the composition of different kinds of manure or litter. Organic matter composition of animal excreta is strongly dependent on animal species, diet composition and digestibility (Velthof et al., 2000). Similarly, differences even between samples of the same type of manure or litter could also possibly occur. These differences may come from distinct feeding of the animal, the housing system, system of manure collection, storage time, and microbiological and chemical processes during storage (Velthof et al., 2000).

In addition to focusing mostly on other types of manure/litter (i.e., beef manure, turkey litter or unspecified poultry manure), existing literature is based on studies conducted for short periods of time (i.e., 7 to 38-day durations). At present, no information is available on the long-term degradation of polyether ionophores from stacked (or stored) broiler litter under similar conditions commonly practiced by farmers prior to broiler litter application to the field. Furthermore, no information is available on how certain recommended management practices such as mixing of alum with broiler litter could influence the degradation of polyether ionophores particularly in broiler litter during stacking.

SORPTION AND DESORPTION OF POLYETHER IONOPHORES IN SOILS AND WOOD CHIP SORBENTS

Sorption isotherms of polyether ionophores follow a general linear trend for most studies done in soils and wood chip sorbents although they are also described using

Freundlich models (Sassman and Lee, 2007; Hussain and Prasher, 2011; Ilhan et al., 2012). Estimated linear partitioning coefficients (Kd), Kd normalized to organic matter (Koc), and Kd normalized to clay (Kclay) are shown in Table 2.4. These sorption parameters differed widely depending on the type of soil used and their properties.

Sassman and Lee (2007) modeled sorption of monensin to soils of different physico-chemical properties using the Koc(pH-pKa) relationship derived by Lee et al. (1990) for the unionized and ionized species of monensin in the aqueous phase. In their study, log Koc of monensin was found to be inversely related to soil pH with 60% of their data adequately described by the model, implying that organic matter and soil pH are controlling sorption of monensin for most of their soils. They attributed the lack of sufficient model fit at pH above monensin's pKa to the complexation of monensin with alkali ions that would lead to metal-complexed species of monensin, which was not accounted for in their model.

Hussain and Prasher (2011) conducted sorption of polyether ionophores on two soils obtained from a constructed wetland. Greater sorption potential was observed for sandy clay loam than for sandy soil for monensin and salinomycin (Table 2.4) whether the soils were unsterilized or were sterilized prior to use. Although Hussain and Prasher (2011) attributed this finding to greater clay and organic matter contents of the former, our normalization of their reported Kd by these soil properties suggests organic matter may be controlling sorption of the compounds more than clay for their soil. Furthermore, a general decrease in portioning of the ionophores was also observed with an increase in pH. However, their magnitudes of decrease in Koc over wide soil pH were not as much as in comparison to those of Sassman and Lee (2007) who observed an almost two orders

of magnitude decrease in Koc between soil pHs ~4 and ~7. These may be due to differences in the quality of organic matter present in each soil used.

In contrast, Ilhan et al. (2012) reported Kd values for monensin from loamy soils from the Midwestern US (Typic Endoaquoll) that were much greater in comparison to the Kds reported by Hussain and Prasher (2011) and in comparison to many of the Kds observed by Sassman and Lee (2007) (Table 2.4). Of the two soils tested, Ilhan et al. (2012) saw smaller monensin partitioning in surface than in the subsurface soils. These differences, however, could neither be explained by the typical pH effect on monensin sorption as observed by Hussain and Prasher (2011) and Sassman and Lee (2007), nor by differences in the soils' organic matter or clay contents. No explanation was provided for this observation. However, it is possible that these could be due to the two soils' differences in their specific surface area, which was not measured in the study of Ilhan et al. (2012). Soil specific surface area were neither measured in the works of Hussain and Prasher (2011) and Sassman and Lee (2007).

Limited information is available on desorption of polyether ionophores from the soils particularly as a function of length of reaction time. Ilhan et al. (2012) evaluated desorption of monensin not from soils but from oak (*Quercus* sp.) wood chips (<2 cm in width or length). After eight days of reaction time, desorption procedures, either in water or organic solvent, desorbed only up to 20% of the initially added monensin. Ilhan et al. (2012) speculated that the 80% unextracted fraction of monensin was retained in the wood chips. While this may be likely, it is possible that some of the monensin had also abioticatically degraded (via acid-catalyzed hydrolysis) with time especially when the sorption solution of the woodchips was acidic. However, no information was reported

with regards to the pH of the reaction solutions of the wood chips before and after eight days of equilibration.

To date, existing literature has focused on sorption and/or desorption of polyether ionophores either in soils taken from wetlands or agricultural soils largely dominated by 2:1 clay minerals. Such soils, however, are not common in inherently nutrient-poor, 1:1 clay mineral-dominated highly weathered soils, which underlie many agricultural grasslands particularly along the Southern Piedmont in the Southeastern United States (West et al., 2008). Information on the sorption and desorption of polyether ionophores in grassland soils receiving long-term litter application could provide important considerations for devising management strategies to reduce ionophore transport from these systems.

Soils receiving years or decades of animal manure have approximately 20% greater soil organic carbon relative to soils not receiving any form of manure fertilizer at all (Edmeades, 2003; Liu et al., 2010; Maillard and Angers, 2014). As mentioned previously, soil organic carbon is one of the main factors controlling sorption of polyether ionophores in several soils studied. Considering this, greater sorption partitioning might be expected to occur in soils receiving long-term manure or litter amendment in comparison to unamended soils. Greater sorption of polyether ionophores in soil may help decrease mobility of these drugs in the environment.

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TABLES AND FIGURE

Table 2.1. Physicochemical properties of polyether ionophores commonly used as anticoccidial drugs in broiler production.

Compound (Chemical Formula)	Molecular Weight	Water Solubility	log Kow‡	pKa
	g mol^{-1}	mg L^{-1}		
Monensin ($C_{36}H_{62}O_{11}$)	671	0.85 - 63† 5 - 63‡	2.8 – 4.2	6.7‡ 4.26§ 4.50#
Salinomycin ($C_{42}H_{70}O_{11}$)	751	17 - 905‡	5.15	4.5, 6.4‡ 4.37#

† Dolliver and Gupta (2008a)

‡ Hansen et al. (2009)

§ Franco et al. (2009)

Sun, 2014

Table 2.2. Water solubility (S_w) of different polyether ionophore species and their metal dissociation constants (K_{diss}) based on the measurements of Sun (2014).

Property	Monensin	Salinomycin
S_w (μM) for:		
Protonated species	76.6	ND
Na-complexed species	2.67	108.1
K-complexed species	199.6	ND
Dissociated species	>1200	
K_{diss} for:		
Na^+	0.058	1.31
K^+	0.573	ND

ND, not determined

Table 2.3. Rate constants (k) and half-lives ($t_{1/2}$) for hydrolytic degradation of polyether ionophores based on the measurements of Sun et al. (2013b).

pH	Monensin		Salinomycin	
	k	$t_{1/2}$	k	$t_{1/2}$
	h^{-1}†	h	h^{-1}	h
3	0.0222 (0.0054)	40.0	0.522	1.3
4	0.0072 (0.0064)	159.8	0.167	4.2
5	0.0029 (0.0088)	NA	0.017	40.8

† Forward reaction rate constant (Backward rate constant)

NA, not available

Table 2.4. Sorption partitioning parameters of polyether ionophores in different soils under different experimental conditions.

Soil	Sorption pH	OC Content	Clay Content	Kd	Koc	Kclay
		%	%	L kg^{-1} soil	L kg^{-1} OC	L kg^{-1} clay
MONENSIN						
Data from Sassman and Lee, 2007						
Drummer-1	7.5	2.91	21	33.7	1160	160
Drummer-30	7.3	2.20	33	4.0	182	12
Raub-12	6.2	1.35	24	6.6	491	28
Toronto-4	4.2	1.34	21	54.3	4050	259
Oakville-24	4.7	0.52	8	12.6	2423	158
Oakville-31	7.0	0.87	11	1.1	125	10
A1A2	5.5	1.38	41	78.6	5700	192
Coloma-32	6.8	0.64	5	0.9	143	18
Data from Hussain and Prasher, 2011						
Sandy clay loam	6.8	5.3	20.0	17.6, 4.9†	334, 93†	88, 25
Sandy soil	6.8	2.7	0.8	8.5, 2.2	316, 83	1063, 275
Data from Ilhan et al. (2012)						
Surface soil	7.7‡	21.15	27	26	1233	96
Subsurface soil	8.2	0.23	23	101	43965	439
SALINOMYCIN						
Data from Hussain and Prasher, 2011						
Sandy clay loam	6.8	5.3	20.0	21.8, 5.8†	412, 110†	109, 29
Sandy soil	6.8	2.7	0.8	10.6, 3.0	391, 110	1325, 375

†Two Kd or Koc values correspond to the unsterilized and sterilized soils, respectively, in the work of

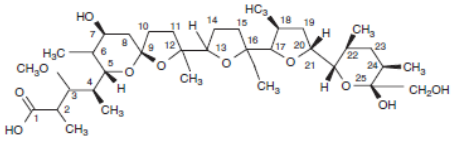
Hussain and Prasher, 2011.

‡ Exact sorption solution pH not reported.

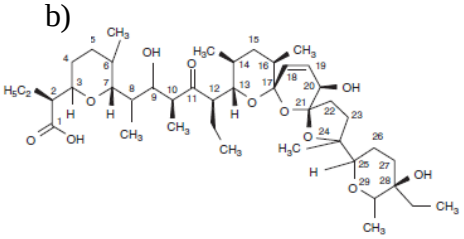
Figure 2.1. Structures of undissociated a) monensin and b) salinomycin and their corresponding metal-complexed forms c) and d), respectively. Sources: Kim and Carlson, 2006; Mollenhauer et al., 1990 and Miao et al., 2003.

Figure 2.1.

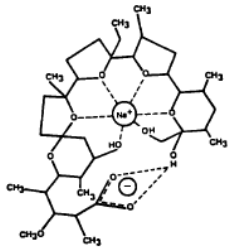
a)



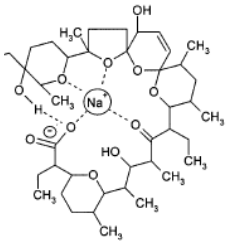
b)



c)



d)



CHAPTER 3

ALUM AND RAINFALL EFFECTS ON IONOPHORES IN RUNOFF FROM SURFACE-APPLIED BROILER LITTER[1]

[1] Sarah A. Doydora, Dorcas Franklin, Peizhe Sun, Miguel Cabrera, Aaron Thompson, Kimberly Love-Myers, John Rema, Vaughn Calvert III, Spyros G. Pavlostathis, and Ching-Hua Huang. Submitted to Journal of Environmental Quality, Feb 2015.

ABSTRACT

Polyether ionophores, monensin and salinomycin, are commonly used as antiparasitic drugs in broiler production and may be present in broiler litter (bird excreta plus bedding material). Long-term application of broiler litter to pastures may lead to ionophore contamination of surface waters. Because polyether ionophores break down at low pH, we hypothesized that decreasing litter pH with an acidic material such as aluminum sulfate (alum) would reduce ionophore losses to runoff. We quantified ionophore loss to runoff in response to (a) addition of alum to broiler litter and (b) length of time between litter application and the first simulated rainfall event. The factorial experiment consisted of unamended (~pH 9) vs. alum-amended litters (~pH 6), each combined with simulated rainfall at 0, 2, or 4 wk after litter application. Runoff from alum-amended broiler litter had 33% lower monensin concentration ($p < 0.01$), 57% lower monensin load ($p < 0.01$), 48% lower salinomycin concentration ($p < 0.01$) and 66% lower salinomycin load ($p < 0.01$) compared to runoff from unamended broiler litter when averaged across all rainfall events. Ionophore losses to runoff were also less when rainfall was delayed for 2 or 4 wk after litter application relative to applying rainfall immediately after litter application. While the weather is difficult to predict, our data suggest that ionophore losses in runoff can be reduced if broiler litter applications are made to maximize dry time after application.

INTRODUCTION

Polyether ionophores are carboxylic compounds originally derived from Streptomyces species that have antimicrobial properties because they transport ions across the lipid bilayer of the cellular membrane leading to impaired osmotic balance, depleted energy and disintegrated cells (Westley, 1982; Bergen and Bates, 1984; Smith and Galloway, 1983). These compounds are used as antiparasitic drugs in poultry production against coccidia, an intestinal protozoa commonly encountered among broilers (*Gallus gallus domesticus*) and turkeys (*Meleagris gallopavo*).

Monensin and salinomycin are among the most commonly used ionophore anticoccidial drugs (against coccidia) (Peek and Landman, 2011; Landoni and Albarellos, 2015) (see Table 3.1 for compound properties). These drugs can pass through the bird's digestive tract and appear in manure, which is used for fertilizer along with discarded feathers and bedding materials, known collectively as broiler litter (Furtula et al., 2009; Biswas et al., 2012; Sun et. al., 2013a). In the state of Georgia, where the broiler industry is the largest in the US (National Agricultural Statistics Service, 2012), as much as 11 Mg of ionophores may be applied to agricultural soils every year, assuming 2.1 million Mg of broiler litter are generated per year (Doydora et al., 2011) with a median ionophore concentration (monensin and salinomycin combined) of 5135 μg kg^{-1} (Sun et al., 2013a).

Runoff water (overland flow) from agricultural fields can contain veterinary drugs and be contaminated with polyether ionophores. In a 1-h simulated rainfall event, Kim et al. (2010) and Davis et al. (2006) demonstrated transport of monensin in surface runoff. In their study, the highest average concentrations and the absolute mass and fraction of amounts applied found in runoff was highest for monensin when compared to a suite of

other veterinary drugs (tetracycline, chlortetracycline, sulfathiazole, sulfamethazine, erythromycin and tylosin). Furthermore, surface runoff can potentially contribute ionophore loads to streams more so than leaching. In the 3-yr field study of Dolliver and Gupta (2008) conducted on a Rozetta silt loam soil, annual runoff losses of monensin generally accounted for <0.05% of the applied monensin, whereas annual leaching losses of monensin accounted for <0.005% from beef manure-amended soils under chisel plow and no-till corn production systems.

Several researchers reported presence of polyether ionophores in natural waters, mostly in areas close to livestock production where these drugs are also used. For instance, monensin and salinomycin (<0.01 to ~0.04 µg L^{-1}) were found in Cache la Poudre River in Northern Colorado (Cha et al., 2005; Kim and Carlson, 2006; 2007). Monensin was also detected (0.02 to 0.21 µg L^{-1}) in the Grand River within Ontario, Canada (Hao et al., 2006), in lagoons (3.91 to 16.24 µg L^{-1}), and in shallow groundwater (0.04 to 0.39 µg L^{-1}) beneath dairy farms located in the Central Valley of California (Watanabe et al., 2008). In Australian waters, monensin and salinomycin were also detected along with 26 other antibiotics, with monensin being the most frequently detected veterinary drug in 92 out of 98 river samples (Watkinson et al., 2009).

The presence of polyether ionophores in the environment may cause harm to native biota. These compounds are known to be toxic to Gram-positive bacteria, protozoa, fungi and even to higher organisms including humans, making these drugs currently unsafe for human medicine (Rutkowski and Brzezinski, 2013; Hansen et al., 2009; Khan et al., 2008). Based on reported ecotoxicological tests, polyether ionophores can be damaging to certain organisms living in aquatic and terrestrial environments

(Žižek et al., 2011; Hillis et al., 2007; European Food Safety Authority, 2004, 2005; Hoagland, 1996). For instance, in a 96-h exposure to monensin and salinomycin at 1.88 and 1.66 mg L-1, respectively, 50% of a fish population (*Oncorhychus mykiss*) was affected (96-h LC_{50}) (European Food Safety Authority 2004, 2005). Harmful ionophore effects to other organisms include decreased zooplankton abundance and richness of specific taxonomic groups (at 500 µg monensin L^{-1}) (Hillis et al., 2007), suppressed earthworm (*Eisenia andrei*) reproduction (EC_{50} of 12.7 mg monensin kg^{-1} soil) (Žižek et al., 2011) and even injury to several plant species (at 10^{-4} mol L^{-1} monensin) (Hoagland, 1996). Although the toxicological concentrations of ionophores are much higher in comparison to their reported occurrence in the environment to date, more information is needed to understand the magnitude and temporal variation in chronic losses of the ionophores from manure-amended soils.

Despite the potential environmental risk associated with ionophores, research remains limited on controlling sources of ionophore contamination to the environment, particularly from surface runoff. Existing literature on ionophore movement in runoff has focused on bare, recently tilled sandy clay loam soils (Aridic Haplustalf), simulating land preparation and liquid manure application for corn production (Davis et al., 2006; Kim et al., 2010). These studies, however, do not represent conditions that are typical of grasslands, which do not get tilled and remain covered with grasses throughout the year. In addition, the soils used in previous studies were alkaline (pH 7.9) (Davis et al., 2006; Kim et al., 2010), which is not characteristic of the acidic, highly weathered soils of many grasslands along the Piedmont in the Southeastern US. Because grasslands (pastures and rangelands) and/or no-till systems receiving broiler litter applications may

become more prevalent with the expansion of sustainable agricultural practices in the future (Vadas et al. 2011; Godfray et al., 2010), further research is needed on these systems to determine if they can be a significant source of ionophore pollution. Given recent findings that monensin and salinomycin were detected in natural runoff from grasslands fertilized with broiler litter (Sun et al., 2013a), the need to develop methods for mitigating ionophore mobility on these systems becomes more important.

One method to curtail ionophore losses in surface runoff from grasslands may be to expose them to acidic conditions (pH 3 to 5), which allows decomposition of the ionophores through hydrolysis reactions (Sun et al., 2013b; Bohn et al., 2013). Aluminum sulfate (alum) is currently used in the production of over one billion broilers grown in the USA to lower the levels of toxic ammonia inside broiler houses and decrease dissolved phosphorus (P) in surface runoff (Shreve et al., 1995; Moore et al. 2000; Moore, 2011). Alum decreases litter pH through production of protons according to the reaction below (Moore et al., 2000) (Eq. 3.1).

$$Al_2(SO_4)_3.14H_2O + 6H_2O \rightarrow 2Al(OH)_{3(s)} + 3SO_4^{2-} + 6H^+ + 14H_2O \qquad \text{Eq. 3.1}$$

Unlike P, which is either precipitated as poorly ordered wavellite or sorbed to precipitated $Al(OH)_3$ with alum (Moore, 2011), we hypothesized that alum will reduce the presence of ionophores in surface runoff mainly by lowering litter pH, thus favoring their hydrolytic degradation.

Another potential method for mitigating ionophores in surface runoff is timing broiler litter applications. Several studies demonstrated decreased runoff losses as time

increases between surface application of broiler litter and occurrence of rainfall for inorganic nitrogen, phosphorus, and other macro- and micronutrients (Sistani et al., 2009; Sauer et al., 1999; Pierson et al., 2001; Schroeder et al., 2004). Longer periods between litter application and rainfall enhance nutrient transformation and sorption to soil such that nutrient losses to runoff are reduced (Sharpley, 1997; Schroeder et al., 2004). In the case of the ionophores, we expect that timing litter applications when rainfall is less likely (normal dry periods) will allow additional time for inonophore degradation in the surface-applied litter to occur.

This study evaluated the effect of alum additions to broiler litter (litter treatment) and the effect of delayed rainfall after broiler litter application (time of rainfall treatment). We hypothesized that adding alum to broiler litter and increasing the length of time between litter application and rainfall would both decrease monensin and salinomycin losses to runoff. To test these hypotheses, we simulated rainfall to grassed plots at 0, 2, or 4 wk after receiving broiler litter with or without alum and measured ionophores in the runoff water.

MATERIALS AND METHODS

Field Site and Plots

Tall fescue (*Festuca arundinacea* Schreb.)/bermudagrass (*Cynodon dactylon* L.) plots located at the University of Georgia Central Research and Education Center near Eatonton, GA (33° 24’ N; 83°29’W; elevation=150 m) were used for this study. Each plot (0.75 m x 2 m) had galvanized, 25-cm steel boundaries (20 cm into the soil, 5 cm above soil surface) and was established on an Altavista sandy loam (fine-loamy, mixed,

semiactive, thermic Aquic Hapludults) with 2 to 6% slope. This soil type (an Ultisol) is common along the Southern Piedmont (West et al., 2008), a geographic area containing most of Georgia's grasslands for livestock production (Hancock et al., 2014). The slope range is also similar to other pastures studied in this region (Franzluebbers and Stuedemann, 2008). The lower end of each plot had a stainless steel gutter that routed surface runoff to the plot's lower outer corners, where runoff was collected.

A 3x3 factorial experiment consisted of three broiler litter treatments (no litter, unamended litter, or alum-amended litter at 200 g alum kg^{-1} fresh litter weight), and three periods of rainfall simulation (once at 0, 2, or 4 wk after litter application) that generated nine treatment combinations. All nine treatments were arranged in a randomized complete block design with three blocks (i.e. groups of plots). A fourth block included only six treatments (3 broiler litter treatments x 2 times of simulated rainfall at 0 or 2 wk) because of lack of plot space. Blocks were established based on similar runoff volumes of plots measured during a background rainfall simulation carried out on all plots prior to conducting the actual study. Blocking was performed in order to account for hydrologic heterogeneity of the plots that could be attributed to soil factors (i.e. porosity, hydraulic conductivity, etc.) not measured in this study. In the background rainfall simulation, one out of four plots sampled had residual ionophore concentrations between 0.1 to 0.2 μg salinomycin L^{-1} while the other plots had undetectable levels of salinomycin. Monensin was not detected in the runoff samples from any of the eight plots tested for background ionophore concentrations.

Broiler Litter Preparation

To prepare the alum-amended broiler litter treatment for the study, ~636 kg of litter was treated with alum (200 g alum kg^{-1} litter) and stored in a wooden bin (1.2 x 1.2 x 1.2 m). Each of the two bins were filled with same total mass of either of the litter treatments, which were stacked to the same height of 1.2 m. The bins containing the unamended litter and the alum-amended litters were stored under a roof to simulate stack house conditions. Stacking means storing or stockpiling of litters under ambient temperature inside a roofed shelter without walls (or stack house). This is typically done by many poultry producers or farmers while waiting for the right time to apply broiler litter on the surface of grasslands as fertilizer (Cunningham, et al., 2012).

After 171 d of storage, alum-amended and unamended broiler litters were sampled from their corresponding bins, and were used for the rainfall simulation study. Treated plots received 1 kg fresh wt. of unamended litter or 1.2 kg fresh wt. of alum-amended litter (1.0 kg fresh wt. of broiler litter plus 0.2 kg of alum). The litter treatments were surface-applied, as this application method is commonly practiced on grasslands (Mitchell and Tu, 2005). This rate was equivalent to 5000 kg dry litter ha^{-1} (Table 3.2), which is approximately equal to 200 kg N ha^{-1} and falls within the typical broiler litter application rate in northern Georgia, USA (Schroeder et al., 2004). The 6000 kg alum-amended litter ha^{-1} rate consisted of 20% alum w/w of dry litter (Table 3.2). We chose an alum application rate of 20% because this rate was shown to decrease soluble reactive phosphorus concentration in runoff by 87 to 97% from plots receiving surface-applied broiler litter (Shreve et al., 1995; DeLaune et al, 2004).

Rainfall Simulation and Sample Collection

After broiler litter was applied, the field plots were covered with clear tarps until the day of simulated rainfall. The tarps were clear to allow light through and were placed about 40 cm above the plots to allow air circulation while preventing natural rain from reaching the plots. Rain was simulated with a rainfall simulator (Tlaloc 3000, Joern's, West Lafayette, IN) at 50 mm h^{-1} and runoff was collected for 30 min after the initiation of runoff. Rainfall event durations ranged from 0.8 to 1.7 h (as described above, blocks were established to account for hydrologic heterogeneity of the plots). The study was conducted in September through October 2012. Although farmers may apply broiler litter anytime during the year (Brink et al., 2002), the timing of broiler litter application was selected to promote growth of tall fescue in the autumn. Runoff samples collected from each plot were analyzed for ionophore concentrations, pH, and dissolved organic carbon (DOC) (see details below). The reported DOC values corresponded to total dissolved C (organic+inorganic), which was assumed to be mostly organic C because the inorganic C constituted a small fraction based on inorganic C analysis of some samples. Runoff volume was measured from each plot by weighing the accumulated 30-min runoff and assuming a runoff sample density of 1 g H_2O cm^{-3}. Average rainfall volume was recorded (taken from five rain gauges inserted on each plot); infiltration volume and ionophore load were calculated for each plot. Infiltration volume was calculated by subtracting runoff volume from the total volume of rainfall applied to each plot. Loads were computed by multiplying the concentration by the runoff volume and expressing the result in terms of mass of ionophores ha^{-1}. Soil temperature in the upper 5 cm throughout

the study is shown in Supplemental Fig. 3.S1 for plots that did not receive simulated rainfall until 4 wk after litter application.

Analysis of Runoff Samples

After collecting 30-min of accumulated runoff from each plot into a 200-L container, the accumulated runoff was stirred inside the container and a 1-L sample was collected by dipping a smaller container and placing the sample in a 1-L glass mason jar for immediate pH measurement and for ionophore measurement later in the laboratory. Another 0.5-L sample of runoff was collected in a similar manner and placed into a wide-mouth, 500-mL polyethylene bottle for DOC analysis in the laboratory.

The pH of runoff samples was measured in the field using a pH electrode (AccuTupH, Fisher Scientific, Pittsburgh, PA) connected to a pH meter (Accumet AB15, Fisher Scientific, Pittsburgh, PA). Immediately after that, the pH values of the runoff samples were adjusted to pH 7 by carefully adding the necessary volume (in µL range) of 1 mol L^{-1} NaOH solution to the 1-L runoff sample while it was constantly stirred on a magnetic stirrer (Corning Model PC 320, Corning, NY) and pH was simultaneously monitored. The pH adjustment was done to prevent hydrolytic degradation of the ionophores in the samples before their analysis. Sodium azide was also added to achieve a concentration of 100 mg L^{-1} to suppress microbial activity prior to ionophore analysis. After the pH adjustment, the runoff samples were brought to the laboratory on ice and where they were kept in the refrigerator (4°C) until transported to Georgia Institute of Technology for ionophore analysis. The time between sample collection and ionophore analysis was 1 to 2 wk.

Samples were analyzed for monensin and salinomycin following the procedure developed by Sun et al. (2013a). Before analyzing for ionophores, 200 mL of each of the runoff samples was filtered through a 0.45-µm glass-fiber filter (Millipore, Billerica, MA). The filtered runoff sample was spiked with 50 µg L-1 nigericin, a surrogate standard, and was subjected to solid phase extraction (SPE) using a 500-mg, 6-mL hydrophilic-lipophilic balance (HLB) cartridge (Waters, Taunton, MA). Prior to SPE, preconditioning of each HLB cartridge was performed by first rinsing it with 5 mL methanol followed by 5 mL deionized water. The filtered runoff sample was then poured into the cartridge and extracted under vacuum at 2 mL min^{-1} with the use of a Visiprep apparatus (Supelco, Bellefonte, PA). Next, the cartridge was rinsed with 5 mL deionized water and dried for 10 min under vacuum. In order to elute the ionophores, 3 mL of methanol was applied to the cartridge and the extract was collected in an amber bottle. The methanol extract was evaporated to dryness under vacuum at room temperature (23ºC) and the analytes were reconstituted by adding 0.5 mL of a buffer solution containing 10 mmol $L^{-1}Na_2HPO_4$ and 5 mL ethyl acetate. The mixture was shaken at 120 rpm for 30 min and the buffer layer was discarded using a Pasteur pipette. The ethyl acetate layer was evaporated to dryness under vacuum and the analytes were finally reconstituted in 1 mL mixture of methanol and 10 mmol L^{-1} Na_2HPO_4 at 1:1 v/v. The mixture (final sample) was transferred into a high-performance liquid chromatography (HPLC) amber vial for analysis through liquid chromatography mass spectrometry (LCMS).

The ionophores were analyzed by a LCMS detection system (Agilent 1100 Series, Agilent, Palo Alto, CA) using a reversed-phase column (2.1 mm × 150 mm, 3 µm

Ascentis RP-amide column, Supelco, Bellefonte, PA), which was heated at 40°C. The mobile phase consisted of (A) a mixture of HPLC-grade water and formic acid (99.9:0.1, v/v) with 25% acetonitrile; and (B) HPLC-grade methanol and acetonitrile (50:50, v/v). The mobile phase was kept at a flow rate of 0.3 mL min^{-1} through the column under a gradient method. The gradient consisted of increasing the mobile phase B from 50 to 90% in the first 8 min, then to 100% at 12 min for a duration of 3 min. After each sample analysis, the mobile phase (50% A and 50% B) was allowed to re-equilibrate the column for 5 min prior to the next sample injection. Each sample injection was 10 µL. The mass spectrometer was fixed under the following settings: positive electrospray ionization mode (ESI+), 190 V fragmentation voltage, and +4500 V capillary voltage. The nebulizer gas (241 kPa psi) and drying gas at 350ºC (6 L min^{-1}) both consisted of nitrogen. Quantification of ionophores was performed under selected ion monitoring (SIM) mode. The molecular ions (*m/z*) of 693 and 773 were used for monensin and salinomycin quantification. The mass spectrometer had a mass resolution of 0.1 *m/z*.

Calibration standards for monensin and salinomycin were made by first adding 20 µL of each of the ionophore stock solutions (50,000 ng mL^{-1}) into 1-mL mixture of methanol and 10 mmol L^{-1} Na_2HPO_4 solution (50:50, v/v) in order to achieve 1,000 ng mL^{-1}. Additional concentrations were prepared by serial dilution of the 1,000 ng mL^{-1} standard in the same methanol-Na_2HPO_4 mixture. Ionophore concentrations in runoff samples were calculated with the use of fitted equations obtained from the calibration curves for monensin and salinomycin. Calibration curves were constructed by plotting the ionophore concentrations versus the peak areas of each of the molecular ions for

monensin and salinomycin. The estimated recoveries of this procedure were 82±19 and 85±13% for monensin and salinomycin in the runoff samples, respectively.

For DOC analysis, runoff samples (with unadjusted pH) from the 500-mL polyethylene bottles were first filtered thru an FP-Vericel^{TM} 0.45 μm filter membrane (Pall Life Sciences, Ann Arbor, MI). The filtered samples were frozen until analyzed for DOC. Prior to DOC analysis, frozen samples were thawed at room temperature. Then, approximately 3 mL of the sample was analyzed for DOC through the combustion-infrared method (American Public Health Association, 1992) using a total C analyzer (TOC-5050, Shimadzu Corporation, Kyoto, Japan).

Analysis of Soil Samples

Surface soils inside the plots were sampled from 0 to 5 cm depth. The samples were air-dried, passed through 2-mm sieve, placed inside plastic bags and stored at room temperature. The soils had a pH of 5.86 ± 0.13 in 1:5 soil to 0.01 mol L^{-1} $CaCl_2$ solution (Minasny et al., 2011), total N content of 2.7±0.2 g kg^{-1} and total C content of 26±1 g kg^{-1} following the micro-Dumas combustion technique for total C and total N analyses (Kirsten, 1983). Soil pH was measured using a combination electrode (Orion 8175BNWP, Beverly, MA) connected to a pH meter (Orion Model SA 720, Orion Research Incorporated, USA). The total C and total N were determined using a CN analyzer (Carlo Erba NA1500, Carlo Erba, Milan, Italy). Soil moisture was also measured prior to simulating rainfall on each plot following the gravimetric soil moisture determination procedure (Gardner, 1986).

Analysis of Broiler Litter Samples

After application of broiler litter on designated plots, samples of the litter materials used were brought to the lab and stored in the refrigerator at 4°C. Measurements on broiler litter were done on the refrigerated samples. Moisture content was determined by drying the litter samples at 60°C for 48 h (Qafoku et al., 2001). Unamended litter had 333±5 g moisture kg^{-1} whereas alum-amended litter had 334±34 g kg^{-1} w/w of dry litter. The unamended litter had a pH of 8.90 ± 0.03, whereas the alum-amended litter had a pH value 6.10 ± 0.03, as determined by 1:5 soil to water ratio (Rothrock et al., 2010). Litter pH was measured as described previously for the soil. The broiler litter used had a total N of 40 ± 3 g kg^{-1} and mean total C of 400 ± 50 g kg^{-1} following the same combustion technique and CN analyzer mentioned above for the analysis of soil samples. Alkalinity was determined by first taking 2 g of broiler litter sample and adding to it 500 mL of 18 MΩ water. The suspension was shaken for 30 min and filtered thru the same FP-Vericel^{TM} 0.45 μm filter membrane already mentioned in runoff filtration for DOC analysis. Fifteen milliliters of the litter water extract (filtrate) was then subjected to potentiometric titration (American Public Health Association, 1992) with 0.025 N HCl to pH 4.5 using the same pH electrode-meter set-up used for the soil and litter pH measurements.

For ionophore analysis, the refrigerated broiler litter samples were further freeze-dried and ground. Ionophore concentrations were measured following the method of Sun et al. (2013a). One gram of sample was placed in a 50-mL centrifuge tube and mixed with an internal nigericin standard at 0.5 μg kg^{-1}, 7.5 mL of McIlvaine's buffer (containing 10 mmol L^{-1} EDTA, Na_2HPO_4 and citric acid), 2.5 mL methanol and 5 mL

hexane. The samples were shaken and centrifuged and the hexane layer was transferred to an amber bottle. This extraction step was repeated one more time by adding another 5 mL of hexane. The two 5-mL hexane extracts were combined and evaporated to dryness under vacuum. After drying, the analyte was reconstituted by adding a 1 mL mixture of methanol and 10 mmol L^{-1} Na_2HPO_4 solution. The samples were then analyzed by LCMS as described previously.

Statistical Analysis

All of the parameters were statistically analyzed using a two-way analysis of variance (ANOVA) for a randomized complete block design taking into account main factors of litter treatment (only plots receiving litter) and time of rainfall as well as their interaction (SAS 9.3, SAS Inst. Inc., Cary, NC). The control plots (i.e. plots not receiving any litter application) were excluded from all statistical analyses because concentrations of ionophores in runoff samples from these plots were below detection (method detection limit = 18 to 19 ng L^{-1}); as such, they provided no information about the response and only affected the variance estimation. Nonlinear regression was also performed by fitting an exponential equation between runoff volume and DOC, and between each of the ionophore concentrations and infiltration volume (SigmaPlot Version 11.0, Systat Software, Inc., San Jose, CA). An exponential equation was chosen for nonlinear curve fitting because this model significantly described the measured data and met the assumptions required for nonlinear regression. Two-way ANOVA and regression models met the necessary assumptions for normality (Shapiro-Wilk test), homogeneity of variance (Brown and Forsythe's test for SAS or the built-in test for constant variance in

SigmaPlot), and independence of residuals (by implementation of appropriate experimental design), except for a few which did not meet homogeneity of variance. Satisfying these assumptions was critical to ensure that inferences derived from each of the statistical tests performed are valid. Observations with studentized residuals outside of the range from -2 to +2 (i.e. outliers) were removed as part of meeting the assumptions. For ANOVAs with normally distributed residuals but with unequal variances, a weighted two-way ANOVA was employed. An alpha level of 0.10 was used to determine all significant effects. Treatment mean comparisons were done based on Tukey's test.

RESULTS AND DISCUSSION

Litter Properties

As indicated previously, the litters were stockpiled inside wooden bins for 171 d under an open-sided roofed shelter prior to their use. Stockpiling or stacking changed some of the alum-amended litter properties as described below. On the first day of stacking, the addition of alum reduced the initial pH of the litter from pH 8.38 ± 0.03 to pH 3.91 ± 0.06. However, as the stacking period advanced, the pH of alum-amended litter increased to 6.10 ± 0.03 at 171 d when the litters were utilized for this study. The pH of the unamended litter was 8.90 ± 0.03 at the time of the study.

The observed increase in pH of alum-amended litter was likely caused by the slow dissolution of carbonate minerals in the litter. Consistent with this, alkalinity in the alum-amended litter increased from 0 g kg^{-1} litter ($CaCO_3$ equivalent) when sampled at 0

d to 81 g kg^{-1} litter at 171 d. Similar pH increases following alum-treatment of litter have been observed by Rothrock et al. (2010) and Cook et al.(2008).

Surface Runoff Volume

A significant interaction was found between litter treatment and time of rainfall on runoff volume ($p = 0.03$). Hence, only the interaction effect and its meaning are described and discussed below for runoff volume.

For rainfall applied immediately after litter application (Week 0), runoff volume was significantly greater from plots receiving unamended litter than from plots with alum-amended litter (Fig. 3.1). Moreover, runoff volume did not change with time for plots receiving alum-amended litter, but did significantly decrease from 0 to 2 and from 0 to 4 wk for plots receiving unamended litter (Fig. 3.1). This could be explained by DOC, which followed the same patterns between litter treatments as that of runoff volume each time simulated rainfall was applied (Fig. 3.1); DOC also increased with increasing runoff volume (Fig. 3.2). Alum reduces DOC losses from broiler litter largely because of decreased litter pH (Moore and Miller, 1994; Shreve et al., 1995). Humic substances (HS) aggregate at low pH, but disperse in solution at high pH (Sparks, 2003). Thus, we suspect that higher DOC concentrations in runoff from plots receiving the unamended litter reflected dispersed litter particles. If these dispersed particles clog the soil pores this might explain the higher runoff volume from plots receiving unamended litter at Week 0. In addition to flocculating the DOC, alum may have flocculated the soil directly leading to lower runoff volumes from plots receiving alum-amended litter relative to plots receiving unamended litter at Week 0.

Main Effect of Alum Addition on Ionophore Losses to Runoff

No significant interactions were found between litter treatment and time of rainfall for concentrations, loads, and percent of ionophores lost to runoff (Supplemental Fig. 3.S2). Therefore, we discuss only the main effects of litter treatment (averaged across all periods of rainfall) and time of rainfall (averaged across the two litter treatments).

Across all rainfall events (0, 2, 4 wk; Table 3.3), ionophore concentrations were significantly lower (33% for monensin and 48% for salinomycin) in runoff from plots receiving alum-amended litter than from plots receiving unamended litter. Runoff from plots receiving alum-amended litter also had a lower pH than runoff from plots with unamended litter (Table 3.3). While acidic conditions can promote monensin and salinomycin degradation via hydrolysis (European Food Safety Authority, 2004; Sun et al., 2013b; Bohn et al., 2013), this mechanism does not likely explain the lower ionophore concentrations in runoff from the alum-amended plots because of the following reasons. Firstly, hydrolysis of these ionophores is minimal at pH 6 (Sun et al., 2013b), which was the pH of alum-amended litter (pH 6.1±0.03) and the pH of runoff from plots receiving alum-amended litter (pH 5.8±0.2). In addition, our rainfall simulations lasted only 0.8 to 1.7 h, which is insufficient time for hydrolysis-driven degradation. According to the first-order kinetic model of Sun et al. (2013b), a 33% (for monensin) and 48% (for salinomycin) decrease in ionophore concentrations would require at least 6 and 13 d, respectively, (see Appendix A to C) at the runoff pH of 5.8 and 22^{o}C.

We suspect that the lower ionophore concentrations in runoff were caused by the coagulating effect of alum in the DOC-rich runoff waters. In a recent review, Davis and Edwards (2014) summarized mechanisms of Al-induced DOC removal as follows: (1) sorption via ligand exchange; (2) charge neutralization/precipitation; (3) coprecipitation; or (4) heterocoagulation either with polymeric or incipiently solid aluminum phases. When alum initially hydrolyzes in water, insoluble aluminum hydroxides form rapidly (see Eq. 3.1; Snodgrass et al., 1984). Maximum rate of aluminum hydroxide precipitation occurs between pH 6 to pH 8 (Cooke et al., 1986), bracketing the starting pH of our runoff waters (pH 6.7, Table 3.3). In the presence of DOC, Al precipitates as amorphous $Al(OH)_3$-HS colloids aggregating into clusters during DOC flocculation at near neutral pH (Lu et al., 1999; Wang et al., 2014; Lin et al., 2014). We believe that Al sequesters ionophores by flocculating it together with DOC. Similarly, DeLaune and Moore (2013) and Nichols et al. (1997) also attributed lower hormone (17β-estradiol) concentrations in runoff to flocculation of DOC from alum-treated plots.

Main Effect of Time of Rainfall

Compared to ionophore concentrations in runoff immediately after litter application, ionophore concentrations in surface runoff at 2 and 4 wk decreased by 47 and 67% (p <0.01), respectively, for monensin, and by 71% in both weeks (p <0.01) for salinomycin (Fig. 3.3), regardless of litter treatment. Significant reductions were also observed for loads and percent of losses of monensin and salinomycin when rainfall occurred at 2 or 4 wk after litter application. Reduced monensin and salinomycin losses in runoff with time can be explained by the increased total infiltration volume (infiltration

between initiation of rainfall until the end of the 30-min runoff collection) (Fig. 3.4). Prior to producing runoff, rainfall must infiltrate into the soil until the soil becomes saturated with water. The increased volume of infiltration at 2 and 4 wk was due to the fact that the soil was drier than at 0 wk (Fig. 3.5) and therefore a larger volume of simulated rainfall had to be applied to each plot to obtain 30 min of runoff. It is possible that more of the ionophores were carried by the infiltrating water into the underlying soils with increased infiltration volume, preventing ionophore losses in surface runoff.

Increased infiltration volume with time was also ascribed for reduced losses of phosphorus in runoff with delayed rainfall after litter application in several phosphorus studies examined by Vadas et al. (2011). Contrary to our hypothesis, degradation does not likely explain the reduced ionophore losses at 2 or 4 wk, as these compounds had not degraded in the unamended litter even after 4 wk on the grassed soil before simulating rainfall (Sun et al., 2014). Although we could not confirm the absence of ionophore degradation in the alum-amended litter with time due to lack of data, it is also likely that the ionophores had not degraded in the alum-amended litter within the 4-wk period prior to the occurrence of rainfall. As mentioned earlier, the two litter treatments had similar moisture contents throughout their storage period (i.e. before they were applied to the soil). Moreover, after they were applied on the plots and prior to rainfall simulation, the surface soils receiving the two litters also had similar moisture contents (22-23% w/w of dry soil, $p = 0.77$).

Ionophore Loss from Surface-Applied Broiler Litter

Our measured ionophore concentrations in runoff (0.8 to 2.1 μg L^{-1}, see Table 3.3) were approximately three orders of magnitude lower than the acute toxicity limits of index aquatic organisms (i.e. *Daphnia magna, Oncorhynchus mykiss,* etc.) (European Food Safety Authority 2004, 2005). Although this suggests that ionophores are not likely to cause acute aquatic impact, the real effect of ionophores in the environment remains uncertain. For instance, information is limited on the potential environmental impacts of chronic low-level exposures to ionophores; the effects of ionophore degradation products; the effects of ionophores when present with other veterinary drugs or other chemical pollutants; and the effect of ionophore presence in the environment on development of pathogenic resistance in the long-run (Boxall et al., 2003; Hansen et al, 2009).

In our study, the fraction of monensin lost to runoff ranged from 1 to 5% of the amount applied (Table 3.3). This is much larger than previous studies, which recovered only <0.1% of applied monensin in runoff (Davis et al., 2006; Kim et al., 2010), despite having on average, more rainfall (61 mm vs 44 mm) and more runoff (35 mm vs 18 mm) than our study. In these previous studies, researchers sprayed aqueous solution of monensin directly on bare, tilled sandy clay loam soils (Davis et al., 2006; Kim et al., 2010). This direct application method apparently allowed ionophores to establish a greater extent of contact with the soil and perhaps facilitate some sorption. Monensin was shown to be able to sorb to some soils (Sassman and Lee, 2007; Hussain and Prasher, 2011; Ilhan et al., 2012). After simulating rainfall, Kim et al. (2010) found most of the recovered monensin remained in the soil at depths below 2 cm, suggesting that underlying soils may have greater sorption capacity than the surface soils. However, the

soils in our study were sandy loams and sorption was likely less in comparison to the soils of Davis et al. (2006) and Kim et al. (2010). We suspect, greater losses of monensin to runoff in our study reflect the combined effect of lower sorption capacity of our surface soils for the ionophores and the reduced contact between the ionophores and the soil prior to rainfall due to the nature of surface application of broiler litter to grassed plots.

CONCLUSION

Reducing ionophore movement with runoff could prevent their contamination of nearby aquatic ecosystems. Our findings on an Altavista grassland soil demonstrated two methods to diminish monensin and salinomycin levels in surface runoff from grassed soils receiving surface-applied broiler litter. One method is adding alum to broiler litter at 20% w/w of fresh litter. Rapid reduction of ionophore losses to runoff with alum, however, could not be explained by ionophore hydrolytic degradation as we previously hypothesized. Instead, reduction in runoff ionophore concentrations was likely due to instantaneous reaction(s) with much faster kinetics than hydrolysis (i.e. ionophore flocculation with DOC in the presence of aluminum sulfate). The other method for decreasing ionophore presence in runoff is to apply litter to maximize dry time after application in order to minimize the potential for ionophore losses in runoff. Although prolonged periods without rain do not seem to cause degradation of the ionophores, the drier soil enhances infiltration and thus lowers ionophore occurrence in runoff. Both of these methods are likely to protect downstream aquatic ecosystems and should be explored as potential best management practices.

FUTURE WORK

Future research is needed to confirm the mechanism of removal of ionophores in the presence of aluminum sulfate. Sorption studies of monensin and salinomycin may be conducted using freshly collected $Al(OH)_3$-HS versus $Al(OH)_3$ colloids as sorbents.

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TABLES AND FIGURES

Table 3.1. Selected chemical characteristics of monensin and salinomycin.

Compound Name	pKa†	Half-life	Partitioning Coefficient
		d	L kg^{-1} soil
Monensin ($C_{36}H_{62}O_{11}$, MW 671)	4.50	22‡	1-101¶
Salinomycin ($C_{42}H_{70}O_{11}$, MW 751)	4.37	4§	11-22#

† Sun (2014)

‡ Monensin degradation half-life in poultry manure from Dolliver et al. (2008)

§ Salinomycin degradation half-life in poultry manure from Ramaswamy et al. (2010)

¶ Combined monensin partitioning constants to soil from Sassman and Lee (2007), Ilhan et al. (2012) and Hussain and Prasher (2011)

Salinomycin partitioning constant to soil from Hussain and Prasher (2011)

Table 3.2. Mean concentrations and application rates of monensin (MON) and salinomycin (SAL) applied to grassed plots with unamended broiler litter or with broiler litter amended with 200 g alum kg^{-1} litter.

Litter Treatment	Litter MON Conc.	Litter SAL Conc.	MON Application Rate	SAL Application Rate	Dry Litter Application Rate‡
	------ μg kg^{-1} ------		------ g ha^{-1} ------		kg ha^{-1}
Unamended Litter	1218 ± 79†	1431 ± 339	6.1 ± 0.4	7.1 ± 1.7	5000 ± 17
Alum-amended Litter	1065 ± 304	1145 ± 340	6.3 ± 1.8	6.7 ± 2.0	6000 ± 155

†Mean values ± standard deviations.

‡ Calculated from the moisture contents of the actual litter treatments.

Table 3.3. Mean values for runoff pH, concentrations, and loads of monensin (MON) and salinomycin (SAL), and percent ionophore lost in 30 min of runoff from grassed plots receiving unamended broiler litter or broiler litter amended with 200 g alum kg^{-1} (averaged across all periods of simulated rainfall applied at 50 mm h^{-1})

Litter	Runoff pH	MON Conc.	SAL Conc.	MON Load	SAL Load	MON Lost to Runoff	SAL Lost to Runoff
		------ μg L^{-1} ------		------ mg ha^{-1} ------		% of Initial Ionophore Applied	
Unamended Litter	6.7±0.1a†	1.2±0.1a	2.1±0.2a	185±17a	340±41a	3±0.3a	5±0.6a
Alum-amended Litter	5.8±0.1b	0.8±0.1b	1.1±0.2b	79±17b	114±41b	1±0.3b	2±0.6b

†Mean values ± standard errors. Values in the same column followed by different letters mean significant difference at 0.10 alpha level.

Figure 3.1. Mean a) 30-min runoff volume and b) concentration of dissolved organic carbon (with standard error bars) from plots receiving unamended or alum-amended litter for rainfall simulations at 0, 2, or 4 weeks after litter application. For each week of rainfall, bars bearing different lower case letters (i.e. a, b, c) indicate significant difference between unamended and alum-amended litter treatments. For each litter treatment, different upper case letters (i.e. A, B) indicate significant difference between weeks. Significance level was set at 0.10.

Figure 3.2. Positive relationship between runoff volume and dissolved organic carbon (p <0.01).

Figure 3.3. Main effect of time of rainfall (0, 2, or 4 weeks after litter application) on a) monensin concentration, b) salinomycin concentration, c) monensin load, d) salinomycin load, e) percent monensin, and f) percent salinomycin lost in 30 min of runoff (with standard error bars) across litter treatments. Bars with different letters (i.e. a, b, c) indicate significant difference between weeks. Significance level was set at 0.10.

Figure 3.4. Inverse relationship between a) monensin concentration vs. total infiltration volume (initiation of rainfall to 30 min of runoff) and between b) salinomycin concentration vs. total infiltration volume. These relationships are significant for monensin ($p < 0.01$) and salinomycin ($p < 0.01$) concentrations.

Figure 3.5. Main effect of time of rainfall (0, 2, or 4 weeks after litter application) on soil moisture (with standard error bars) across the litter treatments. Bars with different letters (i.e. a, b, c) indicate significant difference between weeks. Significance level was set at 0.10.

Figure 3.1

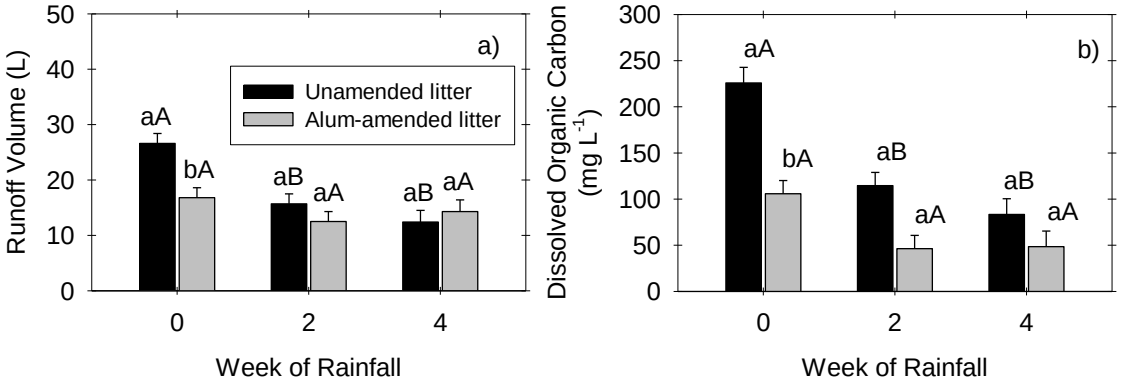

a)
Unamended litter
Alum-amended litter
Runoff Volume (L)
50
40
30
20
10
0
aA
bA
aB
aA
aB
aA
0
2
4
Week of Rainfall
b)
Dissolved Organic Carbon (mg L-1)
300
250
200
150
100
50
0
aA
bA
aB
aA
aB
aA
0
2
4
Week of Rainfall

Figure 3.2

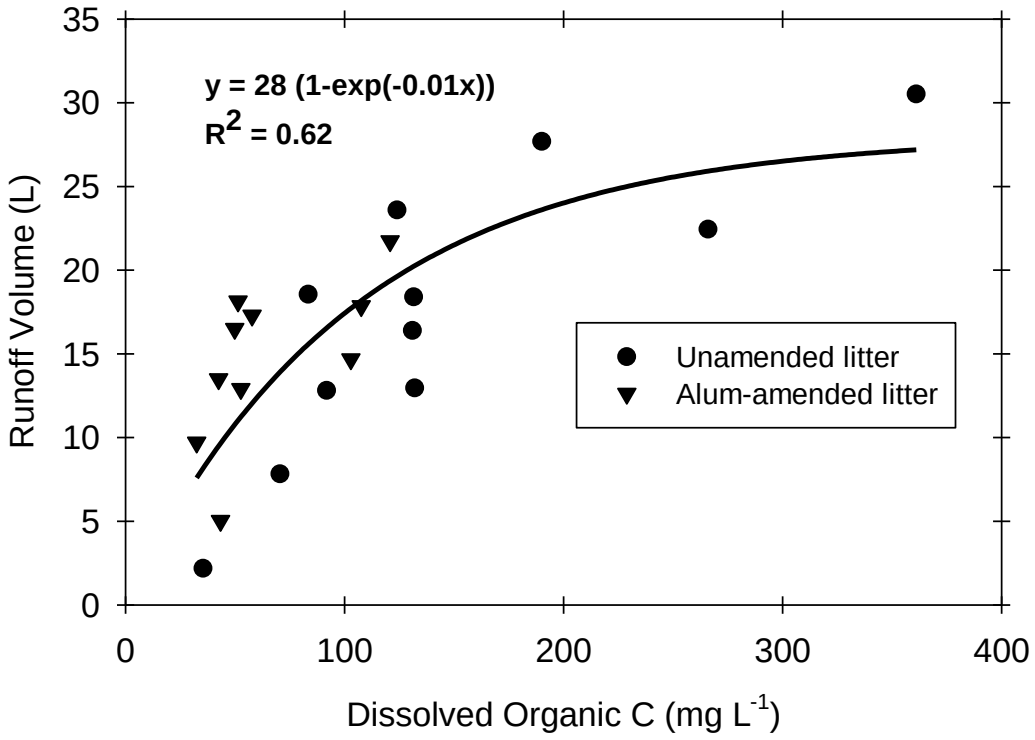

y = 28 (1-exp(-0.01x))
R2 = 0.62
Unamended litter
Alum-amended litter
Runoff Volume (L)
Dissolved Organic C (mg L-1)
0
5
10
15
20
25
30
35
0
100
200
300
400

Figure 3.3

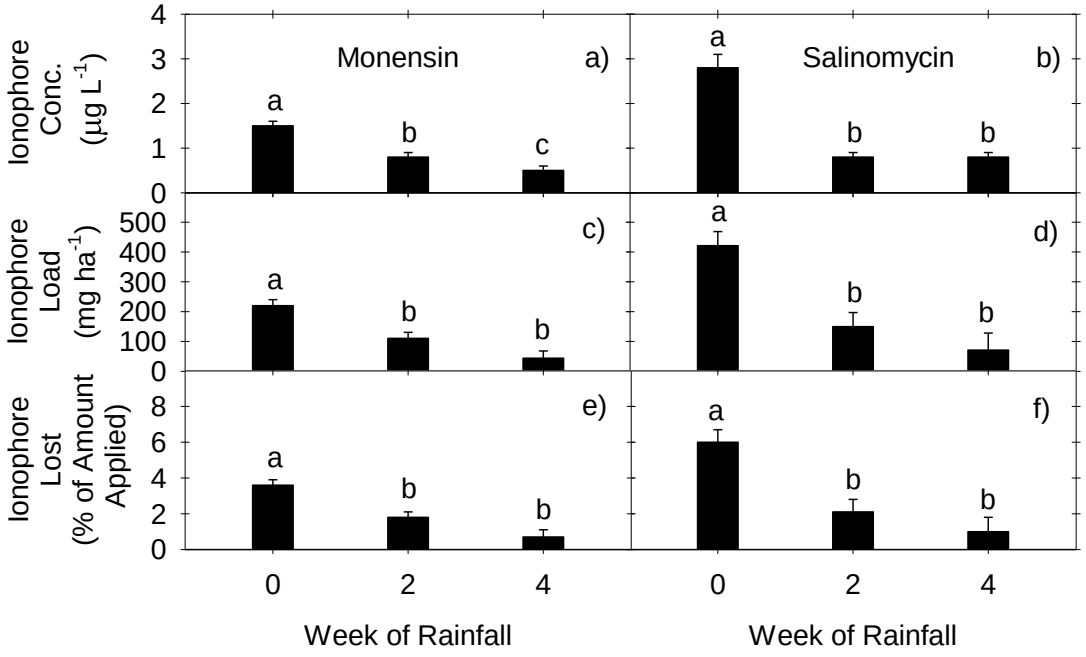

Monensin
Salinomycin
a)
b)
c)
d)
e)
f)
Ionophore Conc. (µg L-1)
Ionophore Load (mg ha-1)
Ionophore Lost (% of Amount Applied)
Week of Rainfall
Week of Rainfall

Figure 3.4

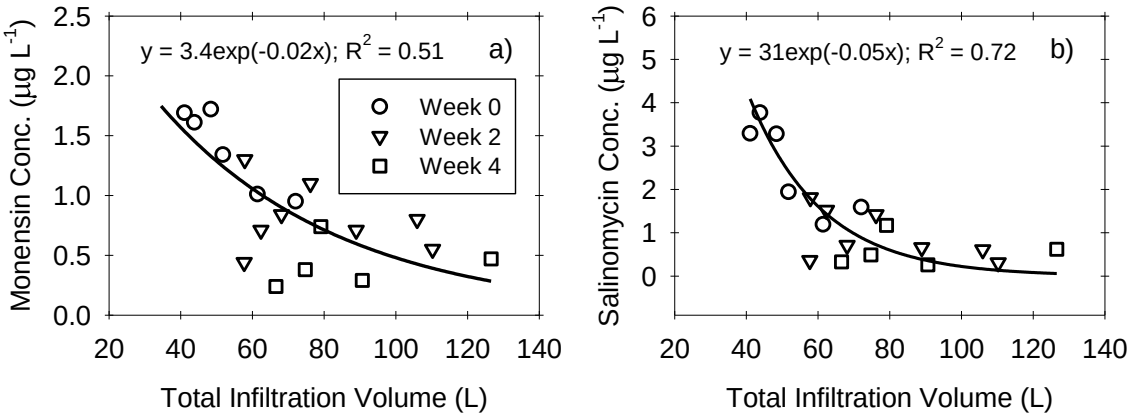
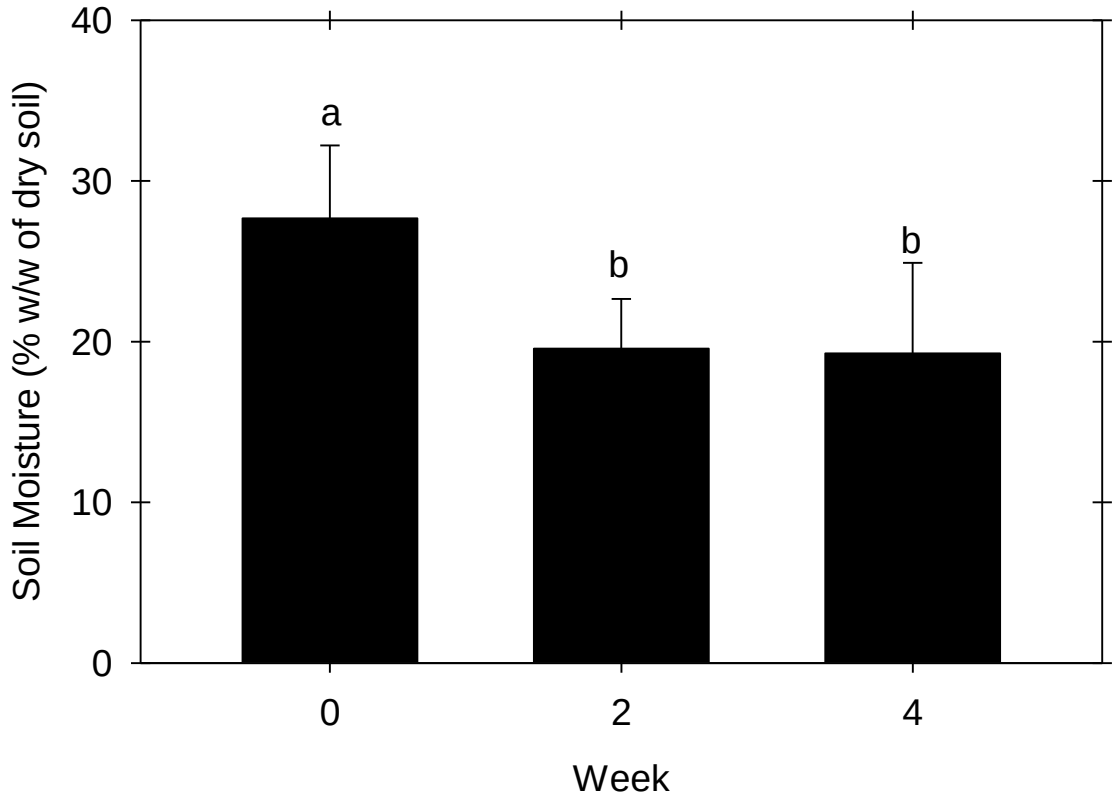

y = 3.4exp(-0.02x); R2 = 0.51
a)
Week 0
Week 2
Week 4
Monensin Conc. (µg L-1)
Total Infiltration Volume (L)
y = 31exp(-0.05x); R2 = 0.72
b)
Salinomycin Conc. (µg L-1)
Total Infiltration Volume (L)

Figure 3.5

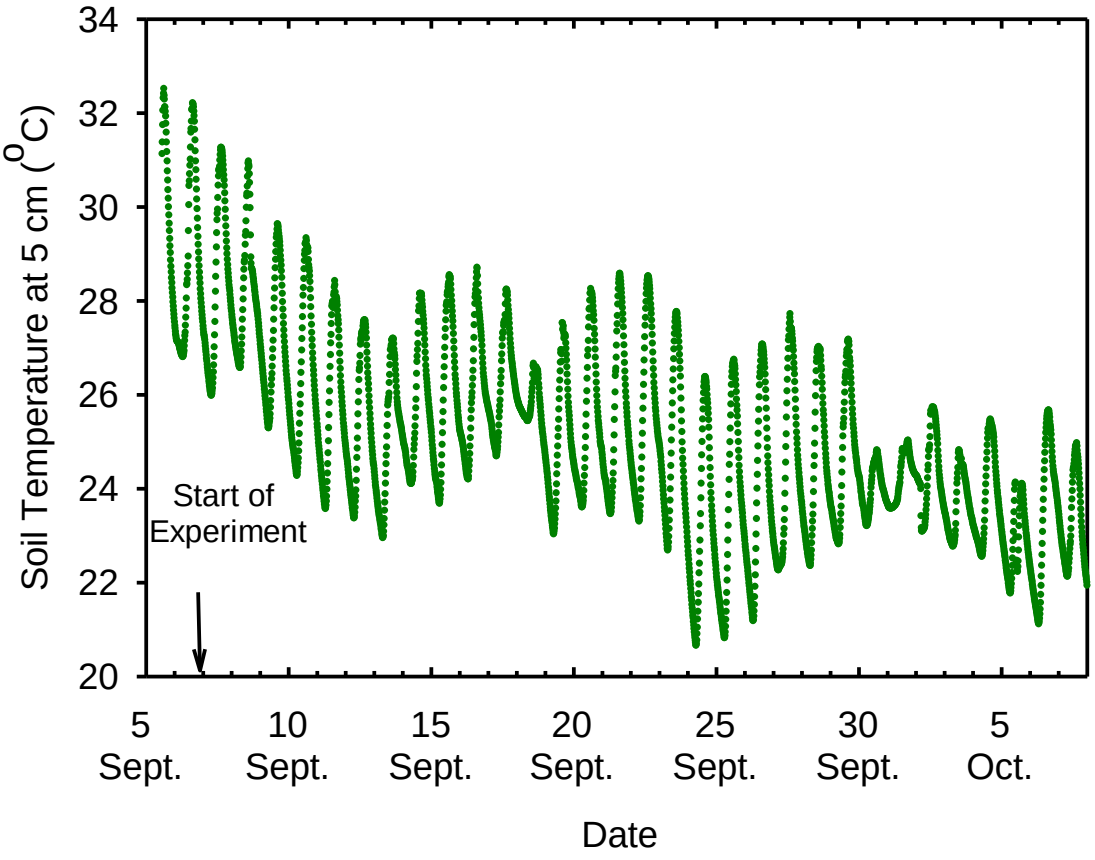


SUPPLEMENTAL FIGURES

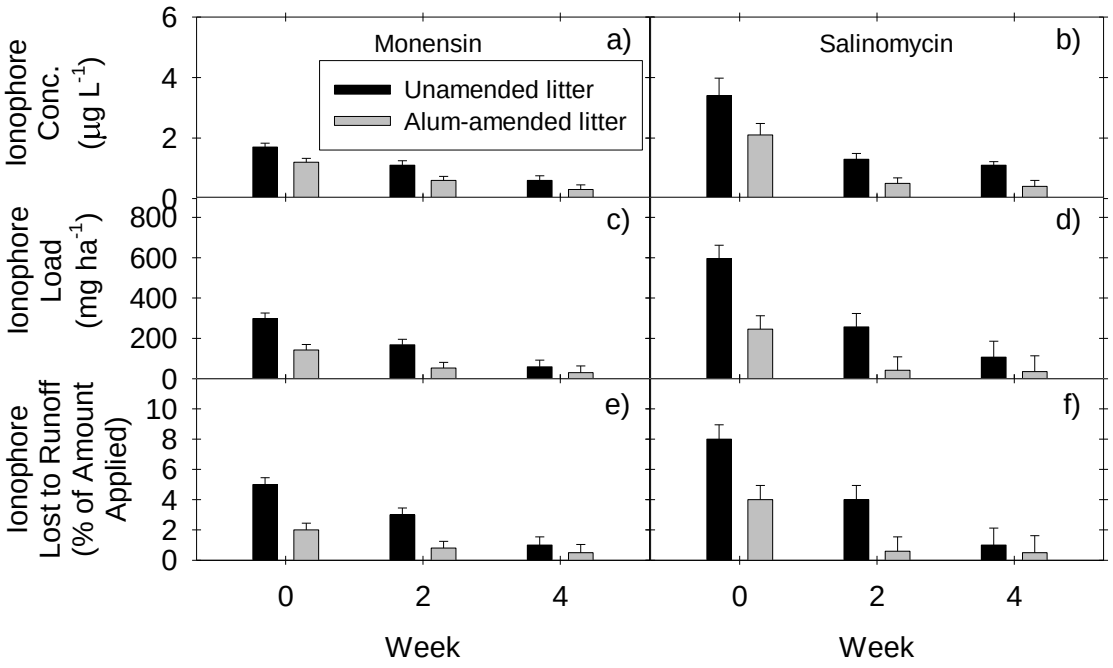
Supplemental Figure 3.S1. Mean soil temperature from two representative plots that did not receive simulated rainfall until 4 weeks after broiler litter application.

Supplemental Figure 3.S2. Mean a) monensin and b) salinomycin concentrations in 30 min of runoff, c) monensin and d) salinomycin loads in runoff and percent of e) monensin and f) salinomycin losses (with standard error bars) to runoff from grassed pasture plots receiving surface-applied unamended or alum-amended broiler litter 0, 2, and 4 weeks prior to rainfall simulation. Significance level set at alpha=0.10. (Note: Insignificant interaction *p* values for each parameter shown above are as follows: monensin concentration 0.89; salinomycin concentration 0.70, monensin load 0.14; salinomycin load 0.19; percent monensin lost 0.14 and percent salinomycin lost to runoff 0.22).

Supplemental Figure 3.S1



Supplemental Figure 3.S2.



CHAPTER 4

STACKING TIME AND ALUM EFFECTS ON POLYETHER IONOPHORES IN BROILER LITTER[2]

[2]Sarah A. Doydora, Peizhe Sun, Miguel Cabrera, Aaron Thompson, Kimberly Love-Myers, John Rema, Vaughn Calvert II, Spyros G. Pavlostathis and Ching-Hua Huang. Submitted to Journal of Environmental Quality, Mar 2015.

ABSTRACT

The use of ionophores as antiparasitic drugs plays an important role in US poultry production, especially in the broiler industry. However, administered ionophores can pass through the bird's digestive system and appear in broiler litter that is applied to agricultural fields, thus presenting an environmental hazard. Stacking (storing or stockpiling) broiler litter for some time might decrease the litter ionophore concentrations before land application. Because ionophores undergo abiotic hydrolysis at low pH, decreasing litter pH with acidic aluminum sulfate (alum) might also decrease ionophore concentrations. We assessed the change in ionophore concentrations in broiler litter in response to (a) the length of time the broiler litter was stored (stacking time) and (b) alum addition. We spiked broiler litter with monensin and salinomycin; placed alum-amended litter (~pH 4 to 5) and unamended litter (~pH 8 to 9) into 1.8 m^3 bins; and repeatedly sampled each bin for 112 d. Our findings showed that stacking broiler litter alone did not have any impact on monensin concentration but it did reduce salinomycin concentration by 55% although salinomycin degradation was slower than previous studies (half-life > 100 d). Adding alum to broiler litter reduced monensin concentration by approximately 20% relative to unamended litter, but it did not change salinomycin concentration. These results call for continued search for alternative strategies that could potentially reduce the concentration of ionophores in broiler litter prior to their application to agricultural soils.

INTRODUCTION

The use of polyether ionophores as antiparasitic drugs plays a vital part in the US broiler (*Gallus gallus domesticus*) industry. Before ionophores were introduced in the

early 1970s, a parasitic protozoan disease, known as coccidiosis had been a major cause of weak poultry performance and productivity (Chapman et al., 2010).

Monensin and salinomycin are among the most commonly used ionophore anticoccidial drugs (Peek and Landman, 2011; Landoni and Albarellos, 2015). Administered monensin and salinomycin in poultry feeds can appear in the litter, a mixture of poultry manure, feathers and bedding material (Furtula et al., 2009; Biswas et al., 2012; Sun et al., 2013a). Thus, broiler litter containing ionophores could potentially contaminate surface water, especially in areas where broiler litter is surface-applied to agricultural fields. In the United States, where poultry production is the largest in the world (USDA-ERS, 2012), 12.8 million Mg of broiler litter are generated per year assuming an average litter production of 1.5 kg per broiler (Vervoort et al., 1998; Endale et al., 2002) and an annual broiler production of 8.52 billion broiler as of 2013 (USDA-NASS, 2014). Based on this estimate, every year up to 64 Mg of ionophores are applied to agricultural soils in the United States, assuming a median ionophore concentration (monensin and salinomycin combined) of approximately 5 mg kg^{-1} litter (Sun et al., 2013a).

The presence of ionophores in the environment has been associated closely with agricultural activities (Davis et al., 2006). Several studies demonstrated ionophore mobility in surface runoff when these compounds were applied to bare soil (Davis et al. 2006; Kim et al. 2010). Recently, monensin and salinomycin were also detected in natural runoff from grasslands fertilized with broiler litter (Sun et al., 2013a). In addition to surface runoff, ionophores were also found in rivers, lagoons and shallow groundwater

that are generally close to livestock production (Cha et al., 2005; Kim and Carlson, 2006; 2007; Hao et al., 2006; Watanabe et al., 2008).

Polyether ionophores are known to possess inhibitory effects to Gram-positive bacteria, fungi, protozoa, and certain earthworm species (Rutkowski and Brzezinski, 2013; Hansen et al., 2009). Their antimicrobial properties lie primarily in their capacity to transport ions across the lipid bilayer of cellular membrane, which leads to impaired osmotic balance, depleted energy, and disintegrated cells (Westley, 1982; Bergen and Bates, 1984; Smith and Galloway, 1983). Hence, the environmental presence of these compounds may cause adverse effects on native biota and potentially increase pathogen resistance to these drugs (Boxall et al., 2003).

The mobility of ionophores in the environment may be minimized if ionophores are allowed to degrade in broiler litter over time prior to application to the field. This may be achieved by stockpiling (or stacking) broiler litter under an open building (roofed but without walls), which many farmers currently do while waiting for the proper time to apply litter to their fields (Cunningham, et al., 2012). Moreover, the use of aluminum sulfate (alum) has been recommended as broiler litter amendment for significantly reducing ammonia gas emissions from broiler houses and dissolved phosphorus in surface runoff from pastures that receive surface-applied broiler litter (Moore et al. 2000; Moore, 2011; Warren et al., 2006; Sistani et al., 2006). Alum decreases the pH of broiler litter and may enhance the abiotic hydrolysis of the ionophores as these compounds degrade at low pH (Bohn et al., 2013; Sun et al., 2013b; European Food Safety Authority, 2004). To our knowledge, no study has evaluated ionophore degradation during broiler litter stacking when broiler litter is amended with alum.

This study evaluated the change in monensin and salinomycin concentrations in broiler litter when the litter was stacked with or without alum. We hypothesized that stacking broiler litter with alum would decrease the concentration of monensin and salinomycin in the litter through hydrolysis. To test this hypothesis, we placed broiler litter with or without alum inside stacking bins and monitored their monensin and salinomycin concentrations over a period of 112 d.

MATERIALS AND METHODS

Two broiler litter treatments, unamended or alum-amended (20% w/w on fresh litter weight basis), were repeatedly sampled after 0, 14, 28, 57, 84, and 112 d of stacking and the concentrations of monensin and salinomycin were measured. Each of the litter treatments was replicated four times and arranged in a completely randomized design. We tested alum because of its benefits in air and water quality and its sustainable impact to soil, broiler, and crop production, which have been documented over the past 20 years (Shreve et al., 1995; Moore et al., 1998; 2000; Moore and Edwards, 2005; Warren et al., 2006; Guo and Song, 2010). In the USA, alum is used in the production of approximately one billion broilers every year (Moore, 2011). The experiment was conducted under an open-air structure, with a high roof (similar to typical stacking houses used by farmers) at the University of Georgia Central Research and Education Center near Eatonton, GA (33° 24’ N; 83°29’W; elevation, 150 m).

Broiler Litter Treatment Preparation

Broiler litter was obtained from a whole house cleanout (no information on number of flocks is available) and was used in the experiment approximately 2 wk after cleanout. Inherent ionophores were not found in the litter. Hence, unamended or alum-amended litter was artificially spiked with the ionophores by adding 7.22 g of a 97% pure Na monensin powder (Sigma Aldrich, USA) and 116.6 g of a 12% salinomycin powder (provided by the department of Poultry Science, University of Georgia) to 2.3 kg of lime (Lowes, USA) and mixing the spiked lime with 136.4 kg of broiler litter using a feed mixer (H.C. Davis Son's Mfg Co., Inc., Bonner Springs, KS). This yielded 46.0 ± 8.1 mg kg^{-1} for monensin and 95.9 ± 9.6 for salinomycin in the stock litter.

The appropriate amount of stock litter was then added to ≈ 180-kg batches of unamended or alum-amended litter (previously combined in the mixer), and the mixture was blended thoroughly using a feed mixer to achieve a target initial concentration of 1000 µg/kg for monensin and 2000 µg/kg for salinomycin. Initially, we targeted equal concentrations for monensin and salinomycin in the litter. However, incorrect labeling of the source of the salinomycin resulted in a double application of salinomycin (116.6 g) to 2.3 kg lime when preparing the stock litter. Nevertheless, this problem was accounted for by testing for Day 0 values as a covariate for all parameters except for litter pH, in our statistical analyses.

After mixing, four wooden bins (1.8 m^3 each) were filled by placing four mixed batches of unamended or alum-amended litter in each bin. To reduce the litter variability between bins of the same treatment, one batch was placed in each of the four bins, followed by a second batch in each of the bins, and so on until the bins were filled to a

height of 1.2 m. A diagram on how the bins were filled is shown in Supplemental Fig. 4.S1. For the alum-amended litter, alum added in the first batch of each bin was from General Chemical USA (Parsippany, NJ), whereas alum added in the last three batches of each bin was from Delta Chemical Corp. (Baltimore, MD). Both sources had similar degree of fineness (100% through 4.76-mm sieve and 90% through 2.0-mm sieve) and chemical composition according to manufacturer's specifications.

The bin weights for unamended litters ranged from 711 to 724 kg, whereas those of alum-amended litters ranged from 718 to 731 kg. All of the litter preparations and bin fillings were completed within the same day. Temperature sensors (Hobo U12, Bourne, MA) were placed immediately after filling at the center of each bin at 45 cm of height from the stack bottom, and one sensor was placed outside the bins to measure air temperature. Temperature served as an indicator of overall microbial activity of the litter inside the bins. Litter in the bins was monitored for up to 112 d for ionophore concentrations, temperature, alkalinity, pH, and gravimetric water content. The study was conducted from 20 Mar 2012 to 10 Jul 2012.

Broiler Litter Sampling and Sample Analyses

At 0, 14, 28, 57, 84, and 112 d of stacking, a sampling tube (4-cm ID) was used to collect a sample from the center of each bin at a height of 30-45 cm measured from the stack bottom. The sample was divided in half, placed into two 500-mL polyethylene bottles, and transported in a cooler to the laboratory. The sample in one of the bottles was freeze dried and stored at -20°C, whereas the sample in the second bottle was stored at 4°C until analysis. Moisture content and litter pH was determined on the 4°C-stored

samples within 1 d of collection. Litter moisture was determined gravimetrically by drying the litter samples at 60°C for 48 h (Qafoku et al., 2001). Litter pH was measured in a 1:5 litter to water w/w ratio (Rothrock et al., 2010). Alkalinity was determined at Days 0 and 112 by first taking 2 g of broiler litter sample and adding to it 500 mL of 18 MΩ water. The suspension was shaken for 30 min and filtered through FP-Vericel™ 0.45-µm filter membrane. Fifteen milliliters of the litter water extract (filtrate) was then subjected to potentiometric titration (American Public Health Association, 1992) with 0.025 mol L^{-1} HCl to pH 4.5 using the same pH electrode-meter setup used for litter pH measurements.

Monensin and salinomycin concentrations were measured on freeze-dried, ground litter samples following the method that our research team had developed earlier (Sun et al., 2013a). One gram of sample was placed in a 50-mL centrifuge tube and mixed with an internal nigericin standard at 0.5 µg kg^{-1}, 7.5 mL of McIlvaine's buffer (containing 10 m mol L^{-1} EDTA, Na_2HPO_4 and citric acid), 2.5 mL methanol, and 5 mL hexane. The samples were shaken 30 min and centrifuged (5 mins at 3000 rpm) and the hexane layer was transferred to an amber bottle. This extraction step was repeated by adding another 5 mL of hexane. The two 5-mL hexane extracts were combined and evaporated to dryness under vacuum. After drying, the residue was reconstituted by adding 1 mL mixture of methanol and 10 m mol L^{-1} Na_2HPO_4 solution. The samples were then analyzed with a liquid chromatography mass spectrometry detection system (Agilent 1100 Series, Agilent, Palo Alto, CA) using a reversed-phase column (2.1 mm × 150 mm, 3 µm Ascentis RP-amide column; Supelco, Bellefonte, PA). The mobile phase consisted of (A) a mixture of HPLC-grade water and formic acid (99.9:0.1, v/v) with 25% acetonitrile;

and (B) HPLC-grade methanol and acetonitrile (50/50, v/v). The estimated recoveries in broiler litter were 79±11 and 82±15% for monensin and salinomycin, respectively. Method detection limits were 18.6 μg kg^{-1} for monensin and 25.1 μg kg^{-1} for salinomycin in broiler litter. Instrument detection limits were 28 and 38 pg for monensin and salinomycin, respectively.

Statistical Analysis

All of the measured variables (except temperature) were analyzed using linear repeated measures models with PROC MIXED in SAS 9.3 (SAS Inst. Inc., Cary, NC) for the effect of days of stacking as a continuous variable, the effect of alum amendment, and their interaction (if significant). All of the models satisfied the required assumption for normality (Shapiro-Wilk test) and were analyzed with a proper correlation structure based on the smallest AICC as a selection criterion. Correlation structures used were heterogeneous compound symmetry for litter pH, spatial power for litter moisture content and monensin concentrations, and compound symmetry for salinomycin concentrations. Satisfying these assumptions was critical to ensure that inferences derived from each of the statistical tests performed were valid. Necessary data transformation was also performed on certain datasets (i.e. fourth power transformation for litter pH and natural log transformation for salinomycin concentration) as part of meeting these assumptions. All of the parameters (except litter pH) were analyzed from Day 14 through Day 112 using Day 0 values as covariate. Note that Day 0 was included as a covariate because this measure was taken immediately after treatment and prior to any effect on the ionophore concentrations and litter moisture. However, in our final linear models, this covariate was

not included in cases where it was not significant. An alpha level of 0.10 was used to determine all significant effects. Among all the parameters, only the concentrations of monensin had a significant Day 0 covariate. Day 0 values were independently tested between the two litter treatments using t-test (PROC TTEST, SAS 9.3), except for litter pH. For litter pH, Day 0 was not a true baseline and hence was not considered a covariate. This is because litter treatment (i.e. alum addition) had already delivered its effect on pH immediately unlike the other parameters.

As mentioned previously, terms for interaction effect between litter treatment and days of stacking were omitted in the final linear models when prior interaction analyses showed they were not significant. Overall, interaction effects were not significant and were therefore not included in the final statistical analyses on all parameters except for litter pH. Hence, an interaction effect was included in our results only for litter pH (i.e. differences in slope for pH between the two litters with days of stacking, Fig. 4.3).

The approximate half-life ($t_{1/2}$) for salinomycin was calculated as $t_{1/2} = \frac{\ln 2}{k}$ where k is the first order degradation constant estimated for salinomycin with a nonlinear regression model, $C_i = C_0 e^{-kt}$, which was fit using PROC NLIN in SAS 9.3. In that model, C_i corresponded to measured salinomycin concentrations at Day 14 thru 112 while C_0 corresponded to the average salinomycin concentrations at Day 0.

RESULTS AND DISCUSSION

Litter Physical and Chemical Properties

Bin temperature for alum-amended litter decreased within 1 d of stacking to near ambient temperature and remained similar to ambient temperature throughout most of the stacking period except after 80 d (Fig. 4.1), when it started to increase. In contrast, the unamended litter temperature rose rapidly to approximately 50°C within 1 d after stacking, remained at levels >40ºC for about 80 d, and then decreased to near ambient temperature (20 to 30ºC) by 112 d (Fig. 4.1). Alum was reported to inhibit certain bacterial populations (i.e., *Clostridium*/*Eubacterium* genera) including potential bacterial pathogens (i.e. *C. jejuni* and *E. coli*) but stimulate fungal diversity and increase fungal abundance in broiler litter with incubation period (Cook et al., 2008; Rothrock et al., 2008, 2010). Thus, a shift in dominant microbial populations likely contributed to depressed temperature throughout most of the stacking period of the alum-amended litter. It also likely caused the observed increase in temperature towards the end of the 112-d stacking of the alum-amended litter.

In terms of the moisture conditions of the litter, unamended and alum-amended litters had similar moisture contents at Day 0 (503±18 vs 482±10 g H_2O kg^{-1}, p=0.36). However, on any given day during stacking (Day 14 thru Day 112), unamended litter had 22 g H_2O kg^{-1} lower moisture relative to alum-amended litter. The relatively drier unamended litter is possibly due to its generally higher temperature in comparison to alum-amended litter throughout most of the stacking period except approximately during the last month of stacking (Fig. 4.1). Stacking had a significant inverse effect on litter

moisture (with litter moisture decreasing at a rate of 0.7 g H_2O kg^{-1} day^{-1}, $p<0.01$; Fig. 4.2), without differences in the rate of decrease between the two litter treatments.

Upon addition of alum, mean litter pH immediately dropped to acid levels (pH 4.0±0.1) whereas that of the unamended litter remained alkaline (pH 8.3±0.1). Over the course of the stacking period, the pH of the alum-amended and the unamended litters were significantly different (Fig. 4.3). The lower pH of alum-amended litter was due to the production of protons when alum is dissociated according to a chemical reaction (Moore et al., 2000):

$$Al_2(SO_4)_3.14H_2O + 6H_2O \Rightarrow 2Al(OH)_3 + 3SO_4^{2-} + 6H^+ + 14H_2O. \quad \text{Eq.[4.1].}$$

Our statistical analysis showed a significant pH increase with stacking time for unamended ($p<0.01$) but not for alum-amended litter ($p=0.14$) (Fig. 4.3). The increase in pH with unamended litter may be due to greater occurrence of hydrolysis of urea, a H^+-consuming reaction. This appears to coincide with the more pronounced decrease in concentrations of urea in unamended than in alum-amended litter (Supplemental Fig. 4.S2). In alum-amended litter, its relatively constant pH with stacking time is likely due to delayed urea hydrolysis (and likely delayed organic N mineralization) caused by a delayed establishment of urease-producing fungal populations (Rothrock et al., 2010). Moreover, the acid-neutralizing capacity of the alum-amended litter was low throughout the 112-d stacking, as evidenced by alkalinity measurement of 0 mg kg^{-1} litter ($CaCO_3$ equivalent) at Day 0 and 1000 mg kg^{-1} at Day 112. We are aware, however, that the effect of alum in decreasing litter pH is temporary. Moore and Edwards (2005) demonstrated higher soil pH for soils under 8 yr of receiving alum-amended litter in comparison to soils not receiving any litter amendment at all.

Effect of Stacking Time

Regardless of the litter treatment, concentrations of monensin did not decrease significantly over the stacking period (p=0.40; see Fig. 4.4a). This finding is different from that of Dolliver et al. (2008) who observed degradation of monensin and calculated a monensin half-life of 22 d in a stockpiled turkey manure at moisture levels similar to our litter (their 400 g H_2O kg^{-1} manure dry wt. vs. our 398 to 503 g H_2O kg^{-1} litter dry wt.). However, Sun et al. (2014), working with the same broiler litter used in our study, found monensin persisted in broiler litter microcosms across a range of litter moisture (from 320 to 2570 g H_2O kg^{-1} dry litter) and temperature (35, 45 or 60°C) levels encompassing those prevailing in our bin treatments (i.e., 393 to 503 g H_2O kg^{-1} dry litter and at 35 to 50 °C). Based on these findings of Sun et al. (2014), the lack of monensin degradation in our bins was likely due to unfavorable moisture and temperature conditions.

In contrast, concentrations of salinomycin decreased significantly with stacking time at a rate of 0.0054 natural log units per day regardless of litter treatment. In other words, each day salinomycin decreased by 0.5 median percent of the previous day's concentration ($p<0.01$; Fig. 4.4b) to a total cumulative reduction of 55% in 112 d. This equates to an estimated salinomycin half-life of >100 d, which is much longer than the relatively shorter half-life of salinomycin in manure reported in other study (Ramaswamy et al., 2010). Ramaswamy et al. (2010) reported a salinomycin half-life of 4 d in an open-mound of poultry manure (at a preadjusted moisture of 50 to 60 g H_2O kg^{-1} of dry manure wt.). The reasons for the slow degradation of salinomycin in our litter compared to other poultry manure are unclear. Sun et al. (2014) predicted a combined moisture content and

temperature scenario wherein salinomycin degradation would not be expected in broiler litter. This scenario matches with the moisture and temperature levels in stacked litters throughout the study period and may explain the slow salinomycin degradation in our study. The possibility that spiked ionophore is not easily degraded can be excluded, considering its relatively faster degradation in other manure (Dolliver et al., 2008; Storteboom et al., 2007).

Generally, degradation of most xenobiotics is faster under aerobic compared to anaerobic conditions (Lee et al., 2007; Thiele-Bruhn, 2003); thus, low oxygen diffusion through the stacked litter may have contributed to slow ionophore degradation. However, Dolliver et al. (2008) have shown only slight difference in the half-life of monensin and no significant decrease in monensin concentration between aerated compost (19 d half-life) vs. unaerated poultry manure pile (22 d half-life) after 35 d. Similarly, in the work of Ramaswamy et al. (2010), differences in half-lives have not been too wide between aerated compost (1.3 d) and an open mound of poultry manure (4 d), despite the significantly different ($p < 0.05$) salinomycin concentrations between their aerated and unaerated manure pile throughout their 38-d study. In contrast, Storeteboom et al. (2007) reported a significantly longer monensin half-life (30.1 d) in unaerated horse manure pile under low-intensity management vs. those that were aerated under high-intensity management (14.7 d). Žižek et al. (2014) also observed much longer half-life (61.8 d) for lasalocid (another ionophore) in unaerated stockpiled chicken manure in comparison to the half-life (17.5 d) in aerated composed chicken manure. Although unaerated manure piles mentioned above have shown longer half-lives for ionophore degradation compared

to aerated piles, none of these reported half-lives are as long as those of our stacked broiler litter.

Effect of Alum

At Day 0, starting mean monensin and mean log salinomycin concentrations were similar for the unamended and the alum-amended litters (1194±144 vs 1137±114 for monensin, p=0.76; 7.42±0.18 vs 7.47±0.20 log concentrations for salinomycin, p=0.87). However, on any given day throughout the stacking period (Day 14 thru Day 112), alum-amended litter had 250 μg kg^{-1} lower monensin concentration relative to unamended litter (p=0.02), which corresponds to a 20% reduction. In contrast, salinomycin concentrations were similar between litter treatments (p=0.55). Both monensin and salinomycin were shown to degrade at acid pH values through acid-catalyzed hydrolysis (Bohn et al., 2013; Sun et al., 2013b, European Food Safety Authority, 2004). At pH 4, the half-lives of monensin and salinomycin are 6.6 (~1 wk) and 0.2 d (~4 h), respectively (Sun et al., 2013b). Given these half-life values reported by Sun et al. (2013b), it appears that monensin underwent limited hydrolysis, whereas salinomycin did not hydrolyze at all. Because hydrolysis must take place in the presence of water, the low moisture content of our alum-amended litter may have inhibited hydrolysis reactions (Fig. 4.2). The occurrence of degradation of monensin but not of salinomycin in alum-amended litter may be due to differences in the extent of contact between the alum-amended litter and the ionophores or differences in each of the ionophore's immediate pH environment. As mentioned previously, the formulation of the monensin spike was nearly pure (97% purity), whereas the salinomycin formulation was only 12% pure with the remainder as

$CaCO_3$. The excess $CaCO_3$ in the salinomycin formulation may have acted as an immediate pH buffer, creating a less acid condition within the immediate vicinity of the salinomycin compound.

CONCLUSIONS

Methods that reduce ionophore concentrations in broiler litter may help control environmental contamination of agricultural fields. Although other studies report ionophore degradation in manure within a few weeks, we found 16 wk (or 112 d) of stacking broiler litter reduced salinomycin concentration by 55%, while monensin concentrations remained unchanged. Adding alum to broiler litter reduced monensin concentration by 20%, but interestingly not salinomycin concentrations. Contrary to our hypothesis, hydrolysis did not occur to a large extent in alum-amended litter likely due to its low moisture, which may have prevented complete solubilization of the ionophores. Moreover, the low moisture in our litter treatments may have possibly limited or caused slow microbial degradation of the ionophores. Overall our work suggests monensin will persist longer than salinomycin in broiler litter.

FUTURE WORK

Future studies are recommended to confirm the limited occurrence of hydrolysis under low moisture content of alum-amended litter. This can be performed by subjecting sterile alum-amended litter to different moisture contents, conducting destructive sampling over time and evaluating the existence of acid-catalyzed hydrolysis products for monensin and salinomycin, which were previously identified by Sun et al. (2013b). Our

proposed hypothesis for the differences in the occurrence of hydrolysis between the two polyether ionophores may also be confirmed by measuring the acid buffering capacity of the used formulations of monensin and salinomycin in this study.

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Figure 4.1. Temperature profile in the stack for a) unamended and b) alum-amended (200 g kg^{-1} fresh litter weight) broiler litters throughout 112 days.

Figure 4.2. Effect of days of stacking on litter gravimetric water contents (by dry litter weight) from Day 14 thru Day 112 ($p < 0.01$), regardless of litter treatment. Significance level was set at 0.10.

Figure 4.3. Litter pH of unamended and alum-amended (200 g kg^{-1} fresh litter weight) broiler litters throughout 112 days of stacking after fourth power data transformation. Significance level was set at 0.10.

Figure 4.4. Effect of days of stacking on the a) concentrations of monensin ($p = 0.40$) and b) natural log concentrations of salinomycin ($p < 0.01$) from Day 14 thru Day 112, regardless of litter treatment. Significance level was set at 0.10.

Figure 4.1

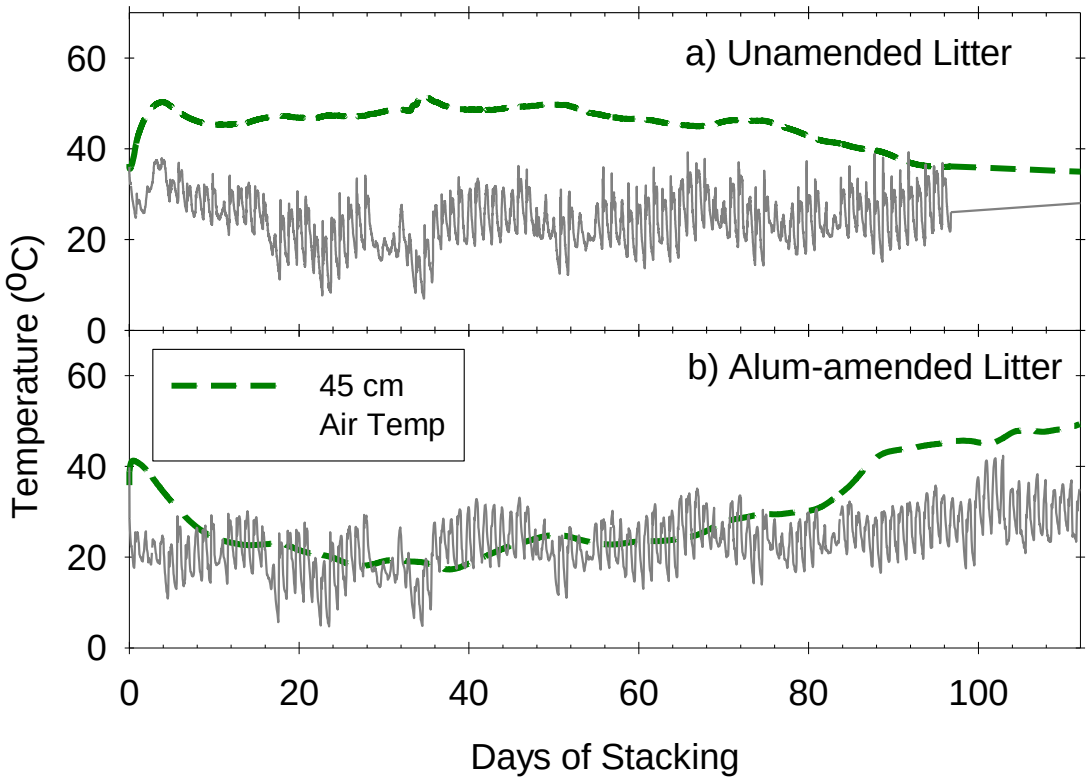

a) Unamended Litter
b) Alum-amended Litter
45 cm
Air Temp
Temperature (°C)
Days of Stacking
0
20
40
60
80
100

Figure 4.2

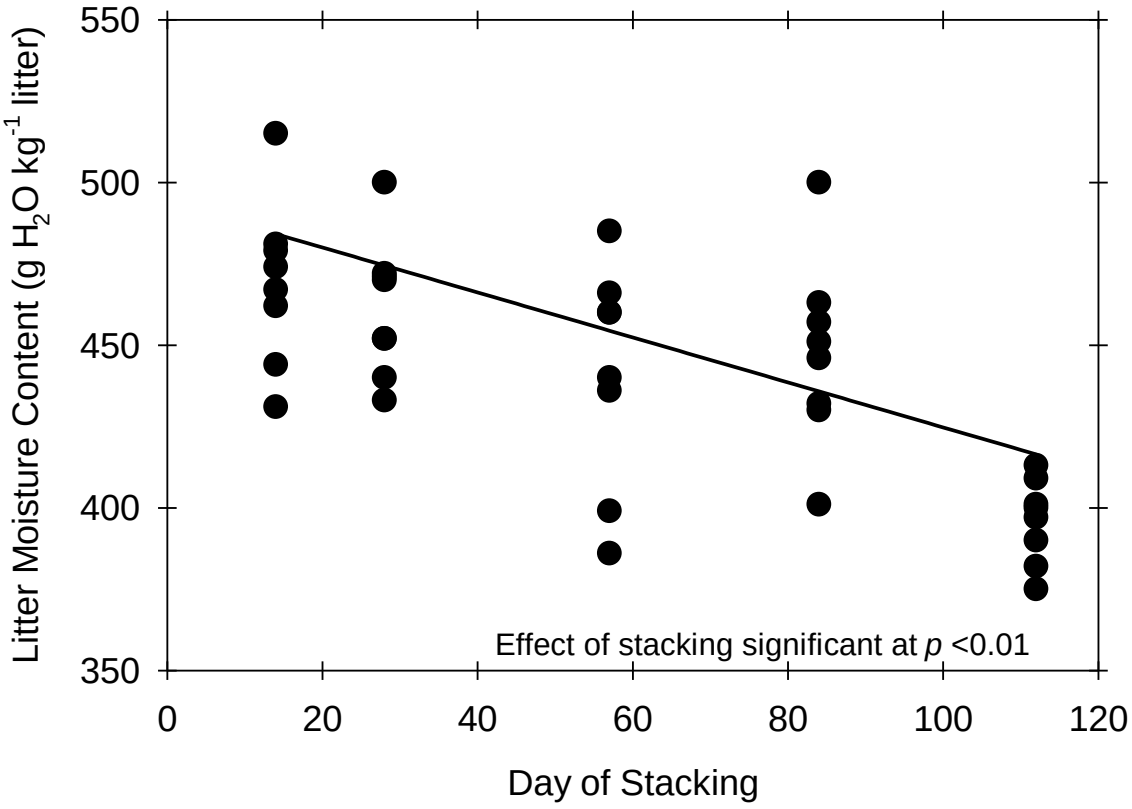

Litter Moisture Content (g H_2O kg^{-1} litter)
550
500
450
400
350
0
20
40
60
80
100
120
Day of Stacking
Effect of stacking significant at p <0.01

Figure 4.3

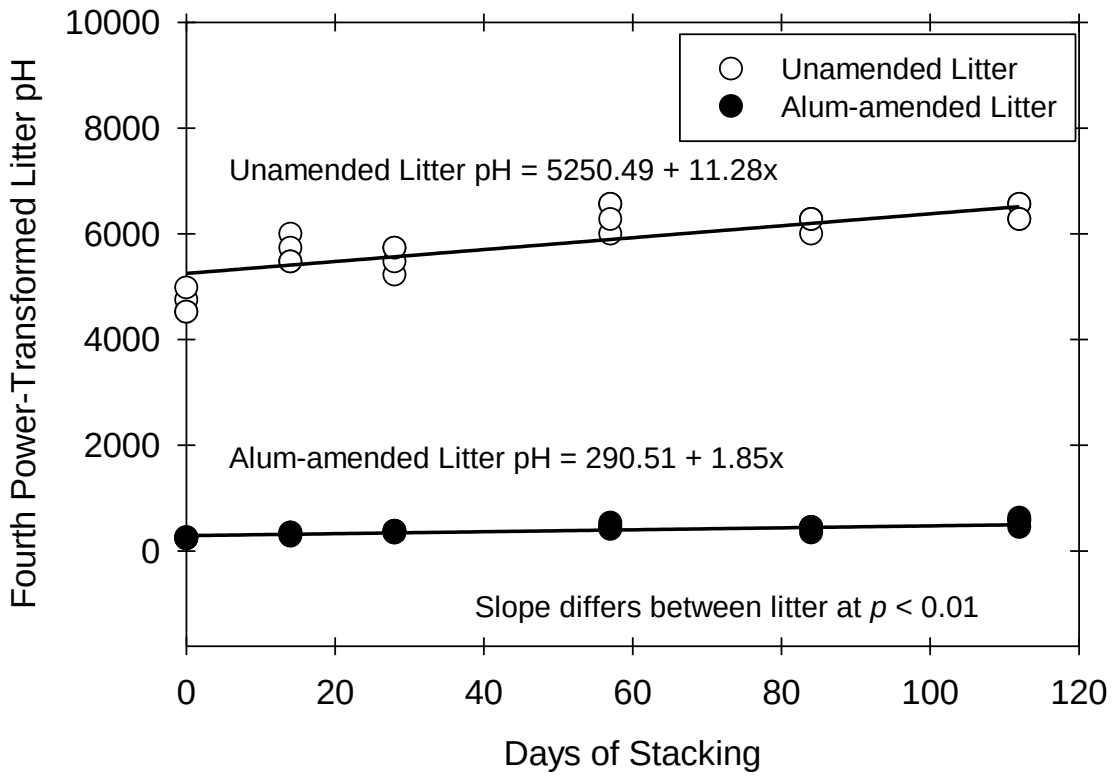
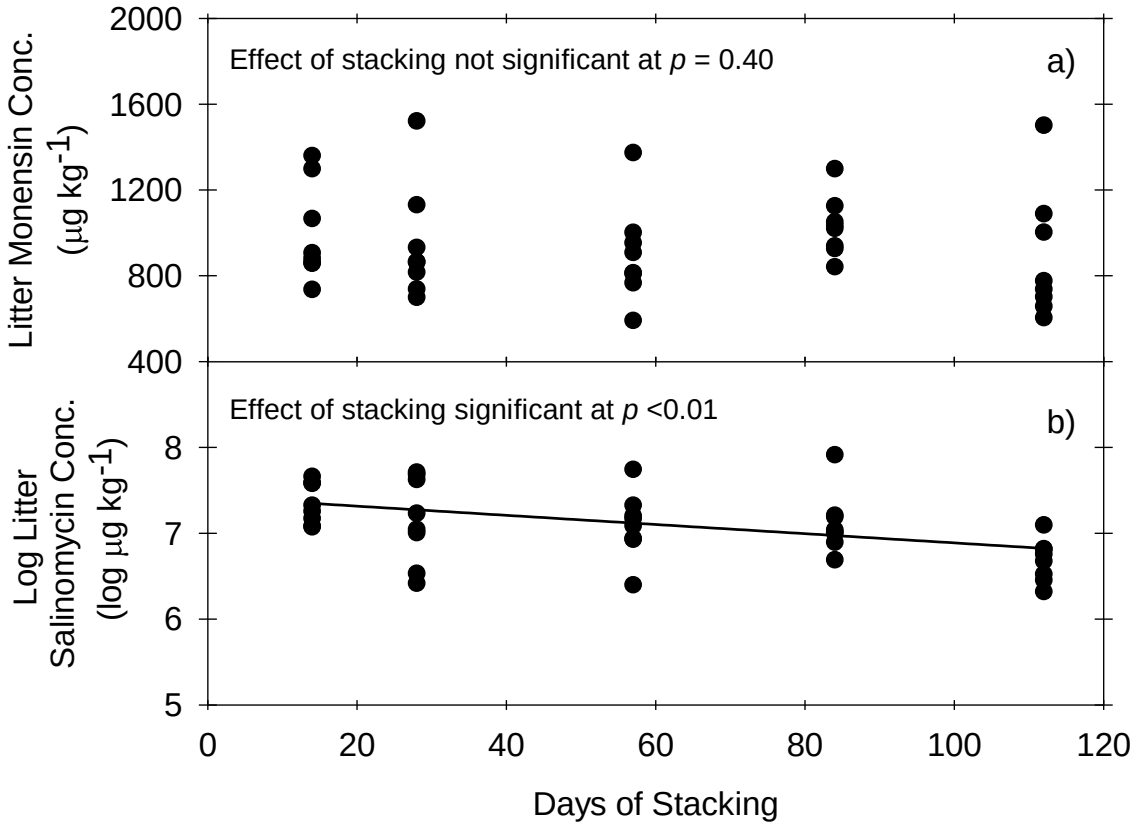

Fourth Power-Transformed Litter pH
10000
8000
6000
4000
2000
0
Unamended Litter
Alum-amended Litter
Unamended Litter pH = 5250.49 + 11.28x
Alum-amended Litter pH = 290.51 + 1.85x
Slope differs between litter at p < 0.01
0
20
40
60
80
100
120
Days of Stacking

Figure 4.4

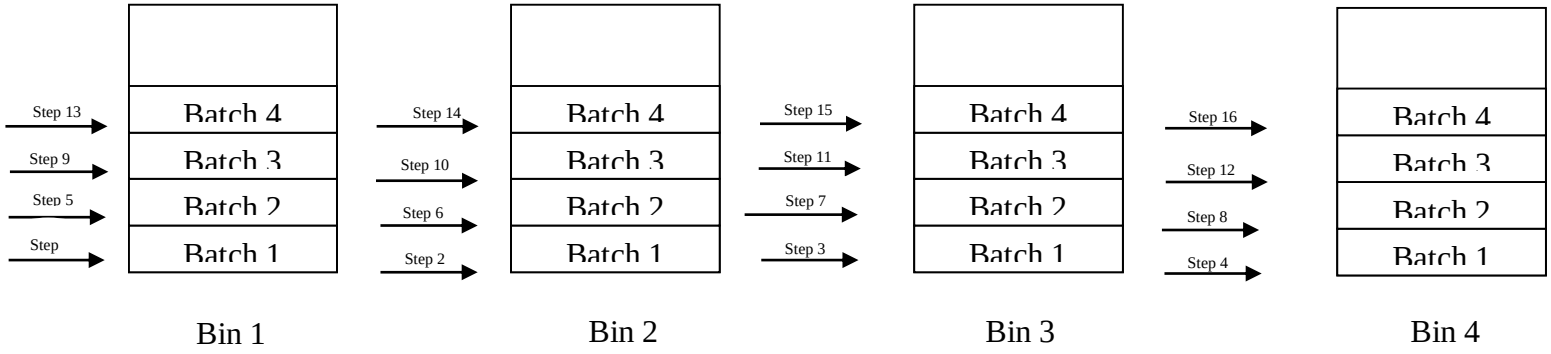

Litter Monensin Conc. (μg kg-1)
2000
1600
1200
800
400
Effect of stacking not significant at p = 0.40
a)
Log Litter Salinomycin Conc. (log μg kg-1)
8
7
6
5
Effect of stacking significant at p <0.01
b)
0
20
40
60
80
100
120
Days of Stacking

SUPPLEMENTAL FIGURES

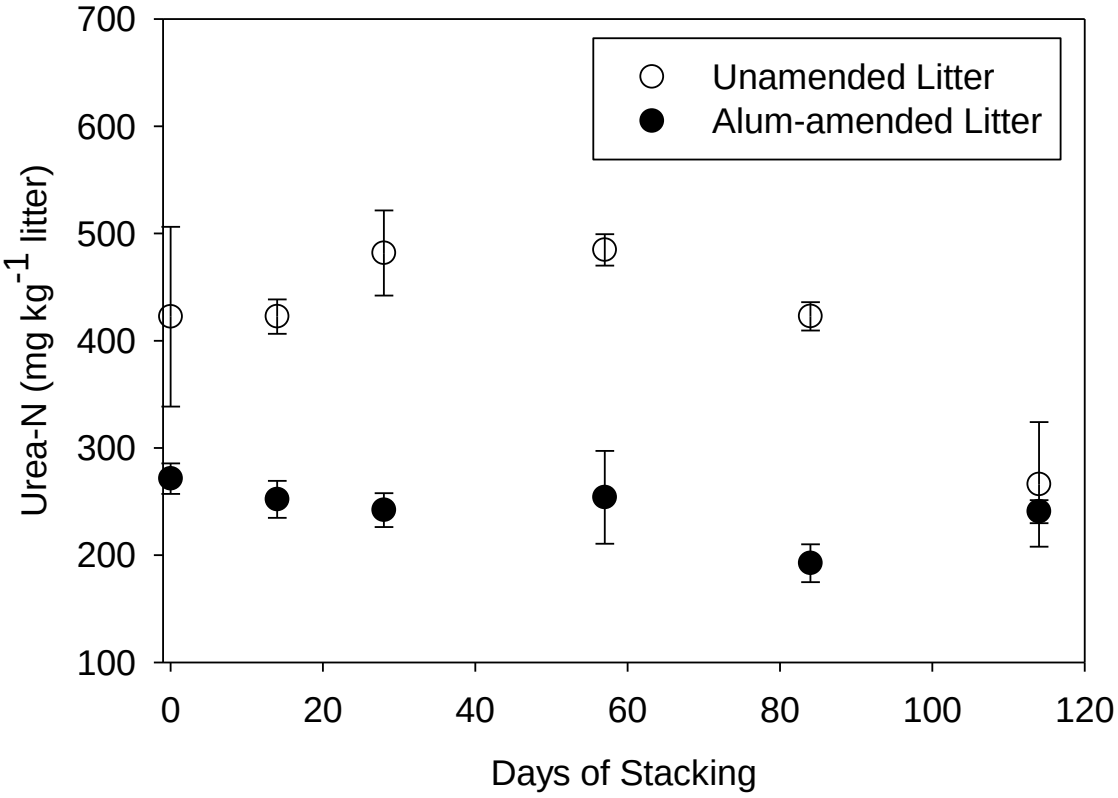
Supplemental Figure 4.S1. Diagram for filling each replicate bin with litter by batch.

Supplemental Figure 4.S2. Change in urea concentration in unamended and alum-amended ((200 g kg^{-1} fresh litter weight) broiler litter in 112 d of stacking.

Supplemental Figure 4.S1.



Supplemental Figure 4.S2.



CHAPTER 5

SORPTION AND DESORPTION OF MONENSIN TO PASTURE SOILS OF NORTHEASTERN GEORGIA[3]

[3] Sarah A. Doydora, Peizhe Sun, Miguel Cabrera, Nehru Mantripragada, Spyros G. Pavlostathis, Ching-Hua Huang and Aaron Thompson. To be Submitted to Journal of Environmental Quality.

ABSTRACT

The affinity of pollutants for soil surfaces is a key factor governing pollutant movement in the environment. Monensin is a common drug given to poultry that can be exposed to the environment when poultry manure and bedding material (broiler litter) is applied to agricultural lands as fertilizer. This study assesses the impact of long-term surface application of broiler litter (116 Mg ha^{-1} litter in 17 yrs) on the capacity of pasture soil to sorb and desorb monensin. We hypothesized that soils that had received long-term applications of broiler litter would retain more monensin than unamended soils because they have higher organic matter concentrations. To test this, we exposed the soils to a range of monensin concentrations (0.18 to 1.81 μmol L^{-1}), solution pH values (pH 4-9) and temperatures (15, 25, and 35°C) and measured monensin loss (sorption). Then after 2 h to 2 wk of exposure, we extracted the soils and measured the release of monensin (desorption). Broiler litter-amended soils sorbed 46% less monensin than the control soils, likely due to the higher native soil pH of the former. The sorption of monensin was strongly influenced by pH, with an order of magnitude greater sorption at pH 4 than at pH 9 and similar sorption capacities for both soils when compared at the same pH. Temperature did not significantly impact sorption for either soil. The broiler litter-amended soil released 25% less monensin than the control soil, when averaged across all weeks of reaction time, with less monensin desorbed for both soils when exposure time was longer (i.e., 2 wk vs. 2 h). Our findings suggest that soils with increased pH could enhance mobility of monensin.

Abbreviations: NaMON, sodium monensin; K_d, monensin sorption partitioning coefficient

INTRODUCTION

In areas close to animal production, monensin has been the most frequently detected veterinary ionophore drug in US water samples (Hansen et al., 2009a). It has been found in Cache la Poudre River (<0.01 to ~0.04 µg L^{-1}) in Northern Colorado (Cha et al., 2005; Kim and Carlson, 2006; 2007); and in lagoons (3.91 to 16.24 µg L^{-1}) and shallow groundwater (0.04 to 0.39 µg L^{-1}) beneath dairy farms in the Central Valley of California (Watanabe et al., 2008). In broiler industry, incorporated monensin in the feed is introduced to the environment through common application of broiler litter (mixture of broiler manure, feathers, and bedding materials) as a fertilizer to pasture lands (Furtula et al., 2009; Biswas et al., 2012; Sun et. al., 2013a). Although monensin is not likely to cause acute effects to index organisms at its reported concentrations, chronic low-level exposure of monensin could harm native biota or fuel pathogen resistance in natural ecosystems (EFSA, 2004, 2005; Boxall et al., 2003; Hansen et al, 2009b).

The behavior of monensin in soil is likely governed by its chemical properties. Monensin consists of a series of oxygenated heterocyclic rings and a single terminal carboxyl group. It is capable of forming lipophilic, quasi-cyclic neutral complexes with alkali metal ions particularly Na^+ at pH above its pKa of 4.5 (Sun, 2014). During complexation, the molecule orients its polar groups around the cation, thereby making the inner surface of the complex hydrophilic and its outer surface hydrophobic. The positive

charge of the engulfed cation and the negative charge of the deprotonated carboxyl are internally compensated, making the cation-monensin complexes electrically neutral (Pressman, 1976). The lipophilic nature of monensin is also apparent from its low solubility limits in water (5-63 mg L^{-1}) and high octanol-to-water partition constants (log Kow = 2.8 to 4.2) (Hansen et al., 2009c).Despite monensin's potential environmental impact, little is known about how it interacts with the soil. Previous work has demonstrated the capacity of monensin to sorb to soils, with partitioning coefficients varying from 1 to 101 L kg^{-1} of soil (Sassman and Lee, 2007; Hussain and Prasher, 2011; Ilhan et al., 2012). Soil organic matter is likely an important factor influencing sorption of monensin to soil (Sassman and Lee, 2007; Hussain and Prasher, 2011) because of monensin's hydrophobic properties. However, several of the soils used in previous studies were dominated by 2:1 clay minerals, which have high cation exchange capacities and which, through cation bridging, could sorb greater soil organic matter. Sorbed soil organic matter could lead to sorption of monensin through hydrophobic interaction. Currently, no information is available on the impact of repeated application of animal manure on the capacity of litter-amended soils to retain monensin.

This study was conducted to evaluate the capacity of soil under long-term (17 yr) broiler litter application to sorb and desorb monensin. We hypothesized that litter-amended soils would retain greater monensin relative to unamended soils given the generally greater soil organic carbon of soils that have been subjected to years of manure amendments (Edmeades, 2003; Liu et al., 2010; Maillard and Angers, 2014). We chose a study site with highly weathered soils, dominated by 1:1 clays as these soils underlie

many agricultural grasslands along the Southern Piedmont in the Southeastern United States likely receiving broiler litter applications (West et al. 2008). To test our hypothesis, we conducted batch sorption experiments of monensin on the amended and control soils as influenced by added monensin concentrations, solution pH, and temperature. We also evaluated the capacity of the two soils to desorb monensin as a function of reaction time. To the best of our knowledge, this was the first study on sorption of monensin conducted on soils under long-term litter application and the first to examine desorption of monensin from soils.

MATERIALS AND METHODS

Soil Sampling and Soil Characterization

Surface soils (0 to 10 cm) were collected from a long-term agricultural research plot that had been amended annually with broiler litter for 17 yr (total of 116 Mg ha^{-1} litter amendment) and from a paired plot on similar soil that had not received any broiler litter over the same period (control soil). These plots are located at the Eatonton Beef Research Unit of the University of Georgia (33° 24' N, 83° 29' W, elevation 150 m). Both soils are classified as Cecil series (fine, kaolinitic, thermic Typic Kanhapludults).

Soil samples collected were analyzed for soil pH in 1:5 soil to water ratio, clay content using the pipette method (Gee and Bauder, 1986), total carbon using a C analyzer (Carlo Erba NA1500, Carlo Erba, Milan, Italy), specific surface area using a BET surface area analyzer (Gemini 2375, Micromeritics Instrument Corp., Norcross, GA), Fe and Al contents analyzed at the ALS Minerals Laboratory (North Vancouver, British Columbia)

using inductively coupled plasma – atomic emission spectroscopy, and dominant clay minerals using x-ray diffractometer (Diffraktometer D8, BrukerAXS, Karlsruhe, Germany). Measurements were also conducted for each soil's point of zero net charge (PZNC) by first preparing a soil paste saturated with LiCl and subjecting the LiCl-saturated soils to a series of solutions consisting of a wide range of pH at a fixed ionic strength (10 mmol L^{-1} LiCl) using a discontinuous titration procedure patterned, in part, after Chorover et al. (2004). After 3 h of equilibrating in these solutions, the soils were centrifuged and the supernatants were measured for Li, Cl and equilibrium pH. Then, the soils were resuspended twice in 1 mol L^{-1} NH_4NO_3, centrifuged and the supernatants were collected. Accumulated supernatants from the NH_4NO_3 extraction were analyzed for Li concentration using an atomic absoption spectrometer (AAanalyst 200, PerkinElmer Inc., Waltham, MA) and Cl concentration using a chloridometer (Labconco Corporation, Kansas City, MO). Retained Li and Cl were determined by subtracting the mass of Li and Cl in the entrained LiCl solution from that of the NH_4NO_3 extract. Retained Li served as a measure for the soil negative charge whereas the Cl served as a measure for soil positive charge. The soil net charge was calculated by subtracting the positive charge from the negative charge for each of the corresponding soils. The PZNC was obtained by fitting an equation to the net negative charge distribution across pH for each soil and solving for the x-intercept or the pH value (PZNC) when the net negative charge of the soil (the y variable) was equal to zero. Procedures for the preparation of the soil paste and determination of PZNC are outlined in Appendix D.

Sorption Isotherm

Stock solutions of sodium monensin (NaMON) (Sigma-Aldrich Co., USA) prepared in MeOH were added to a sorption solution matrix (0.01 mol kg^{-1} NaCl background electrolyte) in order to achieve a series of NaMON concentrations ranging from 0.18 to 1.81 μmol L^{-1}. Each of the NaMON-containing solution was added to each soil (0.15 g soil) in two replicates at 1:10 soil to solution ratio inside a 2-mL siliconized microcentrifuge tube. Blanks were included for each corresponding NaMON concentration. The blanks consisted of similar microcentrifuge tubes without soil but containing the same solution composition as the soil-containing tubes (referred to as sample tubes), consisting of aqueous soil extract to model the dissolved organic matter in the solution. The solution matrices used in the blank tubes were obtained by adding NaMON-free 0.01 mol kg^{-1} NaCl solution to each soil (1:10 soil to solution ratio), shaking the mixture for 2 hr, centrifuging the tubes (14,000 rpm for 30 min; 9.5 cm maximum centrifuging radius) and collecting the supernatants. The supernatants were the solution matrices used for the blank tubes for each respective soil. The blank solution matrices were collected on the same day as the actual sorption experiment was conducted.

In preliminary experiments, no difference in NaMON concentrations were observed between 2 and 72 h of equilibration, nor between sterile and unsterile incubations (Supplemental Fig. 5.S1). Hence, all of the succeeding sorption experiments were reacted for 2 h under unsterile conditions. After 2 h of shaking, the sample tubes were centrifuged (at 14,000 rpm for 30 min; 9.5 cm maximum centrifuging radius) and

sampled for clear supernatants. Five hundred milliliters of each supernatant were added to the same volume of acetonitrile in a 2-mL HPLC vial. The mixture was placed in 10 mmol L^{-1} $NaHCO_3$, with an appropriate volume of 0.5% formic acid to bring the pH to 7; added with 0.13 μmol L^{-1} of nigericin as an internal standard. Calibration standards for each soil were prepared using the corresponding matrices of solutions used for each soil's corresponding blanks. The pH 7-adjusted blanks and supernatants were transferred to HPLC vials and analyzed for NaMON on a liquid chromatography mass spectrometry (LCMS) (Shimadzu, Kyoto, Japan) using a normal-phase column (2.1 mm x 150 mm, 3 μm Atlantis HILIC Silica column, Waters Corp., Milford, MA) heated at 40°C. The mobile phase consisted of A) 0.1% formic acid in acetonitrile and B) 0.1% formic acid in ultrapure water. The mobile phase was kept at an isocratic flow of 0.2 mL min^{-1} with mobile phases A and B constituting 65 and 35% of the total flow, respectively. Each sample injection was 20 μL. The mass spectrometer had an electrospray interface and a nebulizer gas, consisting of nitrogen flowing thru at 4 L min^{-1}. Sodium monensin was quantified under selected ion monitoring mode by relating the known NaMON concentration of the calibration standards versus the ratio of the peak areas of the molecular ion (m/z) of NaMON (m/z of 693) and that of nigericin internal standard (m/z 747).

$$q = \frac{[C_{blank} - C_{soil}]V_{soln}}{M_{soil}} \qquad \text{Eq. [5.1]}$$

Sorbed monensin was calculated by using Eq. 5.1, where q is the concentration of NaMON sorbed in μmol (kg soil)$^{-1}$, C_{blank} and C_{soil} are the equilibrium concentrations of

NaMON in solution (determined after 2 h of equilibration) in μmol L^{-1} in the matrix-matched blank and the soil-containing tubes, respectively, V_{soln} is sorption solution volume (0.0015 L), and M_{soil} is the mass of soil used (0.00015 kg). Sorption of monensin was modeled using linear sorption partitioning, $q = K_d C$ where q and C are as described previously and K_d is the monensin sorption partition coefficient in L kg^{-1} soil. The K_d was estimated by fitting a linear regression between the variables q and C using PROC REG in SAS 9.3 (SAS Inst. Inc., Cary, NC).

Effect of pH on monensin sorption

These studies were done with the same setup mentioned previously, with the exception that 0.1 mol L^{-1} ionic strength was used. Prior to mixing with the soils, the solution matrices were adjusted for their H^+ or OH^- concentrations such that the solution pH of the soil-containing tubes would vary between pH 4 to 9 after the 2-h equilibration. Reaction tubes for the blanks consisted of the same matrix-matched sorption solutions obtained from each corresponding soil after 2-hr equilibration shortly before the actual experiment started. This was done to ensure the same solution pH and composition and to account for any hydrolytic degradation of monensin between the blank and the sample tubes. After the 2-h equilibration, the blanks and the sample tubes were processed and analyzed for NaMON and the amount of sorbed NaMON was calculated using Eq. 5.1.

To measure dissolved organic carbon (DOC) released in solution, the setup for some of the sample tubes (at pH 4, 6 and 8) was repeated on a larger scale (2 g soil:20 mL of monensin-free sorption solutions) for each of the two soils. This was done in order

to collect enough sample for DOC analysis. After shaking for 2 h, the solutions were first measured for pH and afterwards filtered thru 0.45-μm filter membrane (Pall Life Sciences, Ann Arbor, MI). Approximately 5 mL of the sample was analyzed for total C through the combustion-infrared method (American Public Health Association, 1992) using a total C analyzer (TOC-5050, Shimadzu Corporation, Kyoto, Japan). Dissolved organic carbon was obtained by subtracting the inorganic carbon from the total carbon. Inorganic carbon was separately measured in the same instrument by injecting the sample to its inorganic carbon reaction vessel where the sample is decomposed into carbon dioxide under acid condition. The converted carbon dioxide is detected by the same non-dispersive infrared method used to detect combusted carbon from organic carbon.

Effect of temperature on monensin sorption

In addition to the isotherm experiment conducted for the two soils at pH 6 under ambient temperature (25°C), the same isotherm procedures were repeated at 15 and 35°C for the two soils. For these sorption experiments conducted at 15 and 35°C, the blank and sample tubes were first allowed to equilibrate for 1 h at these temperatures inside the incubator, and then the samples were shaken for 2 h. After shaking, the blanks and the sample tubes were processed and analyzed for NaMON and the amount of sorbed NaMON was calculated (Eq. 5.1).

Effect of reaction time on monensin desorption

In this experiment, desorption of monensin was evaluated after the following reaction times: a) 0 wk (i.e, 2 h), b) 1 wk, or c) 2 wk of monensin contact with the soils under aqueous condition. As before, 0.15 g soil was placed inside the 2-mL siliconized microcentrifuge tubes. To each soil, monensin was added at a concentration of 0.58 μmol L^{-1} in 1.5 mL of 0.01 mol kg^{-1} background NaCl solution containing 50 mg L^{-1} $HgCl_2$ as an antimicrobial agent. The added NaCl solution was initially adjusted such that the final or equilibrium solutions containing both soils would have a pH of 6 after the 2-h equilibrium. Each treatment (i.e., reaction time) consisted of three replicates of sample and blank tubes. When the designated reaction time was up, the samples tubes were centrifuged (at 14,000 rpm for 30 min) and sampled for 1 mL of clear supernatant. Half of the supernatant was measured for pH and the other half was processed for analysis of monensin using the same procedures described above. The same measurements were also made with the corresponding blanks. Next, the 1 mL of NaMON-free 0.01 mol kg^{-1} NaCl solution was added to the sample tubes, vortexed for 30 s and shaken again for 30 min. The cycle of steps of centrifugation, sampling and addition of NaMON-free NaCl solution was repeated two more times. In total, three desorption steps were performed on each sample tube. Desorbed amounts of NaMON were calculated by first considering the dilution of the residual NaMON in the sample tubes (initial desorption solution) after addition of the NaMON-free NaCl desorbing solution at each desorption step and by taking the difference of the NaMON concentrations between the initial and final desorption solutions at each desorption step. The quantity of desorbed NaMON was

calculated by adding NaMON released from the three desorption steps and expressing it as percent of monensin initially sorbed at each reaction time.

Statistical Analysis

Statistical comparisons were performed between K_d's obtained for each soil using PROC GLM. These comparisons were done between soils for isotherm experiments conducted at their natural pH, at an adjusted pH of 6 under ambient temperature (25°C) as well as at 15 and 35°C. For the effect of temperature, K_d comparisons were also made across temperatures for each soil. For the effect of pH on monensin sorption, the logarithm of the sorbed to solution ratios across pH was also compared between the two soils using PROC GLM with a significance level of 0.05.

Cumulative amounts of NaMON desorbed were analyzed using a two-way ANOVA, accounting for differences between two soils, the three reaction times, and the combined effect (i.e., interaction) of the soil and the reaction time. Our statistical analysis showed no significant combined effect (i.e. insignificant interaction, $p = 0.26$) between the soil and the reaction time prior to desorption. Hence, only the separate effects of the soil and the reaction time were presented. Sorbed amounts of monensin sorbed at 0, 1 or 2 wk of reaction time were statistically analyzed using PROC GLM.

RESULTS AND DISCUSSION

Soil Properties

The control and broiler litter-amended soils had very similar mineralogical compositions, making the two soils suitable for this study (Supplemental Fig. 5.S2). Both soils are predominantly composed of kaolinite with small amounts of hydro-interlayered vermiculite, goethite and hematite (Supplemental Fig. 5.S2). The broiler litter-amended soil had 68% greater clay contents, 27% higher specific surface area, 25% greater total carbon, and approximately 1 unit higher pH and PZNC relative to the control soil (Table 5.1). Increased total carbon for the broiler litter-amended soil closely agrees with the 26% increase in soil organic carbon for soils that have been fertilized with animal manure relative to soils fertilized with inorganic fertilizers for at least 20 yr based on a meta-analysis from 22 experimental sites throughout the world (Maillard and Angers, 2014). Increased soil organic carbon could be due to a combination of direct input of C from the manure itself and to indirect input from increased net primary production in above- and below-ground crop residues in the manured soils relative to inorganically fertilized soils (Bhogal et al., 2009; Bhattacharyya et al., 2010; Maillard and Angers, 2014). The higher soil pH is also expected following long-term application of broiler litter due to the presence of $CaCO_3$ in the litter (originating from broiler feeds) (Moore and Edwards, 2005; Gaskin et al., 2013).

Total elemental analysis revealed greater Fe and Al contents in broiler litter-amended soil than in the control soil (Table 5.1). This could be due to inherent Fe and Al contents between the two soils. Unintentional scraping of soils in broiler houses during

litter collection could not explain the greater Fe and Al contents in broiler litter-amended soil. Based on our calculation, Al and Fe contents derived from accidental soil scraping could account for only less than 2% Al and 0.3% Fe of the total Al and Fe content of broiler litter-amended soil (see Appendix E). Greater inherent Al and Fe contents explains the higher PZNC measured for the broiler litter-amended soils as Fe and Al oxide minerals have PZNC values >pH 7 whereas those of silicate minerals or organic matter are <pH 4 (Sposito, 2008).

Sorption Isotherm

Sorption isotherms of monensin are shown in Fig. 5.1a for the control and the broiler litter-amended soils with their corresponding Kd values in Table 5.2. Sorption of monensin on the two soils followed a generally linear pattern with Kd values that are generally lower compared to reported Kds (linear or close to linear estimations) for other soils in the literature (Sassman and Lee, 2007). At its natural pH, the broiler litter-amended soil had significantly lower Kd compared to the control soil (Fig. 5.1a, $p < 0.01$). The lesser affinity of broiler litter-amended soil for monensin could not be explained by variation in clay fractions, or by total carbon contents as we previously hypothesized or by differences in specific surface area as shown by unequal Kd values between the two soils after normalizing each soil's Kd for the clay, total carbon and specific surface areas of each soil (Table 5.2). Soil pH is known to affect partitioning of organic acids. To test whether soil pH controlled the differences in monensin affinity of the two soils, we performed a sorption experiment that would bring the equilibrium pH of

the same soils as close as possible. Our analysis showed that the two soils did not differ in their Kd when their pHs were similar (p = 0.20) (Fig. 5.1b), confirming the role of pH in governing the magnitude of sorbed monensin to our test soils.

Effect of pH on monensin sorption

The ratio of sorbed to solution monensin (q/c) followed a negative linear relationship with pH (Fig. 5.2), without significant differences in this trend between the two soils (p = 0.55). In general, at very low pH (i.e., pH 4), sorbed monensin to solution ratios are approximately an order of magnitude greater compared to those at higher pH (i.e., pH > 7). This behavior is quite unexpected for monensin given its predominantly neutral existence (Fig. 5.3). Considering a measured pKa of 4.5 in aqueous solution (Sun, 2014), monensin would exist as a neutral, undissociated compound at pH below 4.5. Above pH 4.5, however, monensin would be expected to exist more as a neutral Na-complexed than as an uncomplexed dissociated anionic species considering our primarily Na-based background electrolyte solutions.

At higher pH, humic substances disperse in solution (Sparks, 2003) due to the repulsion between negatively charged soil surfaces and the ionization of acidic functional groups of DOC such as the carboxyl groups. Dissolved organic carbon has been shown to sorb hydrophobic organic contaminants (Poerschmann and Kopinke, 2001; Pan et al., 2008; Ripszam et al., 2015).We suspect that the higher DOC concentrations at higher pH (Fig. 5.4) is responsible for the lower sorption of monensin. The largely neutral Na-complexed monensin had likely partitioned unto the hydrophobic sites of DOC (i.e.,

aromatic and aliphatic regions), such that at higher pH monensin was released as DOC-monensin complexes into solution.

Apart from the hydrophobic sites of soil organic matter, the siloxane surfaces of the alumino-silicate minerals (e.g., kaolinite) could potentially sorb monensin. However, even as the net charge of these minerals vary from neutral at their PZNCs to negative above their PZNCs, the hydrophobic siloxane sites remain accessible irrespective of pH. Given this consideration, partitioning to the siloxane region of kaolinite may occur and may even comprise a significant portion of the sorbed monensin, but it is unlikely to change as a function of pH and thus cannot explain pH variation of monensin sorption capacity in these soils.

The potential role of DOC on sorption of monensin in our soil experiments is the same mechanism likely affecting the concentration and load of monensin in our runoff experiments. Future studies recommended to understand the release of DOC from broiler litter-amended systems could provide information on limiting transport of DOC-bound ionophores.

Effect of temperature on monensin sorption

The sorption of monensin did not significantly differ with temperature between 15 and 35°C, regardless of soil used (Fig. 5.5 & Table 5.3; overall $p = 0.22$). Lack of significant effect of temperature on sorption was also reported for dissolved organic C and for some pesticides to soil and clay sorbents (Kaiser et al., 2001; Site, 2001). Insignificant effect of temperature on sorption indicates weak sorbate-surface interaction

due to small enthalpy of sorption, a thermodynamic parameter which relates to the sorption affinity of sorbate compounds to the sorbent (ten Hulscher and Cornelissen, 1996). Monensin partitioning neither differed between the two soils (overall $p = 0.22$) when averaged across the three temperatures.

Effect of prior reaction time on monensin desorption

The broiler litter-amended soil had 25% lower amounts of total desorbed monensin relative to the control soil ($p = 0.03$) when averaged across all weeks of reaction periods. Across all reaction periods, the broiler litter-amended soil also had significantly lower equilibrium pH compared to that of the control soil (broiler litter-amended soil pH 6.6 ± 0.2 vs control soil pH 6.9 ± 0.1, $p < 0.01$), despite the same preadjusted pH value (pH 6) of both soil solutions at the start of this experiment. Lower amount of desorbed monensin from broiler litter-amended soil is likely due to its lower equilibrium pH. Decreasing amounts of monensin sorbed with increasing pH were shown previously in Fig. 5.2 for both soils. However, we could not confirm the molecular mechanism responsible for the lower amount of monensin desorbed from broiler litter-amended soil over that of the control soil especially within such a narrow pH difference between the two. At the prevailing desorption pHs of both soils, monensin would exist as neutral Na-complexed species (Fig. 5.3). Apparently, the strength of retention of monensin was different for the two soils at these pHs and the retention mechanism was not governed by electrostatic attraction.

Accumulated amounts of desorbed monensin were smaller when the reaction time was 2 wk compared to 0 wk regardless of the soil used (Fig. 5.6). Decreased amounts desorbed after longer reaction periods have also been observed with other organic compounds (Xu et al., 2008). A commonly held hypothesis for this phenomenon is the slow diffusion of the compounds out of their sorption sites (Alexander, 2000). Increased sorption was not observed when monensin reacted with the soils up to 2 wk (overall ANOVA $p = 0.33$) and is therefore unlikely to explain decreased amounts of desorbed monensin over time.

CONCLUSION

Long-term surface application of broiler litter as fertilizer to pastures could influence the capacity of the soil to retain and release broiler litter-derived monensin. Our findings on a Cecil pasture soil showed significantly smaller sorption affinity for monensin with broiler litter-amended soil (i.e., soil that has been amended with broiler litter in the field for 17 yr) relative to the control soil, contrary to what we had previously hypothesized. Differences in sorption affinity, however, could not be explained by differences in specific surface area, clay, or carbon contents of the two soils but rather by their differences in native solution pHs. The role of solution pH in governing monensin sorption was confirmed by the similar inverse relationship for both soils between pH and the log of sorbed to solution ratio of monensin. Sorption isotherms conducted from 15 and 35^{o}C gave similar sorption capacities under the same preadjusted solution pH (pH 6) for both soils, further suggesting that pH was the dominating factor in controlling the test

soils' monensin sorption capacity. In terms of desorption, however, the broiler litter-amended soil desorbed lower amounts of monensin relative to the control soil, indicating differences between the two soils in the strength of monensin retention when averaged across all tested reaction periods (i.e., 2 h to 2 wk) prior to desorption. Regardless of the soil used, shorter reaction time (i.e., 2 h) of monensin with soil led to significantly greater amounts of desorbed monensin. Overall, our findings showed that mobility of monensin could be expected to be high in soils with increased pH and that a significant amount of this compound is likely to be desorbed from such soils when rainfall occurs after a short period of soil contact (i.e., rainfall immediately after litter application). Influence of long-term litter application cannot be ignored (particularly if it has already resulted to increased soil pH) in devising strategies for minimizing potential transport of potential ionophore contaminants in soils.

FUTURE WORK

Proposed sorption of monensin to DOC is recommended to be studied in the future. This may be conducted using a multiphase system (i.e. water, pre-coated glass fibers, and DOC) through solid-phase microextraction, a procedure that is popularly used for estimating partition coefficients of organic compounds to DOC. Dissolved organic carbon to be used may be obtained from the control and the broiler litter-amended soils. It is also suggested that the DOCs obtained from these two soils be characterized for the types of functional groups present and their relative abundance.

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TABLES AND FIGURES

Table 5.1. Selected properties of soil without litter amendment (control soil) and soil receiving long-term broiler litter amendment (BL soil) (116 Mg ha^{-1} in 17 yr). Errors are 1 SD.

Soil	Soil pH	Clay content	Specific surface area	Total carbon	Total Al	Total Fe	pznc
		g kg^{-1}	m^2 g^{-1}	--------- g kg^{-1} ---------			
Control soil	5.0±<0.01	209±41	11±0.2	24±0.8	41±4	30±2	2.8
BL soil	6.2±0.02	351±31	14±0.7	30±0.4	66±3	38±1	4.1

Table 5.2. Sorption isotherm pH, monensin sorption partitioning coefficient (Kd) values, and Kd values normalized by the clay contents, total carbon and specific surface area for the soil without litter amendment (control soil) and the soil receiving long-term broiler litter amendment (BL soil) (116 Mg ha^{-1} in 17 yr).

Soil	Isotherm pH	Linear Kd†	Clay-normalized Kd	TC-normalized Kd	SSA-normalized Kd
		L $(kg\ soil)^{-1}$	L $(kg\ clay)^{-1}$	L $(kg\ C)^{-1}$	L m^{-2}
Control soil	5.2±0.1	13±2	64±12	551±18	0.0012±<0.0001
BL soil	5.7±<0.1	7±1	20±2	237±3	0.0005±<0.0001

†SAS-Estimated linear Kd ± standard error. The rest of the ± values next to all the other parameters indicate 1 SD.

Table 5.3. Linear sorption partition of monensin (Kd) at different temperatures when averaged between the soil without litter amendment (control soil) and the soil receiving long-term broiler litter amendment (BL soil) (116 Mg ha^{-1} in 17 yr) and for each separate soil. Errors are 1 SD.

Temperature	Linear Kd
°C	L kg^{-1} soil
--------- Averaged Between Control and BL Soils ---------	
15	6.4 ± 1.1
25	7.3 ± 0.8
35	8.3 ± 3.2
--------- Control Soil ---------	
15	6.2 ± 1.9
25	8.0 ± 0.3
35	10.4 ± 3.4
--------- BL Soil ---------	
15	6.6 ± <0.1
25	6.6 ± 0.2
35	6.2 ± 1.0

Figure 5.1. Sorption isotherm of NaMON on soil without litter amendment (control soil) and on soil receiving long-term broiler litter amendment (BL soil) (116 Mg litter ha^{-1} in 17 yr) at their a) natural and b) adjusted sorption pHs.

Figure 5.2. Log of sorbed to solution ratio of monensin on soil without litter amendment (control soil) and on soil receiving long-term broiler litter amendment (BL soil) (116 Mg ha^{-1} in 17 yr) across pH.

Figure 5.3. Speciation of monensin across pH assuming existence of only three species based on measured pKa and MON-Na dissociation constant of Sun, 2014.

Figure 5.4. Dissolved organic carbon in sorption solutions of soil without litter amendment (control soil) and on soil receiving long-term broiler litter amendment (BL soil) (116 Mg ha^{-1} in 17 yr) at increasing pH above the soils' PZNC.

Figure 5.5. Sorption isotherm of monensin on soil without litter amendment (control soil) and on soil receiving long-term broiler litter amendment (BL soil) (116 Mg ha^{-1} in 17 yr) at different temperatures.

Figure 5.6. Accumulated percentage of initially sorbed monensin that desorbed after 0, 1 or 2 weeks of reaction time, averaged across soils.

Figure 5.1.

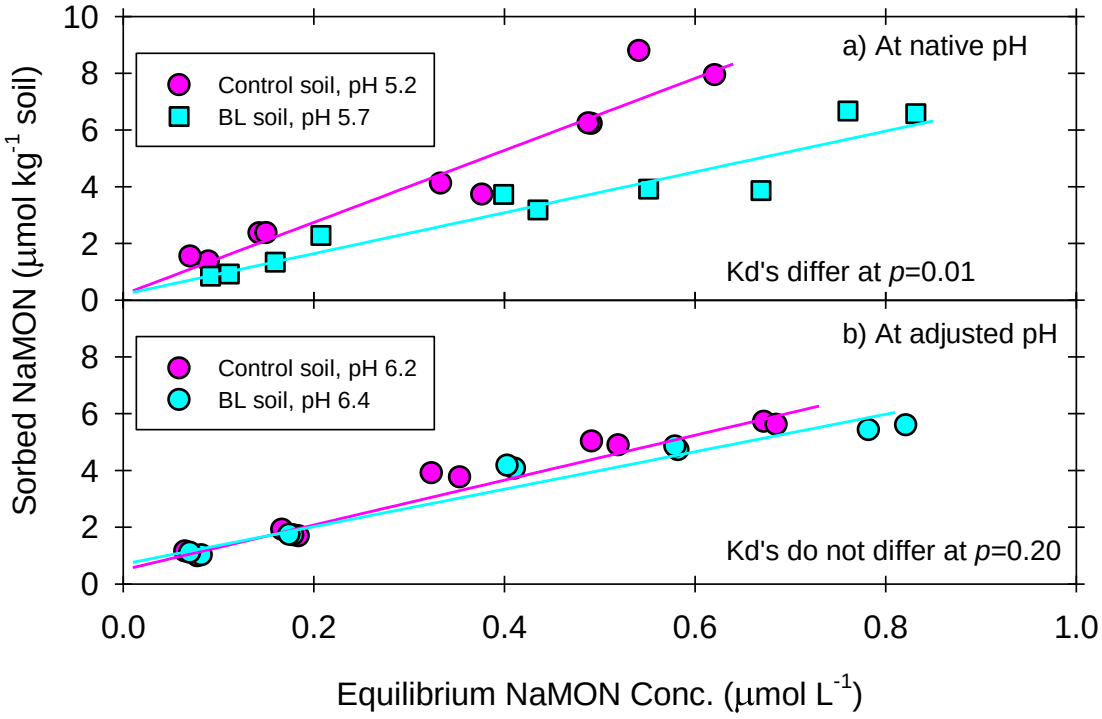

a) At native pH
Control soil, pH 5.2
BL soil, pH 5.7
Kd's differ at p=0.01
b) At adjusted pH
Control soil, pH 6.2
BL soil, pH 6.4
Kd's do not differ at p=0.20
Sorbed NaMON (µmol kg-1 soil)
Equilibrium NaMON Conc. (µmol L-1)
0
2
4
6
8
10
0.0
0.2
0.4
0.6
0.8
1.0

Figure 5.2.

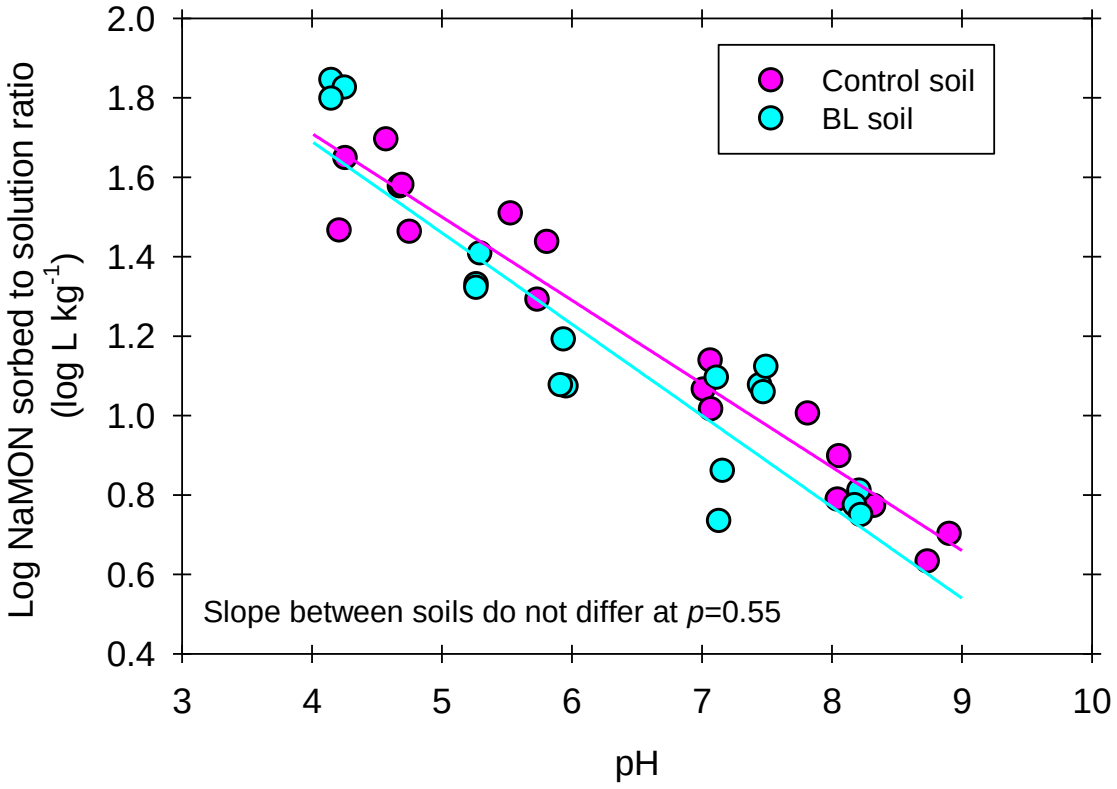

Log NaMON sorbed to solution ratio
(log L kg-1)
pH
Control soil
BL soil
Slope between soils do not differ at p=0.55
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
3
4
5
6
7
8
9
10

Figure 5.3.

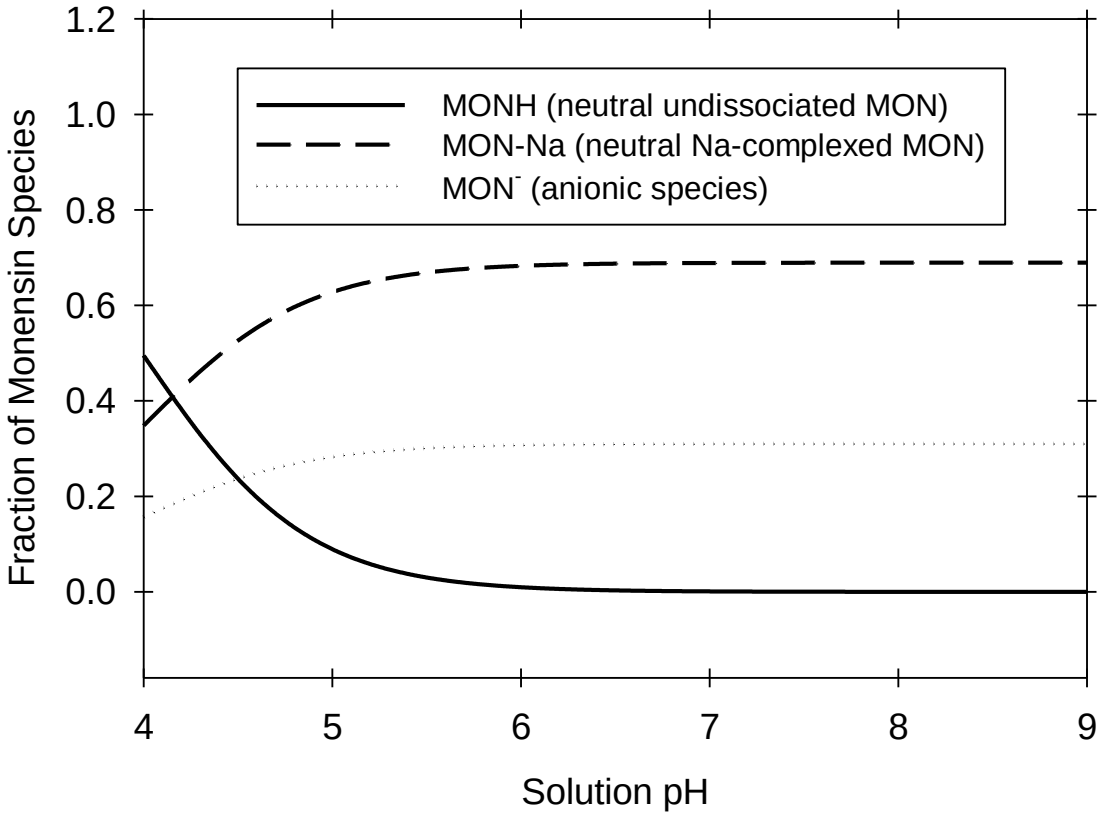

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fraction of Monensin Species
MONH (neutral undissociated MON)
MON-Na (neutral Na-complexed MON)
MON- (anionic species)
4
5
6
7
8
9
Solution pH

Figure 5.4.

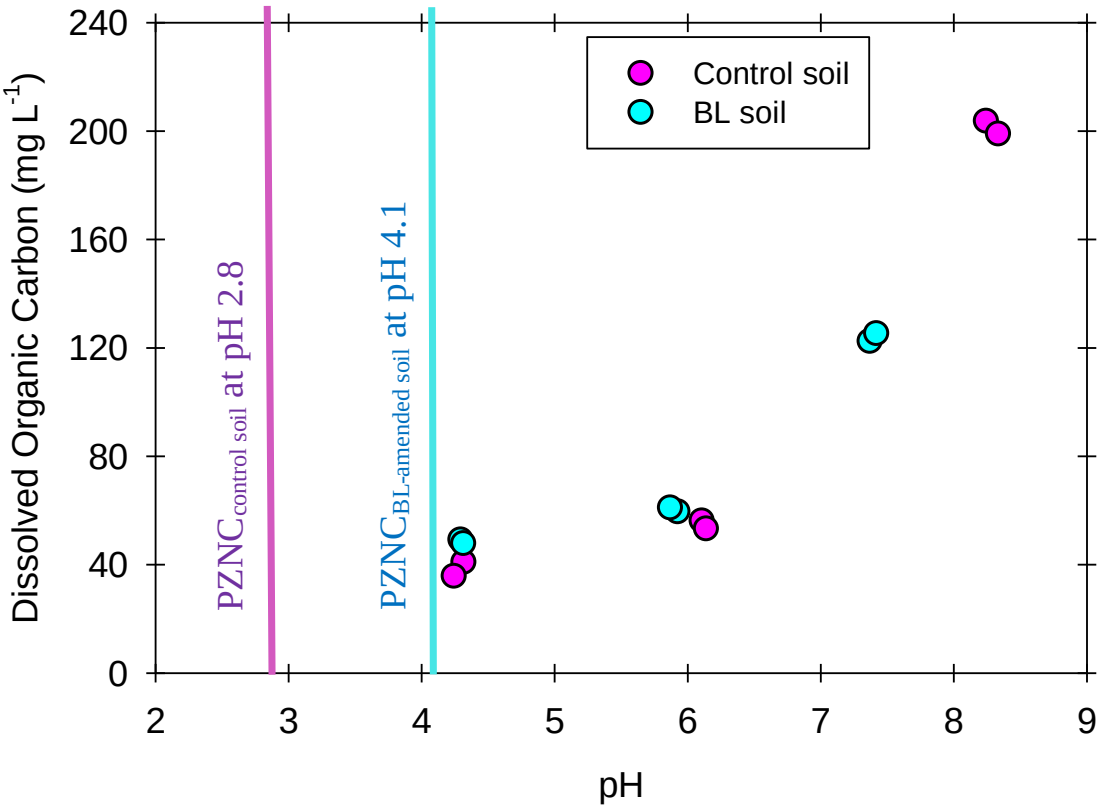

Dissolved Organic Carbon (mg L-1)
240
200
160
120
80
40
0
2
3
4
5
6
7
8
9
pH
Control soil
BL soil
PZNCcontrol soil at pH 2.8
PZNCBL-amended soil at pH 4.1

Figure 5.5.

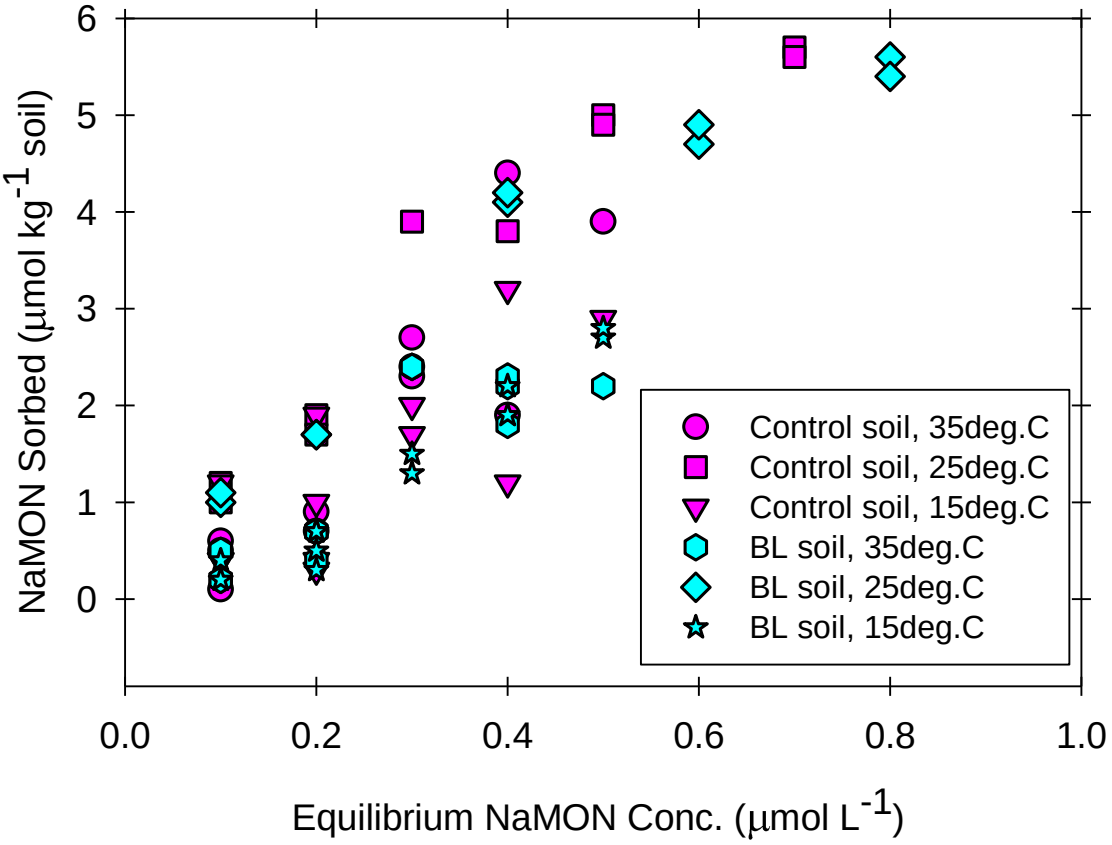
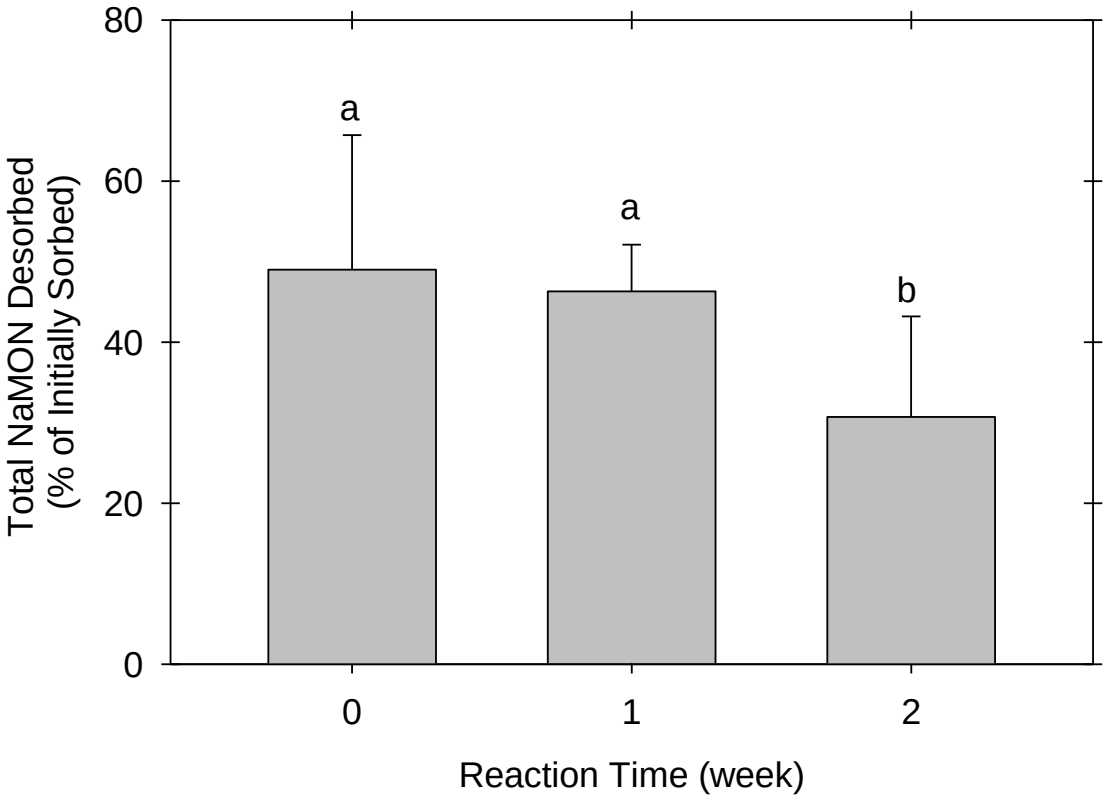

NaMON Sorbed (µmol kg-1 soil)
Equilibrium NaMON Conc. (µmol L-1)
0
1
2
3
4
5
6
0.0
0.2
0.4
0.6
0.8
1.0
Control soil, 35deg.C
Control soil, 25deg.C
Control soil, 15deg.C
BL soil, 35deg.C
BL soil, 25deg.C
BL soil, 15deg.C

Figure 5.6.

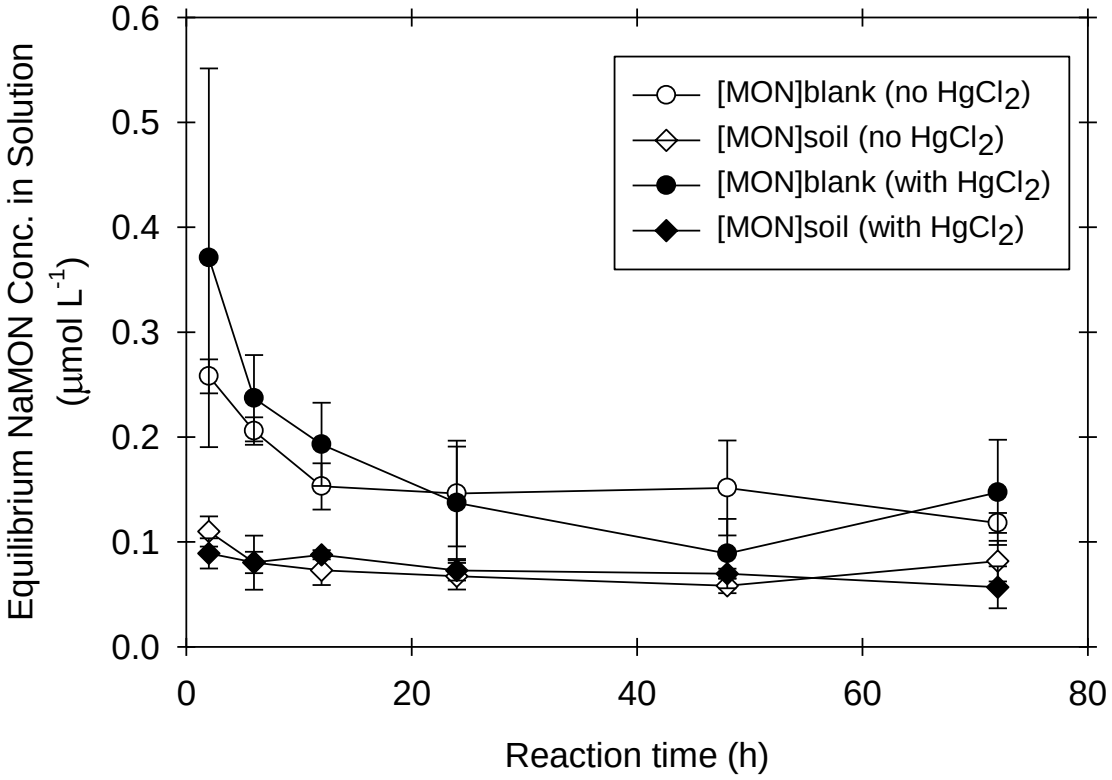

80
60
40
20
0
Total NaMON Desorbed
(% of Initially Sorbed)
a
a
b
0
1
2
Reaction Time (week)

SUPPLEMENTAL FIGURE

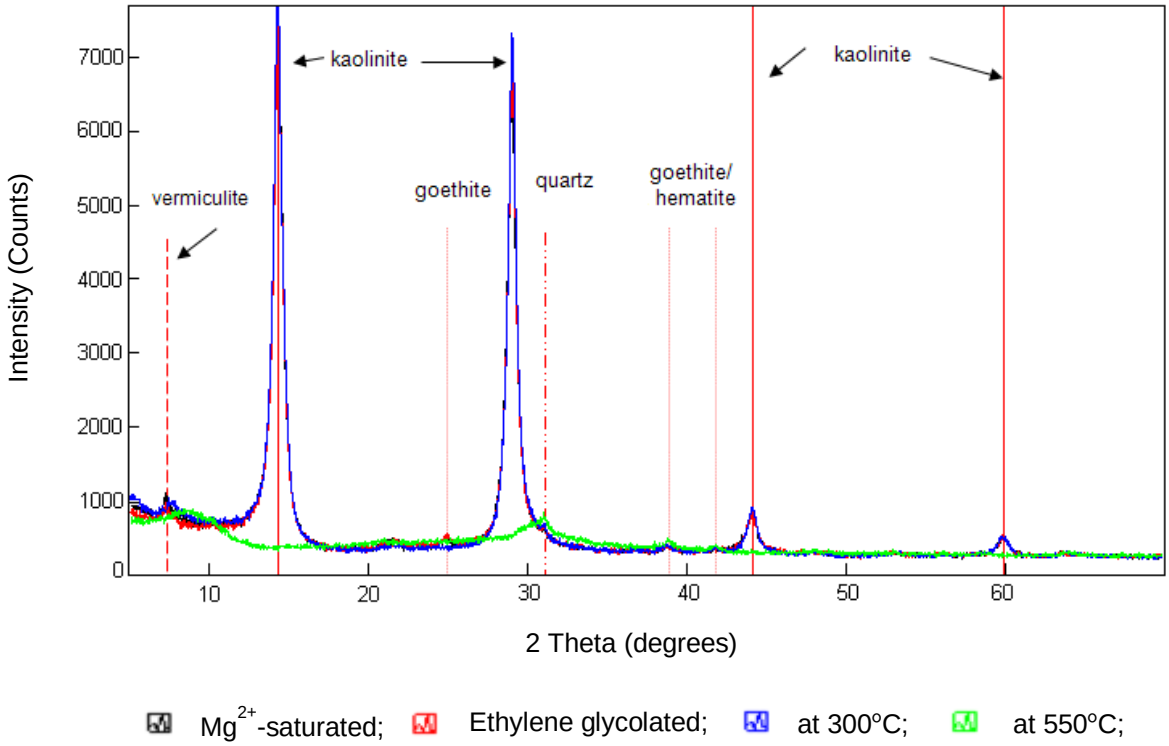
Supplemental Figure 5.S1. Sorption kinetics of sodium monensin on the soil without litter amendment (or control soil) with or without $HgCl_2$ as antimicrobial agent. Errors are 1 SD.

Supplemental Figure 5.S2. X-ray diffraction data on the qualitative determination of the mineral composition of the control and the broiler litter-amended soils. Individual data is not shown due to very similar spectra for each soil.

Supplemental Fig. 5.S1.



Supplemental Fig. 5.S2.



CHAPTER 6

CONCLUSION

Surface application of broiler litter as fertilizer to pastures could introduce polyether ionophores to the environment. Evaluating ionophore occurrence under typical litter management and examining the impact of long-term litter application on ionophore retention of pasture soils could provide important considerations for devising management strategies that could prevent or reduce ionophore contamination of nearby aquatic ecosystems.

On Chapter 3, the simulated rainfall experiment presented aimed to test the effect of aluminum sulfate (alum) addition to broiler litter and the effect of applying broiler litter during longer dry periods on the occurrence of ionophores in surface runoff. Runoff from alum-amended broiler litter (20% w/w) had 33% lower monensin concentration, 57% lower monensin load, 48% lower salinomycin concentration, and 66% lower salinomycin load compared to runoff from unamended broiler litter. Rainfall occurring at 2 or 4 weeks after litter application decreased monensin concentration by 47 and 67%, respectively, and reduced salinomycin concentration by 71%. These two methods can be explored as potential management practices for decreasing the presence of polyether ionophores in surface runoff.

On Chapter 4, the litter stacking study reported aimed to determine the change in ionophore concentrations in broiler litter when stacked with or without alum. Under

representative litter storage conditions, stacking broiler litter for 16 wk (or 112 d) slowly reduced salinomycin concentration by 55% but did not cause any significant change with monensin concentration. Adding alum to broiler litter reduced concentrations of monensin by 20%, but not those of salinomycin. Overall, this work suggests polyether ionophores could persist for long periods during litter stacking regardless of whether the litter is amended with alum or not, with monensin expected to persist longer than salinomycin.

On Chapter 5, sorption and desorption experiments aimed to assess the capacity of Cecil soils under long-term broiler litter application to sorb monensin as influenced by factors such as pH and temperature, and to evaluate the capacity of the soil to desorb monensin as a function of reaction time. There was a smaller sorption affinity for monensin with broiler litter-amended soil (i.e., soil that has been amended with broiler litter in the field for 17 yr) relative to a control soil without broiler litter amendment. This difference was mainly due to the higher pH of the broiler litter-amended soil (5.7 vs 5.2). In the range of 15 to 35^{o}C, temperature did not affect monension sorption, and a reaction time of 2 h led to greater amounts of desorbed monension than a reaction time of 2 wk. These results indicate that mobility of monensin might be expected to be high in soils with increased pH (i.e., in naturally alkaline, intentionally or consequently limed soils) and that a significant amount of this ionophore is likely to be desorbed from these soils when rainfall occurs after a short period of soil contact (i.e., rainfall immediately after litter application). The native monensin retention capacity of these soils would need to be

augmented with management strategies that would help decrease potential movement of monensin from these systems.

Overall, based on the contents of Chapters 3 to 5, a conceptual model is presented in Fig. 6.1 on the impact of moisture and pH as the primary factors that appear to exert the greatest influence on the fate of polyether ionophores in stored animal manure, in surface-applied manure, and in soil. Under dry moisture condition of stored animal manure, polyether ionophores would persist or degrade slowly (either through microbial degradation or acid-catalyzed hydrolysis), regardless of whether the manure has an acid or alkaline pH. This is supported by the findings in Chapter 4 wherein monensin had persisted and salinomycin had degraded slowly (estimated salinomycin half-life of >100 days) in both dry acidic alum-amended and dry alkaline unamended litter. The same findings also support the idea that sorption of polyether ionophores is also not likely important under dry storage condition, regardless of the laboratory-measured pH of the manure. This claim is further supported by the lower monensin concentration (20%) in acidic alum-amended litter than in alkaline unamended litter and by the lack of difference for salinomycin concentrations between the two litter treatments in Chapter 4.

When dry manure is spread on the surface of the soil, the ionophores would not be expected to degrade prior to the occurrence of rainfall, again regardless of the pH of the manure. This is based on our discussion in Chapter 3 on the likely lack of natural degradation of monensin and salinomycin in both surface-applied alum-amended and unamended litter for up to one month under dry condition before application of simulated rainfall.

In contrast, under wet or optimal moisture and at neutral to alkaline pH, polyether ionophores in stored manure are expected to biodegrade at a rate that is relatively faster than would be expected from dry manure. Under such moisture and pH conditions, the same would be expected for ionophores in surface-applied manure (such as may occur after a rainfall) as well as in soil (such as may occur when the ionophores are released to the soil during or after rainfall). This concept is based on the decreased sorption of polyether ionophores at high than at low pH, assuming the trend that we saw in Chapter 5 also holds true for wet manure sorbents. Less sorption would keep the compounds in solution, making them easily accessible to microbial degradation. However, optimum moisture levels may vary for different kinds of manure and/or for different ionophore compounds.

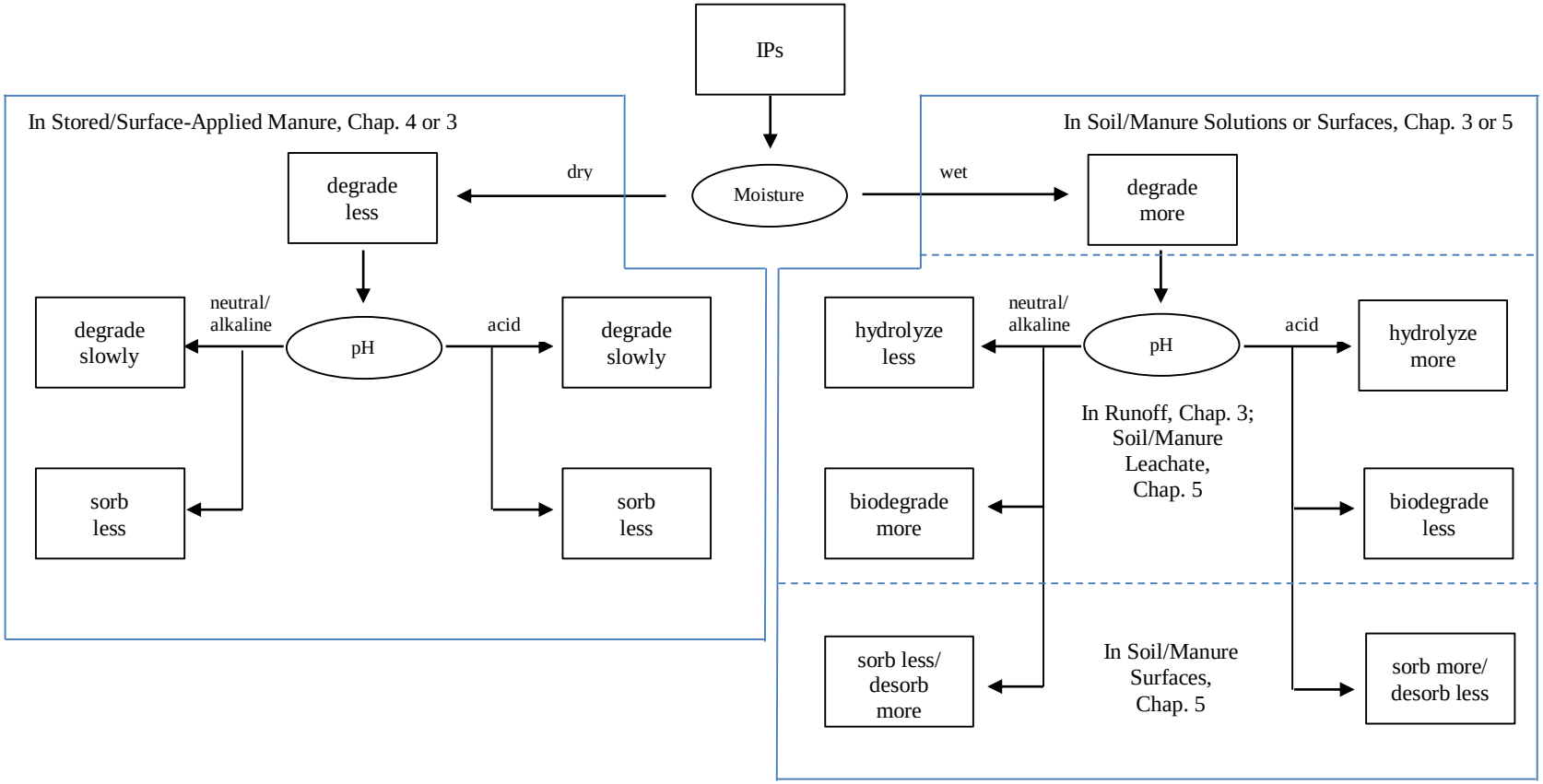
Under wet acid condition, two contrasting processes could occur. On the one hand, some of the ionophores would sorb to the surfaces of stored or surface-applied manure or of the soil, assuming the same inverse effect of pH on ionophore sorption (Chapter 5) applies to all these surfaces. On the other hand, ionophores remaning in solution (i.e., manure or soil leachate or in surface runoff) could abiotically hydrolyze over the long-run. For practical applications, storing of manure or subjecting surface-applied manure over extended periods (i.e., several weeks) under wet acid condition could hydrolyze some fractions of the ionophores but could likely not abiotically degrade all of them as some could partition to the manure surfaces.

Ultimately, when the ionophores are released to the soil, keeping the soil under wet acid condition is expected to decrease the mobility of ionophores from the soils due

to combined effect of abiotic hydrolysis of ionophores in soil solution in the long-run and enhanced ionophore sorption to soil at low pH. However, most/many agricultural soils are intentionally limed to near-neutral pH for improved crop production while some soils are inherently alkaline. Some are also limed consequently from repeated application of some lime-containing manure (such as would occur after long-term surface application of broiler litter). Given these considerations, keeping the soils at low pH may not be a practical option for decreasing the mobility of ionophores from manure-amended soils. Taking this issue into account, decreasing ionophore transport in the environment may be better addressed by finding the best moisture and pH conditions for ionophore degradation in manure prior to manure application to agricultural soils. This concept is therefore recommended for future study. A field-based mass balance study (coupled with laboratory-based experiments on the sorption capacity of different manure for different ionophores) is further recommended to provide information on the actual distribution of these compounds both for surface-applied and incorporated manure-amended systems. Understanding DOC release from these systems would also be important in developing the means to limit transport of potentially DOC-bound ionophores.

Figure 6.1. General conceptual model on the role of moisture and pH on the fate of polyether ionophores (IPs) in manure-amended soils.

Figure 6.1


IPs
In Stored/Surface-Applied Manure, Chap. 4 or 3
In Soil/Manure Solutions or Surfaces, Chap. 3 or 5
dry
Moisture
wet
degrade less
degrade more
neutral/ alkaline
pH
acid
degrade slowly
degrade slowly
hydrolyze less
hydrolyze more
sorb less
sorb less
biodegrade more
biodegrade less
In Runoff, Chap. 3; Soil/Manure Leachate, Chap. 5
sorb less/ desorb more
sorb more/ desorb less
In Soil/Manure Surfaces, Chap. 5

APPENDICES

APPENDIX A. THEORETICAL ESTIMATION OF THE TIME REQUIRED FOR SALINOMYCIN CONCENTRATION TO DECREASE FROM 2.1 μg L^{-1} IN RUNOFF FROM PLOTS RECEIVING UNAMENDED LITTER TO 1.1 μg L^{-1} IN RUNOFF FROM PLOTS RECEIVING ALUM-AMENDED LITTER IF SUCH DECREASE OCCURRED THROUGH ABIOTIC HYDROLYSIS AT pH 5.8 IN THE LATTER.

The first order kinetic model of Sun et al. (2013) for salinomycin hydrolysis is,

$$\frac{d[SAL]}{dt} = -132.8h^{-1}(H^+)^{0.77}[SAL]$$

Its analytical solution would be

$$[SAL]_t = [SAL]_0 e^{-132.8(H^+)^{0.77}t}$$

To solve for t in days,

$$t = \ln\frac{[SAL]_t}{[SAL]_0} \times \frac{1}{-132.8h^{-1}(H^+)^{0.77}} \times \frac{1d}{24h}$$

$$t = \ln\frac{[1.1\mu gL^{-1}]}{[2.1\mu gL^{-1}]} \times \frac{1}{-132.8h^{-1}(10^{-5.8})^{0.77}} \times \frac{1d}{24h}$$

$$t = 6d$$

APPENDIX B. THEORETICAL ESTIMATION OF THE TIME REQUIRED FOR MONENSIN CONCENTRATION TO DECREASE FROM 1.20 µg L^{-1} IN RUNOFF FROM PLOTS RECEIVING UNAMENDED LITTER TO 0.91 µg L^{-1} IN RUNOFF FROM PLOTS RECEIVING ALUM-AMENDED LITTER IF SUCH DECREASE OCCURRED THROUGH ABIOTIC HYDROLYSIS AT pH 5 IN THE LATTER.

The non-first order kinetic model of Sun et al. (2013) for monensin hydrolysis is

$$\frac{d[MON]}{dt} = -k_1[MON] + k_{-1}[MP_t]$$

where $[MP_t] = [MON]_0 - [MON]$

k_1 is the forward rate constant, $0.0029 h^{-1}$ at pH 5

k_{-1} is the backward rate constant, $0.0088 h^{-1}$ at pH 5

The analytical solution of Sun et al. (2014) for monensin hydrolysis is

$$[MON] = [MON]_0 \frac{k_1 e^{-(k_1 + k_{-1})t} + k_{-1}}{(k_1 + k_{-1})}$$

Solving for t in days,

$$t = \ln\left\{\frac{[MON](k_1 + k_{-1})}{[MON]_0} - k_{-1}\right\} - \ln k_1 \times \frac{1}{-(k_1 + k_{-1})} \times \frac{1d}{24h}$$

$$t = \ln\left\{\frac{[0.91\mu gL^{-1}](0.0029 + 0.0088)h^{-1}}{[1.2\mu gL^{-1}]} - 0.0088h^{-1}\right\} - \ln 0.0029h^{-1} \times \frac{1}{-(0.0029 + 0.0088)h^{-1}} \times \frac{1d}{24h}$$

$$t = 13d$$

APPENDIX C. THEORETICAL ESTIMATION FOR MONENSIN CONCENTRATION THAT WOULD PERSIST WITH TIME IN RUNOFF FROM PLOTS RECEIVING ALUM-AMENDED LITTER IF THE INITIAL MONENSIN CONCENTRATION IN RUNOFF WAS 1.2 µg L^{-1}; THE INITIAL MONENSIN CONCENTRATION WAS BASED ON THAT MEASURED IN RUNOFF FROM PLOTS RECEIVING UNAMENDED LITTER.

$$\lim t \to \infty = \left\{ [MON]_0 \frac{k_1 e^{-(k_1 + k_{-1})t} + k_{-1}}{(k_1 + k_{-1})} \right\}$$

$$\lim t \to \infty = \left\{ [1.2 \mu g L^{-1}] \frac{0.0029 h^{-1} e^{-(0.0029 + 0.0088)t} + 0.0088 h^{-1}}{(0.0029 + 0.0088) h^{-1}} \right\}$$

$$\lim t \to \infty = \left\{ [1.2 \mu g L^{-1}] \frac{0.0088 h^{-1}}{(0.0029 + 0.0088) h^{-1}} \right\}$$

$$\lim t \to \infty = 0.9 \mu g L^{-1}$$

APPENDIX D. PROCEDURE FOR PASTE PREPARATION AND PZNC DETERMINATION.

For soil paste preparation, 7 g of oven-dry weight-equivalent for each soil was suspended in 35 g of 0.5 mol kg^{-1} LiCl in 50-mL ocrij centrifuge tubes and shaken for 1 h. After shaking, the suspension was allowed to settle for 15 min and visible organic matter floating on top of the liquid was removed. Then, the tubes were centrifuged (11000 rpm for 15 min) and the clear supernatant was discarded. The soils in the tubes were added with 0.5 mol kg^{-1} LiCl. The 1-hr shaking, centrifugation and supernatant removal were repeated. Next, the soils were resuspended with 35 g of 0.1 mol kg^{-1} LiCl and shaken for 30 min, and the cycle of centrifugation and supernatant removal was repeated using the same concentration of LiCl. Then, the soils were resuspended in 35 g of 0.01 mol kg^{-1} LiCl, shaken for 30 min, centrifuged and the clear supernatant discarded. The remaining soil paste was transferred to a clean petri-dish and stored at 4°C until use.

For the discontinuous titration for each of the two soils, 60 mg fresh weight of soil paste (equivalent to 35 mg oven-dry soil weight) was transferred to each of the 13 pre-weighed centrifuge tubes. The thirteen tubes for each soil were added with the following solutions below in order to achieve a range of soil solution pH after equilibration.

Sample Tube	Acid or Base Conc. Used	Volume Added		
		Acid or Base	Background Electrol.†	Water
		---------- mL ----------		
1	0.005 M HCl	10.000	7.50	2.50
2	0.005 M HCl	6.000	8.50	5.50
3	0.005 M HCl	3.200	9.20	7.60
4	0.005 M HCl	1.600	9.60	8.80
5	0.005 M HCl	0.800	9.80	9.40
6	0.005 M HCl	0.200	9.95	9.85
7	None	0	10.00	10.00
8	0.005 M LiOH	0.200	9.95	9.85
9	0.005 M LiOH	0.800	9.80	9.40
10	0.005 M LiOH	1.600	9.60	8.80
11	0.005 M LiOH	3.200	9.20	7.60
12	0.005 M LiOH	6.000	8.50	5.50
13	0.005 M LiOH	10.000	7.50	2.50

†Background electrolyte concentration used was 0.02 mol L^{-1}. Computed final ionic strength for each solution in each tube was 10 m mol L^{-1}.

After the solutions were added, the suspensions were shaken on an end-over-end shaker for 3 h, centrifuged (15000 rpm for 20 min) and the clear supernatants were

removed for pH, Li and Cl measurements. After removing much of the clear supernatants, each centrifuge tube was weighed to determine the weight of the entrained LiCl solution. Next, the soils were resuspended and shaken in 30 ml of 1 mol L^{-1} NH_4NO_3 for 30 min, centrifuged and the supernatants were collected into HDPE bottles. The 1 mol L^{-1} NH_4NO_3 extraction was repeated and the supernatants from the two steps were combined and analyzed for Li using an atomic absorption spectrometer (AAanalyst 200, PerkinElmer Inc., Waltham, MA) and Cl using a chloridometer (Labconco Corporation, Kansas City, MO). Retained Li and Cl were determined by subtracting the mass of Li and Cl in the entrained LiCl solution from that of the NH_4NO_3 extract. Retained Li served as a measure for the soil negative charge whereas the Cl served as a measure for soil positive charge. The soil net negative charge was calculated by subtracting the positive charge from the negative charge for each of the corresponding soils. The PZNC was obtained by fitting an equation to the net negative charge distribution across pH for each soil and solving for the x-intercept or the pH value (PZNC) when the net negative charge of the soil (the y variable) was equal to zero.

APPENDIX E. CALCULATION ON THE CONTRIBUTION OF ALUMINUM AND IRON DERIVED FROM UNINTENTIONAL SCRAPING OF SOIL (DURING BROILER LITTER COLLECTION IN BROILER HOUSES) TO THE ALUMINUM AND IRON CONTENTS OF BROILER LITTER-AMENDED SOIL AFTER 17 YEARS OF BROILER LITTER APPLICATION.

Assumption: We assume that broiler litter applied to our test site include the accidentally scraped soil in it and that analyzed Al and Fe concentrations in broiler litter is a combined contribution from both the litter material and the accidentally scraped soil.

Cumulative broiler litter application in our study site

= 116 Mg ha^{-1} for 17 years = 116000 kg litter ha^{-1}

Given 1.87% Al and 0.17% Fe in alum-amended broiler litter from Moore (2011) (Note: We based our calculations on that of alum-amended due to its greater Al and Fe concentrations than unamended litter.),

Cumulative Amt. of $Al_{litter+scraped\ soil}$ = 116000 kg litter x $\frac{0.0187\ kg\ Al}{kg\ litter}$

=2169.2 kg $Al_{litter+scraped\ soil}$

Assume: BD = 1.5 g cm^3 = 1500 kg m^3
Soil volume at 10 cm depth over 1 ha = 10000 m^2 x 0.1 m = 1000 m^3 soil
Soil mass at 10 cm depth over 1 ha = $\frac{1500\ kg}{m^3}$ x 1000 m^3 = 1500000 kg soil

Concentration of Al in soil after 17 yr of litter application

$$= \frac{2169.2\ kg\ Al_{litter+scraped\ soil}}{1500000\ kg\ soil} = 1446\ ppm = \frac{1446\ mg\ Al_{litter+scraped\ soil}}{kg\ soil}$$

Percent $Al_{litter+scraped\ soil}$ of the Al_{BLsoil} concentration

$$= \frac{1446\ mg\ Al_{litter+scraped\ soil}\ kg^{-1}\ soil}{66{,}000\ mg\ Al_{BLsoil}\ kg^{-1}\ soil} \times 100 = 2\%$$

Hence, percent $Al_{scraped\ soil}$ of the Al_{BLsoil} concentration is < 2%.

Solution for solving for the contribution of $Fe_{scraped\ soil}$ to Fe_{BLsoil}:

$$\text{Cumulative Amt. of } Fe_{litter+scraped\ soil} = 116000\ kg\ litter \times \frac{0.0017\ kg\ Fe}{kg\ litter}$$

$$= 197.2\ kg\ Fe_{litter+scraped\ soil}$$

Assume: BD = 1.5 g cm^3 = 1500 kg m^3

Soil volume at 10 cm depth over 1 ha = 10000 m^2 x 0.1 m = 1000 m^3 soil
Soil mass at 10 cm depth over 1 ha = $\frac{1500\ kg}{m^3} \times 1000\ m^3 = 1500000$ kg soil

Concentration of Fe in soil after 17 yr of litter application

$$= \frac{197.2\ kg\ Al_{litter+scraped\ soil}}{1500000\ kg\ soil} = 131\ ppm = \frac{131\ mg\ Fe_{litter+scraped\ soil}}{kg\ soil}$$

Percent $Fe_{litter\text{-}derived}$ of the Fe_{soil} concentration

$$= \frac{131\ mg\ Fe_{litter+scraped\ soil}\ kg^{-1}\ soil}{38{,}000\ mg\ Fe_{BLsoil}\ kg^{-1}\ soil} \times 100 = 0.3\%$$

Hence, percent $Fe_{scraped\ soil}$ of the Fe_{BLsoil} concentration is <0.3%.

Note: For complete citation of Moore (2011), see Literature Cited section of Chapter 5.