

## ABSTRACT

Title of Dissertation: QUANTIFICATION OF IONOPHORE  
ANTIMICROBIALS ASSOCIATED WITH  
POULTRY LITTER AND THEIR DYNAMICS IN  
THE SOILS OF THE MID-ATLANTIC USA  
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Anticoccidants, biochemically known as ionophores are added to poultry feed for growth promotion, prophylactic and therapeutic purposes to better sorb nutrients and against coccidiosis caused by parasite *Eimeria sp.* Ionophores belong to the class of emerging contaminants, as they are not regularly monitored in the environment and not specifically treated in the effluents. Potentially, this can cause ionophores to enter into the environment freely. There is little information regarding the dynamics of ionophores in the environment. This has been related to the lack of reliable, sensitive and robust methods that can measure their trace levels from complex environmental matrices like soil, natural water and animal manure. Studies show ionophore toxicity exhibited in flora and fauna, even reported in humans above the dose of  $1 \text{ mg kg}^{-1}$ . Hence accumulation of ionophores in the environment can be detrimental. Our multi-scale investigation of ionophores involved, a) method development for trace analysis of ionophores in poultry manure using liquid chromatography triple quadrupole mass spectrometry (HPLC-MS/MS), b) batch equilibrium studies of ionophores using soils from mid-Atlantic region of the USA and c) influence of soil physico-chemical parameters on dynamics of ionophores in soil-water systems. Our HPLC-MS/MS

method was successful in quantifying ionophores ranging from  $19.19 \pm 6.6 \mu\text{g kg}^{-1}$  to  $97.86 \mu\text{g kg}^{-1} \pm 19.19 \mu\text{g kg}^{-1}$  with concentrations of monensin being the highest. This method was further used to investigate partitioning of monensin in soil-water systems relevant to the occurrence of ionophores in the natural environment. Sorption and desorption isotherms were developed and influence of soil physico-chemical parameters on the sorption-desorption processes were analyzed. C-type linear isotherms were generated with partition coefficients ranging from  $6.41 \text{ L Kg}^{-1} \pm 1.34$  to  $343.83 \text{ L Kg}^{-1} \pm 5.68 \text{ L Kg}^{-1}$ . Soil parameters such as cation exchange capacity, pH, organic matter, sand and silt content were found to correlate with sorption under different conditions. A major focus of this dissertation was to develop novel methodologies and design experiments to execute our research objectives.

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USA

By

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## **DEDICATION**

THIS THESIS IS DEDICATED TO MY GURU, MY MOTHER AND MY GRANDPARENTS, WHO ARE ALWAYS THERE WITH ME IN SPIRIT.

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# **CHAPTER 1. LITERATURE REVIEW: USE OF ANTIMICROBIALS IN ANIMAL REARING PRACTICES AND THEIR DYNAMICS IN THE ENVIRONMENT**

## **1.1. INTRODUCTION**

Veterinary antimicrobials are commonly used at sub-therapeutic or prophylactic and therapeutic levels for growth and development of various livestock animals, like cattle, poultry or swine. In USA, more than 11x10<sup>6</sup> Kg year<sup>-1</sup> of antimicrobials are used at sub-therapeutic levels (Hansen et al., 2009b; Mellon et al., 2001). Macrolides, ionophores and antibiotics like tetracycline are the most commonly used antimicrobials in poultry, dairy and swine production.

Animal manure is commonly used as fertilizer in agriculture throughout the world. This is also a way of reusing the animal waste which otherwise has to be disposed of or destroyed and doing so without impacting the environment might be a difficult and expensive task. Various countries have different recommended rates for manure application depending upon the available nutrient status of the soil, need of the crops, and the nutrient content of the manure. Manure can be beneficial to crop production, improving soil quality and fertility when the manure is land-applied for agricultural purposes. However other undesired constituents such as antibiotics can also enter the agro-ecosystem, and potentially impact soil, ground water, or surface water. United States Department of Agriculture-National Agriculture Statistics Services (USDA-NASS) gives an estimate of cropland and pastureland that is treated with manure in USA in recent years using geographical information system (GIS) aided Ag Atlas maps consisting of statistical data from 2002 and 2007 Censuses of Agriculture. 22749,251

acres of land was reported to be treated with animal manure including poultry manure in 2002. Manure application was significant in the northern states of Minnesota, Iowa, Wisconsin, eastern states of New York, Pennsylvania, Maryland, Delaware, several parts of the mid-west and south-eastern states and some parts of California, Oregon and Washington in the west and north-west. There has been a significant increase in the production of meat animals especially for poultry broilers that went up from twenty-five billion pounds in 1990 to close to 50 billion pounds in 2009. Proportionately, the value of production also increased for the poultry industry. For broilers the value of production increased from fifteen billion dollars in 1999 to above twenty billion dollars in 2009 and for layers it increased from 5 million dollars in 1999 to 7 million dollars in 2008 as per the USDA-NASS data.

Thus billions of pounds of poultry are produced every year in the US and millions of dollars are involved in this industry with increasing production rate. This indicates that use of antibiotics in animal feed especially poultry feed is likely on the rise concomitantly with increasing livestock production in confined animal feed operations (CAFOs).

Significant quantities of antibiotics have been found in manure-amended soils, associated groundwater, surface water systems and sediments and even plants and animals that grow in those soils. This implies that antibiotics from the manure do get transported into various components of the agro-ecosystem and hence have a high potential of entering the food web (Chee-Sanford et al., 2009).

Antibiotics are known to cause antibiotic resistance in bacteria including pathogenic bacteria posing a great human health concern. Also some antimicrobials like ionophores have been found to be toxic to soil dwelling flora and fauna, higher animals

and even human beings (Dowling, 1992; Hansen et al., 2009b). As the potential risks of using antibiotics in animal feed are becoming well known the use of other antimicrobials such as ionophores, will likely increase. Poultry companies classify ionophores as ‘non-antibiotic’ compounds, as they are not used as clinical drugs. This has led to several controversies (Washington Post, May, 2, 2008) as ionophores have been found to be toxic to non-target species, including humans at higher levels (Dowling, 1992). Ionophores have been used for many decades as anticoccidants in poultry feed, coccidia being a major parasitic disease. They are also used as feed additives in cattle as they increase efficiency in post ruminal digestion by altering rumen microbial population through ion transfers across cell membranes and are also known to decrease methane emission from them, methane being a severe greenhouse gas (Russell 2002). Significant levels of ionophores have been found in public waterways, sediments, and soils near confined animal feeding operations (Puginini 2005).

Knowledge on the occurrence, fate and transport of antimicrobials continues to increase, despite a lack of data regarding their use in animal feed (Mellon et al, 2001). However there is still a knowledge gap regarding the fate of ionophores in the environment and what risk, if any, they pose as emerging contaminant. Therefore our objectives were to conduct multi-scale investigations evaluating presence and magnitude of ionophores in poultry manure, and associated soil-water systems to determine if ionophores are an emerging soil contaminant. Moreover, this study will attempt to validate that ionophores may be used as a potential source marker for contamination by livestock manure since they are only used in animal feed.

## 1.2. ANTIMICROBIAL USE IN ANIMAL FEED

Antimicrobials are routinely used in animal feed at sub-therapeutic levels to promote growth and prevent diseases that may occur. Literature shows that antibiotic use for animal rearing has been in practice for several decades. Antibiotics used in the animal feed at sub-therapeutic levels help to increase the animal's ability to absorb feed and thus reach market weight much earlier. They also act as preventive measure to counteract adverse health effects that may occur in the poor hygienic conditions of the CAFOs where they are reared. Antibiotic doses vary from 3-220 gMg<sup>-1</sup> of feed depending on type and size of animal and also the group of antibiotic used (McEwen and Fedorka-Cray, 2002).

In the United States, 25% of swine feed was found to contain antibiotics above recommended levels (Dewey et al., 1997). The animals do not actually absorb most of the antibiotics that they are fed with 50-90% of the antibiotics are reported to be excreted in the manure (Schlüsener et al., 2003). Table 1-1 presents antibiotics commonly used in livestock production.

Table 1-1. Antibiotics used at sub-therapeutic levels for growth improvement in livestock production.

Antibiotic	Concentration in animal feed		
	Swine	Cattle	Poultry
	----- mg kg <sup>-1</sup> -----		
Bacitracin	11-33	35-70	0.8-36.0
Oxytetracycline	11-55	8.3-75	5.5-29.1
Chlorotetracycline	11-55	10-35	0.1-26.3
Penicillin	11-55	nd	0-25.0
Monensin	90	6.0-132	5-400
Lasalocid	30	11-33	5-125
Tylosin	11-110	9-397	20
Virginiamycin	6-11	9-25	10

Miskimins and Neiger, 1996; Qaiyumi et al., 2000; Herrman and Sandberg, 2001; Herrman and Stokaa, 2001; Kumar et al., 2005; Matabudul et al., 2001; Berrang et al., 2007; Kumar et al., 2005a; Dolliver and Gupta, 2008



According to the Food and Drug Administration green book, among the ionophore class of compounds, monensin ionophore is used for chicken and broilers with a dose limit of 90–110 g per ton of feed. Also monensin is used in cattle and dairy feedlots with a dose limit of 5–400 g per ton of feed depending on the species. Salinomycin has similar uses and dose limits. Narasin is also used in broiler chickens with a dose limit of 54–72 g per ton of feed. Also, this ionophore is used for increasing the rate of weight gain and improving feed efficiency for finishing swine. It has been estimated by the Union of Concerned Scientists that approximately 600 Mg of monensin ionophore were used in the beef industry and 900 Mg in poultry production with lasalocid, salinomycin and narasin having slightly less usage (Mellon et al., 2001).

### **1.3. ANTIMICROBIAL PERSISTENCE IN SOIL**

Antibiotics have different modes of interaction with soils based on their distribution coefficient ( $K_d$ ) values. The  $K_d$  provides an idea of how strongly the antimicrobial is bonded with the soil particles. Similarly distribution coefficients for organic carbon ( $K_{oc}$ ) are used to predict antibiotic solubility or retention in organic carbon. Higher  $K_{oc}$  values indicate less mobility in organic carbon. Antimicrobials (including antibiotics and ionophores) have wide range of  $K_d$  and  $K_{oc}$  values (Table 1-2). Compounds having higher  $K_d$  and  $K_{oc}$  values such as tetracyclines are expected to be more associated with soil solids, rather than water, and would be more likely to be transported sorbed to sediments rather than dissolved in surface runoff. Those with lower  $K_d$  and  $K_{oc}$  like sulphonamides would be expected to dissolve in surface run-off water or perhaps leach into ground water. Antimicrobial compounds can be mobilized through sorption to organic carbon, which is then dissolved into runoff waters. Table 1-2

compiles the distribution coefficients of several commonly used antimicrobials in animal feed. Sometimes, when organic matter is determined instead of organic carbon,  $K_{om}$  is used instead of  $K_{oc}$ .

Table 1-2. Distribution coefficients ( $K_d$ ) and organic carbon distribution coefficients ( $K_{oc}$ ) for commonly used antimicrobials.

Antimicrobials	$K_d$	$K_{oc}$
	-----L kg <sup>-1</sup> -----	
Tetracycline	400-1620	23,654-94,310
Oxytetracycline	420-1030	27,800-93,300
Enrofloxacin	260-6310	16,500-770,00
Oxolinic acid	0.3-116	14-4510
Tylosin	8.3-128	550-7990
Sulfamethazine	0.6-31	60-208
Chloramphenicol	0.7-1.7	46-116
Monensin	30 - 10,500	250- 120,000
Lasalocid	31-950	794- 15,000
Salinomycin	16- 4500	158 – 18,738
Narasin	69- 1629	536 – 16,450

Elanco, 1989; Hansen et al., 2009a; Hansen et al., 2009b; Hao et al., 2006; Hussain S.A. and Prasher, 2011; Kim and Carlson, 2006; Lissemore et al., 2006; Sassman and Lee, 2007

Antibiotic chemical structure can also affect its binding with soil. Chlortetracyclines were found to increase the interlayer spacing of 2:1 types clay minerals though tylosin was not found to do so. This can be due to the fact that chlortetracycline has a smaller size compared to tylosin and could sorb within the interlayers. That might be why it has a higher  $K_d$  value than tylosin as well (Gupta et al 2003). pH can also affect the binding with soil. In acidic soils, the basic antibiotics can acquire protons and become cations while acidic antibiotics may remain nonionized. In basic soils on the other hand basic antibiotics remain nonionized while acidic antibiotics may be ionized. The commonly used antibiotics like tetracycline and sulphonamides belong to the amphoteric group and they can exist as zwitterions depending on the soil pH. Suggestions have been made that cationic species bind to soil through ionic

interactions while the anionic species bind through nonionic interactions (Sarmah et al 2006). In relation to this theory, no studies have been found on ionophores but they are known to form zwitterionic complexes and further studies are necessary to understand their interaction with soil under such conditions.

Limited studies have been done on occurrence, fate and transport of ionophores in the soil environment in the past due to lack of sophisticated instruments requiring very low detection limits on the order of  $10^{-9}$  to  $10^{-12}$  m, because of their low concentration in the environment. Recent advances in chromatographic techniques using High Pressure Liquid chromatography in tandem with Mass spectrometry (LC-MS/MS) has made it the most popular choice for measuring ionophores in complex environmental matrix like manure, soil, sediment and water (Petrovic and Barcelo, 2006; Gros et al, 2006; Snow et al, 2007).

Significant amounts of ionophores have been found in agricultural landscapes. Monensin, salinomycin, narasin and lasalocid are most commonly used ionophores in animal feed, hence more likely to be found in animal manure and associated environment where the manure is land applied as fertilizer. According to the United States Geological Survey the most likely pathway for veterinary medicines like ionophores to move from the soil to ground or surface water is from stockpiled or land applied manure. This leads to significant exposure of these antimicrobials to soil and aquatic flora and fauna.

The persistence of ionophores in animal manure has been known for many years. Donoho et al (1984), found monensin in cattle feces and urine and Catherman et al (1991), found narasin ( $1.0-725 \mu\text{g kg}^{-1}$ ) in poultry manure. Cha et al (2005), reported monensin, narasin and salinomycin in surface water samples collected in Colorado, USA.

However these earlier studies were limited by a lack of sophisticated instruments that allow precise quantification of ionophores such as LC-MS/MS. Table 1-3 lists measured concentration of ionophores in different environmental matrices by various research groups in past.

Table 1-3: Summary of ionophore concentrations detected in the environment.	
Ionophore	Concentration range
<u>Swine Manure (<math>\mu\text{g kg}^{-1}</math>)</u>	
Salinomycin	11- 25.7
<u>River water (<math>\mu\text{g L}^{-1}</math>)</u>	
Monensin	0.01- 3.45
Salinomycin	0.001- 0.7
Narasin	0.001- 0.25
<u>Surface runoff from farm field (<math>\mu\text{g L}^{-1}</math>)</u>	
Monensin	0.002- 0.45
Lasalocid	0.001- 0.028
<u>Groundwater (<math>\mu\text{g L}^{-1}</math>)</u>	
Monensin	0.04 – 0.57
<u>River sediment (<math>\mu\text{g kg}^{-1}</math>)</u>	
Monensin	1.5- 31.5
Salinomycin	0.9- 30.1
Narasin	2.5- 16.3
Cha et al., 2005; Hao et al., 2006; Kim and Carlson, 2006; Hansen et al., 2009a	

Degradation rates of antibiotics in soil vary with half-lives ranging from days to years (Table 1-4). Degradation rate can vary with type of antibiotics, soil types, temperature, pH and presence of microbial community. Data in the literature shows that antibiotics such as macrolide biodegrade within 30 days at temperatures ranging from 20 to 30°C. Some antibiotics (eg: sarafloxacin, virginiamycin) can persist up to 80 days (Gavalchin and Katz, 1994). Hence some of the antibiotics are more persistent in the environment than the others. The half-life values may vary in natural settings as they are influenced by different conditions that affect sorption processes, such as matrix type or temperature.

Antibiotics	Matrix	Half-life ----days----	Reference
Bacitracin	Soil-manure	22.5-12	Gavalchin and Katz., 1994
Chloramphenicol	Sediment	<12	Lai et al., 1995
Chlortetracycline	Manure	7	Morrison et al 1969
Cetiofur	Silt clay loam	41	Gilbertson et al., 1990
Erythromycin	Soil-manure	11.5-8	Gavalchin and Katz., 1994
Oxytetracycline	Soil	270	Halling-Sorensen et al., 2005
Sulfadiazine	Marine sediments	50	Hektoen et al 1995
Trimethoprim	Marine sediments	75	Hektoen et al 1995
Monensin	Clay loam	4-15	Sassman and Lee, 2007; Carson and Maybury, 2006
Lasalocid	Clay loam	<4	Sassman and Lee, 2007

Though half-lives of monensin and lasalocid were reported to be as low as four days or less, they were still detected in the environment even after months by other research groups (Davis et al., 2006; Kim and Carlson, 2006). This is because ionophores can ‘pseudo-persist’ in the environment, as they are constantly being added to the environment, due to their continuous use in animal rearing practices. Hence despite of their low half-lives they can still be found in the environment due to constant addition caused by high usage. Also, these half-lives may not actually be field representative, where the compounds may be bound strongly to soil or sediment molecules. The reason for their persistence beyond their laboratory-measured half-lives can vary based on field conditions, including biotic and abiotic factors.

Persistence of antibiotics increases with decrease in temperature and it is likely that many of the antibiotics in fall-applied manure will remain in their original form over

winter in northern latitudes where soils are seasonally frozen. For the same temperature antibiotics are expected to be more persistent in the colder environments of deeper soil layers compared to top soils and likewise in groundwater compared to surface waters (Hektoen et al., 1995). It is expected that overall the half-lives of antibiotics will increase in winter seasons while being lowest in the summers and the persistence will increase with increase in latitude, in other words, antibiotics might persist longer in temperate zones than the tropical zones. This has been observed by researchers studying direct photolysis of selected pharmaceuticals at different seasons such as spring, summer, fall and winter and latitudes (Andreozzi et al., 2003).

Some antibiotics like tylosin are found to degrade in manure compared to soil (Halling-Sorensen et al., 2005) whereas some others like ivermectins have been found to degrade better in soil-manure mixture than only manure, in warmer conditions (Thompson et al., 2009). These observations can be due to the presence of different microbial systems that are present in manure compared to soil-manure mix that are able to better degrade some antibiotics compared to others. The carbon in the manure and manure mixture provide readily available nutrients for the microbes and thus help in the process of degradation. In the case of tylosin the degradation was enhanced with increase in concentration of the manure particles under methanogenic conditions. Cefotaxime a broad spectrum antibiotic belonging to the group of cephalosporin was seen to be quickly degraded to inactive metabolites in presence of excess cattle manure, though sterilized cattle feces failed to cause the same effect (Gilbertson et al., 1990). This suggests that heat-labile microbes in the manure are responsible for the rapid degradation and the rapidity can vary with type of manure and temperature.

Antibiotics can degrade through hydrolysis and photolysis. Both mechanisms were found to be affected by various factors including presence of animal excreta, light and pH (Gilbertson et al., 1990). It is also indicated that surface application of manure can hasten photodegradation of antibiotics due to direct exposure to sunlight while deep injections may increase microbial degradation and hydrolysis. Indirect photolysis was observed in presence of natural sensitizers like nitrate and humic acids that were either found to hasten or slow down the degradation process. Degradation of diclofenac and carbamazepine in another study was reduced by the presence of humic acids but that of sulfamethoxazole, ofloxacin and propranolol was increased. Nitrate on the other hand reduced the half-lives of all the above but increased that of propranolol and hastened its photodegradation (Andreozzi et al., 2003). This also indicates that photodegradation of antibiotics in streams can be affected by the natural sensitizers like nitrates, chlorides and humic acids.

Out of all the ionophores, environmental fate of monensin has been studied the most to date, because it is most widely used, not only in poultry feed, but also in dairy and swine feed. When monensin is released into the environment, it has the potential to persist and reach aquifers because hydrolysis is not typically observed and photolysis is slow (Elanco Product Company, 1989). However the reported biodegradation rates indicate that rapid biological attenuation is possible. Furthermore it was reported in another study that abiotic processes might contribute significantly to monensin dissipation (Sassman and Lee, 2007). In the same study reported half-lives ranged from 1.4 - 4.1 days for lasalocid and monensin half-lives ranged from 1.2 to 1.9 days in the previous study, compared to 13.5 days by Carlson and Mabury (2006). The reported

sorption co-efficient ( $K_d$ ) ranged from 0.915 to 78.6 ( $LKg^{-1}$ ) for various soils at an aqueous phase concentration of  $0.05 \mu mol L^{-1}$ . Based on the  $K_d$  values, monensin is expected to be more mobile than tetracyclines and similar or less mobile than sulfamethazine in water systems (Tolls 2001). Comparable results were obtained on measuring first order half-lives of salinomycin under anaerobic conditions in swine manure, reported to be 6 days (Schlusener and Bester, 2006). A recent study on California dairy farms (Watanabe et al, 2008) found that monensin persisted at relatively high concentration in the manure transport and storage system and there was significant attenuation in the waste handling and storage. Monensin was also detected in flush lane water samples, lagoon water samples and ground water samples within the farms at concentrations of  $10^{-1}$  to  $10 \mu g L^{-1}$ . Data indicated that monensin attenuation might be higher under predominantly aerobic subsurface conditions than under anoxic conditions associated with lagoons monitoring wells.

A study was recently conducted on abiotic degradation of ionophores under controlled laboratory conditions (Pernille Bohn, 2013). Both photolytic and hydrolytic degradation for monensin, salinomycin, narasin and lasalocid were assessed. Half-lives found for hydrolytic degradation of monensin, salinomycin and narasin were 13, 0.6 and 0.7 days respectively. Lasalocid was not found to hydrolyze at the experimental temperatures ranging from 6-28<sup>0</sup>C and pH ranging from 4-9. However, lasalocid was found to be sensitive to photolysis and photolytic degradation was rapid with half-life of less than 1 hour. The other ionophores were resistant to photolysis at 190-1100 nm wavelength range.



An agricultural run-off study done with six antibiotics and monensin ionophore revealed that antimicrobials had different transport characteristics and their type significantly impacted the partitioning of their losses between water and sediments. Monensin had the highest concentration in runoff and second highest in the sediment (Davis et al, 2006). Thus if agricultural runoff is proven to result in development of multi-drug resistant genes or toxicity to aquatic organisms, then management practices will be needed to reduce ionophore run-off.

A study by Villalba et al, (2009), indicated that some of the fragmentations that can occur in ionophores include, loss of water, decarboxylations, ketone  $\alpha$ -cleavages and rearrangement of cyclic ethers and amide groups (Villalba et al, 2009). Depending on the species, interactions with soil can occur through electrostatic interaction, surface bridging, hydrogen bonding or hydrophobic interactions. The sorption behavior in soil can also be influenced by the properties of soil including pH, organic carbon content, metal oxide content, ionic strength and cation-cation exchange. Manure and slurry may also alter the behavior of ionophores in soil system and affect its persistence. These effects have been attributed to changes in pH or nature of dissolved organic carbon in the soil-manure system (Boxall A.B.A. et al, 2003; Boxall A.B.A., 2008).

#### **1.4. ANTIMICROBIALS AND SOIL MICROBIOTA**

Presence of antibiotics, especially with a broad-spectrum mode of action like tetracyclines and sulphonamides, in the soil environment can adversely affect the soil microbial populations, which can have significant impact on the soil ecology, ultimately disrupting many important microbially mediated soil processes, like the nitrogen cycle (Halling-sorenson et al 2002). One example, of such an impact would be decreases in

nitrifying bacteria populations due to antibiotic exposure. However the narrow spectrum antibiotics like sefadiazine, oxolinic acid and tylosin favored the nitrification process (Halling-Sørensen, 2001). They concluded narrow spectrum antibiotics applied selective pressure on microbes that did not participate in the nitrification process, limiting competition on nitrifying bacteria. Likewise, Jacobsen et al. (2004) found that certain antibiotics influenced sulfate reduction and organic matter decomposition in soil and manure by affecting the microbial population present.

Ionophores are a natural product of *Streptomyces sp.*, the largest genus of Actinomycetes predominantly dwelling in the soil, but there is hardly any knowledge about their interaction with the soil microorganisms. This is not the case for other antibiotics as there is lot more evidence on increasing antibiotic resistance of soil microorganism highlighted by the importance of horizontal gene transfer by the presence of mobile genetic carriers such as plasmids and transposons. Several studies have documented conjugal transfer of plasmids between introduced and indigenous strains of soil bacteria. The rate of conjugal gene transfer seems to be higher in nutrient-rich environments relative to oligotrophic ones (Nwosu, 2000). Higher rates of transfer were found in the rhizosphere relative to bulk soil. Daane et al, (1996) also observed a relatively high rate of transfer of plasmid to indigenous soil bacteria in earthworm-containing soils and found earthworms facilitate conjugal transfer because they help disperse bacteria in soil. Earthworms may also modify the physicochemical and biological properties of the soil and in doing so provide a conducive environment for bacterial growth and gene transfer.

Antibiotics are known to cause antibiotic resistance in bacteria some of which are pathogenic and a great human health concern. Possible pathways for spread of antibiotic-resistant bacteria in terrestrial environment due to their use in agriculture are selection of resistant microbial population in the animal gut and shedding through their feces; transfer of resistance genes (plasmids, integrons etc.) from bacteria in manure to native soil and water microbial populations when land-applied and also accumulation of antibiotics in animal and plant tissues that we consume (Kumar et al., 2005).

Selection of tetracycline resistant genes in various soil bacteria has been found to be quite wide spread due to horizontal gene transfer and these resistant genes have been found to mobilize and persist. Also increasing evidence of horizontal transfer of these resistant genes into human pathogens is a cause of grave concern in human health (Chee-Sanford et al., 2009), (Sarmah et al., 2006; Sengeløv et al., 2003).

Potency of antibiotics and their residues might be reduced if they were bound with organic or inorganic substances. For example, tetracyclines and their biodegradation products lose their potency quickly when chelated with divalent and trivalent metals like  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Al^{3+}$  (Halling-Sorensen et al., 2002). This also indicates that presence of these specific metals not only affects antibiotics' potency but also their degradation products. Soil adsorbed tetracyclines have also been found to reduce both resistant and sensitive strains of *Salmonella*, and the resistance decreased with decrease in its concentration of tetracyclines in soil (Chander et al., 2005). Similar trends are expected for ionophores and further studies are needed in this area. Several antibiotics like tetracyclines and tylosin are found to have similar potency against soil microorganisms for both parent and degraded products, though by different modes. With certain soil

bacteria antibiotic potency decreased with time as the antibiotics degraded. Conversely, ciprofloxacin and oxytetracycline had high potency and remained as long as 100 days (Halling-Sorenson et al 2002).

Ionophores like monensin were found to reduce methane production and excretion of nitrogenous compounds like ammonia in cattle hence affecting N concentration in manure (Tedeschi et al., 2003). Thus decrease in use of monensin and similar antimicrobials can lead to increase in nitrogen load of manure and more nitrogen can be derived by applying lesser amount of manure in soil which also decreases the potential of antimicrobials to get into the soil environment through land application of the manure. This also benefits phosphorous management.

#### **1.5. FATE OF ANTIMICROBIALS IN GROUND AND SURFACE WATER**

Antibiotics loosely bound to soil (having lower  $K_d$  and  $K_{oc}$  values) tend to be transported to ground or surface water as dissolved constituents in leachate or runoff. Highly mobile antibiotics may leach into groundwater first, before lateral transport into surface water. Small amounts of highly sorbed antibiotics with high  $K_d$  and  $K_{oc}$  values like tetracyclines can enter ground and then surface water through preferential flow paths such as macropores.

Studies in Germany suggested tetracycline and tylosin were fairly immobile with none found in groundwater at 80 cm depth (De Liguoro et al., 2003; Hamscher et al., 2002). This lack of deep percolation was mainly due to high sorption of these antibiotics to clay, due to high  $K_d$  and  $K_{oc}$  (Kumar et al 2002). This may be the reason why in Table 1-2, tetracyclines were found mostly associated with soil and sediments in comparison to water where they were either absent or below the limit of detection.

Hydrophobic partitioning was also inferred by Sassman and Lee in 2007, when they found that linear isotherms adequately modeled the sorption by eight soils of two ionophores monensin and lasalocid ionophores. Log K<sub>oc</sub> values ranged from 2.1 to 3.8 for monensin and 2.9 to 4.2 for lasalocid and generally decreased with increasing soil pH (pH range 4.2 to 7.5), which would be expected, as carboxylic acid groups are deprotonated under alkaline conditions. As carboxyl and ether O atoms in the molecule can chelate environmentally relevant cations (eg. Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>), this may increase the apparent hydrophobicity of the molecules and possibly alter their sorption and mobility by reducing their net charge.

Kim and Carlson (2006), found ionophores occur solely in agriculture specific sites. Song et al. (2007) and Zang et al. (2007) monitored antimicrobials, including monensin, in surface runoff from a livestock farm and near a suburban sewage outfall. Both studies found monensin only to occur in the farm samples. This was expected since ionophores are only known to be used to treat livestock. As a result they have been suggested as a source marker for contamination from agricultural non-point sources. Similarly, researchers in Alberta and Ontario, Canada found ionophores at ng L<sup>-1</sup> concentrations near agricultural areas (Hao et al, 2006; Lissemore et al, 2006; Thompson et al, 2008).

In USA, tetracyclines, sulphonamides, and several ionophores were found in Poudre River, Colorado (Yang and Carlson 2003; (Kim and Carlson, 2006), in wells and streams near poultry and dairy farms (Campagnolo et al., 2002; Watanabe et al., 2008), and other surface and groundwater (Lindsey et al., 2001). collected 144 ground and surface water samples throughout USA and found sulphonamides, tetracyclines,

trimethoprim, streptozotocin and sulfadimethoxine in more than 75 % of them, with concentrations ranging from 0.06-0.24  $\mu\text{g L}^{-1}$ , with the ionophores typically associated with sediments in the samples. In Iowa streams, antibiotics were present in concentrations ordered as following: sulfonamides > trimethoprim > erythromycin > tetracyclines, with greater concentrations of antibiotics under low-flow compared to high flow conditions, probably due to dilution effects (Kolpin et al., 2004). Small amounts of highly sorbed antibiotics like tetracyclines can enter ground and then surface water through preferential flow macropores.

Kim and Carlson (2006) found monensin, narasin, and salinomycin at significant concentrations in water and sediment samples in close proximity to poultry farms in their study area in Colorado. They also found that greater concentrations of these ionophores were found in the sediment compared to the overlaying water matrix, which validates its chemical nature, as its hydrophobic from outside. Also high partitioning was observed at a low flow condition compared to the high flow and it was assumed that ionophores not only sorb to the sediments but also to suspended solids. Their high association with sediments and solids indicates that presence of ionophores in surface water can significantly affect the stream benthic biota.

### **1.6. ANTIBIOTIC MOBILITY IN THE FOOD WEB**

Several studies have found that plants can take up antibiotics at levels causing toxicity problems (Boxall, 2006; Boxall et al., 2003; Boxall et al., 2004; Boxall et al., 2006). Some of the commonly studied antibiotics including tetracyclines, sulfonamides and macrolides that have been found at trace levels or higher in cabbage, lettuce, carrots, wheat, soybeans, tomatoes, aster plants green onions (Boonsaner and Hawker, 2010;

Boxall et al., 2006; Brian, 2003; Ellis, 1963; Kumar et al., 2005b; McCoy, 1975; PRAMER, 1954; R. C. Sinha and Peterson, 1972). From literature review tetracyclines were found to be most commonly taken up by plants followed by sulfamethazine. Boxall et al. (2006) found that antibiotics having dissipation time of around 2.5 months in potting soil had significant uptake by plants like lettuce and carrots, which showed plant uptake could occur even if the antibiotics are less persistent in soil. Kumar et al. (2006a) found that chlortetracycline concentrations in plant tops increased with increasing concentrations in a potting mix that was a manure-soil mixture, with chlortetracycline concentrations in the pots 100 times more than what was found in soil environment.

The presence of tetracyclines in soil has been found to inhibit plant growth. Oxytetracycline uptake by alfalfa has been found to decrease shoot growth 61% and root growth by 85% (Kong et al., 2007). (Boonsaner and Hawker, 2010) did a study where oxytetracyclines and NaCl in soil was found to decrease the rate of seed germination and growth of soybeans in soils with elevated salinity but still could be used to phytoremediate the soil as soybean roots could take up significant amount of antibiotics without major physiological imbalance. This was because antibiotics were not observed to translocate from roots upwards into the plant system. Significant oxytetracycline residues of 20 ug g<sup>-1</sup> were found in coconut fronds and even translocating into the fruits at lower concentrations (McCoy, 1975). Patten et al. (1980) found pinto bean shoot and root dry matter decreased up to 87% and 94%, nutrient uptake inhibited, and 52-67% fewer root nodules when grown in tetracycline-rich sandy-loam soil. Conversely, no adverse effects were detected in clay loam soils. (Batchelder, 1982) found radish, wheat and corn had greater nutrient uptake in presence of tetracycline in the clay loam soils,

compared to controls. This could create selective pressure on the nitrogen-fixing bacteria that have symbiotic relations with leguminous crops, thereby inhibiting nitrogen fixation. While with the non-leguminous plants the antibiotics might have suppressed microbes outside the rhizosphere, allowing microbes within the rhizosphere to facilitate improved nutrient uptake. Effects of antibiotics on plants thus depend on soil characteristics and plant sensitivities.

A number of studies have indicated that the plants might take up antimicrobial residues that are reversibly adsorbed to soil. This has been demonstrated by hydroponic cultures using tetracycline antibiotics (Schneider, 2008). Greenhouse studies using corn took up ionophores such as monensin and lasalocid (King et al. 1983). Though few studies have been done to show if such uptake and accumulation of antimicrobials in plants is detrimental and at what concentrations toxicity might occur, but studies have indicated that the risk may not be negligible.

Pharmacokinetics and toxicity studies have indicated that ion transport capacity of ionophores does not discriminate between membranes of bacteria and higher organisms. Hence ionophores have been found to be toxic to higher animals including mammals and in fact ionophores are most toxic among all antimicrobials applied to animal feed. LD<sub>50</sub> for monensin and lasalocid in rats have been found to be 35 mg kg<sup>-1</sup> and 100 mg kg<sup>-1</sup>, which are much lower than LD<sub>50</sub> for chlortetracycline (10,800 mg kg<sup>-1</sup>) or sulfamethazine (1,060 mg kg<sup>-1</sup>) and approaches that of the well-known poison potassium cyanide (10 mg kg<sup>-1</sup>) (Hardmann et al, 1997). Moreover, presence of other antibiotics (e.g. tiamulin, erythromycin, sulfamethazine, chloramphenicol) intensifies ionophore toxicity (Mazlum et al, 1985).



The concentration at which ionophore toxicity occurs is species dependent. For example, amounts fed to chickens at therapeutic levels would be toxic to horses or turkeys (Volmer et al, 1998). The therapeutic range is narrow, with a lethal dose being two-to threefold the recommended dosage level. Toxic doses of ionophores have adverse effects on the heart, skeletal muscles, and liver tissues (Galitzer et al, 1984; Friedman et al 1998; Oehme et al, 1999). Ionophore toxicity to poultry farm workers, in some cases causing death has been reported (Story and Doube, 2004; Sharma et al 2005). Therefore the persistence of ionophores in the soil systems may present health hazard to higher order species if found at elevated concentrations. Several studies were done on ecotoxicity of different antibiotics on species inhabiting soil-plant environment and in many cases toxic levels were found to be just 10 times or 100 times lower than known occurrence of those antibiotics in the soil environment (Boxall et al., 2004).

### **1.7. BIOCHEMISTRY OF IONOPHORES**

Biochemically the ionophore class of compounds, are lipid soluble selective biological ion carriers having liganding oxygen due to the presence of lone electron pairs in various functional groups like hydroxyls (-OH), carboxyls (-COOH), ethers (-O-), amides (-CONR (Pressman, 1967). Ionophores are relatively large molecules (> 600g/mol and above) comprised of a backbone consisting of tetrahydropyran or tetrahydrofuran groups with multiple carboxylic acid, keto and ester groups (Carmosini and Lee, 2008) Their basic structures consists of multiple cyclic ethers, a free carboxylic acid group at one end of the molecule and a terminal alcohol group at the other such that they are described as polyether antibiotics (Cha and Carlson, 2005). They alter the natural flow of cations across the cell membranes by chelating with them selectively, thereby

altering the membrane permeability and hence disrupting the normal physiological functions of the target species. At the molecular level ionophores lower the energy barrier necessary for the membrane transport of ions and catalyze an electroneutral cation-proton exchange across the barrier. Consequently they abolish the gradients of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and  $\text{Na}^+$  causing cell death of mostly gram-positive bacteria. The cell walls of gram negative bacteria do not permit the penetration of hydrophobic molecules with high molecular weights as that of the ionophores and hence not susceptible to ionophore actions (Westley 1983). When ionophores are applied as coccidiostat, in poultry feed, they affect both asexual and sexual cycles of coccidia by disrupting normal transport of essential metals like  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ .

On the basis of the transport mode, ionophores can be classified in 3 broad categories such as neutral ionophores, carboxylic ionophores and channel forming Quasi-ionophores (Butaye et al, 2003). Neutral ionophores are not used in animal feed as they do not have antimicrobial properties and same for Channel forming Quasi ionophores as they have a different mechanism of transmembrane transport not favorable for the purpose. Thus all the ionophores incorporated in the animal feed belong to the group of carboxylic ionophores and further subdivided into monovalent and divalent polyether ionophores, depending on their preferential transport of monovalent or divalent cations. These ionophores are toxic to many bacteria, fungi, protozoa and even higher animals, due to their three-dimensional conformation creating a highly hydrophobic exterior and hydrophilic interior, enabling the binding of one or more cations. The lipophilic nature allows ready penetration of cell membranes, enabling uncontrolled influx and/or efflux of

selected ions, such as potassium and sodium, from the cell. This osmotic interference often leads to cell death.

The most common ionophores used in animal feed are monensin, lasalocid, salinomycin and narasin. Monensin, a monovalent carboxylic ionophore produced by *Streptomyces cinamonensis* transports  $\text{Na}^+$  more efficiently than  $\text{K}^+$ . Salinomycin, another monovalent carboxylic ionophore produced by *Streptomyces albus* transports  $\text{K}^+$  more efficiently than  $\text{Na}^+$ , same for Narasin produced by *Streptomyces aureofaciens*. Lasalocid is a divalent carboxylic ionophore that transports  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  very well (Butaye et al, 2003). The chemical structures of monensin, lasalocid, salinomycin and narasin are illustrated in (Hansen et al, 2009). Chemical properties of ionophores, compiled from literature review have been presented in Table 1-5.

Table 1-5. Select chemical properties of ionophores reported in the literature.

Ionophore	Molecular Weight kg mol <sup>-1</sup>	pKa	Aqueous Solubility mg L <sup>-1</sup>	Log Kow			Log Koc	Half-life days	Reported Poultry Feed Concentrations mg kg <sup>-1</sup>
				pH 5	pH 7	pH 9			
Monensin	670.9	4.5 - 6.65	0.003 – 6.3	4.2	2.75	3.79	2.1 - > 6.3	Soil: 4-10 Manure: 13 - 30	100
Lasalocid	590.8	2.6 - 4.4	0.25 - 1	nd	2.8	nd	2.9 – 4.2	4 - 15	75
Salinomycin	751	4.5 - 6.5	3.4 - 905	3.3	2.9	2.6	2.2 – 4.36	5 – 18	120
Narasin	765	7.9	102 - 681	102	681	nd	4.9 – 6.89	5 - 30	80

Hansen et al., 2009a; Hussain S.A. and Prasher, 2011; Sassman and Lee, 2007; Pressman, 1976; EFSA 2004; Elanco, 1989; Furtula et al., 2009; Dolliver et al., 2008

## **1.8. RESEARCH OBJECTIVES AND HYPOTHESES**

Multiple studies have been done on the occurrence, quantification, dynamics, mobility, degradation, and speciation of antibiotics. More studies have been conducted on antibiotics belonging to the sulphonamide and tetracycline groups and there is a lack of research dealing with the ionophore groups of antimicrobials. This could be due to the lack of a good analytical method to quantify ionophores from different environmental matrices. Therefore, our first objective was to develop an improved analytical method for determining the presence and quantifying ionophores in aged poultry litter using LC/MS/MS. We hypothesized that LC/MS/MS could be used to precisely quantify ionophores in aged poultry litter utilizing an optimized method. Our second objective was to study ionophore sorption-desorption in mid-Atlantic soils and to more fully understand their partitioning behavior in soil and water systems. We hypothesized that different soil properties such as texture, pH and mineralogy would influence ionophore-soil sorption and desorption characteristics influencing ionophore partitioning between solid and solution phases in soil-water systems. Our final objective was to better understand which soil properties influenced ionophore sorption and desorption and to what degree. We hypothesized that ionophore sorption and desorption in soil would be influenced by soil texture compounded with organic matter content, soil pH and other soil parameters.

## **CHAPTER 2. QUANTIFYING IONOPHORES AGED IN POULTRY**

### **LITTER**

#### **2.1. INTRODUCTION**

Coccidiosis is a major protozoan disease found in Confined Animal Feed Operations (CAFOs) raising poultry. Anticoccidials are used at therapeutic levels against this disease and sub-therapeutic levels for growth promotion (Hansen et al., 2009a). Anticoccidials are biochemically known as ionophores due to their ‘ion-bearing’ properties while crossing the biological membranes (Pressman, 1976) and play a major role in the biochemical processes of a living system. There are  $11.2 \times 10^4$  Mg of non-therapeutic antimicrobials used in the USA for livestock production, with  $4.8 \times 10^3$  Mg used for poultry production (Mellon et al., 2001). Commonly, compounds that are part of the ionophore class of antimicrobials are used in poultry production. Some of these ionophores are excreted, without undergoing any metabolism, and have been found in agricultural watersheds, presumably from land application of the manure. There is an increasing concern regarding the persistence of these anticoccidials in the environment. The most commonly used ionophores, as animal feed-additives are monensin, salinomycin, narasin and lasalocid. The use of ionophores in poultry feed varies by country. Typical feed amounts reported in the literature for North America average 99 mg kg<sup>-1</sup> for monensin, 120 mg kg<sup>-1</sup> for salinomycin, 80 mg kg<sup>-1</sup> for narasin (Furtula et al., 2009) and 75 mg kg<sup>-1</sup> for lasalocid (Sassman and Lee, 2007).

Previous studies have found significant quantities of various ionophores including the aforementioned in feed, chicken tissues, liver, eggs and associated soil and water bodies around poultry houses (Cha et al., 2005; Coleman et al., 1997; Dubois et al., 2004;

Kim and Carlson, 2006; Olejnik et al., 2009; Shao et al., 2009). As ionophores are exclusively used in animal rearing operations especially poultry and cattle but not used clinically, they can prove to be good markers for transport of veterinary antimicrobials in the watershed and hence account for pollution from agricultural practices (Kim and Carlson, 2006). Ionophores have been found to be toxic to animals and humans causing deaths at high levels and at present there exists no antidote or treatment for ionophore toxicity. Reported toxicity of ionophores (LD50 mg kg<sup>-1</sup> of body weight) in chicken are 200, 44.3, and 71.5 for Monensin, Salinomycin and Narasin respectively (Al-Dobaib and Mousa, 2009; Kart and Bilgili, 2008; Story and Doube, 2004).

Little attention has been directed to method development for quantitatively measuring ionophores from complex matrices without using complicated sample clean-up techniques. Poultry litter is one of the least studied environmental matrices for analyses of ionophores, even though significant amounts of ionophores are fed to chickens. Earlier studies that have focused on quantifying low levels of ionophores in challenging environmental matrices have used complex derivatization techniques for UV detection after HPLC separation (Coleman et al., 1997). Post column derivatization was necessary as ionophores have poor chromophore characteristics (Kim and Carlson, 2007). More recently, an increasing number of studies have relied on liquid chromatography tandem mass spectrometry (LC-MS/MS) based methods to quantify ionophores because LC-MS/MS provides better precision and linearity along with a more robust quantitative analysis that does not require pre- or post-derivatization (Dai and Herrman, 2010; Soler et al., 2005). These studies have successfully shown high recovery of ionophores with freshly spiked samples. It is less clear how such methods would perform in analyzing

complex and aged poultry litter. Finally, the use of simatone as an internal standard, a common practice in these studies, has also been criticized due to its very low molecular weight and lack of chemical resemblance to the ionophores, posing significant challenges in reliable quantification. A more suitable approach would be to use Nigericin as an internal standard, since Nigericin, a type of ionophore, has not been used as animal feed additives (Hansen et al., 2009a).

Therefore, our objective was to develop and test a rapid and sensitive liquid chromatography tandem mass spectrometry based method for simultaneous quantification of monensin, lasalocid, salinomycin, and narasin in aged poultry litter samples using HPLC-MS/MS.

## **2.2. MATERIALS AND METHODS**

### **2.2.1. Sample and standard preparation**

The ionophores used in this study, monensin (Mon: cat# 46468), lasalocid (Las: cat# 33339), salinomycin (Sal: cat# S4526), and narasin (Nar: cat# N1271), were purchased from Sigma Aldrich Co. (St. Louis, MO) as sodium salts. The erythromycin-N-methyl-<sup>13</sup>C<sub>3</sub> (Eryd3: cat # 663506) and nigericin (Nig: cat # N7143) to be used as internal standard and surrogate standard were also purchased from Sigma Aldrich Co. (St. Louis, MO). The HPLC grade methanol and water were purchased from Burdick and Jackson (MI, USA) and acetonitrile from EMD chemicals Inc. (NJ, USA). Water (ACS grade), 0.1% formic acid, and phosphoric acid were purchased from JT Baker (NJ, USA). Hydrophilic-lipophilic-balanced solid phase extraction cartridges (HLB: cat# WAT094226) were purchased from Waters Co (Miliford, MA).



Each of the ionophores was diluted in methanol to make 100- $\mu\text{g ml}^{-1}$  of stock solution. Fresh stocks were made bi-monthly and stored at less than 4<sup>0</sup>C. Using this standard, 1 $\mu\text{g ml}^{-1}$  of working stock solution consisting of monensin salinomycin, lasalocid, narasin and the surrogate nigericin was prepared. From this working stock, 7 calibration curve standards were prepared with concentrations in the range of 0 - 300 ng mL<sup>-1</sup>. These dilutions were made in 1:1 acetonitrile and 0.1% formic acid, to match with the mobile phase composition. 10- $\mu\text{l}$  aliquot of 2- $\mu\text{g ml}^{-1}$  internal standard, Eryd3 was added to all standards.

Poultry litter (a mixture of excreta and wood shavings) was collected from poultry houses located in the mid-Atlantic region. The litter samples were stored at < 5 °C for more than three years. The samples were ground, homogenized and sieved (2 mm). A 0.5 g portion of this sieved sample was weighed into 15 ml polypropylene centrifuge tubes. The poultry litter samples were spiked with 10  $\mu\text{l}$  of 5- $\mu\text{g ml}^{-1}$  surrogate Nigericin. The poultry litter was treated with 12 ml of 20% aqueous phosphoric acid solution (v/v), sonicated (Branson 3510, CT, USA) at room temperature for 15 minutes followed by 15 minutes centrifugation at 10,000 xg (Beckman Coulter, CA, USA). The phosphoric acid extract was analyzed and not found to contain any of the analytes, and discarded. After acid treatment, 12 ml aliquot of 1:1 (v/v) methanol: water solution was added to the litter sample that was followed by sonication and centrifugation process as described previously. The supernatant obtained were loaded onto HLB cartridges that were pre-conditioned with 3 ml methanol and 3 ml de-ionized (DI) water. The cartridges were mounted on a vacuum manifold (Sigma Aldrich Co., St. Louis, MO) and extracted under a steady vacuum pressure of 5kPa. The HLB cartridges were then washed with 9 ml of DI

water to remove any impurities and traces of phosphoric acid. The ionophores were eluted with 5 ml of methanol and the extracts were concentrated at 50 °C under gentle flow of nitrogen using an evapo-heater (Thermo Scientific, MA, USA). The samples were reconstituted in 1 mL of 1:1 acetonitrile and 0.1% formic acid. To the 1 mL sample, a 10- $\mu$ l aliquot of 2- $\mu$ g ml<sup>-1</sup> internal standard (Eryd3) was added.

### **2.2.2. Sample analysis**

Ionophore concentrations were determined using a Shimadzu HPLC 10 AVP Series combined with API 3000 mass spectrometer (Applied Biosystems Sciex, USA), which was operated and controlled by the Analyst software (version 1.4.1). Chromatographic separation was achieved using 10x2.1 mm C18 Aquasil column (Thermo Scientific, WI, USA) with 3- $\mu$ m particle size. The mobile phase consisted of 1:1 mixture (v/v) of acetonitrile and 0.1% formic acid for 1 minute that was ramped to 90% acetonitrile in 8 minute and holding it at 90% for an additional minute, with a flow-rate of 0.25 ml/min. The injection volume was 10  $\mu$ l and each chromatographic run was 10 minutes long. The mass spectrometer was operated in positive electrospray ionization mode with source temperature at 400 °C and electrospray capillary voltage at 5 kV. The MS/MS parameters were optimized by constant infusion of the standard solution of concentration 1  $\mu$ g ml<sup>-1</sup> at the flow rate of 10- $\mu$ l min<sup>-1</sup>. The optimized compound parameters of the multiple reaction monitoring transitions used for the analyses are presented in the Table 2-1.

Table 2-1: Optimized LC-MS/MS parameters of ionophores used for quantification in the instrument.

Analyte	Parent ion	Daughter ion	Declustering Potential	Focusing Potential	Collision Energy	Cell exit Potential
	m/z		ev			
Monensin	693.7	675.6	50	150	55	24
Salinomycin	773.8	755.8	79	200	45	25
Narasin	787.6	769.6	90	220	45	25
Lasalocid	613.60	577.6	73	187	45	26
Nigericin	747.5	703.4	85	210	75	22
Erythromycin - N-methyl- <sup>13</sup> C,d3	738.5	580.4	50	160	30	20

Since all poultry litter samples contained ionophores, it was necessary to generate a “clean” poultry sample for determining method detection limit (MDL). Therefore, we extracted 10 grams poultry litter sample using the methanol extraction method as previously described until no ionophores of interest were detected in the extract. After this final extraction, the resulting poultry sample was dried and used for spiking experiment to determine MDL and recovery rate. Recovery rate was determined by spiking seven of 0.5 g of “clean” poultry litter sample with 10  $\mu$ l 5- $\mu$ g mL<sup>-1</sup> (50 ng) standard. Additionally, seven of 0.5 g of the “clean” poultry samples were spiked with each ionophore by adding 10  $\mu$ l of 2- $\mu$ g mL<sup>-1</sup> (20 ng) of ionophore standards for determining the MDL. These samples were extracted and analyzed using the method described previously. Recovery efficiency was determined as the ratio of the amount recovered to that of the amount spiked. The method detection limit (MDL) was determined according to the Code of Federal Regulations, Part 136, by multiplying the standard deviation of the calculated concentration of the seven spiked samples (20 ng spike) with the Student’s t-value associated with the 99% confidence interval at six

degrees of freedom. For field samples, non-detect observations were assigned a concentration equivalent to one-half of the MDL according to established practices (Clarke, 1998; Hornung and Reed, 1990). Table 2-2 presents regression parameters of the standard curves used for calibration in the HPLC-MS/MS.

Table 2-2: Regression equations for standard curves of ionophores in HPLC-MS/MS

Analyte	Line Equation	R <sup>2</sup>
Monensin	y= 24.6 x+2.82 e <sup>-005</sup>	0.99
Lasalocid	y= 5.64 x+1.87 e <sup>-005</sup>	0.99
Salinomycin	y= 0.95 x+7.5 e <sup>-007</sup>	0.99
Narasin	y= 1.22 x+0.013	0.99

### 2.3. RESULTS AND DISCUSSION

The MDL for LC-MS/MS ranged from 0.67  $\mu\text{g kg}^{-1}$  for nigericin to 2.02  $\mu\text{g kg}^{-1}$  for salinomycin, with a rather small sample mass of 0.5 g. The seven point calibration curves were linear over a fairly wide range of 0-300  $\text{ng mL}^{-1}$  with r-square value greater than 0.99 in all cases. The recovery for the ionophores ranged from 92% for monensin to 104.4 % for salinomycin.

Without the phosphoric acid cleanup, we observed a significant shift in retention time as well as split peaks for ionophores in poultry samples (Figure 2-2) compared to the standard solutions (Figure 2-1). The issue of shift in retention time and split peaks, particularly for salinomycin and narasin, have not been discussed previously in the method development literature. The chromatogram for the duplicate sample that underwent phosphoric acid sample treatment prior to the SPE cleanup is shown in Figure 2-3. The phosphoric acid treatment resulted in distinct improvement in retention time shift (Table 2-3) as well as the split peak observed previously. The shift in retention that we observed was likely due to a higher pH as well as the presence of protein in the litter

samples. Previous studies have reported that even with a change of 0.1 pH units an approximate 10% shift in retention time can happen for reverse-phased chromatography (Nueu, 2001). So the use of phosphoric acid in our case resulted in denaturing of the proteins as well as adjusting the pH.

Table 2-3: LC-MS/MS retention time (RT) before and after inclusion of phosphoric acid treatment in the extraction method with the standards.

Analyte	RT of standards	RT before acid treatment	RT after acid treatment
		minutes	
Monensin	5.62	6.15	5.62
Lasalocid	4.95	5.29	4.92
Salinomycin	4.21	5.95-6.35 (split-peaks)	4.21
Narasin	4.49	6.36-7.09 (split-peaks)	4.50
Nigericin	7.57	Na	7.56

The use of hydrophilic-lipophilic balanced cartridges that have already been recommended for solid phase extraction of ionophores from complex matrices like sediments and soil (Hansen et al., 2009a; Kim and Carlson, 2007) proved to be efficient for poultry litter as well. The cartridges were activated with 3 ml of methanol and DI water that also removed impurities after loading the analytes. 9 mL of DI water was used after loading for washing to remove loosely bound impurities for cleaning the analytes locked within the cartridges.

The measured concentration of ionophores in the aged poultry litter sample is presented in the Table 2-4, adjusted for the recovery of surrogate by normalization. Out of all the ionophores in our study, monensin was found to be present at the highest mean concentration of  $97.86 \pm 3.18 \mu\text{g kg}^{-1}$ , followed by salinomycin at  $70 \pm 2.74 \mu\text{g kg}^{-1}$ , and narasin at  $57.31 \pm 2.57 \mu\text{g kg}^{-1}$  and finally lasalocid was present at a much lower

concentration of  $19.19 \pm 6.6 \mu\text{g kg}^{-1}$ . The poultry litter samples were ground and sieved before sample analyses as that gave more consistent data than using fresh poultry litter.

Table 2-4: Measured Ionophore concentration in poultry litter normalized by percent recovery of nigericin surrogate.

Replicate	Monensin	Lasalocid	Salinomycin	Narasin
	----- $\mu\text{g kg}^{-1}$ -----			
1	95.93	28.18	68.37	56.42
2	99.95	13.42	67.62	60.98
3	91.67	15.37	67.82	56.2
4	99.04	26.27	67.75	52.93
5	98.02	23.66	71.64	57.76
6	99.26	14.87	74.04	57.4
7	101.1	12.54	72.78	59.48
Mean	97.86	19.19	70	57.31
Standard Deviation	3.18	6.6	2.74	2.57

Our findings regarding relatively high monensin concentration compared to other ionophores is consistent with the usage data. Monensin is one of the most extensively used ionophore in animal feed due to its anticoccidial property. In the US, monensin is almost exclusively used as cattle and poultry feed additives (Chapman et al., 2010; Watanabe et al., 2008) . The usage of Monensin at sub-therapeutic levels in cattle and poultry production has been reported to be approximately 1500 Mg (Mellon et al., 2001) which is higher than the use of other ionophores. Although no specific usage data are reported, poultry industries on the Mid-Atlantic region are known to be using ionophores such as salinomycin, narasin and monensin in their poultry. Our findings are consistent with the reports of these ionophore usage in these areas (Fritz, 2008; Shin, 2008). A recent study (Kim and Carlson, 2006; Kim and Carlson, 2007) reported presence of

monensin, salinomycin and narasin, in stream water and sediments, with the highest monensin concentration reported near animal rearing operations. In a separate study, monensin was found to be present in highest concentration amongst a mixture of antimicrobials in run-off samples, indicating that these analytes can be easily transported to surrounding soil and water systems (Davis et al., 2006). A recent study in Canada on ten veterinary antimicrobials, detected monensin at the highest frequency and also at the highest concentration that significantly correlated with manure production in the area (Forrest et al., 2011).

A separate Canadian study, (Furtula et al., 2009) analyzed poultry litter piles from farms in British Columbia and reported significant quantities of narasin, salinomycin and monensin (4.1, 6.5 and 0.06 mg kg<sup>-1</sup> respectively) with overall ionophore concentrations ranging from 10-11,000 µg kg<sup>-1</sup>. The comparatively lower concentration of monensin was reported to be due to low usage in feed in the area. This large concentration range of ionophores indicates heterogeneous nature of poultry litter that has been analyzed, mainly because litter is a mixture of manure, bedding and woodchips. Nonetheless, this study is important, as there is hardly any data on the concentration of ionophores in poultry litter. Previous studies have shown that just after 35 days of stockpiling manure on farm in an unattended condition, 54-76 % of monensin were found to be degraded (Dolliver et al., 2008). In our case the poultry litter collected from litter piles were stockpiled and stored in unattended conditions for over three years, which should have led to further biotic and abiotic degradation of the ionophore analytes. Degradation of the ionophores in our samples during storage is one possible explanation for the lower concentrations detected in our samples compared to those reported by Furtula et al., (2009).

Available literature shows that half-lives of these ionophores can range from 3 to 17 days with almost 99% degradation in 36 to 38 days depending if it is soil or manure matrix (Carlson and Mabury, 2006; Dolliver et al., 2008; Furtula et al., 2009; Sassman and Lee, 2007). However, our findings show that relatively high levels of these ionophores in the range of  $\mu\text{g kg}^{-1}$  (ppb levels) can be present in aged poultry litters (>3 years old). This suggests that aged poultry litters may represent a unique matrix where the half-life of these ionophores can be substantially longer, for reasons currently unknown. Therefore, persistence of these compounds in aged poultry litters needs to be further investigated to understand how persistence and bioavailability may change during storage and after subsequent land application.

Lasalocid in animal manure and associated water and sediments has not been studied much extensively, most likely because it is less likely to be used in animal feed as compared to other ionophores (Furtula et al., 2009; Kim and Carlson, 2006). Usage of lasalocid in animal feed have been reported to be much lesser than monensin (Mellon et al., 2001). Our results for lasalocid are consistent with these observations. In addition to the US and Europe, extensive use of ionophores has been reported in parts of Asia including China, Korea and India with ionophore toxicity reported to be a potential occupational hazard for poultry farm workers due to accidental consumption during feed mixing (Shao et al., 2009; Sharma et al., 2005).

#### **2.4. CONCLUSIONS**

In summary, we have developed a rapid and improved HPLC-MS/MS based method for detection of ionophores in aged poultry litter samples that uses appropriate internal standard and a surrogate with similar chemical properties as the analytes to



account for signal suppression and recovery loss. Many of the previous studies have chosen simatone, a common pesticide, as their internal standard, even though it has very low molecular weight and quite different chemical properties than the ionophores. This modification was based on previous suggestions (Hansen et al., 2009a). Using this method, we successfully measured concentration of commonly used ionophores in aged poultry litter. The inclusion of phosphoric acid cleanup significantly improved the shift in chromatographic retention time as well as split peaks. Results from our studies show significant presence of ionophores monensin ( $97.8 \pm 3.2 \mu\text{gkg}^{-1}$ ), lasalocid ( $19.2 \pm 6.6 \mu\text{gkg}^{-1}$ ), salinomycin ( $70 \pm 2.7 \mu\text{gkg}^{-1}$ ) and narasin ( $57.3 \pm 2.6 \mu\text{gkg}^{-1}$ ) in poultry litter stored for unknown period outdoors and then over three years at less than 5°C. Our findings suggest that ionophores can persist in stored poultry litter longer than previously thought.

Our findings indicate that even after several years of unmanaged storage of poultry litter, the ionophores continues to persist in the matrix, exhibiting incomplete degradation. However our study is limited by the fact we do not know under what conditions the poultry litter was stored prior to collection from the field. Furthermore, the litter samples used in this study were collected from a facility that processed and resold litter from multiple farms. Therefore the results of our analyses cannot be related to specific poultry rearing practices. The poultry samples were taken as composite samples from very selective poultry farms on the Delmarva Peninsula. Hence these concentrations cannot be related to feed management practices for specific farms.

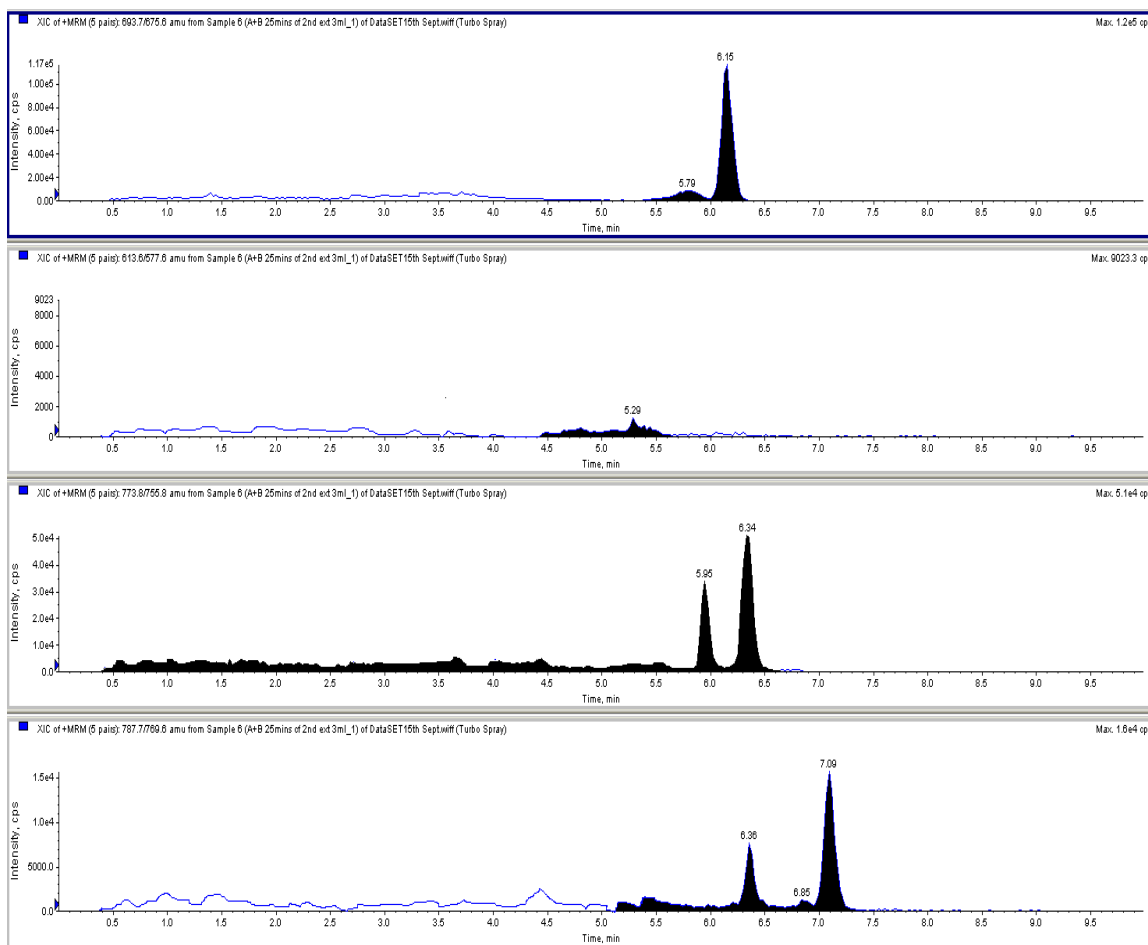


Figure 2-1. High performance liquid chromatography tandem mass spectrometer extracted-ion chromatogram for ionophores extracted from poultry litter before phosphoric acid treatment was used in extraction, causing split peaks to occur. Panels from top to bottom: monensin, lasolocid, salinomycin, and narasin.

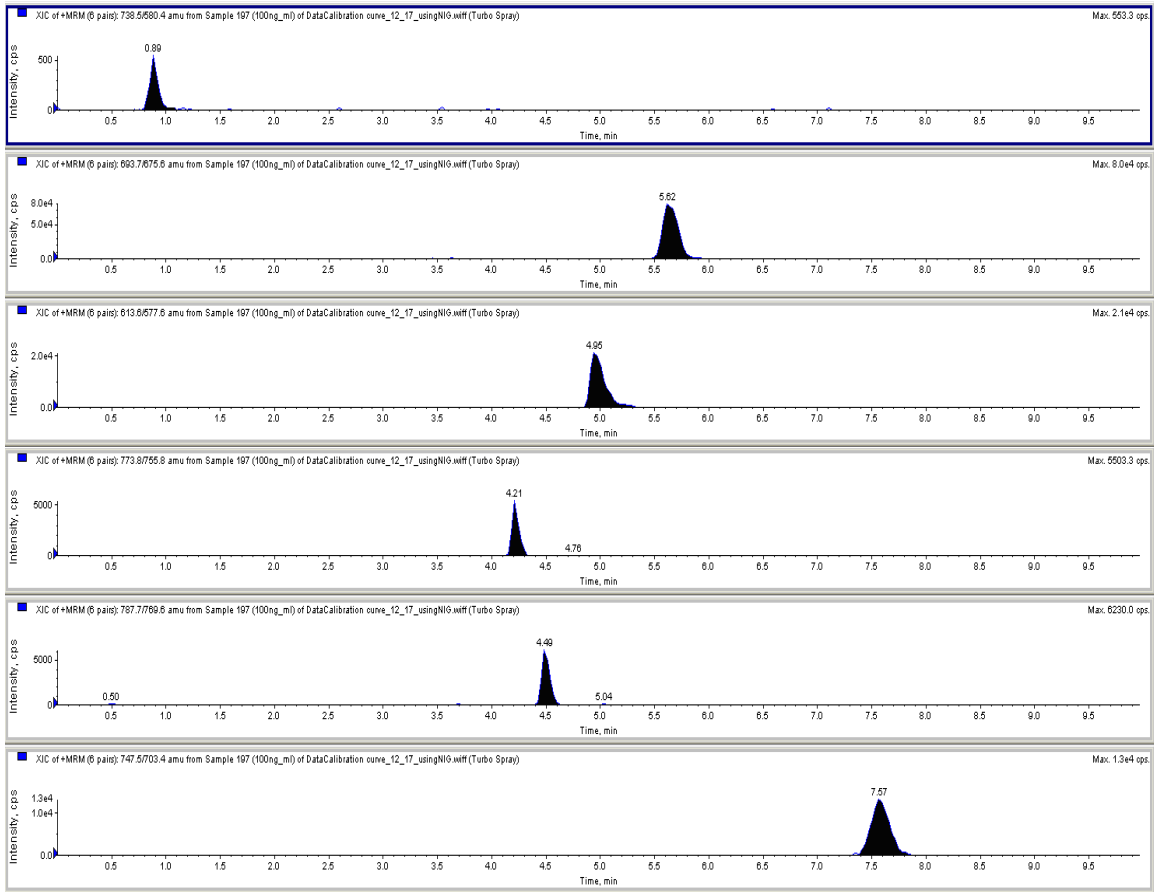


Figure 2-2. High performance liquid chromatography tandem mass spectrometer extracted-ion chromatogram for ionophore standard solutions erythromycin-d3, monensin, lasalocid, salinomycin, narasin, and nigericin (presented top to bottom).

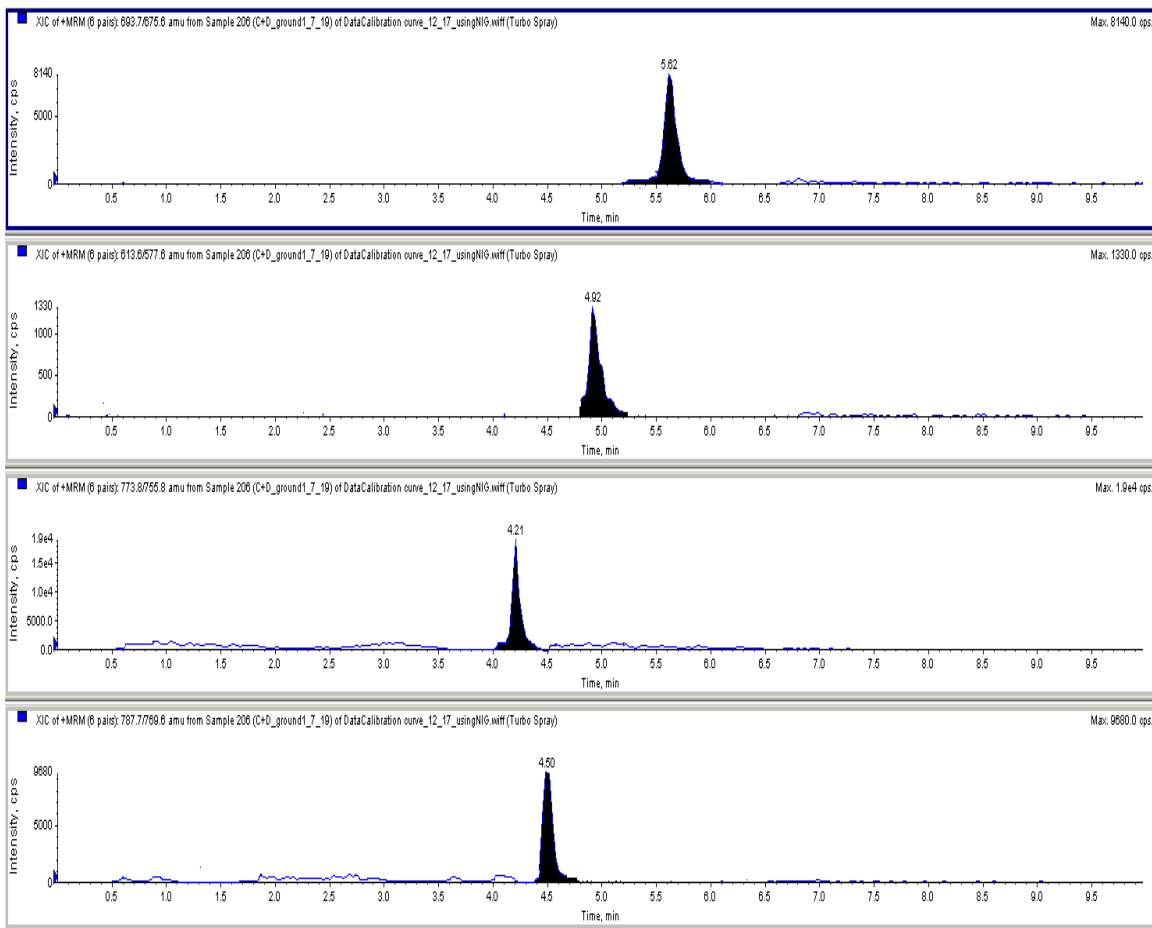


Figure 2-3. High performance liquid chromatography tandem mass spectrometer extracted-ion chromatogram for ionophores extracted from poultry litter after phosphoric acid treatment, resolving split-peak issues. Panels from top to bottom: monensin, lasalocid, salinomycin, and narasin.

## CHAPTER 3. MONENSIN SORPTION AND DESORPTION IN SOIL

### 3.1. INTRODUCTION

Anticoccidials or ionophores are non-clinical antimicrobials that are most frequently used as feed-additives to prevent and treat coccidiosis, a major protozoan disease occurring in commercial poultry production, and to promote growth (Hansen et al., 2009a; Hansen et al., 2009b). Ionophores are only used in animal production. Typical feed concentrations for poultry range from 100 – 200 mg kg<sup>-1</sup> depending upon animal rearing practices (Furtula et al., 2009). Poultry production has continued to increase as global demand for meat has increased, with production increasing from 15.5 to 28 million Mg between 1990 and 2009, globally (Mellon et al.; 2001).

Livestock manure is considered the most likely source of ionophores in the soil environment as most in feed are excreted undigested. Around 80% of the ionophore, lasalocid fed to poultry was found to be excreted in the manure (EFSA 2004). More than 13 Mg of poultry litter is produced across the U.S. per year and greater than 90% is applied in agriculture (Moore, et al., 1995). Poultry litter is composed of manure, bedding materials and feathers and is a good source of crop nutrients like nitrogen (N), phosphorus (P) and potassium (K). Unlike human waste, there are no requirements for processing of poultry litter before discharge into the environment. Typically, manure application is based on nutrient requirements of the crop and does not consider content of emerging contaminants like ionophores.

Ionophores have been quantified in manure, soil, and water at concentrations of 0.01 – 20 mg kg<sup>-1</sup>, 0.9 – 31.5 µg kg<sup>-1</sup> and 0.001 – 0.038 ng L<sup>-1</sup>, respectively. (Dolliver and Gupta, 2008; Hansen et al., 2009a; Kim and Carlson, 2006, Biswas et al., 2012).

Specifically, ionophores have been found in poultry manure ranging from 10  $\mu\text{g kg}^{-1}$  to 200,000  $\mu\text{g kg}^{-1}$  (Biswas et al., 2012; Furtula et al., 2009; Halling-Sorensen et al., 1998; Hansen et al., 2009a; Hansen et al., 2009c; Kumar et al., 2005). Hansen et al. (2009b) ionophores in sediments above predicted no-effect concentrations and opined them to pose environmental risk due to their toxicity.

Anticoccidials are biochemically known as ionophores due to their ion-bearing properties and crossing biological membranes where they affect major physiological systems such as the cardiac, nervous and muscular systems (Dowling, 1992; Oehme and Pickrell, 1999). Ionophores toxicity occurs in animals, including humans at tissue concentrations above 1  $\text{mg kg}^{-1}$ , with lethal doses in the range of 100-200  $\text{mg kg}^{-1}$  (Al-Dobaib and Mousa, 2009; Story and Doube, 2004). The medial lethal dose ( $\text{LD}_{50}$ ) for monensin is 35  $\text{mg kg}^{-1}$  in tissue for adult rats and 100  $\text{mg kg}^{-1}$  lasalocid (Sassman and Lee, 2007). At present there exists no antidote or treatment for their toxicity (Al-Dobaib and Mousa, 2009; Kart and Bilgili, 2008).

It is important to understand ionophore persistence and mobility in the soil system due to potential environmental risks as discussed above. Though ionophores have been found to have degradation half lives of ~17 days in manure, some studies have shown that even after composting manure for 35 days, 24 - 45% of ionophores present persisted (Dolliver and Gupta, 2008). This suggests that these compounds can persist in the environment, longer than their half-lives would indicate. The degraded metabolites can also be transformed back to the parent product, thereby increasing their persistence as reported for other antimicrobials (Boxall et al., 2003). Though degradation and dissipation half-lives of 2-5 days have been found in various soil types, ionophores are

still a concern as they have been found to ‘pseudo-persist’ in the environment due to constant introduction to the soil and water systems through current manure management practices (Carlson and Mabury, 2006; Halling-Sorensen et al., 1998; Sassman and Lee, 2007).

Few data exist on the mobility of ionophores due to lack of an efficient method to quantify them from complicated environmental matrices like manure. Watanabe et al. (2008) studied the persistence of monensin in soil-manure systems on dairy farms and found monensin concentrations of up to  $0.39 \mu\text{g L}^{-1}$  in ground water near the dairy farms. They suggested that further studies on transport and soil sorption mechanisms of ionophores were warranted.

It is hard to predict sorption processes of chemicals from octanol - water partitioning ( $\log K_{ow}$ ) and organic-carbon normalized soil sorption ( $\log K_{oc}$ ) coefficients for ionophores due to the chemical complexity of soil systems (Boxall et al., 2003; Tolls, 2001). Hence other parameters need to be studied that affect the ionophore-soil sorption processes. Studies on other antimicrobials have shown that soil sorption mechanisms are related to factors like pH, cation exchange capacity (CEC), soil texture compounded with % organic matter, and manure chemistry (Boxall et al., 2003).

Sassman and Lee (2007) studied sorption and degradation patterns of monensin and lasalocid in eight different soil types. They found monensin in the sediments as well as aqueous phase of natural waters, while lasalocid was associated with soil (Sassman and Lee, 2007). Similarly, Davis et al (2006), investigated several different classes of antimicrobials and found monensin at the highest concentration in the field run-off, showing that monensin was more hydrophilic than lasalocid in those soils. In the same

study, Log K<sub>oc</sub> of monensin was found to be inversely proportional to soil pH and monensin dissipation was found to be effected by abiotic conditions. Conversely, biotic factors such as microbial degradation controlled lasalocid persistence (Sassman and Lee, 2007). It is important to note that in the above study, monensin and lasalocid were studied separately in the soils, hence the possibility of competitive sorption or interactions between the ionophores if any, were not evaluated. Another study on monensin, salinomycin, and narasin using soils collected from constructed treatment wetlands found sandy clay loam to have higher K<sub>oc</sub> values for all three ionophores compared to sandy soil (Hussain S.A. and Prasher, 2011). Narasin had the highest hydrophobicity amongst them, while monensin had the lowest, the latter in line with the findings of Sassman and Lee (2007). Once again, ionophore sorption was inversely proportional to pH, but at a very high pH of 8.5 this was reversed, probably due to complexation of the anionic ionophores with the metal ions when pH exceeded the pK<sub>a</sub>. The authors reported that this result contradicted trends expected from Log K<sub>ow</sub> values, which was not the case as already reported by Sassman and Lee (2007) where Log K<sub>ow</sub> of monensin increased instead of decreasing from pH 7 to 9. The latter study on constructed wetland soils had other discrepancies as well. No reasoning was given on the selection of sandy soil (99% sand) as representative of a wetland soil, as wetlands are generally known to contain hydric soils. Furthermore, the experimental design was an incomplete factorial, which led to a lack of statistical significance in the results. (Hansen et al., 2009a) hypothesized that metal concentration might relate to ionophore sorption in soil, since metal-ionophore complexes occur at higher pH.



There is a clear need in the literature to better understand ionophore mobility in soil and water systems. Fundamental to this understanding would be the sorption and desorption behavior of ionophores in soil systems. Therefore our objective was to determine soil sorption and desorption characteristics for monensin in mid-Atlantic soils.

## **3.2. MATERIALS AND METHODS**

### **3.2.1. Soil Collection and Preparation**

Five farms located across the Delmarva Peninsula were selected for sampling (Figure 3-1 The Delmarva Peninsula was selected because of the presence of large, concentrated poultry industry that could potentially be a source for large quantities of ionophores in the environment. Therefore, it is important to understand ionophore behavior in soils of this region. Four of the farms were university research farms and one was a privately held research farm. These sites allowed the greatest access to field management history. The soils on these farms generally represent common soils used for crop production across the Delmarva that might receive poultry litter application. In order to represent a broader range of soil series in this study samples were collected from each map unit present on each of the farms. Details of the sampling sites, along with geographical co-ordinates, map units and site collected have been tabulated in Table 3-1.

Table 3-1. Map unit descriptions and sampling locations for soils used in sorption-desorption studies.

Sample No.	Farm <sup>†</sup>	Map Unit Description	Latitude	Longitude
1	CF	Colts Neck loam , 5-10% slope	39°11'31.06"N	76°10'6.84"W
2	CF	Elkton silt loam	39°11'10.09"N	76°11'17.17"W
3	CF	Sassafras loam, 2-5 % slope	39°11'39.20"N	76°10'19.07"W
4	CF	Sassafras gravelly loam, 10-15% slope	39°11'38.99"N	76°10'7.20"W
5	CF	Butlertown-Mattapex silt loam, 2-5% slope	39°10'6.25"N	76°10'53.14"W
6	CF	Keyport fine sandy loam, 0-2% slope	39°09'40.87"N	76°11'11.85"W
7	CF	Keyport silt loam, 5-15% slope	39°09'48.54"N	76°11'0.30"W
8	CF	Mattapeake silt loam, 5-10% slope	39°10'12.13"N	76°11'0.44"W
9	CF	Mattapex silt loam, 2-5% slope	39°09'52.95"N	76°11'7.33"W
10	CF	Mattapex variant silt loam, 0-2% slope	39°10'46.65"N	76°11'0.07"W
11	CF	Sassafras sandy loam, 2-5% slope	39°11'2.41"N	76°10'50.07"W
12	CF	Woodstown sandy loam, 2-5% slope	39°09'83.56"N	76°11'1.24"W
13	LESREC	Evesboro sand, 2-5% slope	38°22'13.88"N	75°39'19.50"W
14	LESREC	Fort Mott loamy sand, 0-2% slope	38°22'25.94"N	75°39'38.83"W
15	LESREC	Pepperbox-Rockawalkin complex, 0-2%	38°22'33.88"N	75°39'28.73"W
16	LESREC	Rosedale loamy sand, 0-2% slope	38°22'28.88"N	75°39'18.95"W
17	LESREC	Zekiah silt loam, frquently flooded	38°22'32.24"N	75°39'46.59"W
18	PH	Evesboro sand, 5-10% slopes	38°21'42.83"N	75°47'8.25"W
19	PH	Hambrook sandy loam, 0-2% slope	38°21'35.08"N	75°46'27.93"W
20	PH	Mattapex silt loam, 0-2% slope	38°21'23.23"N	75°46'41.56"W
21	PH	Nassawango silt loam, 0-2% slope	38°21'31.39"N	75°46'40.96"W
22	PH	Othello silt loam, 0-2% slope	38°21'13.05"N	75°46'48.46"W
23	PH	Runclint- Cedartown complex,2-5% slope	38°21'39.59"N	75°47'5.29"W
24	UD	Delanco silt loam, 3-8% slope	39°40'06.50"N	75°44'42.15"W
25	UD	Elsinboro silt loam, 3-8% slope	39°58'00"N	75°44'38.10"W
26	UD	Keyport silt loam, 0-2% slope	39°39'39.40"N	75°44'33.07"W
27	UD	Mattapex silt loam, 0-2% slope	39°40'17.32"N	75°44'16.41"W
28	UD	Othello silt loam,0-2% slope	39°40'10.59"N	75°44'36.20"W
29	UD	Sassafras sandy loam, 2-5% slope	39°40'11.04"N	75°44'3.61"W
30	UD	Zekiah sandy loam, frequently flooded	39°40'13.58"N	75°44'7.38"W
31	WREC	Downer and Unicorn soil, 10-15% slope	38°54'2.05"N	76°8'28.83"W
32	WREC	Ingleside sandy loam, 0-2% slope	38°54.5'69"N	76°8'24.26"W
33	WREC	Mattapex-Butlertown silt loam, 0-2% slope	38°53'55.08"N	76°8'13.13"W
34	WREC	Unicorn silt loam, 2-5% slope	38°53'58.06"N	76°8'17.69"W
35	WREC	Unicorn-Sassafras loam, 0-2% slope	38°54'3.55"N	76°8'6.69"W
36	WREC	Nassawango silt loam-0-2% slope	38°54'44.50"N	76°8'47.85"W
37	WREC	Whitemarsh silt loam	38°55'0.74"N	76°8'56.09"W

<sup>†</sup>CF: Chesapeake Farms; LESREC: Lower Eastern Shore Research and Education Center; PH: Poplar Hill Farm; UD: University of Delaware; WREC: Wye Research and Education Center.

Soil samples were collected from 37 different soil map units across the five farms. Soils were collected separately from the A and B horizons. Three replicate cores were collected at each sample location using a Giddings hydraulic probe measuring 3.81 cm in diameter to a 1 m depth. The soil cores were divided at the interface of the A and B horizons. The three A horizon cores and the three B horizon cores were then mixed together to form a single composite sample for each horizon at each sample location. The composite samples were then sieved in the field to pass a 7 mm wire mesh to remove debris and organic detritus. This resulted in a total of 74 soil samples (37 map units by two horizons). The samples were transported to the lab in cloth sample bags and then laid out on paper plates to be air-dried. After drying, the samples were ground to pass through a 2-mm sieve.

### **3.2.2. Evaluation of background ionophore content**

After collection, the soils were screened for background ionophore concentrations. The soil samples (0.5 g) were weighed into 15 ml polypropylene centrifuge tubes and then spiked with 10  $\mu\text{l}$  of 5- $\mu\text{g ml}^{-1}$  surrogate Nigericin. After spiking, the soil samples were treated with 12 ml of 20% aqueous phosphoric acid solution (v/v), and sonicated (Branson 3510, CT, USA) at room temperature for 15 minutes followed by 15 minutes centrifugation at 10,000  $\times g$  (Beckman Coulter, CA, USA). The phosphoric acid extract was not found to contain any of the analytes, and discarded. After the acid treatment, the sample was extracted in 12 ml of 1:1 (v/v) methanol to water solution and then sonicated and centrifuged as before. The extracts thus obtained were loaded onto HLB cartridges that were pre-conditioned with 3 ml methanol and 3 ml de-ionized (DI) water. The cartridges were mounted on a vacuum

manifold (Sigma Aldrich Co., St. Louis, MO) and extraction done by loading under a steady vacuum pressure of 5kPa. The HLB cartridges were then washed with 9 ml of DI water to remove any impurities and traces of phosphoric acid. The ionophores were eluted with 5 ml of methanol and the extracts were concentrated at 50 °C under gentle flow of nitrogen using an evapo-heater (Thermo Scientific, MA, USA). The samples were reconstituted in 1 mL of 1:1 acetonitrile and 0.1% formic acid. To the 1 mL sample, a 10- $\mu$ l aliquot of 2- $\mu$ g ml<sup>-1</sup> Eryd3 internal standard was added.

Extract analysis was performed using a Shimadzu HPLC 10 AVP Series combined with API 3000 mass spectrometer (Applied Biosystems Sciex, USA), operated and controlled by the Analyst software (version 1.4.1). Chromatographic separation was achieved using 10x2.1 mm C18 Aquasil column (Thermo Scientific, WI, USA) with 3- $\mu$ m particle size. The mobile phase consisted of a 1:1 mixture (v/v) of acetonitrile and 0.1% formic acid in the first minute, this was then ramped up to 90% acetonitrile over the next 8 minutes and then held at 90% for an additional minute, with a flow-rate of 0.25 ml min<sup>-1</sup>. The injection volume was 10  $\mu$ l and each chromatographic run was 10 min long. The mass spectrometer was operated in positive electrospray ionization mode with source temperature at 400 °C and electrospray capillary voltage at 5 kV. The MS/MS parameters were optimized by constant infusion of the standard solution of concentration 1  $\mu$ g ml<sup>-1</sup> at the flow rate of 10- $\mu$ l min<sup>-1</sup>. The optimized compound parameters and instrument parameters of the multiple reaction monitoring transitions used for the analyses were kept as before.

### **3.2.3. Preparation of monensin solution for method development**

Monensin is sparingly soluble in water with solubility ranging from 0.003-10 mg L<sup>-1</sup> (Hansen et al.; 2009, Kim and Carlson, 2006). Therefore, in order to make a solution for use in the sorption and desorption studies it was necessary to initially dissolve the monensin in methanol and then dilute it further to the target concentration. To make the monensin-methanol solution, 1 g monensin (Sigma Aldrich Co. cat# 46468, St. Louis, MO) was dissolved in 20 mL methanol and then 2 mL of this solution was brought to a volume of 100 mL with DI water. During method development a fresh stock solution was prepared monthly. Additional concentrations of monensin solution were prepared from this stock solution by dilution with DI water for the methods detailed in subsequent sections. For the purpose of our studies, a background salt concentration (e.g. 0.01 M CaCl<sub>2</sub> or 0.01 M NaNO<sub>3</sub>) was not used as seen in the literature for similar studies. Instead the monensin solution used in our sorption and desorption studies was prepared using DI water to avoid introduction of highly charged ions like Na<sup>+</sup> and Ca<sup>2+</sup> in the mass spectrometer, as they are hard to remove and cause ion suppression, affecting the chromatograms and the machine. In addition, washing away these ions without losing any ionophores from the extracts would be time consuming, especially when handling a large number of samples in a time-bound experiment.

### **3.2.4. Determination of monensin concentration using HPLC-MS/MS**

For the method development and the complete batch equilibrium study, monensin concentrations were determined in filtrate using a Shimadzu HPLC 10AVP Series combined with the API 3000 mass spectrometer (Applied Biosystems Sciex, USA), which was operated and controlled by the Analyst software (version 1.4.1.) The method

was similar to that developed for analyzing poultry litter extracts (Chapter 2). Chromatographic separation was achieved using 10x2.1mm C18 Aquasil column (Thermo Scientific, WI, USA) with 3- $\mu\text{m}$  particle size. After centrifugation and filtering as described above, 1 mL of the filtrate was transferred into amber HPLC vial, and 10  $\mu\text{L}$  of 2  $\mu\text{g mL}^{-1}$  internal standard lasalocid was added to it. HPLC-MS/MS parameters were set to what was used in Chapter 2, to measure monensin in poultry manure. These parameters included time program of mobile phases that were HPLC grade acetonitrile and 0.1% aqueous formic acid solution, declustering potential, focusing potential, cell exit potential and collision energy for monensin to determine parent-daughter ion. The mobile phase consisted of 1:1 mixture (v/v) of acetonitrile and 0.1% formic acid for 1 minute that was ramped to 90% acetonitrile over eight minutes and then held at 90% for an additional minute, with a flow-rate of 0.25  $\text{ml min}^{-1}$ . The injection volume was 10  $\mu\text{l}$  and each chromatographic run was 10 minutes long. The mass spectrometer was operated in positive electrospray ionization mode with source temperature at 400  $^{\circ}\text{C}$  and electrospray capillary voltage at 5 kV. The MS/MS parameters were optimized by constant infusion of the standard solution of concentration 1  $\mu\text{g mL}^{-1}$  at the flow rate of 10  $\mu\text{l min}^{-1}$ . The optimized compound parameters of the multiple reaction monitoring transitions used for the analyses are presented in Table 2-1. Extracted -ion chromatogram from filtrates of batch study is presented in Figure 3-2.

Quality assurance/quality control (QA/QC) samples were run after every 15 experimental samples. The QA/QC samples included a check monensin solution not reacted with soil; a check soil where DI water was equilibrated with soil and no monensin was added; and a DI water blank without soil or monensin. During the complete batch

equilibrium study, each experimental sample was run in duplicate. Table 2-2 presents the regression equation and  $R^2$  of the calibration curves for the HPLC-MS/MS.

### **3.2.5. Development of batch equilibrium methods**

Batch equilibrium experiments are commonly used to study how compounds interact with their environment (e.g., soil, water, air). Data generated from these studies can be used to predict chemical degradation, transformation of chemicals or uptake by organisms, potential for leaching, volatility from soil (especially for low molecular weight chemicals), or run-off from land to surface water bodies. The adsorption-desorption data generated can also be used for comparative or modeling purposes. The objectives of such studies are to obtain a sorption value that can be used to predict partitioning under a variety of environmental conditions. Equilibrium adsorption coefficients for a chemical on various soils are determined as a function of soil characteristics (e.g., organic carbon content, soil texture, pH). The desorption values along with the sorption values can show hysteresis patterns thereby furthering our knowledge of the dynamics of the chemical in a system.

In order to evaluate the sorption and desorption characteristics of monensin, the ionophore found in the greatest concentration in Delmarva poultry litter (Biswas et al.), a batch equilibrium method was developed. Our method was developed through a series of pilot studies, based on available literature (Sassman and Lee, 2007; Hussein S.A. and Prasher, 2011) and EPA guidelines (EPA, 2008) for performing batch equilibrium. A subset of six soil samples from the 74 collected were selected for method development. The six samples represented the A and B horizons of three of the 37 locations sampled. The samples included an Evesboro sand (Mesic, coated Lemellic Quartzipsamments), a

Mattapex silt loam (Fine-silty, mixed, active, mesic Aquic Hapludults) and a Sassafras sandy loam (Fine-loamy, silicious, semiactive, mesic Typic Hapludults), sampled from Lower Eastern Shore Research and Education Center, Poplar Hills and University of Delaware Research Farm, respectively. .

The objective of the pilot study was to determine optimum (i) soil to solution ratio; (ii) equilibration time – sorption phase; (iii) time required for desorption phase; and (iv) initial concentration for sorption study. These steps were performed iteratively to evaluate each parameter in the context of the others.

#### *3.2.5.1. Optimization of soil to solution ratio*

Trials were initially conducted to determine the soil to solution ratio. 55% to 65% of monensin in solution would be sorbed to the soil. This range was selected to ensure that the analyte concentration in both the sorbed and desorbed phase was high enough for detection. Sassman and Lee (2007) found that if less than 20% of the analyte was sorbed then it was difficult to detect in the solid phase and if more than 70% was sorbed it was difficult to detect in the solution phase.

An aqueous solution of  $1 \mu\text{g mL}^{-1}$  of monensin was added to 1 g of soil to obtain target soil to solution ratios of 1:5, 1:10, 1:20, 1:30, 1:50, 1:75 and 1:100. The tubes were placed on end-to-end shaker for 12 hours. The supernatant was then decanted into centrifuge tubes, centrifuged for 15 minutes at 1500 g and then filtered through a  $0.45 \mu\text{m}$  filter paper. The filtrates were stored at  $4^{\circ}\text{C}$ , until analyzed using the HPLC-MS/MS method as discussed in Section 3.2.4.



### *3.2.5.2. Equilibration time optimization-sorption study*

The shake time for the batch equilibrium experiment was optimized so that the soil-solution system reached equilibrium, allowing equilibrium partition coefficients ( $K_d$ ) to be accurately estimated. If the system were reacted for too long, past the sorption maximum ( $S_{max}$ ), desorption would start to occur, and give inaccurate estimates. Likewise, using a shake time that was too short would result in the soil-solution system not reaching equilibrium, and  $K_d$  estimate would be inaccurate. According to USEPA protocol (EPA-OPPTS 835.1230,2008) the optimum equilibration time should be determined at the time point where analyte concentration in solution changes less than 5%. Therefore, a series of time trials were conducted by shaking 1 g of soil in 20 mL of 1  $\mu\text{g mL}^{-1}$  of monensin solution. The shake times evaluated were 1, 6, 12, 18, 24, 48 and 72 hours. After shaking, the supernatant was decanted into centrifuge tubes, centrifuged for 15 minutes at 1500  $\times g$  and then filtered through a 0.45  $\mu\text{m}$  filter paper. The filtrates were stored at 4<sup>0</sup>C, until HPLC-MS/MS determination of monensin concentration remaining in solution (Section 3.2.4).

### *3.2.5.3. Equilibration time optimization- desorption study*

As with sorption study, shake time for the desorption study was determined as the time when increase in shake time led to 5% decrease in monensin concentration in the solution (EPA-OPPTS 835.1230, 2008). In addition, with the desorption study it was important to consider other potential losses, like degradation. Dissociation, hydrolysis and photolysis that could take place due to prolonged shaking.

To determine the shake time for the desorption study, first a sorption experiment was conducted using optimized parameters and the solids were retained, then 20 mL DI

water was added to the centrifuge tubes. The tubes were shaken on an end-to-end shaker (Burrell, wrist action shaker, Model number 76) for 1, 6, 12, 18, 24, 48, and 72 hours. After each of these time periods, the corresponding tubes were taken off the shaker and the samples processed in the same way as before and analyzed in HPLC-MS/MS to determine the amount of monensin remaining in the filtrate. After conducting the sorption and desorption steps the solids were retained and analyzed as described in Section 3.2.4. This final step was done to be able to calculate a complete mass balance and determine if other potential losses occurred during the complete batch equilibrium experiment.

#### *3.2.5.4. Optimization of initial concentration*

It was important to select an initial monensin concentration for the batch equilibrium procedure high enough that after equilibration the concentrations in solution and solid phases were detectable. Similarly, the initial concentration could not be too high, otherwise it would be difficult for the soil-solution system to reach equilibrium and the solid phase would get saturated too quickly. In addition high concentrations of analyte can cause problems in sensitive detection and ion suppression with the HPLC-MS/MS system. During method development several trial concentrations were tested and signal to noise ratio and peak resolution were evaluated.

To determine the optimum initial concentration, 1 g of soil was equilibrated 20 mL of aqueous solution for 18 hours. Solution concentrations tested included 5, 10, 25, 50, 75, 100  $\mu\text{g mL}^{-1}$  of monensin. After shaking, the supernatant was decanted into centrifuge tubes, centrifuged for 15 minutes at 1500  $\times g$  and then filtered through a 0.45  $\mu\text{m}$  filter. The filtrates were stored at 4<sup>0</sup>C, until HPLC-MS/MS determination of monensin concentration remaining in the solution (Section 3.2.4).

### 3.2.6. Sorption-desorption pilot studies

After parameter optimization was completed a pilot sorption-desorption study was conducted in two phases. The first phase was a single time, multiple concentration sorption study and subsequent desorption. The second phase was a single concentration, multiple time sorption study with subsequent desorption. Each of these studies was conducted using the same six samples described above for the parameter optimization studies.

The single-time, multi-concentration sorption study was conducted at seven monensin concentrations, 0.25, 0.5, 1, 1.5, 2.5, 3.75 and 5  $\mu\text{g mL}^{-1}$ . Where 1 g of soil was reacted with 20 mL of the solution on an end-to-end shaker for 18 hours. After shaking, the supernatant was centrifuged for 15 minutes at 1500 xg and then filtered through a 0.45  $\mu\text{m}$  filter paper. The filtrate was stored at 4 °C until analysis on HPLC-MS/MS. The single-concentration, multi-time sorption study followed the same procedure except 1 g of soil was reacted with 20 mL of 1  $\mu\text{g mL}^{-1}$  monensin solution for 1, 6, 12, 18, 24, 48, and 72 hours.

For both the single time and single concentration sorption experiments, all solids were retained and a desorption experiment was conducted. The retained solids were reacted with 20 mL of DI water for 12 hours on an end-to-end shaker. After shaking, the supernatant was centrifuged for 15 minutes at 1500 xg and then filtered through a 0.45  $\mu\text{m}$  filter paper. The filtrate was stored at 4 °C until analysis on HPLC-MS/MS. The retained solids were then extracted as described in Section 3.2.2 to determine residual monensin not desorbed and calculate a mass balance that included monensin retained on the soil, desorbed in DI, and potentially lost from the system.

### **3.2.7. Complete batch equilibrium study**

After the parameter optimization and pilot studies were completed a single time, single point isotherm was performed on all the 74 soil samples, to study the partitioning behavior of monensin across a range of soils. The complete batch equilibrium study included a sorption and desorption phase as was done in the pilot study. For the sorption study, 20 mL of 1  $\mu\text{g mL}^{-1}$  of monensin solution was added to 1 g of each of the 74 soil samples in 50 mL centrifuge tubes. The tubes were then agitated on an end-to-end shaker for 18 hours. After shaking, the supernatants were decanted, centrifuged at 1500  $\times g$ , filtered through 0.45  $\mu\text{m}$  filter, and stored at 4  $^{\circ}\text{C}$ . The solids from the sorption phase were retained and 20 mL of DI water was added to them in the centrifuge tubes and then they were shook on the end-to-end shaker for 12 hours. After shaking, the supernatants were decanted, centrifuged at 1500  $\times g$ , filtered through 0.45  $\mu\text{m}$  filter paper and stored at 4 $^{\circ}\text{C}$ . After the sorption and desorption phases were complete the supernatant from both were analyzed for monensin concentration using the HPLC-MS/MS method described in section 3.2.4. Monensin concentration in the solids was determined in the HPLC-MS/MS after pre-treating with sonication, centrifugation and solid phase extraction as detailed in Section 3.2.2, allowing for calculation of complete mass balance.

### **3.2.8. Statistical Analysis**

SAS analytical software package 9.3 was used to perform statistical analyses. Analysis of variance (ANOVA) was used to test statistically significant differences among regression line parameters, using estimate statements to determine difference between each slope. Means and standard deviations were used to summarize the data from monensin sorption and desorption isotherms, where applicable.

### **3.3. RESULTS AND DISCUSSION**

#### **3.3.1. Soil Characteristics**

For our pilot study, our sample set contained three soil series, Evesboro (Mesic, coated Lemellic Quartzipsamments), Mattapex (Fine-silty, mixed, active, mesic Aquic Hapludults) and Sassafras (Fine-loamy, silicious, semiactive, mesic Typic Hapludults). No measurable amount of monensin, salinomycin, narasin or lasalocid was detected in the soil samples. Hence the soil samples were concluded to have no significant background ionophores and were not pre-treated before conducting further experiments.

#### **3.3.2. Batch Equilibrium Method Development Study**

Using monensin solution dissolved in methanol, we got a good signal to noise ratio, greater than 1:3 and good peak resolution. During the optimization phase of our study a soil to solution ratio of 1:20 was found to be optimum, with about 60-70 % of the analyte found to be sorbed onto the solid phase, ensuring that the analyte concentration in both the sorbed and desorbed phase was high enough for detection. In Figure 3-3, the amount of monensin on the Y-axis, corresponds to the percent sorbed at equilibrium, while the X-axis denotes the amount of monensin in the original solution. The shake time for the batch equilibrium experiment was optimized to 18 hours so that the soil-solution system reached equilibrium, allowing  $K_d$  to be accurately estimated. Figure 3-6, shows the shake time vs. monensin sorbed at equilibrium. At 18 hours of shake time, the sorption curve reached a plateau, after which less than 5% sorption occurred. Hence 18 hours was selected as shake time for monensin sorption.

The initial concentration of monensin solution was optimized as  $1 \mu\text{g mL}^{-1}$ . This was done so that adequate amount of monensin was allowed to be in the system to have

detectable concentrations of the analyte, after equilibrium partitioning, but not too high to over burden the system.

For the desorption study, the shake time was optimized to 12 hours. This optimization was done to ensure that complete desorption of monensin from the solids was allowed but at the same time, loss of monensin was prevented due to excess shaking.

### **3.3.3. HPLC-MS/MS Analysis**

The LC-MS/MS method had a method detection limit (MDL) ranging from 0.67  $\mu\text{g Kg}^{-1}$  for Nigericin to 2.02  $\mu\text{g Kg}^{-1}$  for Salinomycin, with a rather small sample mass of 0.5 g. The seven point calibration curves were linear over a fairly wide range of 0-300  $\text{ng mL}^{-1}$  with r-square value greater than 0.99 in all cases. The regression equation parameters are presented in Table 2-2.

### **3.3.4. Determination of distribution coefficients through batch equilibrium study**

In batch equilibrium study, at equilibration, the amount of monensin that disappeared from the solution phase is expected to be sorbed to the solid phase, which is the soil, in our experiment. Theoretically concentration of monensin sorbed to soil has been calculated as:

$$C_s = V (C_i - C_w) / M.$$

Here  $C_s$  ( $\mu\text{g g}^{-1}$ ) is the concentration of monensin sorbed to soil at equilibrium.  $C_i$  ( $\mu\text{g mL}^{-1}$ ) is the initial concentration of monensin in solution phase.  $C_w$  ( $\mu\text{g mL}^{-1}$ ) is the final concentration of monensin at equilibrium.  $V$  (mL) is the volume of the solution phase.  $M$  (g) is the mass of soil. The accuracy of this calculation has been determined by mass balance analyses, where the mass of monensin present in the soil after the experiment has been analyzed using HPLC-MS/MS, using the method in section 3.2.3.4.

The mass balance account for loss of monensin either during sorption or desorption process. Hence the experimental mass balance is calculated as:

Initial amount of monensin in the system (g) = mass of monensin (g) (in filtrate after sorption + in filtrate after desorption + retained in soil at the end) + mass of monensin lost in the process (g).

The mass balance for all our samples accounted for > 90 % of the initial mass of monensin. Hence < 10 % accounted for some form of loss during the process.

This is quite expected during sorption or desorption batch experiments due to biotic and abiotic degradation, such as photolytic, hydrolytic and microbial degradations. Along with that, underestimation of final mass of analyte in the retained solids is possible, due to irreversible bonding of the analyte to the solids that prevented it from being extracted for final analyses.

Literature reviews suggest that < 10% loss of total mass of analytes in these kinds of experiments is acceptable and in such cases, the analyte is considered to be stable (EPA, 2008; Sassman and Lee, 2007). However, if the mass balance accounts for > 10 % loss of total mass of analyte, then the analyte is deemed as unstable to be analyzed using batch equilibrium techniques. In our experiments, mass balance for B horizon samples accounted for > 94 % of the initial concentration, compared to that of 90 - 96 % for A horizon samples. This may be because of organic matter present in the A horizon samples that is not expected to be present in B-horizon samples, that may have supported microbial activities and losses related to biotic degradation. Further investigation on organic matter and other soil parameters and their effects on the sorption-desorption processes have been presented in Chapter 4.

The mass balance procedures in the current literature have been criticized as it only included theoretical value of  $C_s$ , without practically extracting the solid phases to compare the results (Sarmah et al., 2006). Hence true sorption values may not be estimated in such cases, where such indirect methods have been used. Due to such differences in practices it is extremely difficult to compare sorption and desorption pattern between studies.

The primary objective of conducting batch equilibrium study was to understand the partition behavior of the analyte in different solid solution phases. The partition (or distribution) coefficient,  $K_d$ , is a measure of sorption of analyte to soils and is defined as the ratio of the quantity of the analyte adsorbed per unit mass of solid to the amount of the analyte remaining in solution at equilibrium. It is the most simplest and cost-effective method available that can provide valuable information regarding mobility of the analyte.

$$K_d (\text{mLg}^{-1} \text{ or } \text{LKg}^{-1}) = \frac{\text{concentration in solid phase at equilibrium } = C_s (\mu\text{gg}^{-1})}{\text{concentration in solution phase at equilibrium } C_w(\mu\text{gmL}^{-1})}$$

$K_d$  is used in contaminant transport model, to study how far and in what ways the analyte moves through the areas under risk.

In our pilot study of isotherm, we found partition coefficient or  $K_d$  values of Evesboro soil, Mattapex soil and Sassafra soil to range from  $(6.41 \pm 1.34)$  to  $(93.11 \pm 3.58) \text{ L Kg}^{-1}$ ,  $(29.49 \pm 2.56)$  to  $(343.83 \pm 5.68) \text{ L Kg}^{-1}$  and  $(25.07 \pm 2.78) - (244.49 \pm 5.43) \text{ LKg}^{-1}$ , at isotherm pH 6.2, 5.1 and 5.9 respectively. Evesboro had the smallest  $K_d$  values and hence showed more preference in partitioning into the solution phase. Mattapex had the highest  $K_d$  values, partitioning more into the soil phases.  $K_d$  values of Sassafra was in-between these two. Figure 3-4 compares sorption isotherms of the A



horizons of all the three soils. All the isotherms were of the linear C-type. C type isotherm is also known as constant partitioning isotherm. It suggests a constant relative affinity of the analyte to the partitioning phases. Hydrophobic molecules have been found to produce these types of isotherms at their lower concentrations. From Table 1-5 we can see that Log Kow which is the measure of hydrophobicity of the analyte is highest at pH 5, around 4.2 and it decreases to below 3 with pH above 7. Hence in the pH range below 7, monensin is expected to be hydrophobic, thus justifying this kind of isotherm. C-type isotherm indicate preference of physical adsorption mechanism over chemisorption, though isotherm shapes can never be proved by mechanisms and further investigation is required to find out the processes, like molecular spectroscopy.

Sorption experiments were followed by desorption to study the reversibility of sorption processes in both A and B horizons. USEPA test guidelines states that an analyte must desorb > 75% from sorbent in atleast twice the time of sorption equilibrium to be considered reversibly sorbed (EPA, 2008). Hysteresis is the deviation between desorption and sorption isotherm. Here the pathway for sorption and desorption are different. Irreversible sorption can be verified by hysteresis in the isotherm where the sorption branch deviates from desorption as seen in all the 3 soils.

For A horizons, sorption isotherms for all the three soils were significantly different ( $p = 0.05$ ). The sorption isotherms for the A horizons of the three soils are presented in Figure 3-4. Hysteresis was observed in all the three soils due to irreversible sorption of monensin. Evesboro soil showed highest desorption (Figure 3-5), followed by Sassafras (Figure 3-6), and then Mattapex (Figure 3-7). For B horizons soils, the sorption isotherms were not significantly different in the three soils ( $p = 0.05$ ). The sorption

isotherms of the B horizon soils are presented in Figure 3-8. Desorption of Evesboro (Figure 3-9) and Sassafras (Figure 3-10) soils were higher than Mattapex (Figure 3-11) soils. Hysteresis was observed in all three soils of the pilot study presumably due to irreversible sorption of monensin. Though we found a desorption pattern in the pilot study as described here, in the main study desorption was more random and so was hysteresis. Sorption and desorption data for all 37 samples are presented in Table 3-2 for the A-horizon and Table 3-3 for the B-horizon. The regression equations tend to negative intercept, as with increase in concentration of initial solution, amount of monensin sorbed also increased, decreasing the concentration of monensin in the filtrate at equilibrium. It is to be noted that by the way this batch study was designed, monensin was not allowed to reach saturation that is  $S_{max}$ . That was done in a separate kinetic study as described in the following section.

Table 3-2. Monensin sorption and desorption parameters for A-horizon soil samples.

Sample No.	Cs	Cw	Isotherm pH	Kd	Kom	Dw	IR
	$\mu\text{g g}^{-1}$	$\mu\text{g mL}^{-1}$		-----L kg <sup>-1</sup> -----		$\mu\text{g L}^{-1}$	$\mu\text{g g}^{-1}$
1	14.25	0.22	5.41	65.37	2439.07	0.62	1.93
2	11.12	0.34	6.21	32.37	3518.76	0.48	1.56
3	13.98	0.22	5.51	64.72	3852.51	0.54	3.09
4	14.35	0.22	5.41	66.44	3148.59	0.61	2.21
5	16.09	0.09	4.81	180.79	6255.59	0.66	2.84
6	14.55	0.16	5.61	90.65	4316.87	0.67	1.1
7	16.23	0.09	5.11	171.75	14077.54	0.73	1.67
8	16.34	0.09	4.81	172.91	6427.88	0.72	1.98
9	15.92	0.11	5.21	150.9	5115.27	0.69	2.05
10	15.89	0.11	5.41	149.2	4782.11	0.68	2.33
11	13.56	0.16	5.81	83.45	4661.8	0.54	2.67
12	14.21	0.18	5.71	77.44	5694.02	0.64	1.32
13	11.43	0.34	5.81	33.72	1756.08	0.38	3.75
14	13.89	0.26	6.01	54.36	3054.16	0.56	2.68
15	13.14	0.23	6.01	56.27	4263.19	0.56	1.96
16	14.89	0.21	5.61	70.74	2997.3	0.63	2.33
17	14.31	0.23	5.71	61.95	3560.23	0.6	2.33
18	13.73	0.28	6.11	48.35	4933.17	0.56	2.45
19	15.62	0.16	6.01	99.49	4670.91	0.65	2.68
20	16.49	0.11	5.01	151.98	5314.04	0.72	2.17
21	16.02	0.07	4.71	220.97	7753.18	0.68	2.46
22	16.34	0.11	5.01	149.91	5614.54	0.69	2.45
23	12.67	0.28	5.81	44.69	4138.09	0.51	2.56
24	11.76	0.31	6.11	38.43	3525.81	0.47	2.36
25	16.21	0.1	4.51	167.98	5752.71	0.66	2.93
26	16.38	0.1	5.01	156.75	5638.36	0.71	2.17
27	13.65	0.26	6.01	52.4	4330.52	0.61	1.54
28	15.92	0.14	4.41	110.17	3825.45	0.62	3.48
29	14.21	0.16	5.71	91.38	5133.86	0.66	1.1
30	14.65	0.22	5.71	67.82	5652.01	0.64	1.76
31	14.23	0.2	5.71	71.51	4583.82	0.64	1.34
32	14.2	0.19	5.61	73.01	3493.19	0.57	2.86
33	15.89	0.15	5.41	108.84	3714.53	0.66	2.71
34	15.89	0.14	5.11	109.97	4013.34	0.61	3.79
35	14.21	0.19	5.41	74.2	4818.42	0.6	2.23
36	11.21	0.34	5.81	33.17	3219.97	0.49	1.32
37	16.28	0.11	5.21	154.31	5530.92	0.71	2.17

Table 3-3. Monensin sorption and desorption parameters for B-horizon soil samples.

Sample No.	Cs	Cw	Isotherm pH	Kd	Kom	Dw	IR
	$\mu\text{g g}^{-1}$	$\mu\text{g mL}^{-1}$		-----L kg <sup>-1</sup> -----		$\mu\text{g L}^{-1}$	$\mu\text{g g}^{-1}$
1	13.12	0.24	5.71	55.13	N/A	0.62	0.67
2	13.45	0.28	5.71	48.38	N/A	0.6	1.52
3	11.28	0.35	6.41	32.51	N/A	0.54	0.52
4	13.28	0.27	5.81	49.74	N/A	0.6	1.3
5	12.34	0.27	6.21	46.22	N/A	0.6	0.37
6	12.34	0.23	6.01	52.85	N/A	0.58	0.78
7	13.09	0.26	5.81	51.03	N/A	0.6	1.06
8	12.89	0.24	6.01	54.25	N/A	0.59	1.1
9	12.34	0.12	5.91	100.73	N/A	0.59	0.45
10	13.12	0.24	5.81	54.15	N/A	0.59	1.25
11	11.46	0.32	6.31	35.37	N/A	0.54	0.68
12	11.87	0.31	5.81	38.17	N/A	0.51	1.65
13	12.89	0.21	6.31	61.24	N/A	0.56	1.78
14	13.21	0.21	6.11	62.61	N/A	0.6	1.12
15	13.34	0.27	5.61	50.02	N/A	0.62	0.91
16	13.65	0.29	5.61	47.23	N/A	0.59	1.76
17	13.67	0.28	5.71	49.53	N/A	0.59	1.78
18	11.21	0.38	5.61	29.5	N/A	0.51	1.09
19	12.65	0.26	6.51	48.47	N/A	0.55	1.66
20	13.45	0.27	5.81	49.45	N/A	0.6	1.37
21	13.21	0.28	5.71	46.6	N/A	0.62	0.78
22	12.76	0.29	6.01	43.33	N/A	0.59	0.89
23	13.11	0.21	5.21	62.28	N/A	0.59	1.22
24	13.23	0.26	5.71	50.59	N/A	0.59	1.45
25	11.12	0.37	6.11	30.47	N/A	0.52	0.78
26	13.23	0.16	5.61	80.67	N/A	0.6	1.21
27	12.14	0.23	6.01	52.55	N/A	0.56	0.89
28	13.54	0.27	5.81	50.71	N/A	0.62	1.11
29	12.98	0.28	5.71	46.36	N/A	0.56	1.87
30	12.98	0.31	5.81	41.6	N/A	0.56	1.77
31	12.43	0.24	6.01	51.11	N/A	0.56	1.15
32	12.34	0.3	6.11	41.27	N/A	0.58	0.67
33	13.38	0.21	5.41	63.71	N/A	0.61	1.27
34	12.34	0.27	5.91	46.57	N/A	0.57	0.89
35	12.34	0.22	6.41	55.24	N/A	0.57	0.89
36	12.65	0.34	5.81	36.99	N/A	0.54	1.76
37	13.26	0.24	5.61	56.19	N/A	0.59	1.5

In the Tables 3-2 and 3-3, the equilibrium concentration of monensin sorbed is denoted as  $C_s$  ( $\mu\text{g g}^{-1}$ ), the equilibrium concentration of monensin in solution is denoted as  $C_w$  ( $\mu\text{g mL}^{-1}$ ), the equilibrium partition co-efficient in soil-solution system as  $K_d$  ( $\text{Kg L}^{-1}$ ), the equilibrium partition co-efficient in organic matter-solution system as  $K_{om}$  ( $\text{Kg L}^{-1}$ ), concentration of monensin desorbed in the solution phase as  $D_w$  ( $\mu\text{g mL}^{-1}$ ) and concentration of monensin irreversibly sorbed to the solid phase after complete desorption as IR or irreversible sorption.

The isotherm parameters averaged across all samples for each soil series are presented in Table 3-4 by soil horizon. The C-type isotherms for both A and B horizons can be modeled using Freundlich isotherm. Freundlich adsorption isotherm is a non linear equation defined as

$$q_i = K C_i^n,$$

where  $q_i$  is the amount adsorbed at equilibrium,  $C_i$  is the equilibrium concentration,  $K$  and  $n$  are adjustable positive parameters. Where  $K$  is the slope of the isotherm and  $n$  ranges from 0 to 1. Though  $K$  and  $n$  have no physical meaning, Sposito (1980) presented  $n$  as a measure of heterogeneity of the adsorption sites on the solid phase. When  $n=1$ , the linear C-type isotherm is formed. Thus for isotherms in our study that belong to the C-type linear category,  $K$  is the slope of the isotherm, equivalent to  $K_d$ .

Table 3-4: Isotherm parameters for the 34 sample locations averaged across soil series and soil horizon.

Soil	Horizon	Mean $K_d$	Isotherm	Regression equation	$R^2$
		$\text{LKg}^{-1}$	pH		
Evesboro	A	62.99	6.2	$y = 121.76x - 18.09$	0.96
	B	29.03	6.3	$y = 58.583x - 14.42$	0.95

Sassafras	A	137.64	5.9	$y = 382.16x - 44.39$	0.98
	B	36.58	6.1	$y = 65.16x - 11.81$	0.98
Mattapex	A	201.59	5.1	$y = 532.48x - 46.73$	0.93
	B	43.61	5.6	$y = 90.911x - 19.21$	0.97

For the kinetics study, the sorption maxima or the  $S_{\max}$  was reached around 24 hours for all the soil samples. This was when maximum sorption of the analyte on the sorbent which is our soil, had taken place. Monensin sorption was highest in Mattapex soil, followed by Sassafras soil and Evesboro soil as it reached  $S_{\max}$ . The sorption was linear as also found in the adsorption isotherm study. After reaching  $S_{\max}$  the sorption curves plateaued and remained a straight line till around 72 hours. At the end of 72 hours, a decrease in sorption was noticed for all three soils. This may be because of continuous shaking for a long period of time that might have caused reversibility in sorption, letting some of the analytes desorb back into the solution or loss of analyte in the soil-solution system due to biotic or abiotic degradation. Shaking was not continued any longer after this point. The sorption kinetics for B-horizon soils, followed the same pattern, though the sorption was lower for all three soils, compared to their respective A horizons.

In the main study on all the 74 soil samples from the five farms, single concentration, single time point sorption and desorption experiments were performed. Both the  $K_d$  and hysteresis pattern of these samples was within the range of what was found for the three soils from the pilot study at isotherm pH ranging from 5.5-6.5 as shown in Table 3-2 and Table 3-3.

### 3.4. CONCLUSIONS

Our sorption and desorption studies showed difference in behavior in the A and B horizons of the soils, especially significant difference in the isotherms of our 3 representative soils. Hence further studies were conducted to see how the soil physico-chemical parameters, in each of these soils might have influenced these processes.

The importance of sorption-desorption batch equilibrium studies lies in its foundation to further understand how ionophores may behave in the soil systems where land application of poultry manure containing ionophores may occur. These studies helped us understand the partitioning behavior of monensin in soil and water that may affect its mobility in the soil-water system. Future studies may include estimating availability of monensin for degradation in soils, its chemical transformations, uptake by biota, leaching through soil profiles, volatilizations from soil and run-off from soils into natural waters.

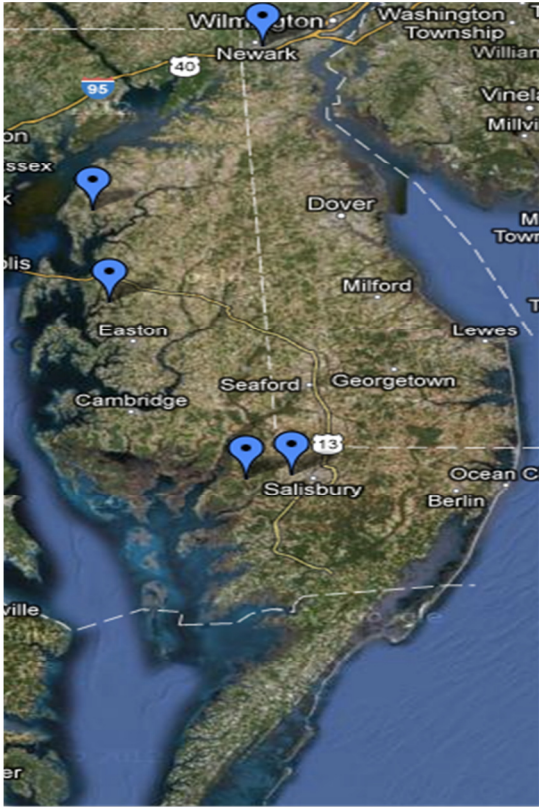


Figure 3-1. Soil samples were collected from five farms on the Delmarva Peninsula.



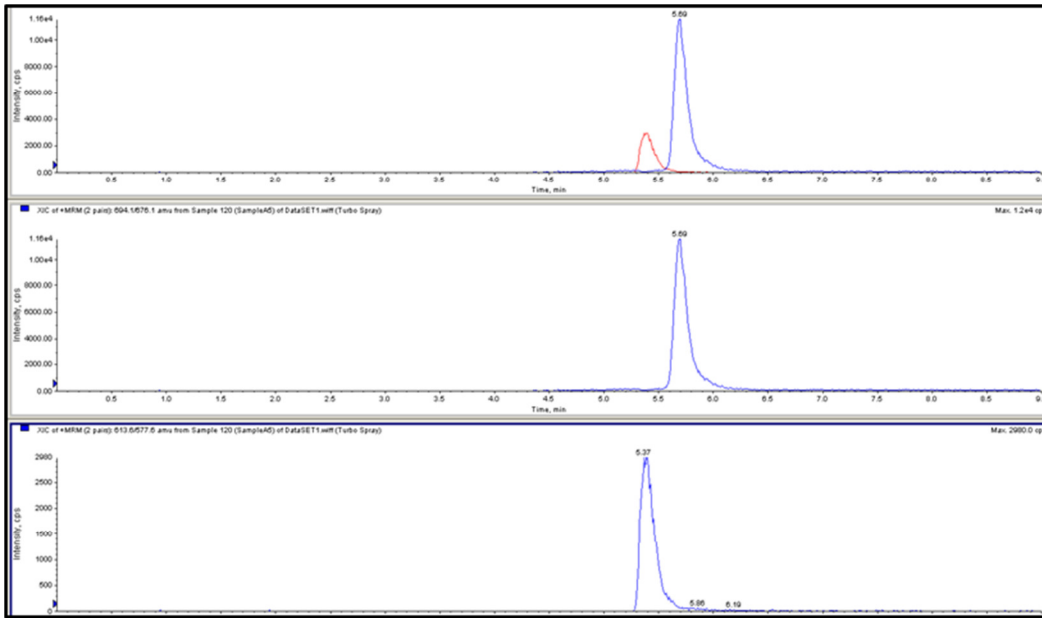


Figure 3-2. Example of high performance liquid chromatography tandem mass spectrometer extracted-ion chromatogram for monensin (middle pane) and internal standard lasalocid (bottom pane) for one of the samples included in the batch equilibrium study.

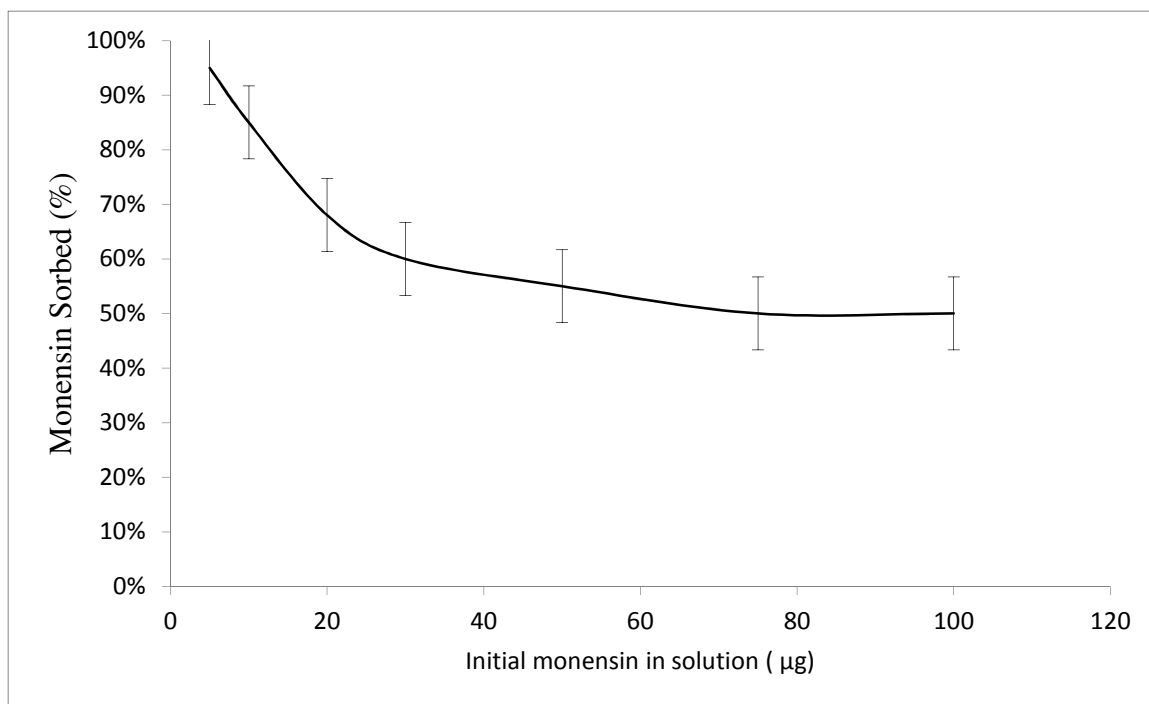


Figure 3-3. Relative portion of monensin sorbed (%) versus the initial mass of monensin in solution for the batch equilibrium method development conducted over a range of soil to solution ratios using 1 g of soil and 1 µg mL<sup>-1</sup> monensin solution.

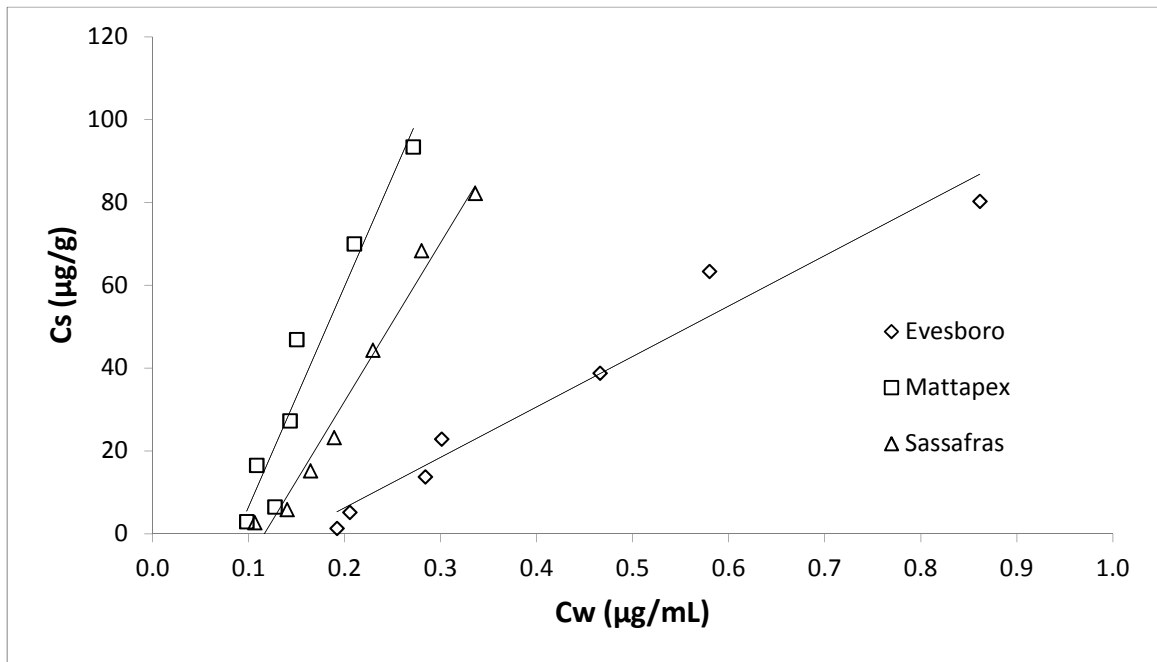


Figure 3-4. Effect of solution equilibrium concentration ( $C_w$ ) on solid phase equilibrium concentration ( $C_s$ ) for the A-horizon samples evaluated during batch equilibrium method development.

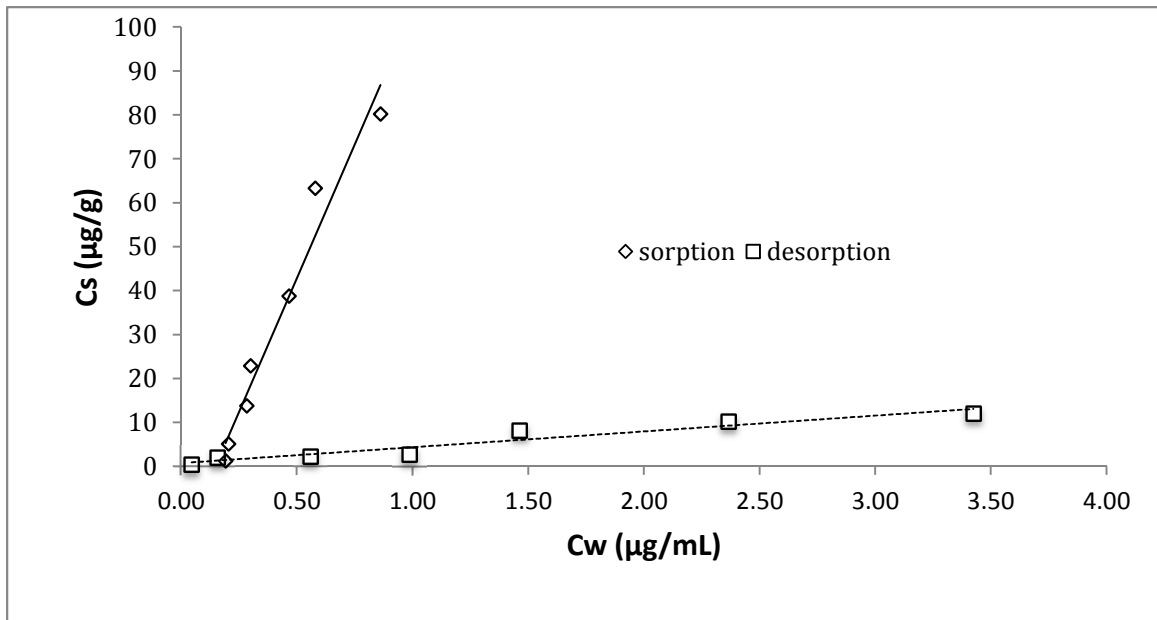


Figure 3-5. Sorption and desorption isotherms for the Evesboro A horizon presented as the equilibrium concentration of monensin in solution ( $C_w$ ) versus the equilibrium concentration on the solid ( $C_s$ ).

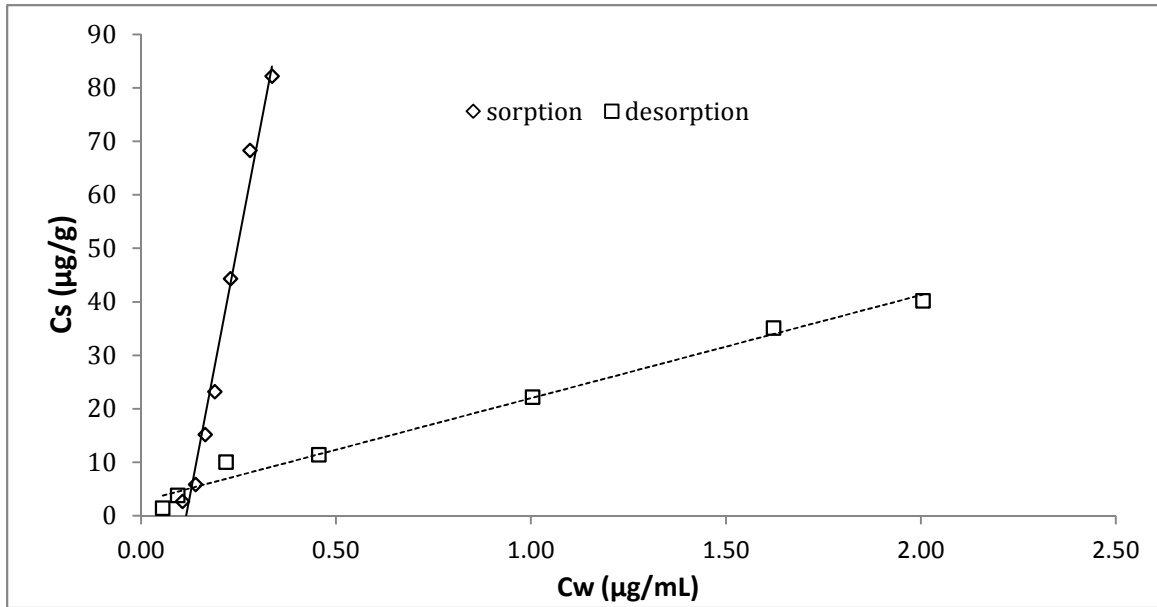


Figure 3-6. Sorption and desorption isotherms for the Sassafra A horizon presented as the equilibrium concentration of monensin in solution ( $C_w$ ) versus the equilibrium concentration on the solid ( $C_s$ ).

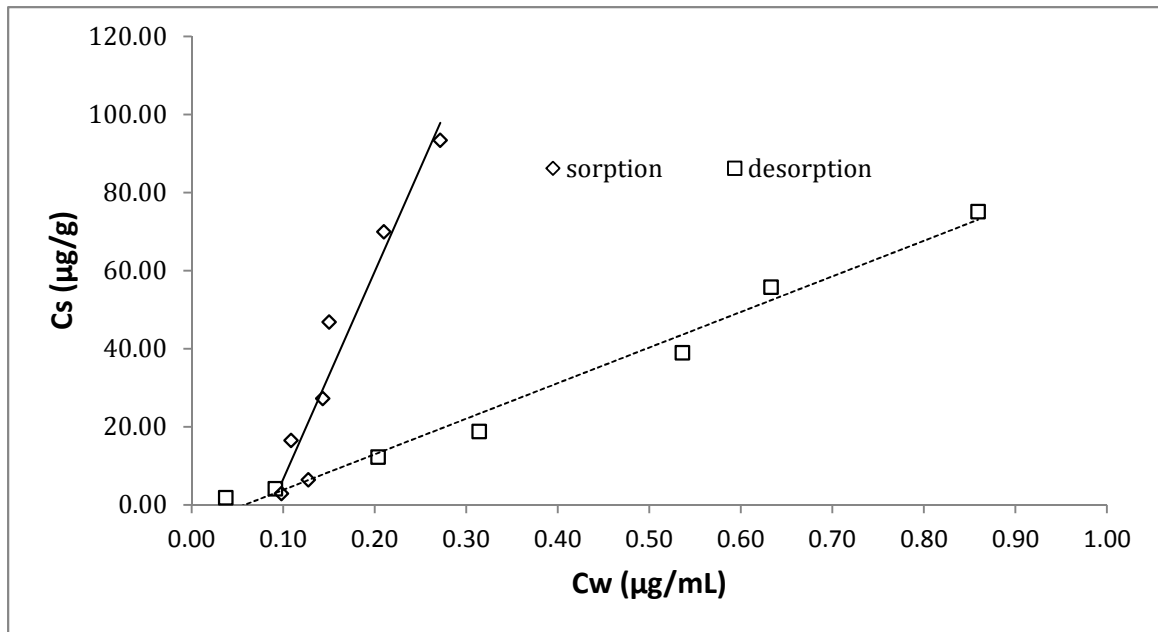


Figure 3-7. Sorption and desorption isotherms for the Mattapex A horizon presented as the equilibrium concentration of monensin in solution ( $C_w$ ) versus the equilibrium concentration on the solid ( $C_s$ ).

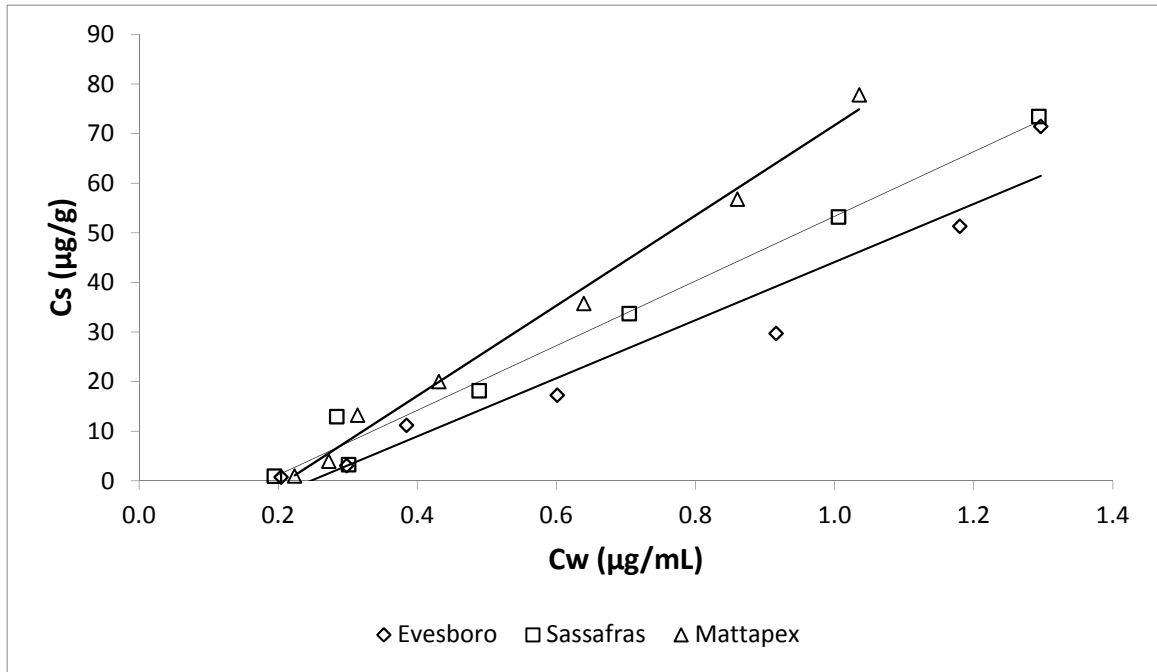


Figure 3-8. Effect of solution equilibrium concentration ( $C_w$ ) on solid phase equilibrium concentration ( $C_s$ ) for the B-horizon samples evaluated during batch equilibrium method development.

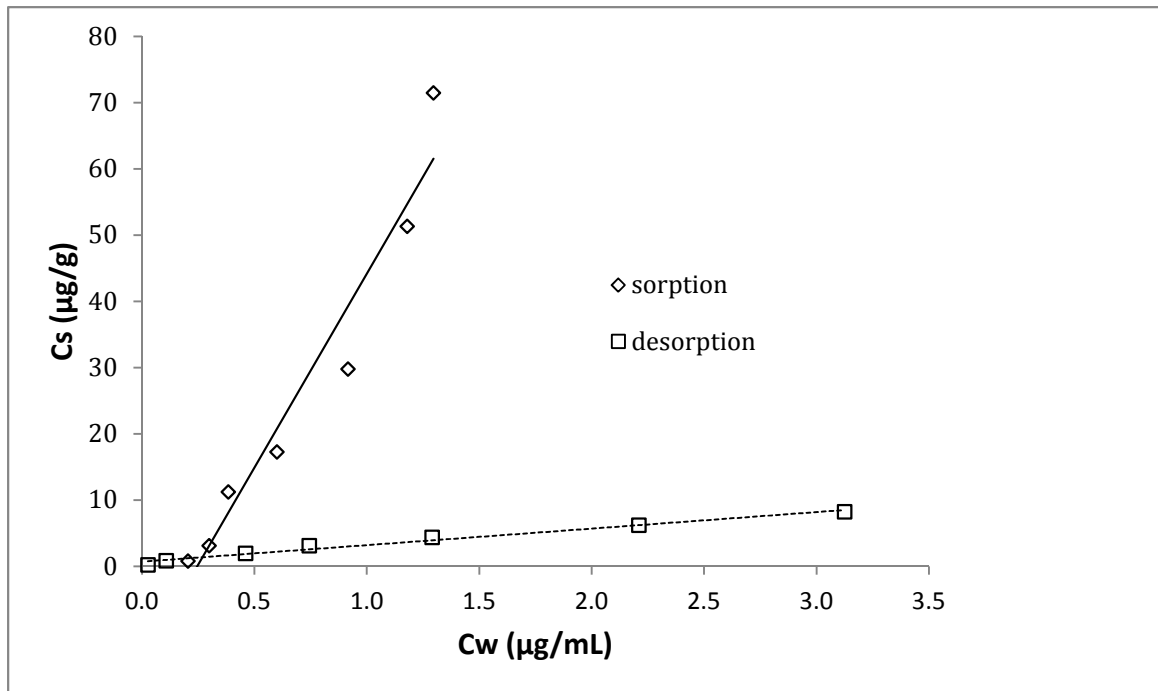


Figure 3-9. Sorption and desorption isotherms for the Evesboro B horizon presented as the equilibrium concentration of monensin in solution ( $C_w$ ) versus the equilibrium concentration on the solid ( $C_s$ ).



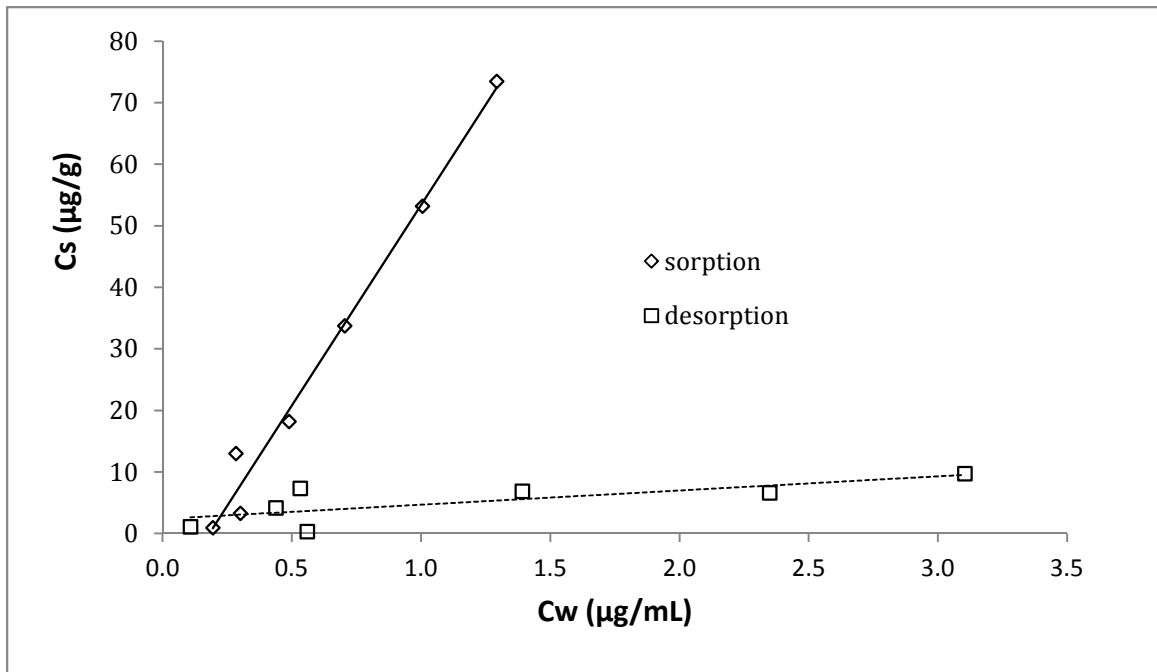


Figure 3-10. Sorption and desorption isotherms for the Sassafra B horizon presented as the equilibrium concentration of monensin in solution ( $C_w$ ) versus the equilibrium concentration on the solid ( $C_s$ ).

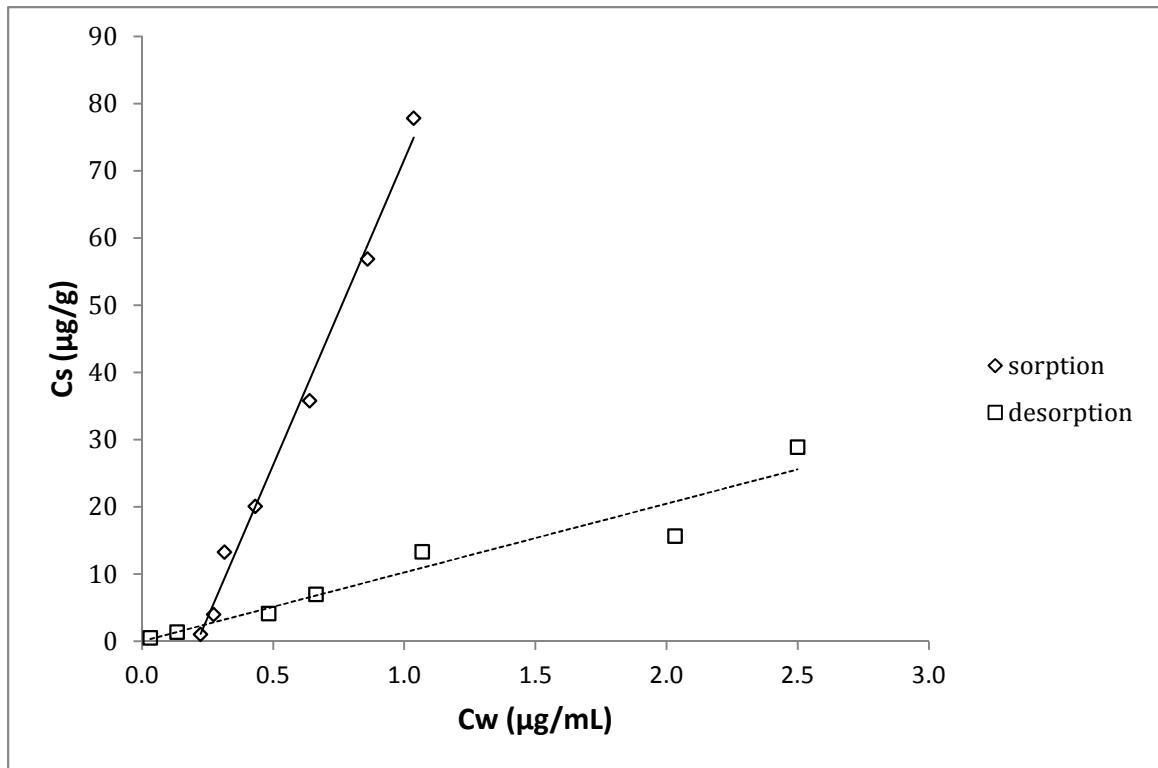


Figure 3-11. Sorption and desorption isotherms for the Mattapex B horizon presented as the equilibrium concentration of monensin in solution ( $C_w$ ) versus the equilibrium concentration on the solid ( $C_s$ ).

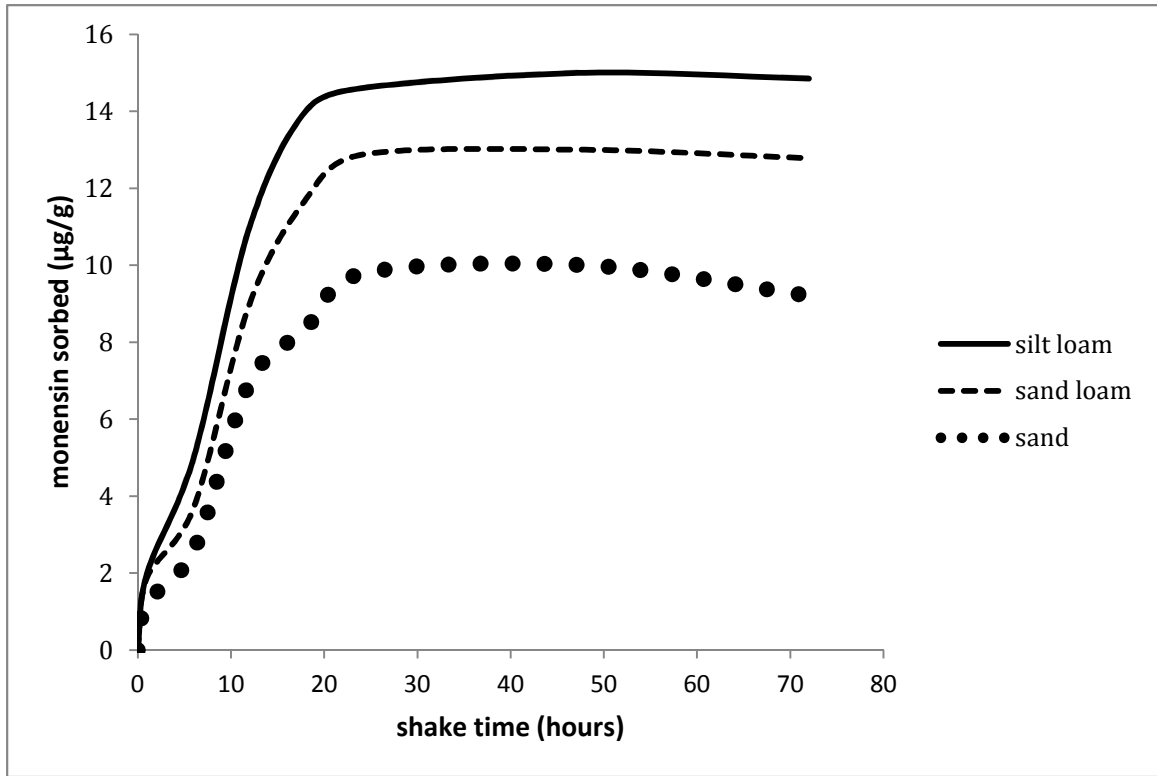


Figure 3-12. Sorption kinetics of A horizon soil of Evesboro (sand), Mattapex (silt loam) and Sassafras (sandy loam) soils with X axis as shake time (equilibration time) and Y axis the concentration of monensin ( $\mu\text{g/g}$ ) sorbed at equilibrium.

## **CHAPTER 4. EFFECT OF SOIL CHARACTERISTICS ON IONOPHORE SORPTION AND DESORPTION**

### **4.1. INTRODUCTION**

Soil properties are known to affect the mobility of chemicals through the soil system (Alcock et al., 1999; Halling-Sørensen, 2001). There is a lack of literature review on the relationship of soil physical and chemical parameters and ionophore sorption and desorption studies. In fact, we did not find any desorption studies on ionophores and no study exploring interaction of ionophores in the B horizons of soils. Hence this is one of the first studies in this area of research. Literature review on other antimicrobials in the soil systems showed that physical and chemical properties such as organic carbon content, cation-exchange capacity, texture and pH, to have significant influence over mobility of antimicrobials (Aga, 2008; Kumar et al., 2005).

Depending on the chemical species, interactions with soil can occur through electrostatic interaction, surface bridging, hydrogen bonding or hydrophobic interactions (Martinez-Villalba et al., 2009). The sorption behavior in soil can also be influenced by the properties of soil including pH, organic carbon content, metal oxide content, ionic strength and cation-cation exchange. Manure and slurry may also alter the behavior of antimicrobials in soil system and affect its persistence. These effects have been attributed to changes in pH or nature of dissolved organic carbon in the soil-manure system (Boxall A.B.A. et al, 2003; Boxall A.B.A., 2008). The chemical nature of the analyte has a significant contribution towards its interaction with the soil system. Certain chemicals that have ionizable functional groups are pH dependent in terms of their mobility in the soil system. Hence when their pH is below their pKa value, they are expected to be

protonated and associated with negatively charged particles including clay and organic matter, while, at pH above pKa they may be deprotonated and have weak association with soil (Boxall, 2008).

Recently the importance of cation exchange capacity in sorption processes have been studied in clay, soil and humic acids in different pH conditions. The range of three pKa values ranging from 3.3-9.3 has resulted in large shifts in ionic speciation of tetracyclines in environmental relevant pH range from cationic to neutral to anionic species (Martinez-Carballo et al., 2007). Brambilla et al.; 2007 studied effect of cation exchange capacity (CEC) of soil on oxytetracycline sorption at pH of 5 and found CEC to be a weak predictor of sorption with correlation co-efficient or  $r = 0.35$  (Brambilla et al., 2007).

Cation exchange and cation bridging can influence the binding of antimicrobials to the dissolved organic carbon in aqueous system that may enhance their mobility through the soil profile and also through the surface run-off as seen in fluoroquinolone group of antimicrobials (Carmosini and Lee, 2008).

Hydrophobic partitioning was found to contribute to the sorption in soil by tylosin, a basic macrolide having pKa of 7.7. The sorption was strongly correlated to cation exchange capacity with correlation co-efficient ( $r$ ) = 0.77, clay content with  $r=0.86$  and surface area with  $r = 0.91$ . Organic matter was found to have a lesser influence with  $r=0.46$ , may be because of its cationic nature in environmental relevant pH conditions (Schlusener and Bester, 2006).

Sulphonamide class of antibiotics was found to sorb lesser to clay and organic matter. The low sorption may be due to its negatively charged state and high polarity

under environmentally relevant soil conditions. These compounds tend to be in cationic forms at pH below 4.5, neutral forms at pH between 4.5 to 5.5 and anionic above 5.5. Hence at environmentally relevant conditions, that is in the pH ranging from 5.5- 7.5, they were either in anionic or neutral forms that made them sorb considerably less to soils and have been detected in surface water in the range of 0.003- 0.25  $\mu\text{g L}^{-1}$  (Carmosini and Lee, 2008).

Limited studies were found on ionophores related to their behavior under the influence of soil parameter. A study of ionophore sorption in the wetland soils at different pH such as 4.5, 6.5 and 8.5 was performed and soil organic carbon was found to have a strong influence over the sorption processes with Log Koc decreasing with increase in pH. This trend was different above pH 8.5 where contribution of clay fraction in soil had a stronger influence (Hussain S.A. and Prasher, 2011).

In another study Log Koc values ranged from 2.1 to 3.8 for monensin generally decreased with increasing soil pH (pH range 4.2 to 7.5). This was suggested to be because carboxylic acid groups are deprotonated under alkaline conditions. As carboxyl and ether O atoms in the molecule can chelate environmentally relevant cations (eg.  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ), this may increase the apparent hydrophobicity of the molecules and possibly alter their sorption and mobility by reducing their net charge (Sassman and Lee, 2007).

Five different physico-chemical soil parameters have been analyzed in our soils. They are soil texture, soil organic matter, cation exchange capacity, pH in water and electrical conductivity. Soil texture is a physical property of soil that influences several processes like water holding capacity of soil, percentage of plant available water, cation

exchange capacity and other soil processes. Determination of soil texture as % sand, silt and clay, the primary particulate components of soil is also known as mechanical or particle size analysis. The different particle sizes in the soil influence various soil behaviors including sorption and desorption. The hydrometer method is based on change of density of soil and water suspension upon settling of soil particles.

Soil organic matter (SOM) influences many physical, chemical, biological processes of soil like soil structure, water holding capacity, water and air infiltration rate and activities of organic contaminants. Amount of soil organic matter (SOM) present in soil can be influenced by various factors such as climate, water regime, soil texture, vegetation, cropping practices, tillage, drainage, irrigation and erosion to name some of them.

Cation exchange capacity is a measure of exchangeable bases and soil acidity and relates to the concentration of negatively charged sites on soil colloids that can adsorb exchangeable cations. Cation exchange capacity is also used for regulatory purposes in monitoring land application of biosolids, pesticides and may influence activity of organic contaminants present in the soil.

The pH of the soil is a measure of the active acidity of the soil that results from free  $H^+$  ions in the soil solution. Soils also have reserve acidity that includes exchangeable  $H^+$  and hydrolysable  $-OH$  groups on clays and organic matter. The aluminum ions also react with water to release hydrogen ions. It is also useful in assessing potential availability of essential nutrients and toxic elements to plants. For agricultural and nutrient management purposes, relevant soil-water pH ranges from 5.5-7.5.

Soil electrical conductivity (EC) is the measure of salt amounts in soil that correlates with soil properties that affect crop productivity, including soil texture, soil structure, soil aggregation, water potential, electrolytes in soil-water, cation exchange capacity (CEC), drainage conditions, organic matter level, salinity, soil nutrients, contaminants, and subsoil characteristics.

Our sorption-desorption batch equilibrium studies, showed significant differences in partitioning behavior in different soil types, collected from the different farms. This lead to the formation of our third objective, to study physico-chemical properties of the soil samples collected from the 5 different farms on the Delmarva Peninsula and hence analyze the partitioning behavior of monensin as a function of these properties. The methods used for soil physico-chemicals have been adapted from standard procedures.

## **4.2. MATERIALS AND METHODS**

### **4.2.1. Soil texture by hydrometer method**

Each of the 74 soil samples was split into triplicate sub-samples and analyzed to determine sand, silt, and clay content, organic matter content, cation exchange capacity, pH, and electrical conductivity. The methods for each are described below.

In order to classify the soil texture for each sample, sand, silt, and clay content was determined using the soil hydrometer method. Fifty grams air-dried soil was weighed into a 250 mL beaker. Then 100 mL of distilled water and 25 mL, 1M sodium hexametaphosphate solution were added to the beaker and stirred for five minutes. Using distilled water the contents of the beaker were completely transferred to a metal blender cup, such that it was half full. The metal cup was placed in a blender (Hamilton Beach Commercial, Model number 230057800) set such that the blender blades did not touch



the inside walls of the cup. After blending for seven minutes, the contents from the metal cup were transferred to the hydrometer cylinder along with distilled water so that the cylinder was full to the 1000 mL mark. The cylinder was sealed with a rubber stopper and shook vigorously end to end for 10 times. Immediately after the 10<sup>th</sup> time, the cylinder was put back upright on the bench. The hydrometer (VWR Scientific, model number 34792-001) was carefully and swiftly inserted into the cylinder and allowed to rest for 40 seconds. After 40 seconds the hydrometer reading was taken at the water level. The process of shaking the cylinder and recording the hydrometer reading after 40 seconds was repeated three times. The average of the three values was used in the calculations to determine soil texture. Temperature of the suspension was also recorded for temperature corrections.

The 40 seconds hydrometer reading estimates the amount of silt and clay suspended after the sand particle have settled. The cylinder was kept undisturbed and suspension further allowed to settle for six hours. After this period, the hydrometer was gently suspended into the cylinder and reading recorded. This was used to determine the amount of clay in suspension.

To correct for temperature effect on density 0.4 units was added to the reading of the sample for every 1<sup>0</sup>C above 20 <sup>0</sup>C and 0.2 unit subtracted for every 1<sup>0</sup>C below 20<sup>0</sup>C. A blank was prepared the same way as above by adding 950 mL of distilled water to 25 mL sodium hexametaphosphate solution in the 1000 mL cylinder and hydrometer readings taken in the similar procedure as before. The hydrometer reading from the blank was subtracted from each of the soil sample reading for density corrections.

Clay content was calculated as percent clay that is the corrected hydrometer reading at 6 hours X 100/ wt. of the sample. Silt content was calculated as percent silt that is the corrected hydrometer reading at 40 seconds X 100/ wt. of the sample. Sand content was calculated as 100 - % Silt - % Clay.

For soil texture analyses using hydrometer method samples were treated with sodium hexametaphosphate, a dispersion agent to complex  $\text{Ca}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$  and other cations that bind clay and silt particles into aggregates. Organic matter is suspended in this solution. Dispersion of soil clays are very crucial to the accuracy of the results as they are tightly cemented together by cations and organic matter. Incomplete clay dispersion can lead to low readings for clay and high for sand and silt. Temperature and density corrections are also necessary as it affects rate of sedimentation. For quality control, a standard soil with known texture was analyzed after every batch of 20 samples to check for instrument calibration and procedural accuracy. The results are reported as mineral fractions, i.e. % sand, % silt and % clay as a standard reporting procedure that corresponds to the USDA texture triangle (diagram not reproduced as permission from USDA has not been taken yet). Method adapted from (Bouyoucos, 1962).

#### **4.2.2. Soil organic matter content by loss on ignition**

Ring stand with ceramic triangle were set up over the burner. With tongs, the ceramic crucible was placed over the triangle on the burner till the crucible glows red hot for 2 minutes. Then burner was turned off and crucible cooled. The weight of the cooled crucible was recorded as (B). 5 g of air-dried soil was loosely placed in the crucible such that it was about 1/3 full. It was weighed accurately and recorded as (C). The crucible containing the soil was placed in the microwave oven and heated on high for 3 minutes.

The crucible was taken out, cooled in the desiccator and weighed accurately and recorded as (D). The crucible with the soil was then placed over the burner and gradually heated until the ceramic glowed red. It was continuously heated in this red hot condition for 2 hours occasionally stirring the soil with the glass rod very gently. Care was taken such that no soil was spilled in this process. After 2 hours it was visible that the dark colored soil sample became almost discolored. The burner was turned off and crucible cooled in the desiccator. After cooling it was weighed accurately and data recorded as (H). At this stage the crucible contains only the inorganic portion of the soil that resembled color of ash. The weight lost during ignition is mostly that of the organic matter.

Loss on ignition was calculated as the difference between D and H in grams.

Grams of oven dried soil was the difference between D-B. Organic matter content, was calculated as a percent as  $\text{Loss on ignition} / \text{g of oven dried soil}$ .

For soil organic matter analyses, introduction of moisture in the sample is a potential cause of error. Hence sample was initially heated in microwave oven in a hot crucible to remove moisture. Also in between the crucible was never kept exposed to open air for cooling. It was always kept in a desiccator with dry agents to avoid adding moisture to the soil sample. This procedure was found to be used, very popularly, in routine analyses of soil for organic matter content, due to the simplicity and cost effectiveness of the method. The method allowed for complete loss of organic matter from the sample but the temperature was not allowed to go very high so that the inorganics did not get a chance to decompose. The common balance was tested for accuracy before using it for this method and the crucible weights in each case was taken within 0.001 g of accuracy. Method adapted from (Schulte 1996).

### 4.2.3. Cation Exchange Capacity

250 mL beaker, balance to weigh to the nearest 0.01 gm, 7.0 cm Buchner funnel, Filter paper (7 cm Whatman #1 or #42), 250 mL suction flask connected to vacuum pump, 250 mL volumetric flasks, stir plate, stir bars and container for reagents, Apparatus and instrumentation for  $\text{NH}_4^+$  analysis (Spectrophotometer: Fisher Scientific Education Model number FS0306039), chemicals from JT Baker (NJ, USA) that are prepared as below.

To prepare, 1 M  $\text{NH}_4\text{OAc}$  (ammonium acetate) at pH 7.00, 580 mL of glacial acetic acid (99.5%) was added to approximately 5 L of water. 680 mL of concentrated ammonium hydroxide (58%  $\text{NH}_4\text{OH}$ ) was added to it. Volume was made up with distilled water to approximately 1900 mL. Adjusted pH to 7.00 with dropwise additions of either ammonium hydroxide or acetic acid. Diluted to 10 L. Solution was made in a fume hood to avoid breathing vapors of ammonia and acetic acid. To prepare, 1 M KCl, dissolved 745 g KCl (potassium chloride) in ~ 8 L of water. Diluted to 10 L. To prepare, Phenol- Nitroprusside Reagent, dissolved 7 g of phenol and 34 mg of  $\text{Na}_2[\text{Fe}(\text{NO})(\text{CN})_5] \cdot 2\text{H}_2\text{O}$  (sodium nitroprusside) in 80 mL of deionized water and diluted to 100 mL. It was mixed well and stored in a dark-colored bottle in a refrigerator.

Buffered Hypochlorite Reagent was prepared, by dissolving 1.48 g of NaOH in 70 mL of deionized water and 4.98 g of  $\text{Na}_2\text{HPO}_4$  and 20 mL of sodium hypochlorite (5% NaOCl) solution was added. The pH was checked to insure a value between 11.4 and 12.0. A small amount of additional NaOH was added as required to raise the pH. This was diluted to a final volume of 100 mL. EDTA Reagent was prepared by dissolving 6 g

of disodium EDTA (ethylene- diamine-tetraacetic acid) in 80 mL of deionized water and adjusted to pH 7. Mixed well and diluted to a final volume of 100 mL.

10 grams of air-dried soil was weighed and ground to less than 2 mm and placed into a 250 ml beaker. Then added 25 mL of  $\text{NH}_4\text{OAc}$  to the soil. It was covered to let it set overnight. For each sample, a 7 cm Buchner funnel was prepared by fitting it with a 7 cm Whatman #42 filter paper. The filter was wetted with a minimum amount of  $\text{NH}_4\text{OAc}$ . The funnel was then inserted into a 250 ml suction flask. Vacuum pump was turned on to seat the moistened filter. The soil- $\text{NH}_4\text{OAc}$  mixture was stirred and transferred into the filter.

75 mL  $\text{NH}_4\text{OAc}$  for each sample was measured into a plastic squirt bottle with one bottle for each sample. 10 mL of the  $\text{NH}_4\text{OAc}$  was used in the bottle to transfer all of the soil to the Buchner funnel. The soil was covered with a 7.0 cm Whatman #1 filter paper to keep the soil moist between leachings. The soil was leached 5 to 7 times with 10 to 15 ml increments of  $\text{NH}_4\text{OAc}$ . The soil was not allowed to dry between leachings. To remove excess  $\text{NH}_4\text{OAc}$  in the soil, leached the soil with about 25 mL portions of ethanol five to six times for a total volume of about 150 mL.

To remove adsorbed  $\text{NH}_4^+$  in the soil, the soil was leached with 25 mL of 1 M KCl four to five times for a total volume of about 125 mL. The leachate was transferred to a 250 mL volumetric flask and brought to volume using 1 M KCl. The solution was analyzed for  $\text{NH}_4$  concentration using colorimetric analyses.

For Colorimetric analyses, 5 ml volume of the 1M KCl extract was pipetted into a 25 mL volumetric flask. 1 mL of the EDTA reagent was added and the contents of the flask mixed. The mixture was allowed to stand for at least 1 min. Added 2 mL of the

phenol-nitroprusside reagent followed by 4 mL of the buffered hypochlorite reagent. Immediately diluted the flask to volume with deionized water, and mixed the flask contents by inverting several times. Placed the flasks in a water bath maintained at 40°C and allow 30 minutes for color development. Removed flasks from bath, cooled to room temperature for 10 minutes and determined the absorbance of the colored complex at a wavelength of 636 nm against a reagent blank solution.

Determined the  $\text{NH}_4^+$  concentration of the sample by reference to a standard curve based on analysis of standard solutions containing 0, 2, 4, 6, 8, 10, and 12  $\mu\text{g NH}_4^+ \text{mL}^{-1}$ . To prepare this curve, added 1M KCl solution using same volume as used for aliquots of soil extract, to a series of 25-mL volumetric flasks. Then added 0, 1, 2, 3, 4, 5, and 6 mL of the 2  $\mu\text{g NH}_4^+ \text{mL}^{-1}$  standard solutions to separate flasks and measured the intensity of blue color developed with these standards by the procedure described for analysis of the extract. Cation exchange capacity, can be calculated as,

$$(\text{mg NH}_4 / \text{L}) (0.25 \text{ L} / 10 \text{ g soil}) (1 \text{ meq NH}_4 / 18 \text{ mg NH}_4) \times 100$$

Cation exchange capacity can be overestimated if not all of the excess  $\text{NH}_4^+$  is leached out of the sample with ethanol or it can be underestimated if some of the  $\text{NH}_4^+$  is displaced during washing. Hence care has been taken to follow these steps in the method protocol precisely. Cation exchange capacity can be underestimated if calcium carbonate or gypsum exists, by using this method. When soil colloids are saturated with  $\text{NH}_4^+$  from  $\text{NH}_4\text{OAc}$ ,  $\text{Ca}^{2+}$  can neutralize some of the negative charge on the soil colloids resulting in incomplete saturation of exchange sites with  $\text{NH}_4^+$ . Calcium carbonate is likely to exist when soil-water pH is greater than 7.5. Our pH measurements confirmed that our soils were below pH 7 hence this limitation is not applicable in our case.

Soil texture has an important impact on CEC since negatively charged colloids dominate in the clay-sized fraction. In general, sandy loams have CEC less than 8 meq  $100\text{ g}^{-1}$ , silt loams have CEC between 8 and 15 meq  $100\text{ g}^{-1}$ , clay loams have CEC between 10 and 20 meq  $100\text{ g}^{-1}$ , and clays have CEC greater than 20 meq  $100\text{ g}^{-1}$ . Organic matter content also has a strong influence on soil CEC since organic colloids have a greater CEC compared to clay minerals. The  $\text{NH}_4\text{OAc}$  solution was prepared in a fume hood to avoid breathing ammonia and acetic acid vapors. The method adapted for this study is from the works of (ASA, 1998), (Sumner, 1996) and (Dorich, 1983).

#### **4.2.4. $\text{pH}_w$ analyses of soil samples**

pH meter capable of reading + 0.01 pH units, Plastic water cups, 90 mL (3 oz.), Glass stirring rod. Deionized (DI) water, Buffer solutions, at pH 4.00 and pH 7.00 for standardization Soil pH was determined by weighing 10 g of air-dried and (< 2 mm) into a cup then adding 10 mL of DI H<sub>2</sub>O. The mixture was stirred with a glass rod until homogeneous slurry is achieved and then allowed to equilibrate for 15 min before stirring again. The stirring rod was rinsed with DI H<sub>2</sub>O between samples. The cup was allowed to sit for another 15 min, but no more than a total of 60 min. The pH meter (Seven Multi Mettler Toledo, Model number LP11000 with built-in combination glass indicator electrode and automatic temperature control [ATC] or, Accumet 13-620-530 which contains KCl with Ag/AgCl reference) was standardized per the manufacturer's instructions. Immediately before reading, the samples were stirred once again. The pH was measured by placing the electrodes so that the glass electrode dipped into the soil slurry. Slightly swirled the pH cup to rinse the electrode with the suspension. Allowed the pH meter reading to stabilize before recording the value; the letter "S" in a black block

(for stable) appeared in about 20 seconds. Recorded pH value to one decimal place. Checked the pH meter against both buffers every 20 samples and re-adjusted if necessary. Re-read any samples with pH <4 or >7.5. Allowed electrodes to soak briefly in either the 4.00 or 7.00 buffers before re-reading. Determined the pH of all the samples.

For pHw analyses to maintain optimum performance of the pH meter after every sample analyses, the electrodes were washed well with deionized water and excess water was shaken off. The electrode was stored in saturated KCl solution. Care was taken so that the electrode was inserted carefully into the soil cups in a way that prevented insertion to the very bottom of soil cups that would cause abrasion of the sensing surface, decreasing electrode life and causing inaccurate readings. Care was also taken so that the body of the electrode was not allowed to touch the side or bottom of the cup containing the sample. The buffers of 4.00 and 7.00 cannot properly provide for samples with pHs out of this range, but we expected the pH of our soils to be within this range as they were collected from non-agricultural fields, that did not have recent history of liming and manure treatment. Differences in pH may occur with electrode placed in a soil-slurry or in the supernatant after the soil has settled. The differences are more pronounced with soil pH in water compared to electrolyte solutions. To avoid this variability in pH, it was important to stir the soil slurry right before measurement. With sandy soils, the settling time of soil particles was rapid and continuous stirring during measurement is recommended. Method adapted from (Kalra, 1995).

#### **4.2.5. Electrical Conductivity Analyses**

To determine EC 10 g of air-dried and sieved (< 2 mm) soil was weighed into a cup and then 10 mL of DI H<sub>2</sub>O was added. The mixture was stirred with a glass rod until



homogeneous slurry was achieved and then allowed to equilibrate for 15 min and then stirred again. The stirring rod was rinsed with distilled water after stirring each sample. The cup was allowed to sit for another 15 min but not more than a total of 60 min. The meter (Seven Multi Mettler Toledo, Model number LP11000 Conductivity/pH meter) was standardized as for the pH method. Rinsed the electrode well with DI water into a waste container. Blotted the electrode dry with a wipe. Immediately before reading, the samples were stirred once again. Determined the electrical conductivity of soil samples by placing the electrodes so that the glass electrode dipped into the soil slurry. Slightly swirled the cup to rinse the electrode with the suspension. Then allowed the EC meter reading to stabilize before recording the value. Recorded pH value to one decimal place. Determined the EC of all the samples.

To maintain optimum performance of the EC meter after every sample analyses, the electrodes were washed well with deionized water and excess water was shaken off. Care was taken so that the electrode is inserted carefully into the soil cups in a way that prevents insertion to the very bottom of soil cups that will cause abrasion of the sensing surface, decreasing electrode life and causing inaccurate readings. Care was also taken so that the body of the electrode was not allowed to touch the side or bottom of the cup containing the sample. A good soil EC level will be somewhere above  $200 \mu\text{S cm}^{-1}$  and  $1200 \mu\text{S cm}^{-1}$  ( $1.2 \text{ MS cm}^{-1}$ ). Any soils below  $200 \mu\text{S cm}^{-1}$  indicate there is not enough nutrients available to the plant and could perhaps show a sterile soil with little microbial activity. An EC above  $1200 \mu\text{S cm}^{-1}$  may indicate too much high salt due to fertilizer or salinity problem from lack of drainage. Method adapted from (Kalra, 1995).

#### **4.2.6. Experimental Design and Analysis**

In order to evaluate the influence of soil physical and chemical properties on monensin sorption and desorption a simple linear correlation analysis was used. This approach was observational as variables were not controlled. Therefore, causal relationship between the soil parameters and the sorption-desorption parameters could not be established, as the dependent and independent variable relationship criteria could not be met. Many examples were found in the literature where correlation analyses were used to evaluate sorption and desorption processes (Boxall, 2008; Kumar et al., 2005). In fact some studies have argued that controlling soil parameters to study soil sorption or desorption may alter the soil properties and ultimately influence the results. For example, to study the effect of soil pH on monensin sorption, changing native pH of the soil by acid or base treatment, may impact other properties. Unless it is ensured by analyses that other soil parameters remain the same, the sole causal effect of any explanatory variable over the response variable cannot be concluded. No literature studies on controlled soil parameters for sorption of these kinds of analytes have been found, so far, to explicitly mention analyzing these conditions. Hence adding significant amounts of acids or bases to counteract soil buffering capacity in order to change the pH, may change the cation exchange capacity of soil, or the sorption properties of the organic matter present in the soil. Studying those effects would be a significant contribution to the scientific knowledge in this area of research that we highly recommend.

SAS 9.3 was used to perform PROC CORR method to generate descriptive statistics and Pearson Correlation Coefficients for pH, CEC, sand, silt, organic matter,

monensin sorbed (Cs), partition coefficients (Kd) for both A and B horizons along with an added variable Kom for A horizon.

### 4.3. RESULTS AND DISCUSSION

#### 4.3.1. Soil physical and chemical properties

The 74 soil samples collected and included in the batch equilibrium study were diverse in chemical and physical properties (Table 4-1). Even across the relatively small geographic area covered by our sampling, only including five different farms, sand content ranged from 10.5 to 93.7%, silt from 3.7 to 79.1, and clay from 0.7 to 25.4. Soil OM covered a narrow range, but still showed diversity with a mean of 1.13%, but a standard deviation of 1.06. Much of this diversity was due to the inclusion of both A and B horizon soil samples. Average sand, silt, clay, and OM contents were 39.67%, 8.69%, 51.64%, and 2.05% for the A horizon compared to 44.85%, 7.97%, 47.18%, and 0.21% for the B horizon soils (Table 4-2 and Table 4-3).

Table 4-1: Summary of soil parameters<sup>†</sup> for all 74 samples evaluated.

	pHw	CEC	sand	silt	clay	OM	EC
		cmol/kg	-----%				μScm <sup>-1</sup>
Min	4.5	1.2	10.5	3.7	0.7	0.01	204.6
Max	6.6	15.6	93.7	79.1	25.4	3.12	293.4
Mean	5.77	8.21	49.41	42.26	8.33	1.13	223.77
Standard Deviation	0.44	3.68	25.82	23.56	4.8	1.06	17.14

<sup>†</sup>pHw: pH in water; CEC: cation exchange capacity; OM: organic matter content by loss on ignition; EC: electrical conductivity.

Soil chemical properties were also diverse across the sample set, with pH ranging from 4.5 to 6.6 and EC ranging from 204.6 to 293.4 μScm<sup>-1</sup>. Cation exchange capacity is typically highly correlated to soil texture, with higher CECs found in conjunction with

higher clay and OM contents, but they also provide insight into the soil chemistry. The samples evaluated in the current study had CEC ranging from 1.2 to 15.6 cmol<sub>e</sub>/kg.

Table 4-2. Soil pH, cation exchange capacity (CEC), sand, silt, and clay content, organic matter (OM), and electrical conductivity (EC) for the A-horizon samples.

Sample No.	pHw	CEC cmol kg <sup>-1</sup>	sand	silt	clay	OM	EC μS cm <sup>-1</sup>
			-----%-----				
1	5.5	14.2	45.7	28.9	25.4	2.68	239.9
2	6.3	4.2	89.4	7.4	3.2	0.92	217.64
3	5.6	15.6	47.2	44.2	8.6	1.68	217.54
4	5.5	9.86	46.7	42.5	10.8	2.11	212.3
5	4.9	13.7	14.2	70.6	15.2	2.89	247.34
6	5.7	9.3	60.9	28.3	10.8	2.1	221.18
7	5.2	11.3	40.5	52.8	6.7	1.22	217.97
8	4.9	10.7	16.5	68.5	15	2.69	216.43
9	5.3	13.8	17.3	70.3	12.4	2.95	248.96
10	5.5	11.3	10.5	78.7	10.8	3.12	282.3
11	5.9	8.1	66.3	24.5	9.2	1.79	204.6
12	5.8	7.5	64.9	24.4	10.7	1.36	211.34
13	5.9	4.8	93.7	3.7	2.6	1.92	210.4
14	6.1	6.2	80.2	12.8	7	1.78	232.6
15	6.1	4.6	82.4	13.5	4.1	1.32	211.23
16	5.7	15.6	40.8	45.8	13.4	2.36	228.65
17	5.8	8.5	63.7	26.4	9.9	1.74	217.88
18	6.2	6.7	90.7	7.1	2.2	0.98	220.3
19	6.1	8.6	60.3	27.4	12.3	2.13	223.4
20	5.1	12.3	22.3	66.8	10.9	2.86	293.4
21	4.8	11.7	28.5	64.1	7.4	2.85	212.4
22	5.1	10.3	14	79	7	2.67	252.9
23	5.9	6.4	91.2	5.8	3	1.08	228.7
24	6.2	5.7	91.8	7.5	0.7	1.09	216.53
25	4.6	14.8	21.7	68.5	9.8	2.92	256.54
26	5.1	13.5	36.4	57.3	6.3	2.78	256.43
27	6.1	8.4	81.5	14.6	3.9	1.21	218.38
28	4.5	12.9	14	79	7	2.88	243.12
29	5.8	6.8	66.3	24.5	9.2	1.78	218.25
30	5.8	7.5	66.3	29.4	4.3	1.2	212.45
31	5.8	7.8	68.5	26.8	4.7	1.56	213.24
32	5.7	10.5	66.8	22.5	10.7	2.09	230.3
33	5.5	13.6	15.6	70.2	14.2	2.93	257.89
34	5.2	14.3	35.4	58.3	6.3	2.74	238.98
35	5.5	15.6	43.7	43.9	12.4	1.54	238.67
36	5.9	5.3	93.7	3.7	2.6	1.03	217.84
37	5.3	12.4	20.9	68.1	11	2.79	268.43

Table 4-3. Soil pH, cation exchange capacity (CEC), sand, silt, and clay content, organic matter (OM), and electrical conductivity (EC) for the B-horizon samples.

Sample No.	pHw	CEC	sand	silt	clay	OM	EC
		cmol kg <sup>-1</sup>	-----%-----				μS cm <sup>-1</sup>
1	5.8	7.4	31.8	64.9	3.3	0.25	212.36
2	5.8	8.6	22.5	66.5	11	0.11	218.96
3	6.5	2.5	92.1	6.8	1.1	0.1	212.35
4	5.9	8.5	40.3	57.4	2.3	0.18	208.45
5	6.3	7.3	45.3	43.7	11	0.25	219.67
6	6.1	2.5	64.8	30.4	4.8	0.28	215.67
7	5.9	9.4	36.5	58.5	5	0.14	217.65
8	6.1	7.5	18.2	73.9	7.9	0.16	213.43
9	6	7.6	63.9	27.6	8.5	0.01	218.45
10	5.9	8.3	17.8	76.5	5.7	0.22	214.89
11	6.4	1.2	93.5	5.8	0.7	0.19	211.78
12	5.9	3.5	55.4	36.9	7.7	0.25	217.54
13	6.4	1.6	77.5	13.5	9	0.1	210.45
14	6.2	3.2	78.6	17.8	3.6	0.27	210.87
15	5.7	7.4	26.4	56.9	16.7	0.12	219.56
16	5.7	9.8	28.4	63.2	8.4	0.18	210.25
17	5.8	8.5	38.7	52.4	8.9	0.13	219.59
18	5.7	4.3	48.4	46.2	5.4	0.34	227.89
19	6.6	3.8	65.8	25.6	8.6	0.17	211.34
20	5.9	8.8	31.5	57.5	11	0.1	220.56
21	5.8	7.5	25.8	62.4	11.8	0.24	219.54
22	6.1	3.6	59.2	29.7	11.1	0.21	218.45
23	5.3	7.8	28.5	59.3	12.2	0.16	218.2
24	5.8	8.6	14.3	65.4	20.3	0.18	217.89
25	6.2	2.1	91.5	6.8	1.7	0.31	217.87
26	5.7	9.5	36.5	58.6	4.9	0.13	222.56
27	6.1	3.2	57.8	38.7	3.5	0.32	216.75
28	5.9	9.4	26.8	72.4	0.8	0.36	218.34
29	5.8	9.8	30.8	63.6	5.6	0.24	213.43
30	5.9	8.5	32.6	54.3	13.1	0.17	212.76
31	6.1	3.4	66.3	21	12.7	0.42	212.33
32	6.2	4.2	81.4	13.6	5	0.2	212.35
33	5.5	8.4	12.5	70.6	16.9	0.15	213.26
34	6	4.2	42.5	42.7	14.8	0.23	229.76
35	6.5	3.6	85.4	11.3	3.3	0.2	214.76
36	5.9	7.9	47.8	43.6	8.6	0.21	219.87
37	5.7	9.5	28.7	63.6	7.7	0.32	212.73

As with soil physical properties, the diversity in soil chemical properties were most noticeable between A and B horizon samples. Cation exchange capacity of A horizon was much higher with mean of  $10.12 \text{ cmol kg}^{-1}$  and standard deviation of 3.5, compared to B horizon, where the mean cation exchange capacity was  $6.29 \text{ cmol kg}^{-1}$  and standard deviation of 2.79. For soil organic matter (OM), A horizon had a mean of 2.05% OM with standard deviation of 0.72, while the B horizon had negligible organic matter content with mean of 0.21% and standard deviation of 0.08. Mean pH of A horizon was 5.56 with standard deviation of 0.47, while that of B horizon was 5.98 with standard deviation of 0.29.

#### **4.3.2. Effect of soil properties on monensin sorption**

Based on correlation analyses, several observations were made. Organic matter, silt content, and CEC positively correlated with  $C_s$  in A horizon soils, with Pearson correlation coefficients of 0.77, 0.88 and 0.71 (p-value < 0.0001), respectively. pH<sub>w</sub> and sand content negatively correlated in A horizon soils with Pearson correlation coefficients of -0.77 and -0.89 (p-value < 0.0001), respectively. Cation exchange capacity and silt content positively correlated with  $C_s$  in the B horizon, with Pearson correlation coefficients of 0.75 and 0.71 (p-value < 0.0001), respectively. pH<sub>w</sub> and sand content negatively correlated with B horizon with Pearson correlation coefficients of -0.52 and -0.72 (p-value < 0.0001), respectively.

The organic matter distribution coefficient ( $K_{om}$ ) is used to estimate the extent of sorption of the analyte in organic phase. It is commonly used in place of  $K_{oc}$ , the organic carbon – water partition co-efficient. Both  $K_{om}$  and  $K_{oc}$  indicate the sorption characteristics of the analyte in the organic phase of soil.

$K_{om}$  can be calculated as the partition coefficient or  $K_d$  normalized by the fraction of organic matter in soil or  $f_{om}$ .

$$K_{om} = K_d / f_{om}$$

Figures 4-1 to 4-13 present the comparative correlation of  $C_s$  and  $K_d$  with soil parameters in A and B horizons as scatter plots along with trendlines. The magnitude of correlation coefficients for A horizon variables were found to be higher than for B horizons, suggesting that influence of soil parameters on partitioning behavior may be stronger in A horizon than B horizon soils. The correlation trends found in our study are comparable to other studies in the literature (Boxall et al., 2004; Hussain S.A. and Prasher, 2011; Sarmah et al., 2006; Sassman and Lee, 2007).

As the  $pK_a$  of monensin ranges from 4.5-6.5 as described in Table 1-5 and the native soil pH was found within the same range, it is expected that monensin will be in its protonated form. Hence monensin is expected to be strongly sorbed to the negatively charged clay or organic matter in the soil. This might be the cause of considerably higher  $K_d$  and  $K_{om}$  values in A and B soil horizons, compared to literature, The sorption may significantly decrease in agricultural soils, that is limed causing pH levels to be higher than 7 that is above the  $pK_a$  of monensin. Hence monensin may be negatively charged in such conditions and more associated with the solution phase and may be found more in ground water, surface water or accumulate in B horizons by preferential flow during high water conditions in the field.

Presence of organic matter has been related with lower pH, due to acidic degradation products by microbial activities in soil. This may be the reason why soils with higher sand percentages had higher pH and in general B horizon soils had higher pH



than A horizon soils. As sandy soils, generally has lower organic matter and clay content that sorbs monensin, soils with higher sand percentages were negatively correlated with monensin sorbed and  $K_d$ . For the same reason soils with higher silt percentages were positively correlated with sorption. The  $K_d$  and  $K_{om}$  values found in our study as presented in Table 3-2, Table 3-3, and Table 3-4, correspond well with the Log  $K_{ow}$  and Log  $K_{oc}$  values of monensin as presented in Table 2-1. Based on the log  $K_{ow}$  values found in the literature, where it suggests hydrophobicity is highest when  $pH \sim 5$ , where log  $K_{ow}$  is 4.2, monensin is most likely hydrophobic in nature in the  $pH$  range of our study. Log  $K_{ow}$  was found to decrease with increase in  $pH$ , to less than 3 at  $pH$  above 7. Hence the correlation trend is expected to change above the  $pH$  range of our study.

Cation exchange capacity (CEC) has been found to increase with increase in the active cation exchange sites, especially in organic phases and clay. Hence CEC is positively correlated with soils with higher clay and organic matter and hence with monensin sorption. Organic matter was also found to have positive correlation with sorption and  $K_{om}$  values ranging from 1756 – 14,077  $LKg^{-1}$  in our study. This indicates that monensin at our given  $pH$  and other soil conditions are very lipophilic in nature.

Based on the Pearson correlation coefficients,  $C_s$  and  $K_d$  were strongly correlated with  $pH_w$ , sand and silt content, and closely followed by organic matter in A horizon. Pearson correlation coefficients between  $pH$  and  $C_s$ ,  $K_d$ , and  $K_{om}$  were -0.77 ( $p < 0.0001$ ), -0.83 ( $p < 0.0001$ ) and -0.41 ( $p < 0.01$ ), respectively. Pearson correlation coefficients between sand and  $C_s$  and  $K_d$  were -0.89 ( $p < 0.0001$ ) and -0.84 ( $p < 0.0001$ ) respectively. Pearson correlation coefficients between silt and  $C_s$  and  $K_d$  were 0.88 ( $p < 0.0001$ ) and 0.85 ( $p < 0.0001$ ) respectively. Pearson correlation coefficients between

organic matter and  $C_s$  and  $K_d$  were 0.77 ( $p < 0.0001$ ) and 0.75 ( $p < 0.0001$ ) respectively. Overall,  $C_s$  was not as strongly correlated to soil physical and chemical properties in the B horizon compared to the A horizon.  $K_{om}$  was found to correlate with pH, CEC, sand, silt and organic matter with Pearson correlation coefficients of -0.41 ( $p=0.01$ ), 0.18 ( $p=0.27$ ), -0.34 ( $p=0.04$ ), 0.38 ( $p=0.02$ ) and 0.03 ( $p=0.87$ ). In the B horizon,  $C_s$  was strongly correlated with CEC and sand and silt content with Pearson correlation coefficients of 0.75 ( $p < 0.0001$ ), -0.72 ( $p < 0.0001$ ), and 0.7 ( $p < 0.0001$ ) respectively. There was a weak correlation in the B horizon between  $C_s$  and pH ( $r=-0.52$ ;  $p < 0.001$ ). In the B horizon  $K_d$  was weakly correlated with all the soil parameters with Pearson correlation coefficients less than 0.39;  $p < 0.01$ . Hence soil texture may not have a major influence on the sorption processes compared to CEC and pH in the B horizon. For B horizon soils the organic matter content was close to or below detection, as a result  $K_{om}$  could not be calculated.

#### **4.3.3. Effect of soil properties on monensin desorption**

The desorption parameters and irreversible sorption are presented in Table 3-2 for the A horizon and Table 3-3 for the B horizon. Desorption was weakly correlated with  $pH_w$  and cation exchange capacity ( $r = < \pm 0.25$  at  $p < 0.001$ ). Desorption was found to correlate with sand, silt, and organic matter content. The relationships between desorption and sand and silt content were higher in the A horizon, ( $r = 0.75$  and  $0.73$  at  $p < 0.01$ , respectively) compared to B horizon ( $r = 0.54$  and  $0.46$  at  $p < 0.01$ , respectively). The relationship between desorption and OM in the A horizon was not as strong as for other physical properties, with  $r = 0.59$ ,  $p < 0.001$ ). Scatter plots comparing desorption vs. sand, silt and organic matter content in A and B horizons have been presented in Figures 4-12

and 4-13. Overall a stronger correlation existed between desorption and sand, silt and organic matter content in A horizon compared to B horizon. Desorption exhibited weaker correlation to soil properties than sorption. Other factors might have a stronger influence on desorption; such as sorption processes and dynamic equilibrium state of the soil-solution system.

Understanding the sorption and desorption processes in the B horizon was an important part of the study. The B horizon has been neglected in the study of occurrence, fate and transport of pharmaceuticals in the environment. A general understanding is that these chemicals have less probability of reaching the sub-surface regions or B horizons due to either sorption, degradation, or surface run-off from the A horizons. Nonetheless, studies have found traces of ionophores in groundwater and have suggested their presence in the deeper soil horizons as well (Davis et al., 2006; Kim and Carlson, 2006). Also, as nitrogen loss due to volatilization from manure added as soil fertilizers is becoming an issue, precision agriculture techniques are being developed to use new technologies, such that fertilizers and manure can be added to the sub soils, instead of the soil surface. These techniques would definitely minimize volatilization, but at the same time introduce the ionophores deeper in the soil profile than surface application. Therefore, it is necessary to study the interactions of the ionophores with the B horizon soils.

Another way that ionophores can enter into the ground water and subsurface soils, is through the solution phase. Even though the  $K_d$  and the  $K_{om}$  values of monensin were in the higher range in the native pH of our study, suggesting its association with the solid phase, there is always a probability that in real field conditions they may sorb to the

dissolved organic matter or soil colloids immersed in the solution phase. Thus they may be transported into the ground water along with preferential flow of water, especially when farms are inundated. In this process they can also interact with sub-horizons and preferentially sorb to the solid phases of those horizons as water percolates down.

Irreversible monensin sorption was found on analyses of the residual monensin in solids in both A and B horizon soils, after the desorption study. Irreversible sorption may be related to the soil factors. In our mass balance experiments, 4-10% of initially added amount of monensin was irreversibly sorbed in the A horizon, compared to less than 6 % in the B horizon as also shown in Table 3-2 and 3-3. As organic matter, texture and cation exchange capacity was found to have a stronger correlation with sorption in A horizon compared to B horizon, the irreversible sorption may also be due to these soil factors.

#### **4.3.4. Limitations of the study**

Batch equilibrium studies are not an exact representation of field conditions, hence the data should not be extrapolated to understand the results in the field, but should be used as tools to design precise field experiments. The results discussed in Chapter 3 and Chapter 4 are mechanistic in nature. A few  $K_d$  values are often not sufficient for an entire study site and may change with environmental conditions. It is therefore important to be able to identify and measure the effect of ancillary environmental parameters that influence contaminant sorption. It is important to note that the interpretation of results from batch sorption tests generally allow no distinction to be made on how the analyte is associated with the sorbent (i.e., soil). The sorbate may be truly adsorbed by ion exchange, chemisorption, bound to complexes that are themselves sorbed on the solid, or

precipitated. Along with the physico-chemical soil parameters that have been correlated here to study its relatedness to the sorption and desorption processes, there are physical parameters in the field conditions, such as bulk density, moisture content, soil temperature and other factors that can influence these processes and cannot be accounted batch equilibrium laboratory studies. Other chemical parameters that were not included in the analyses were cations like magnesium ( $Mg^{2+}$ ), calcium ( $Ca^{2+}$ ), potassium ( $K^+$ ), or sodium ( $Na^+$ ). Monensin is known to chelate with cations like sodium, so the presence of these cations might influence the sorption processes.

The scope of inference for the  $K_d$  and  $K_{om}$  values and soil parameters were limited to our experimental design and experimental units that were studied. Doing more similar studies on different soil systems, under different biotic and abiotic conditions, would yield a better understanding of the dynamics of ionophores in the ecosystem. Hence the information generated by this study and the conclusions drawn cannot answer all the questions related to fate and transport of ionophores in the environment.

Due to the large sample size, and some of the experiments being timed, the filtrates from the batch studies had to be stored at  $4^{\circ}C$  for 2-3 months, before transporting it to the HPLC-MS/MS laboratory facilities for quantification of monensin. HPLC-MS/MS is a highly sensitive and time consuming instrument that requires frequent optimization and re-conditioning, especially if multiple projects are going on at the same time. Hence a better alternative to this would be to use radio-labeled isotopes of monensin that can be quantified in the soil and solution matrices by using scintillation chambers or radio-active counting instruments. But radio-labeled isotopes need to be custom synthesized and are extremely expensive. Hence they are not commonly used in

research purposes. However for mass scale environmental fate analyses in industries, this is a common technique.

Due to lack of time and resources, it was not possible to further this study and perform controlled experiment, to see the causal effects of the soil parameters especially pH, CEC, sand, silt and organic matter content on the sorption and desorption processes, though it is highly recommended as a future work.

#### **4.4. CONCLUSIONS**

Monensin sorption was found to be more strongly correlated with the physicochemical parameters of soils such as sand and silt content, pH<sub>w</sub>, and organic matter content in the A horizon soils than in B horizon soils. Monensin partitioning coefficients were less influenced by the soil parameters in B horizon soils with cation exchange capacity, sand and silt content having a greater influence on sorption compared to others. Desorption was influenced mainly by sand, silt and organic matter content in the A horizons but none of the parameters were found to have a strong influence in the B horizon soils. As several soil physico-chemical parameters strongly influenced sorption and desorption in A and B horizons, it may be expected that they may have a compounded influence on these processes under field conditions.

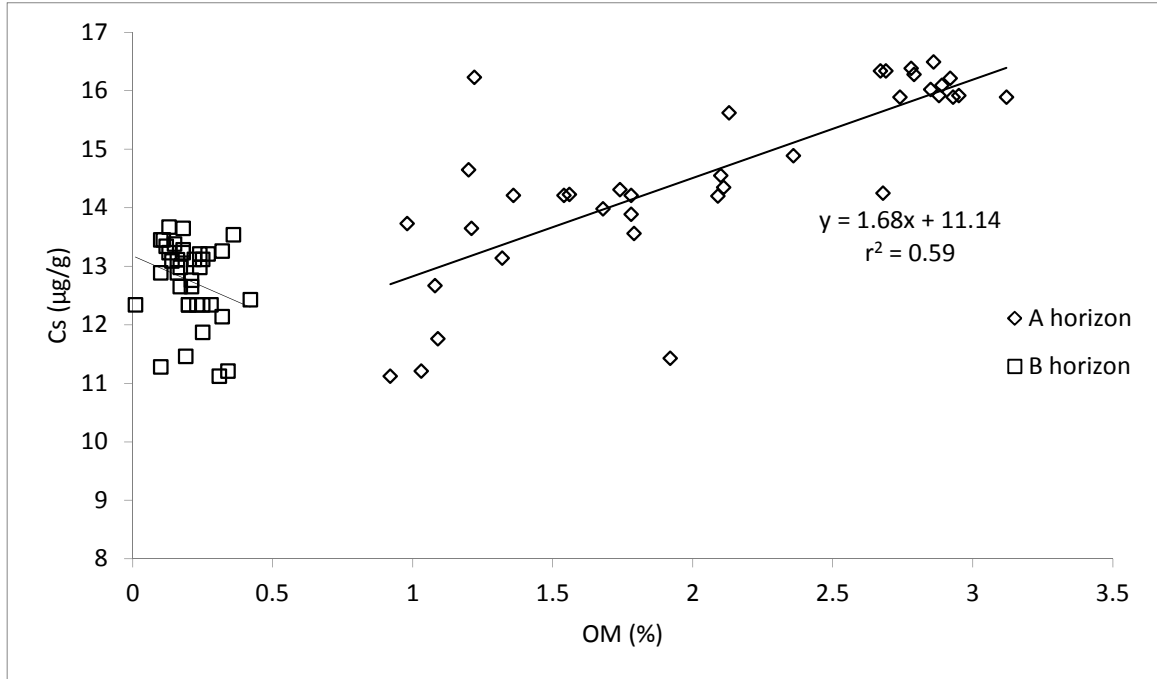


Figure 4-1. Relationship between monensin sorbed to the solid phase ( $C_s$ ) at equilibrium and soil organic matter content (OM) in A and B horizons of 37 soil samples evaluated.

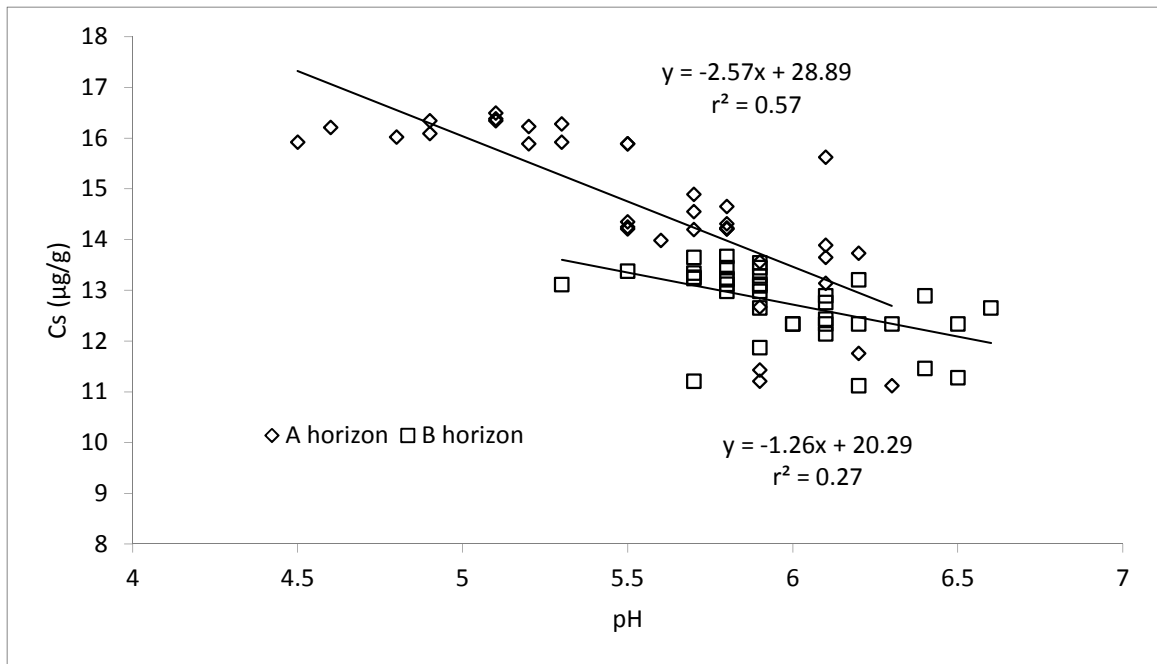


Figure 4-2. Relationship between monensin sorbed to the solid phase (Cs) at equilibrium and soil pH in A and B horizons of 37 soil samples evaluated.



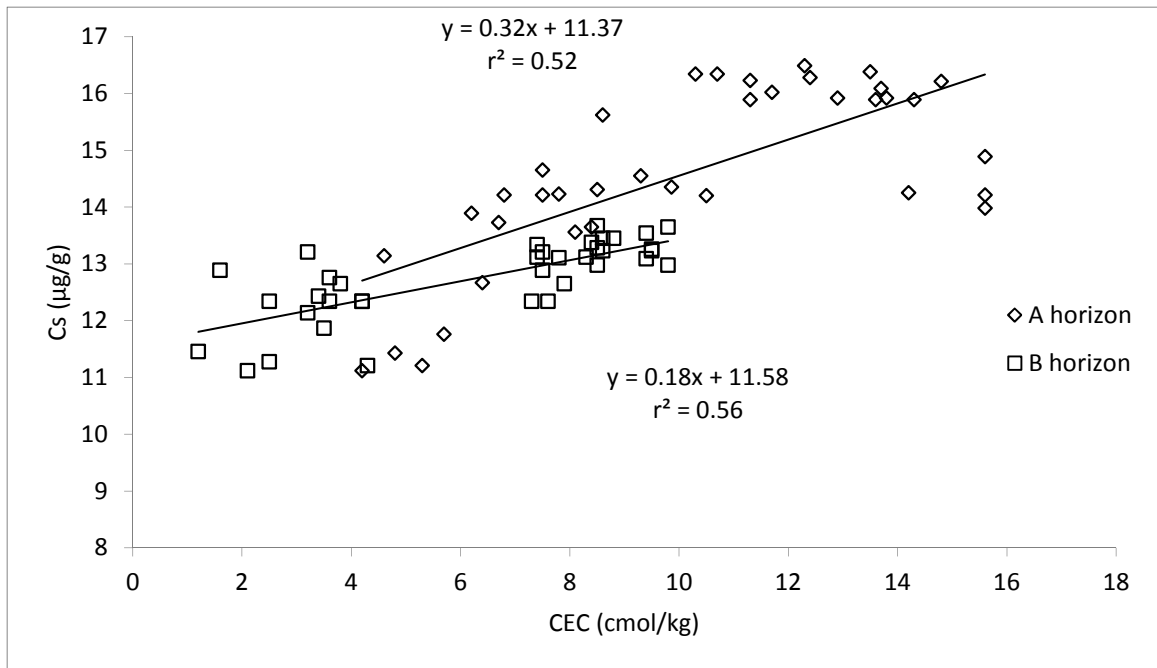


Figure 4-3. Relationship between monensin sorbed to the solid phase ( $C_s$ ) at equilibrium and soil cation exchange capacity (CEC) in A and B horizons of 37 soil samples evaluated.

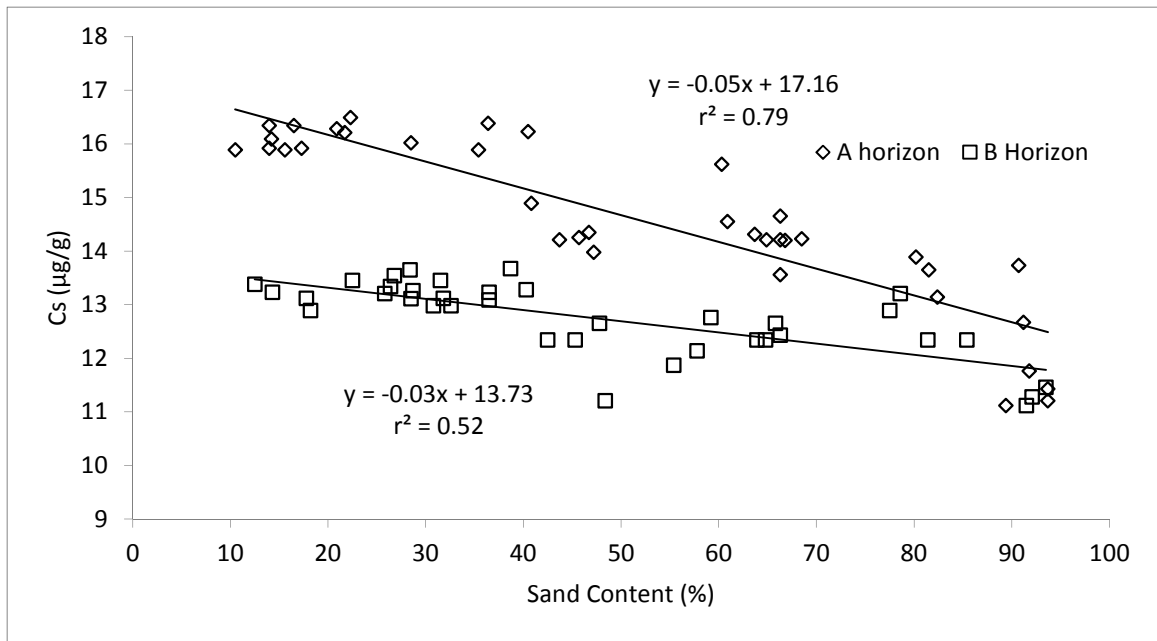


Figure 4-4. Relationship between monensin sorbed to the solid phase ( $C_s$ ) at equilibrium and sand content in A and B horizons of 37 soil samples evaluated.

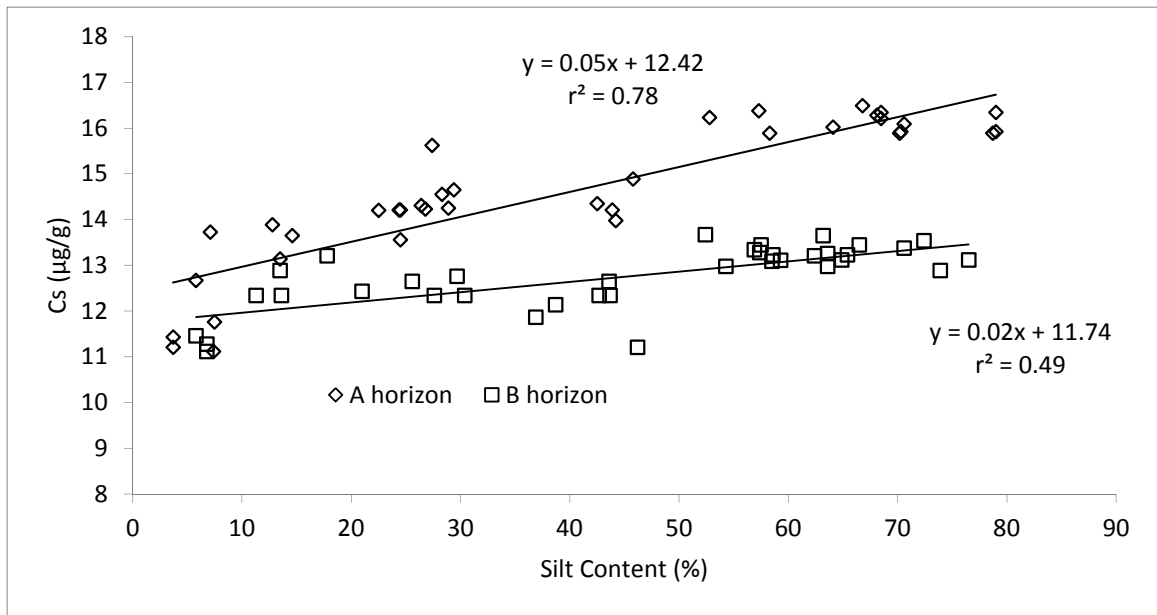


Figure 4-5. Relationship between monensin sorbed to the solid phase ( $C_s$ ) at equilibrium and soil silt content in A and B horizons of 37 soil samples evaluated.

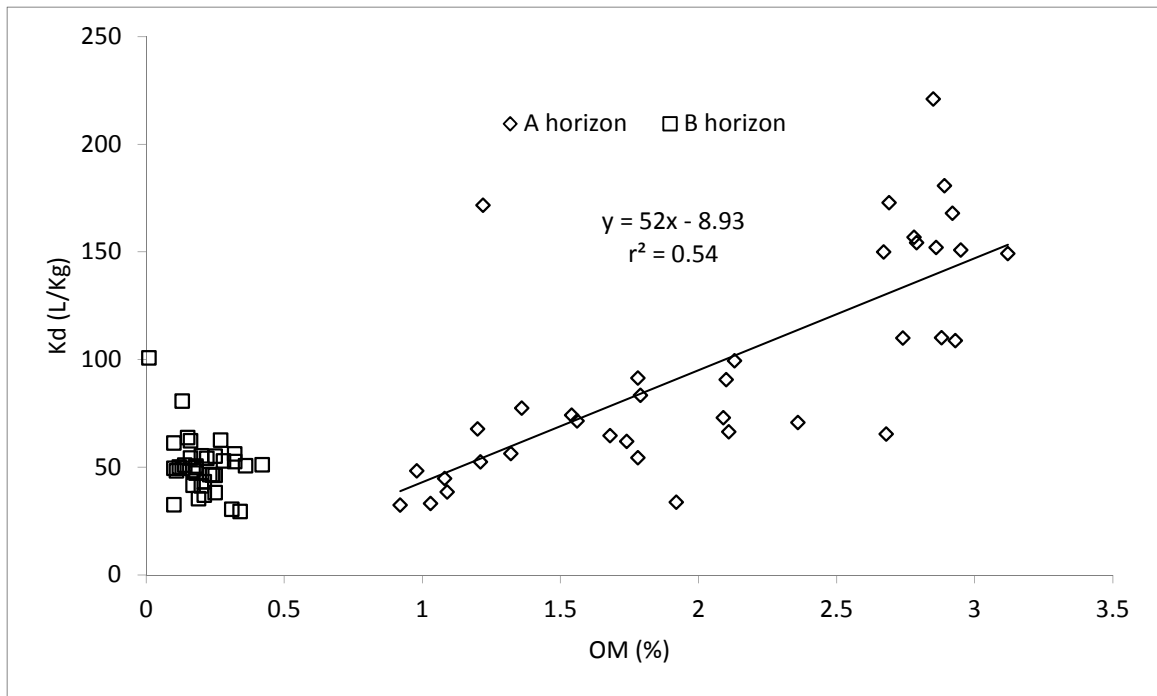


Figure 4-6. Relationship between monensin sorbed to the distribution coefficient (Kd) and soil organic matter content (OM) in A and B horizons of 37 soil samples evaluated.

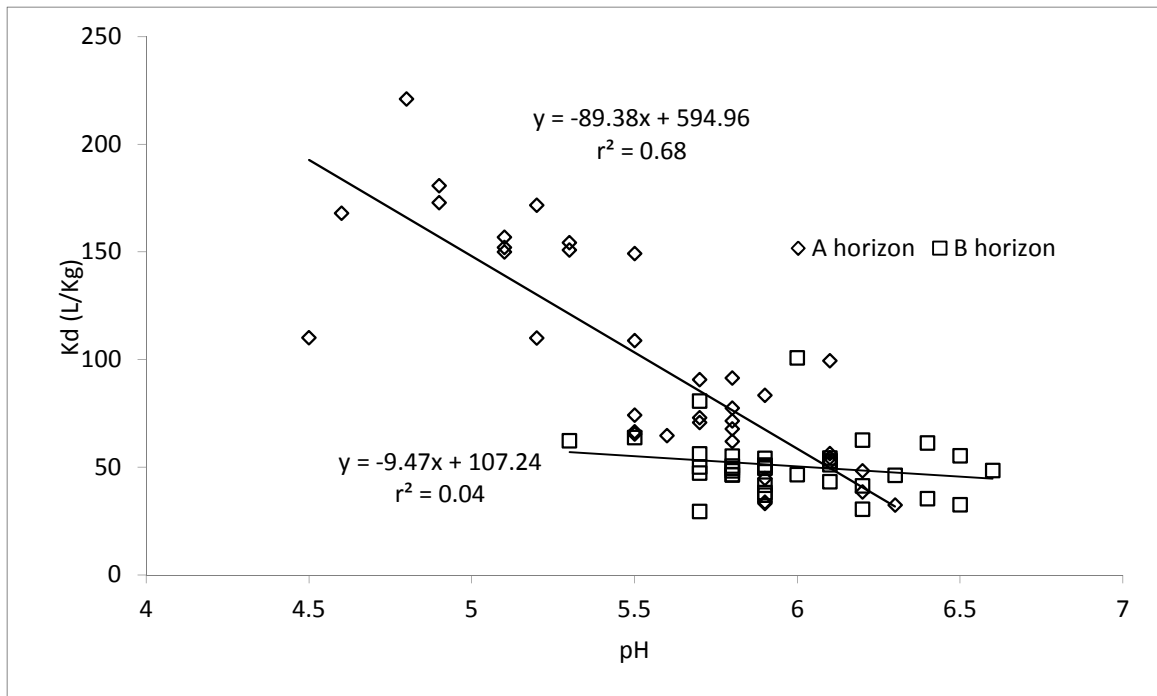


Figure 4-7. Relationship between distribution coefficient (Kd) and soil pH in A and B horizons of 37 soils evaluated.

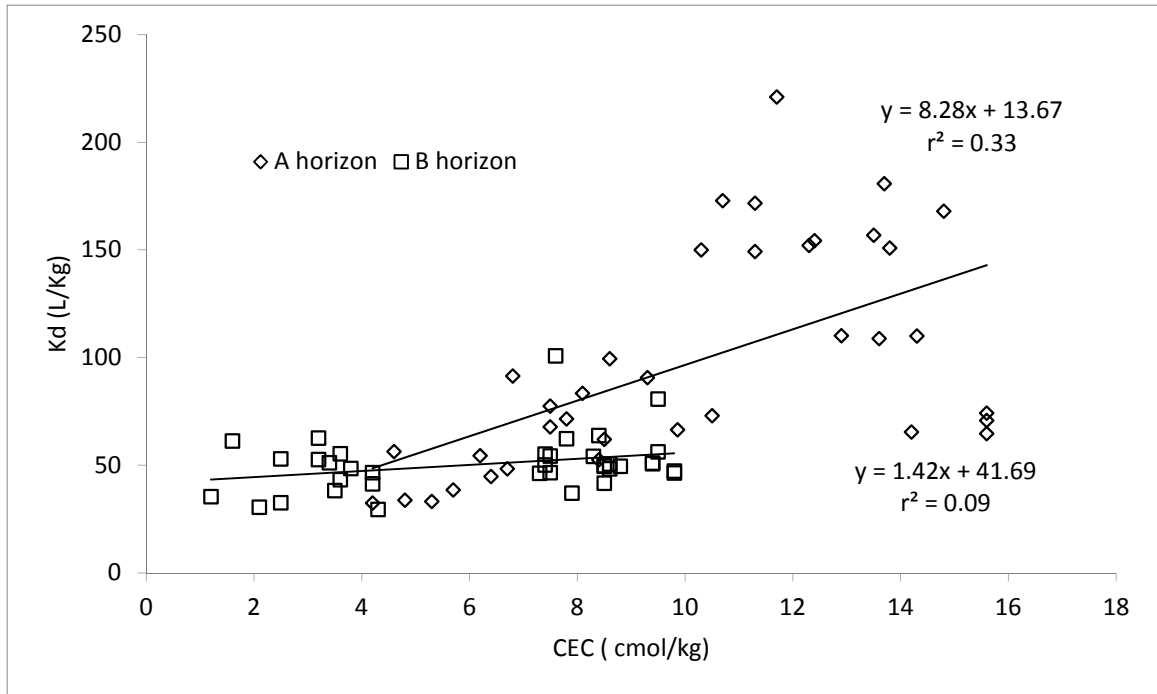


Figure 4-8. Relationship between distribution coefficient (Kd) and soil cation exchange capacity (CEC) in A and B horizons of 37 soils evaluated.

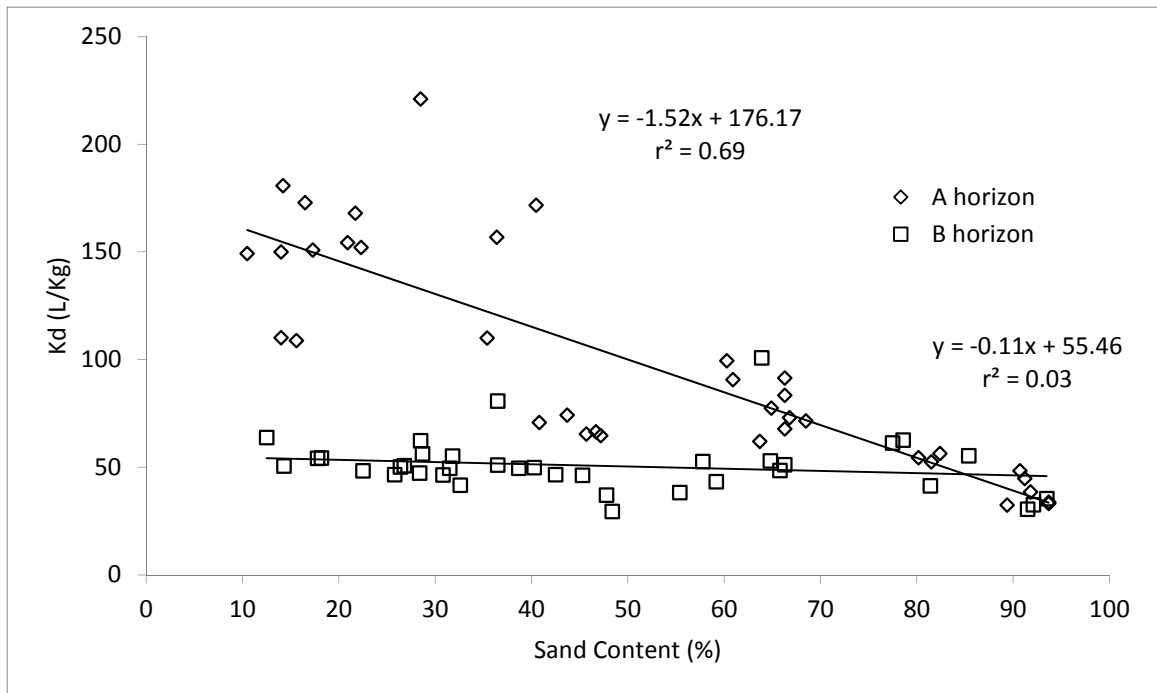


Figure 4-9. Relationship between distribution coefficient (Kd) and soil sand content in A and B horizons of 37 soils evaluated.

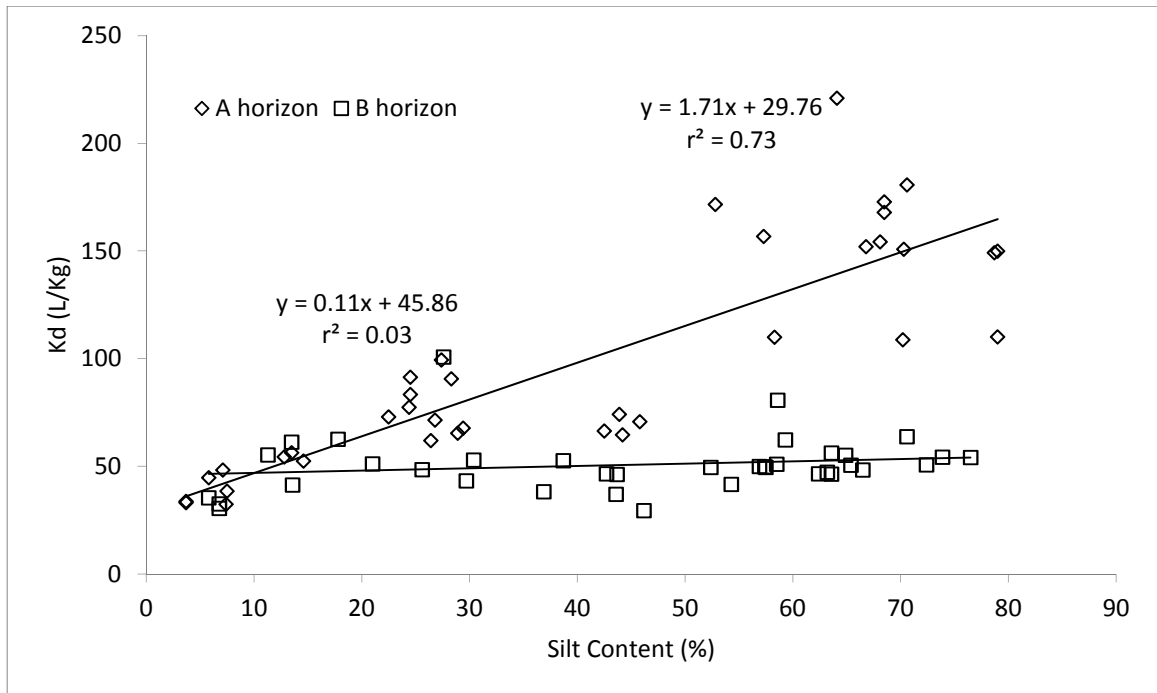


Figure 4-10. Relationship between distribution coefficient (Kd) and soil silt content in A and B horizons of 37 soils evaluated.



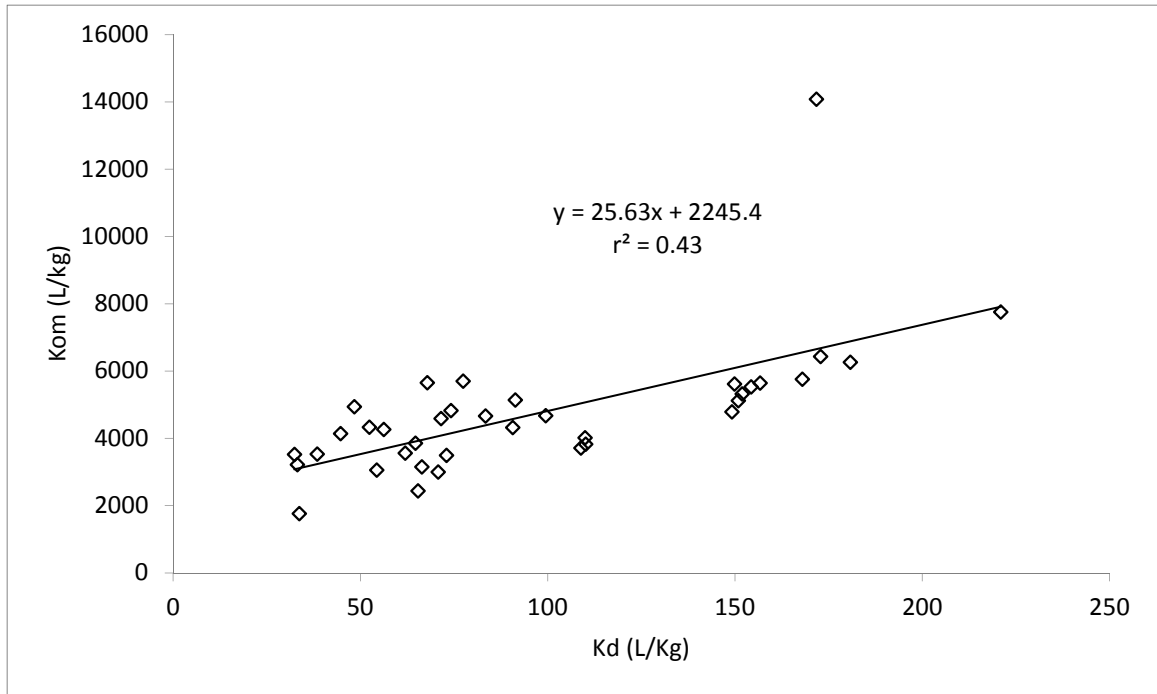


Figure 4-11. Relationship between distribution coefficient (Kd) and organic matter distribution coefficient (Kom) for 37 A-horizon soils evaluated..

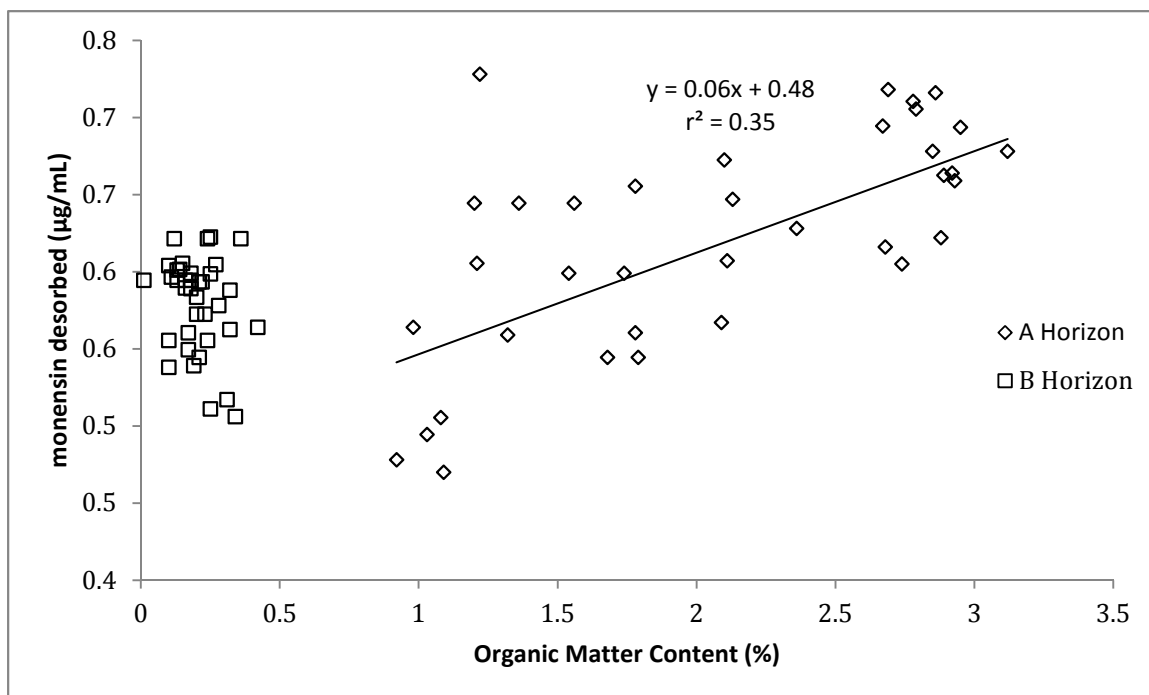


Figure 4-12. Relationship between monensin desorbed and soil organic matter content (OM) in the A and B horizons of the 37 soils evaluated.

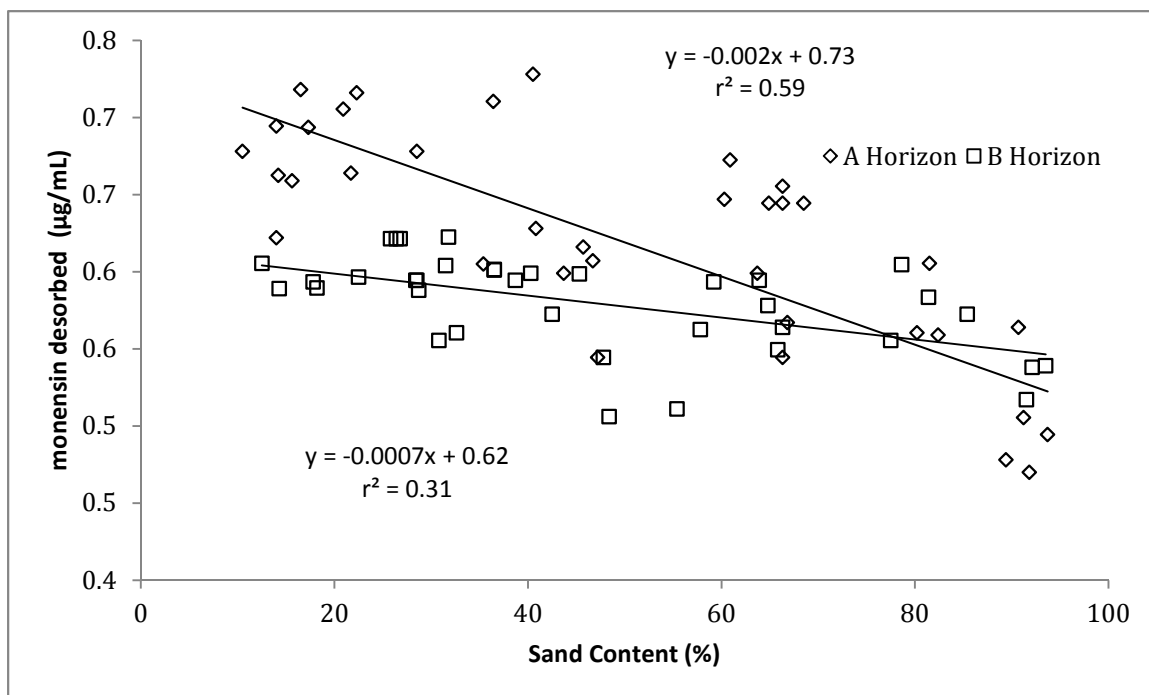


Figure 4-13. Relationship between sand content and monensin desorbed after batch equilibrium sorption study in the A and B horizon of 37 soils evaluated.

## **CHAPTER 5. CONCLUSIONS AND RECOMMENDED FUTURE RESEARCH**

This dissertation describes a multi-scale study on the trace analyses of ionophores in poultry litter and their behavior in soils. This is the first study on the dynamics of ionophores in soils of the Mid-Atlantic region of the US, and specifically the Delmarva Peninsula. A reliable and sensitive method using liquid chromatography triple quadrupole mass spectrometer was developed to quantify trace levels of monensin, salinomycin, narasin, and lasalocid aged in poultry litter. This method has been further used to quantify unknown concentrations of monensin in different soil types, sampled from the Mid-Atlantic region of the US, with minimum method modification. Studies have suggested that using high-pressure liquid chromatography for analyte separation and triple quadrupole mass spectrometer for detection and quantification is the most preferred technique for trace analyses of emerging contaminants from environmental matrices. This technique is preferred over using ELISA bio-assay kits, which can cause overestimation of analyte due to cross-reactivity, or UV detectors, which are not very analyte-specific. Using HPLC-MS/MS for trace analyses of ionophores greatly improved the quality of our results.

Furthermore, we developed our batch equilibrium study methodology according to EPA guidelines for parameter optimization. In addition, mass balances were calculated to confirm sorption parameter estimates. Other similar studies presented in the literature did not present their methodology for parameter optimization.

The results presented in this dissertation provide foundational data for further research on pH dependent sorption and desorption in sterilized and non-sterilized

environment. In addition ionophore degradation studies in soil are needed and should be evaluated under multiple conditions, including sterilized and non-sterilized soils; with and without manure addition; abiotic degradation (e.g., photolysis and hydrolysis); and transformation of product studies using high resolution mass spectrometer and collision induced dissociation. These controlled laboratory studies would provide important mechanistic information and should be followed by laboratory columns studies to understand the transportation mechanism of the analyte through the soil profile or field column studies using lysimeters to provide information regarding the fate and transport of the analytes at the field level.

Finally all these results should be used to model the transportation and dissipation pathways for monensin and other ionophores in the soil-water system. Further information is needed to ascertain how far the analyte can disperse in the environment and how long may it take. For contaminant transport modeling, apart from acquiring the above results, one needs to procure information on the partition coefficients between mobile and immobile regions, fractions of sorption sites, boundary layer transfer coefficient, liquid dispersion constant, and gas diffusion constant and flux.

This research generated critical information regarding the occurrence, quantification, and dynamics of monensin. This information can be used to support and design future large scale field studies and also contaminant transport modeling, which would contribute to a more complete understanding of the fate and transport of ionophores in the agricultural environmental. This would in turn allow further risk assessment studies to be performed to determine if ionophores are indeed an emerging contaminant of concern.

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