ESTRADIOL AND TESTOSTERONE SORPTION AND TRANSPORT IN SOIL WITH AND WITHOUT POULTRY LITTER

by

MONALISA BERA

(Under the direction of David E. Radcliffe)

ABSTRACT

The estrogenic hormones 17-β estradiol and testosterone are naturally occurring steroids found in poultry litter. The effects of litter on transport of these hormones are not known. Adsorption isotherms and breakthrough curves with packed soil columns were developed for ^{14}C-labeled testosterone and ^{3}H-labeled estradiol in a Cecil soil with and without poultry litter addition. The effect of applying the hormones alone or together (as they occur when litter is the source) was also investigated. Linear sorption coefficients (K_d) decreased significantly ($p<0.05$) from 19.7 to15.9 mL g^{-1} for testosterone and significantly ($p<0.05$) increased from 18.6 to 24.3 mL g^{-1} for estradiol in the presence of litter. In column experiments, estradiol peak concentrations occurred immediately after chloride (a conservative, slightly sorbed tracer) and the breakthrough curves were highly skewed. Testosterone peak concentrations occurred almost 12 pore volumes after chloride and the breakthrough curves were more symmetrical. Litter had no effect on the percentage of testosterone leaching from the columns (on average 18.0%) but estradiol leaching significantly decreased from 28.2 to 25.4% with litter. Applying the hormones together increased the mobility of estradiol and decreased the mobility of testosterone. Although the sorption coefficients of both hormones were high, appreciable amounts of estradiol moved through soil quickly due to chemical non-equilibrium. Litter may facilitate the movement of testosterone due to co-transport by fine particulate organic matter.

INDEX WORDS: Hormones, Poultry litter, Soil transport parameters, Packed soil columns, HYDRUS-1D

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DEDICATION

Dedicated to my parents

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CHAPTER 1

INTRODUCTION

According to the National Agricultural Statistic Service, the United States is the world's largest poultry producing country. Broiler production in the United States has increased from 4.1 in 1968 to 22.68 billion kg in 2008 (USDA-NASS, 2009). Georgia, which is the largest producer of broilers, produced 1.4 billion broilers in 2009. This rapid growth of broiler production automatically increases the production of poultry waste which is used as fertilizer as a source of nitrogen, phosphorous and potassium (Moore et al., 1995).

In addition to nutrients, 17-β estradiol and testosterone have been found as naturally occurring estrogens in poultry litter. These hormones have the ability to affect endocrine systems of humans and wild life. The amount of hormone in litter depends on sex and type of the bird. Lorenzen et al. (2004) estimated the content of estradiol and testosterone were 55 and 30 μg kg^{-1} in broiler litter and 70 and 25 μg kg^{-1} in breeder litter. Shore et al. (1993) found that the testosterone content in male and female broiler litter was approximately 14 μg kg^{-1} but the amount of estrogen was higher in layer litter (approximately 55 μg kg^{-1}). Both hormones can pose a potential health hazard to human and wild life when they contaminate the surface and subsurface water as a result of runoff and leaching from pastures.

Some studies have reported the sorption and transport characteristics of estrogenic hormones through soil. Sorption and transport parameters of estradiol and testosterone

were determined using disturbed and packed soil column (Casey et al., 2003; Casey et al., 2004; Casey et al., 2005; Das et al., 2004; Fan et al., 2008; Sangsupan et al., 2006). But these studies have not shown the effect of poultry litter in the transport of hormones.

No studies have examined the effect of poultry litter on the transport of 17-β estradiol and testosterone. Organic matter in litter could retard the movement of the hormones due to strong sorption, or could enhance movement due to co-transport with fine particulate matter. Also, when poultry litter is the source for these hormones, they have the potential to compete for sorption sites. The objective of our research was to evaluate the effect of poultry litter on the sorption and transport of 17-β estradiol and testosterone in packed soil columns when the hormones were applied together and when they were applied alone.

CHAPTER 2

LITERATURE REVIEW

Land application of poultry litter, animal manure and sewage sludge as fertilizer increases the amount of estrogenic hormones in soil and can lead to surface and subsurface water contamination. Kolpin et al. (2002) sampled surface water from 139 streams of 30 states in the U.S. and found that reproductive hormones were present in approximately 40% of the 139. The presence of these hormones in the environment, even in very low concentrations, can cause infertility, abnormal physiological processes and reproductive impairment in humans and wildlife (Nichols et al., 1997). Some chlorinated hydrocarbons, pesticides and industrial byproducts display estrogenic activity and they can cause infertility, deformed reproductive organs and other biological defects, as well (Peat, 1997). Estrogenic activity of some xenobiotic compounds impaired the embryonic development of wild alligators in Lake Apopka in Florida (Guillette et al., 1994).

Increasing population and consumer demand have led to the expansion of the poultry industry in several states in the U.S. (Moore et al., 1995). According to the National Agricultural Statistics Service, (USDA-NASS, 2009) in 2006 the poultry industry in Georgia, the top broiler-producing state, generated 1.3 billion broilers with about 13 million Mg of litter. Annual broiler production in the U.S. increased from 4.1 billion kg in 1968 to 22.7 billion kg in 2008 (USDA-NASS, 2009) leading to increased production of litter.

According to the U.S. Department of Agricultural, the number of layers in United States was 333 million in 2009. Poultry litter is considered "the best organic fertilizer" (Moore et al., 1995) and applied in agricultural land. Though poultry litter is a good source of nutrients (N, P, K) for plants, testosterone and 17-β estradiol have also been found in litter (Das et al., 2004). As such, the traditional land application of poultry litter poses a potential environmental hazard due to the presence of these estrogenic hormones which may reach surface or drinking waters.

Testosterone and estradiol are naturally occurring steroids which are responsible for the growth and development of reproductive organs in vertebrates . Estrogen in women helps to develop secondary female sex characteristics. Estrone, estradiol and estriol are three naturally occurring forms of estrogen in women. Apart from secondary sex characteristics, estrogen accelerates skeletal maturation without accelerating growth, reduces muscle mass, and stimulates endometrial uterine growth (Barnard et al., 2002). Estrogen can also be present in male tissues. (Hess et al., 1997) estimated a concentration of 250 pg mL^{-1} estrogen in rete (delicate network of tubules of the testicle) testis fluid.

Testosterone is an androgenic steroid which plays a vital role in the development and maintenance of the male sex organ. It is responsible for the normal virilization in males which includes increased musculature, bone maturation, deepening of the voice, thickness of the jaw and broadening of the shoulders. Testosterone helps to prevent osteoporosis in males. (Olsson et al., 2000) found that testosterone implantation significantly increased plasma testosterone levels which helped to increase movement in sand lizards.

Elevated levels of both testosterone and estradiol have led to tumorigenic and carcinogenic effects in human cells. Estrogen promotes the growth of cancers in mammary glands (National Cancer Institute, 2008). (Sinha et al., 1972) reported that the growth of mammary tumors increased with increased levels of estrogen. Administration of estrogen promoted the secretion of prolactin which hastened the growth of tumors in the mammary gland of female rats. Testosterone deficiency can causes hypogonadism in males which is associated with damage to the hypothalamus, pituitary gland or testicles. Low levels of both hormones may include poor libido, sleep disorder, glucose intolerance, and high cholesterol (Testosterone deficiency, Urology channel).

Environmental exposure of naturally occurring estrogen and xenoestrogen (synthetic estrogen) has been associated with a number of physiological abnormalities in humans and wildlife. Physiological abnormalities include infertility, deformed reproductive organs, tumors, immune deficiency and other biological defects (Peat, 1997). Some of the most widely reported evidence of endocrine disruption has been found in aquatic environments, especially in fish (Jafari et al., 2009). Plasma vitellogenin of rainbow trout increased 1000-times in three weeks after sewage treatment (Purdom et al., 1994). Nakamura et al. (1984) reported that 17β-estradiol exposure to masu salmon induced gonadal feminization when treated with 0.5-5 μg L^{-1} estradiol. Estradiol administered at different doses to alevins (Atlantic salmon) resulted in feminization of male fish (Sower et al., 1984)

The effect of testosterone has been reported in only a few studies. Premdas et al. (2001) injected testosterone into white suckers to promote the development of papilloma. Ninety three per cent of white suckers either retained or developed papilloma and 85% of

non-papillomatous white suckers developed papilloma with 5 -25% growth in tumors for all the white suckers which had papilloma initially. Buhler et al. (2000) has shown that testosterone treatment of male trout increased the level of P450 enzyme by 42%.

Studies such as Kolpin et al. (2002) have found wide spread evidence of hormones in the environment. Environmental exposure to estrogenic compounds, even in very low concentrations, has become a growing cause of concern. Jafari et al. (2009) investigated the concentration of different endocrine disrupting chemicals (EDC) in surface waters and wastewaters of Iran. EDCs were higher in sewage than other sampling sites such as surface water and drinking water. Huang et al. (2000) collected wastewater from four municipal wastewater treatment plants in California. The highest concentrations of 17β-estradiol and 17α-ethinyl estradiol in the effluent were 3.8 and 2.0 ng L^{-1}, which are above the concentrations reported to cause endocrine disruption (Desbrow et al., 1998).

The major sources of estrogen in the environment are waste water treatment effluent, land applied animal waste (which includes poultry litter), and feces and urine excreted by humans and wildlife (Khanal et al., 2006). Both estradiol and testosterone are found in human and other livestock. The amount of hormone present depends on sex and species. Men and women excrete estradiol at the rate of 1.6 and 3.9μg day^{-1}, respectively. But the amount excreted by pregnant women is 259 μg day^{-1} (Ying et al., 2002). 17-α estradiol, 17-β estradiol, estrone and estriol have been found in the excreta of dairy cows, whereas other animal species excrete 17- β estradiol, estrone, estriol. It has been shown that a pregnant dairy cow excretes more 17- α estradiol than a non-pregnant dairy cow (Khanal et al., 2006). Broiler litter is one of the main sources of estradiol and

testosterone in agricultural fields. Poultry litter contains 17- β estradiol and testosterone. Concentrations of these hormones depend on the sex and type of bird. Male broiler litter contains 14 and female broiler litter contains 65 μg $kg^{-1}$of estrogen. The concentration of these hormones in breeder litter is much higher than broiler litter. The amount of estradiol and testosterone present in breeder litter is 533 μgkg^{-1} and 254 μgkg^{-1}, respectively (Hemmings and Hartel, 2006).

Addition of litter and manure as fertilizer may contaminate both surface and subsurface waters and ultimately impact biota in the aquatic environment. The degree of water contamination will depend on the amount of hormones that are sorbed by soil. Steroids are hydrophobic in nature so the persistence and bioavailability of these hormones depends on their sorption to particulate matter (Emmerik et al., 2003). Their high sorption coefficients (K_d) indicate that estradiol and testosterone are strongly bound to soil. Holthaus et al. (2002) reported linear sorption coefficients (K_d) ranging from 4-74 mL g^{-1} for bed sediments and 21-122 mLg^{-1} for suspended sediment in the River Thames. Linear (K_d) and non-linear (K_f) sorption coefficients for testosterone range from 5 to 13.5 mL g^{-1} and 13.6 to 29.4 μg^{1-n} mL ng^{-1} and for estradiol 9.6 to 21.9 mL g^{-1} and 24.3 to 36.9 μg^{1-n} mL ng^{-1} (Sangsupan et al., 2006). A runoff study by Nichols et al. (1997) has shown that concentration of estradiol in runoff increases with the application rate of litter. Finley-Moore et al. (2000) estimated the concentration of estradiol and testosterone in flow-weighted runoff of a non-grazed paddock. The concentrations were 60 ng L^{-1} for estradiol and 15 ng L^{-1} for testosterone before litter application. These concentrations increased to 2530 ng L^{-1} for estradiol and 1830 ng L^{-1} for testosterone after the third application of poultry litter. Estradiol was degraded to estrone even in sterile soil after 70

days incubation (Ying and Kookana, 2005). But the degradation rate of 17-β estradiol was lower in sterile soil (Colucci et al., 2001). Casey et al. (2005) could not find any metabolites of 17-β estradiol in the aqueous phase in their adsorption experiment.

Several column studies have simulated the transport of steroids through soil (Casey et al., 2003; Casey et al., 2004; Casey et al., 2005; Das et al., 2004; Fan et al., 2008; Sangsupan et al., 2006). Casey et al. (2004) reported column transport experiments for a wide range of soil textures. Low concentrations of ^{14}C-labeled testosterone in column effluent indicated a high sorption affinity of hormone to soil. The lowest peak concentrations occurred for the organic C rich (7.5%) Bearden soil. They also showed that most of the testosterone was adsorbed in the upper 1-3 cm of packed soil columns. Casey et al. (2003) found no 17-β estradiol in column effluent and the recovery was 100% which indicated very high sorption. In another column study, 26% of ^{14}C-labeled 17-β estradiol and 36.7% of estrone (applied as ^{14}C-labeled 17-β estradiol) were eluted. Twenty two percent of ^{14}C was recovered through oxidation which contained ^{14}C as 17-β estradiol (16.9%), estrone (5.08%), estriol (0.18%) and a very low amount of another unidentified metabolite (0.08%) (Casey et al., 2005). Transport and sorption of these hormones and their metabolites depend on the polarity of the compounds. Higher polarity metabolites of estradiol can be rapidly transported through soil, compared to lower polarity metabolites (Casey et al., 2003). Packed soil columns may not be sufficient to simulate field conditions. Packing destroys soil structure and large soil pores which are natural and essential for liquid flow. An intact soil column study by Sangsupan et al. (2006) demonstrated that the transport of chloride and hormones were similar. Continuous macropores in undisturbed soil allowed rapid infiltration .

Liquid flow and solute transport in variably saturated porous media can be simulated by the HYDRUS-1D software package (Šimůnek et al., 1998) which includes one-dimensional movement of water and solutes. This model numerically solves the Richards (1931) equation for saturated-unsaturated water flow and the advection dispersion equation (ADE) for solute transport.

HYDRUS-1D can simulate transport of solutes involved in sequential first-order decay reactions, such as estradiol (solute 1) transforming to estrone (solute 2). This is known as a chain model. HYDRUS-1D also simulates chemical non-equilibrium due to sorption kinetics. The chemical non-equilibrium model assumes that total sorption (s) [M M^{-1}] is divided into type-1 sites (s^e) [M M^{-1}] and type-2 sites (s^k) [M M^{-1}]:

$$s = s^e + s^k$$

Equation 1

Type-1 sites include instantaneous equilibrium sorption while the adsorption in type-2 sites is assumed to be kinetic. At equilibrium, the fraction of the total sorption that occurs on type-1 sites is described by f (unit less):

$$f = \frac{s^e}{s}$$

Equation 2

where linear kinetic adsorption is assumed, the change in sorption on type-2 sites is described by the equation:

$$\frac{\partial s^k}{\partial t} = \omega\left[(1-f)K_d c - s^k\right] - (\mu_s + \mu_s')s^k$$

Equation 3

where $K_d c$ is the final sorbed concentration at equilibrium, ω is the kinetic rate constant for sorption on type-2 sites [T^{-1}], μ_s is a first-order solid-phase transformation constant [T^{-1}], and μ_s' is a first-order solid-phase transformation providing a connection between species in a chain model [T^{-1}].

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CHAPTER 3

ESTRADIOL AND TESTOSTERONE SORPTION AND TRANSPORT IN SOIL WITH AND WITHOUT POULTRY LITTER[1]

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Abstract

The estrogenic hormones 17-β estradiol and testosterone are naturally occurring steroids found in poultry litter. The effects of litter on transport of these hormones are not known. Adsorption isotherms and breakthrough curves using packed soil columns, were developed for ^{14}C-labeled testosterone and ^{3}H-labeled estradiol in a Cecil soil with and without poultry litter addition. The effect of applying the hormones alone or together (as they occur when litter is the source) was also investigated. Linear sorption coefficients (K_d) decreased significantly ($p<0.05$) from 19.7 to 15.9 mL g^{-1} for testosterone and significantly ($p<0.05$) increased from 18.6 to 24.3 mL g^{-1} for estradiol in the presence of litter. In column experiments, estradiol peak concentrations occurred immediately after chloride (a conservative, slightly sorbed tracer) and the breakthrough curves were highly skewed. Testosterone peak concentrations occurred almost 12 pore volumes after chloride and the breakthrough curves were more symmetrical. Litter had no effect on the percentage of testosterone leaching from the columns (on average 18.0%) but estradiol leaching significantly decreased from 28.2 to 25.4% with litter. Applying the hormones together increased the mobility of estradiol and decreased the mobility of testosterone. Although the sorption coefficients of both hormones were high, appreciable amounts of estradiol moved through soil quickly due to chemical non-equilibrium. Litter may facilitate the movement of testosterone due to co-transport by fine particulate organic matter.

Introduction

The presence of estrogenic hormones in land-applied animal manures, poultry litter, and domestic and industrial waste, even in very low concentrations, has become a

growing concern due to their adverse effect on endocrine systems of humans and wildlife. Although estradiol and testosterone help to regulate secondary sex characteristics under normal conditions, chronic exposure to these steroids has been associated with abnormal physiological processes and reproductive abnormalities in birds (Moccia et al., 1986), turtles (Bishop et al., 1991) and mammals (Martineau et al., 1988). A number of studies have reported endocrine disruption in aquatic species as most land applied domestic and industrial effluents have the potential to go to surface and subsurface waters (Jafari et al., 2009). Routhledge et al. (1998) reported that the vitellogenin level in male rainbow trout and roach was increased during three weeks exposure period of estradiol.

According to the National Agricultural Statistic Service, the United States is the world's largest poultry producer. Broiler production in the United States has increased from 4.1 in 1968 to 22.68 billion kg in 2008. Georgia, which is one of the highest poultry producing states, produced 1.4 billion broilers in 2009 (USDA-NASS, 2009). This rapid growth of broiler production has increased the production of poultry waste, which is being applied to agricultural land as fertilizer.

In addition to nitrogen, phosphorus and potassium, poultry litter contains appreciable amounts of 17-β estradiol and testosterone. Lorenzen et al. (2004) have shown that pullet layer litter contains approximately 400 ng g^{-1} of estradiol and of 15 ng g^{-1} of testosterone. They estimated the content of estradiol and testosterone were 55 and 30 ng g^{-1} in broiler litter and 70 and 25 ng g^{-1} in breeder litter (Lorenzen et al., 2004). Significant amounts of steroid hormones can reach surface water by runoff and this poses a potential hazard to humans and wildlife, even in concentrations at the ng L^{-1} level (Routledge et al., 1998).

The degree of surface and subsurface water contamination depends on the sorption of estrogen to soil. Steroids are hydrophobic in nature; water solubility of estrogen is 13 mg L^{-1} (Ying et al., 2002). Their high sorption coefficients (K_d) indicate that estradiol and testosterone are strongly bound to soil. Holthaus et al. (2002) reported linear sorption coefficients (K_d) ranging from 4-74 mL g^{-1} for bed sediments and 21-122 mLg^{-1} for suspended sediment in the River Thames. In work by Sangsupan et al. (2006) linear (K_d) and non-linear (K_f) sorption coefficients for testosterone ranged from 5 to 13.5 mL g^{-1} and 13.6 to 29.4 μg^{1-n} mL ng^{-1}. For estradiol, the values ranges were 9.6 to 21.9 mL g^{-1} and from 24.3 to 36.9 μg^{1-n} mL ng^{-1}. The high sorption coefficients of steroids indicate that the transport and bioavailability of these hormones depend on their sorption to soil particles. Several studies have reported a correlation between sorption and the total organic carbon content of the soil. Higher organic matter content of the soil results in higher sorption (Casey et al., 2003; Lai et al., 2000). Casey et al., (2003) determined the K_d values (ranging from 135 to 6670 mL g^{-1}) for a broad range of soil textures (that varied in organic C content from 3.3 to 9.2%). The highest sorption was observed for the soil with highest organic C content. Casey et al. (2005) reported K_d values that varied from 3.56 to 83.2 mL g^{-1} for estradiol and 4.47 to 42.7 mL g^{-1} for testosterone. However, the log K_{oc} values were consistent, in the range from 3.19 to 3.46.

The fate and transport of steroid hormones in the environment have been simulated using column studies (Braga et al., 2005; Casey et al., 2003; Casey et al., 2004; Casey et al., 2005; Das et al., 2004; Fan et al., 2008; Sangsupan et al., 2006). Casey et al. (2004) reported column transport experiments of ^{14}C-labeled testosterone for a wide range of soil textures that showed a high sorption affinity of the hormone to soil. They

also showed that most of the testosterone was adsorbed in the upper 1-3 cm of packed soil columns. Casey et al. (2003) found no 17-β estradiol in column effluent and the recovery was 100% which indicated very high sorption. Packed soil columns may not be sufficient to simulate field conditions. Packing destroys soil structure and large soil pores which are natural and essential for liquid flow. Das et al., (2004) has shown that the estradiol and testosterone breakthrough curves in a packed soil column are strongly asymmetrical indicating nonequilibrium sorption with two sorption sites. Fan et al. (2008) has reported the early peak of 17-β estradiol (and metabolites) than chloride in an undisturbed column study. An intact soil column study by Sangsupan et al. (2006) demonstrated that the transport of chloride and hormones were similar. Continuous macropores in undisturbed soil allowed rapid infiltration. So far, no studies have examined the effect of poultry litter on the transport of estrogenic hormones through soil.

No studies have examined the effect of poultry litter on the transport of 17-β estradiol and testosterone. Organic matter in litter could retard the movement of the hormones due to strong sorption, or could enhance movement due to co-transport with fine particulate matter. Also, when poultry litter is the source for these hormones, they have the potential to compete for sorption sites.

The objective of our research was to evaluate the effect of poultry litter on the sorption and transport of 17-β estradiol and testosterone in packed soil columns. Both sorption kinetics and batch equilibrium sorption experiments were performed to determine sorption characteristics. The hormones were applied alone and together. Hormone transport was modeled using the HYDRUS-1D model (Šimůnek et al., 1998).

Materials and Methods

Soil Samples

Soil Samples and Poultry Litter

Bulk soil samples for sorption experiments and packed columns were collected from the 0-15 and 15-30 cm soil depths at the University of Georgia Central Branch Research Station near Eatonton, Georgia. The soil at the site was a Cecil sandy loam (fine, kaolinitic, thermic Typic Kanhapludult) which is a common soil in the Southern Piedmont physiographic region. The plots were selected from pastures that have received broiler litter for approximately 12 years. Soil samples were air-dried, ground, passed through a 2-mm sieve and stored at 20˚C. Table 1 gives the soil physical properties.

Poultry litter was obtained from a poultry farm located in North-East Georgia. Litter was air dried, ground and stored at 20˚C.

Hormones

Unlabeled estradiol and testosterone (both >98% purity) were obtained from Sigma-Aldrich (St. Louis, MO). Unlabeled hormones were diluted to 100 μg mL^{-1} with HPLC grade methanol. Desired concentrations of estradiol and testosterone were made from 100 μg mL^{-1} with 0.01 M $CaCl_2$ for sorption isotherms. Solutions were stored in a refrigerator. Radio-labeled 6,7-^{3}H-estradiol (specific activity 50 Ci $mmol^{-1}$) and 4-^{14}C-testosterone (specific activity 55 mCi $mmol^{-1}$) were obtained from American Radiolabeled Chemicals (St. Louis, MO). Labeled hormone stock solution of the desired concentration were made using HPLC grade methanol and kept in a refrigerator. In this study, we refer to the ^{3}H label as “testosterone” and the ^{14}C as “estradiol”, but recognize

that the label may actually be a metabolite of these hormones. Where it is appropriate, we discuss the metabolites.

Equilibrium Sorption Isotherms

Batch equilibrium sorption experiments were performed for soil, soil mixed with litter, and litter alone. Sorption experiments were conducted for estradiol and testosterone separately as well as with both hormones together for soil from the 0-30 cm depth to determine the interaction of these two hormones. Unlabeled hormones were diluted to 100 μg L^{-1} with HPLC grade methanol to avoid precipitation due to the inorganic solvent 0.01M $CaCl_2$. Then it was diluted to 0.001, 0.01, 0.1, 0.5, 1 μg L^{-1} with 0.01M $CaCl_2$ solution for sorption isotherms following the procedure used by (Sangsupan et al., 2006). Ten mL of unlabeled estradiol or testosterone of each concentration were added to 1 g air-dried soil or litter in a 15-mL glass centrifuge tube. For soil mixed with litter, 0.007 g of litter was added to each g of soil. Test tubes were sealed by Teflon-lined caps. In each test tube, 5μL of labeled testosterone and estradiol (0.059 KBq 6,7-^{3}H-estradiol and 0.015 KBq 4-^{14}C-testosterone) were added. Samples were shaken in a reciprocating shaker for 2, 24, and 48 hours at 20˚C. The same experiment was done for both hormones together. One g of air-dried soil was shaken with 10 mL of unlabeled solution (5 mL unlabeled estradiol and 5 mL unlabeled testosterone of corresponding concentrations). Five μL of labeled estradiol and testosterone were added to each test tube and shaken for 2, 24, 48, 72, 120, and 144 hours at 20˚C. Samples were centrifuged for 15 minutes at 3500 rpm at each time interval. Then 1 mL supernatant was taken for analysis of radioactivity by a liquid scintillation counter (LSC). Three replications of

each experimental unit were performed. The difference in initial and final radioactivity was considered as that adsorbed by 1 g of soil.

Sorption experiments were performed in sterile and non-sterile medium. Mercuric chloride ($HgCl_2$) was used for sterilization. $HgCl_2$ effectively destroys the microbial population without changing soil physical and chemical properties (Wolf et al., 1989). The application rate of $HgCl_2$ was 0.12 mg mL^{-1}.

Batch equilibrium sorption experiments were also performed to determine sorption coefficients for chloride in soil from the 0-30 cm depth. Isotherms were performed using $CaCl_2$ solutions of 0.005, 0.03, 0.37, 0.59 and 0.70 mg mL^{-1}. Ten mL of each solution concentration was added to 1 g of air-dried soil in a 15 mL glass centrifuge tube and capped. There were three replications of each concentration. The samples were shaken in a reciprocating shaker for two hours and centrifuged for 15 minutes at 3500 rpm. The chloride concentration in the supernatant was determined by a digital chloridometer (Labconco, Kansas city, MO).

Batch equilibrium sorption data were fit with the linear Freundlich (1909) sorption equation:

$$s = K_d c$$

Equation 4

where s is the concentration of hormone in the sorbed phase (μg g^{-1}), c is the concentration of hormone in solution (μg mL^{-1}), and K_d is the sorption coefficient (mL g^{-1}).

The sorption data were also fit with the nonlinear Freundlich (1909) sorption equation:

$$s = K_f c^n$$

Equation 5

where K_f is the non-linear Freundlich sorption coefficient (μg^{1-n} mL^n g^{-1}), and n is the Freundlich exponent.

Desorption isotherms were also developed. Ten mL of unlabeled estradiol or testosterone (1 μg mL^{-1}) were added to 1 g air-dried soil or litter in a 15-mL glass centrifuge tube. For soil mixed with litter, 0.007 g of litter was added to each g of soil. Test tubes were sealed by Teflon-lined caps. In each test tube, 5μL of labeled testosterone and estradiol (0.082 KBq 6,7-^{3}H-estradiol and 0.019 KBq 4-^{14}C-testosterone) were added. Samples were shaken in a reciprocating shaker for 24 hours, centrifuged for 15 minutes at 3500 rpm and then 1 mL of supernatant was taken for analysis of radioactivity by a liquid scintillation counter (LSC). The supernatant was discarded and 10 mL of unlabeled estradiol or testosterone (1 μg mL^{-1}) were added to the same test tube and it was shaken for 24 more hours. This process was repeated four times.

Column Transport

Nine hundred and sixty g of soil were packed in a plexiglass flow cell, 15 cm in length with an internal diameter of 7.5 cm for each packed column such that a bulk density of ~1.4 g cm^{-3} was maintained. Estradiol and testosterone breakthrough curves were run with hormones applied separately and together with three replications. In another triplicate set of columns, 2.21 g of ground, air-dried poultry litter was added on the top of each column. This amount of litter was equivalent to a litter application rate of

5 Mg ha^{-1} (Shore et al., 1995). The columns were turned upside down and flow entered from the bottom. A dilute solution of calcium nitrate (0.01 M) was run very slowly through the column to minimize entrapped air in the column. The flow rate was controlled by a peristaltic pump. When the column was saturated the flow was adjusted to a steady rate of about 15mL min^{-1}.Volumetric water content was determined by weighing the column. Once steady state was achieved, the flow of background solution was stopped. A pulse of 170 mL (~0.52 pore volume) of 0.01 M $CaCl_2$ and hormone was passed through the column. The pulse consisted of 1.5 μg estradiol (21.5 KBq 6,7-^{3}H estradiol and 1.497 μg unlabeled estradiol) and 3 μg 4-^{14}C testosterone (21.2 KBq) in 0.01 M $CaCl_2$ solution. Applied estradiol and testosterone concentrations were high compared to a typical litter application rate (5 Mg ha^{-1}) (Shore et al., 1995) to ensure that concentrations could be accurately measured in the effluent. After the pulse, flow was re-established using the background solution (0.01 M $Ca(NO_3)_2$) until 23 pore volumes (7500 mL) were eluted. Column effluents were collected every minute (15 mL) by a fraction collector. A 1-mL sample was taken from each 15-mL effluent fraction and added to 15 mL of Scintiverse BD (Fisher Scientific, Pittsburgh, PA) and analyzed for radioactivity by liquid scintillation counter.

Column Hormone Distribution and Transport Analysis

At the end of the breakthrough curve experiments, the $Ca(NO_3)_2$ solution was allowed to drain and each soil column was taken from the flow cell. Columns were divided into 1-cm depth increments. Each section was air-dried and ground. A 1-g sample from each section was oxidized using a biological oxidizer (OX-500, R.J. Harvey Instruments, Hillsdale, NJ). ^{3}H and ^{14}C present in the soil were released during oxidation

and captured by the scintillation cocktails (R.J. Harvey, Tappan, NY). Then OX-161 and OX-162 cocktails were used to estimate concentrations of 6,7-^{3}H-estradiol and 14-^{14}C-testosterone, respectively. Cocktails were analyzed for radioactivity by liquid scintillation spectroscopy.

Column Transport Models

Hormone transport was modeled with the HYDRUS-1D software package (version 4.14; Šimůnek et al., 1998). This program simulates the one-dimensional flow of water and solutes through variably saturated porous media. It can also simulate transport of solutes involved in sequential first-order decay reactions, such as estradiol (solute 1) transforming to estrone (solute 2). HYDRUS-1D uses an inverse modeling approach to fit observed data and estimate transport parameters.

Chemical non-equilibrium was applied to fit hormone breakthrough curves using the two-site sorption model (Šimůnek et al., 1998). This model assumes that sorption can be divided into two fractions:

$$s = s^e + s^k$$

Equation 3

where s^e is the sorbed concentration [M M^{-1}] in the type-1 sites and is assumed to be instantaneous; s^k is the sorbed concentration [M M^{-1}] in the type-2 sites and assumed to be kinetic. At equilibrium, the fraction of total sorption sites that can be considered as type-1 site is given by f (dimensionless):

$$f = \frac{s^e}{s}$$

Equation 4

Sorption on type-2 is modeled as a first-order rate process (Šimůnek et al., 1998). The mass balance equation for the type-2 sites is given by:

$$\frac{\partial s^k}{\partial t} = \omega\left[(1-f)K_d c - s^k\right] - (\mu_s + \mu_s')s^k$$

Equation 5

where ω is the kinetic rate constant for sorption on type-2 sites [T^{-1}], μ_s is a first-order solid-phase transformation constant [T^{-1}], and μ_s' is a first-order solid-phase transformation constant providing a connection between species in a chain model [T^{-1}],.

Fractionation

To determine the extractability of resident 4-^{14}C testosterone and 6,7-^{3}H estradiol, 20 mL of water was combined with 10 g of soil from each of the bottom four 1-cm depth increments (where a majority of the radioactivity was sorbed since flow entered the bottom). The water and soil were placed in a 50-mL centrifuge tube and capped with a Teflon lined cap. The samples were shaken 24 hours in a reciprocating shaker at 20˚C. They were removed and centrifuged for 12 minutes at 3500 rpm. One mL of supernatant was analyzed for radioactivity by liquid scintillation counter. The same procedure was done with acetone as an extractant after water.

Non-extractable 4-^{14}C testosterone and 6,7-^{3}H estradiol associated with different organic matter fractions (humic acid, fulvic acid and humin) of the soil was then determined. The remaining soil after centrifuging was air-dried, ground, and washed with 20 mL of 0.1N HCl. Twenty five mL of 0.5N NaOH was added and the sample was

shaken for 3 hs, centrifuged, and the supernatant placed in a beaker. Then 25 mL 0.1N $Na_4P_2O_7.10H_2O$ was added and the same procedure followed as with NaOH. The portion in the fulvic acid was analyzed for radioactivity by LSC. The insoluble part was humic acid. The radioactivity associated with humic acid and humin was determined by combustion with a biological oxidizer (OX-500, R.J. Harvey Instruments, Hillsdale, NJ) (Fan et al., 2007). A schematic diagram was shown in Figure 10.

Results

Sorption Kinetics

In non-sterile medium, both estradiol and testosterone showed evidence of sorption kinetics in soil and in soil mixed with poultry litter. Testosterone sorption in soil increased from 2 to 48 h and remained relatively afterwards (Figure 1). The same trend was seen when testosterone with soil mixed with poultry litter for all time intervals, but sorption was less than soil alone. This may have been due to competition of organic matter present in poultry litter with testosterone for adsorption sites. The greatest sorption occurred in poultry litter alone kinetics were not observed for testosterone. Testosterone sorption attained equilibrium within 2 hours (3.9 $\mu g\ g^{-1}$) with constant sorption from 2 to 144 h.

The kinetic sorption pattern of testosterone for soil in our study was similar to that observed by Casey et al. (2004) and Sansupan et al. (2006), but the time for maximum sorption differed. Casey et al. (2004) found that maximum sorption of testosterone occurred within 1-5 h of shaking for most of the soils examined. Sangsupan et al. (2006) found that maximum sorption for testosterone occurred at 72 h.

Estradiol demonstrated different sorption kinetics from testosterone in soil and soil mixed with poultry litter (Figure 2). Sorption in soil was relatively constant from 2 to 24 h (3.2 μg g^{-1}), and then decreased from 24 to 72 h. Sorption then increased from 72 to 120 h and stayed relatively constant thereafter. Contrary to testosterone, initial estradiol sorption did not decrease in the presence of poultry litter (Figure 2). Maximum sorption occurred at 24 h (3.4 μg g^{-1}), then decreased until 120 h, and subsequently it remained constant. The difference in sorption in the presence of litter was much greater at 48 and 72 h than at other time intervals. Lee et al. (2003) found estrone (as a metabolite) within 11 minutes in the sorption study of estradiol but Casey et al. (2003) reported the presence of appreciable amount metabolite after 169 h. Estradiol may be oxidized to estrone even in sterile soil indicating that microorganisms are not required for the transformation (Colucci et al., 2001).

The decrease in sorption of ^{14}C-labeled hormone may be due transformation of estrogen to one or more of its metabolites (Lai et al., 2000). The methodology could not differentiate whether the ^{14}C came from estradiol or its metabolites. No kinetics were observed for estradiol in poultry litter alone. The sorption data were similar for testosterone and estradiol in sterile medium (data not shown).

Equilibrium Sorption Isotherms

Both testosterone and estradiol isotherms were linear in the concentration range examined when the hormones were applied separately (Figures 3 and 4) or together (data not shown). In Table 2, the r^2 values (mean of three replications) for the fit of the linear sorption model (Equation 1) at different shaking times for soil, soil plus litter and litter alone are shown when the hormones were applied separately. When the r^2 values were

averaged over shaking times, they were 0.99 for testosterone and estradiol applied separately. When both hormones were applied together r^2 for estradiol was 0.96 and for testosterone was 0.99 (data not shown). Mean r^2 values over shaking times for the fit of the nonlinear sorption model (Equation 2) were also 0.99. (data not shown). Nonlinear exponent (n) values for both hormones with and without litter ranged from 0.92 to 0.98 for 2, 24, 48 hours time intervals but in most cases n values were greater than 0.95 (data not shown). These values are comparable to values for n reported (Casey et al., 2003). Since the linear model provided an adequate fit to the data, we used this simpler model in the discussion of our results and in modeling the column data.

The average linear sorption coefficient (mean of three time intervals) for testosterone (18.3 mL g^{-1}) was significantly ($p<0.05$) greater than estradiol (15.6 mL g^{-1}) (Table 2). Das et al. (2004) also reported higher testosterone linear sorption coefficients compared to estradiol. Log K_{oc} values were determined for estradiol and testosterone (Table 2 in parenthesis) and our results are comparable to values reported in the literature (Casey et al, 2005; Das et al., 2004; Lee et al., 2003). Sorption coefficients for both hormones varied for soil with and without poultry litter. For testosterone, K_d decreased significantly in the presence of litter for 2 hours (14.9 to11.8 mL g^{-1}, $p<0.001$), 24 hours (19.7 to 16.0 mL g^{-1} $p<0.001$) and 48 hours (20.2 to 17.0 mL g^{-1}, $p<0.001$) (Table 2). These results showed that the organic matter or inherent testosterone in poultry litter may compete with testosterone for adsorption sites.

The opposite trend was seen for labeled estradiol. Linear sorption coefficients increased with soil in the presence of litter for all time intervals. For two hours K_d increased from 19.6 to 23.1 mL g^{-1} and for 24 hours the value increased from 18.6 to 24.3

mL g^{-1}, but these changes were not statistically significant The change was significant for 48 hours, from 8.7 to 19.6 mL g^{-1} with litter ($p<0.001$). Essentially, this showed that when ^{3}H estradiol was in soil, a reduction in sorption occurred after 24 hours (which might be due to transformation to a less sorbed metabolite such as estrone). But, when estradiol was in the presence of soil and litter, this reduction did not occur. The reason for this difference in the presence of litter is unclear.

Sorption isotherms were also performed to determine K_d in the presence of both hormones. The results for hormones applied to soil with a shaking time of 24 hours are compared to the values for hormones applied alone are shown in Table 3. The testosterone K_d (12.5 mL g^{-1}) was nearly twice as large as the estradiol K_d (7.4 mL g^{-1}) when both hormones were present. Lower values of K_d in the presence of both hormones may have been due to their competition for sorption sites.

Adsorption-desorption hysteresis was observed for both estradiol and testosterone on soil (measurements were not made for litter alone) and indicating that a degree of irreversible sorption occurred for both hormones (Figure 3 and 4). The linear sorption coefficient for testosterone (a linear model was used to allow comparison with the sorption data) was significantly higher (19.7 to 24.4 mL g^{-1}) for desorption than for adsorption. For estradiol, K_d for desorption increased approximately three fold (18.6 to 67.5 mL-g^{-1}) compared to the sorption coefficient. Desorption experiments were done for soil with litter, however a significant change was not observed for estradiol and testosterone K_ds (data not shown).

Sorption isotherms for Cl were linear. The mean value for K_d was 0.07 mL g^{-1}, which is typical for soils with a small capacity for anion adsorption (due to variable charge clay mineralogy).

Column Transport Analysis

Breakthrough of detectable concentrations of both 6,7-^{3}H-estradiol and 4-^{14}C-testosterone occurred for each of the three replicate soil columns without litter and with litter when the hormones were applied together. Peak concentrations of chloride occurred within 18 to 30 min for all columns (approximately 0.8 to 1.4 pore volumes). Estradiol peak concentrations occurred immediately after Cl (within 1.2 to 1.4 pore volumes) but testosterone breakthrough curves reached peak concentrations after 9.8 to 11.7 pore volumes, almost 3 to 4 h after Cl and estradiol peaks occurred. The early peak concentrations with estradiol indicated rapid movement of a fraction of the ^{14}C label through the columns, compared to the testosterone label. This can be explained in part by the lower linear sorption coefficient of estradiol (7.4 mL g^{-1}) than testosterone (12.4 mL g^{-1}) in the presence of both hormones (Table 3). The retardation coefficients ($R = 1 + \rho_b K_d/\theta$) calculated from these K_d's were 39.1 for testosterone and 23.7 for estradiol. This would represent the number of pore volumes when the peak concentrations should occur under chemical equilibrium transport. Hence, the early peak in estradiol (and to a lesser extent in testosterone) was also due to an apparent higher degree of chemical non-equilibrium in estradiol transport compared to testosterone.

Chloride was chosen as tracer because of its low sorption affinity to soil and conservative nature (no transformations). Symmetrical breakthrough curves of chloride for all columns indicated the lack of soil structure in these packed columns. A chemical

equilibrium model with K_d and dispersivity as the only fitted transport parameters accurately described the Cl breakthrough curves (Figure 5) (r^2 = 0.98 to 0.99) (Table 4). Dispersivity was low (< 1 cm), as would be expected for packed soil columns, and K_ds were just slightly higher than the mean value (0.07 mL g^{-1}) measured in the sorption isotherm experiments.

The chemical non-equilibrium two-kinetic site model was used to describe the breakthrough curves of the hormones using dispersivity (λ) values from the Cl equilibrium model. HYDRUS-1D determined the fraction of type-1 exchange sites (f), the first-order sorption rate coefficient (ω), and the first-order transformation constant for solid phase (μ_s) which represented degradation (probably to CO_2) losses in our columns. This was a total of three fitted parameters for testosterone without a litter layer. The mean K_d measured in isotherms after 24 hours when hormones were applied together (Table 3, 12.5 mL g^{-1}) was used in the model. The fitted fraction of non-kinetic sorption sites (f = 0.02 to 0.10) and rate constant for kinetic sorption (ω = 0.01 to 0.02 min^{-1}) were low, indicating an high degree of chemical nonequilibrium. The estimated f values were higher than previously reported values by Casey et al. (2005). The estimated ω values fell within the range of ω values reported by Casey et al. (2004). Degradation losses were small and consistent with our findings of less than 100% total recovery of radio label (discussed later). The values for μ_s were approximately five times lower than the reported value by Casey et al. (2004). Overall, the fit to the testosterone breakthrough curves in soil without a litter layer was good (r^2 = 0.80 to 0.87).

In the columns where a litter layer was present, the column was modeled as two materials: a litter layer that was approximately 0.03 cm thick underlain by a soil layer

14.97 cm in thickness. In these columns, f, K_d, and ω for the litter layer were also fit (a total of 7 fitted parameters for testosterone). In these columns, the fitted parameters in the soil layer (f, μ_s, and ω) were similar to the values found in the columns without a litter layer, except for K_d which was lower in the column with litter. The litter layer parameters indicated an intermediate level of chemical nonequilibrium with low values for f (0.001 to 0.04) and intermediate values for ω (0.06 to 0.28 min^{-1}). The fit to the testosterone breakthrough curves in soil with a litter layer was also good (r^2 = 0.84 to 0.95).

We had a more difficult time fitting a parsimonius model to the estradiol breakthrough curves for soil without a litter layer. We tried to use sorption K_ds from the batch experiments but could not get an adequate fit with these values. Since the sorption kinetics of estradiol showed a significant decrease of K_d after 24 hours in the absence of poultry litter, a chain model with two solutes was considered in modeling estradiol in soil without litter (as was done by Casey et al., 2005). In this case there were eight fitted parameters: f, K_d, and ω for solute 1 (presumed to be estradiol) and solute 2 (presumed to be estrone or another less sorbed metabolite), μ_s for solute 2 (degradation losses), and μ_s' (the first-order transformation constant from solute 1 to solute 2) for solute 1.In the columns without litter, the fitted K_ds for solute 1 were much larger (270 to 1700 mL g^{-1}) than we measured in the sorption isotherm experiments even at the shortest time when hormones were applied alone (Table 2, 15.6 mL g^{-1}). The fitted K_ds for solute 2 were much lower (0.11 to 0.14 mL g^{-1}) which supported the idea that estradiol is being transformed to a less adsorbed metabolite.

A larger number of model parameters were also required to fit the breakthrough curves for estradiol in soil with a litter layer. Since we saw less of a reduction in estradiol

sorption with time when soil was mixed with litter compared to soil alone (Figure 2), we did not use a chain model with two solutes for estradiol with a litter layer. In this case, there were seven fitted parameters: f, K_d, and ω for the litter layer and f, K_d, μ_s, and ω for the litter layer. Overall, significant differences were found between estimated parameters for estradiol in presence of poultry litter. Fractions of equilibrium sites (f) significantly decreased from 0.069 to 0.007 ($p<0.08$) in presence of poultry litter (Table 4). Estradiol ω values fell within the range reported by (Casey et al. (2003). Lower ω for solute 2 in absence of litter may be responsible for rapid leaching and early breakthrough of ^3H estradiol. There were no significant differences between the estimated K_ds of estradiol with or without a litter layer (Table 4).

Total estradiol and testosterone in the soil within each 1-cm increment near the bottom of the column is shown in Figure 6 with and without poultry litter. The fit of the predicted total soil hormone content is also shown using the parameters values obtained from the breakthrough curves. The fit of the HYDRUS predictions were poor, but the concentrations were of the right order.

Breakthrough curves when the hormones were applied separately are shown in Figure 7. For comparison purposes, the breakthrough curves when hormones were applied together (from Figure 5) are shown again. Although the differences are not large, they are consistent. Peak concentrations of estradiol were higher when the hormones were applied together. The opposite was true of testosterone. It appears that when the hormones are both present, estradiol becomes more mobile. This supports our data on sorption isotherms which showed a lower K_d for estradiol under these circumstances (Table 3).

The effect of applying the hormones together or separately on the distribution in the soil column at the end of the breakthrough curves is shown in Figures 8 and 9. Further movement into the column (flow was from the bottom) of testosterone was observed.

Recovery of Hormones

In transport experiments, 18.0 % of the testosterone initially present in the pulse solution broke through soil columns in the absence of litter (Table 6). When poultry litter was present, the percentage that broke through was approximately the same, 19.0%.

Comparatively higher amounts of estradiol were eluted from the columns (Table 6). Of the estradiol initially present in the pulse solution 25.4 % and 28.2 % broke through soil columns with and without litter, respectively. These results support the sorption data.

Oxidation of soil samples from the columns recovered an average of 62.8% of the testosterone and 63.8% of the estradiol (Table 6). The distribution of the hormones within the columns was not affected whether the hormones were applied together or separately. The distribution with depth is shown in Figures 7 and 8 when estradiol and testosterone were applied together. The majority of the hormones were sorbed to the bottom 5-6 cm of the columns where the pulse entered.

When poultry litter was present, more hormones were recovered from the columns. On average 70.0 % of the testosterone and 69.6% of the estradiol were recovered from the columns when broiler litter was present (Table 6). The estradiol distribution was similar in the presence or absence of a litter layer (Figure 7). But testosterone moved further into the columns when a litter layer was present (Figure 8). Higher amounts of testosterone were present in the 8-11 cm depth increment in the

presence of poultry litter. This may have been due to the competition for sorption sites by the organic matter present in poultry litter, which increased the mobility of testosterone or it could have been due to co-transport of sorbed testosterone on fine organic matter from the litter layer. The average recovery of both hormones from the columns was significantly higher in the presence of litter at the $p = 0.08$ level for testosterone and 0.01 level for estradiol. Addition of litter increased the total recovery from 92.0 to 95.0% for estradiol and 80.8 to 89.7% for testosterone ($p<0.07$) (Table 6).

Fractionation

Fractionation was used to determine the radioactivity associated with different organic fractions (humin, fulvic acid and humic acid) of soil. Humic substances (humic acid, fulvic acid and humin) are large organic molecules with various functional groups. Humic acids have greater molecular weight than fulvic acids. Humins have the greatest molecular weight. The bottom four 1-cm sections of each soil column, with and without litter, were fractionated as the majority of the hormone was sorbed in these sections.

The distribution of both ^{14}C and ^{3}H sorption within the different organic fractions were significantly different in the presence of poultry litter (Table 7). Overall, the addition of poultry litter made estradiol more extractable (by water or acetone) and testosterone less extractable. Poultry litter significantly increased the amount of water extractable estradiol from 15.3 to18.4% ($p<0.05$) and testosterone from 9.5 to 14.9% ($p<0.05$). This may have been due to competition of organic matter present in the poultry litter for sorption sites and consequently less sorption to soil. The opposite trend was observed for acetone extraction of both hormones though the difference for estradiol was not significant. However, significantly ($p<0.05$) less testosterone (50.3 vs. 64.3%) was

extracted with acetone in the presence of litter. Addition of litter may bind testosterone in a way that it cannot be extracted with acetone after water extraction. Litter did not affect the total extractable estradiol but the total extractable testosterone decreased significantly ($p<0.05$) from 73.8 to 65.2% in the presence of litter (Table 7). Poultry litter did affect non-extractable testosterone and estradiol associated with humin and fulvic acid for both hormones. Non-extractable estradiol associated with humin decreased significantly ($p<0.05$) from 24.7 to 14.1% but testosterone increased significantly ($p<0.05$) from 17.7 to 20.9% in the presence of litter. The amount of non-extractable estradiol and testosterone increased significantly ($p<0.05$) for fulvic acid from 11.8 to 20.6% and from 4.0 to 8.9%, respectively, in the presence of litter. No significant change was observed for testosterone or estradiol associated with non-extractable humic acid in the presence of litter. The total amount of non-extractable testosterone increased significantly ($p<0.05$) from 26.2 to 34.8% with litter. Although, the total non-extractable estradiol decreased slightly with litter, the difference was not statistically significant. Higher amounts of estradiol were bound to humic acid while most of the testosterone was associated with the humin fraction. The probable cause may be the presence of a phenolic group on estradiol (Fan et al., 2007), which is more reactive compared to the keto group in testosterone. This phenolic group of estradiol can attach with a carboxyl group of humic acid via hydrogen or covalent bonds. Hydrolysis of phenolic groups also differentiates the sorption behavior of estradiol from testosterone (Yu et al., 2004).

The percentage of radioactivity associated with different organic fractions was also analyzed by 1-cm depth increments. The layers started 4 cm from the bottom of the column where the solute entered and continued to the bottom of the column. When litter

was present, it was part of the 3-4 cm layer. Water extractable ^{3}H was higher in each layer in the presence of litter but significantly ($p<0.05$) increased from 13.3 to 23.5% in the 1-2 cm layer (measured from the bottom of the column where the solute entered) (Table 8). For testosterone, water extractable ^{14}C significantly ($p<0.05$) increased in three layers in the presence of litter. Poultry litter had no significant effect on acetone extractable ^{3}H but significant ($p<0.05$) increases were observed for ^{14}C in each layer with litter with the maximum difference (43.2 to 63.6%) occurring in the 3-4 cm layer. The probable cause may the presence of organic matter in the poultry litter which increased the extractability of ^{3}H and ^{14}C due to competition for sorption sites.

Non-extractable radioactivity (^{3}H) associated with humin significantly ($p<0.05$) decreased in each layer in the presence of poultry litter with the maximum decrease (42.3 to 18.1%) in the 0-1 cm layer. Decreased percentages of ^{3}H in humin in the presence of litter may be due to the occupancy of humin functional groups by organic matter in the litter and consequently less sorption of the added hormone. An increased percentage of ^{14}C was found with litter for humin in the 0-1 cm and 3-4 cm layer. Fulvic acid bound radioactivity was higher for both ^{3}H and ^{14}C in the presence of litter. This increase was significant for ^{3}H in each layer and for ^{14}C in the 1-2 and 3-4 cm layers. This may also be explained due to competition for sorption sites of organic matter present in the poultry litter. Tritium and ^{14}C associated with humic acid did not show significant differences for layers but a significant increase (14.4 to 27.1%) was found for ^{3}H in the 0-1 cm layer.

These results have shown that most of the ^{3}H label for estradiol was associated with different non-extractable fractions of humic substances. The reason may be the presence of phenolic groups in estradiol which can form hydrogen or covalent bonds with

different functional groups of humic substances (Yu et al., 2004). Changes in pH during the fractionation procedure may hydrolyze the phenolic group. This may be responsible for the different distribution pattern of estradiol and testosterone in humic substances (Fan et al., 2007b).

Table 1. Soil physical properties for the 0-15, 15-30 and combined 0-30 cm soil depths.

Depth (cm)	pH	Particle size distribution (%)			Base Saturation (%)	Total Carbon (%)	CEC (cmol kg^{-1})	Soil Texture
		Sand	Silt	Clay				
0-15	6.06	42	30	28	87.3	2.31	10.68	Sandy clay loam
15-30	6.17	40	26	34	86.4	1.07	7.18	Sandy clay loam
0-30	6.15	40	28	32	87.3	1.68	9.19	Sandy clay loam

Table 2. Mean linear sorption coefficients (K_d) and log organic C sorption coefficients (K_{oc}) for soil, soil with litter and litter in sterile medium as a function of time. Values are for hormones applied separately.

	Estradiol K_d (mL g^{-1}) and log K_{oc} (in parenthesis)				Testosterone K_d (mL g^{-1}) and log K_{oc} (in parenthesis)			
Time (hours)	2	24	48	Mean	2	24	48	Mean
Soil	19.6 (3.07)	18.6 (3.04)	8.7 (2.72)	15.6 (2.94)	14.9 (2.95)	19.7 (3.07)	20.2 (3.08)	18.3 (3.03)
r^2	0.96	1.00	1.00	0.99	0.99	0.99	0.98	0.99
Soil + litter	23.1	24.3	19.6	22.3	11.8	16.0	17.0	14.9
r^2	0.99	0.99	1.00	0.99	1.00	1.00	0.99	1.00
Litter	55.5	59.8	59.5	58.3	35.3	36.1	38.0	36.5
r^2	0.99	1.00	1.00	1.00	0.99	1.00	1.00	1.00

Table 3. Comparison of K_ds for estradiol and testosterone alone and in presence of both hormones for soil.

Hormone	Alone	Estradiol + Testosterone
Estradiol K_d (mLg^{-1})	18.6a†A‡	7.4bB
Testosterone K_d (mLg^{-1})	19.7aA	12.5bA

† within a row, means followed by different lowercase letters are significantly different according to Fisher's protected LSD of p=0.05.

‡ within a column, means followed by different uppercase letters are significantly different according to Fisher's protected LSD of p=0.05.

Table 4. Fitted hormone transport parameters from chemical non-equilibrium model with litter and without litter in soil columns where hormones were applied together.

		Column without litter				Column with litter			
		1	2	3	Mean	1	2	3	Mean
Chemical Nonequilibrium Model									
Chloride									
λ (cm)		0.98	0.64	1.00	0.87	0.52	0.54	0.51	0.52
K_d (mL g^{-1})		0.11	0.12	0.07	0.10	0.11	0.11	0.10	0.11
r^2		0.98	0.98	0.98	0.98	0.99	0.99	0.98	0.99
Testosterone									
Litter layer	F					0.0390	0.0058	0.0009	0.0152
	ω (min^{-1})					0.280	0.061	0.070	0.1370
Soil	F	0.1060	0.00002	0.0320	0.05	0.1800	0.0100	0.0001	0.063
	K_d (mL g^{-1})					8.0	9.0	13.0	10.0
	μ_s (min^{-1})	0.002	0.002	0.002	0.002	0.003	0.003	0.002	0.0027
	ω (min^{-1})	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.017
	r^2	0.87	0.85	0.80	0.84	0.95	0.93	0.84	0.91

Estradiol									
Litter layer	F					0.0002	0.0002	0.0160	0.0055
	K_d (mL g^{-1})					340	1023	642	668.3
	ω (min^{-1})					0.020	0.013	0.013	0.0155
Soil solute 1	F	0.110	0.077	0.020	0.07	0.009	0.007	0.004	0.007
	μ'_s (min^{-1})	0.120	0.005	0.007	0.04	0.044	0.049	0.045	0.046
	K_d (mL g^{-1})	290	270	1700	753	0.95	0.50	0.67	0.071
	ω (min^{-1})	0.68	0.95	2.71	1.45	0.07	0.10	0.09	0.087
Soil solute 2	F	0.110	0.077	0.020	0.07				
	μ_s (min^{-1})	1.08	1.09	1.43	1.20				
	K_d (mL g^{-1})	0.11	0.11	0.14	0.12				
	ω (min^{-1})	0.16	0.17	0.27	0.20				
	r^2	0.89	0.76	0.69	0.78	0.78	0.87	0.88	0.84

Table 5. Square of regression of sorption of both hormones to soil predicted by HYDRUS-1D

	Column with no litter				Column with litter			
	1	2	3	Mean	1	2	3	Mean
Estradiol r^2	0.37	0.56	0.54	0.49	0.96	0.71	0.79	0.82
Testosterone r^2	0.84	0.87	0.98	0.90	0.49	0.55	0.73	0.59

6. Percent mass of applied estradiol and testosterone recovered from soil and effluent in presence of both hormones.

	Soil	Soil + Poultry litter
Estradiol		
Recovered in effluent, %	28.2*	25.4*
Recovered in soil, %	63.8*	69.6*
Total recovered, %	92.0	95.0
Testosterone		
Recovered in effluent, %	18.0	19.0
Recovered in soil, %	62.8	70.0
Total recovered, %	80.8	89.7

* indicates significant differences between soil and soil plus poultry litter at the 0.05 level.

Table 7. Fractionation of extractable and non-extractable ^{14}C and ^{3}H recovered from soil (%).

	Soil	Soil + litter	Soil	Soil + litter
Extractable	---------- Estradiol----------		----------Testosterone----------	
Water	15.3b	18.4a	9.5a	14.9b
Acetone	22.9a	22.7a	64.3a	50.3b
Subtotal	38.2a	41.1a	73.8a	65.2b
Non-extractable				
Humin	24.7a	14.1b	17.7b	20.9a
Fulvic acid	11.8b	20.6a	4.0b	8.9a
Humic Acid	25.4a	24.1a	4.6a	5.0a
Subtotal	61.8a	58.8a	26.2b	34.8a

Within a hormone and row, means followed by different letters are significantly different according to Fisher's protected LSD at p=0.05.

Table 8. Percentage of radioactivity (^{3}H and ^{14}C) associated with different fractions in the presence and absence of litter by depth in soil. Poultry litter was added to the layer 3-4 cm layer.

	Water		Acetone		Humin		Fulvic acid		Humic acid	
Layer (cm)	No litter	Litter	No litter	Litter	No litter	Litter	No litter	Litter	No litter	Litter
Estradiol										
0-1	12.0	14.4	20.9	16.2	42.3a	18.1b	10.4b	24.1a	14.4b	27.1a
1-2	13.3b	23.5a	21.7	17.3	26.5a	15.5b	12.3b	20.8a	26.1	22.9
2-3	16.2	18.9	26.8	24.5	21.4a	14.2b	11.0b	18.6a	24.7	23.8
3-4	17.4	17.3	20.7	27.3	21.6a	11.2b	12.3b	20.6a	28.0	23.6
Testosterone										
0-1	9.5b	16.4a	50.5b	63.2a	17.2b	21.7a	4.9	6.5	5.1	5.0
1-2	10.5	13.3	55.0b	63.1a	17.0	18.1	4.1b	8.8a	5.2	4.9
2-3	10.2b	16.7a	53.5b	66.0a	15.3	17.6	4.0	6.6	4.6	5.7
3-4	7.8b	13.7a	43.2b	63.6a	20.8b	25.2a	3.4b	12.9a	4.3	5.1

Within a row, means followed by different letters are significantly different according to Fisher's protected LSD at p=0.05.

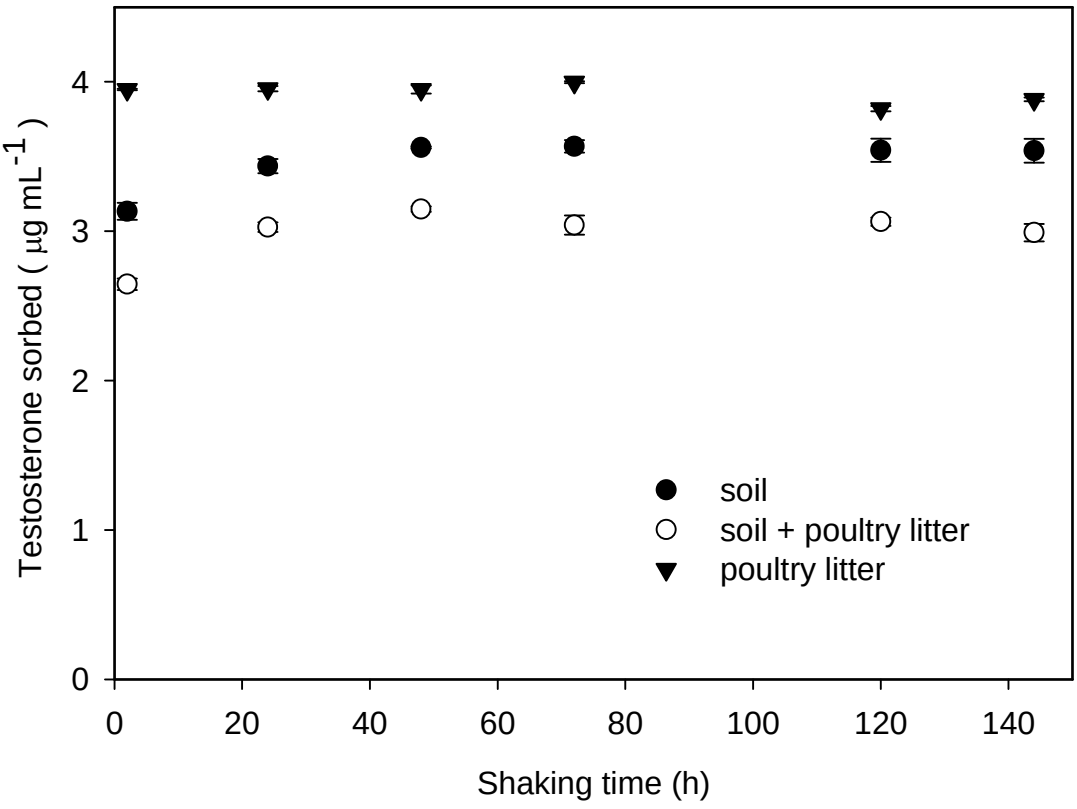


Figure 1. Effect of shaking time on sorption of testosterone in poultry litter and in soil with and without poultry litter.

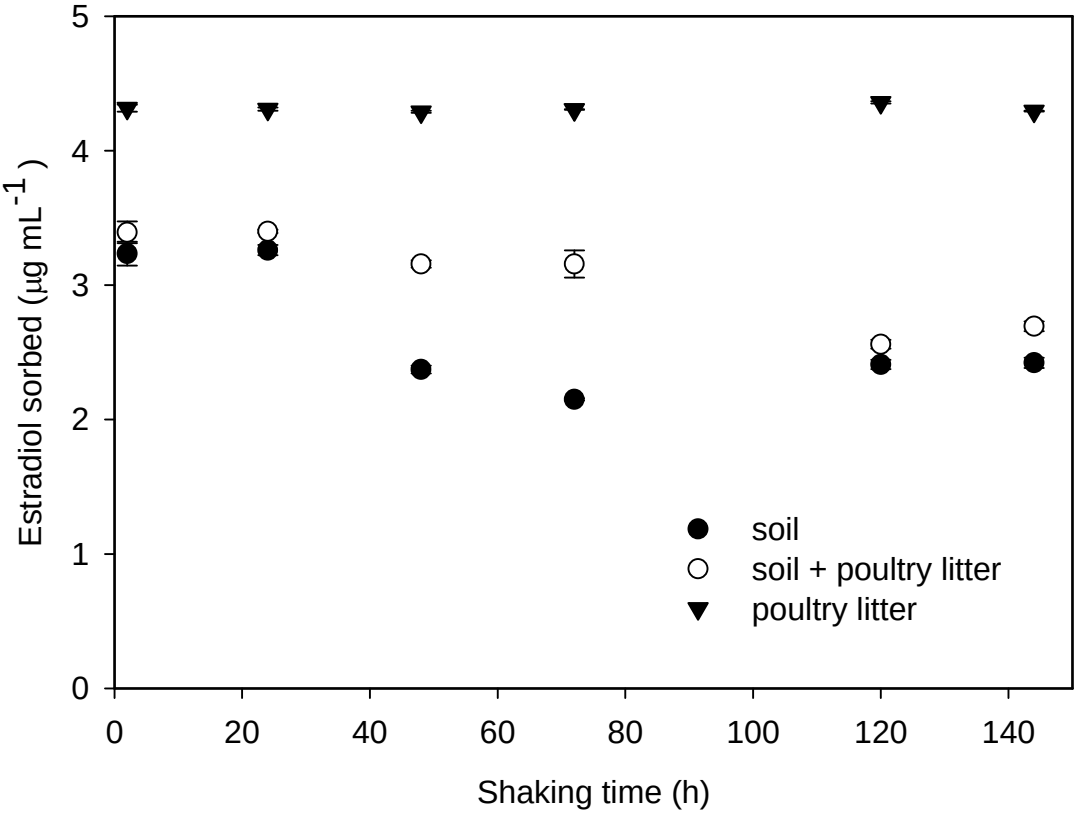


Figure 2. Effect of shaking time on sorption of estradiol in poultry litter and in soil with and without poultry litter

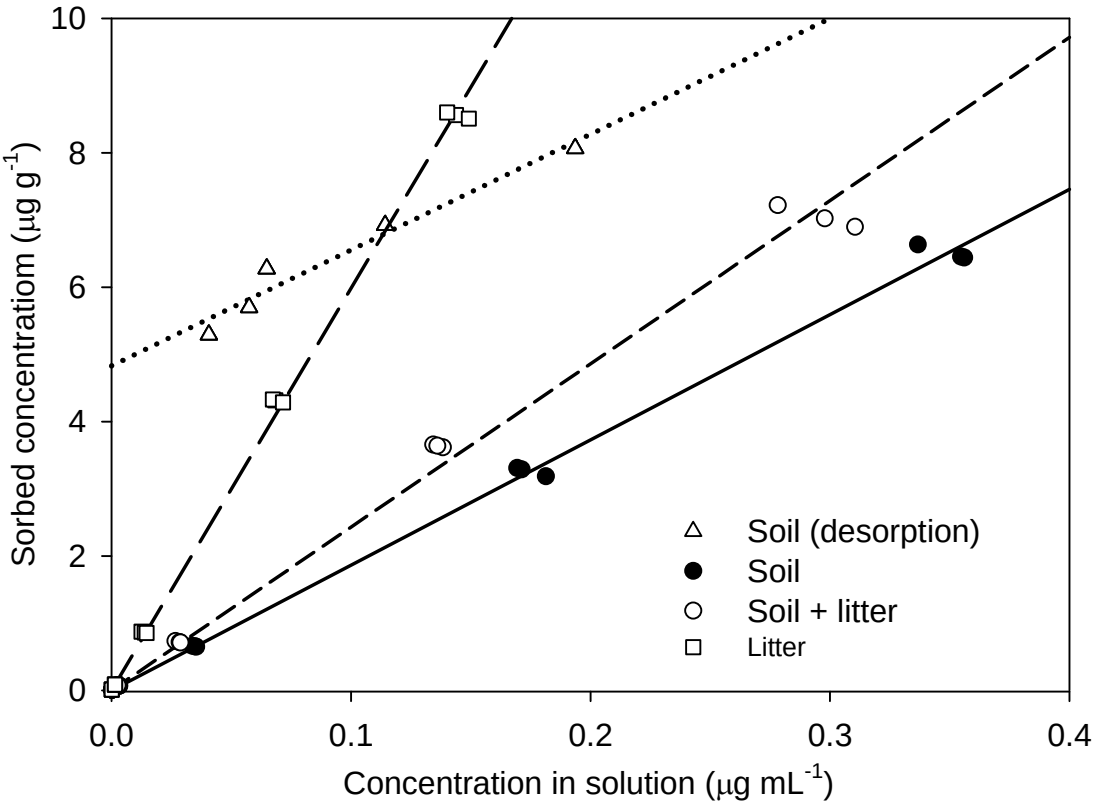


Figure 3. Fitted estradiol linear sorption (and desorption for soil) isotherms for soil, soil with litter and litter with and without poultry litter using estradiol alone.

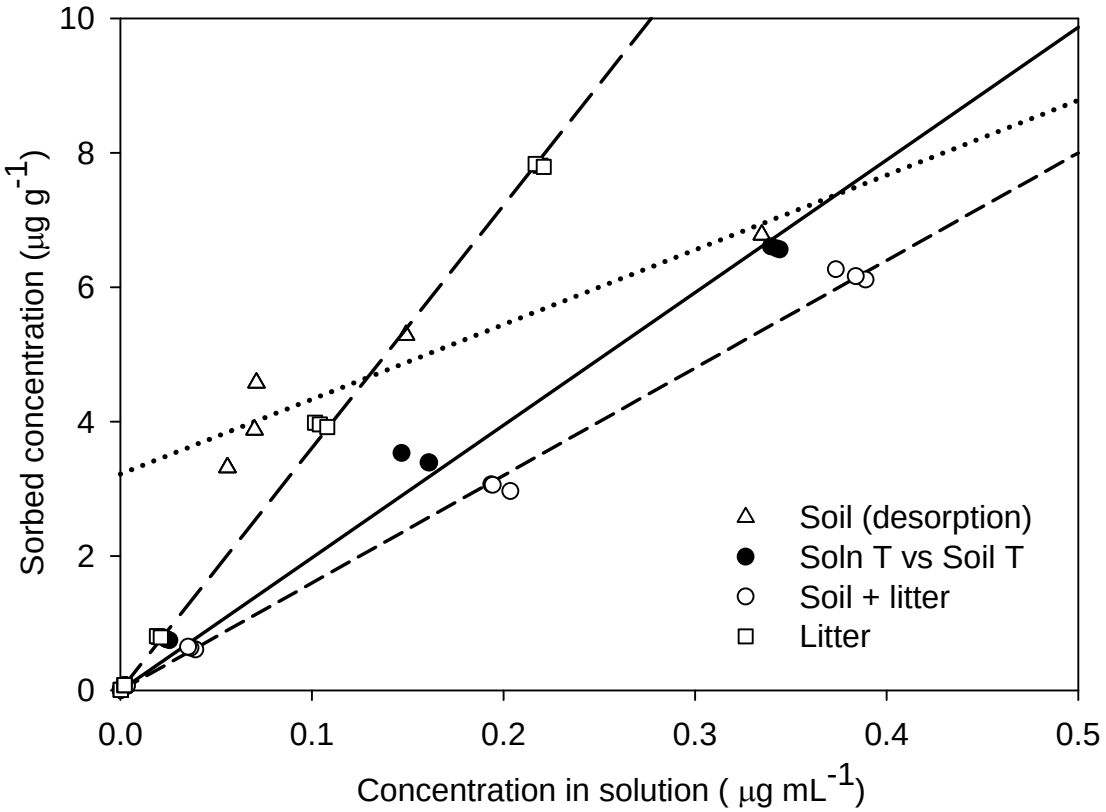


Figure 4. Fitted testosterone linear sorption (and desorption for soil) isotherms for soil, soil with litter and litter using testosterone alone.

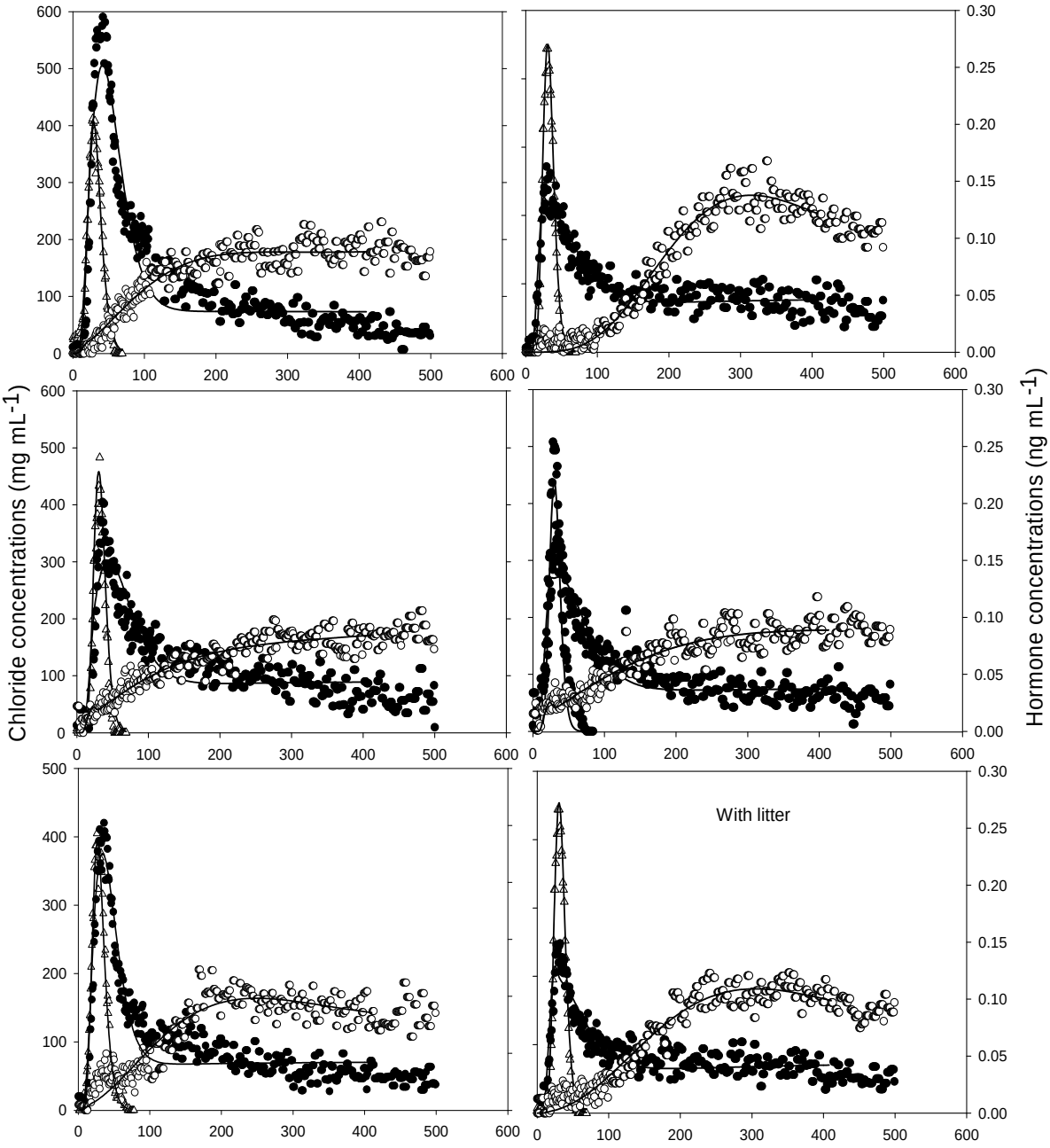


Figure 5. : Breakthrough curves of Cl, estradiol and testosterone in packed soil columns with and without poultry litter in the presence of both hormones. Solid lines are fitted curves generated by HYDRUS-1D.

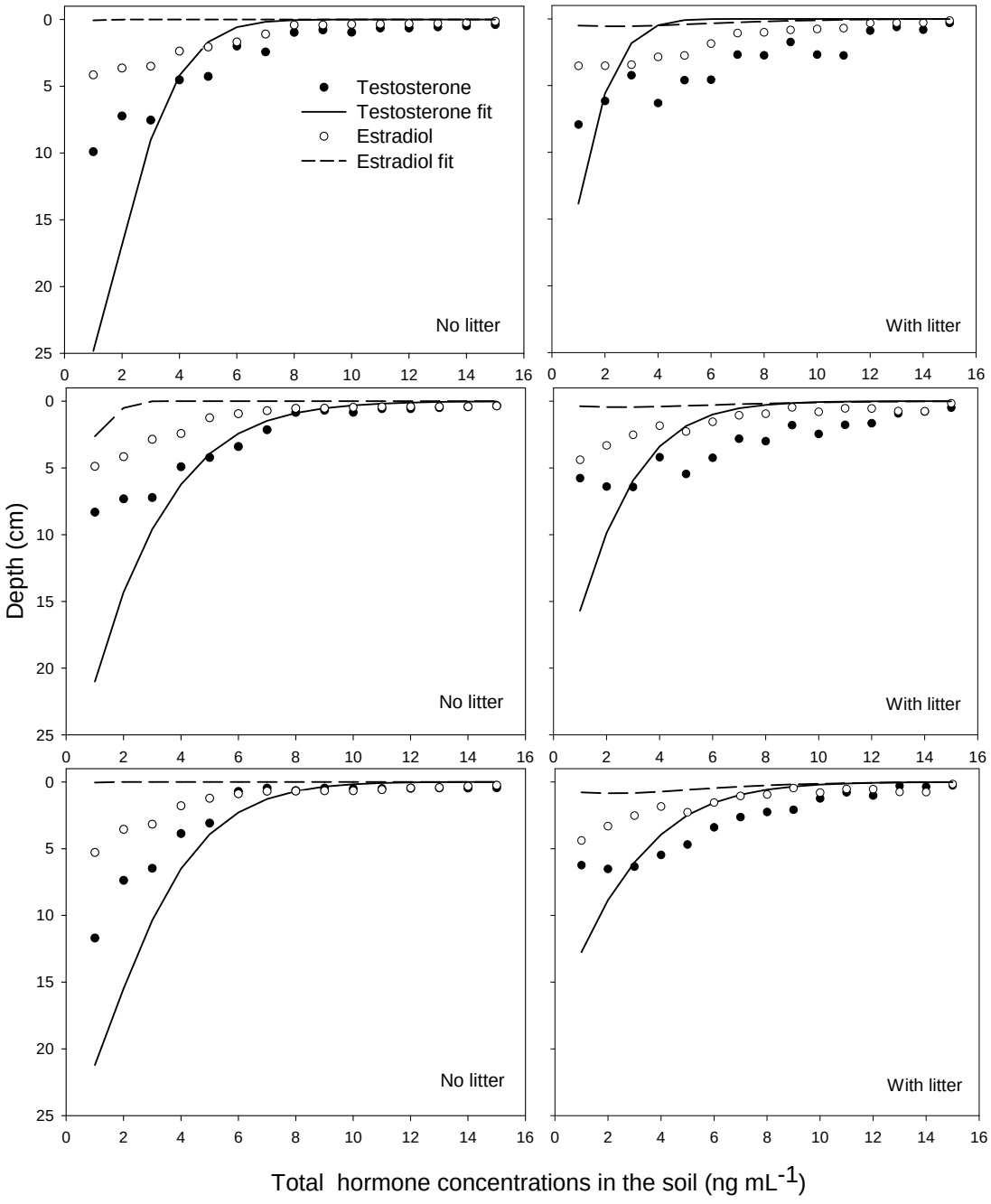


Figure 6. Hormone distribution with depth in soil column with and without poultry litter in presence of both hormones. Solid lines are fitted curves generated by HYDRUS-1D.

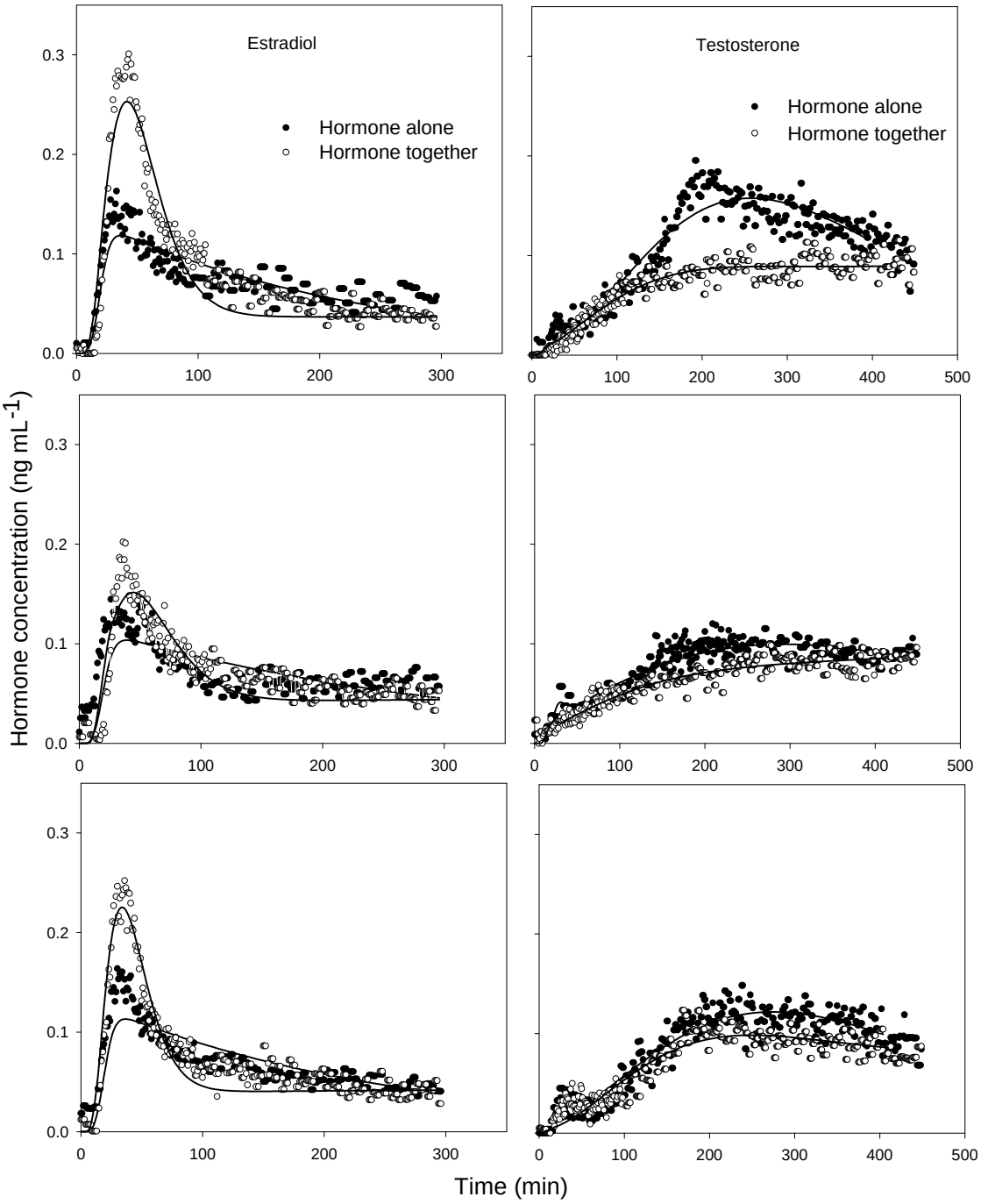


Figure 7. Breakthrough curves of estradiol and testosterone alone and together in packed soil columns without poultry litter. Solid lines are fitted curves generated by HYDRUS-1D.

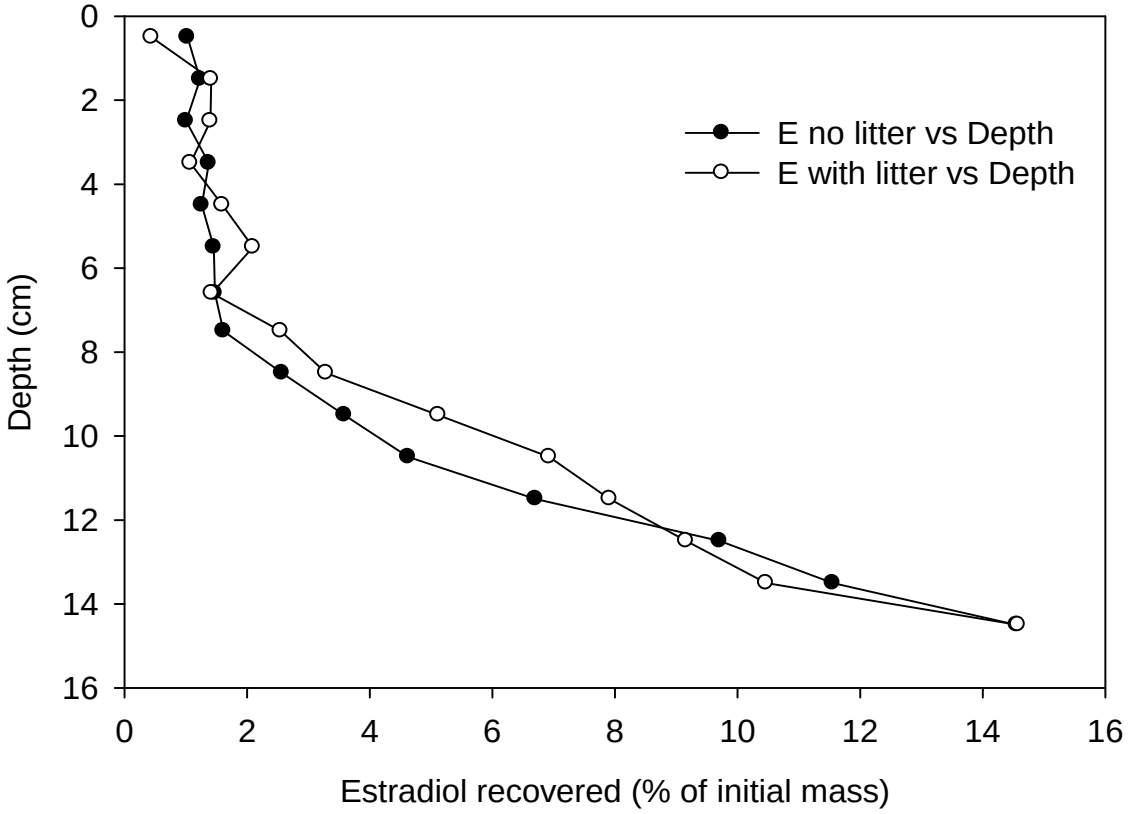


Figure 8. Mean distribution with depth of estradiol in soil columns with and without poultry litter (in the presence of both hormones). The estradiol recovered at each depth is the percentage of the total mass of estradiol initially present in the pulse solution.

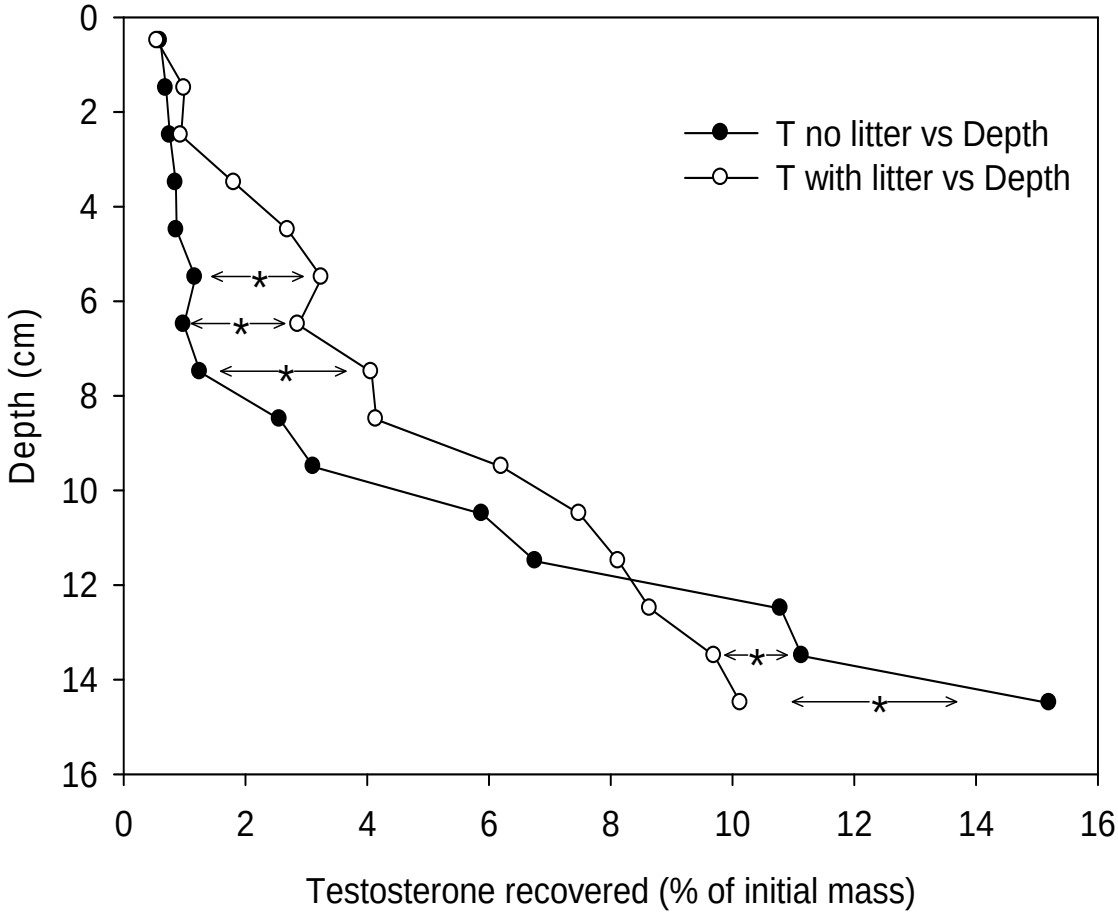
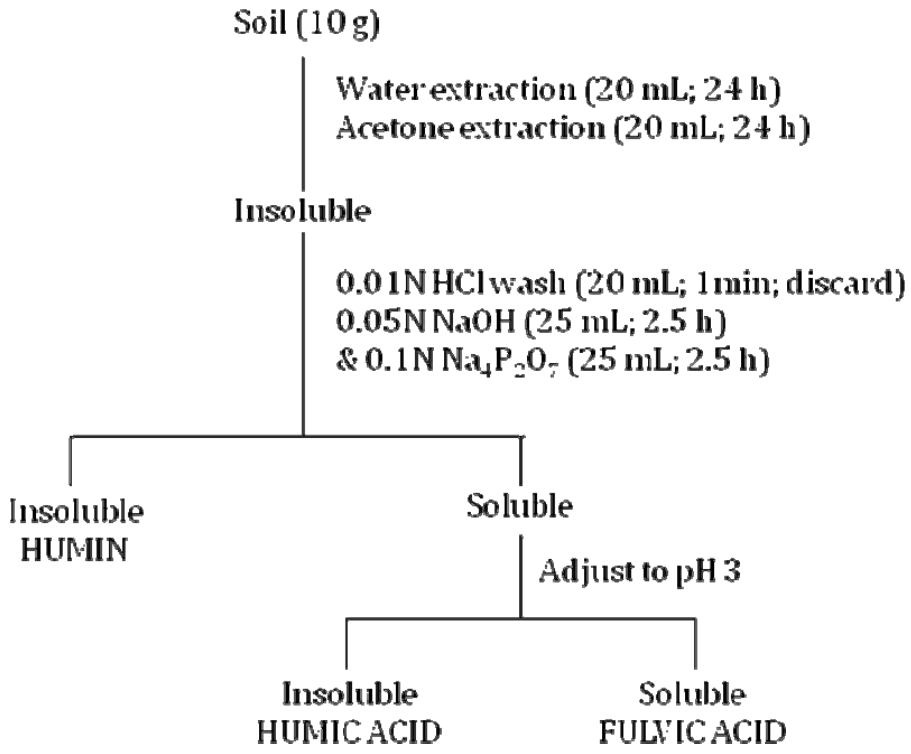


Figure 9. Mean distribution with depth of testosterone in soil columns with and without poultry litter (in the presence of both hormones). The testosterone recovered at each depth is the percentage of the total mass of testosterone initially present in the pulse solution. * indicates significant difference at depth same depth in presence and in absence of litter.

Figure 10. Schematic diagram for fractionation of radio labeled hormones into different soil fractions.



(adopted from Kaplan and Kaplan, 1982).

CHAPTER 4

CONCLUSIONS

Batch sorption isotherms showed that both hormones were strongly bound to soil in the presence or in the absence of poultry litter and the isotherms were linear. In the presence of litter, sorption of estradiol increased but sorption of testosterone decreased. Testosterone sorption reached equilibrium within 24 hours. But estradiol sorption was consistent till 24 hours and decreased significantly at 48 hours. Adsorption-desorption hysteresis was observed for both hormones indicating irreversible sorption.

Detectable concentrations of chloride, estradiol and testosterone reached peak concentrations in each of the soil columns within the total time that the columns were leached (500 minutes or approximately 23.24 pore volumes). Estradiol peak concentrations occurred immediately after Cl, but testosterone reached peak concentrations 4.2 hours after the Cl peak. The early breakthrough of estradiol indicated more rapid movement of a fraction of the estradiol than testosterone.

On average 28.2% of the estradiol and 18.0% of the testosterone applied in the pulse solution were leached through the columns. Poultry litter significantly decreased the leaching of estradiol from 28.2 to 25.4%. But no significant change was observed for testosterone. HYDRUS-1D modeling of chemical non-equilibrium transport using two sorption sites has shown that there is a difference in estimated parameters for estradiol in presence of poultry litter but no significant difference was found for testosterone with litter. Chemical nonequilibrium provided a good fit of observed data for chloride,

estradiol and testosterone. Lower ω values indicate slower transfer of adsorbed testosterone to type-2 sites.

Overall recovery was higher for both hormones with litter. For testosterone poultry litter significantly increased overall recovery from 80.8 to 89.7%. For estradiol recovery was 92% without litter and 95% with litter. The movement of estradiol within the soil column was not affected by litter, but testosterone moved deeper into the column when litter was present.

Instead of higher sorption of estradiol in the presence of poultry litter as expected, the increase in peak concentrations of both hormones suggests that there is a greater chance to contaminate groundwater due to leaching in the presence of litter. Testosterone leaching is more affected by litter than estradiol.

Fractionation study demonstrated that the distribution of both estradiol and testosterone was different among various organic fractions because of the structural differences between estradiol and testosterone. The distribution of these hormones in some of the fractions was significantly affected by the addition of poultry litter.

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