The Occurrence and Fate of Steroid Hormones from Manure Amended Agriculture Fields

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Abstract

Hormones are endocrine disrupting compounds, which have been shown to alter the sexual development of aquatic organisms. Animal manure applications to agricultural fields for nutrient management can be a source of environmental hormones. This dissertation investigates the occurrence of hormones in fields applied with various manures and their adjacent streams, as well as the effect of manure application technologies on the fate of hormones in soils, sediments, and runoff. A total of 11 hormone compounds were studied. All studied analytes were quantified using liquid chromatography and triple-quadrupole mass spectrometry following various sample extraction and clean-up strategies.

The spatial and temporal distribution of manure-associated hormones in a manure surface applied agricultural field and adjacent stream was studied at time points up to 7.5 months after a routine manure application. Hormones were detected mainly in the top 0-5cm soils. Significantly higher levels of hormones were found in the drystack applied area of the field when compared to dairy manure slurry applied portion.

New technologies for the subsurface application of poultry litter show promise as a tool to reduce the transportation of environmental hormones in surface runoff. Once adequate

sampling protocols were established; it was determined that subsurface injection of both dairy manure and poultry litter reduced the impact of manure surface runoff. Hormones also showed little vertical and lateral movement in the soil.

The transformation rates of 1,4-androstadiene-3,17-dione, 4-androstene-3,17-dione and estrone were studied comparing the effects of temperature, soil type, and application type. The calculated half-life of 1,4-androstadiene-3,17-dione in poultry litter surface-applied soils was 1.9 times higher than that in the poultry litter subsurface-injected soils, indicating a faster dissipation rate in the injection slits. Estrone persisted at detectable levels for the duration of the study in all treatments.

The continued use of best management practices and innovative manure management techniques for the reduction of nutrients, sediment and other contaminants has the potential to also reduce hormone transport to the natural environment. Monitoring many different types of hormones in all areas of an environmental system will continue to provide better information on the occurrence and fate of hormones sourced from manure amended soils.

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Abstract: General Audience

Hormones can contaminate streams and cause harm in the environment by interfering with the sexual development of aquatic organisms. Hormones are naturally occurring in animal manure, which is applied to agricultural fields for nutrients. Animal manures are usually spread on the surface of the soil and hormones can travel from the field to the streams when it rains. There are new technologies where the manure is injected into the soil instead of spread on the surface that could reduce the environmental impact of hormones in manure. This dissertation investigated the how hormones behave in fields applied with animal manures using the surface application technique and the subsurface injection technique. We investigated how long hormones persist in the soil, and their levels in manures, runoff from precipitation, and in waterways. Hormones were shown to stay in the top 0-5cm of soil, and to remain in the manure injection slit, showing that they do not easily move in the environment. Hormones were only detected in runoff water from soils that had surface applied manures. The transformation rate of hormones that were found at the highest levels in all the manures analyzed in this research was studied. It was determined that manure subsurface injection caused one of the compounds to transform at a faster rate in the soil. Overall, hormones were detected for up to 9 weeks in soils that were applied with animal manures. Best management practices such as manure subsurface injection and using a buffer zone between an agricultural field and a stream are promising tools for preventing hormones in animal manure from entering the natural environment.

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Chapter 1: Background

1.1 Introduction

Steroid hormones are emerging environmental contaminants that fall under the category of endocrine disruptors. While all vertebrates excrete hormones, the majority of the input of hormones in the environment can be traced to livestock production. The USEPA (2012) estimates that livestock produce 13 times more manure than human sewage produced in the United States (USEPA, 2012). Hormones from animal manure and hormones from human waste need to be handled differently because human waste is almost always treated by a wastewater treatment plant before being discharged into the environment, while it is very possible and likely that animal manure will be applied to land and environmentally accessible with little to no treatment. Manure is a renewable resource and an excellent source of macronutrients and micronutrients for crop production and has been used as an organic fertilizer for centuries. Other than its nutrient benefits, manure is known to improve soil structure, increase organic matter content, and increase water holding capacity in soils (Edwards and Someshwar, 2000; Haynes and Naidu, 1998; Khaleel et al., 1981).

Due to financial pressures, US livestock farming is becoming increasingly industrialized. Between 1987 and 2002, the production locus (number of animals per farm) increased by 60 percent in broiler, 100 percent in cattle, 240 percent in dairy and 2000 percent in hog production (MacDonald and McBride, 2009). In 2007, a total of 2.2 billion cattle, swine, and poultry was produced in the US and generated 1.1 billion tons of manure (USDA, 2009; USEPA, 2012). There is currently a trend of geographical concentration in US livestock farms, with farms tending to be in areas with common providers and processors, which they are linked to through

formal contracts. While this has increased productivity and lowered production and food costs, larger farms in closer proximity means that manure is also being concentrated (MacDonald and McBride, 2009). These new large scale agricultural productions are called Concentrated Animal Feeding Operations (CAFOs) and are designated as point source polluters. These CAFOs are a major cause for concern for the input of hormones into the environment because as land applied manure and feedlots become more concentrated, the potential for environmentally relevant concentrations of hormones in waterways increases. The overall objective of this research is, therefore, to understand the fate and transport of steroid hormone compounds sourced from animal manure applications on agricultural fields.

Steroid hormones are part of a class of potent emerging contaminants called endocrine disrupting compounds, which interfere with the endocrine system of an organism. Hormones are chemicals that are excreted by glands in the endocrine system when an external or internal stimuli prompts their production (NIH). The endocrine system regulates important process in an organism such as sexual function, reproduction, growth, metabolism, and even mood and stress response. Steroid hormones regulate the behavior, development and function of the reproductive system in vertebrates, with androgens produced mainly by males and estrogens and progestogens produced mainly by females (Lange et al., 2002). The hormones of interest in this proposal are listed in *Table 1.1*.

Table 1.1: Hormones of interest in this project	
Hormone Class	Hormones
Estrogens	Estrone (E1) 17α-estradiol (17α-estradiol) 17β-estradiol (17β-estradiol) Estriol (E3)
Androgens	1,4-Androstadiene-3,17-dione (1,4-ADD) 4-Androstene-3,17-dione. (4-AD)
Progestagens	Progesterone (P)
Estrogen-Conjugates	Estrone-3-Sulfate (E1-3S) Estrone-3-Glucorinide (E1-3G) Estradiol-3-Sulfate (E2-3S) Estradiol-3-Glucorinide (E2-3G)

1. 2 Occurrence of Hormones in Animal Manure

It is estimated that livestock in the US excrete 49 tons of estrogens, 4.4 tons of androgens, and 80 tons of progestogens annually (Lange et al., 2002). Very often only a few hormones of interest are monitored in livestock manures, making comparisons between the hormone content in different types of manures complicated.

Poultry

In a survey for hormones (17β-estradiol, estrone, estriol, testosterone, estrone -3-sulfate and estradiol-3-sulfate) in fresh broiler litter collected from the brooding area and other areas of the house at broiler farms on the Delmarva peninsula, only progesterone and estrone were in poultry litter samples at an average of 63.4and 44.1 µg/kg dry-weight, respectively (Bevacqua et al., 2011). Hutchins et al. (2007) measured total estrogen (estrone, 17α-estradiol, 17β-estradiol, estriol) in laying chicken lagoons to be 1.8-4.0 µg/L (Hutchins et al., 2007).

Implanted cattle

In cattle implanted with trenbolone acetate or estradiol, hormones (estrone, 17α-estradiol, 17β-estradiol, estriol) were measured in manure pit wastes with the highest observed estrogens

being estrone: 0.99 µg/L, estriol: 2.73 µg/L, 17α estradiol: 0.27 µg/L, and 17β-estradiol 0.01 – 0.06 µg/L (Khan and Lee, 2012).

Dairy

In fresh dairy manure less than 2 hours from excretion, Zheng et al. (2007) measured 17α-estradiol at 1416±104 µg/kg, 17β-estradiol at 153±25 µg/kg, estrone at 535±62 µg/kg, progesterone was below the LOQ, and estriol was not detected (Zheng et al., 2007). Dairy manure is seldom applied to a field without dilution. An example of concentration ranges of free estrogens in fresh dairy shed effluent (1/3 fecal matter, 2/3 urine, 10% excrement, 90% water) before any storage was measured from 18 farms, and was found to be at 0.111-11.000 µg/L for 17α-estradiol (median 0.730µg/L), 0.010-0.580 µg/L for estradiol (median 0.100 µg/L), and 0.001-0.310 µg/L for 17β-estradiol (median 0.024 µg/L)(Gadd et al., 2010). Mina et al. (2016) detected 17α-estradiol, estrone, and estriol at 91 µg/kg, 130 µg/kg, 150 µg/kg dry weight respectively in dairy manure slurry (Mina et al., 2016).

1.3 Fate of Hormones in Stored and Treated Manure before Land Application

Factors that can alter the hormone content of manure are ages of the animals, the sex and reproductive state of the animal, and also the way the manure is stored and treated before application (Bartelt-Hunt et al., 2013; Lange et al., 2002; Shore and Shemesh, 2003). Before manure is applied on an agricultural field, it is common practice to store manure in a holding facility and apply most of the manure at one time, generally in the spring before planting and again in the fall (DCR, 2005). Solid manure is either stacked on a concrete pad with at least three side walls or in the case of poultry litter, stored in the house with the animals in the bedding (Jones and Sutton, 2007). Liquid manure is stored in either underground or above ground pits or

in treatment lagoons that are earthen or concrete where the manure is slurried (Jones and Sutton, 2007). Manure treatment can be an important tool for farmers to manage their manure stores. Manure can be treated physically by separation or combustion, chemically by process such as coagulation and lime stabilization, or biologically through anaerobic or aerobic composting (Ogejo, 2009). Benefits of manure treatment are decreased pathogens and antibiotics, reduced mass, and reduced nutrients which when applied in excess can pollute the environment; however, treatment of manure can be expensive (Ogejo, 2009).

Composting

Composting not only reduces odor and eliminates pathogens in decomposing organic material, it has also been shown to alter the fate and reduce hormone input in the environment. Aerobic composting reduced the amount of 17β-estradiol in poultry litter from 83 µg/kg to 13 µg/kg (84% reduction) and reduced the amount of testosterone from 115 µg/kg to 11 µg/kg (90% reduction) but the formation of metabolites from these compounds was not taken to account (Hakk et al., 2005). Cost can come into play; when composting poultry litter, heated composting was shown to reduce estradiol concentrations by 97% (48% recovered as estrone), while ambient temperature composting reduced the estradiol concentrations by 85% (57% recovered as estrone) after 24 days, but at a large scale heated composting is more expensive (Hakk and Sikora, 2011). Furthermore, research has shown that estrone can be transformed back to estradiol within 24 hours in the presence of swine manure colloids under anaerobic conditions after oxygen was depleted by microbial activity in a closed vessel (Prater et al., 2015). While composting for 76 days decreased the total hormone content by 79-87% in a study by Bartelt-Hunt et al. (2013) for beef cattle manure, the concentration of 4-androstene-3,17-dione remained stable over the course of the study. It is hypothesized that 4-androstene-3,17-dione may have been transformed from

progesterone, which was supported by the fact that concentrations of progesterone, estrone, 17β-estradiol and testosterone and androsterone all decreased in concentration by a factor of 15 (Bartelt-Hunt et al., 2013).

Residence Time

The residence time of wastewater and manure from livestock operations can also play a large role in the concentrations and distribution of hormones in the final product to be applied to a field. Zheng et al. (2007) showed that in dairy milking parlor effluent, as the wastewater moved further from the milking parlor, the concentration of 17α-estradiol decreased while the concentration of estrone increased. In the same study, the concentration of steroid hormones (estrone, 17α-estradiol, 17β-estradiol, estriol, progesterone) in the manure lagoon was compared to the concentrations of hormones in the original wastewater and the lagoon concentrations were found to be 1-3 orders of magnitude less than at their origin (Zheng et al., 2007). In another study on dairy manure lagoons, it was found that total estrogen content (estrone, 17α-estradiol, 17β-estradiol, estriol) decreased by 99.8% after 8 months of lagoon storage (Zhao et al., 2010). In a simulated anaerobic digester for 42 days, the chemical fate of radiolabeled estradiol was determined to be primarily oxidation to estrone, followed by 5.7% of the initial estradiol forming methane, and 0.3-0.5% of the initial 17β-estradiol being mineralized to CO_2, with the total estrogenicity calculated to be reduced by 98% (Hakk et al., 2014). Residence time has a similar effect on the fate of estrogens and estrogen conjugates in municipal wastewater treatment: from WWTP influent to effluent, estrone was removed by 61%, 17α-estradiol by 85%, estriol by 97%, estrone-3-sulfate by 64%, estrone-3-glucorinide by 84%, estradiol-3-sulfate by 100%, and estradiol-3-glucorinide by 100% (D'Ascenzo et al., 2003).

1. 4 Occurrence of Hormones in the Animal Manure Affected Environment

Occurrence in surface water

Comparing watershed hormone contents can be a challenge because often only a few hormones are chosen as a representative sample of hormones in a watershed among many other pharmaceutical and personal care products. In the US, a 1999-2000 nationwide survey of 139 streams in 30 states observed 95 organic contaminants sourced from wastewater. Six of the contaminants of interest were hormones and they were detected in 20% of the streams surveyed. Median values for reproductive hormone concentrations were found to be 0.03 μg/L for 17α-estradiol, 0.16 μg/L for 17β-estradiol, 0.019 μg/L for estriol, 0.027 μg/L for estrone, 0.11 μg/L for progesterone, and 0.116 μg/L for testosterone (Kolpin et al., 2002). More specifically some work has already been completed on the occurrence of hormones in the waterways in the mid-Atlantic region of the US, the region of interest in the current proposed study. A survey by Vaicunas et al. (2013) of hormones in the 50 surface waters of the state of Delaware reported concentration ranges of 0-0.004 μg/L estrone, 0-0.005 μg/L for estrone-3-sulfate, and 0-0.006 μg/L for 17β-estradiol in mid spring after manure applications. estrone-3-sulfate and 17β-estradiol was not detected in the early fall, but estrone was detected at 0-0.002 μg/L (Vaicunas et al., 2013).

The concerning environmental concentrations of hormones are not a problem that is limited to the United States; hormone loading in rivers is a global problem. Much like within the United States, hormone concentrations vary by study area and by the compounds of interest in the specific studies. In a study by Lei et al. (2009) of three rivers in the Tianjin region of China, rivers with high contents of industrial, domestic and agricultural waste loads showed high amounts of natural estrogens: in the Beitang river estrone, 17β-estradiol and E3 were detected at

mean concentrations of 0.023, 0.009 and 0.010 µg/L respectively, and in the Dagu River at levels of 0.020, 0.010 and 0.012 µg/L respectively. The Yongading New River, a river with somewhat lower pollutant loads still had detectable estrogen concentrations at 0.011, 0.007, and 0.006 µg/L respectively. For all rivers studied, Estrone had the highest mean concentration in the river water (Lei et al., 2009). Also in China, in the Yangtze River Estuary, only estrone was detected out of 4 endocrine disrupting compounds at a mean concentration of 0.0002 µg/L in the wet season and at 0.00019 µg/L in the dry season (Shi et al., 2014). In a study in the Llobregat River Basin in Spain, a river with a high concentration of agricultural and industrial activities that is the main source of drinking water for Barcelona, estrogens were monitored and only estrone and estrone-3-sulfate were detected across 16 sites in the watershed. Estrone concentrations ranged from 0.00082 – 0.00581 µg/L. Estrone-3-sulfate concentrations ranged from 0.00025 – 0.00146 µg/L (López-Roldán et al., 2010).

While the input of hormones to the environment is widely believed to be mainly sourced from agriculture, there is supporting evidence that a combination of urban runoff and wastewater treatment effluents contribute environmentally relevant concentrations of hormones to the environment. A study on the river Weynyu in Beijing that is predominantly urban with no agricultural inputs reported concentrations of androgens up to 0.480 µg/L, progestogens: up to 0.050 µg/L and estrogens: up to 0.0098 µg/L with androgens detected in over half of the samples collected (Chang et al., 2009).

A few studies have been completed for specifically targeting the occurrence of steroid hormones in watersheds directly affected by agricultural runoff. Only estrone was detected at a range of 0.0026 µg/L to 0.058 µg/L, 24 hours after a manure (hog and dairy) field application during 5mm of precipitation at 7 adjacent water sampling sites and no estrogens were detected

from rainfall events at 12 days, 2 weeks, and 3 weeks after application (Lafrance and Caron, 2013). In a year-long monthly sampling of a stream adjacent to an organic dairy CAFO, estrogens (estrone, 17α-estradiol, 17β-estradiol, estriol) were consistently below detection limit, though this is attributed to an 8 month residence time in lagoon storage (Zhao et al., 2010). In an extreme case in Taiwan, very high estrogen (estrone, 17β-estradiol, estriol) concentrations were observed in receiving river discharge from one CAFO (1,030,000 broiler chickens, 934,000 laying hens, 85,000 pigs, and 1500 cattle) Winter concentrations for estrone ranged from 0.0811-0.755 µg/L, 17β-estradiol ranged from 0.0115-0.1443 µg/L and estriol ranged from 0.020-0.1356 µg/L. Summer concentrations for estrone ranged from 0.0111-0.1573 µg/L, 17β-estradiol ranged from 0.0038-0.0243 µg/L and estriol ranged from 0.0063-0.0375 µg/L (Chen et al., 2010). The authors projected that higher concentrations in the winter season were thought to be caused by a low dilution effect because of decreased precipitation combined with low microbial activity.

Table 1.2: Summary of hormone occurrence in surface water.

Location	*Watershed Type*	*Hormones Present*	*Concentration (ng/L)*	*Source*
United States (Nationwide)	Downstream of urbanization and agriculture	estrone 17α-estradiol 17β-estradiol estriol progesterone testosterone	27.0 (median) 30.0 160.0 19.0 110.0 116.0	(Kolpin et al., 2002)
United States, Delaware	Areas with high N and P concentrations	estrone 17β-estradiol estrone-3-sulfate	ND-3.7 (spring) ND-1.5 (fall) 6.7 (spring) ND (fall) ND-4.7 (spring) ND (fall)	(Vaicunas et al., 2013)
China, Tianjin Region	Beitang River (high pollution loads)	estrone 17β-estradiol estriol	23.4 (mean) 8.7 10.3	(Lei et al., 2009)
	Dagu River (high pollution loads)	estrone 17β-estradiol estriol	17.9 (mean) 10.3 12.4	
	Yongading New River (lower pollution loads)	estrone 17β-estradiol estriol	10.5 (mean) 7.3 5.8	
China, Yangtze	Surface runoff and WWTP effluent from Shanghai	estrone 17β-estradiol	2.0 (mean) ND	(Shi et al., 2014)
Spain, Llobregat River Basin	Concentrated population, Agriculture and Industrial	estrone estrone-3-sulfate	0.8-5.8 0.3-1.5	(López-Roldán et al., 2010)
China, River Weynyu, Beijing	Urban runoff and WWTP effluent	progestogens estrogens androgens	50.0 (max) 9.8 480.0	(Chang et al., 2009)
Canada, Quebec, Bras d'Henri Watershed	Agricultural field runoff	estrone 17β-estradiol estriol	2.6-58.0 ND ND	(Lafrance and Caron, 2013)
USA, New York, Catskill/Delaware Watershed	CAFO Runoff (mild)	estrone 17α-estradiol 17β-estradiol estriol	ND ND ND ND	(Zhao et al., 2010)
Taiwan	CAFO Runoff (severe)	Estrone 17β-estradiol estriol	11.1- 157.3 (summer) 81.1-755.0 (winter) 3.8- 24.3 (summer) 11.5-144.3 (winter) 6.3- 37.5 (summer) 20.0-135.6 (winter)	(Chen et al., 2010)

1. 5 Biological Impact of Hormones.

Sub part-per-billion levels of steroid hormones have been shown to affect aquatic wildlife at the cellular, behavioral, and population level. Increased vitellogenin production and testicular oocytes in fish species are common tracking tools wildlife biologists use to identify endocrine disruption in the environment. Vitellogenin is a precursor to the production of yolk proteins that depends on estrogen as a hormonal signal and can be easily detected in an enzyme-linked immunosorbent assay (ELISA) (Tyler et al., 1999). Intersex characteristics can be manifested in Testicular oocytes (in males) and atrecal follicles (in females) and can be physically observed in the gonadal tissue when testes take on ovary-like character (Kidd et al., 2007). Vitellogenic responses were observed in Atlantic salmon (*Salmo salar*) when exposed at 0.0119 and 0.118 µg/L 17β-estradiol (Duffy et al., 2014). When exposed to 3.9725 µg/L of 17β-estradiol, populations of Japanese Medaka (*Oryzias latipes)* reacted with a 50% mortality rate, and surviving subjects did not regain any reproductive capability after dosing ceased, signaling permanent damage of the reproductive system (Jukosky et al., 2008). Behavioral impacts have also been linked to estrogen exposure in aquatic life. The reproductive hierarchy of dominant Zebrafish (*Danio rerio*) males was disrupted when exposed to 17α-ethinylestradiol (EE2), commonly used in contraceptive medication, at 0.002 and 0.010 µg/L (Coe et al., 2008; Coe et al., 2009). Fathead minnows (*Pimephales promelas*) exposed to 100 ng/L estrone, 28ng/L 17α-estradiol and 0.010 µg/L 17α-ethinylestradiol in the larval and in the embryonic stage showed decreased escape performance (McGee et al., 2009). These behavioral changes could impact the overall health of the species population over many generations.

Few long term studies of the effects of steroid hormones on aquatic life have been completed but in a study by Kidd et al (2007), chronic exposure of fathead minnow (*Pimephales*

promelas) to 17α-Ethinylestradiol at 0.005-0.006 µg/L over the course of 7 years was shown to induce vitellogenin production in males, impact gonadal development, cause intersex individuals and ultimately push the fathead minnow population to near-extinction in the lake observed (Kidd et al., 2007). While not a long term study, long-term effects on an entire ecosystem can be implied from a study that showed 17α-ethinylestradiol bioaccumulating in freshwater benthic organisms *C. tentans* (midge) and *H. Azteca (*amphipod*),* which indicates that ingestion can be a pathway for estrogenic compounds to enter organisms that consume benthic invertebrates (Dussault et al., 2009).

When assessing the impacts of hormones sourced from agriculture, in-situ observations are useful tools for investigating the big-picture environmental impacts. One example of an in-situ observation of the effects of CAFO ditchwater on fish populations was completed by Leet et al. and it was observed that multiple aquatic species exposed to CAFO ditchwater exhibited lower richness, faster stomatic growth, and lower reproductive conditions when compared to fish not exposed to CAFO Ditchwater. In the same study, populations of fathead minnows were exposed to ditchwater from CAFO sites and it was observed that sex ratios were skewed towards males. It is speculated that this is due at least in part by the maximum hormone concentrations reaching 0.021µg/L 17β-estradiol, 0.040 µg/L estrone, 0.012 µg/L estriol, 0.008 µg/L 4-androstene-3,17-dione, and 0.015 µg/L testosterone (Leet et al., 2012). In an example of endocrine disruption in the Mid-Atlantic region, 200 male specimens of 6 fish and 2 frog species from the Delmarva Peninsula, a region dominated by poultry production and agricultural land use, were surveyed for intersex individuals. During 2008 and 2009 testicular oocytes were encountered in largemouth bass with prevalence ranging from 33-88% (weighted mean 57%),

and it was hypothesized that this was due in part to intensive row crop agriculture and the associated fecal estrogens from manure applications (Yonkos et al., 2014).

While it is known that hormones and other endocrine disrupting compounds cause reproductive complications in aquatic organisms, a complication of in-situ studies is that often contaminated sites from agriculture do not contain only one type of contaminant, and excess nutrients and other chemicals from agricultural runoff could be interfering with the results. Studies completed in the Platte and Elkhorn rivers in Nebraska came to inconclusive results when analyzing for a large array of hormones and agricultural chemicals in the agriculture affected streams. Sellin et al (2009). observed reduced vitellogenic and estrogen receptor α responses in fathead minnows subject to the water, but could not pinpoint a specific chemical responsible (Sellin et al., 2009). In a later study, Sellin et al (2009). determined that the sediment was responsible for the altered female fish behavior rather than the water alone, but still could not find a chemical concentration pattern, indicating that it could be multiple inputs that are affecting the fish species, or it was a compound that was not being sought out in the study (Sellin et al., 2010). A similar conclusion was determined in a study in the same watershed, i.e. that agricultural runoff adversely effects the aquatic organisms, but the exact mechanism is unknown (Knight et al., 2013). Many studies focus on the effect of estrogen on the feminization of male aquatic species, but androgenic responses from feedlot effluent may also have this effect on male organisms (Orlando et al., 2004).

Despite previously mentioned endocrine disruption activities of endogenous hormones, it has been demonstrated that reproductive defects (low progeny survival) from trout exposed to EE2 were not inherited from parent Male Rainbow Trout (Oncorhynchus mykiss) with defects, indicating that controlling EDCs in the environment could help a fish population recover with

normal, healthy individuals (Brown et al., 2009). Endocrine disruption in one generation does not mean that future generations will not recover. If the input of endocrine disrupting compounds to a specific ecosystem is stopped, then the future generations of organisms will not experience the consequences.

1. 6 Fate of Hormones in the Animal Manure-Affected Environment

1.6.1 Sorption, Leaching, and Transformation: Laboratory Studies

In studying sorption of organic chemicals to soils or sediments, it is important to note that a higher K_{oc} value indicates that the compound will be more strongly retained in the soil or sediment, while a low K_{oc} value indicates higher mobility in the soil. It is common to track the sorption, mobility and transformation of a compound by using radiolabeled compounds and scintillation counting, combined with a quantitative tool like LC/MS, because then the specific molecules that were deposited into the soil can be tracked. Using this method, Casey et al (2005) was able to track metabolite formation and even determined the log K_{oc} of 17β-estradiol to be 3.2 and estrone to be 3.3, and from that it was determined that the log K_{ow} values were 3.25 and 3.28 respectively (Casey et al., 2005). Included in the study, 17β-estradiol was tracked through a soil column, and only 26% was recovered as 17β-estradiol in the effluent, while 36.7% was recovered as estrone, demonstrating a fast transformation of the compound (Casey et al., 2005). The concentration of estrogen in the soil may influence the sorption, as it was demonstrated that as the concentration of estrogen decreases, the log K_{oc} increases, meaning that lower concentration levels of estrogens means that they may exhibit greater sorption to the soil particles(Yu et al., 2004). The Yu et al (2004). study showed log K_{oc} values of 3.14-3.94 for 17β-estradiol and 3.23-3.72 for estrone. Log K_{oc} values for estrone, 17β-estradiol and estriol were

determined to be 3.49, 3.56, and 3.14 respectively (Ying and Kookana, 2005). Log K_{oc} values of 3.20 and 3.34 for estrone and 17β-estradiol respectively have also been reported (Lee et al., 2003).

The degradation and transformation of estrogen compounds has been studied in a laboratory setting, and it was found that soil conditions will affect degradation rates of hormones. Estrone, 17β-estradiol, and estriol were all degraded rapidly under aerobic conditions, but showed little to no degradation over 70 days under anaerobic conditions (Ying and Kookana, 2005). 17β-estradiol has reported half-lives ranging from 1.1-9.1 days (Lee et al., 2003). Estrogens have also exhibited stereo-selective degradation rates, with 17β-estradiol degrading at a faster rate by soil bacteria than 17α estradiol in sediments, which could lead to the increased environmental persistence of 17αestradiol (Zhang et al., 2016).

Relationships between free estrogens and conjugated estrogens are of interest because hormones are excreted from an animal in their conjugated form, often glucorinated or sulfonated. Conjugated estrogens have the potential to be more mobile in the environment, namely in groundwater, due to their high water solubility (Shrestha et al., 2012). In a study by Shrestha et al. (2012), hydrolysis of estradiol-3-glucorinide and estrone-3-glucorinide is biologically driven, transforming the compounds to 17β-estradiol and estrone respectively, indicating that conjugated estrogens are a source of free estrogens in the environment. Even with the biologically driven hydrolysis, estradiol-3-glucorinide has the ability to persist for up to 28 days where estradiol-3-glucorinide was shown to be more readily hydrolyzed in the higher organic matter topsoil than in the lower organic matter subsoil, which could impact the persistence of hormones from subsurface manure injections(Shrestha et al., 2012). Free estrogens can be derived from conjugated estrogens, but conjugated estrogens can also be created from free estrogens. In a

column test it was shown that in non-autoclaved soil, estrone and estrone-3-sulfate can both be formed from 17β-estradiol, however estradiol is only transformed to estrone in autoclaved soil, indicating the transformation of estrone-3-sulfate from estradiol is biologically driven, but transformation to estrone is not (Goeppert et al., 2014). In agreement, Colucci et al (2001). also determined that 17β-estradiol could be transformed to estrone abiologically, but in addition added that estrone needed microbial activity to be degraded further (Colucci et al., 2001); though sterile soil seldom exists in the natural environment, especially in soils that contain hormones sourced from livestock, reduced biological activity can be observed under very cold or very anaerobic conditions.

Studies on the sorption of androgen compounds are scarcer than studies on estrogen sorption and transformation. A column study reported K_{oc} values of 2.97-4.56 for testosterone, and demonstrated that testosterone was not as strongly sorbed to the soil as 17β-estradiol, meaning that testosterone could travel to greater depths in the soil where low biological activity may not sufficiently degrade the compound (Casey et al., 2003). Likewise, log K_{oc} values of 3.72 and 3.34 for 4-androstene-3,17-dione and Testosterone respectively have also been reported (Lee et al., 2003).

In soil, it has been shown that testosterone, much like 17β-estradiol, transforms to metabolite compounds. In a loam soil incubated at 30ºC, 48.7% of the testosterone transformed to 4-androstene-3,17-dione, 23.7% to 1,4-androstadiene-3,17-dione, and 9.6% to 5α-androstane-3,17-dione after only 6 hours (Lorenzen et al., 2005). Lee et al. reports a half-life of testosterone at 0.3-7.3 days in soil (Lee et al., 2003). Fan et al. (2007) determined that 23.4% of testosterone was mineralized to CO_2 during a 168 hour column experiment, and 4.48-113.25% of the testosterone or metabolites were recovered in the column eluent (Fan et al., 2007)

Hormones that enter the environment from agriculture are sourced from manure; therefore it is important to study their fate when applied to the soil as part of the manure matrix. Addition of poultry litter may decrease the transformation rate of parent hormones. It was observed that with the addition of poultry litter, the applied estradiol sorbed more strongly and testosterone did not sorb as strongly to the soil (Bera et al., 2011), which is consistent with the relative affinities of their primary transformation products. However noted in the same study, there were no significant differences in sorption kinetics between sterile and non-sterile soil, though the authors attribute this to the short duration of the study (48 hours) (Bera et al., 2011). When observing manure applications over the long term, it is apparent that the soil organic carbon is the main parameter that affects sorption of estrogens (Stumpe and Marschner, 2010). Transformation of estrogen compounds has been studied in applications of swine manure and it was determined that when applied with swine manure, 17β-estradiol was converted to estrone and testosterone was transformed to 4-androstene-3,17-dione more rapidly than when applied to soil without any manure, but with or without manure amendment, the testosterone and its degradation product were not detectable within a few days. (Jacobsen et al., 2005).

1.6.2 Runoff

Because hormones are more likely to be associated with soil particles than in aqueous concentrations, travel via runoff is a common fate for these compounds.

Runoff from Feedlots

An analysis of runoff from beef heifer feedlot soils had 4-androstene-3,17-dione and progesterone in 100% of the runoff samples and androsterone in 96% of runoff samples with average concentrations of 0.102, 2.660, and 0.0991 µg/L respectively (Bartelt-Hunt et al., 2012). Heifers are not expected to excrete much testosterone, so the androgens were likely sourced from

the high amounts of progesterone, adding to evidence that animal waste is a source of large amounts of progesterone and androgens due to transformation (Bartelt-Hunt et al., 2012).. Similarly, in a rainfall simulation on a steer feedlot, higher concentrations of progesterone and 4-androstene-3,17-dione was detected. 17α-estradiol, 4-androstene-3,17-dione, and progesterone concentrations ranged from 0.050-0.250 µg/L and 17β-estradiol, estrone, and testosterone were mostly less than 0.050 µg/L in the steer feedlot runoff. Only 17α-estradiol (15 µg/kg), testosterone (2 µg/kg) and progesterone (below LOQ) were detected in fresh manure, but, 17β-estradiol, estrone, testosterone, 4-androstene-3,17-dione and progesterone were all detected in feedlot runoff. When plots were aged for 7 days, they contained more 1,4-androstadiene-3,17-dione, progesterone and estrone but less 17α-estradiol and testosterone when compared to the runoff from the initial sampling event. Also noted in the experiment was rapid loss of androgens and progestagens in the soil, after the simulated rainfall, possibly attributed to the increased microbial activity after wetting (Mansell et al., 2011).

Field Management Impacting Runoff

Field management type has the potential to reduce or increase runoff hormone concentrations. In a comparison of hormone (testosterone, estradiol) runoff from poultry litter application at agronomic rates between till and no-till field management it was found that no till contributed less hormones to the runoff than tillage, but the poultry litter in either treatment did not add to the background loading of hormones (Jenkins et al., 2009). It was found that pelletized litter (estrone, 2.1 µg/kg. 17β-estradiol 0.5 µg/kg) has slightly lower total estrogen content than raw (estrone, 3.7 µg/kg. 17β-estradiol, 0.4 µg/kg), most likely due to the heat treatment to create pelletized poultry litter. In the same study, it was also found that reduced till produced more estrogens (estrone, 17β-estradiol, estriol) in runoff than no till. It was also discovered that the

size of the rainfall event was more important than time from litter application. Larger rainfall events later in the season still produced significant amounts of estrogens in the runoff (Dutta et al., 2010).

The runoff potential of hormones from fields managed with different cattle manure application techniques has also been studied recently. Mina et al. (2016) conducted a study of natural rainfall runoff occurrence of estrogens in dairy manure amended soil comparing surface application and subsurface injection of the manure. Not only were estrogens detected in the runoff, it was observed that the surface applied plots showed significantly greater concentrations of estrogens in the runoff than the subsurface injected plots. However, the hormones in the subsurface injected plots were released over a longer period of time, where the hormones from the surface applied plots were detected mainly in the first runoff sampling event. (Mina et al., 2016). In a simulated rainfall study of till and no-till plots of cattle manure, less than 10% of the theoretically applied hormone mass was detected in the runoff from all simulated rainfall plots, indicating that runoff water was not the primary transport pathway for hormones, with limited effects of tillage treatment. (Biswas et al., 2017). In both aforementioned studies, hormones were shown to persist in the soil for longer than 30 days.

1.6.3 Fate in the Natural Environment

Interaction with Streambed Materials

Partitioning of estrogens (estrone, 17β-estradiol, estriol) between water and sediment with organic carbon content as a variable was investigated and it was observed that the higher the K_{ow} value, the more readily removed the estrogen was from the water, with the maximum sorption obtained after 1 hr. greater sediment:estrogen ratios improved the removal and a correlation between total organic carbon and estrogen sorption was apparent (Lai et al., 2000).

Endocrine disrupting compounds have been shown to accumulate in stream biofilms. In a study of biofilm and sediment matrices before and after discharge of a wastewater treatment plant effluent, 17β-estradiol mineralization was not significantly different, but over time in a laboratory study, the microbes extracted from the biofilm and sediment after the WWTP effluent discharge, were more efficient at mineralizing 17β-estradiol (Writer et al., 2011a). The type of sediment in a body of water can also influence the impact of hormones on organisms. Sangster et al. (2016) exposed fathead minnows to sand and silty loam sediment, both spiked with progesterone. The fish which were exposed to progesterone-spiked silty loam sediment did not exhibit as severely reduced vitellogenin and androgen receptor expression as fish which were exposed to progesterone-spiked sand, indicating that the physical properties of the sediment can influence how a population of organisms responds to hormone-sediment content (Sangster et al., 2016). Even with the attenuation of estrogens in the biofilm and sediment, the estrogens have the potential to travel kilometers downstream from a source. In a study tracing the same set of compounds in WWTP effluent down the Redwood river in Minnesota, it was found that estrogen from the effluent (estrone 0.00138 ± 0.00004 µg/L, 17β-estradiol 0.00009±.0.00002µg/L) was transported 2.1 km downstream, increasing the concentrations of estrogens there (estrone 0.00116 µg/L, 17β-estradiol 0.00096 µg/L) (Writer et al., 2011b).

Photodegradation

Once a free hormone enters a waterway, light has been shown to influence the transformation of hormones in a waterway, reducing endocrine disrupting activity. When exposed to direct sunlight, 4-androstene-3,17-dione and testosterone were shown to photodegrade readily with half-lives ranging from 3.7-10.8 hours. Within the same investigation, it was shown that the addition of dissolved organic matter reduced the amount of sunlight

reaching the hormones and slowed the photodegradation, increasing the half-life by 11-35% (Young et al., 2013). It was reported that the half-life of estrogens in river water undergoing photodegradation was 2.0 ± 0.14 hours for 17β-estradiol 2.9 ±0.16 hours for estriol, and 2.3 ± 0.07 hours for estrone. (Lin and Reinhard, 2005).

1.7 Analytical Techniques for Quantifying Hormones

Liquid chromatography tandem mass spectrometry (LC/MS/MS), gas chromatography tandem mass spectrometry (GC/MS/MS), or an enzyme linked immunosorbent assay (ELISA) are commonly used for analysis of hormones in environmental samples. While ELISA is simple, quick, and user-friendly, it has a tendency to report higher values of estrogens due to interference with other metabolites of estrogen or other structurally similar compounds (Dutta et al., 2010). Chromatography tandem mass spectrometry is considerably more sensitive and selective. Chromatography tandem mass spectrometry also enables the researcher to quantify multiple compounds simultaneously from one sample, which is valuable when considering the numerous metabolites and transformation products of the hormones being studied. LC/MS/MS is superior to gas chromatography in analyzing steroid hormones in that in order to be analyzed by GC/MS/MS, a compound must be fairly volatile or must undergo chemical derivatization to become volatile, adding analysis time, resources, and potential operational errors. LC/MS/MS analysis allows the steroid hormone compound to remain in its natural state.

1.8 Summary

The increasing industrialization and concentration of farming is putting stress on the environment by condensing agricultural waste products to the same area. These animal manures contain hormones naturally excreted by all livestock that are considered endocrine disrupting

compounds once in the environment. Hormones are likely to enter the environment through agricultural manure applications and their subsequent runoff during rainfall events. Hormones are already prevalent in surface waters of the US and around the world, though manure treatment techniques and manure application best management practices show promise for reducing the environmental hormone input. In this literature review it is apparent that while some research has been completed in the field of agricultural environmental hormones, there are gaps and shortcomings that still need to be filled. Firstly, many studies only analyze a few hormones, or use limiting immunosorbent assays that can only identify the most popular and potent hormones such as estradiol or testosterone and cannot properly quantify the metabolites. Secondly, new manure soil application technologies, such as subsurface injection of solid manures has not been adequately investigated, which could help reduce runoff and thus hormone input into the environment. Finally, as analytical technology advances, detection limits are getting lower, and changes in environmental concentrations of hormones and the subsequent biological impacts could be investigated even further.

1.9 Objectives

The overall goal of this study is to understand the effect of animal manure application on the fate of hormones in manure amended soils.

Objective 1: To investigate the spatial and temporal movement of hormones in a field receiving animal manure surface application.

Objective 2: To understand the effect of animal manure application technologies on the fate of hormones in manure amended soils.

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Chapter 2: The Spatial Distribution and Temporal Change of Hormones in a Manure Surface Applied Corn Field Adjacent to a Stream.

Abstract

Hormones are endocrine disrupting compounds which can be detrimental to the sexual development of aquatic organisms, with exposure sometimes resulting in population losses and individual deformities. Manure amendments to agricultural fields concurrently apply hormones with beneficial plant nutrients. The main goal of this investigation is to understand the spatial distribution and temporal change of manure-associated hormones in a manure surface applied agricultural field that is adjacent to a stream. In this study a total of 11 compounds including 2 hormone precursors, 5 hormones, and 4 conjugated hormones were tested in soils from a corn field receiving manure surface application for decades. Two different types of manure (drystack and dairy slurry) were applied to two different zones of the field. Soil samples (0-5cm and 5-20cm in depth) were collected at 6.5 months and one week before the scheduled yearly manure application, three times during the two weeks of manure applications, and 1 week, 2 weeks, 6 weeks, 3 months, and 7.5 months after the manure application. The samples were collected from 18 locations along the drainage swales and the ridges of the field and 2 locations in the grass buffer zone between the field and the adjacent surface stream. Water samples from the stream were also collected during each soil sampling event. The levels of all 11 hormone compounds in all the water samples tested were below their detection limits. Although all 11 compounds were detected in both types of manure, only 1,4-androstadiene-3,17-dione, estrone, and progesterone were detectable in the environment, mostly in the 0-5cm soils, with only 13.7% of samples with detectable levels of 1,4-androstadiene-3,17-dione, estrone, and progesterone in the 5-20cm soil. Observed greater concentrations of hormones were found in the swales of the manure-applied

field, though not statistically greater than the hormone concentration in the swales. There were higher levels of detectable hormones in the dry stack-applied zone compared to the dairy slurry-applied zone of the field. Overall, hormone concentrations in the soil peaked on the day of dairy slurry application, but all tested hormones dissipated to levels below their detection limits in the field 6 weeks after the manure application.

2.1 Introduction

Hormones are included in a class of compounds called endocrine disrupting compounds, which can interfere with the endocrine system of an organism, the system which regulates many sexual, reproductive, and developmental functions. Hormones can impair the sexual development of aquatic organisms at sub ppb levels, putting them at risk for population deficits, altered mating behavior, and deformities of the reproductive system (Jukosky et al., 2008; Kidd et al., 2007; Leet et al., 2012). Hormones are often transformed in the environment to compounds which can still cause environmental impacts, therefore it is important to simultaneously detect hormone parent compounds and their metabolites (Jacobsen et al., 2005; Lee et al., 2003).

Hormones are naturally excreted by livestock; therefore, manure amendments to agricultural fields concurrently apply steroid hormones in addition to beneficial plant nutrients (Lange et al. 2002). When manure is applied to the field it is not only the nutrients and organic matter that are applied, but also other compounds present in the manure, such as hormones.

There are two major transport pathways that manure-borne contaminants such as hormones can take once applied to the soil, surface runoff and leaching, and both are driven by precipitation. Surface runoff occurs when the contaminant of interest is soluble in the water or is sorbed to soil particles and then is transported to a stream or waterway, and leaching takes place

when the contaminant of interest becomes desorbed from its substrate and then is transported through the soil profile by water movement. Field studies show that many factors could alter hormone degradation rates such as levels of organic matter, precipitation, and soil microbial populations (Mansell et al., 2011; Ying and Kookana, 2005). The chemical nature of hormone compounds causes the molecules to tend to associate with organic matter and soil particles, therefore the hypothesized primary transport mechanism for hormones from manure to waterways is mainly via surface runoff of eroded particulates (Bera et al., 2011; Casey et al., 2005; Lee et al., 2003). In turn, it has been demonstrated that surface runoff from manure amended agricultural fields can contain environmentally relevant hormone concentrations (Dutta et al., 2010; Mina et al., 2016).

The purpose of this investigation was to monitor the spatial and temporal occurrence of hormones sourced from manure applied to a field with an adjacent surface stream. This task was accomplished by monitoring the concentration of 11 hormones in surface soil, subsurface soil, and adjacent stream water during and following a routine manure application.

2.2 Materials and Methods

2.2.1 Chemicals and Materials

Hormone standards for 1,4-Androstadiene-3,17-dione (1,4-ADD), 4-Androstene-3,17-dione (4-AD), Progesterone (P), Estrone (E1) (≥99%), 17α-Estradiol (αE2) (≥98%) , 17β-estradiol (βE2) (≥98%), and Estriol (E3) (≥97%) were purchased from Sigma-Aldrich. (Saint Louis, MO). Hormone standards for 1,3,5(10)-Estratrien-3-ol-17-one glucosiduronate, Sodium Salt (≥98%) (E1-3G), 1,3,5(10)-Estratrien-3, 17β-diol 3-sulphate, Sodium Salt (≥98%) (E2-3S), and 1,3,5(10)-Estratrien-3, 17β-diol 3- glucosiduronate, Sodium Salt (≥98%) (E2-3G) were purchased from Steraloids (Newport, RI). Hormone standard for 1,3,5(10)-Estratrien-3-ol-17-

one Sulphate, Sodium Salt (>98%) (E1-3S) was purchased from Alfa Chemistry, (Protheragen Inc., Stony Brook, NY). HPLC grade acetonitrile, HPLC grade ethyl acetate, HPLC grade methanol, HPLC grade acetone, ammonium hydroxide, hydrochloric acid, and ammonium fluoride were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium acetate was purchased from Amresco (Dallas, TX). De-ionized water and Milli-Q water was made using a Millipore water purification system (Millipore, Billerica, MA).

2.2.2 Site Characteristics, Manure Application, and Sampling Scheme

This study was conducted on a no-till corn field with a history (20+ years) of routine annual manure applications. The studied field has 2%-15% slopes with soils types: Duffield (Fine-loamy, mixed, active, mesic Ultic Hapludalfs), Ernest (Fine-loamy, mixed, superactive, mesic Aquic Fragiudults), Guernsey (Fine, mixed, superactive, mesic Aquic Hapludalfs), Berks (Loamy-skeletal, mixed, active, mesic Typic Dystrudepts) and Groseclose (Fine, mixed, semiactive, mesic Typic Hapludults). Sampling sites were selected based on the terrain of the field (*Fig. 2.1*). Sites A1-A5 were situated on the main ridge of the study area. Sites B1-B4 and sites D1-D4 were located along the drainage swales of the field. These two drainage swales converged at site A6 to form a combined swale that contained sites A7-A9 with sites A8 and A9 in the grass buffer zone between the field and a surface stream. Sites C1-C3 were located on one of the hillsides in the study area. The water samples were collected at the outlet point of the combined swale to the surface stream.

Two types of manure were surface-applied to two different zones of the studied field (*Fig. 2.1*). Drystack manure, a partially composted manure from various livestock animals stored in a covered shed, was first applied at a rate of 2.2kg/m^2 to the zone including sampling

sites A5-A7, B4, and D4. Dairy manure slurry was applied 12 days later at a rate of 9.8 L/m^2 to the zone that was upslope of the drystack manure-applied zone and included sampling sites A1-A4, B1-B3, C1-C3, and D1-D3. Sampling sites A8 and A9 were not treated with any manure and were located in a riparian buffer zone between the manure applied field and the stream.

Manure was collected on the day of application for each manure type. Water and soil samples were taken on 196 and 6 days prior to the application of drystack manure (Day -196 and day-6) and days 0 (day of drystack application), 8, 12 (day of dairy slurry application), 20, 27, 54, 111, 238 following the application of drystack manure. A composite soil sample was collected at each designated site (*Fig. 2.1*) at a depth of 0-5cm and then at a depth of 5-20cm. All samples were immediately packed on ice until the end of the field day (maximum 4 hours) and then stored at -10ºC until analysis.

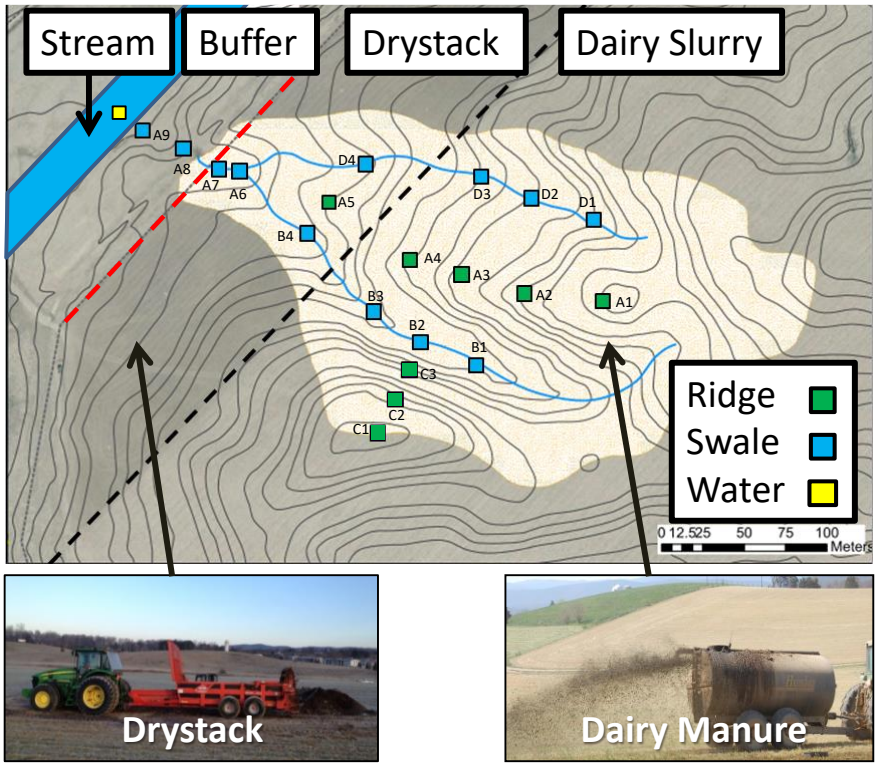

Fig. 2.1: Sampling locations (numbered letters) in the field applied with two different manures at two zones. The field topography, outline of the drainage swales and ridges, the grass buffer zone, and the stream are shown

2.2.3 Sample Preparation and Extraction.

Soil samples were freeze dried, lightly ground, and then subject to the following accelerated solvent extraction and solid phase extraction protocol. Five grams of freeze dried soil or 1.00g of freeze dried manure were mixed with 1g of hydromatrix and then transferred into an 11ml stainless steel extraction cell (Büchi, New Castle, DE). The remaining space in each cell was filled with Ottawa Sand standard of 20-30 mesh (Agilent Technologies, Santa Clara, CA)with 1cm of headspace. The prepared extraction cells were load to an accelerated solvent extraction system (E916 Speed Extractor, Buchi, New Castle, DE) and the contents of the extraction cells were extracted at 100bar and 75°C using a two 3-minute cycles of

methanol/acetone (50:50 v/v) followed by two 3-minute cycles with water/ methanol (50:50 v/v) (Nieto et al., 2008). Soil and manure extracts were diluted to 50mL and centrifuged at 2054xg for 40 minutes to remove excess solids. An aliquot of 25 mL of the soil or manure extract was then diluted to 500mL with DI water and acidified with 5 M HCl to ~pH 2.= to prepare for the solid phase extraction. Stream water samples were measured to 500mL then vacuum filtered through a Millipore 0.45µm HNWP nylon membrane filter (Millipore, Billerica, MA) then acidified to pH 2 with 5M HCl.

2.2.4 Solid Phase Extraction for Soil, Manure and Stream Water Samples

Oasis HLB cartridges (60mg/3cc, Waters) were arranged in a 20-position Sampli-Q SPE Vacuum Manifold (Agilent Technologies, Santa Clara, CA) and conditioned with 3 mL 9:1 ethyl acetate/methanol, 3mL methanol, then 6 mL water. Diluted extract samples (500 mL) or 500mL filtered creek water samples were loaded onto the Oasis HLB cartridges at a flow rate of 4-5 mL/min. SPE cartridges were then rinsed with 5 mL of 95:5 water/methanol and allowed to dry under gentle vacuum for 20 min. Hormones were eluted from the SPE cartridges with 3mL 9:1 Ethyl Acetate/Methanol followed by 3mL 2% NH_4OH in methanol. Primary secondary amine (50mg) (Agilent Technologies, Santa Clara, CA) was added to the manure samples as an extra clean-up step. Sample solvent was evaporated to dryness under a gentle stream of nitrogen at 40ºC (TurboVap, Labconco). Sample residue was reconstituted in 0.5 ml of acetonitrile followed by 0.5 mL water and filtered through a 0.22 µm Thermo PTFE filter (Thermo Scientific, Rockwood, TN) and stored at -80°C until LC/QQQ analysis. To overcome matrix effects, matrix matched standards were made for soil samples and a standard addition method was used for manure samples.

2.2.5 UPLC-MS/MS Analysis

An Agilent 1290 series UPLC coupled to an Agilent 6490 triple-quadrupole mass spec, (Agilent Technologies, Santa Clara, CA) was used to separate and quantify 11 hormone compounds in each sample from the experiment. Analytical columns used were Zorbax Eclipse Plus C18 rapid resolution HT 2.1x100mm, 1.8µm, 600 bar preceded by a Zorbax Eclipse Plus C18 2.1x5mm 1.8µm UHPLC Guard Column (Agilent Technologies, Santa Clara, CA). The sample injection volume was 10µL, the column temperature was 40°C and the mobile phase flow rate was 0.300ml/min. Mass spectrometer parameters were sheath gas temperature at 200 ^{o}C with a flow rate of 8L/min, drying gas temperature at 250 ^{o}C with a flow rate of 14L/min, Nebulizer pressure 45 psi, capillary voltage 3500(+)V 3000(-)V.

For the analysis of E3, αE2, βE2, and E3, the mobile phases were A: 0.2mM ammonium fluoride in water, and B: acetonitrile/water (95:5, v/v), and the QQQ was run in negative mode electrospray ionization. The mobile phase gradient was 0-1 minutes, 10% B; 9 min, 85% B; 9.5 min, 98% B; 10.5 min, 98% B; 11 min, 10% B; 14 min, 10% B. Retention times of each compound are as follows: E3, 4.94min; βE2, 6.82min; αE2, 7.07min; E1, 7.35min.

For the analysis of E2-3G, E1,3G, E2-3S, E1-3S, 1,4-ADD, 4-AD, and P, the mobile phases were A: 5mM ammonium acetate in water, and B: acetonitrile/water (95:5, v/v). The mobile phase gradient was 0-1 minutes, 10% B; 9 min, 85% B; 9.5 min, 98% B; 10.5 min, 98% B; 11 min, 10% B; 14 min, 10% B. The mass spectrometer electrospray ionization was run with negative mode for E2-3G, E1-3G, E2-3S and E1-3S then with positive mode for 1,4-ADD, 4-AD, and P. Retention times of each compound are as follows: E2-3G, 4.04min; E1-3G, 4.34; E2-3S, 4.93min; E1-3S, 5.29 min; 1,4-ADD, 6.90 min; 4-AD, 7.44 min; P, 8.70 min. Soil and

creek water method detection limits t(n-1=6, 1-α=0.99), for each compound can be found in *Table 2.1*.

Table 2.1: Soil and stream water method detection limits t(n-1=6, 1-α=0.99)

	Soil		Water	
Compound	MDL (µg/kg)	Recovery (%)	MDL (µg/L)	Recovery (%)
1,4-ADD	0.35	96.4	0.8	120.3
4-AD	0.43	94.7	0.66	86
P	0.39	97.9	1.39	139.1
E2-3G	1.03	112	0.66	17.2
E1-3G	0.66	93.4	0.8	21.4
E2-3S	0.82	99.7	0.11	36.2
E1-3S	0.41	88.2	0.22	63.2
E3	0.76	93.2	0.22	82.3
β-E2	0.61	83.7	0.34	52.5
α-E2	0.65	79.8	0.21	62.3
E1	0.33	68.4	1.77	117.0

2.2.6 Statistical Analysis

Statistical analysis was completed in JMP Pro 13. An ANOVA and post-hoc Tukey's HSD test was used to compare the concentration of soil hormones in different regions of the field.

2.3 Results and Discussion

2.3.1 Concentrations of Hormones in Drystack and Dairy Slurry Manure Applied to the Field

The dry weight-based concentrations of the 11 tested hormones in the drystack and dairy slurry manure applied were determined in this study (*Fig 2.2a*). Overall E1 was detected in the highest dry weight concentration in both the drystack and in the dairy slurry, at 141.3 µg/kg and 190.4 µg/kg respectively. All of the hormones studied were detected in the drystack manure. E2-3S was detected at 64.2 µg/kg, followed by E2-3G at 50.2 µg/kg. The remaining estrogen

conjugates were detected at 33.1 µg/kg and 23.8 µg/kg for E1-3S and E1-3G respectively; androgens at 30.4 µg/kg and 29.9 µg/kg for 1,4-ADD and 4-AD; P at 39.5 µg/kg; and the remaining estrogens, E3, α-E2 and β-E2 at 4.75 µg/kg, 5.21 µg/kg, and 23.1 µg/kg. In addition to E1, P was also detected at a relatively high concentration in the dairy slurry at 94.4 ug/kg. The remaining 6 hormones detected in the dairy slurry make up only 34% by weight of the hormones detected. 4-AD at 37.0 µg/kg, 1,4-ADD at 13.7 µg/kg, E2-3G at 42.4, E1-3G at 19.1 µg/kg, E2-3S at 22.3, and E1-3S at 9.6 µg/kg . E3, βE-2, and α-E2 were below their detection limits in the dairy slurry.

The dairy manure in this study had been stored in a lagoon for up to 12 months before application. When compared to values for extremely fresh manure collected within 2 hours of excretion reported previously, the dairy manure used for this study had 10 times lower estrogen content (α-E2, β-E2 and E1) (Zheng et al., 2007). Mina et al. (2016) detected similar E1 levels in the lagoon-stored dairy manure used in their study, but also detected α-E2 and E3, which were below detection limits in the dairy manure used in the current study (Mina et al., 2016).

The drystack and dairy slurry manure used in this study were very different in character. The drystack manure originated from many different animals, including cow, sheep and horse, and was kept in its solid form in a partially enclosed area for up to 12 months before land application. The dairy slurry originated only from dairy cattle and had been stored in a lagoon for up to 12 months before land application. This led to drastically different water content in each manure. Drystack manure had a 50% water content by weight and the dairy slurry contained 97% water by weight. When taking into consideration the application rates of 2.2 kg/m^2 for drystack and 9.8L/m^2 for the dairy slurry (estimating 1L manure = 1kg manure), it was estimated that 1.1 kg and 0.31 kg/m^2 of manure solids were applied with drystack and dairy slurry, respectively,

resulting in higher amount of each hormone applied to the field with the drystack than with the dairy slurry (*Fig 2.2b*).

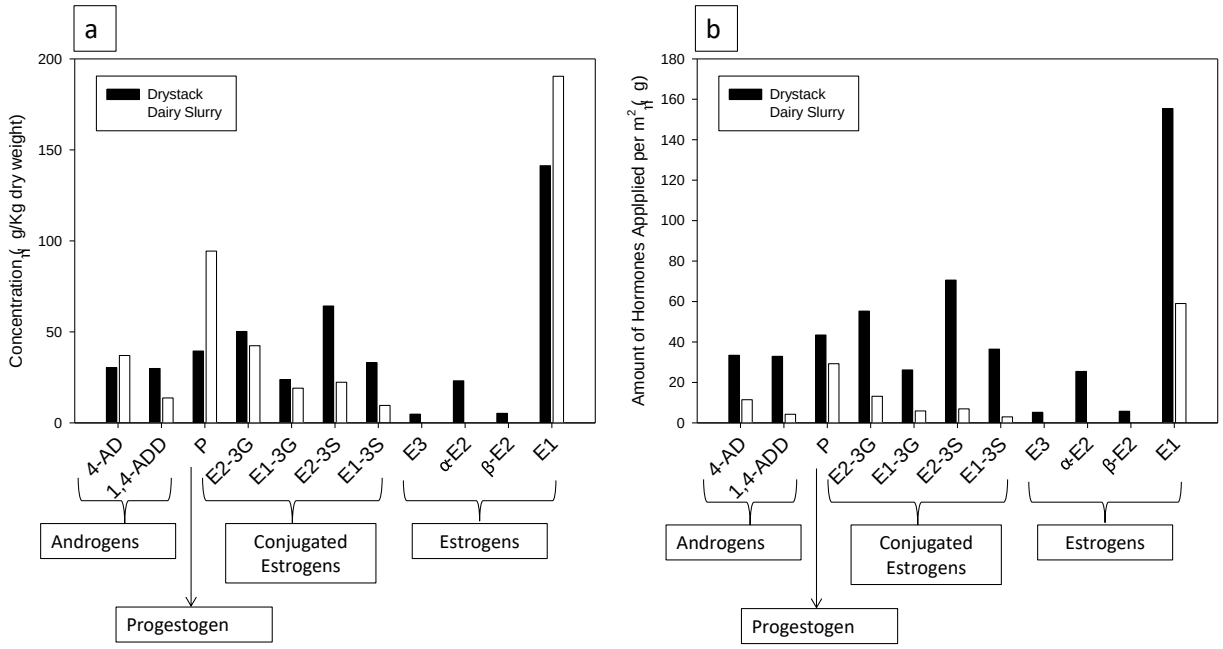

Fig. 2.2: The concentration of individual hormones in the drystack and dairy manure applied to the field for this study (a) and the estimated amount of hormones applied per m^2 with each type of manure (b)

2.3.2 Vertical Distribution of Hormones in the Field

Of the 11 hormones studied, only 1,4-ADD, E1 and P were at detectable levels in any soil samples. It is not surprising that estrogen conjugates were below their detection limits in any soil samples because they tend to deconjugate rapidly to free estrogens in the soil (Shrestha et al., 2012). E2 also tends to transform readily to E1 (Goeppert et al., 2014). The hormones were mainly detected in the 0-5cm soil and sporadically detected in the 5-20cm soil. Only 13.7% of the total number of soil samples with detectable hormones were from the 5-20cm depth. Even though hormones were detected more frequently in the 0-5cm soil, the average concentration of total soil hormones in detectable samples in the 0-5cm soil was only slightly greater than in the

5-20cm soil at 1.22 µg/kg and 0.95 µg/kg respectively. The 0-5cm soils had a broader range of detected concentrations, from 0.39-7.38 µg/kg where 5-20cm soils only ranged from 0.43-1.74 µg/kg total hormones among samples with detectable levels of hormones (*Fig. 2.3*). Hormones, with log K_{ow} values between 3 and 4, tend to associate with soil and organic matter particles and not readily move downward through the soil profile (Casey et al., 2005; Lee et al., 2003; Ying and Kookana, 2005; Yu et al., 2004).

For the hormones which were detectable in the 5-20cm soil, P was detected at 0.49 µg/kg at site A9 196 days prior to the application of drystack manure and at site A8 at 1.07 µg/kg 6 days prior to the application. Both sites are located within the buffer area between the field and the stream (*Fig 2.1*), where ducks and geese were observed to congregate. The sporadic detection of P in this area before the manure application might be due to the waste from the wildlife. All hormones were below their detection limits in all the 5-20cm soil on the day of drystack application, but 1,4-ADD, P, and E1 were then detected again at a concentration range of 0.39-2.51 µg/kg 8 days later at 5 sampling points: A6 and A7 (within the drystack application area), A8 (buffer), B2 and D2 (in the dairy slurry application area, but prior to the application of dairy slurry). After day 8, no more hormones were detectable in the 5-20cm soil. An explanation for the detection of hormones in the 5-20cm soil on day 8 can be offered by noting that a substantial rain event occurred on day 6-7, where approximately 17.78mm of rain fell over the course of 12 hours. The rain event may have caused some downward movement of the hormones in the freshly applied drystack manure, though those hormones were short lived in the soil, as they were below their detection limits in all 5-20 cm soils on day 12 and thereafter. The karst topography of the sampling region could have also contributed to the downward movement of

hormones in the soil, as the fissures and pores associated with Karst which aid water in permeating to the subsurface. (VA DCR, 2008)

Because most of the soil samples with detectable hormones were from the top 0-5 cm soil depth (*Fig 2.3*), the following discussion on the lateral distribution and temporal change of hormones in the manure-applied field is therefore, focused on the surface soils.

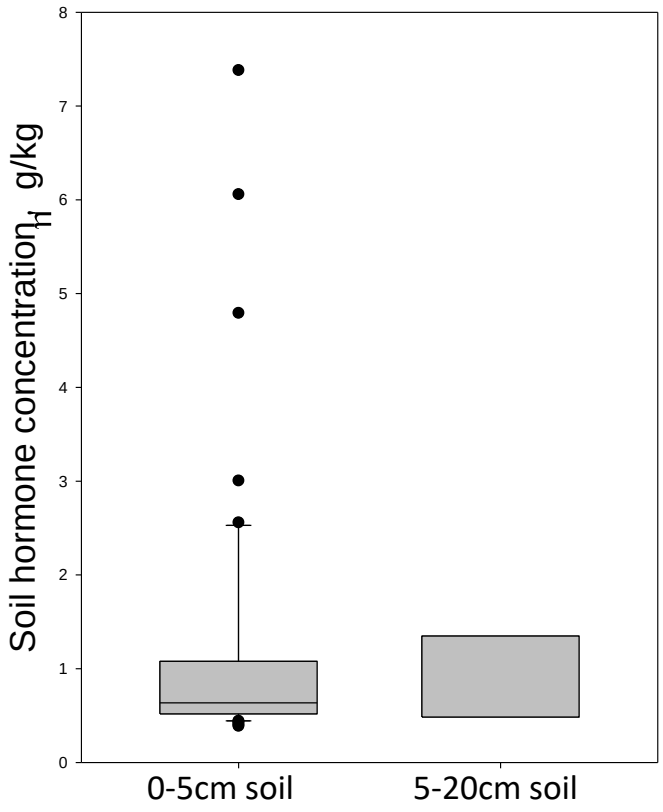

Fig. 2.3: Box and whisker plot of total hormone concentrations in samples with detectable levels of hormones in the 0-5cm soil and the 5-20cm soil.

2.3.3 Lateral Distribution of Hormones in the Field.

At any time point, the levels of total detectable hormones in the surface soils from the drainage swales were not statistically different from those in the surface soils from the ridges

(*Fig 2.4*), although the average of total detectable hormones in the ridge soils was below that in the swale soils at each time point. This result suggests that lateral transport of hormones from the ridges to the swales of the field is occurring during the 2 month period after manure application, however not in statistically significant observable quantities.

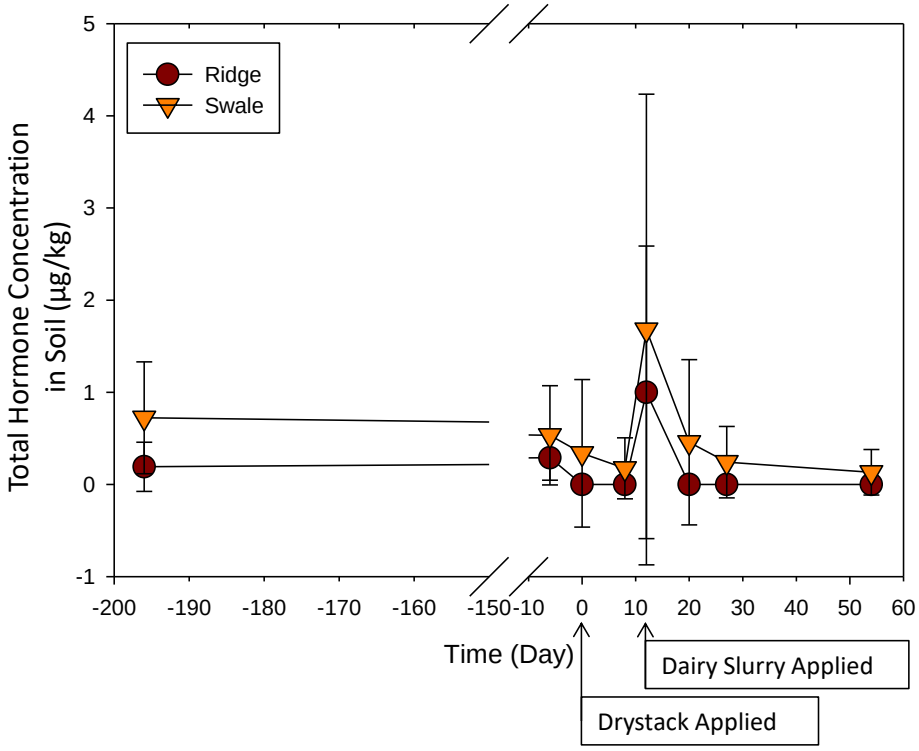

Fig. 2.4: Total hormone concentrations in the top 0-5cm soil samples collected from the ridges (n=8) and the drainage swales (n=12) before and after manure application. Symbols represent the average and the vertical bars indicate the standard deviation.

The total hormone concentrations in the 0-5cm soils from the drystack-applied zone, the dairy slurry-applied zone, and the no manure-applied grass buffer zone between the field and the stream show more distinct trends than the ridge and swale comparisons (*Fig 2.5*). 196 days prior to the application of manure, and again 6 days prior to the application of manure, the surface

soils within the buffer zone contained a significantly ($p < 0.05$) greater concentrations of total hormones the surface soils from the field up slope. At the time when the manure was applied to the field, the levels of total hormones in the buffer zone decreased 5 times as compared to pre-manure application, remained constant within 1 month thereafter, and increased two fold 54 days post manure application.

Within the first month of manure application, the levels of total hormones in the 0-5 cm soils of dairy slurry-applied zone were not significantly different from that of the buffer zone even on the day of dairy slurry application (*Fig 2.5*). On the day of drystack application and 12 days (the day of dairy slurry application) and 27 days after the application of drystack, the 0-5 cm soils in the drystack-applied zone had significantly ($p < 0.05$) greater concentrations of total hormones comparing to the dairy slurry-applied zone and the buffer zone (*Fig 2.5*). The most apparent reason that more hormones were detected in the drystack manure during the periods of manure application was because of the greater amount of hormones applied with the drystack manure than with the dairy slurry (*Fig 2.2b*). On day 8 and day 20 following the application of drystack manure, no significant differences were found for the total hormone levels in the surface soils of all three zones. On the 54 days following the application of drystack manure, the concentration of total hormones in the 0-5cm soil was once again significantly ($p < 0.05$) greater in the buffer zone than other two up slope zones where manure was applied roughly two months before.

The soil in the buffer zone is undisturbed and shaded by grasses and wetter than the up slope agricultural field where it is heavily trafficked and aerated for planting and harvesting, as well as was relatively exposed to sunlight and dryness during the growing season, especially early on in the season when the corn plants were small. The buffer zone therefore, had wetter

areas, while the other two up slope zones had more aerobic and dryer areas. Previous research has shown that hormones tend to degrade more rapidly under aerobic conditions: for instance, it has been studied that the estrogens E1, β-E2, and E3 will degraded rapidly under aerobic conditions, but can be persistent for months under anaerobic conditions (Ying and Kookana, 2005).If the manure-associated hormones reach the buffer zone through runoff after manure application, it may take longer for them to dissipate. It has also been determined that manure could accelerate dissipation rates of hormones, when compared to hormones applied to soil without any manure (Jacobsen et al., 2005). The hormones that had moved to the buffer zone would be in a different biological environment than those remaining in the two manure-applied zones. The karst topography of the sampling region could have also aided in the transport of hormones to the buffer area. Some hormones which had percolated downward through the soil profile with the groundwater could have been deposited back at the surface downslope and nearer to the stream. The hormones could have been re-deposited in the soil of the buffer region, where it was contained from moving further to the creek by dense vegetation and organic matter. In all potential cases, the buffer zone could ultimately prevent the movement of loose soil and manure particles from the manure-applied field, and re-deposited hormones from groundwater into the stream, where aquatic organisms are most vulnerable to the exposure to exogenous hormones.

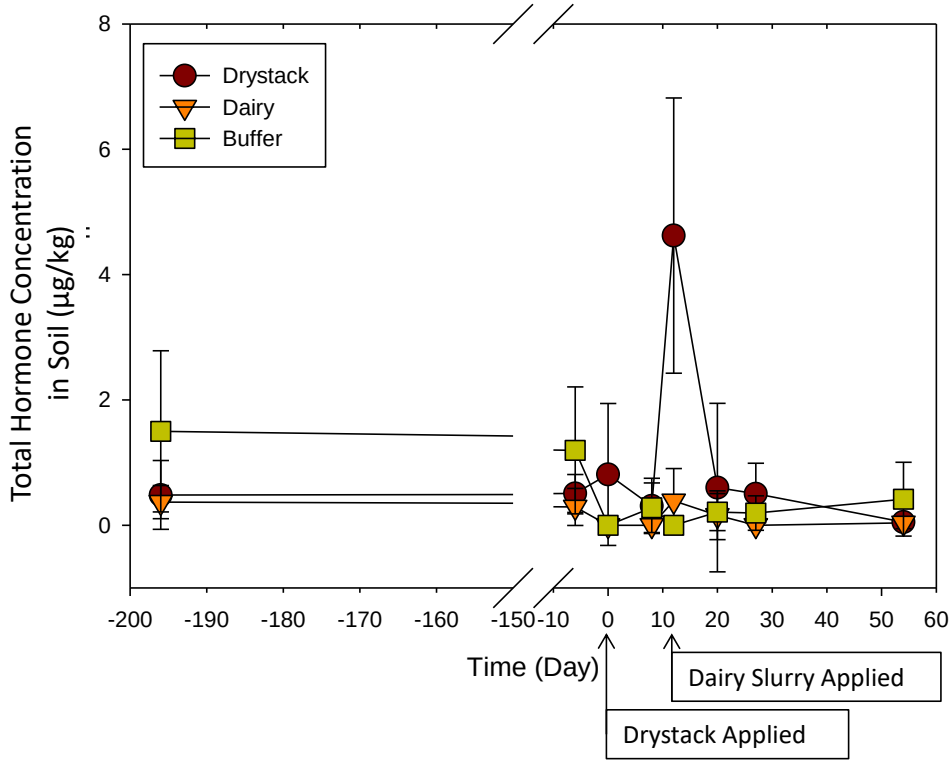

Fig. 2.5: Total hormone concentrations in the top 0-5cm soil samples collected from the dairy manure-applied zone (n=13) and the drystack manure- applied zone of the field (n=5) before and after manure application. Symbols represent the average and the vertical bars indicate the standard deviation.

2.3.4 Temporal Distribution of Hormones in the Field.

As discussed early on, the hormone compounds at detectable levels in the soils were 1,4-ADD, E1 and P. *Figs. 2.6, 2.7, and 2.8* shows the temporal change of the concentrations of those compounds in the 0-5 cm soil at each sampling point during this study. 1,4-ADD was not detectable before the manure application (*Fig. 2.6*). It was first detected at two swale sites one in the drystack-applied zone and one outside but close to the edge of this zone at 0.47-0.53 µg/kg on the day of drystack application (day 0). On the day of dairy manure application (day 12), 1,4-ADD was detectable in many swale and ridge soils and its concentrations peaked to a range of

0.40-2.17 µg/kg. It remained detectable at overall lower concentrations in soils of only 2-3 swale sites until day 27 after the drystack application. 1,4-ADD is a transformation product of testosterone, P, and cholesterol and, therefore, could be more abundant than its parent compounds in the manure-applied soil (Jenkins et al., 2004; Lorenzen et al., 2005; Marsheck et al., 1972).

Similar to 1,4-ADD, E1 was also first detectable on the day of drystack application (day 0) at concentration ranging from 0.73-1.15 µg/kg in the soils from a couple of swale sites in the drystack-applied zone (*Fig. 2.7)*. On the day of dairy slurry application (day 12), it became detectable at more swale and ridge sites in both manure-applied zone and peaked to a concentration range of 0.40-3.88 µg/kg. On the 20th day after drystack application, E1 was detectable only at two swale sites, one each in the two manure-applied zones. On the 27th day and thereafter the drystack application, E1 fell below its detection limit at all sites, while 1,4-ADD were below its detection limit on day 54 after the drystack application. E1 is a transformation product of β-E2 (Goeppert et al., 2014) and was applied at the highest amount to the field with both drystack manure and dairy slurry comparing to other compounds (*Fig 2.2b*).

Progesterone (P) was present at a concentration range of 0.4-2.41 µg/kg in the 0-5 cm soils of almost all swale and ridge sites at 196 days and 6 days prior to the application of drystack manure (day 0) (*Fig 2.8*). However, its levels fell below the detection limit at all sites but two in the drystack-applied zone on the day of drystack application. On the 8th day thereafter, only one site buffer zone and one site in the in the drystack-applied zone had detectable P in the soils. On the day when dairy slurry was applied (day 12), higher levels of P (0.62-1.97 µg/kg) were detectable at three swale sites and one ridge site in the drystack-applied zone and one swale site in the dairy slurry-applied zone. On the 20th day only one site in the

buffer zone had detectable P (0.42 µg/kg). The level of P at this site remained the same on the 27th day and increased slightly to 0.83 µg/kg on the 54th day. The levels of P at all sites in the buffer zone became non-detectable again on the 111th and 238th day. On the 20th and 27th days, none of the sites in the dairy slurry-applied zone had detectable P. The P detectable in many sites of the entire field before the application of manure may have been either leftover from the previous season's manure application, or deposited by wildlife which over-winter in the field. The fact that it was not detected on the 111th and 238th day after the manure application, wildlife was highly likely major source of P in this field. Geese have been known to frequent the field, and their feces have been shown to contain significant concentrations of P up to 9.4 µg/kg (Hirschenhauser et al., 1999), much higher than that in the manure that was applied to the field (*Fig 2.2a*).

At each sampling site with detectable hormones in the field, the total hormone concentration peaked significantly ($p < 0.05$) on the 12th day after drystack application which was also the day dairy slurry was applied. However, the majority of the hormones detected on day 12 were found at sampling points in the drystack-applied zone. A possible explanation for the increased detection of hormones in the soils of drystack-applied zone on day 12 is that after several rainfall events between the drystack application and the dairy slurry application, other hormones or sterols may have transformed into detectable levels of P, E1 and 1,4-ADD contributing to the total detectable hormones. It is also known that hormones could be biotically and abiotically transformed into other hormones (Colucci et al., 2001; Goeppert et al., 2014). Jenkins et. al determined that P can be microbially derived from naturally sourced phytosterols and that P can transform into 1,4-ADD and other androgen compounds (Jenkins et al., 2004). It is also well documented that estradiol and testosterone can transform into E1 and 1,4-ADD

respectively (Colucci et al., 2001; Lorenzen et al., 2005). 1,4-ADD is also a degradation byproduct of cholesterol and other natural sterols (Marsheck et al., 1972).

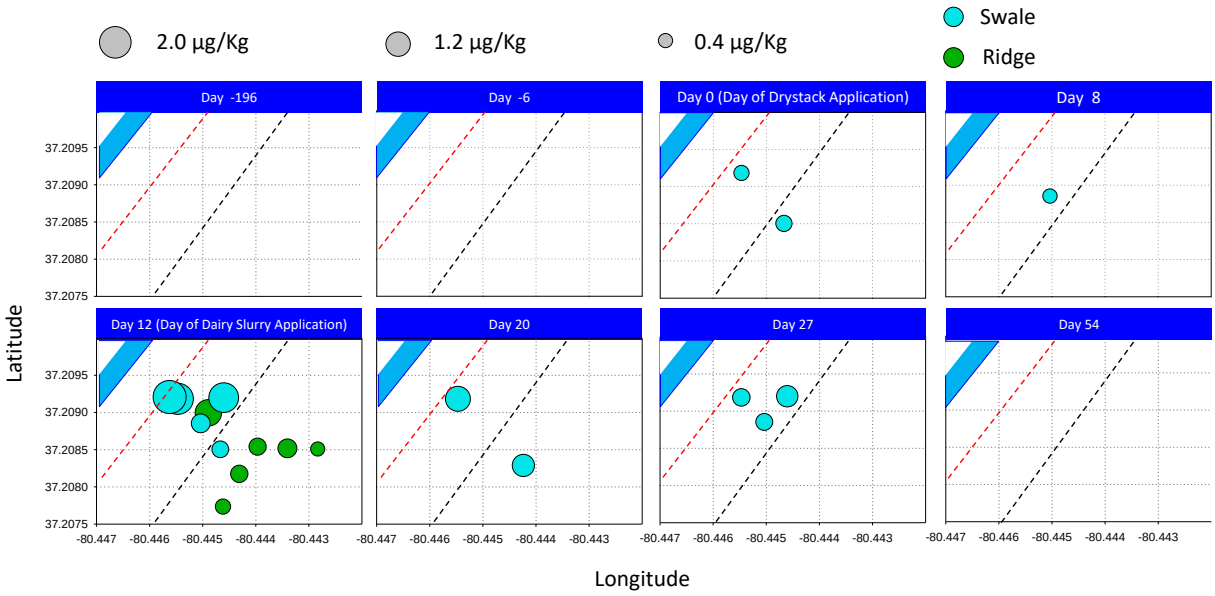

Fig. 2.6: Concentration of 1,4-ADD in 0-5cm depth soil at 8 time points before and after manure application. 1,4-ADD was below the detection limit in surface soil samples collected on 54, 111, and 238 days after the manure application. Size of bubble indicates the concentration of 1,4-ADD in soil. The stream is represented by the blue colored strip at the top left corner of a field plot. The area between the stream and the red dashed line is the buffer zone. The area between the red dashed line and the black dashed line is the drystack-applied zone. The area on the right side of the black dashed line is the dairy slurry-applied zone.

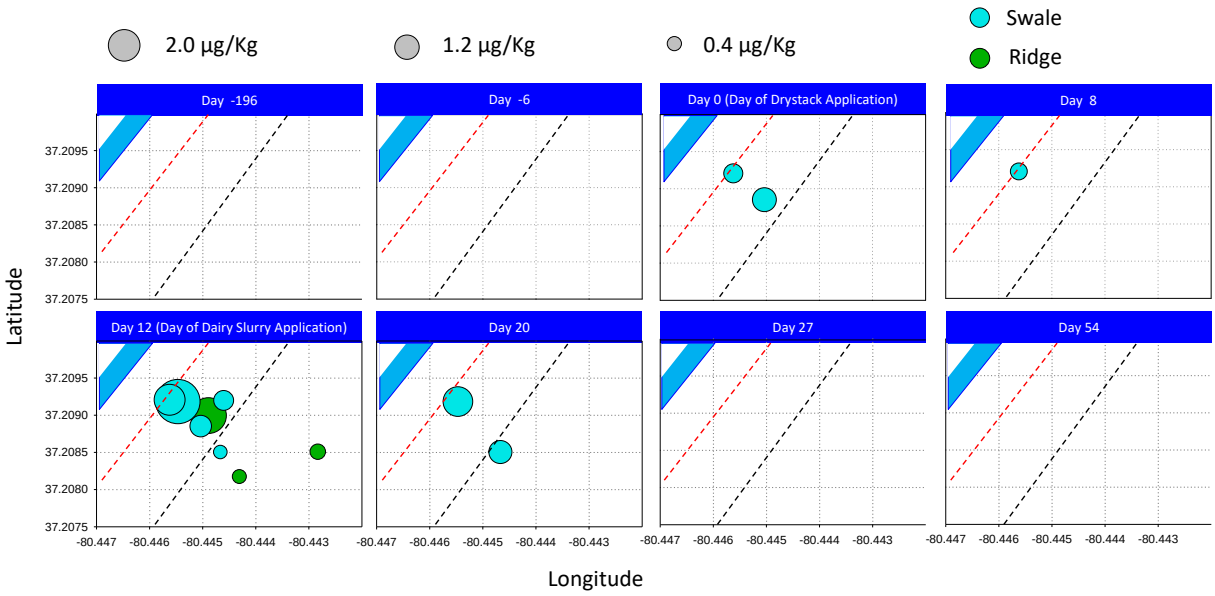

Fig. 2.7: Concentration of E1 in 0-5cm depth soil at 8 time points before and after manure application. E1 was below the detection limit in surface soil samples collected on 27, 54, 111,and238 days after the manure application. Size of bubble indicates the concentration of E1 in soil. The stream is represented by the blue colored strip at the top left corner of a field plot. The area between the stream and the red dashed line is the buffer zone. The area between the red dashed line and the black dashed line is the drystack-applied zone. The area on the right side of the black dashed line is the dairy slurry-applied zone.

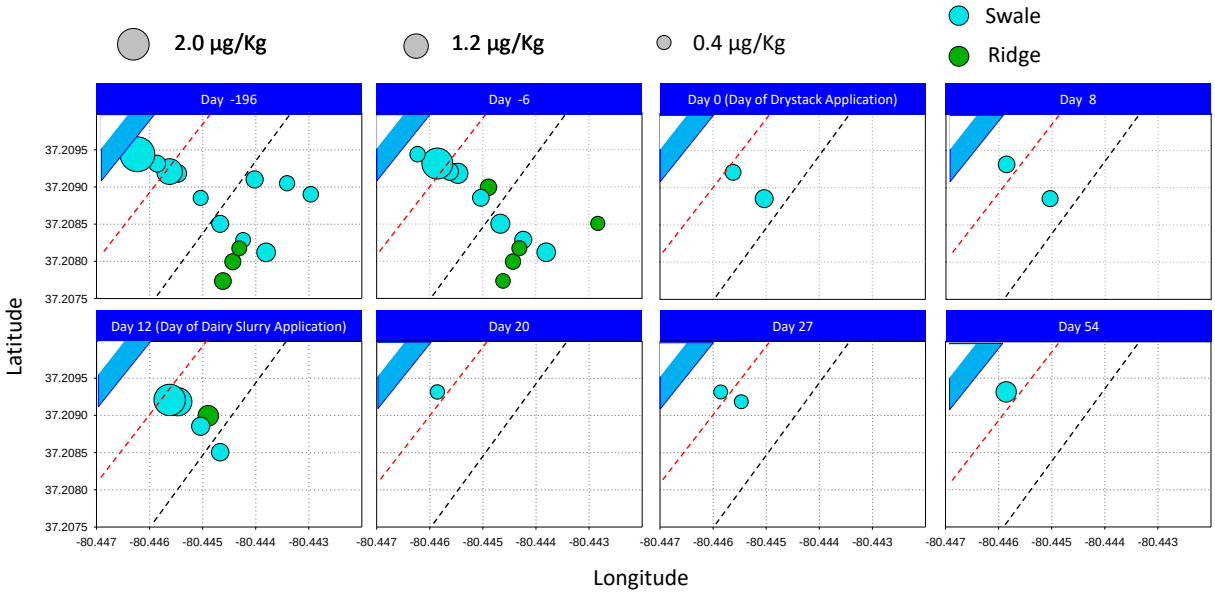

Fig. 2.8: Concentration of P in 0-5cm depth soil at 8 time points before and after manure application. P was below the detection limit in surface soil samples collected on 111 and 238 days after the manure application. Size of bubble indicates the concentration of progesterone (P) in soil. The stream is represented by the blue colored strip at the top left corner of a field plot. The area between the stream and the red dashed line is the buffer zone. The area between the red dashed line and the black dashed line is the drystack-applied zone. The area on the right side of the black dashed line is the dairy slurry-applied zone.

2.3.5 Hormones in the Stream Adjacent to the Manure-Applied Field

All 11 targeted hormones were below their detection limits in all water samples collected at the point of drainage from the studied field for the duration of the study. Samples were collected during stream baseflow. There are a few explanations for the lack of detection of hormones. Firstly the manure in this study was managed properly, as soil test guidelines were followed for determining the proper application rate of manure for the state of Virginia (DCR, 2005). In addition to responsible manure management, a buffer zone of natural, dense vegetation between the field and the stream, designed to reduce sediment input to the stream (VTBSE,

2017), may have effectively prevented further transport of manure and manure-associated contaminants to the stream.

Similar observations have been made by others in similar studies. Lafrance and Caron only detected trace levels of E1 at 0.0026 µg/L to 0.058 µg/L 24 hours following the application of hog and dairy manure to a field during a rain event, no more estrogens were detected in the stream after 12 days (Lafrance and Caron, 2013). Estrogens were below the detection limits in a stream adjacent to a properly managed organic dairy farm (Zhao et al., 2010). Hormones have also been shown to photodegrade upon entrance into a waterway further reducing the hormone content in stream water (Lin and Reinhard, 2005; Young et al., 2013). It has been shown that 4-AD and testosterone rapidly degrade in water within a few hours of exposure to direct sunlight. (Young et al., 2013). The streambed sediments were not sampled in this study; however, it is possible that hormones could be contained in the streambed sediments during baseflow sampling. Hormones are also known to partition with streambed sediments, therefore reducing the concentrations of hormones in the aqueous phase of a stream (Lai et al., 2000).

2.4 Conclusion

Immediately following the application of manure, the drystack-applied zone tended to have a significantly greater concentration of hormones, due to more hormones per surface area applied with the drystack manure than with the dairy manure, which indicates that the type of manure applied to a field and how that manure is handled could greatly impact the amount of hormones present in the soil. Observed higher concentrations of hormones in drainage swales indicate that there is some lateral movement of hormones on the surface of the soil, however the lack of statistically significant differences from the ridges of the field, combined with the observance of some hormone movement to the 5-20cm soil horizon indicates other processes

than only surface transport occurring. The karst topography of the sampling region could contribute to the vertical transport of hormones, and the occurrence of hormones in the buffer area, therefore sampling of groundwater could give a more complete picture of the spatial distribution of hormones. Sampling streambed sediments could also contribute to better understanding the spatial and temporal distribution of hormones. This study suggested that the movement of hormones to streams can be reduced by combining proper manure management with additional reductions in sediment movement to the stream, such as using a buffer zone.

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Chapter 3: Occurrence of Hormones in Corn and Orchardgrass Fields receiving Poultry Litter Application via Surface Application and Subsurface Injection.

Abstract

Steroid hormones can cause negative reproductive physical and behavioral traits in aquatic wildlife, and are endocrine disrupting compounds (EDCs). Hormones in natural systems can be sourced from poultry litter and other manures which are applied to agricultural crops for nutrients. A major pathway for manure-borne hormones to natural environmental systems is through surface runoff. New technologies for the subsurface application of poultry litter show promise as a tool to not only reduce nutrient runoff from agricultural fields, but can also reduce other manure-borne contaminants like hormones. A field scale study was set up to compare the soil concentrations of hormones over time in plots cultivated with corn and orchardgrass, for two application rates of manure, for surface applied and subsurface injected poultry litter. Of the 11 hormones studied, four of them (1,4-Androstadiene-3,17-dione, 4-Androstene-3,17-dione, Estrone and Progesterone) were detected over the course of the experiment. No correlation was found between manure application rate and hormones detected in the 0-5cm soil on the day of manure application. 1,4-Androstadiene-3,17-dione was detected in the 0-5cm soil up to 21 days following the application of poultry litter, while 4-Androstene-3,17-dione, Progesterone, and Estrone was detected for 8 days in the 0-5cm soil following the application of poultry litter. Unusually high concentrations of total hormones were found in the 5-20 cm soil on day 0, before leaching could have occurred, with hormones detected in the 5-20cm soil up to 64 days. Regardless of poultry litter application rates and type of crops, no distinct trends were found in individual and total soil hormone concentrations in comparing poultry litter subsurface injection

with surface application. On the day of poultry litter application between 10.59% and 49.33% of the predicted hormones applied with the manure were detected in the total soil for each treatment. Inadequate representative sampling of the injection slit and the areas between may have been the cause of the lack of conclusive results from the treatments in this study.

3.1 Introduction

Hormones are endocrine disrupting compounds, and it has been demonstrated that exposure to hormones can result in negative behavioral and physical effects for certain aquatic organisms (Kidd et al., 2007, Jukosky et al., 2008). Hormones are naturally excreted from livestock and have been shown to be present in poultry litter, soil which has been amended by poultry litter, and the runoff from manure amended soil (Bevacqua et al., 2011; Dutta et al., 2010; Jenkins et al., 2009; Jenkins et al., 2006)

Poultry litter is a byproduct of poultry farming, and it includes the manures and bedding materials from the flock when the poultry house is cleaned out. Poultry litter is applied to agricultural lands as a soil amendment to add nutrients such as phosphorus and nitrogen to supply crop needs. Poultry production in the US has become increasingly concentrated in recent years, with producers consolidating resources to particular regions in order to reduce costs and resources (MacDonald and McBride, 2009) (USEPA, 2012). In Virginia, one of the major poultry producing regions is located in the Shenandoah Valley, where this study took place, in the northwestern portion of the state. Virginia farms sold over 237 million broiler chickens in 2012, with poultry production consolidated to 9 counties in the state, and 4 of those counties located in the Shenandoah Valley region (USDA, 2012a; USDA, 2012b). Concentrating poultry farms also concentrates the waste production by poultry. Increasing the land area concentrations of manures and poor manure management has been linked to increasing nutrient and manure

contaminant pollution in nearby waterways (Ogejo, 2010). Concentrated regions of manure can also cause increased occurrence of hormones in waterways due to runoff from manure. (Chen et al., 2010; Gall et al., 2011; Lafrance and Caron, 2013; Vaicunas et al., 2013).

New technologies in poultry manure management have emerged due to concern over nutrient pollution. The use of no-till agriculture has demonstrated reduced nutrient losses from agricultural fields, and manure subsurface injection can follow no-till agriculture requirements. (Maguire et al., 2011). The challenge of incorporating manure in no-till systems has been addressed with the innovation of a manure subsurface injector for dry manures, such as poultry litter. Reducing nutrient pollution may also have an added effect on reducing the transport of hormones from the manure applied to agricultural fields to nearby streams and waterways. Hormones sourced from manure applications have been shown to persist in the soil, and do not readily leach through the soil profile, as the chemical behavior of hormones lends them to be associated with soil and organic matter fractions (Casey et al., 2005; Lee et al., 2003). Not exposing manure to land surface could have an effect on the behavior of hormones in soils. Although some information is available on the occurrence of hormones in soils sourced from manure, new sensitive analytical techniques such as liquid chromatography and mass spectrometry, can deliver more complete information about the occurrence of a wider array of hormones in soil.

This project sought to determine if there are any significant changes to the occurrence of 11 hormones in soil over time following the surface application and the subsurface injection of poultry litter. Two common cropping systems for the region (corn and orchardgrass) were studied, as well as different poultry litter application rates.

3.2 Materials and Methods

3.2.1 Chemicals and Standards

Hormone standards 1,4-Androstadiene-3,17-dione (1,4-ADD), 4-Androstene-3,17-dione (4-AD), Progesterone (P), Estrone (E1) (≥99%), 17α-Estradiol (αE2) (≥98%) , 17β- estradiol (βE2) (≥98%), and Estriol (E3) (≥97%) were purchased from Sigma-Aldrich. (Saint Louis, MO). Hormone standards 1,3,5(10)-Estratrien-3-ol-17-one glucosiduronate, Sodium Salt (≥98%) (E1-3G), 1,3,5(10)-Estratrien-3, 17β-diol 3-sulphate, Sodium Salt (≥98%) (E2-3S), and 1,3,5(10)-Estratrien-3, 17β-diol 3- glucosiduronate, Sodium Salt (≥98%) (E2-3G) were purchased from Steraloids (Newport, RI). Hormone standard 1,3,5(10)-Estratrien-3-ol-17-one Sulphate, Sodium Salt (>98%) (E1-3S) was purchased from Alfa Chemistry, (Protheragen Inc., Stony Brook, NY). HPLC grade acetonitrile, HPLC grade ethyl acetate, HPLC grade methanol, HPLC grade acetone, ammonium hydroxide, hydrochloric acid, and ammonium fluoride were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium acetate was purchased from Amresco (Dallas, TX). De-ionized water and Milli-Q water was made using a Millipore water purification system (Millipore, Billerica, MA).

3.2.2 Treatment Design and Manure Application

This study was completed near Mauzy, VA on an orchardgrass field and a corn field under no-till production. The soil type was a mix between Frederick (fine, mixed, semiactive, mesic typic, paleudult) and lodi (fine, mixed, semiactive, mesic typic, hapludult) soil series. Each field of orchardgrass was not mowed prior to the application of poultry litter and plot size was 2.5m x 6.1 m. Each corn plot size was 5.0m x 30.5m, and manure was applied prior to planting.

Plots were arranged in a split plot design. There were 4 treatments with poultry litter application rates based on difference in the projected plant N uptake for orchardgrass and corn production (VADCR, 2014). The rates of poultry litter application are listed in *Table 3.1*. There were 4 replicates plots/treatment. There was one reference plot where no litter was applied for both orchardgrass and corn which had not been applied with manure for at least approximately 2 years prior to the beginning of the study.

The poultry litter for all application treatments was from a broiler production facility in the Shenandoah Valley region of Virginia. Application of poultry litter to all treatments was completed on the same day. The same equipment (Subsurfer poultry litter injector) was used for both surface application and subsurface injection. For the subsurface injection, slits (5cm in depth, 5 cm in width) were cut with the soil cutting discs attached to the equipment followed by injection of ground poultry litter (Pote et al., 2009). For the surface application, the soil cutting discs were lifted off the soil surface and the ground poultry litter was surface spread in bands with the injector. The space between the surface-spread manure bands was therefore the same as that between the injection slits.

Table 3.1 Manure application rate and plot area for each treatment

Plot Type	Manure Application Rate (Kg/M^2)	Plot Area (M^2)
High Rate Corn	0.59	150.5
Low Rate Corn	0.29	150.5
High Rate Orchardgrass	1.08	15.25
Low Rate Orchardgrass	0.54	15.25

3.2.3 Sample Collection

The poultry litter samples were collected on the day of application. Composite soil samples from each plot were collected at 0-5cm and 5-20cm at 32 days prior to the day of poultry litter application, on the day of poultry litter application, and at 8 days, 21 days, and 64 days following the poultry litter application. Five 0-5cm soil samples and three 5-20cm soil samples were taken throughout the collection area and mixed thoroughly prior to subsampling. All poultry litter and soil samples were packed on ice until the end of the field day (maximum 8 hours) and then stored at -10ºC until analysis.

3.2.4 Sample Preparation and Accelerated Solvent Extraction.

Soil and manure samples were freeze dried prior to analysis. A subsample of 5g of freeze dried soil or 0.5g of freeze dried poultry litter were mixed with 1g of hydromatrix and placed in an 11ml stainless steel extraction cell (Buchi, New Castle, DE). The remaining space except for 1cm headspace in each cell was filled with Ottawa Sand standard 20-30mesh (Agilent Technologies, Santa Clara, CA). Cells were placed in a Buchi E916 speed extractor (Buchi, New Castle, DE) and soils were extracted using a method of two 3-minute cycles of methanol/acetone (50:50 v/v) followed by two 3-minute cycles with water/ methanol (50:50 v/v), which resulted in approximately 40ml of extract per sample. (Nieto et al., 2008). Soil and poultry litter extracts were diluted to 50mL and centrifuged at 2054xg for 40 minutes.

3.2.5 Solid Phase Extraction for soil and manure

An aliquot of 25 mL of the soil or poultry litter extract was diluted to 500mL with DI water then acidified with 5 M HCl to ~pH 2. The Oasis HLB cartridges (60mg/3cc, Waters) arranged in a an 20-position Sampli-Q SPE vacuum manifold (Agilent Technologies, Santa Clara, CA) then conditioned with 3 mL 9:1 ethyl acetate/methanol, 3mL methanol, then 6 mL water . The prepared extracts were loaded onto the SPE cartridges at a flow rate of 4-5 mL/min. After loading samples, SPE cartridges were washed with 5 mL of 5% methanol in water and dried under gentle vacuum for 20 min. The analytes were eluted from the SPE cartridges with 3mL 9:1 ethyl acetate/methanol followed by 3mL 2% NH_4OH in methanol and collected into glass test tubes. At this point manure extracts had an additional clean-up step by adding 50mg of primary secondary amine (Agilent Technologies, Santa Clara, CA) to each sample, then the eluents were evaporated to dryness under a gentle stream of nitrogen at 40ºC. (TurboVap, Labconco). The sample residue was then reconstituted in 0.5 ml of acetonitrile followed by 0.5 mL water and filtered through a 0.2 μm PTFE filter (Thermo Scientific, Rockwood, TN). Final sample extracts were then stored at -80°C until LC/QQQ analysis. Matrix matched standards from control soils were made for soil samples to overcome their complex matrix in the LC/QQQ analysis (Pihlström, 2015). Manure was quantified using standard addition to account for complex matrix (Pihlström, 2015).

3.2.6 UPLC-MS/MS Analysis

Separation and quantification of 11 hormone compounds was achieved using an Agilent 1290 series UPLC coupled to an Agilent 6490 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). The analytical columns used were Zorbax Eclipse Plus C18

rapid resolution HT 2.1x100mm, 1.8µm, 600 bar preceded by a Zorbax Eclipse Plus C18 2.1x5mm 1.8µm UHPLC Guard Column (Agilent Technologies, Santa Clara, CA) with a sample injection volume of 10µL, a column temperature of 40°C and a mobile phase flow rate of 0.300ml/min. For the analysis of E3, αE2, βE2, and E3, the mobile phases were A: 0.2mM ammonium fluoride in water, and B: acetonitrile/water (95:5, v/v), and the mass spectrometer was run in negative ion mode electrospray ionization. The mobile phase gradient was 0-1 minutes, 10% B; 9 min, 85% B; 9.5 min, 98% B; 10.5 min, 98% B; 11 min, 10% B; 14 min, 10% B. Retention times of each compound are as follows: E3, 5.70min; βE2, 7.61min; αE2, 7.86min; E1, 8.14min. For the analysis of E2-3G, E1,3G, E2-3S, E1-3S, 1,4-ADD, 4-AD, and P, the mobile phases were A: 5mM ammonium acetate in water, and B: acetonitrile/water (95:5, v/v). The mobile phase gradient was 0-1 minutes, 10% B; 9 min, 85% B; 9.5 min, 98% B; 10.5 min, 98% B; 11 min, 10% B; 14 min, 10% B. The electrospray ionization was run with negative ion mode for E2-3G, E1-3G, E2-3S and E1-3S then with positive ion mode for 1,4-ADD, 4-AD, and P. % after this week's analysis. Retention times of each compound are as follows: E2-3G, 4.82min; E1-3G, 5.06; E2-3S, 5.70min; E1-3S, 6.04 min; 1,4-ADD, 7.72 min; 4-AD, 8.27 min; P, 9.53 min.

Mass spectrometer parameters were sheath gas temperature at 200 ^{o}C with a flow rate of 8L/min, drying gas temperature of 250 ^{o}C with a flow rate of 14L/min, nebulizer pressure 45 psi, capillary voltage 3500(+)V 3000(-)V for both methods. Method detection limits and recovery are listed in *Table 3.2.*

Table 3.2: Method detection limit (MDL) and recovery% for individual compounds in soil for this study.

Compound	MDL (µg/kg)	Recovery (%)
1,4-ADD	0.40	129
4-AD	0.51	126
P	0.61	148
E2-3G	0.88	71.7
E1-3G	0.47	89.2
E2-3S	0.33	100
E1-3S	1.22	95.9
E3	0.42	72.2
β-E2	0.33	65.9
α-E2	0.32	63.9
E1	0.34	73.8

3.2.7 Statistical Analysis

Statistical analysis was completed in JMP Pro 12. Analysis of Variance and a Post-Hoc Tukey's HSD test were used to compare treatments within cropping system. Mass balances were calculated using the manure application rates, soil bulk density (1.33 g/cm^3), detected amount of hormones in each applied manure type, depth/volume of sampling area, and assumed uniformity of composite soil samples.

3.3 Results and Discussion

3.3.1 Hormones Detected in the Poultry Litter

Of the 11 hormones tested, only 1,4-ADD, 4-AD, P, E1-3G, and E1 were detectable in the poultry litter used for this study (*Fig. 3.1*). The level of 1,4-ADD was the highest at 162.33 µg/kg (dry weight basis). The levels of P, 4-AD, E1-3G, and E1 followed the decreasing order at 96.23, 40.3, 29.91, and 7.59 µg/kg, respectively. Bevacqua et al. detected similar levels of P and E1 in a poultry litter at 63.4 and 44.1 µg/kg dry weight respectively, while 4-AD, 1,4-ADD, and E1-3G were not detectable in the study (Bevacqua et al., 2011). Poultry litter hormone content can vary greatly between litters because different litters from different flocks can have varying amounts of manure:bedding content. The age, sex ratios, and storage technique of the manure can also alter the hormone content between manures (Bartelt-Hunt et al., 2013; Lange et al., 2002; Shore and Shemesh, 2003).

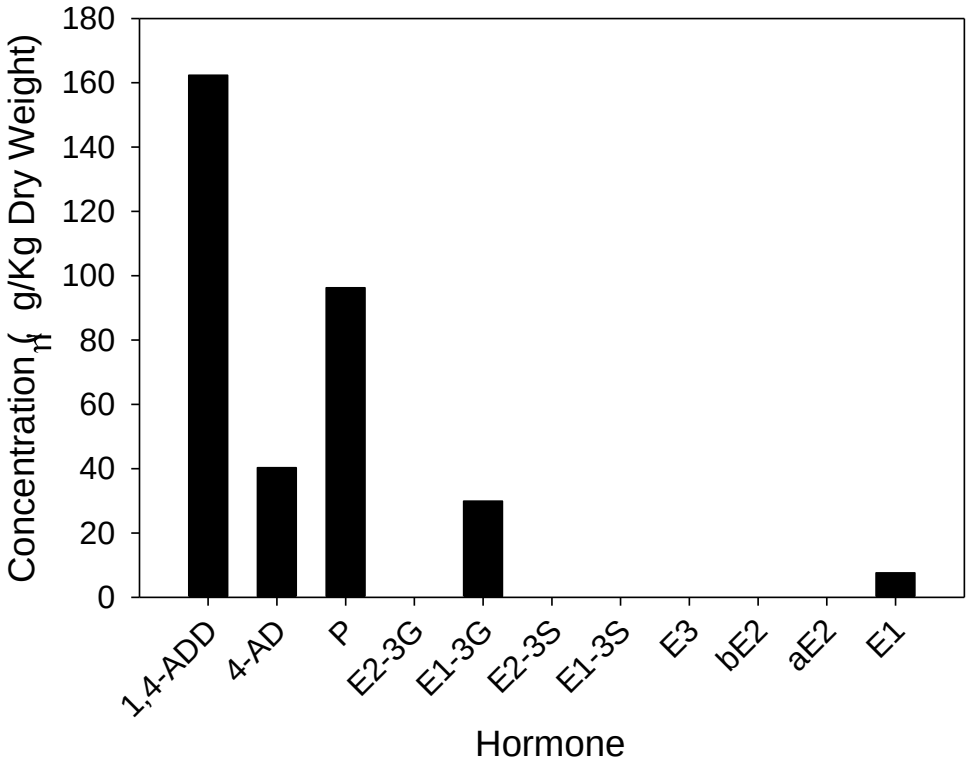
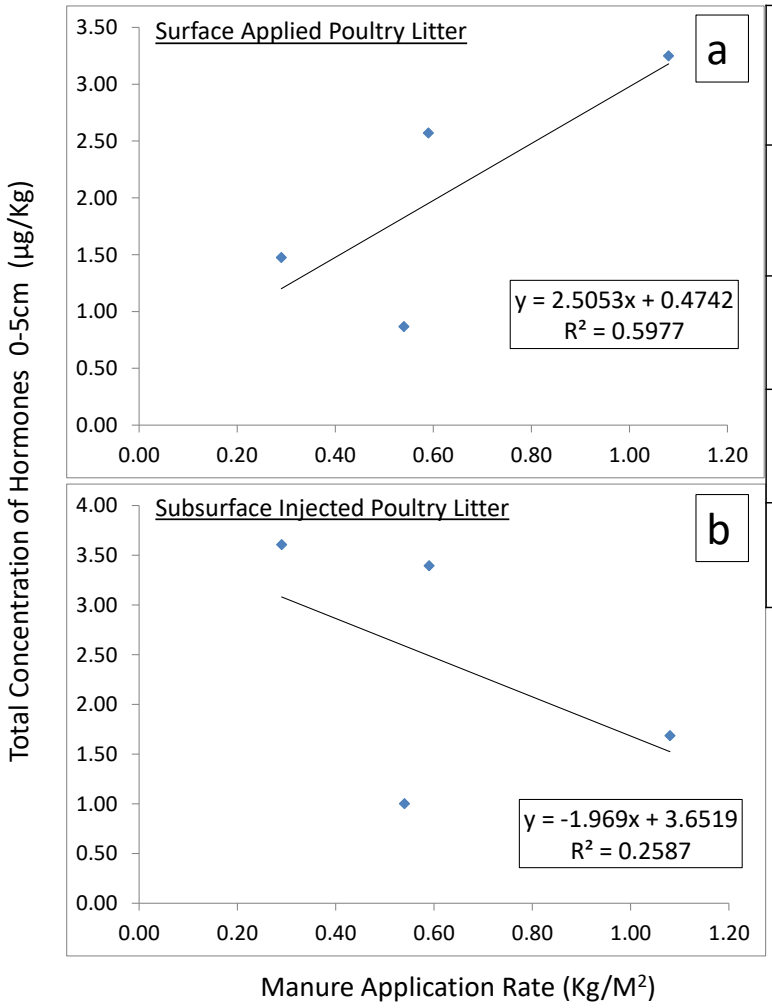


Fig. 3.1: The concentration of hormones in the poultry litter used for this study.

3.3.2 Detectable hormones in poultry litter applied soil

The poultry litter was applied at 4 different rates depending on the application rate (high, low) and crop type (corn, orchardgrass). As the application rate increased, a greater concentration of hormones in the 0-5cm soil would be expected. For the surface application, the general trend shown in *Fig 3.2a* suggests higher application poultry litter rate resulted in higher concentration of hormones in the 0-5 cm soil ($R^2 = 0.6$). However, for the subsurface injection plots, there seems to be a slight negative correlation between the two (*Fig 3.2b*). The differences in hormone response to application rate could be caused by inadequate sampling. The poultry litter was applied in bands for both the surface applied plots and for the subsurface injected plots, meaning there was stratification of the poultry litter in the plots. Perhaps the poorer correlation in the injected plots was caused by even tighter bands of poultry litter, as not only was the poultry litter applied in bands, the slits where the poultry litter was applied were then sealed. The surface applied poultry litter had the option to spread out a little more in the plot.



Plot Type	***Manure Application Rate (Kg/M^2)***
High Rate Orchardgrass	1.08
High Rate Corn	0.59
Low Rate Orchardgrass	0.54
Low Rate Corn	0.27

Fig. 3.2: Relationship between application rate of poultry litter and total concentration of soil hormones in the 0-5cm soil on the day of manure application. Table on the right includes 4 rates of poultry litter application rates used in this study.

Of the 11 hormones tested, only 1,4-ADD, 4-AD, E1, P, all of which were detected at high levels in the poultry litter, were detectable in the poultry litter-applied soils over the course of the experiment. The E1-3G which had been detected in the poultry litter was not detected in any soil samples. E1-3G is known to deconjugate to E1 in soils, though it is not likely that it could have been deconjugated quickly in the soil on the day of application (Shrestha et al., 2012). The E1-3G could have been deconjugated in the soil during storage. The hormones that

were detected in the soil were likely detected because they are generally transformation products of active biological hormones. 1,4-ADD and 4-AD are biological precursors of testosterone, and testosterone is known to transform into 1,4-ADD and 4-AD within hours of exposure to soil (Lorenzen et al., 2005). Estrone is an environmental transformation product of α-E2 and β-E2, a transformation that is both biologically and abiologically driven. (Colucci et al., 2001; Goeppert et al., 2014). P has been previously detected both poultry litter and in soil amended with poultry litter (Ho et al., 2014). All tested hormones were below their detection limits in the soils from the control plots.

The individual hormones were detected in similar proportions for all treatments in the 0-5cm soil, with 1,4-ADD detected in the highest concentrations for all manure application rates, which is related to 1,4-ADD being detected at the highest concentration in the poultry litter (*Fig. 3.3*). E1 was detected at the lowest concentration in the 0-5cm soil in day 0 for all but one manure application rate for the poultry litter applied soil, which also is related to E1 being detected in the lowest concentration in the poultry litter. In the poultry litter, 4-AD was present in the poultry litter at less than half the concentration of P, however 4-AD was detected near or above the concentrations of P in the 0-5cm soil on the day of poultry litter application.

There were unusually high concentrations of hormones in the 5-20cm soil. Hormones in the 5-20cm soil were not expected on the day of poultry litter application, as no leaching should have occurred yet because there had been no rainfall events. These unusual concentrations of hormones could be caused by carryover in sampling. The soil sampled was often hard and filled with coarse gravel, and a hammer was required to drive the sampler. Poultry litter and surface soil could have been disturbed and mixed into 5-20cm soil samples with the vibrations and movement of the hammering. A soil probe cleaning could be needed between sampling depths,

additionally between treatments, to avoid possible contamination. 1,4-ADD and P were mainly detected in the 5-20cm soil samples, and those were the hormones detected in the greatest concentrations in the poultry litter. 4-AD was only detected in the 5-20cm soil with the greatest manure application rate, and no E1 was detected in the 5-20cm soil.

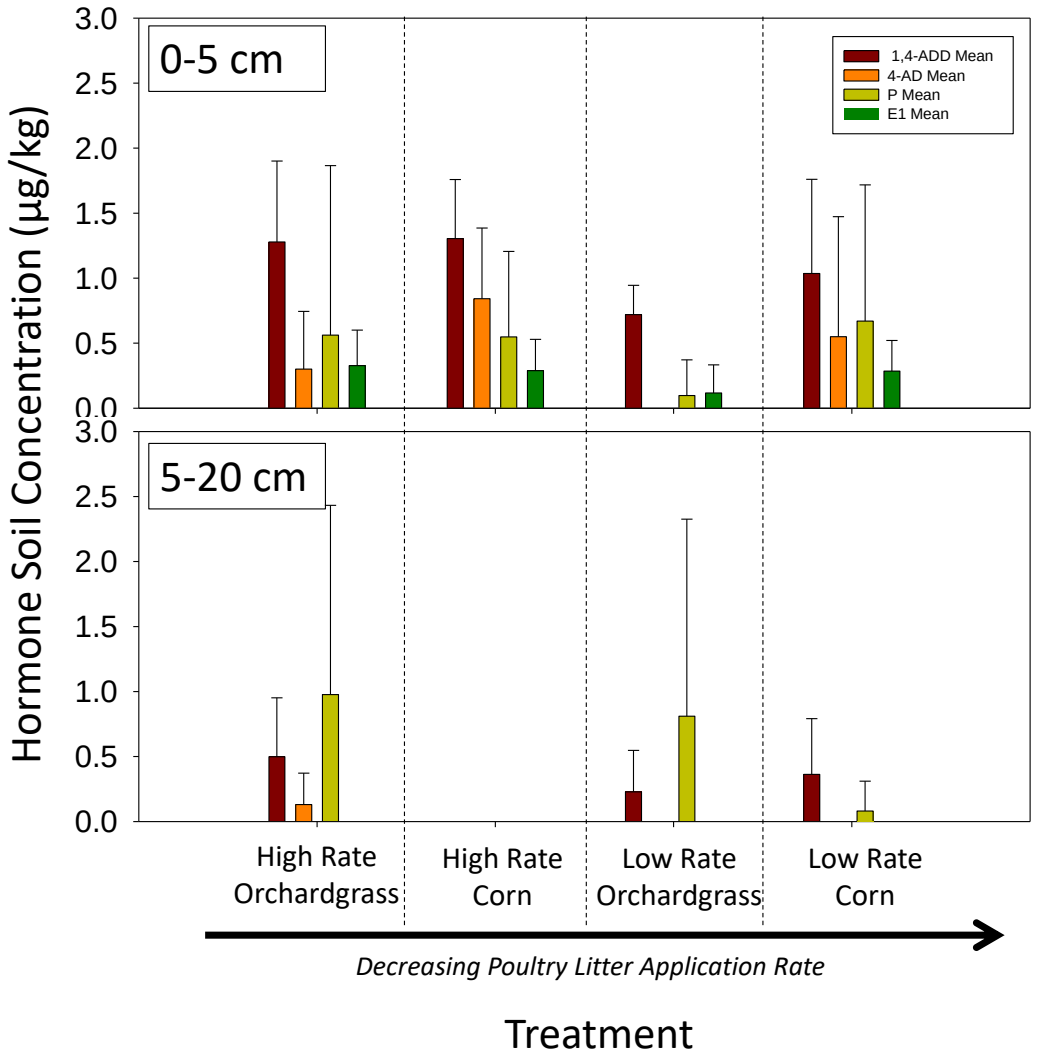


Fig. 3.3: Individual soil hormone concentrations in the 0-5cm and 5-20 cm soil on the day of poultry litter application for surface application and subsurface injection combined. Error bars indicate 1 standard deviation from the mean.

3.3.3 Temporal change of hormones in poultry litter applied soil

Individual hormones exhibited different behavior in their dissipation over time in the corn and orchardgrass fields. The soil concentrations of 1,4-ADD over time are depicted in *Fig. 3.4*. 1,4-ADD was not at detectable levels in the 0-5cm soil and only detected slightly in the 5-20 cm

soil 32 days prior to the application of poultry litter. Following the application of poultry litter 1,4-ADD was detected in the greatest concentrations among tested hormones and across all treatments, decreased to 70% its detectable day 0 levels on day 8. By day 21, total 1,4-ADD across all treatments decreased to 6% its detectable day 0 levels in the 0-5cm soil. 1,4-ADD was not at detectable levels 64 days after the application of poultry litter.

4-AD was not detectable on the site 32 days prior to the application of poultry litter in both the 0-5 and the 5-20cm horizons, but was detected on the day of poultry litter application across all treatments (*Fig. 3.5*). By the eighth day following the application of poultry litter, the initial detectable levels of 4-AD across all treatments had decreased to 22% of its initial hormone levels. Detectable levels of 4-AD could not be found in the 0-5cm soil at 21 and 64 days following the application of poultry litter. Over all treatments 4-AD was only detected in the 5-20cm soil on day 0 and day 64.

Like 4-AD, P was only detected in 0-5cm soil on day 0 and day 8 in the 0-5cm soil (*Fig. 3.6*). The 0-5cm soil levels of P across all treatments decreased by 53% between day 0 and day 8. P was detected at high concentrations in the 5-20cm 32 days prior to the application of poultry litter and on the day of manure application when compared to other hormones detected in this study. In fact, 95% of the total hormones detected before the application was P, with the remaining being 1,4-ADD. A possible reason for the detection of P in the soil before any manure was applied this season could be carryover of instances of manure applications from previous growing seasons. Another possible reason for detected P in the soil could be interference of wildlife. P has been detected in the feces of both geese and white tailed deer in varying amounts, generally depending on the reproductive state of the animal (Hirschenhauser et al., 1999; Kapke et al., 1999). White tailed deer and geese are both present in the Shenandoah

Valley, and tend to frequent agricultural fields, especially during winter and early spring, where food sources are limited. Depositions of wildlife feces could have contributed to extra P in the soil that was not applied with the poultry litter. P was not detected in the 5-20 cm soil 8, 21 and 64 days following the poultry litter application.

E1 was detected in the lowest total concentrations of any hormone over the course of the study (*Fig 3.7*). No detectable levels of E1 were found in the 0-5cm soil 32 days before the application of poultry litter. On day 8 the detectable levels of E1 were only 10% of the initial detectable levels of E1 on day 0 in the 0-5 cm soil. E1 was not at detectable levels on day 21 or 64 in the 0-5cm soil. E1 was not detected in the 5-20cm soil 32 days before poultry litter application and on day 0 and day 8, but was detected on day 21 and day 64 in the corn plots.

Few studies exist on the degradation rate of 1,4-ADD, 4-AD, E1, and P in soil and poultry litter systems, as research tends to focus on testosterone and estradiol. However some studies focusing on other types of animal manure have studied these compounds. A soil application of swine manure, was proven to produce 4-AD as well as E1, and both compounds were only determined to only persist in the soil for a few days. (Jacobsen et al., 2005). In a study detecting hormones in the soil beneath beef feedlots, both E1, 4-AD, and P had been detected in the soil for at least 14 days, when the study concluded (Mansell et al., 2011). In both studies, 1,4-ADD was not included.

Individual hormones were detected 2.8 to 7 times more frequently in the 0-5cm soil samples than in the 5-20cm soil samples for 1,4-ADD, 4-AD, E1. P was detected at relatively the same frequency in the 5-20cm soil and 0-5cm soil, however all of the soil samples which detected P were either detected 32 days prior to manure application, or on the day of manure application, when it would not be expected for leaching to have occurred (*Fig. 3.6*).

The hormone concentrations of 0-5cm soil samples were significantly greater ($p<0.1$) in the injection plots on day 21 for 1,4-ADD than in the surface applied plots, but did not show any significant differences for any other day. 1,4-ADD was the only hormone detected in the 0-5cm soil on day 21, and was only detected in the injected plots, suggesting that soil subsurface injection can cause 1,4-ADD to persist longer in the soil. No significant differences ($p<0.1$) were seen for 0-5cm soil between subsurface injection and surface application for 4-AD, E1, and P for any sampling time-points. No significant differences were found between the injection plots and the surface applied plots for any individual hormone on any day for the 5-20cm soil.

Crop type only had a significant effect on 4-AD on day 0 for the 0-5cm soil where the plots planted with corn had a significantly greater ($p<0.1$) concentration of 4-AD in the 0-5cm soil than the plots which were planted with orchardgrass. It was not expected for crop type to have any significant effect on the soil hormones on day 0, especially when the average poultry litter application rate was lower than the orchardgrass application rates, subsequently applying fewer hormones in the corn plots. Crop type had more instances of significant effect for the 5-20cm soil. On day -32 and on day 0, orchardgrass was found to have a significantly greater ($p<0.1$) concentration of P than corn in the 5-20cm soil. On day 8, orchardgrass was found to have a significantly greater ($p<0.1$) concentration of 1,4-ADD than corn. For the 5-20cm soil, all instances of significant difference had orchardgrass at higher concentration than corn, which likely relates to a greater application rate of poultry litter for the orchardgrass plots than for the corn plots.

When comparing the poultry litter application rate soil hormone response by each day, the concentration of 4-AD in 0-5cm soil on day 0 is also the only instance of significance ($p<0.1$) between treatments in the 0-5cm soil, where the high rate corn poultry litter application had the

highest concentration of 4-AD, and was significantly different from the low rate orchardgrass plot, which was the plot with the lowest concentration of 4-AD. For the 5-20 cm soil there were significant differences between poultry litter application rate soil hormones on day 0, for 1,4-ADD. The high rate orchardgrass was significantly greater than the high rate corn, which had the lowest 1,4-ADD concentration. In both instances of significant differences, the plot with the significantly greater concentration of individual hormone was applied with a greater amount of poultry litter than the plot with the significantly lower concentration of individual hormone.

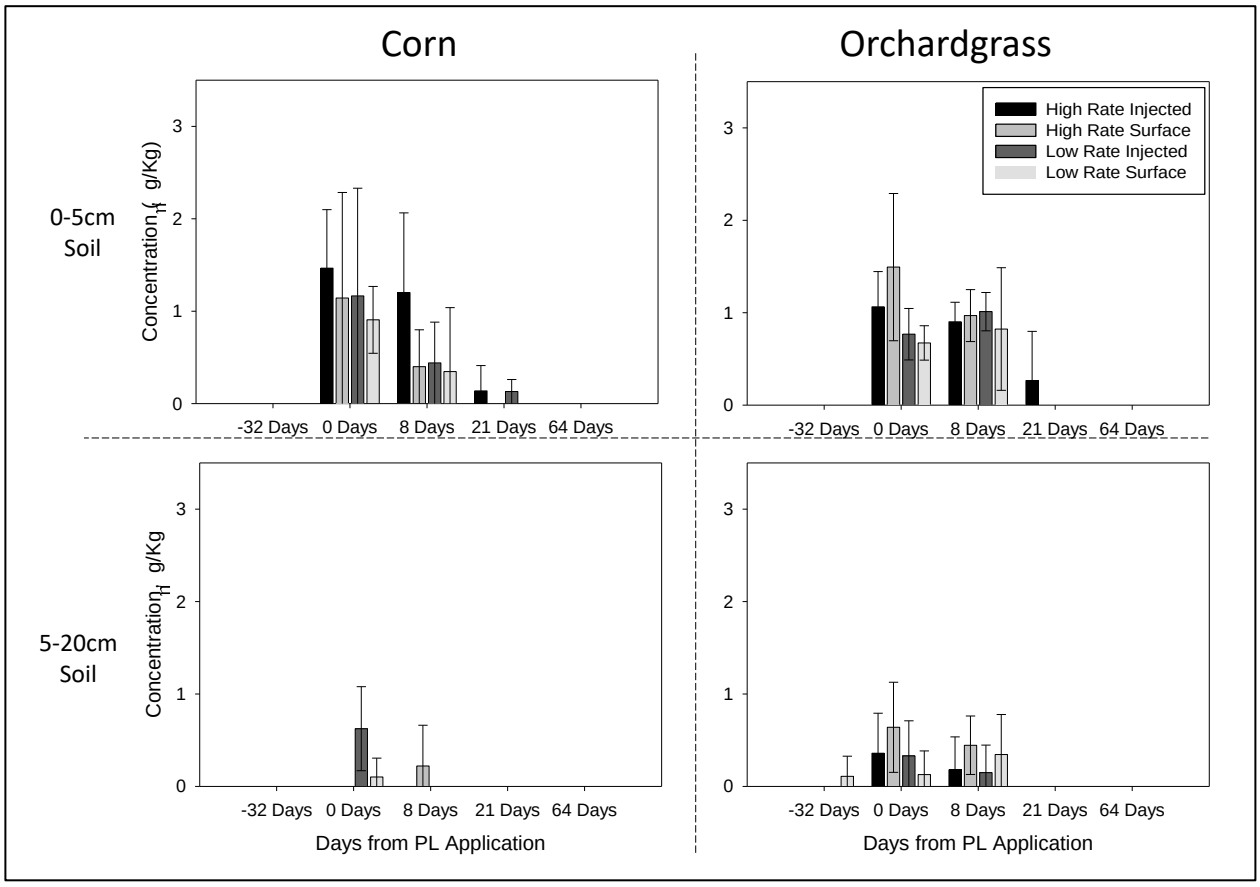


Fig. 3.4: Soil Concentration of 1,4-ADD in soil from 32 days prior to manure application, to 64 days post-application.

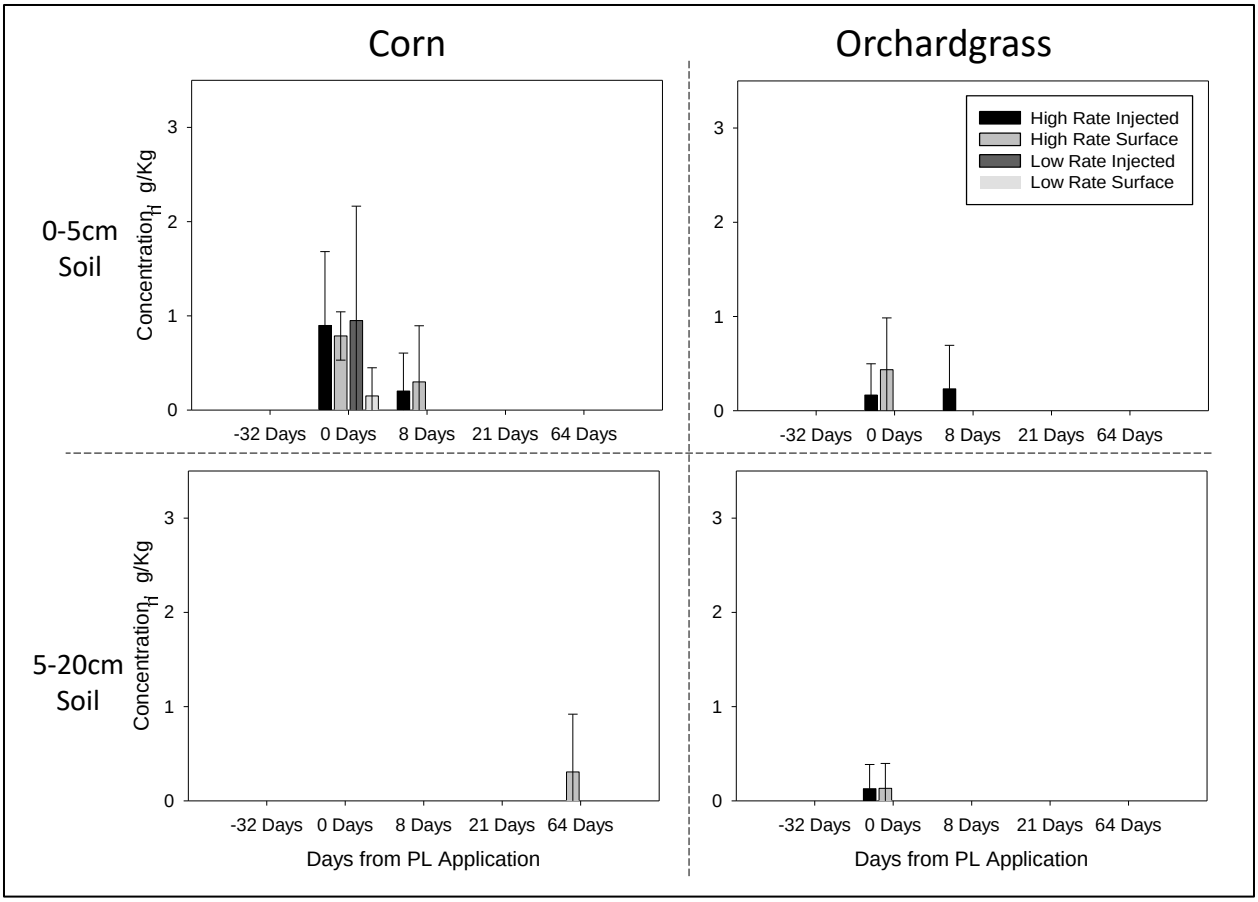


Fig. 3.5: Soil Concentration 4-AD in soil from 32 days prior to manure application, to 64 days post-application.

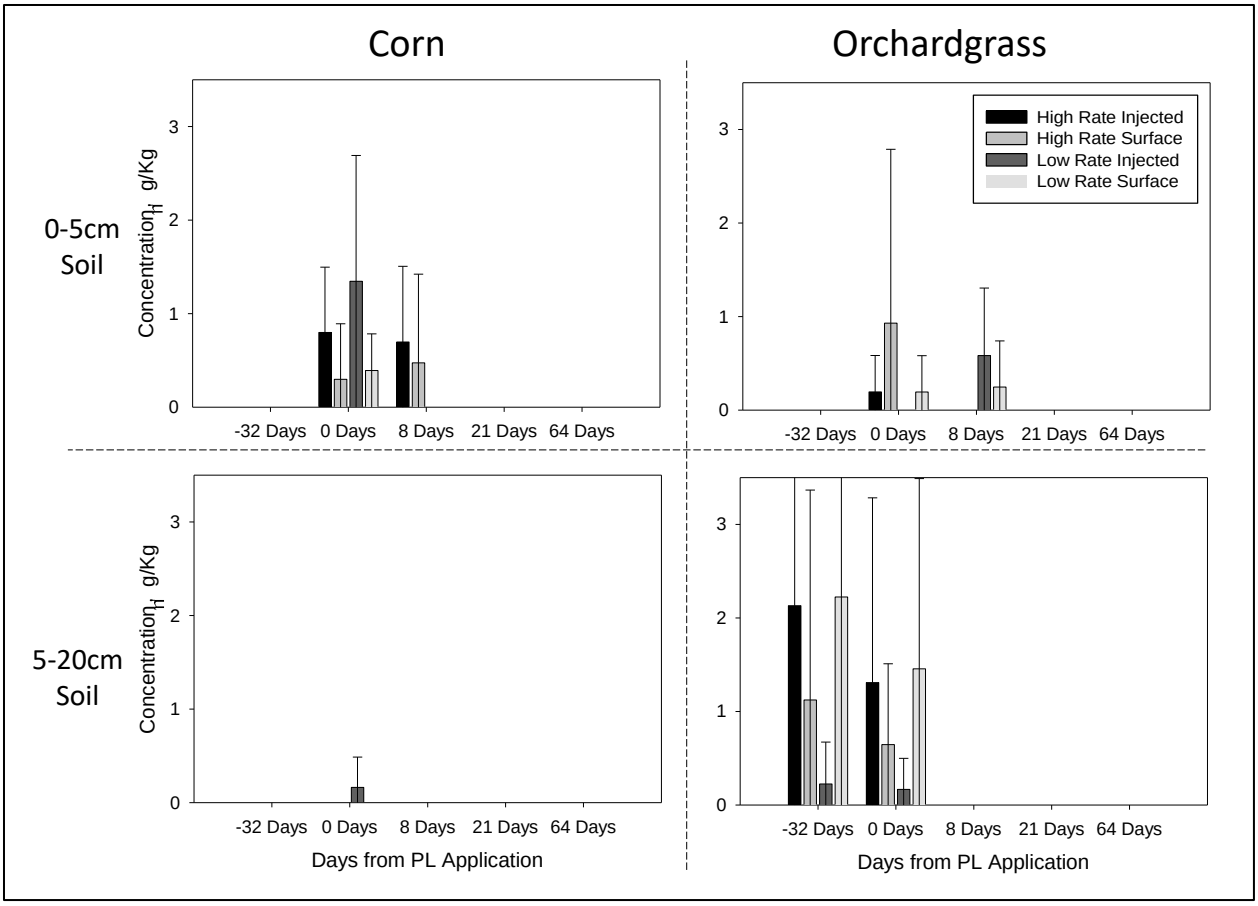


Fig. 3.6: Soil Concentration of Progesterone (P) in soil from 32 days prior to manure application, to 64 days post-application.

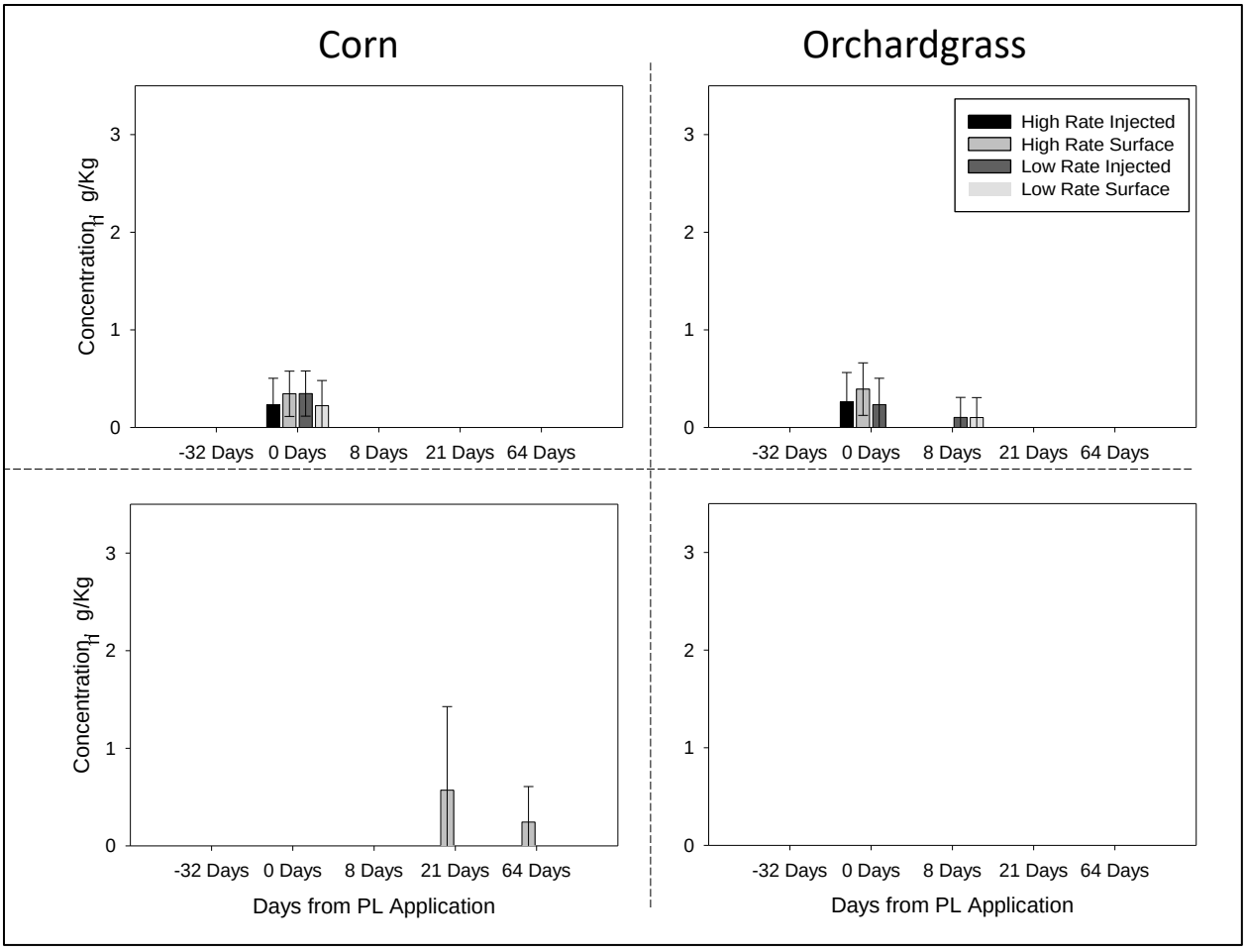


Fig. 3.7: Soil Concentration of E1 in soil from 32 days prior to manure application, to 64 days post-application.

Combining the individual hormones, similar trends occur in the total hormone results from the study (*Fig. 3.8).* Hormones were only detected in the 5-20cm soils of the orchardgrass plots at an average soil concentration range of 0.45 µg/kg, and detected in the low rate injection plots to 3.68 µg/kg detected in the low rate surface plots 32 days prior to the application of poultry litter. On day 0, the day of poultry litter application, the greatest concentrations of total hormones were observed in the low rate injected corn plots, which was surprising because the low rate corn plots actually had the lowest rate of applied manure overall between corn and orchardgrass plots.

Between day 0 and day 8, total soil hormones decreased to 50% of the initial concentration in the 0-5cm soil and to 22% initial concentration in the 5-20cm soil, indicating rapid degradation of hormones in the soil. At 21 days following the application of poultry litter, the concentration of total hormones in the soil had decreased to 3% of the initial concentration in the 0-5cm soil and to 6.9% initial concentration in the 5-20cm soil and to indicating that the majority of the hormones detected in this study had transformed At the final sampling date, 64 days following the application of poultry litter, no hormones were detected in the 0-5cm soil. The hormones which were detected in the 5-20cm soil had actually increased slightly from day 21 to 7.9% initial soil hormone concentrations on day 64. Overall total hormones were detected more frequently in the 0-5cm soil samples than in the 5-20cm soil, with 1.8x more 0-5cm soil samples containing hormones.

No significant differences were seen between injected and surfaced applied plots for days -32, 0, 8, and 64 in the 0-5cm soil. Because 1,4-ADD was the only hormone to be detected on day 21, the total hormone concentration is also significantly higher for the injected plots when compared to the surface applied plots for the 0-5cm soil. There were only significant differences for crop type on day 0 for the 0-5cm soil, with corn having a higher concentration of hormones than orchardgrass. The same trend as 4-AD on day 0 for poultry litter application rate soil hormone response by each day, where the total concentration of hormones in 0-5cm soil for the high rate corn poultry litter application rate on day 0 is significantly higher ($p<0.1$) than the low rate orchardgrass.

For the 5-20cm soil, significant differences between treatments were driven by previously mentioned significant differences in individual hormones. For crop type on day -32, day 0, and day 8, orchardgrass had a significantly greater concentration of hormones in the 5-20cm soil

when compared to corn. Also similar to the individual hormones, no significant differences were found between the injection plots and the surface applied plots on any day for the 5-20cm soil. Again, significant differences between poultry litter application rate soil hormones for the 5-20cm soil were driven by significant differences in individual hormones. Like the 1,4-ADD on day 0, the high rate orchardgrass was significantly greater than the high rate corn.

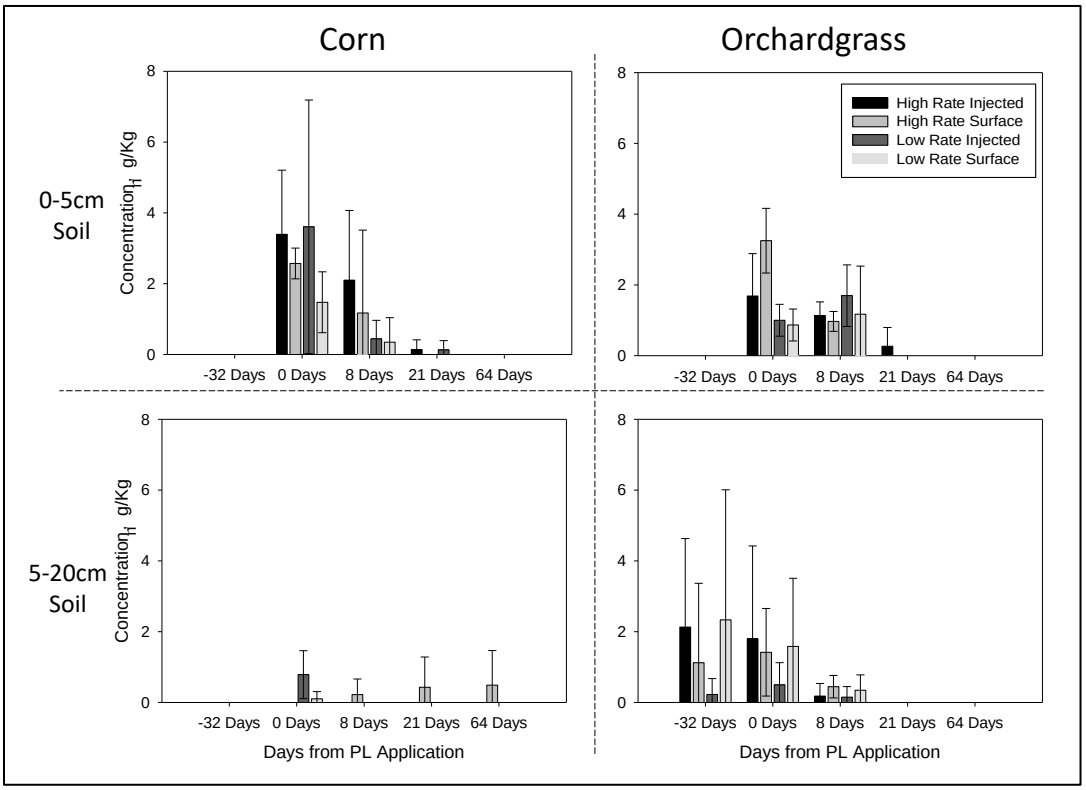


Fig. 3.8: Soil Concentration of total hormones in soil from 32 days prior to manure application, to 64 days post-application.

3.3.4 Calculated amount of hormones applied comparing to the detected hormones in soil.

When comparing the theoretical amount of hormones applied to manure as outlined in *Table 3.3*, it is apparent that fewer hormones were detected in the soil than were theoretically applied as an extrapolation of the concentrations of hormones found in the manure. On the initial sampling day, only between 10.59% and 49.33% of the predicted hormone concentrations were

detected in the total soil (both 0-5cm and 5-20 cm samples) for each treatment. These low mass balance figures for this study could have been caused by hormone transformation. Lower than expected soil hormone concentrations may have also been caused by inadequate sampling; either too few subsamples, or too large of a sampling area could have led to an inaccurate estimate of the soil hormone concentration. Also the poultry litter likely remained in the injections slits or in strips in the surface applied plots, contributing to high variation in sampling. The sampling technique also may have not accurately been able to sample the injection slit, as it is only 5cm wide and not as likely to be randomly sampled as the areas of soil between the injection lines. In future work, it is recommended to mark the location of the poultry litter injection slit and sample both within the slit and between the slit, to get a more accurate estimate of soil hormones sourced from manure.

Even though hormones had been detected more frequently in the 0-5cm soil samples, the hormone distribution changed slightly when calculating the mass balance. The 0-5cm soil contained a greater proportion of the hormones applied than the 5-20cm soil for corn treatments. For the orchardgrass treatments, however, a greater proportion of the hormones were contained in the 5-20cm soil. This is unexpected because on day 0, the poultry litter had just been applied so there was no chance for the hormones to leach out of the litter and into the lower soil profile, and hormones tend to not exhibit much leaching behavior through the soil profile, as noted in previous studies. Steroid hormones tend to have high log K_{oc} values, experimentally determined to be between 2 and 4, which defines them as molecules which would not readily leach through a soil profile. (Casey et al., 2005; Lee et al., 2003; Ying and Kookana, 2005; Yu et al., 2004). The additional hormones on day zero are mainly P, and as discussed earlier, there were multiple reasons for the phenomena of increased P concentration in the soil both on the day of the poultry

litter application, as the P was present in the soil 32 days prior to the application. There is also more extrapolation involved in converting soil hormone concentrations to soil hormone amounts because of the greater volume of soil contained in the 5-20cm soil sampling area.

Table 3.3 Mass balance of total amount of hormones detected at initial sampling on the day of manure application.

Table 3: Mass balance of the total amount of hormones detected at initial sampling on the day of manure application.					
			Total detected Hormones/Total Hormones Applied in Poultry Litter (%)		
Treatment	Crop	App. Rate	0-5cm Soil	5-20cm Soil	Total detected
Surface	Corn	High	10.59	0.00	10.59
		Low	12.20	2.53	14.72
	OG	High	7.25	9.48	16.73
		Low	3.87	21.19	25.06
Inject	Corn	High	13.99	0.00	13.99
		Low	29.83	19.50	49.33
	OG	High	3.76	12.02	15.78
		Low	4.47	6.63	11.10

3.4 Conclusions

Of the 11 analytes studied, only 1,4-ADD, 4-AD, E1 and P were found at detectible levels in the soil. No correlation was found between manure application rate and hormones detected in the 0-5cm soil on the day of manure application, and unusually high concentrations of hormones were found in the 5-20 cm soil on day 0, before leaching could have occurred which could be attributed to the sampling protocol for this study. 1,4-ADD was detected in the 0-5cm soil up to 21 days following the application of poultry litter, while 4-AD, P, and E1 was detected

for only 8 days in the 0-5cm soil following the application of poultry litter, indicating that the most important time period for managing soil hormones is within weeks following the application of manure. For the majority of the days, no significant differences were found between soil hormones concentrations for crop type, manure injection or surface application protocol, and poultry litter application rate, with no distinct trends in soil hormone concentrations. Issue may have arisen from incomplete representative sampling, and it is advised in future poultry litter injection studies to sample within the injection slit and between the injection slit separately, as well as taking more subsamples.

3.5 References

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Chapter 4: Fate of Hormones during Simulated Rainfall in a Field Receiving Dairy Manure and Poultry Litter: Effect of Surface-Application and Subsurface Injection

Abstract

Steroid hormones are naturally found in manure and little is known of their environmental persistence and transformation following the application of manure to agricultural fields. Therefore, a field-scale rainfall simulation study was conducted to investigate the impact of manure surface application and subsurface injection on the occurrence and fate of 11 hormones, hormone precursors, and conjugated hormones in soil and surface runoff. Field plot treatments included poultry litter subsurface injection and surface application, dairy manure slurry subsurface injection and surface application, and control without manure application. Poultry litter was applied at 6.72 Mg/Ha and dairy manure slurry was applied at 56.12 Mg/Ha, typical application rates in Virginia. Thirty-minute runoff samples were collected from each plot during the simulated rainfall (70 mm/h) conducted on the day of manure application and 7 days thereafter. Immediately after each surface runoff collection, soil samples from 0-5cm and 5-20cm depths were collected from each plot. The target compounds were extracted from the soil using high pressure solvent extraction followed by cleaning up with solid phase extraction. Runoff samples were extracted and cleaned up with solid phase extraction. The soil and water extracts were analyzed for the target compounds on an ultra-performance liquid chromatography/tandem mass spectrometry. Of the 11 hormone compounds tested, androgen compounds had the highest concentrations in both poultry litter and dairy manure. For the manure subsurface injected plots, hormones were mostly concentrated in the injection slits, and showed little lateral and vertical movement within 7 days. Similarly for the manure surface applied plots, the hormones were concentrated in the 0-5cm soil and also showed little vertical

movement within 7 days. On the 7th day after manure application, 1,4-androstadiene-3,17-dione, 4-androstene-3,17-dione, progesterone, estriol, α-estradiol, β-estradiol and estrone were detectable at average 0-5cm soil concentrations ranging from 4.2 to 0.13 µg/kg. Overall total average hormones were reduced by 17.6 to 47.9% on the 7th day after manure application comparing to that on the day of application. Of the 11 tested hormones, only 1,4-androstadiene-3,17-dione was detected in the surface runoff of surface-applied plots, while none were above detection limits in that of the subsurface injected plots. Comparing to the mass of 1,4-androstadiene-3,17-dione removed from the plots via surface runoff, significantly higher amounts were detected in the soil of both surface applied and subsurface injected plots during the 7-day investigation. This result suggests that for the soil type of this study, manure-borne hormones are not susceptible to loss in surface runoff. Manure subsurface injection further decreased their likelihood of exporting off the field via surface runoff.

4.1 Introduction

Manure is a valuable renewable resource which is used to add nutrients to croplands and to help enhance soil organic matter content (Khaleel et al., 1981). In order to reduce costs, agriculture in the United States is becoming increasingly industrialized and concentrated, with larger farms contributing to the instance of concentrated animal feeding operations, also known as CAFO's (MacDonald and McBride, 2009; USDA, 2009; USEPA, 2012). While beneficial nutrients are applied to fields with manure applications, excess nutrients and other compounds such as hormones concurrently applied with manure can be detrimental to water quality when lost via surface runoff and/or leaching.

Hormones, naturally occurring in animal manure, are endocrine disrupting compounds. They interfere with an organism's endocrine system, which is responsible for many sexual and

developmental functions. Hormones at sub part-per-billion concentrations have been known to cause adverse effects in aquatic organisms, such as changing the physical reproductive structure of fish, and sometimes causing intersex characteristics or impotence (Kidd et al., 2007, Jukosky et al., 2008). Exposure to hormones can also cause behavioral changes in the social reproductive structure of aquatic organisms (Coe et al., 2008; Coe et al., 2009).

Runoff from agricultural fields amended with animal manures have been shown to contain significant levels of hormones up to 4 $\mu g\ L^{-1}$ for some individual hormone compounds (Dutta et al., 2010; Mina et al., 2016). Additionally, aquatic organisms which have been exposed to agricultural runoff known to contain hormones have been shown to have skewed sex-ratios of their offspring, decreased reproductive fervor, and intersex reproductive configurations (Leet et al., 2012). Hormones are a difficult class of compounds to generalize in manure application studies because the amounts and types of hormones in manure varies with the type, age, and reproductive state of the herd or flock, as well as the storage technique of the manure. (Bartelt-Hunt et al., 2013; Lange et al., 2002; Shore and Shemesh, 2003).

New developments in manure land application techniques have shown promise in pollution reduction, especially for nitrogen and phosphorus, from manure-applied fields. No-till and reduced tillage cropping systems restrict the movement of sediments and their related pollutants from agricultural fields, however, they require surface application of manure, as to not disturb the soil (Maguire et al., 2011). Subsurface application of animal manure is becoming an increasingly popular technique to avoid the pollution associated with surface application while still maintaining no-till character. Due to the relatively low water solubility and high octanol water partition coefficients of steroid hormones, these molecules are more likely to remain in the soil by sorption with soil components and less likely to leach with soil water through the soil

profile. (Casey et al., 2005; Lee et al., 2003; Ying and Kookana, 2005; Yu et al., 2004) By reducing the manure contact with sediment laden runoff, the inputs of hormones from manure-applied fields into the aquatic environment could also be further reduced.

Up to date, there have been limited studies systematically comparing the fate of a wide range of animal hormones in fields receiving surface applied and subsurface injected manure. This is especially true for poultry litter, as poultry litter subsurface injection is a relatively new technique. The objective of this study was to determine if subsurface shallow disc injection of poultry litter or dairy manure slurry reduces surface runoff and enhances soil dissipation of a wide range of hormones when compared to manure surface application. To achieve this objective, simulated rainfall plots were set up to quantitatively characterize 11 hormone compounds in two subsequent runoff events, 7-days apart, and in soils following the application of manure.

4.2 Materials and Methods

4.2.1 Chemicals and Materials

Hormone standards 1,4-Androstadiene-3,17-dione (1,4-ADD), 4-Androstene-3,17-dione (4-AD), Progesterone (P), Estrone (E1) (≥99%), 17α-Estradiol (αE2) (≥98%) , 17β- estradiol (βE2) (≥98%), and Estriol (E3) (≥97%) were purchased from Sigma-Aldrich. (Saint Louis, MO). Hormone standards 1,3,5(10)-Estratrien-3-ol-17-one glucosiduronate, Sodium Salt (≥98%) (E1-3G), 1,3,5(10)-Estratrien-3, 17β-diol 3-sulphate, Sodium Salt (≥98%) (E2-3S), and 1,3,5(10)-Estratrien-3, 17β-diol 3- glucosiduronate, Sodium Salt (≥98%) (E2-3G) were purchased from Steraloids (Newport, RI). Hormone standard 1,3,5(10)-Estratrien-3-ol-17-one Sulphate, Sodium Salt (>98%) (E1-3S) was purchased from Alfa Chemistry (Protheragen Inc., Stony Brook, NY). HPLC grade acetonitrile, ethyl acetate, methanol, and acetone, ammonium

hydroxide, hydrochloric acid, and ammonium fluoride were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium acetate was purchased from Amresco (Dallas, TX). de-ionized water (18.2 MΩ•cm at 25°C) was produced from a Millipore water purification system (Millipore, Billerica, MA).

4.2.2 Rainfall Simulation Plots Set-up

This investigation was conducted simultaneously with a previous study on the fate of an antibiotic compound (Kulesza et al., 2016). Detailed experimental set up was described in that study. The rainfall simulation plots (2m x 2m on a 9-11% slope) were setup using a standardized protocol (SERA-17, 2008) on a no-till corn field which had been harvested and seeded with barley prior to the start of the experiment. The soil was classified as Braddock Loam (Fine, mixed, semiactive, mesic, typic, hapludult). For each plot, an area of 1.5 m x 2 m was designated by an iron frame with a hose attached to a pan at the downhill end, which enabled all of the runoff from the plot to be collected. The treatments for this experiment were: surface applied poultry litter, subsurface injected poultry litter, surface applied dairy manure slurry, subsurface injected dairy manure slurry, and control where no manure was applied. Treatments were arranged in a randomized complete block set-up with one treatment per block, and blocks arranged horizontally on the slope.

4.2.3 Manure application

Poultry litter was collected from a Virginia broiler farm and applied at a rate of 6.72 Mg/Ha wet weight. Surface applied poultry litter was manually spread on the surface of the plot. Subsurface injected poultry litter was applied to a 5-cm deep slit created 20 cm apart in each plot and closed by hand. Dairy manure was collected fresh from a Virginia dairy barn and was

diluted to 95% water in order to replicate dairy lagoon conditions. This simulated dairy lagoon slurry was applied at a rate of 56.12 Mg/Ha wet weight. Dairy manure slurry was manually surface applied with the aid of a flat pan to help even spreading and subsurface injected to 15-cm deep slits that were 60cm apart in each plot and closed by hand. Both manure application rates are typical agronomic application rates of poultry litter and dairy manure slurry for Virginia soils (Maguire, 2009). For each treatment plot, manure was applied to extra 0.25m x 2m area on each side of the framed plot for soil collection after the first rainfall simulation without soil disturbance inside the framed area before the second rainfall simulation.

4.2.4 Rainfall Simulation and Sample Collection

Two consecutive rainfall simulations were performed on each plot. The first rainfall simulation was performed within 30 minutes of manure application. The second rainfall simulation was performed on the same plots 7 days after the manure application. Rainfall simulation plots were protected from any natural precipitation between the first and second simulated rainfall events.

Rainfall was applied to each 2m x 2m plot at a rate of 70mm/hr until 30 minutes of runoff was collected from the 1.5m x 2m framed area of each plot (SERA-17, 2008). The collected runoff was weighed, and thoroughly mixed before a 1-L subsample was immediately transferred into a glass jar. Approximately 0.3 mL 6M HCl was added to each glass jar to preserve the sample before packing on ice and transported back to the lab to be stored in -10^{o}C for later analysis. Soil samples were collected within the two 0.25m x 2 m areas on each side of the framed plot after the first rainfall event and then inside the framed area after the second rainfall event. For each surface applied plot, soil samples at depths of 0-5cm and 5-20cm were collected at 7 and 3 random spots, respectively. For each subsurface injected plot, soil samples were taken

at 7 spots in the injection slit and 7 spots halfway between the injection slit for the 0-5cm soil depth, and at 3 spots each for the 5-20cm soil depth. Replicate soil samples for each depth and treatment were composited, packed on ice and transported back to the lab to be stored in -10^{o}C for later analysis.

4.2.5 Surface runoff sample preparation

Exact volume of 500mL surface runoff sample was filtered using a pre-weighed Millipore 0.45µm HNWP nylon membrane filter (Millipore, Billerica, MA) and with the aid of a vacuum pump. Filters with runoff sediment were frozen at -80°C, freeze dried, and weighed before extraction and cleanup using the protocols as described below. The runoff water samples (filtrates) were immediately subject to extraction and cleanup using the solid phase extraction method described below.

4.2.6 Manure, soil, and runoff sediment sample extraction

An accelerated solvent extraction system (E916 Speed Extractor, Büchi, New Castle, DE) was used for the extraction of manure, soil, and runoff sediment samples. Briefly, 5g of freeze dried soil, 0.5 g of freeze dried poultry litter, 1g of freeze dried dairy manure, or pre-weighed freeze dried sediment with filters were mixed with 1g of hydromatrix (Agilent Technologies, Santa Clara, CA) and placed in an 11-ml stainless steel extraction cell (Büchi, New Castle, DE). The remaining space in each cell was filled with Ottawa Sand standard (20-30 mesh, Agilent Technologies, Santa Clara, CA) to 1-cm headspace. The extraction cells were placed on the extraction system and the samples were extracted using a method of two 3-minute cycles of ~10 mL methanol/acetone (50:50 v/v) followed by two 3-minute cycles with ~10 mL water/ methanol (50:50 v/v)(Nieto et al., 2008). Soil, sediment, and manure extracts were then brought

to volume to 50 mL using ultrapure de-ionized water and centrifuged at 2054xg for 40 minutes before being cleaned up using the solid phase extraction method as described below.

4.2.7 Solid Phase Extraction (SPE) for runoff water samples and soil, manure, and runoff sediment extracts

A volume of 25 mL of each soil, manure, or sediment extract was further diluted to 500 mL with ultrapure de-ionized water and acidified with 5 M HCl to ~pH 2 before the SPE. For the runoff water samples, 500ml of runoff water was acidified with 5 M HCl to ~pH 2 before the SPE. The Oasis HLB cartridges (60mg/3cc, Waters, location) were conditioned first with 3 mL Ethyl Acetate/Methanol (9:1,, v/v), then 3mL methanol, and lastly 6 mL water after being arranged in a 20-position Sampli-Q SPE Vacuum Manifold (Agilent Technologies, Santa Clara, CA). The above prepared 500-mL samples were each loaded onto an Oasis HLB cartridges at a flow rate of 4-5 mL/min. After loading the samples, each cartridge was washed with 5 mL of 5% Methanol in water (v/v) and allowed to dry under a gentle vacuum for 20 min. The hormone compounds were then eluted from each cartridge first with 3mL ethyl acetate/methanol (9:1, v/v) and then with 3mL 2% NH_4OH in Methanol (v/v) into a 10 mL glass test tube. After the SPE, the manure extracts were subject to an additional cleanup step with addition of 50mg primary secondary amine (PSA) (Agilent Technologies, Santa Clara, CA) into each test tube followed by vortexing for 2 min. The final extract with or without PSA in each test tube, was then evaporated to dryness under a gentle stream of nitrogen at 40ºC. (TurboVap, Labconco, Kansas City, MO). The dried residue was reconstituted by first adding 0.5 ml acetonitrile and then 0.5 mL water. Each reconstituted sample was filtered through a 0.22 μm polytetrafluoroethylene (PTFE) filter (Thermo Scientific, Rockwood, TN) into a 2 mL glass vial, capped tightly, and stored at -80°C if

needed before analysis on an ultra-performance liquid chromatography/triple-quadrupole tandem mass spectrometer (UPLC/MS/MS).

4.2.8 UPLC/MS/MS Analytical method

Matrix matched standards were used for the quantitative analysis of the soil and runoff sediment extracts to overcome their complex matrix interferences during the UPLC/MS/MS analysis (Pihlström, 2015). To overcome complex matrix effects in the manure extracts, the standard addition technique was employed for the quantitative analysis (Pihlström, 2015). There was minimum matrix effect for the runoff water sample extracts and therefore, external standards prepared in acetonitrile/water (1:1, v/v) were used for the quantitative analysis.

An Agilent 1290 series UPLC coupled to an Agilent 6490 triple-quadrupole tandem mass spectrometer (Agilent Technologies, Santa Clara, CA) was used to separate, qualify, and quantify 11 hormone compounds in each sample extract. Analytical columns used were Zorbax Eclipse Plus C18 rapid resolution HT 2.1x100mm, 1.8µm, 600 bar preceded by a Zorbax Eclipse Plus C18 2.1x5mm 1.8µm UHPLC Guard Column (Agilent Technologies, Santa Clara, CA). The sample injection volume was 10µL, the column temperature was 40°C and the mobile phase flow rate was 0.300ml/min. The parameters for the mass spectrometer were: sheath gas temperature at 200 ^{o}C with a flow rate of 8 L/min, drying gas temperature at 250 ^{o}C with a flow rate of 14L/min, nebulizer pressure 45 psi, capillary voltage 3500V(+); 3000V(-). For the analysis of E3, αE2, βE2, and E3, the mobile phases were A: 0.2mM ammonium fluoride in water, and B: acetonitrile/water (95:5, v/v), and the mass spectrometer was run in negative mode electrospray ionization. The mobile phase gradient was 0-1 minutes, 10% B; 9 min, , 85% B; 9.5 min, 98% B; 10.5 min, 98% B; 11 min, 10% B; 14 min, 10% B. Retention times of each compound are as follows: E3, 5.70min; βE2, 7.61min; αE2, 7.86min; E1, 8.14min. For the

analysis of E2-3G, E1-3G, E2-3S, E1-3S, 1,4-ADD, 4-AD, and P, the mobile phases were A: 5mM ammonium acetate in water, and B: acetonitrile/water (95:5, v/v). The mobile phase gradient was 0-1 minutes, 10% B; 9 min, 85% B; 9.5 min, 98% B; 10.5 min, 98% B; 11 min, 10% B; 14 min, 10% B. The mass spectrometer electrospray ionization was run in negative mode for E2-3G, E1-3G, E2-3S and E1-3S then with positive mode for 1,4-ADD, 4-AD, and P. Retention times of each compound are as follows: E2-3G, 4.82min; E1-3G, 5.06; E2-3S, 5.70min; E1-3S, 6.04 min; 1,4-ADD, 7.72 min; 4-AD, 8.27 min; P, 9.53 min. Method detection limit (MDL) and recovery % information for compounds of interest can be found in *Table 4.1*.

Table 4.1: Method Detection Limit (MDL) and Recovery for each compound in each matrix studied.

	Soil		Runoff Sediment		Runoff Water	
Compound	MDL (µg/kg)	Recovery (%)	MDL (µg/kg)	Recovery (%)	MDL (µg/L)	Recovery (%)
1,4-ADD	0.40	129	9.22	70.63	0.26	99.7
4-AD	0.51	126	9.44	81.11	0.63	112
P	0.61	148	9.62	76.91	0.83	147.0
E2-3G	0.88	71.7	8.68	61.9	0.22	73.8
E1-3G	0.47	89.2	9.00	61.5	0.16	82.7
E2-3S	0.33	100	5.22	39.8	0.32	56.4
E1-3S	1.22	95.9	5.45	41.5	0.06	28.5
E3	0.42	72.2	9.19	29.5	0.25	80.2
β-E2	0.33	65.9	7.36	30.5	0.28	78.3
α-E2	0.32	63.9	6.70	22.5	0.25	76.2
E1	0.34	73.8	11.0	45.3	0.26	74.2

4.2.9 Statistical Analysis and Calculations

Statistical analysis was completed in JMP Pro 12. ANOVA and a post-hoc mean comparison for all pairs using Tukey-Kramer HSD were used to compare treatments within manure type. Mass Balance for total hormones were calculated using the manure application rates, soil bulk density (1.33 g/cm^3), detected amount of total hormones in each applied manure type, depth/volume of sampling area, volume of runoff, and assumed uniformity of sample.

4.3 Results and Discussion

4.3.1 Concentrations of 11 Hormones in Poultry Litter and Dairy Manure

Both androgens have the highest dry weight-based concentrations (109-144 µg/kg) in the poultry litter (water content: 24%), followed by progestogen, all four estrogen glucosiduronates and sulphates, and two of the estrogens (E1 and E3) at levels ranging from 15.0-57.1 µg/kg (*Table 4.2*). The other two estrogens (α- and β-E2) were below their detection limits. A previous study reported similar levels of P and E1 at average concentrations of 63.4 and 44.1 µg/kg (dry weight basis), respectively, in fresh poultry litter samples from 12 different poultry farms in Delaware and Maryland, USA, though 4-AD, 1,4-AD, E2-3G and E1-3G were not included in the comparative work (Bevacqua et al., 2011). 4-AD, though not frequently studied, has been previously detected in poultry litter sourced from 4 different broiler farms in Spain at levels below the LOQ (22.9 µg/kg) up to 504 µg/kg (Valdehita et al., 2016).

Different from the poultry litter, all 11 hormones were detectable in the dairy manure slurry. For the dairy manure slurry (water content: 95%), although 4-AD has the highest dry weight-based concentration (702 µg/kg), the concentration of other androgen compound (1,4-AD) was about 100 times lower (*Table 4.2*). The dry weight-based concentrations of progestogen, two estrogen glucosiduronates (E1-3G, E2-3G), and E1 and E3 ranged from 32.5 to 72.8 µg/kg, while the two estrogen sulphates and α- and β-E2 were detected as concentrations similar to that of 1,4-AD. Comparing to the levels detected in the dairy manure used for our study (*Table 4.2*), higher dry weight-based concentrations of α-E2 (1416µg/kg), β-E2 (153µg/kg), and E1 (535µg/kg) were detected in a fresh manure that was analyzed within 2 hours of collection, however, the levels of P and E3 were below the detection limits (Zheng et al., 2007). Comparing to our test results, Mina et al (2016) detected higher levels of α-E2, E1, and

E3 at 91, 130, and 150 μg/kg (dry weight basis), respectively, in a dairy manure slurry (Mina et al., 2016).

Up to date, the most commonly studied environmental hormones are estrogen compounds including E1, β-E2, α-E2, and E3. However, in the poultry litter and dairy manure analyzed for the current study, the four estrogen compounds combined make up only 11% and 16% of the detectable hormones, respectively. On the other hand, androgen compounds such as 4-AD and 1,4-ADD has seldom been included in previous investigations on manure hormones, most likely due to analytical limitations. Our study has clearly shown that both androgen compounds make up 56% and 72% of the detectable hormones in the poultry litter and dairy manure, respectively (*Fig. 4.1*). Similarly, P and conjugated estrogens including E2-3G, E1-3G, E2-3S, and E1-3S have also been ignored in many previous studies, but our study shows that they collectively make up 12-33% of total hormones in the two manures used for this study (*Fig. 4.1*). The result from this study strongly suggests the importance of understanding the environmental occurrence and fate of manure-borne androgens, P, and conjugated estrogens in addition to estrogens.

Based on the dry weight hormone concentrations for the two manures and their application rates, the mass amount of each hormone applied to each 1.5m x 2m plot were calculated (*Fig. 4.2*), showing 4.6x more androgens, 11.6x more P, 19x more conjugated estrogens, and 4.2x times more estrogens were applied to the plots with agronomic rate of poultry litter than those applied with agronomic rate of dairy manure slurry.

Table 4.2: Concentrations of individual hormones in the dairy manure and poultry litter used in this study.

		Concentration (µg/Kg, dry weight)	
Class	Compound	Poultry Litter	Dairy Manure
Androgens	4-AD	144	702
	1,4-ADD	109	7.64
Progestogen	P	42.3	47.1
Conjugated Estrogens	E2-3G	56.8	32.5
	E1-3G	57.1	66.9
	E2-3S	20.7	4.08
	E1-3S	24.8	4.36
Estrogens	E3	15.0	57.6
	β-E2	<DL	12.0
	α-E2	<DL	8.72
	E1	34.6	72.8

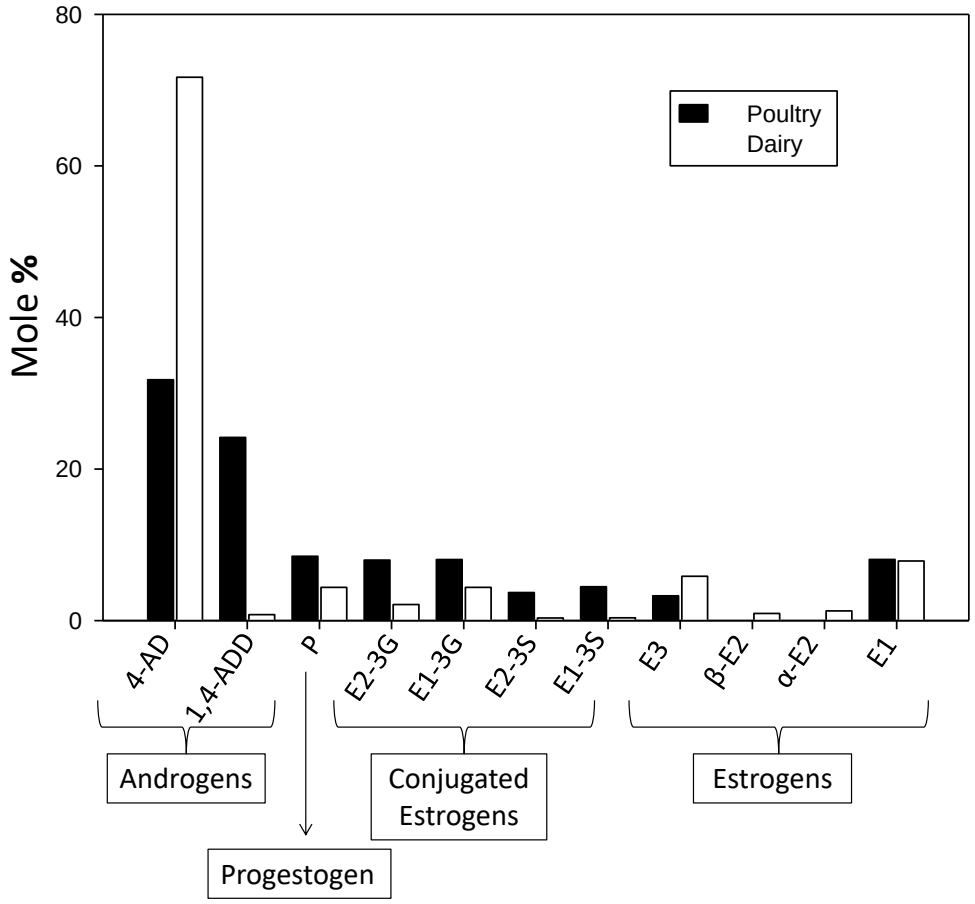

Fig. 4.1: Relative molar concentration of individual detectable hormones in the poultry litter and dairy manure for this study

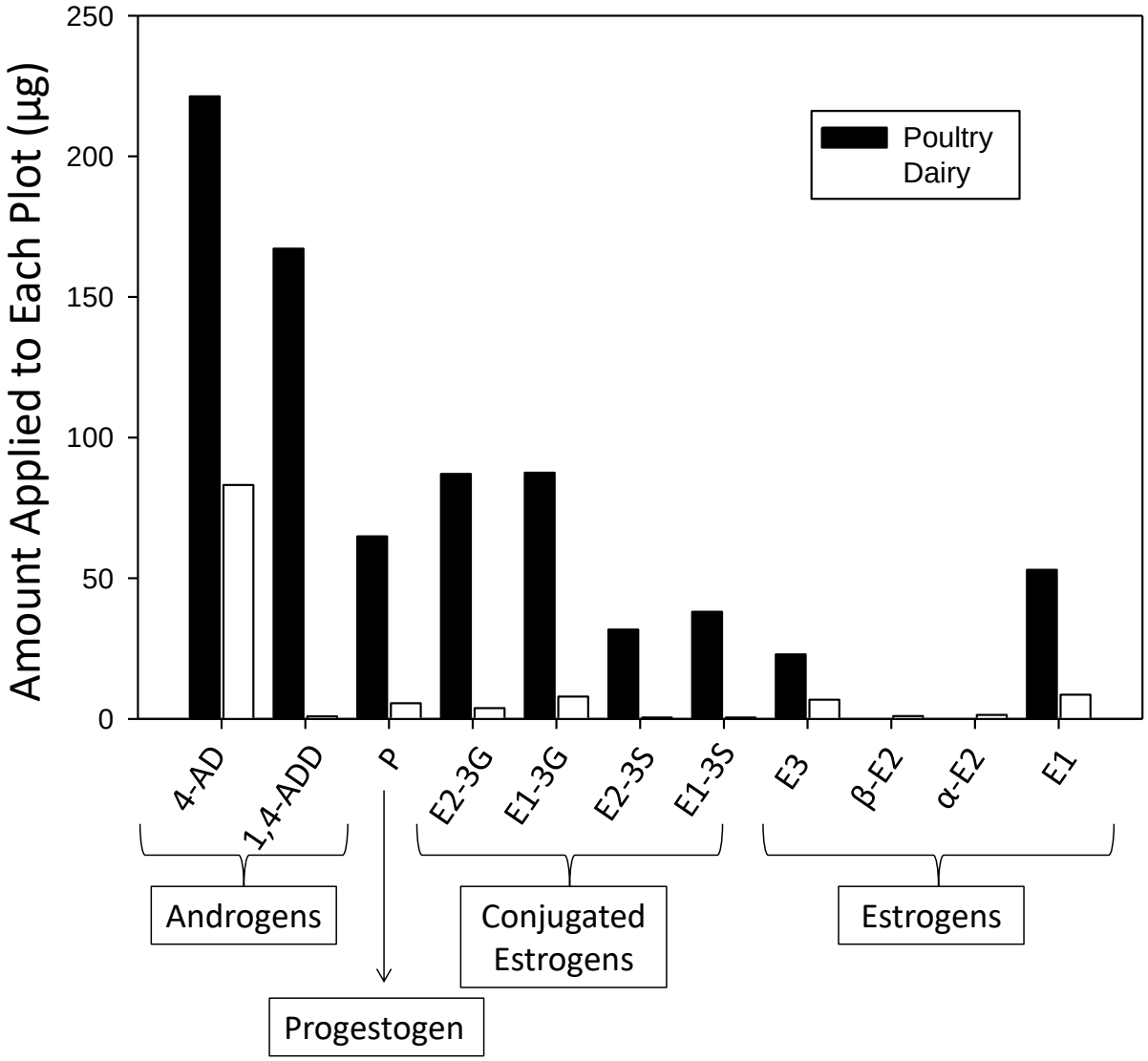

Fig. 4.2: Calculated mass input of hormones to each 1.5m x 2m plot applied with poultry litter or dairy manure slurry.

4.3.2 Runoff Water and Sediment Collection

Within the same day of simulated rainfall and for reach manure type there were no significant differences between the two application methods for the following: time to start runoff after the simulated rainfall had begun, the volume of 30-min runoff collected, and the sediment concentration in the runoff (*Table 4.3*). On day 0, less water was applied to the control plots as compared to that applied to the plots amended with poultry litter, however, when the poultry litter was surface applied, more water was needed to be applied to the plots than when the poultry litter was subsurface injected. This could be due in part to the dry poultry litter on the surface of the soil absorbing a greater amount of water than the injected poultry litter before start of the runoff, which meant that more water was applied before the observed runoff started. Although overall less water was needed on day 7 for the control plots and the poultry litter subsurface injected plots comparing to on day 0, on the 7th day the difference between the poultry litter application methods diminished because the applied poultry litter was already wetted from the first simulated rainfall event. This could be due in part to the intense rainfall event on day 0, compacting the soil and creating pathways or channels for the subsequent rain event on the 7th day. A concern of subsurface application of animal manures is that the slight soil disturbance from the disc injector could cause more sediment to be lost in the runoff when compared to surface application or no application of manure (Maguire et al., 2011); this however, was not an apparent issue as shown by the result of this study.

Table 4.3: Time to start runoff, amount of water applied to a plot, amount of 30-min runoff collected from each plot after the initial runoff, and sediment concentrations in the runoff. The data was for the two simulated rainfall events (70mmh^{-1}) conducted 0 and 7 days after application of manures to a loam soil in Virginia

Poultry Litter Applied Plots

Treatment	Time to start runoff (min)		Water applied to plot (kg) 2m x 2m plot		Runoff collected (kg) 1.5m x 2m plot		Sediment in runoff (g/kg) 1.5m x 2m plot	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Control	12.3Aa	8.6Ab	62.4Ba	57.7Aa	25.2Aa	37.6Aa	0.85Aa	0.40Aa
Poultry Injection	15.9Aa	7.6Aa	69.3ABa	56.8Aa	15.4Aa	17.5Aa	1.56Aa	0.81Aa
Poultry Surface	20.3Aa	14.1Aa	81.8Aa	67.0Aa	14.7Aa	15.4Aa	0.59Aa	0.92Aa

Dairy Manure Applied Plots

Treatment	Time to start runoff (min)		Water applied to plot (kg) 2m x 2m plot		Runoff collected (kg) 1.5m x 2m plot		Sediment in runoff (g/kg) 1.5m x 2m plot	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Control	12.3Aa	8.6Ab	62.4Aa	57.7Aa	25.2Aa	37.6Aa	0.85Aa	0.40Aa
Dairy Injection	32.7Aa	12.0Ab	86.7Aa	62.2Aa	12.3Aa	12.4Aa	0.87Aa	1.17Aa
Dairy Surface	6.90Aa	9.40Aa	54.4Aa	58.7Aa	23.4Aa	35.1Aa	0.80Aa	0.76Aa

*Means in the same column followed by different capital letters are significantly different ($p<0.05$) between treatments.
*Means in the same row and same column category followed by different lowercase letters are significantly different ($p<0.05$) between sample collection days.

4.3.3 Concentrations of Hormones in Runoff Water and Sediment

All 11 tested hormones were below their detection limits (*Table 4.1*) in the runoff sediment samples of all treatments. It is because the sediment concentrations in the runoff (*Table 4.3*) were too low to adsorb significant amount of hormones to be detectable. The average sediment concentrations in the runoff ranged from 0.40 g/kg to 1.56 g/kg (*Table 4.3*). The highest amount runoff sediment calculated based on data in *Table 4.3* would be around 27 g/plot. Assuming the average detection limit for each compound was 8 µg/kg for the runoff sediment *(Table 4.1*), the amount of each hormone leaving each plot with runoff sediment would be less than 0.21 µg/plot, or 2.3 µg/plot for the sum of 11 hormones. Comparing to the total amount of 11 hormones applied to each plot (*Fig. 4.2*), no more than 0.15% or 0.95% of the total hormone

applied with poultry litter or dairy manure, respectively, would leave each plot with runoff sediment during each of the two simulated rainfall events.

Of all 11 hormones analyzed, only 1,4-ADD was detected in the runoff water samples from both poultry litter and dairy manure surface applied plots but not in the subsurface injected plots (*Fig 4.3*). All other compounds were below their detection limits (*Table 4.1*). Although not statistically significant comparing to the levels detected in the runoff water during the first simulated rainfall, 1,4-ADD levels fell below its detection limit in the runoff water during the second simulated rainfall on the dairy manure surface applied plots, while it was still detectable at an average concentration of 0.008 µg/L in the runoff from the poultry litter surface applied plots. Even so, hormone concentrations in water less than 0.04µg/L have been shown to have negative effects on aquatic wildlife (Coe et al., 2008; Coe et al., 2009; Duffy et al., 2014). On average 12.3-37.6 kg of runoff was collected per plot, if all the hormones applied with the poultry litter and dairy manure (*Fig. 4.2*) was collected in the runoff, the maximum concentration of hormones would be 9.8 – 62.8 µg/kg in the runoff. Based upon highest concentration of 1,4-ADD detected in the surface applied plots in *Fig 4.3*, only 0.14-0.02% of the hormones applied to the plots were detected in the runoff.

The hormones 4-AD was the most abundant hormone in both the poultry litter and the dairy manure analyzed in this study, but 1,4-ADD was the only hormone at detectable levels in the runoff water. Both 1,4-ADD and 4-AD are transformation products of testosterone in the environment, and 4-AD will preferentially form under sterile conditions. (Lorenzen et al., 2005) It is also known that 1,4-ADD and 4-AD are formed preferentially from other sterols under controlled conditions in growing media depending on many factors arising from the type of bacteria exposed to and the conditions of the transformation (Shao et al., 2015). 1,4-ADD is

also a transformation product of 4-AD and P when digested by certain microorganisms. (Hafez-Zedan and Plourde, 1971). Detected 1,4-ADD in the runoff could have originated from further degradation of testosterone, 4-AD or another sterol, or with the changing conditions of the water and storage, though this is unlikely to happen completely on the day of application. Most steroid hormones are similar in character and structure and have K_{ow} values ranging from 3 to 4. Little is known about the solubility of 1,4-ADD, however the Log K_{ow} can be calculated using US EPA EPISuite KOWWIN program, which estimates the Log K_{ow} for 1,4-ADD to be 2.54 and for 4-AD to be 2.76. This estimates that 1,4-ADD is slightly more soluble in water than 4-AD, which makes it more likely to be detected in the runoff water. This is confirmed in our own analytical method, as 1,4-ADD elutes from the hydrophobic C-18 analytical column earlier than 4-AD during our LC/MS/MS analysis.

A few studies on hormones runoff from manure applied fields have been published, though none tested 1,4-ADD (Jenkins et al., 2006, Mina et al, 2016). The most comparable study compared estrogen content in runoff from natural rainfall events using surface broadcast and subsurface injection of dairy manure (Mina et al, 2016). While we did not detect any estrogens in our runoff samples, they had detected E1 at concentrations ranging from 0.720-4.0μg/L and α-E2 concentrations ranging from 0.310 – 2.4 μg/L in the runoff following a rainfall event 48 hours after application, which is much greater than the concentration of 1,4-ADD detected in this study. This could be due to their higher estrogen content in applied manure, as the application rates of manure were similar; their study detected 2x more E1, 3x more E3 and 9x more α-E2 in the manure. Similar to our study, no hormones were detected in the runoff from subsurface injected plots (Mina et al., 2016).

Other studies found few hormones in the runoff from manure applied agricultural fields. In a study detecting α-E2l and testosterone in surface applied poultry litter, average runoff concentrations from runoff events 3 weeks after poultry litter applications ranged from 0.04 µg/L to 0.20 µg/L for α-E2l and ranged from 0.003 to 0.007 for testosterone, similarly found no significant differences from background watershed were determined (Jenkins et al., 2006). When comparing concentrations of α-E2 and testosterone in tilled and no tilled crop land applied with poultry litter, again no differences between the control and treatment plots were detected (Jenkins et al., 2009). In both studies, lack of significant detection of hormones were attributed to the broad range of hormones that exist in different batches of poultry litter, and the occurrence of the hormones in the background due to avian and wildlife activity.

In comparison to manure field application, simulated runoff studies have also been completed on beef feedlots. A feedlot will contain not only fresher manure, but a greater volume of manure on the surface of the feedlot. Feedlots also tend to have more compacted, more impervious soil underneath the lot, allowing less water to infiltrate the soil, and more water to flow over the surface. In a study conducted on heifer feedlots, 1,4-ADD was detected in 14% of all runoff samples with median concentration lower than 0.005 µg/L. The same heifer feedlot produced runoff containing 4-AD and P in 100% of the runoff samples, androsterone in 96% of runoff samples, βE2 in 77% of the runoff samples, and estrone in 46% of the runoff samples from untreated cattle with average concentrations of 0.102, 2.660, 0.0991, 0.103, and 0.243 µg/L respectively. Therefore in comparison, other hormones were detected in much greater amounts than 1,4-ADD from beef heifer feedlots. (Bartelt-Hunt et al., 2012). In another feedlot rainfall simulation study, E2, 4-AD, and P were detected in concentrations ranged from 0.05-0.250 µg/L and β-E2 and E1 were mostly less than 0.050 µg/L in the steer feedlot runoff. Aging the plots

for 7 days showed an increase in 4-AD, P and E1 but a decrease in α-E2 and testosterone in the runoff (Mansell et al., 2011). One must also take into account that the hormone profile from adolescent beef cattle will be different from that of dairy manure and poultry litter. Compared to these studies, it should be noted that the impact of hormones via runoff from feedlot CAFOs have a greater input of hormones to environment than either surface or subsurface application of manure to soil.

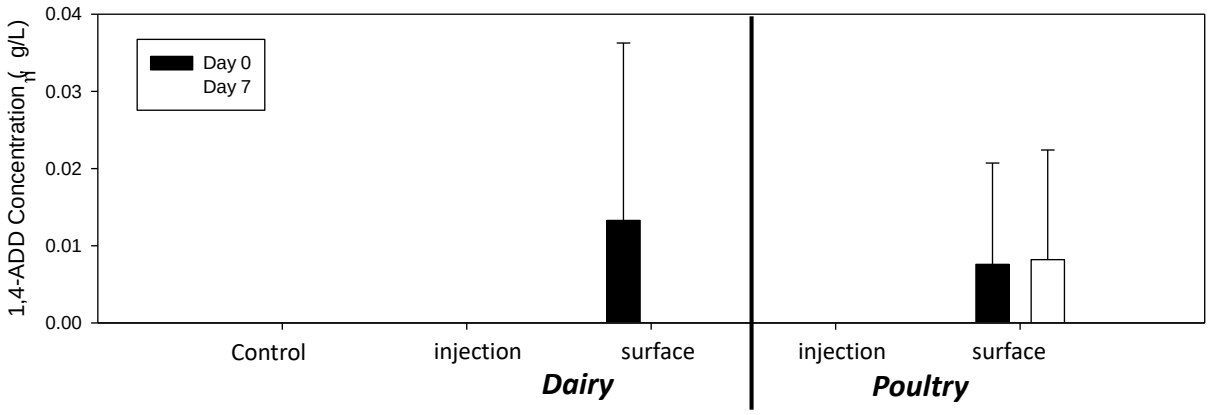

Fig. 4.3: Average concentration of 1,4-ADD in surface runoff water collected from two simulated rainfall events, one on the day of manure application and one on the 7th day after the application. Error bars signify standard deviation

4.3.4 Distribution of detected hormone compounds in soils

No estrogen conjugates (E2-3G, E1-3G, E2-3S, and E1-3S) were detected in any soils samples. Conjugated estrogens tend to deconjugate rapidly after excretion, and their transformation has been shown to be biologically driven, as conjugated estrogens have been

shown to deconjugate even more rapidly in surface soils where organic matter is more abundant. (Shrestha et al., 2012)

The distribution and types of hormones in the surface soil between treatments are demonstrated in *Fig 4.4*. E3 was the most prevalent estrogen detected in the surface soil and was regularly detected in poultry litter injection slit and surface samples on day 0 and day 7. Additionally, the concentration of E3 remained relatively constant over 7 days while androgen concentration decreased in both poultry slit and surface soil samples. E3 was detected at concentrations of 4.61 µg/kg in the poultry litter injection slit and 2.18 µg/kg in the surface applied poultry litter 0-5cm soil on day 0. The concentration of E3 only decreased by 8.2% in the poultry litter injection slit and by 10.6% in the poultry surface applied 0-5cm soil between day 0 and day 7. This phenomenon could be described with findings by Ying and Kookana (2005), which showed rapid degradation of estrogens, including E3 and E1 in aerobic soil, but little degradation over two months in anaerobic conditions (Ying and Kookana, 2005). The wetting of the soil during the rainfall simulations could have contributed to reduced aerobic, or more anaerobic-like conditions in the soil. E1 was also regularly detected in poultry litter Day 1 and day 7 surface soil samples, and the concentration of E1 actually increased by 50% in the poultry litter injection slit and by 61% in poultry litter surface applied 0-5cm soil samples between day 0 and day 7. E2 is known to be transformed to E1 in both biotic and abiotic conditions (Goeppert et al., 2014). There could have been estradiols below the detection limit, compounding to transform to detectable E1 after a week in the soil. Bartelt-Hunt et al., (2012) detected E3 in the surface soil of beef cattle feedlot pens for up to 46 days following the removal of cattle, and E1 for up to 109 days, and in once instance, E1 was detected for the first time on

day 109 (Bartelt-Hunt et al., 2012). β-E2 was not detected on day 0, but was detected in the dairy injection slit, the poultry injection slit, and the poultry surface applied soil on day 7.

While androgen compounds were applied to each plot in the greatest amounts based on the loading rates of manure and their concentrations in the manure, they were neither the most frequently detected hormone nor the most abundant in the manure applied soil. 1,4-ADD and 4-AD were generally detected in second and third highest concentrations, respectively, for poultry litter applied soil samples, though they decreased in concentration over 7 days more rapidly than E3. 1,4-ADD and 4-AD were detected at concentrations of 3.28 µg/kg and 2.04 µg/kg in the poultry litter injection slit and 1.13 µg/kg and 0.88 µg/kg in the surface applied poultry litter 0-5cm soil on day 0. The concentration of 1,4-ADD and 4-AD decreased by 64.3% and 62.7% in the poultry litter injection slit and by 30.1% and 100% in the poultry surface applied 0-5cm soil between day 0 and day 7. Androgens were also seldom detected in surface soil samples which were treated with dairy manure. In the dairy manure injection slit on day 0, which contained the greatest concentration of total hormones among dairy manure treated samples; P was detected in the greatest concentration, at 1.32 µg/kg. Little information exists on half-lives of the androgen compounds studied in this experiment in soil, as most published studies focus on the transformation of testosterone

It is interesting, though not surprising, that the hormone distribution in the soil *(Fig. 4.4)* is different from the relative distribution of hormones in the manure used in this study *(Fig 4.2).* When hormones are exposed to different environments or different microbial communities, the preferred transformation products can change. For instance, when exposed to different microbes, P, E2 (no distinction of stereochemistry), 4-AD, and testosterone will transform into different transformation products (Hafez-Zedan and Plourde, 1971). Presence or absence of

microbes can also alter the transformation products of hormones: testosterone will transform to 1,4-ADD and 4-AD in soils, but will only transform to 4-AD in sterilized soil (Lorenzen et al., 2005). Soil and soil-water systems will have different types of microbial communities present, which could rapidly alter the present hormones during the rainfall simulation, or even possibly in storage.

The types of hormones found in the poultry litter injection slits on day 0 and day 7, are the same types of hormones and relative concentrations of hormones found in the surface applied poultry litter soil. In contrast, the surface applied dairy manure had fewer different types of hormones in the surface soil when compared to the types of hormones found in the injection slit. The surface applied dairy manure only contained E1, while the injection slit contained 1,4-ADD, P, E3, and α-E2. There was also one instance of lateral movement in the soil where on day 7, E3 was detected between the injection slits at an average concentration of 0.13 µg/kg and had not been detected between the injection slits on day 0.

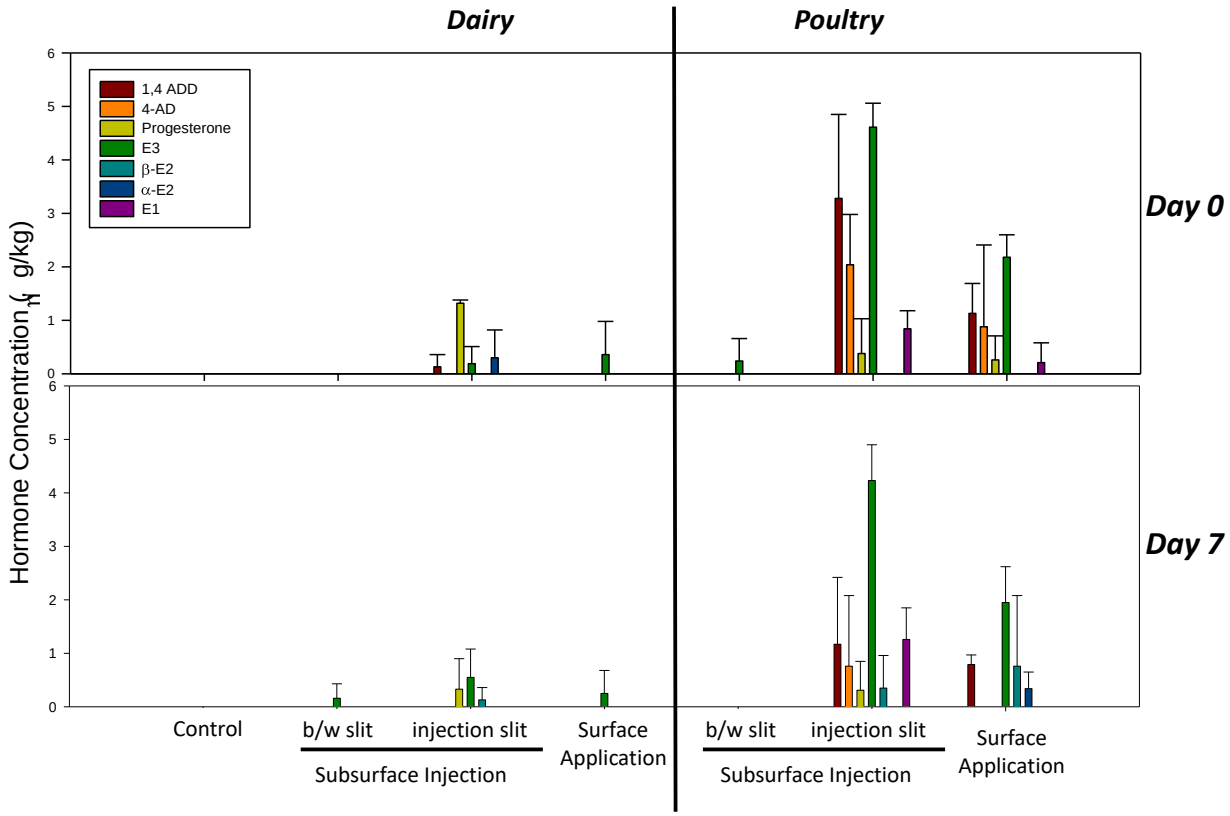

Fig 4.4: Distribution of individual detected hormones in 0-5 cm soils collected after two post manure application simulated rainfall events, 7 days apart.

4.3.5 Total concentrations of hormones in soils

No hormones were found above their detection limit in any samples from the control plots (*Fig. 4.5*). For the poultry litter applied plots, on both day 0 and day 7, the greatest total concentration was found to be in the top 0-5cm soil of the poultry litter injection slit, followed by the top 0-5cm soil of the surface applied poultry litter (*Fig. 4.5*). . The 0-5cm soils located between injection slits of poultry litter, and in the surface applied poultry litter plots were not found to be significantly different from the control on both day 0 and day 7. On day 7, the total hormone concentrations in the 0-5cm soil of the poultry litter surface applied plots were slightly significantly greater than the control. None of the total hormones detected in the 5-20cm soil of

the poultry litter applied plots were determined to be statistically different from their detection limits. These findings strongly suggest that the hormones do not tend to leach downward through the soil profile regardless of application methods. In addition, the findings show that hormones do not tend to move laterally through the soil either, as when the manure was injected, the hormones tended to remain in the injection slit and not transport to areas between the injection slits in instances of heavy rainfall.

Similarly for dairy manure, the greatest total concentration of hormones was found to be in the dairy injection slit on both day 0 and day 7 (*Fig. 4.5*). The concentrations of total hormones detected in the top 0-5cm soil of the dairy manure injection slit on day 0 and day 7 were significantly different from all of the other treatments for each simulated rainfall event. For both day 0 and day 7, the 0-5cm soils located between dairy manure injection slits and in the surface applied dairy manure plots were not to be significantly different from the control. None of the total hormones detected in the 5-20cm soil in dairy manure applied plots were determined to be statistically different from the detection limits. This result again indicates simulated rainfall resulted in limited lateral or vertical transport of manure-borne hormones when applied to soil, either in the injection slit or in the top layer of the soil depending on the manure application methods.

Immediately following the application of poultry litter, the total concentrations of hormones in the surface soil was 11.15 µg/kg, and 4.66 µg/kg respectively for injection slit and surface applied plots, respectively. In the samples collected 7 days following the application of poultry litter, the concentration of total hormones in the surface soil was 8.08 µg/kg, and 3.84 µg/kg respectively for injection slit, and surface applied plots respectively, corresponding to 27.53%, and 17.60% hormones dissipated in seven days and one rainfall event. A similar trend

is seen in the dairy manure applications, with the day 0 hormone surface soil concentrations being 1.94 µg/kg, and 0.36 µg/kg for injection slit, and surface applied, respectively. The concentration of total hormones in the surface soil 7 days following the dairy manure application was 1.01 µg/kg, and 0.25 µg/kg respectively for injection slit, and surface applied respectively, resulting in a loss of hormones of 47.94%, and 30.56% between collections. No significant differences were found for any treatment between day 0 and day 7 total soil hormone concentrations for both the 0-5cm soil and the 5-20cm soil. This observation shows that hormones persist in the soil for at least 7 days, which shows that soil management within the first week of manure application is essential for preventing the loss of hormones from agricultural fields to the surrounding environments.

It is apparent that dissipation rates for hormone compounds have broad ranges that last from a few hours up to a few days, and much of this can likely be attributed to the differing conditions of each experiment conducted (Das et al., 2004; Lee et al., 2003; Ying and Kookana, 2005). The hormones in this study persisted for at least 7 days, and this could be due to anaerobic conditions created by the two heavy rainfalls, leaving the soil wet for a period of time after each rainfall simulation. Ying and Kookana (2005) showed that while E1, 17β-E2, and E3 had been degraded within 7 days under aerobic conditions, the compounds persisted for over 70 days under anaerobic conditions (Ying and Kookana, 2005).

Regardless of manure application methods used in this study, a greater levels of hormones were detected in the 0-5cm soil than in the 5-20 cm soil (*Fig.4.5*), showing that hormones do not readily leach through the soil profile during the simulated rainfall immediately or 7 days after manure application. Hormones tend to have Log K_{oc} ranging between 3 and 4, meaning that they are more likely to be associated with the soil and organic matter phase than in

an aqueous phase (Casey et al., 2005; Lee et al., 2003; Ying and Kookana, 2005; Yu et al., 2004). Any downward movement through the soil profile would be attributed mainly to leaching with water, which is unlikely to occur to the somewhat hydrophobic hormone compounds. The results from our study further support this argument.

Greater concentrations of hormones were detected in the soils that were treated with poultry litter than soils that were treated with dairy manure. This is likely due to greater hormone loading in poultry litter plots (*Fig. 4.2*), as more than 6x more hormones were applied with the poultry litter than were applied with dairy manure. It is difficult to compare the total hormones detected in soil, as the different types of hormones studied, and the amount of hormones studied varies greatly between research groups; however we believe that the range of types of hormones tested in this experiment is representative of natural hormones expected to be found in poultry litter and dairy manure.

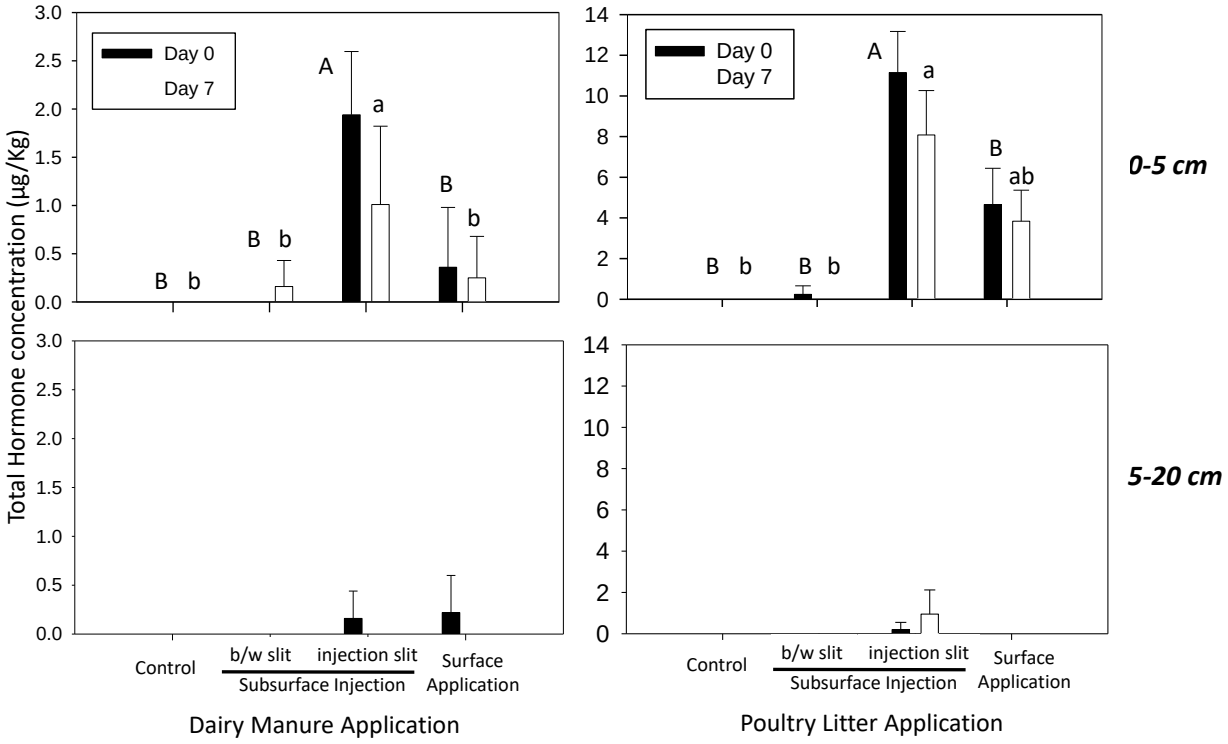

Fig 4.5. Concentrations of total hormones in 0-5 cm and 5-20 cm soil depths after two post poultry litter application simulated rainfall events, one on the day of manure application and one on the 7th day after the application.. Error bars signify standard deviation. Capital letters were used to show significant difference ($p < 0.1$) between means for Day 0 rainfall simulations. Lowercase letters were used to show significant difference($p < 0.1$) between means for Day 7 rainfall simulations. No significant differences were found between any treatments at the 5-

4.3.6 Mass Fluxes of Hormones in Soils Amended with Poultry Litter and Dairy Manure Using Surface Application and Subsurface Injection

Table 4.4 shows the mass balance of the total hormones detected in each major component of a soil system amended with animal manure (*Fig. 4.7).* For day 0, greater than 100% of the hormones applied were detected for each treatment, at a range of 100% to 169%. For poultry litter injected and surface applied plots and the dairy manure injected plots, 35-100% of the manure-associated hormones were detected in the 0-5cm soil. The only instance where there was higher amount of total hormones in the 0-5 cm soil than the 5-20cm soil was for the dairy manure surface applied plots on the day of manure application. Only up to 3.10% of the manure-associated hormones were detected between the injection slits on day 0, further highlighting the lack of lateral movement of hormones through the soil.

There are several reasons for why more hormones were detected than the estimated total input of hormones with the manure applied. Firstly, variations in a compound's analytical method recovery between the different matrices changed its detected amount in each subsample, which is scaled up and compounded during mass balance calculations. Estimations existed in the mass balance calculations, such as bulk density, uniformity in slit width, and sample homogeneity. General variations come into play when scaling up near the detection limits of compounds, especially when dealing with non-homogenous sampling sources, although care was taken to homogenize each sample. For instance, poultry litter is chunky due the beddings mixed

with the manure at the poultry house and was difficult to maintain uniform proportions during application and sampling.

For Day 7, the percentage of hormones detected when compared to the estimated loading rates decreased by 75.5% for the surface applied and 38.4% for the injected plots in the dairy manure applied plots. The percentage of hormones detected when compared to the estimated loading rate only decreased slightly for the poultry litter applied plots, decreasing by 7.45% for the surface applied and 17.6% for the injected plots from day 0. This indicated that the hormones entered to soil with dairy manure dissipated more rapidly than those with the poultry litter, strongly suggesting that the matrix and characteristics of a manure applied to the soil can significantly affect the overall fate of hormones

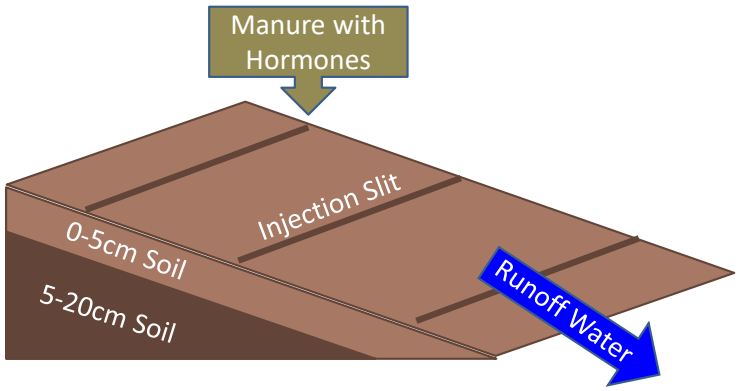

Fig. 4.7: Conceptual depiction of the fate of hormones in a manure-soil system. Manure is applied to the soil and the hormones tend to remain in 0-5cm soil and in the injection slit, and not lost to leaching to the 5-20cm soil and surface runoff.

Table 4.4: Total hormones detected relative to the total amount of manure-associated hormones

		Total detected Hormones/Total Manure Associated Hormones Applied			
		0-5cm Soil	5-20cm Soil	Water	Total detected
Treatment		Day 0			
Dairy Manure Injection	Between Injection Line	0.0	0.0	0.0	0.0
	Injection Line	80.3	19.9		100
Dairy Manure Surface		59.7	109	0.0	169
Poultry Litter Injection	Between Injection Line	3.10	0.0	0.0	3.10
	Injection Line	144	7.75		152
Poultry Litter Surface		120	0.0	0.0	120
		Day 7			
Dairy Manure Injection	Between Injection Line	19.9	0.0	0.0	19.9
	Injection Line	41.8	0.0		41.8
Dairy Manure Surface		41.5	0.0	0.0	41.5
Poultry Litter Injection	Between Injection Line	0.0	0.0	0.0	0.0
	Injection Line	104	36.8		143
Poultry Litter Surface		99.2	0.0	0.0	99.2

4.4 Conclusion

The inputs of hormones from poultry litter to an agricultural field were greater than that from dairy manure in this study, indicating that tools such as the prototype poultry litter injector used are needed for reducing the surface transport of hormones sourced from poultry litter applied to agricultural fields. Injecting the poultry litter and dairy manure was effective for reducing the hormone concentrations detectible in the runoff water from the manure applied plots, highlighting the value of manure injectors. Hormones detected in the soil from the injection plots were confined to the injection slits, limiting their exposure to surface transport, which is important because the hormones persisted at detectible concentrations in the manure applied soil for longer than the 7 day study. Injecting dairy manure and the recent technological

advancement of injecting poultry litter can be an important tool for mitigating environmental hormones, especially when combined with other benefits of injecting manure such as reducing nutrient losses and reducing erosion.

4.5 References

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Chapter 5: Transformation of Hormone Precursors in Soils Amended with Poultry Litter

Abstract

Hormones are emerging environmental contaminants and endocrine disrupting compounds which can be sourced naturally from animal manures. When manures are applied to agricultural fields, surface runoff provide a pathway to the adjacent aquatic environment, where hormones in concentrations at low as ng/L can have detrimental effects on certain aquatic organisms. Previous investigation has shown significantly higher levels of hormone precursors than hormones in a wide range of animal manures. It is unknown if those precursors can be further transformed into hormones once they are in the environment with manure land application. The objective of this study was therefore to study the dissipation of manure-borne hormone precursors 1,4-androstadiene-3,17-dione (1,4-ADD) and 4-androstene-3,17-dione (4-AD) as well as Estrone (E1), a known metabolite of 4-AD. A soil microcosm experiment with poultry litter was conducted to mimic two manure land application methods: surface application and subsurface injection. The effect of temperature (20^{o}C and 10 ^{o}C) and soil type (a silt loam-silty clay loam and a fine sandy loam) on the dissipation of the 3 target compounds was investigated. This study showed that dissipation of 1,4-ADD and 4-AD followed first order kinetics in all treatments. The half-lives of 1,4-ADD and 4-AD ranged 3.56-7.69 and 3.91-4.99 days, respectively. Within 30 days of poultry litter application, both surface applied or subsurface injected, 1,4-ADD and 4-AD dissipated to nearly undetectable levels. The half-life of 1,4-ADD in the poultry litter surface-applied soils was 1.9 times higher than that in the poultry litter subsurface-injected soils, indicating faster dissipation rate in the injection slits. However, poultry litter soil application methods did not have a significant effect on the half-lives of 4-AD.

On day 0 and day 1, the silt loam soil was determined to have significantly greater concentrations of 1,4-ADD and 4-AD, but after day 1 soil type no longer had any significant effect on the concentration of both hormone precursors. Temperature had no significant effect on the half-lives of 4-AD and 1,4-ADD. E1 persisted at detectable and slightly increased levels for the duration of the study, with sandy soils having a slightly higher overall concentration of E1 than the silt loam soil, suggesting its persistence and its transformation from 4-AD. This study provided insight to the behavior of two testosterone precursors 1,4-ADD and 4-AD, as well as estradiol metabolite E1 when applied concurrently with poultry litter.

5.1 Introduction

The biological transformation pathways of steroid hormones are shown in *Fig. 5.1*, and contributes to what would be excreted in the manure. In biological systems, compounds 1,4-ADD and 4-AD are precursors to testosterone as well as E1, therefore they are likely to be present in both male hormone dominated and female hormone dominated manures. Further, E1 is a precursor compound to α-E2 and β-E2 (*Fig. 5.1*).

Once hormones are excreted in the manure, they are subject to an entirely different environmental degradation pathway. The environmental degradation pathway of hormones is not entirely understood because changes in temperature, moisture content, and sterility of the matrix studied can alter the specific degradation pathway of each hormone (Goeppert et al., 2014; Shrestha et al., 2012; Ying and Kookana, 2005). Because of its known negative effects on fish wildlife and humans, the degradation and dissipation rates of testosterone in the environment are often studied (Jacobsen et al., 2005; Lee et al., 2003). However it is known that in the environment, testosterone can transform into 1,4-ADD and 4-AD also contribute androgenicity, or testosterone-like behavior (Lorenzen et al., 2005). The abundance of 1,4-ADD and 4-AD are

enhanced in concentration, as they are also microbial degradation by products of cholesterol and other naturally sourced sterols (Marsheck et al., 1972). The estrogen compounds α-E2 and β-E2 tend to transform rapidly in animal manures and soil to E1, a compound which is still an endocrine disrupting compound, estrogenic in character, and exhibits similar negative effects on environmental organisms. (Colucci et al., 2001).

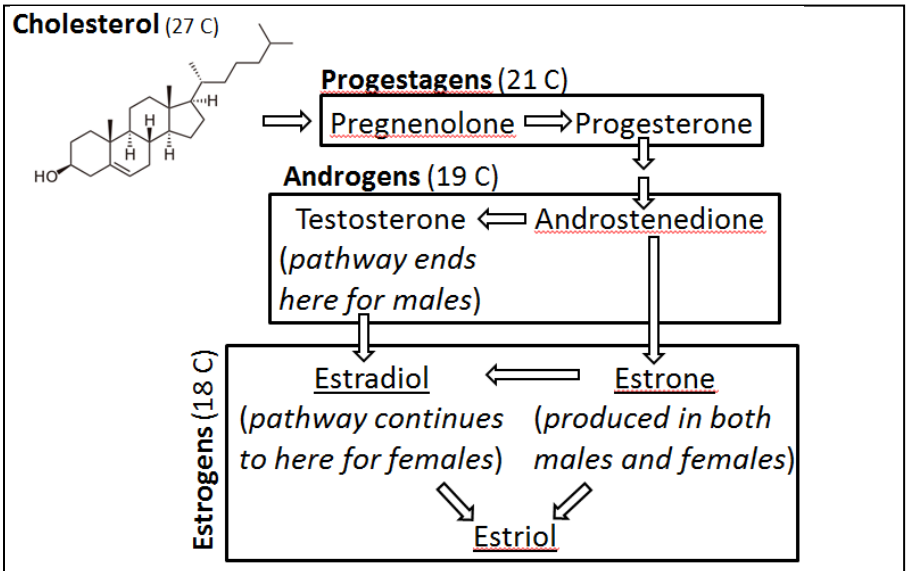

Fig. 5.1. Simplified biosynthesis pathways for major steroid hormones in organisms. # of C in parenthesis indicate basic carbon skeleton of each hormone group: progestogens, androgens, and estrogens.

Manure is applied as a soil amendment to provide valuable nutrients to crops. The application of animal manure has many other benefits such as increasing organic matter and water holding capacity of the soil (Edwards and Someshwar, 2000; Haynes and Naidu, 1998; Khaleel et al., 1981). In recent years, no-till agriculture has become a major part of best management practices for cropping systems in order to reduce sediment runoff and to retain

more soil organic matter. Nutrient loss from manure can occur from surface runoff and can contribute to increased nutrient loading in surrounding waterways and in severe and chronic cases, algal blooms (Mueller and Helsel, 1996) To reduce nutrient loss, manure can be incorporated into the soil, but this is then no longer considered no-till. Manure subsurface injection can maintain the no-till characters of the soil management while incorporating the manure, and new advances in the subsurface injection of solid manures has allowed for this to be done with poultry litter (Maguire et al., 2011).

Nutrients are not the only environmental concern when applying manures to agricultural fields; hormones are naturally excreted from livestock in their manure, and are environmental contaminants of emerging concern. (MacDonald and McBride, 2009; USDA, 2009; USEPA, 2012). Various studies show that hormones can be sourced from poultry litter and can persist in the environment (Bevacqua et al., 2011; Dutta et al., 2010; Gall et al., 2011; Jenkins et al., 2009; Jenkins et al., 2006). Hormones are endocrine disrupting compounds, which negatively affect the endocrine systems of certain vulnerable wildlife, leading to reproductive, developmental, and behavioral challenges (Kidd et al., 2007, Jukosky et al., 2008 Coe et al., 2009).

When applied to agricultural fields concurrently with animal manures, a major pathway for hormone entrance into waterways is in surface runoff (Dutta et al., 2010; Gall et al., 2011; Mina et al., 2016). Multiple field and laboratory studies have shown that hormones tend to be associated with soil particles, and therefore their risk of leaching is minimal (Casey et al., 2005; Lee et al., 2003). Therefore, manure injection can be a viable alternative for reducing the entrance of hormones from manure applications into the environment. The unique conditions that the manure is subject to during subsurface manure application, such as lack of sunlight and

less drying out as would happen on the surface raises interesting questions on the changes in hormone behavior between surface application and subsurface injection of manure.

. The environmental behavior of 1,4-ADD and 4-AD is not as extensively studied as that for Testosterone. 1,4-ADD and 4-AD have been found in high concentrations in various animal manures, and have been found to be persistent in the soil following manure applications (Sosienski, Chapter 2,3,4). In addition, the degradation rates of 1,4-ADD and 4-AD in soil-water-manure systems under various environmental conditions is relatively unknown. Estrogen compounds are also persistent in the environment because E1 is both a biological precursor, and also an environmental degradation product of α-E2 and β-E2. Therefore, this study was designed to determine degradation rates of 1,4-ADD and 4-AD in poultry litter under varying conditions of soil type, temperature, and manure application technique. The occurrence of estrogen compounds α-estradiol, β-estradiol and estrone was also studied under these conditions.

5.2 Materials and Methods

5.2.1 Chemicals and Materials

1,4-Androstadiene-3,17-dione (1,4-ADD), 4-Androstene-3,17-dione (4-AD), Estrone (E1) (≥99%), 17α-Estradiol (αE2) (≥98%) , 17β- estradiol (βE2) (≥98%) were purchased from Sigma-Aldrich. (Saint Louis, MO). HPLC grade acetonitrile, ethyl acetate, methanol and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium hydroxide, ammonium fluoride and hydrochloric acid were also purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium acetate was purchased from Amresco (Dallas, TX). De-ionized water and Milli-Q water was made from a Millipore water purification system (Milford, MA).

5.2.2 Soil and Poultry Litter Used for the Soil Microcosm Study

Two soils were used in this study, a Poplimento silt loam-silty clay loam (Fine, mixed, subactive, mesic Ultic Hapludalfs) from Blacksburg, VA and Nansemond fine sandy loam (Coarse-loamy, siliceous, subactive, thermic Aquic Hapludults) from Suffolk, VA. The soils are referred to as silt loam and sandy loam throughout the experiment and were chosen on the basis that they are typical soil types in Virginia in poultry producing regions, where poultry litter would be applied as a nutrient amendment. Soils were collected from the 0-12" depth and air-dried prior to use in the experiment. The field water holding capacity of soils was determined using the method described by Cassel and Nielsen (1986) by saturating 100g of each soil in triplicate with water and allowing each pot to drain freely for 2 days, then determining the amount of water lost by weighing (Cassel and Nielsen, 1986). Poultry litter was collected from a large broiler farm near Staunton, VA and was stored at 6°C until the start of the experiment.

5.2.3 Soil Microcosm Study Set-up

In addition to the 1,4-ADD and 4-AD in the poultry litter, appropriate amounts of 1,4-ADD and 4-AD were spiked to the poultry litter to achieve a starting concentration of 100 µg/kg for each compound the poultry litter-applied soils. The poultry litter applied to the sandy loam soil was spiked with a 30.3µg of each hormone and the poultry litter applied to the silt loam soil was spiked with 27.8 µg of each hormone. A different spiking amount for each soil resulted from soil bulk density variation, because of requiring a greater mass of the sandy soil in the testing beaker to achieve the 5cm injection depth.

To set up the soil microcosms, 275g of air-dried silt loam soil or 300g of air dried sandy loam soil were added to 400mL beakers which were used as microcosm vessels. Two different

soil masses were chosen because the density of the clay loam was greater than that of the sandy loam, and the goal was to have soil at a depth of 6cm. Poultry litter was applied at a rate of 3 tons per acre, or 2.73g of poultry litter per microcosm vessel (surface area 40.72 cm). For each of the two soils, poultry litter was surface applied and subsurface applied as shown in *Fig. 5.2a* and *Fig. 5.2b*. To mimic conditions for poultry litter application in the field, poultry litter was hand spread on the surface of the soil or was placed in a 5cm slit created by two small pieces of plastic and then the slit was manually pressed closed over the applied poultry in the slit, as shown in *Fig 5.2a* (Kulesza et al., 2014). A control without poultry litter was also set up.

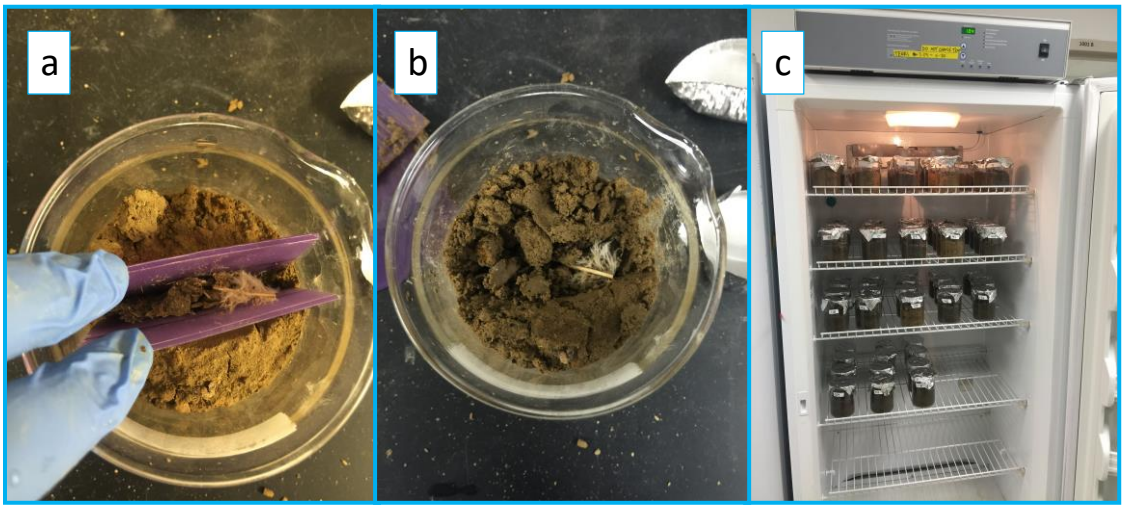

Fig.5.2: Soil-manure microcosm set-up showing (a) the simulated poultry litter subsurface application process, (b) a soil microcosm with manure surface applied, and (c) the set of soil microcosms randomly arranged in the incubator.

At the completion of the microcosm set up, all microcosm vessels were loosely covered in perforated foil and placed at either 10°C or 21°C in darkness for up to 30 days (*Fig. 5.2c*). There were three replications for each treatment. Every 5 days, each microcosm vessel was weighed and the soil was brought to 70% field capacity with de-ionized water to compensate the weight loss due to water evaporation. Microcosm vessels were destructively sampled on 0, 1, 3,

10 and 30 days of the study. Upon collection, each sample was thoroughly mixed and immediately frozen at -10°C for later analysis.

5.2.4 Sample Extraction and Clean-up

Soil and manure samples were extracted using an accelerated solvent extraction system (E-916 Speed Extractor system, Buchi, New Castle, DE), with a method modified from Nieto et al. (Nieto et al., 2008). 5.00g of soil or 1.00g of manure was mixed with 1.00g hydromatrix (Agilent Technologies, Santa Clara, CA) which had been previously heated at 200°C for 12h, and placed in an 11-ml stainless steel cell, the remaining space of the cell was filled with Ottawa Sand standard 20-30mesh (Agilent Technologies, Santa Clara, CA) to reach approximately 1cm headspace. The accelerated solvent extraction was operated at 75°C and 100bar. Four extraction cycles were performed: two cycles using methanol/acetone (50:50 v/v) followed by two cycles with water/ methanol (50:50 v/v). Each cycle was preceded by 1 minute of heating, followed by 3 minutes holding at operating temperature and pressure, followed by 1 minute of discharge. The final extracts were diluted to 50 ml with de-ionized water and centrifuged at 2054 x g for 40 minutes in glass centrifuge tubes. 25 ml of each extract was diluted with de-ionized water to 500ml and adjusted to pH 2 with hydrochloric acid for the following solid phase extraction clean up.

Soil and poultry litter samples were cleaned up using solid phase extraction. Oasis HLB cartridges (60mg/3cc HLB sorbent, 30µm particle size, Waters, Milford, MA) were arranged in an 20-position Sampli-Q SPE Vacuum Manifold (Agilent Technologies, Santa Clara, CA) and conditioned with 3ml of ethyl acetate/methanol (9:1, v/v), then 3ml of methanol, followed by 3 ml milli-Q water. Samples were loaded to the cartridges at a flow rate of 4-5 ml/min. SPE cartridges were then rinsed with 5ml of water/methanol (95:5, v/v) and dried under gentle

vacuum for 20 min. Analytes were eluted from the cartridge with 3ml of ethyl acetate/methanol (9:1, v/v) followed by 3ml of methanol/ammonium hydroxide (98:2, v/v). At this step manure samples had an additional clean-up step with the addition of 50mg of primary secondary amine (Agilent Technologies, Santa Clara, CA) to each sample. Samples were evaporated to dryness under a gentle stream of nitrogen at 45°C (TurboVap, Labconco, Kansas City, MO) The resulting sample residue was dissolved in 0.5 ml of acetonitrile followed by 0.5 mL water, then filtered with a 0.22um PTFE syringe filter (Thermo Scientific, Rockwood, TN). Samples were stored at -80°C before LC/MS analysis.

5.2.5 UPLC/MS/MS Analysis

Samples were analyzed using an ultra-performance liquid chromatography in tandem with triple quadrupole mass spec (UPLC/MS/MS) (1290 Infinity UPLC, 6490 triple-quadrupole mass spectrometer, Agilent Technologies, Santa Clara, CA). The analytical column was Zorbax Eclipse Plus C18 rapid resolution HT 2.1x100mm, 1.8µm, 600 bar with a Zorbax Eclipse Plus C18 2.1x5mm 1.8µm UHPLC Guard Column (Agilent Technologies, Santa Clara, CA). The column temperature was 40°C and the flow rate was 0.300ml/min. The injection volume was 10µL. For the analysis of E1, αE2 and βE2, the mobile phases were A: 0.2mM ammonium fluoride in water, and B: acetonitrile/water (95:5, v/v). The mobile phase gradient was 0-1 minutes, 10% B; 9 min, 85% B; 9.5 min, 98% B; 10.5 min, 98% B; 11 min, 10% B; 14 min, 10% B. The mass spectrometer was run with negative mode electrospray ionization. For the analysis of 1,4-ADD and 4-AD, the mobile phases were A: 0.2mM ammonium acetate in water, and B: acetonitrile/water (95:5, v/v). The mobile phase gradient was 0-0.5 minutes, 20% B; 3.5 min, 100% B; 5.5 min, 100% B; 6 min, 20% B; 9 min, 20% B. The mass spectrometer was run with positive mode electrospray ionization. For all compounds the triple-quadrupole mass

spectrometer parameter were sheath gas temperature at 200 ^{o}C with a flow rate of 8L/min, drying gas temperature at 250 ^{o}C with a flow rate of 14L/min, nebulizer pressure 45 psi, capillary voltage 3500(+)V 3000(-)V. Liquid chromatography method retention times of each compound are as follows: βE2, 7.84min; αE2, 8.11min; E1, 8.40min; 1,4-ADD, 4.40min; 4-AD, 4.65min. Matrix effects were overcome by using matrix matched standards for soil samples (Pihlström, 2015) . Standard addition method was used for the quantification of hormones in manure samples (Pihlström, 2015). Soil method detection limit (MDL) and recovery % for each compound is shown in *Table 5.1*.

Table 5.1: Soil method detection limit and recovery

Table 1: Soil method detection limit and recovery.		
Compound	MDL (µg/kg)	Recovery (%)
1,4-ADD	0.40	129
4-AD	0.51	126
β-E2	0.33	65.9
α-E2	0.32	63.9
E1	0.34	73.8

5.2.6 Statistical Analysis and Calculations

Statistical analysis was completed in JMP Pro 12. Analysis of variance, fit least squares, and in appropriate cases, a Kruskal-Wallis test was used to compare means. First order degradation kinetics was used to determine half lives in MS Excel. The degradation rate constant was determined using first order kinetic equation $[C]_t = [C]_0 e^{-kt}$ where $[C]_t$ is the concentration of the analyte, $[C]_o$ is the initial concentration of the analyte, k is the rate constant and t is time. The natural log of the concentration of each analyte in the soil was plotted over time, resulting in the linear equation, $ln[C]_t = -kt + ln[C]_0$. Half-life was determined using the equation $t_{\frac{1}{2}} = \frac{ln\,2}{k}$

5.3 Results and Discussion

5.3.1 Hormone Profile and Levels in Poultry Litter Spiked with 1,4-ADD and 4-AD.

When 2.73g poultry litter was applied to the soils, 24.1 µg 1,4-ADD, and 24.5 µg 4-AD were applied to the sandy loam soil, while 14.6 µg 1,4-ADD and 11.8 µg 4-AD were applied to the silt loam soil (*Fig 5.3*). The 2.73g of poultry litter contained 0.03 µg of α-E2 and 0.28 µg of E1. β-E2 was not detectable in the poultry litter (*Fig 5.3*). On average only 47.4% of the spiked 1,4-ADD and 45.0% of the spiked 4-AD was detected in the spiked poultry litter. This may have been caused by rapid dissipation of 1,4-ADD and 4-AD once spiked into poultry litter, or incomplete transfer of the spiking solution to the poultry litter. 4-AD has been detected in un-spiked poultry litter from less than 22.9 µg/kg up to 504 µg/kg (Valdehita et al., 2016). In other studies completed in this dissertation (Sosienski, Sections 3.3.1 and 4.3.1) 1,4-ADD has been detected in un-spiked poultry litter at a range of 162-109 µg/kg, 4-AD has been detected at a range of 40.3-144.3 µg/kg, E1 has been detected at a range of 7.6-34.6 µg/kg, and α-E2 and β-E2 have not been detected (Sosienski, Sections 3.3.1 and 4.3.1). The amount of hormones in a manure can vary based on many factors such as the age of the animal or the age of the litter. (Lange et al., 2002; Shore and Shemesh, 2003)

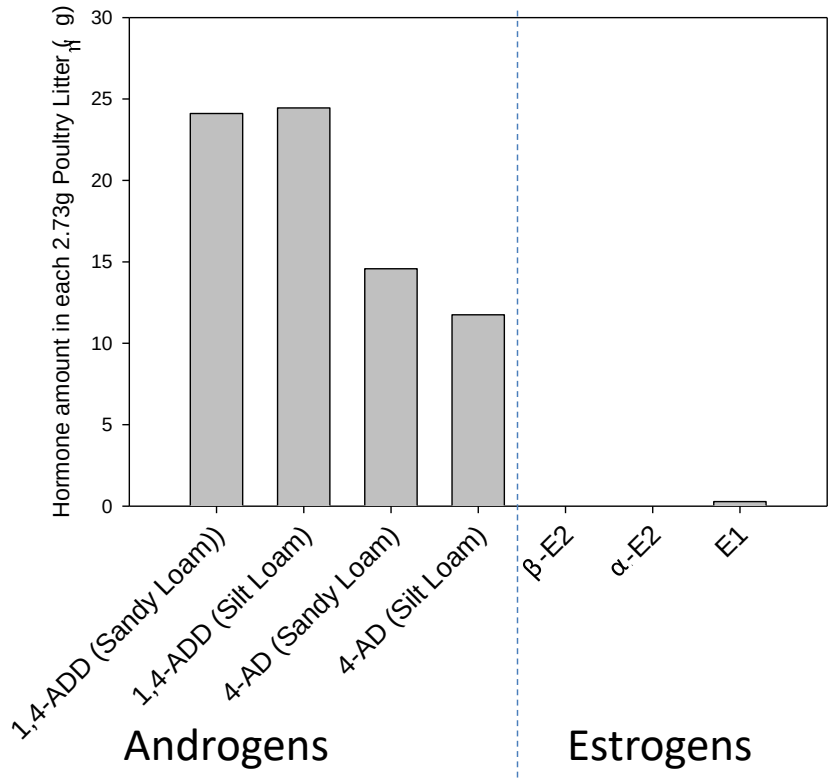

Fig 5.3. Amount of hormones in the poultry litter spiked with 1,4-ADD and 4-AD applied to each soil-microcosm.

5.3.2 Changes of 1,4-ADD and 4-AD with time in poultry litter amended soils

Overall there was great variation in the initial soil concentrations for all treatments, though they should have theoretically been the same. Some loss occurred during the spiking and the application of the manure, and the variation and size of poultry litter pieces may have played a role in the variation of the measurements. The measured initial concentration C_0 was used for the kinetic curves shown in *Fig. 5.4* and *Fig. 5.5* because the measured initial soil concentrations for 1,4-ADD and 4-AD were 20.8 to 86.1% lower than the expected initial soil concentration. *Fig. 5.4* shows the change with time of 1,4-ADD and *Fig. 5.5* shows the changes in 4-AD over

time in the silt loam and sandy loam soils, either surface applied or subsurface injected over the course of the 30 day study at two incubation temperature of 10°C and 20°C.

There was an increase in average concentration of 1,4-ADD and 4-AD for the majority of the treatments between day 0 and day 1, which had not been anticipated. This could be due to hormones becoming wet as the water from the soil is soaked into the manure and being released from the organic matter. It is also hypothesized that this could be due to the poultry litter being more easily incorporated into the soil during mixing when it is wet. This enabled more consistent levels of poultry litter to be collected in the subsample, and therefore more hormones also collected.

Overall, time was the significant main treatment factor, with time*treatment as a significant interaction for 1,4-ADD over the course of the 30 day experiment. Each day's soil 1,4-ADD totals were also significantly different from any other day. When broken down in to individual days, on day 0 and day 1, the only significant treatment was soil type, with the concentration of 1-4ADD in the silt loam soil was significantly greater than that of the sandy loam soil. At day 3 there were no significant differences between any treatments. On day 10, treatment type became a significant factor, with surface application having a significantly higher concentration of 1,4-ADD than subsurface injection. By day 30, no more 1,4-ADD was detected in the soil, with the exception of surface applied sandy loam soil at 10°C, where one replicate contained 4.13% of the initial detected concentration of 1,4-ADD in the soil.

For the overall 4-AD soil concentrations over time, day was a significant effect, and day*soil type was a significant interaction. On day 0 and day 1, soil type was a significant treatment, with silt loam soil having a greater concentration than sandy loam soil. By day 3 there were no significant differences between any of the treatments, but by day 10, application method was

significant, with surface applied poultry litter having a greater concentration of 4-AD than the injected poultry litter. Again by day 30 there were no significant differences in soil 4-AD concentration between treatments, as no more 4-AD could be detected in any treatment. Similarly to the behavior of 1,4-ADD, temperature had no effect on the concentration of 4-AD in the soil.

No significant differences were seen between temperatures for any treatment or day for the duration of this study for either 4-AD or 1,4-ADD. Few studies have been completed on temperature dependence of dissipation rates of 1,4-ADD specifically, however it has been shown that the dissipation rate of testosterone parent compound of 1,4-ADD and 4-AD, was slowed at cooler temperatures. A study by Lorenzen et al. (2005) determined that testosterone was rapidly dissipated at various environmentally relevant temperature and moisture contents, and indicated that the dissipation of testosterone was temperature dependent (Lorenzen et al., 2005). At 23-30°C the dissipation rates did not change, but the dissipation rate showed slowing at temperatures lower than 12°C, therefore we would have expected to see some differences between the dissipation rates of 1,4-ADD and 4-AD at 10°C and 20°C. It should be noted that in the same study it was determined that the change in concentration of testosterone was also biologically driven, with the dissipation rates of testosterone significantly lengthened by the sterilization of the soil. (Lorenzen et al., 2005) This was similar to an investigation where testosterone was transformed to 4-androstene-3,17-dione more rapidly than when applied to soil without any manure. (Jacobsen et al., 2005)

In an investigation of the persistence of hormones in beef feedlot soil no 4-AD in fresh manure was detected, but during the sampling of a fresh beef manure feedlot over 14 days, 4-AD had the highest mass of any hormone studied. In the same study, there was a rapid dissipation of

4-AD to 75% of its initial concentration once no more fresh manure was being added to the system. The rapid dissipation of 4-AD was hypothesized to be accelerated by wetting and drying cycles of the soil when bringing the soil up to field capacity every 5 days, as demonstrated in the rainfall simulation study of beef feedlots. In that study, researchers also observed a stability of estrogens in the feedlot soil over the course of the study (Mansell et al., 2011). Maintaining the field capacity at 70% by watering every 5 days could have contributed to more rapid dissipation of androgens, while preserving estrogen concentrations in the soil.

For both 1,4-ADD and 4-AD day*soil type was a significant interaction, meaning that while each variable had no overall effect, soil type had a significant effect on the concentration of 1,4-ADD and 4-ADD when taking day into account. The main differences between the two soils used in this study were particle size, organic matter content, and water holding capacity. In a study to determine the half-lives of estrogens and testosterone (1,4-ADD and 4-AD were not included in this study), it was determined that soil type was a significant factor in determining the half-lives of the hormones β-E2, 17α-ethinyl estradiol, and testosterone in various soil types, determining that sandier soil tended to lengthen the half-life of the hormones (Lee et al., 2003).

Many factors could have led to sample variation. Sampling time often took up to a few hours, leading to variations of a few hours between replicates of samples within the same time-point, which would be especially important for the early time-points, where the compounds were disappearing more rapidly. The application of poultry litter also posed challenges, as each pre-weighed poultry litter dose for each incubation beaker was spiked with hormone individually in order to avoid stratified hormone dosages in the litter, however individually spiking each poultry litter sample could have also lead to more hormones left behind on the weighing dish in which the samples were spiked in. Incomplete homogenization of the soil is likely the greatest source

of error in this experiment. Poultry litter is cakey and not easily broken apart with hand mixing, leading to high variation between subsamples.

The calculated half-lives in *Table 5.2* were longer for 1,4-ADD in manure applied to the surface of the soil when compared to the half-lives of 1,4-ADD for subsurface applied soil. In fact, ANOVA and a Wilcoxin/Kruskal-Wallis test performed using JMP software proved that treatment was a significant variable in determining the half-life of 1,4-ADD. With surface applied poultry litter contributing to a longer half-life of 1,4-ADD. The half-life of 4-AD in soil treatments were not affected by treatment. Temperature and soil type did not have significant effects on the half-life of either compound. The half-lives determined in this study are similar for the half-lives for testosterone applied without manure determined at 22°C, which ranged from 0.3-7.3 days among various soil types (Lee et al., 2003).

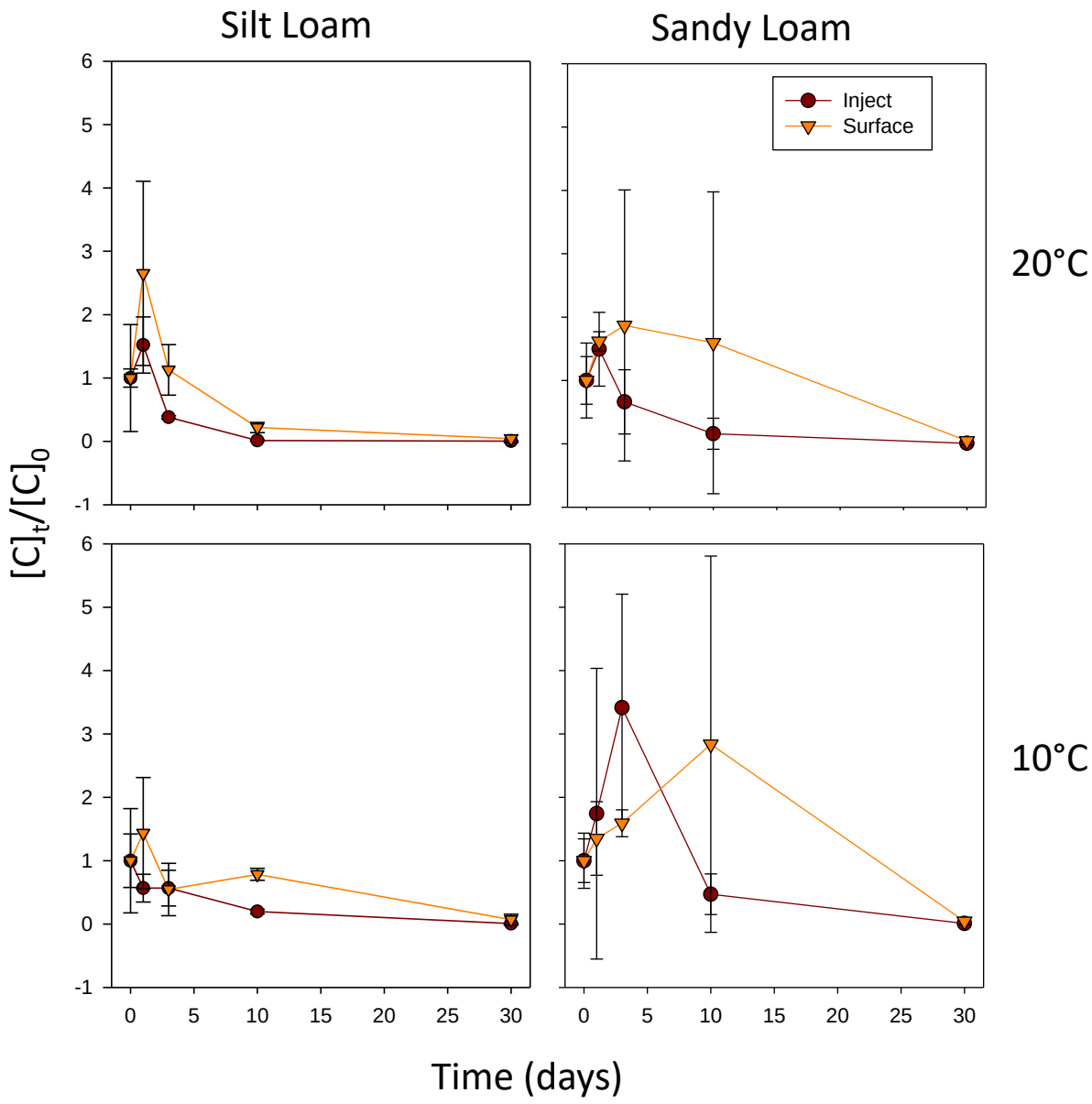

Fig. 5.4: Relative changes of 1,4-ADD levels with time in two soils amended with a poultry litter using two applications methods, surface application and subsurface injection.

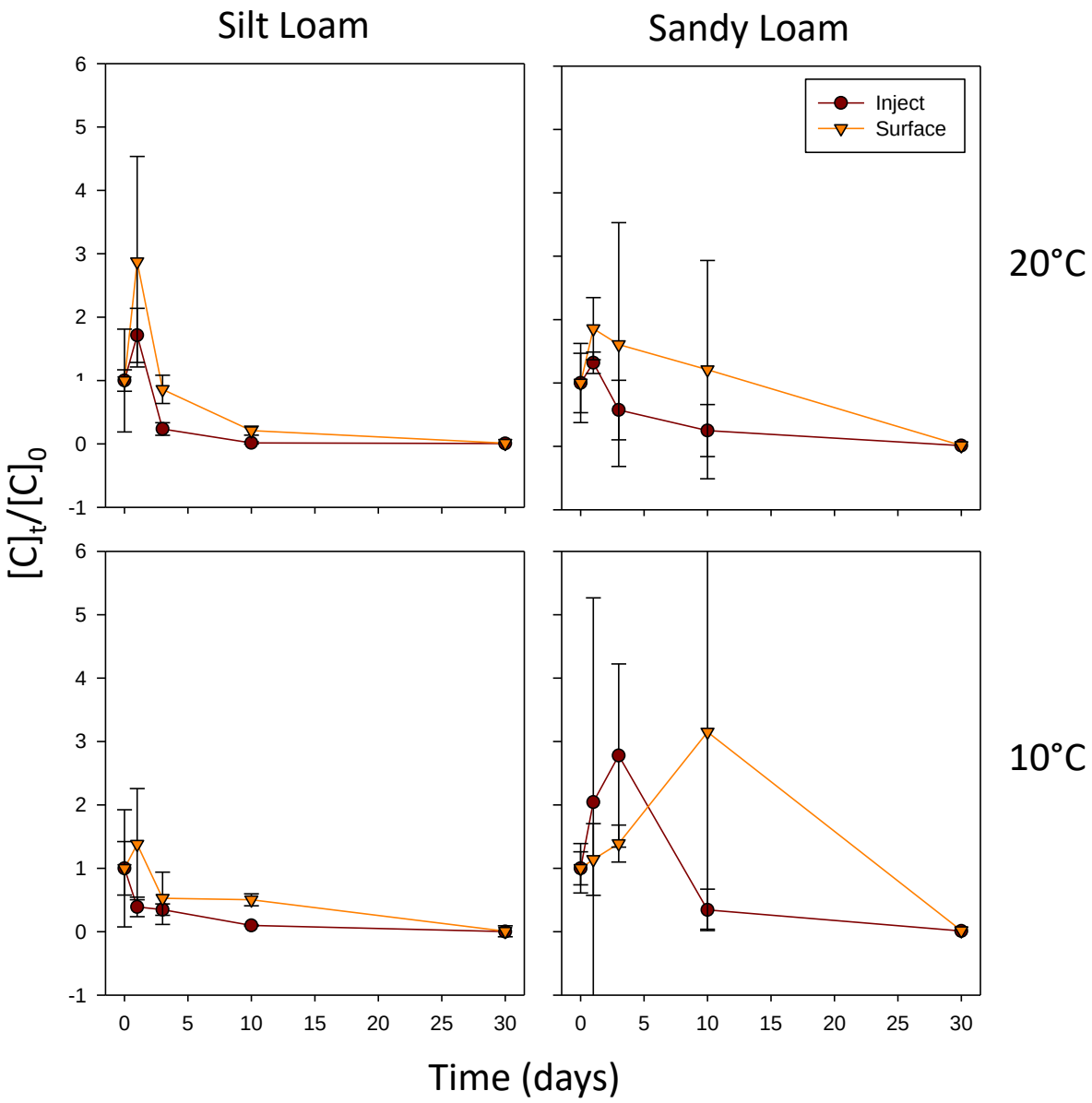

Figure 5.5: Relative changes of 4-AD levels with time in two soils amended with a poultry litter using two applications methods, surface application and subsurface injection.

Table 5.2: Calculated first order transformation kinetics of soil incubation experiments of 1,4-ADD and 4-AD when applied concurrently with poultry litter. First order equation y = ax + b, is relative to the equation: $ln[C]_t = -kt + ln[C]_0$.

1,4-androstadiene-3,17-dione

Soil Type	Treatment	Temperature	First Order Equation	R^2	K	t=1/2
Clay	Inject	21°C	y = -0.1945x + 3.5963	0.81	0.19	3.56
		10°C	y = -0.1902x + 4.203	0.99	0.19	3.64
	Surface	21°C	y = -0.1229x + 3.5046	0.89	0.12	5.64
		10°C	y = -0.0901x + 3.8361	0.89	0.09	7.69
Sand	Inject	21°C	y = -0.167x + 3.3044	0.98	0.17	4.15
		10°C	y = -0.1747x + 3.7721	0.92	0.17	3.97
	Surface	21°C	y = -0.1146x + 3.6711	0.85	0.11	6.05
		10°C	y = -0.1124x + 3.3609	0.73	0.11	6.17

4-androstene-3,17-dione

Soil Type	Treatment	Temperature	First Order Equation	R^2	K	t=1/2
Clay	Inject	21°C	y = -0.1773x + 3.3269	0.77	0.18	3.91
		10°C	y = -0.1791x + 3.9655	0.98	0.18	3.87
	Surface	21°C	y = -0.1643x + 3.4668	0.95	0.16	4.22
		10°C	y = -0.1668x + 3.8401	0.95	0.17	4.15
Sand	Inject	21°C	y = -0.1518x + 3.1853	0.99	0.15	4.57
		10°C	y = -0.169x + 3.7491	0.94	0.17	4.10
	Surface	21°C	y = -0.1559x + 3.6525	0.90	0.16	4.45
		10°C	y = -0.1388x + 3.3835	0.73	0.14	4.99

5.3.3 Changes of E1 with time in the poultry litter amended soils

The estrogen compounds α-E2 and β-E2 were below the detection limit in all soil samples. The initial E1 concentrations in the poultry litter applied soils ranged from 0.20-0.94 µg/kg and 0.15-0.86 µg/kg for the silt loam and the sandy loam, respectively. Unlike the spiked compounds 1,4-ADD and 4-AD, E1 soil concentrations remained at detectable levels throughout the entire 30 day study (*Fig 5.6*). When taking into account the amount of E1 detected in the

poultry litter used in the study, the calculated initial concentration of E1 in the soil microcosms should have been 1.04 µg/kg in the silt loam soil, and 0.95 µg/kg in the sandy loam soil. When compared to the calculated concentration of E1 in the soil microcosms based on the amount of hormones applied in the manure, the actual measured concentration of E1 in the initial soil concentrations 10-80% lower in concentration for the silt loam soil, and a 9.5-84% lower in the sandy loam soil. Because the actual soil E1 concentration ranges from very similar to the calculated initial concentration to surpassing the calculated initial concentration on a few days, it could be likely that the E1 is transforming from the spiked 4-AD and 1,4-ADD.

Until day 10, no significant differences in the concentration of E1 were seen between treatments, however from day 10 on, soil type was a significant factor, with sandy loam soil having a greater concentration of E1 than the silt loam soil. Soil type was also a significant overall effect for the concentration of E1 entire 30-day study, with sandy soils having a slightly higher concentration of E1 than the silt loam soil.

A previous study has shown that E1 could be transformed from β-E2 with or without the presence of microbial activity, however once transformed into E1, the compound needed microbial activity in order to allow for any further degradation (Colucci et al., 2001). Having more organic matter in the silt loam soils could have contributed to the lower E1 concentrations in those soils in this study when compared to the E1 concentrations in sandy soil. E1 can also be formed from conjugated estrogens, which are also naturally excreted from livestock. E1 is known to be formed from estrone-3-glucorinide (Shrestha et al., 2012). α-E2 and β-E2 are known to have relatively short half lives in soil and manure systems, with studies showing maximum half-lives at 4 days to 9.1 days, and even as low as 0.17 days (Carr et al., 2011; Lee et al., 2003; Xuan et al., 2008) in soil-water systems. Alternatively, a previous study by Carr et al.

(2011) has shown that E1 has a half-life of 27.5-56.8 days in saturated soil, though a different study by Xuan et al. (2008) reports E1 to have a half-life less than 3 days (Carr et al., 2011; Xuan et al., 2008).

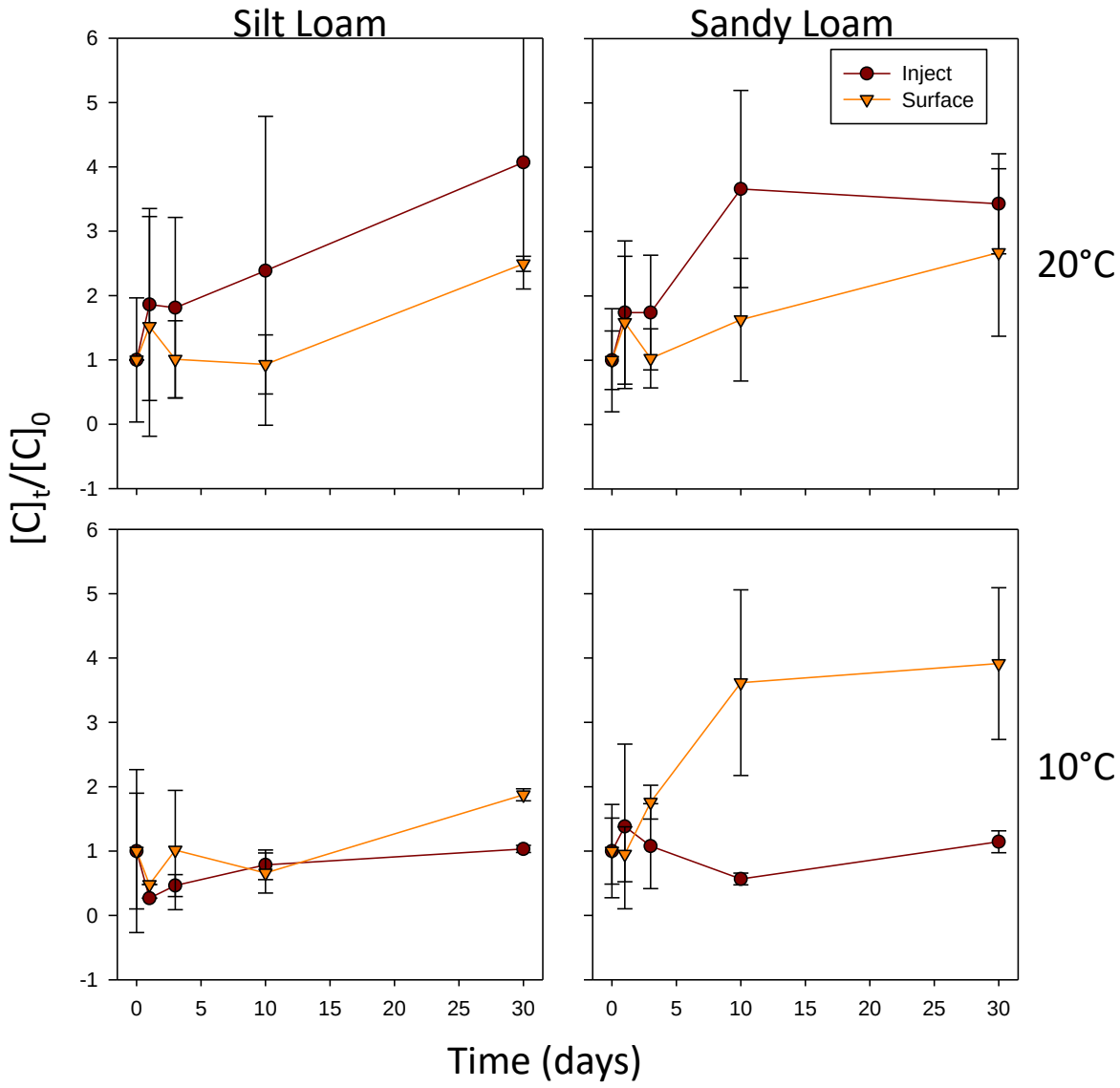

Fig. 5.6: The changes of E1 with time in the poultry litter amended soils incubated at two temperatures over 30 days.

5.4 Conclusion

The hormone precursors 1,4-ADD and 4-AD have been detected at proportionally greater levels in poultry litter than other hormones compounds, though little about their environmental persistence has been investigated. The half-life of 1,4-ADD ranged from 3.56-7.69 days, and the half-life of 4-AD ranged from 3.87-4.99 days, which provides some of the first information on the transformation rates of these compounds in soil-manure systems. Poultry litter subsurface injection is an emerging tool that can be used to mitigate nutrients and other contaminants from entering the environment through surface runoff; additionally, it was found that the subsurface injection of poultry litter allowed 1,4-ADD to degrade faster than when the poultry litter was surface applied. E1 was persistent in the soils and even slightly increased in concentration for the duration of the study, indicating that E1 could be transforming from other hormone compounds such as the spiked 1,4-ADD or the 4-AD in the soil.

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Chapter 6: Implications of Presented Research

Best management practices and new manure management techniques such as manure subsurface injection, show promise for reducing hormone transport into the natural environment. The implementation of these new manure management technologies is a complex problem that needs to be addressed in the near future. Unfortunately, the effects of hormones on the environment are not immediately visible. However, the effects of excess nutrients and sediments from manure runoff have more immediately apparent effects on aquatic wildlife. Implementing manure management guidelines such as manure injection or riparian buffer zones for reducing nutrient and sediment transport can in turn manage the environmental input of hormones. Surface application of manure is generally cheaper than subsurface injection and costs will likely need to be reduced to generate more interest in the adoption of the technology. Generating public interest could also be a good pathway for driving policy changes to promote the adaption of best management practices and adoption of new manure management technologies.

Research studying the concentrations of hormones in groundwater, streambed sediment, and stream water during storm events is needed to answer some remaining questions on the spatial and temporal distribution of hormones from a manure applied fields. Stream water samples taken during baseflow did not contain any hormones their detection limits, however this does not mean that there were no hormones in the water; hormones could have been contained in the sediment samples or were at very low levels. Sensitive and current analytical techniques need to be used for the analysis of environmental hormones, as hormones have been shown to cause negative impacts on aquatic organisms below part-per-billion levels in water. The majority of the studies of hormone environmental impact have been completed on animals. The human effects of long term exposure to exogenous hormones would be very challenging to directly

study, but by monitoring low levels of environmental hormones this information could be used to retroactively determine the effects of chronic low-level hormone exposure in humans. By reducing the input of environmental hormones from animal agriculture, the burden of finding adequate ways to remove hormones from drinking water supplies can be reduced.

Subsurface injection of manures reduced hormone content in the runoff when compared to runoff from surface applied manures. The manure subsurface injection confined the hormones to the manure injection slits, which reduced the surface area of manure that could be subject to surface transport. The poultry litter injector is a new technology, and with greater inputs of hormones from poultry litter when compared to dairy manure, the poultry litter injector can be a valuable tool for reducing hormone input to streams in poultry producing regions. It is advised that future studies involving manure subsurface injection set a proper soil sampling protocol in place which samples both within the injection slit and between injections slits, as the banding of the manure injection slits in the soil render traditional random sampling ineffective. Sampling a larger volume of runoff water is also advised, in order to collect enough sediment from the runoff for analysis.

By studying the hormone precursors 1,4-androstadiene-3,17-dione and 4-androstene-3,17-dione which have been found at high levels in poultry litter, some of the first information on the transformation rates of these compounds in manure and soil systems was provided, while also observing the persistence of another hormone, estrone. Because hormones will transform into other hormones or endocrine disrupting compounds in the environment, monitoring only one hormone per study is often not sufficient to provide enough data for any further projections. Monitoring environmental hormones from a holistic standpoint by studying many different types

of hormones, and as many locations and substances as possible is needed, as this will offer more complete information on the occurrence and fate of environmental hormones.