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Fate of Endogenous Steroid Hormones in Runoff from Cattle Feedlots

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Fate of Endogenous Steroid Hormones in Runoff from Cattle Feedlots

by

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Professor David Sedlak, Chair
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Professor Celine Pallud

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Abstract

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Steroid hormones, including estrogens, androgens, and progestogens, pose potential risks to sensitive aquatic organisms at extremely low concentrations. These compounds have been detected at concentrations high enough to affect aquatic life in water bodies impacted by animal agriculture. However, the mechanism through which steroids from animals reach surface waters and the factors affecting the transformation of the compounds after excretion are not well understood. To provide new insight into these issues, the occurrence, transformation, and partitioning of steroid hormones in cattle feedlot soil and runoff were studied at the laboratory, test plot, and field scales.

The current state of the science regarding steroid hormones from animal agriculture and the factors that could affect their fate and transport were reviewed (Chapter 1) To assess steroid fate and transport under controlled conditions, rainfall, runoff and soil samples were collected after simulated rainfall on a research steer feedlot under different rainfall rates and aging periods (Chapter 2). While only 17α-estradiol, testosterone, and progesterone were detected in fresh manure, 17β-estradiol, estrone, and androstenedione were consistently detected in the surficial soil (0-3 cm) after two weeks. Evidence of steroid transformation after excretion was observed in the feedlot soil, where concentrations of 17α-estradiol decreased by approximately 25% accompanied by an equivalent increase in estrone and 17β-estradiol. Further aging of the feedlot soils for an additional 7 days had no effect on estrogen and testosterone concentrations. In contrast, concentrations of androstenedione, a known metabolite of testosterone decreased substantially, while progesterone concentrations increased. Androstenedione and progesterone concentrations in the surficial soil were much higher than could be accounted for by transformation from testosterone, suggesting that other potential precursors, such as sterols, were converted after excretion. Concentrations of androgens and progesterone in the soil decreased by approximately 85% after simulated rainfall, while the estrogen concentrations remained approximately constant. The decreased masses could not be accounted for by runoff, suggesting rapid microbial transformation of the androgens and progesterone upon wetting. All six steroids in the runoff, with the exception of 17β-estradiol, were detected in both the filtered and particle-associated phases at concentrations well above thresholds for biological responses indicating that steroid hormones runoff at environmentally significant concentrations, and that they may be

transported in both phases.

To provide a better understanding of the interplay between microbial transformation reactions and partitioning, microcosms consisting of steer manure, soil, and water were studied (Chapter 3). Results indicated that the presence of manure caused rapid microbial transformation of steroid hormones, with nearly complete transformation of testosterone and progesterone and partial transformation of 17β-estradiol within 24 hours. After 24 hours, the transformation of 17β-estradiol ceased whenever more than 400 mg/L of manure was present. Stabilization of 17β-estradiol may have been due to partitioning of the compound into organic matter in the manure or changes in the microbial community. The rate of transformation of all three classes of steroids was faster in steroid-amended microcosms, suggesting that, under field conditions, steroids may be more stable than predicted by studies employing steroid amendments. Under conditions encountered in feedlots and manure-applied fields, androgens and progesterone are likely to be transformed in the soil or in runoff while estrogens likely persist long enough to be released to surface waters.

To determine the efficacy of a vegetated treatment system typical of those used to control nutrients for steroid hormone removal, samples were collected from a solids settling basin, vegetated infiltration basin, and a vegetated treatment area before and after ten storms over six months at a cattle feedlot (Chapter 4). The solids settling basin removed approximately 70% of the measured steroid hormones in the feedlot runoff except for estrone and progesterone, which were unaffected. Discharges from the solids settling basin contained steroid hormone concentrations that were several orders of magnitude above thresholds for biological responses. Steroid hormone concentrations were much higher in the sediments of the solids settling basin relative to those detected in the feedlot soil suggesting that the removal was caused primarily by settling. The next step in the treatment train, the vegetated infiltration basin decreased most of the steroid hormones below thresholds for biological response, except for 17β-estradiol and estrone, which exhibited increased concentrations. In the next step, the vegetated treatment area, the concentrations of all steroid hormones decreased below thresholds for biological responses. Soil from the vegetated infiltration basin and the vegetated treatment area contained very low concentrations of steroid hormones, suggesting that biotransformation was the dominant mechanism of removal in these systems.

To determine the relative steroid hormone contributions of tile drains from manure-applied fields and feedlot runoff, feedlot runoff from a commercial feedlot was analyzed for steroid hormones after winter storms for 3 years, and tile drain discharges from an area containing dairies and manure-applied fields was analyzed for steroid hormones over a 2-month period (Chapter 4). Steroid hormone concentrations in the feedlot runoff were similar to those observed from our plot-scale studies described in Chapter 2, and contained steroid hormone concentrations that were several orders of magnitude above thresholds for biological response. Only one of the tile drains sampled ever contained steroid hormones, and estrone was the only steroid detected above quantification limits. Tile drains are unlikely to contribute a significant mass of steroids to surface waters relative to feedlot runoff unless they exhibit significant macropore flow.

This research made a significant contribution towards understanding how steroids from feedlots reach surface waters and the factors controlling their stability and transport. We determined that steroids were much more stable in the presence of manure, and that their sorption does not follow simple equilibrium partitioning which helps explain their observed transport.

This dissertation is dedicated to my wife for all of her support and sacrifices that made it possible.

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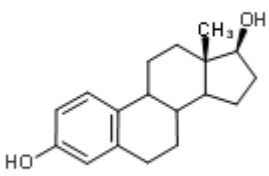
Chapter 1. Introduction

1.1 Steroid Hormones as Pollutants

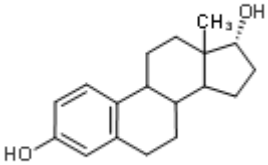
1.1.1 Introduction to steroid hormones

Steroid hormones are used to regulate metabolism, reproduction, development, and immune function in all vertebrates [Sandor & Mehdi, 1979]. Steroid hormones include compounds produced within the body (i.e., endogenous steroid hormones) and synthetic steroids used for growth promotion, contraception, and other medical purposes. Categories of steroid hormones include estrogens, the female sex hormones, androgens, the male sex hormones, and progestogens, the gestational hormones (Figure 1-1). Steroids are excreted as free-forms in the feces and as glucoronide and sulfate conjugates in the urine [Sandor & Mehdi, 1979]. Species, sex, age, pregnancy status, life stage, and drug administration all affect the types and concentrations steroid concentrations in tissues and fluids as well as excretion rates.

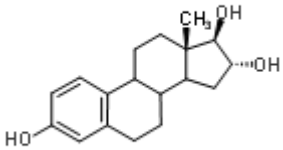
Estrogens



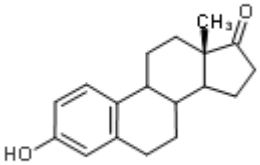
17β-estradiol (17β-E2)



17α-estradiol (17α-E2)

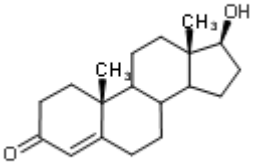


Estriol (E3)

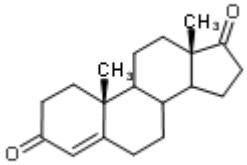


Estrone (E1)

Androgens

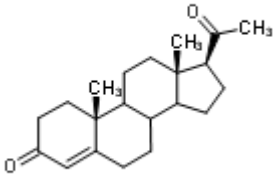


Testosterone (T)



Androstenedione (AD)

Progestogens



Progesterone (PR)

Figure 1-1. Structures of some common estrogens, androgens, and progestogens.

1.1.2 Steroid hormones in municipal wastewater effluent

In the early 1990s, anglers reported unexpectedly high occurrences of hermaphroditic fish near wastewater treatment plant outfalls in Great Britain [Purdom et al. 1994, Tyler et al. 1996, Tyler & Routledge 1998, Jobling et al. 1998]. Concurrent research showed that estrogens present in wastewater effluent could feminize certain species of fish at concentrations as low as 1 ng/L [Purdom et al. 1994, Kidd et al. 2007, Routledge et al. 1998]. Bioassays and direct measurements suggested that steroidal estrogens were often present in wastewater effluent at concentrations well above this threshold [Desbrow et al. 1998]. These findings have spurred research that has led to many studies examining the occurrence of steroid hormones in wastewater effluent and surface waters which have found them to be relatively widespread in the environment [e.g., Kolodziej et al. 2003, Chang et al. 2011, Kolok et al. 2007, Liu et al. 2009]. Other studies have reported the occurrence of feminized fish in surface waters [Jobling et al. 1998, Hinck et al. 2007, Jeffries et al. 2008], and have observed feminizing effects of steroid hormones and wastewater effluent on other aquatic species [Panter et al. 2000, Lai et al. 2002, Damman et al. 2011].

In addition to feminization of male fish by estrogens, masculinization of fish has been attributed to the presence of androgenic hormones, such as androstenedione, in wastewater effluent [Chang et al. 2011] and effluent from pulp and paper mills [Stanko & Angus 2007]. Androgens and progestogens also are present in municipal wastewater effluent at concentrations high enough to affect pheromonal communication and behavior of fish [Kolodziej et al. 2003, Sorenson & Stacey 1999, Murphy et al. 2001, Moore & Scott 1991, Sorenson et al. 1987].

The potency of each steroid with respect to its ability to induce biological and behavioral responses in aquatic life varies by species and by steroid. Among the steroids present in municipal wastewater, 17β-estradiol and 17α-ethinyl estradiol are the most potent endogenous compounds at feminizing fish, and are therefore the most studied. 17α-estradiol, estriol and estrone are typically less potent [Liu et al. 2009]. For example, 17β-estradiol is 8-9 times more potent than 17α-estradiol at feminizing male fathead minnows [Shappell et al. 2010] and 2-3 times more potent than estrone at inducing vitellogenin (a female egg yolk protein) expression in male rainbow trout [Thorpe et al. 2003].

While testosterone has the highest affinity for androgen receptors of the androgenic steroids [Liu et al. 2009], it has not been detected at concentrations capable of masculinizing fish in municipal wastewater effluent. Its primary metabolite, androstenedione, is approximately 300 times less potent with respect to binding affinity to androgen receptors [Fang et al. 2003]. Androstenedione has been shown to induce masculinization of fish at concentrations as low as 40 ng/L [Stanko & Angus 2007].

Testosterone and androstenedione are potent odorants in fish. Testosterone illicits pheromonal responses in Atlantic Salmon parr at concentrations as low as 0.003 ng/L [Moore & Scott 1991], and androstenedione can elicit behavioral responses in goldfish at concentrations as low as 0.3 ng/L [Sorenson & Stacey 1999]. Progesterone suppresses mRNA production and causes decreased fecundity in fathead minnows at concentrations as low as 10 ng/L [DeQuattro 2012]. While no threshold data are available for behavioral effects of progesterone on aquatic life, other progestogens, such as 17-α,20-β-dihyroxy-4-pregnen-3-one, have been shown to elicit pheromonal and odorant responses in goldfish at concentrations as low as 3 ng/L and 0.03 ng/L, respectively [Sorenson et al. 1999].

Municipal wastewater treatment plants (WWTPs) vary widely in their ability to

remove steroid hormones. [Baronti et al. 2000, Drewes et al. 2005, Chang et al. 2008, Chimchirian et al. 2007]. Average removal efficiencies for 17β-estradiol in WWTPs have been reported to be as high as 99.9% [Ternes et al. 1999] and as low as 28% [Svenson et al. 2003]. Reported concentrations of steroid hormones in municipal wastewater effluent are typically between 1 and 10 ng/L [Liu et al. 2009]. When dilution of the effluent by the receiving water is sufficient to lower steroid hormone concentrations below thresholds for biological effects (i.e. ~tenfold dilution), secondary biological treatment is usually sufficient for treating steroid hormones. If dilution is inadequate, advanced treatment processes, such as ozonation [Huber et al. 2003] and advanced oxidation processes [Rosenfeldt & Linden 2004], have been shown to decrease steroid hormone concentrations.

1.2 Animal Agriculture as a Source of Steroid Hormones

While municipal wastewater effluent has been the most studied source of steroid hormones to surface waters, elevated concentrations have also been detected in watersheds not affected by effluent discharges [Kolpin et al. 2001, Kolodziej et al. 2007, Jenkins et al. 2001, Kolok et al. 2007]. Because steroids are excreted by all vertebrates, any source of concentrated vertebrate waste that reaches surface waters has the potential to contribute to the concentration of steroid hormones in the environment. This has led to the study of other sources of steroid hormones, especially animal agriculture.

1.2.1 Animal waste production and steroid content

Livestock in the United States produce approximately 60 billion metric tons (6×10^{13} kg) of manure annually [US EPA 2000]. Manure often contains relatively high concentrations of steroid hormones. Steroid excretion rates and types vary by species, and cattle excrete relatively high concentrations, making them a potentially significant source to the environment. For example, a pregnant dairy cow excretes more estrogen per body mass than a pregnant human [Johnson et al. 2006, Ying et al. 2002]. Furthermore, steroids are effectively removed from municipal sewage in wastewater treatment plants while manure often receives little or no treatment prior to land application. Watersheds containing large numbers of concentrated animal feeding operations (CAFOs) are often polluted by pathogens and nutrients released from manure-treated fields or waste from feedlots. Therefore, it is likely that steroid hormones are also released to surface waters by animal agriculture.

Cattle, poultry, and swine are the most common livestock in the United States and produce the most steroid hormones [Lange et al. 2002]. They differ in the types of steroid hormones they excrete, and the concentration of steroid hormones in their wastes. For example, the dominant estrogen excreted by cattle is 17α-estradiol, while pigs and chickens excrete mostly 17β-estradiol and estriol [Hanselman et al. 2003]. In addition, cattle excrete steroids mostly in the feces (60-70%) while chickens and pigs excrete them mostly in the urine (70% and 96%, respectively) [Hanselman et al.2003, Lange et al 2002]. This is significant because steroids excreted in urine are conjugated with glucoronide or sulfate groups on the phenolic functional groups which makes them more soluble and less biologically active until deconjugated [Lee et al. 2007].

Among livestock, cattle likely contribute the largest amount of steroid hormones to the environment [Johnson et al. 2006]. An estimated 90% of the total estrogen load in the

environment potentially comes from livestock manure [Khanal et al. 2006]. Cattle have the largest mass, a very large population in the United States, and excrete large quantities of endogenous steroid hormones. In addition, they are often implanted with both synthetic and endogenous steroid hormones as growth promoters. The most common steroids used for growth promotion in the United States include combinations of various androgens, such as trenbolone acetate and testosterone; estrogens, such as 17β-estradiol and zeranol; or progestins, such as progesterone and melengestrol acetate [Lee et al. 2007].

Steroid excretion rates by cattle vary widely by sex, age, pregnancy status, and implant type. Concentrations of steroids in manure from steers (castrated males) are typically low with estimated average concentrations of 3 ng/g and 30 ng/g for estrogens and androgens, respectively [Arts et al. 1991, Lange et al. 2002, Johnson et al. 2006]. Manure from bulls (uncastrated males) contains roughly 3 times as much steroid hormones as manure from steers [Lange et al. 2002]. As with humans, pregnant cattle excrete about 100 times as much estrogen as males or non-pregnant females [Ying et al. 2003]. As a result, concentrations of estradiol in dairy cattle manure can be as high as 240 ng/g. [Hanselman et al. 2003]. Implants can also increase the concentration of steroid hormones in cattle waste. For example, a common implant containing trenbolone acetate and 17β-estradiol increased androstenedione, 17β-estradiol, and estrone concentrations in manure relative to non-implanted cattle by approximately 200, 500, and 700 %, respectively [Sellin et al. 2009]. Many researchers do not routinely measure 17α-estradiol concentrations, but cattle convert 17β-estradiol to 17α-estradiol prior to excretion [Rico 1983, Hanselman et al. 2003], so they likely excrete higher concentrations of 17α-estradiol, as well, when implanted with 17β-estradiol.

1.2.2 Impacts on aquatic ecosystems due to steroid hormone releases from CAFOs

The potential pathways for steroid hormones to reach surface waters from animal feeding operations include drainage from feedlots [Kolok et al. 2007, Lee et al. 2007, Matthiessen et al. 2006], rainfall- or irrigation-induced runoff from pastures and crops fertilized with manure [Finlay-Moore et al. 2000], groundwater discharges from waste lagoons [Arnon et al. 2008], groundwater discharges from waste-fertilized crops or pastures [Kjaer et al. 2007], and direct excretion into surface waters by animals with direct access to surface waters [Kolodziej & Sedlak 2007]. Steroid hormone concentrations in surface waters affected by these sources can be several orders of magnitude above thresholds for biological responses for sensitive aquatic organisms (Table 1-1). However, concentrations detected in surface waters are usually much lower due to dilution, with only sporadic detections of high steroid concentrations [Kolodziej et al. 2007, Velicu & Suri 2009, Chang et al. 2009, Lavado et al. 2008].

Table 1-1. Maximum concentrations of steroid hormones detected in surface waters affected by animal husbandry.

Location	Maximum Concentration (ng/L)					
	17β-E2	**17α-E2**	**E1**	**T**	**AD**	**PR**
Dairy farms (surface waters)[a]	0.7	NA	17	1.9	NA	NA
Streams receiving CAFO runoff[b]	290	NA	NA	NA	NA	NA
Streams near grazing cattle[c]	1.7	25	38	2.3	44	27
Aquaculture drainage[a]	NA	NA	1	1	1	NA
Beef cattle waste lagoons[d]	0	7	30	NA	NA	NA
Dairy waste lagoons[d]	150	200	90	NA	NA	NA
Dairy washwater[e]	150	1400	540	NA	NA	NA
Leachate from pig slurry-fertilized field[f]	0.2	NA	68	NA	NA	NA
Pasture runoff[g]	150	NA	NA	120	NA	NA
Poultry litter-fertilized pasture runoff[g]	2500	NA	NA	1800	NA	NA
Threshold for sensitive species	~1[h]	9[i]	5[j]	0.003[k]	0.3[l]	10[m]

17β-E2=17β-estradiol. 17α-E2=17α-estradiol, E1=estrone, T=testosterone, AD =androstenedione, PR=progesterone, NA=not analyzed. a) Kolodziej et al. 2004, b) Matthiessen et al. 2006, c) Kolodziej & Sedlak 2007, d) Hutchins et al. 2007, e) Zheng et al. 2008, f) Kjaer et al. 2007, g) Finlay-Moore et al. 2000, h) Purdom et al. 1994, i) Shappell et al. 2010, j) Liu et al. 2009, k) Moore & Scott 1991 (odorant), l) Sorenson & Stacey 1999 (odorant), m) DeQuattro 2012

1.2.3 Waste management strategies

Due to the potential harmful impacts of discharges from animal agriculture on surface waters, CAFOs are often required to have waste management plans. Most of these plans are focused on nutrients and pathogens [US EPA 2000]. The most common approaches for minimizing impacts of CAFOs include application of manure to crops and pastures, infiltration of liquid waste into soil, composting, and treatment of waste in lagoons or wetlands [USDA 2002, US EPA 2000, Lee et al. 2007]. In many cases, a combination of approaches is used with water and solids from waste lagoons and composted solids being spread on crops and pastures [Combalbert et al. 2010]. Although these approaches can be effective with respect to nutrients and pathogens, it is unknown whether they reduce concentrations of steroid hormones below thresholds for biological responses.

The detection of elevated concentrations of steroid hormones in surface waters near CAFOs suggests that current waste management practices are not always effective. Most previous studies on waste management strategies have not included steroid hormones, so their fate in treatment systems is not well understood. A better understanding of how steroid hormones are affected by current treatment technologies is needed to help determine the need for improvement or modification of current practices.

1.3 Steroid Fate on Feedlots

Previous research has documented the presence of elevated concentrations of steroid hormones in surface waters near feedlots. However, the transport of steroids between its source and surface waters is poorly understood. For example, it is known that the mass of steroid hormones in runoff from feedlots represents a small fraction of the mass of excreted steroids [Lange et al. 2002, Arts et al. 1991]. Therefore, most of the steroids excreted by cattle either remain associated with soils on the feedlot or undergo biotransformation. By understanding the partitioning of steroid hormones between solids and water as well as the rate at which steroid hormones are transformed it may be possible to design strategies to decrease their release to surface waters.

1.3.1 Fates of excreted steroid hormones on feedlots

In cattle, steroids are excreted primarily in feces -a matrix that is approximately 20% solids by mass. The solids consist largely of organic matter (~90%) -a mixture of mostly carbohydrates, lipids, and microorganisms. The high concentration of solids and organic matter implies that the steroid hormones are likely to be associated with particles, and the large amount of microbial activity in the manure makes it likely that they will undergo biotransformation. After the manure dries and is mixed with the feedlot soil by the hoof action of the animals, it is often spread on fields. Under these conditions, the steroids and organic matter are likely to remain in association with soil organic matter.

When rainfall or irrigation occurs, some of the soil-associated steroids may desorb from the particles and undergo transport in the dissolved phase in runoff or groundwater. In addition, the particles that steroids are associated with may be transported in runoff. It is also possible that some of the steroids undergo transport in association with colloidal or dissolved organic matter. Most feedlots are lined with 1' thick clay layers or built in areas with soils containing high clay fractions which limits movement of runoff through the soil. Therefore, transport to groundwater is more likely once the manure has been applied to fields. Land-applied steroids may be transformed by microorganisms, remain associated with organic matter or mineral surfaces in the soil, or undergo transport in runoff or groundwater. An understanding of how steroids interact with soils and organic matter, how they are transported in runoff and groundwater, and how they are transformed by microorganisms is essential to predicting their fate in the environment and the fraction of the excreted mass of steroids that reaches surface waters.

1.3.2 Partitioning to solids and organic matter

Steroid hormones absorb readily to organic matter in manure and soils through hydrophobic partitioning [Lee et al. 2003]. Hydrophobic partitioning to organic matter in soil is usually described with simple, linear partitioning models using a partitioning coefficient, K_d, that relates the fraction of compound in the dissolved and sorbed phases:

$$C_s = K_d C_{aq} = K_{oc} f_{oc} C_{aq} \quad (1)$$

where C_s is the concentration of the compound associated with the soil (mass of compound/mass of soil), K_{oc} is the organic-carbon-normalized partitioning coefficient (volume of water/mass

of organic carbon in soil), f_{oc} is the fraction of the soil that is organic carbon, and C_{aq} is the concentration of the compound in the dissolved phase (mass of compound/volume of water) [Schwarzenbach et al., 2003). The partitioning coefficient, K_{oc}, is often correlated with the octanol-water partitioning coefficient, K_{ow}.

According to this model, the fraction of a compound associated with the solid phase will increase as the concentration of solids increases or as the fraction of the soil that is organic matter increases. By summing the mass of compound in the two phases:

$$M_{total} = M_{part} + M_{diss} \quad (2)$$

where M_{total} is the total mass of the compound, M_{part} is the particle-associated mass, and M_{diss} is the dissolved mass, and using the relationships between concentration and mass:

$$M_{part} = C_s * S \quad (3)$$

$$M_{diss} = C_{aq} * V \quad (4)$$

where S is the mass of soil and V is the volume of water, all four equations can be combined to predict the fraction of the total mass of a compound in the dissolved phase, f_{diss}, under different conditions:

$$f_{diss} = \frac{1}{1 + K_{oc} f_{oc} \frac{S}{V}} \quad (5)$$

Observed log K_{oc} values in soil and sediment are typically between 3 and 4 L/kg [Lee et al. 2003, Das et al. 2004, Schiffer et al. 2004, Holthaus et al. 2002]. As a result, under conditions encountered in rivers or streams, where suspended solids concentrations are typically less than 50 mg/L ($5x10^{-5}$ kg/L), nearly all of the steroids should be in the dissolved phase. In saturated soil, where solids concentrations would be in the range of 1-10 kg/L, nearly all of the steroids should be associated with soil organic matter.

In feedlot runoff, suspended solids concentrations typically range between 1-10 g/L (10^{-3}-10^{-2} kg/L) with organic carbon fractions typically between 10-20%. Under these conditions, the fraction of steroid hormones in the dissolved phase is expected to range between approximately 5 and 90% (Figure 1-2). Therefore, the more hydrophobic steroids may be transported mostly associated with suspended solids while the less hydrophobic steroids may be transported mostly in the dissolved phase.

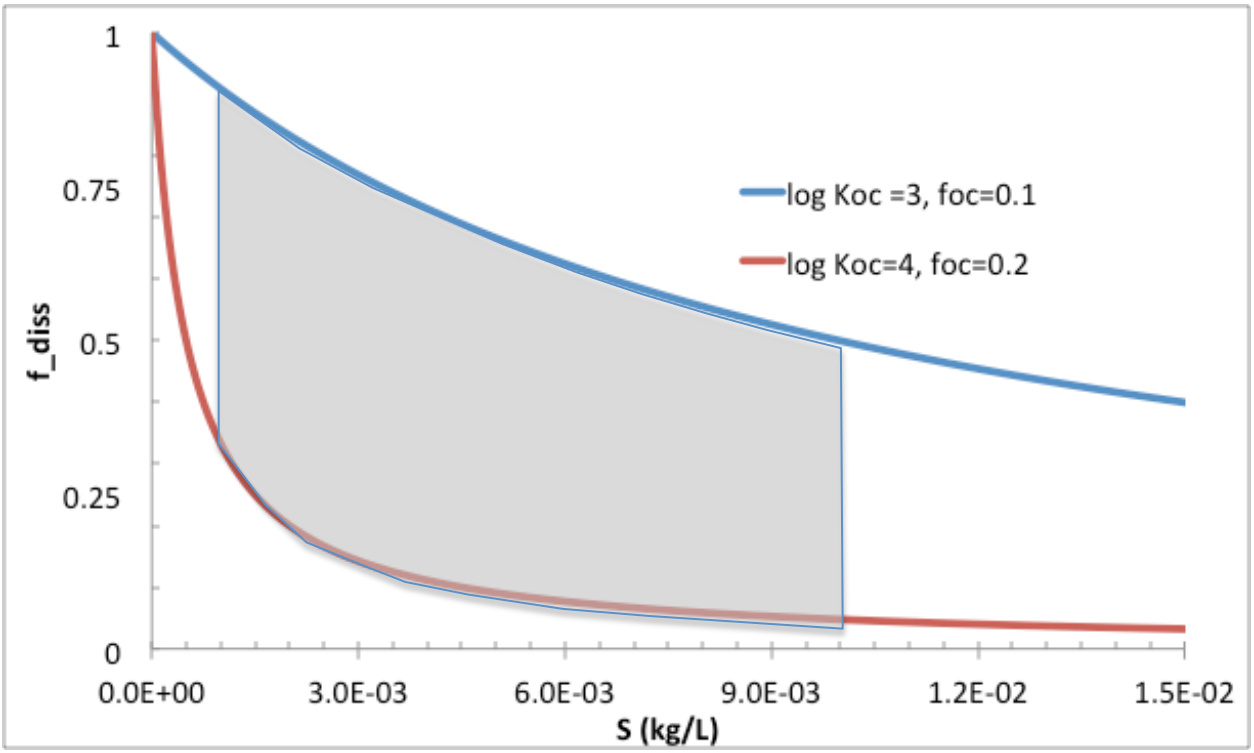


Figure 1-2. Fraction of steroid hormones in dissolved phase as a function of suspended solids concentrations. Shaded portion represents conditions typical of steroid hormones in feedlot runoff.

Attempts to model the transport of steroid hormones from feedlots is further complicated by the fact that partitioning of steroids to soil particles does not always behave as predicted by equilibrium partitioning models [Das et al. 2004, Casey et al. 2008, Steiner et al. 2010, Fan et al. 2008]. Observed K_{oc} values for steroid hormone partitioning to soil are almost never proportional to the K_{ow} values for steroids. For example, androstenedione has the lowest K_{ow} of the endogenous steroids (Table 1-2), but is often reported to have the highest K_{oc} in experiments observing steroid hormone partitioning to soil [Lee et al., 2003, Das et al. 2004]. Because K_{ow}'s are based solely on hydrophobic partitioning, the difference between these two partitioning coefficients implies other kinds of partitioning such as H-bonding and π-bonding may also be important [Lee et al. 2003, Das et al. 2004, Schiffer et al. 2004, Holthaus et al. 2002]. Steroids also adsorb to clays and iron oxides -a factor that complicates attempts to model their partitioning to solids [Shareef et al. 2006, Van Emmerik et al. 2003]. In addition, the organic matter from fresh manure is heterogeneous and complex, containing numerous types of compounds including lipids, aliphatic compounds, starches, cellulose, proteins, aromatics, etc. As a result, partitioning of steroids to manure could be affected by factors associated with the manure, such as the amount of time it has been subjected to microbial transformation because this affects the types of compounds present in the organic matter as more labile types are transformed.

Table 1-2. Octanol-water partitioning coefficients for steroid hormones [Liu et al. 2009]

Steroid	17α–E2	17β-E2	E1	E3	T	AD	PR
log K_{ow}	4.0	4.0	3.1	2.5	3.3	2.8	3.9

17α-E2=17α-estradiol. 17β-E2=17β-estradiol, E1=estrone, E3=estriol, T=testosterone, AD=androstenedione, PR=progesterone.

Partitioning isotherms, like those derived from equation 1, assume that the soil-water system has reached equilibrium. Over the timescales relevant to rainstorm-runoff or irrigation-runoff events on feedlots and fields, equilibrium may not be achieved. Equilibration times for sorption of steroid hormones to soil vary widely, with some investigators reporting equilibration times of a few hours [Lee et al. 2003, Lai et al. 2000, Mansell & Drewes 2004] while others have reported times between 2 and 14 days [Yu et al. 2004, Schiffer et al. 2004, Bowman et al. 2002]. Runoff events are likely to occur on the timescale of hours. Therefore, equilibrium-based models may not accurately predict steroid behavior during runoff events.

When transport and partitioning models fail, they almost always underestimate the extent of transport in the subsurface and/or the fraction of steroid hormones in the mobile phase [Das et al. 2004, Casey et al. 2008, Steiner et al. 2010, Fan et al. 2008]. This implies that steroids may be more mobile than expected based on equilibrium hydrophobic partitioning models. It also helps explain why steroid hormones are often detected in runoff and groundwater leachate from manure-applied fields [Finlay-Moore et al. 2000, Kjaer et al. 2007, Lucas et al. 2009, Laegsmand et al. 2009] and in surface waters near feedlots and CAFOs [Matthiessen et al. 2006, Jeffries et al. 2011].

1.3.3 Effect of colloidal organic matter on transport of steroid hormones

Colloidal organic matter is often invoked as an explanation when higher-than-expected concentrations of steroids are observed in the filterable phase [Casey et al. 2003, Bowman et al. 202, Stumpe & Marschner 2007, Zitnick et al. 2011] or when faster-than-expected transport is observed in the subsurface [Stanford et al. 2010, Steiner et al. 2010, Fan et al. 2008]. Organic colloids are particles that are large enough to have a hydrophobic microenvironment where other compounds can partition (>~1 kDa), but small enough to not undergo gravitational settling (<~1 μm) [Gustaffson & Gschwend 1997]. This colloidal organic matter consists of humic acids, cellulose, lipids, and other biological mass from the partial breakdown of plants and animals. It has been shown that organic colloids can increase the transport of hydrophobic contaminants, such as polyaromatic hydrocarbons (PAHs) [Bergendahl 2005, Laor & Rebhun 2002, Schlautman & Morgan 1993] and dichlorodiphenyltrichloroethane (DDT) [Carter & Suffet 1982], radionucleotides [Sen & Khilar 2006], pesticides [Means & Wijayaratne 1982, Crossan et a;. 2002], herbicides [Irace-Guigand & Aaron 2003], and nutrients [Dao et al. 2008] in both groundwater and surface water.

Colloidal organic matter acts as a third phase into which hydrophobic organic compounds can partition. In the presence of colloidal organic matter, equation 2 becomes:

$$M_{total} = M_{part} + M_{diss} + M_{coll} \quad (6)$$

where M_{coll} is the mass of the compound associated with colloidal organic material. Assuming the compound partitions between the colloid-associated and the dissolved phases in a similar manner to the soil organic matter, the relationship can be written as:

$$C_c = K_c C_{aq} \tag{7}$$

where C_c is the concentration of the compound associated with colloidal organic matter (mass of compound/mass of colloidal organic matter) and K_c is the partitioning coefficient for a compound and colloidal organic matter (vol water/mass of colloidal organic matter). Equation 5 can then be adjusted to incorporate the colloidal phase as:

$$f_{diss} = \frac{1}{1 + K_c\frac{O}{V} + K_{oc} f_{oc} \frac{S}{V}} \tag{8}$$

where O is the mass of colloidal organic material. Both the dissolved phase and the colloidal phase can pass through most types of filters employed prior to quantitative analysis. Therefore, the fraction of the compound passing through a filter (f_{filt}) can be written as:

$$f_{filt} = \frac{1 + K_c\frac{O}{V}}{1 + K_c\frac{O}{V} + K_{oc} f_{oc} \frac{S}{V}} \tag{9}$$

Because both types of filterable organic compounds can be transported in groundwater and runoff, colloidal organic matter increases steroid transport by increasing the fraction of the total mass in the mobile phase.

Steroid hormones exhibit an affinity for organic colloids equal to or greater than that of organic matter-rich suspended particles and soils; reported log K_c values range between 3 and 6 L/kg [Neale et al. 2009, Yamamoto et al. 2003, Liu et al. 2005, Zhou et al. 2007]. Manure is approximately 90% organic matter, and therefore is a potentially important source of colloidal organic matter. Dissolved organic carbon concentrations in feedlot runoff are typically between 0.3-1 g/L). Using equations 8 and 9 along with reported K_c values from the literature, between 37% and 99% of the steroids in the filterable phase are predicted to be associated with colloids.

1.3.4 Microbial transformation of steroid hormones in soil, manure, and water

Steroid hormones undergo a number of biological transformation reactions *in vivo*. The inter-conversions of steroids involve transformation of cholesterol by enzymatic reactions (Figure 1-3). Many microorganisms in manure and soil contain enzymes capable of causing the transformation of steroid hormones. For example, Lee & Liu (2002) exposed 17β-estradiol to bacteria isolated from sewage sludge and observed a metabolic pathway where the alcohol moiety on 17β-estradiol was oxidized to form estrone, which was further oxidized through several non-steroidal compounds as it underwent mineralization (Figure 1-4)[Lee & Liu 2002].

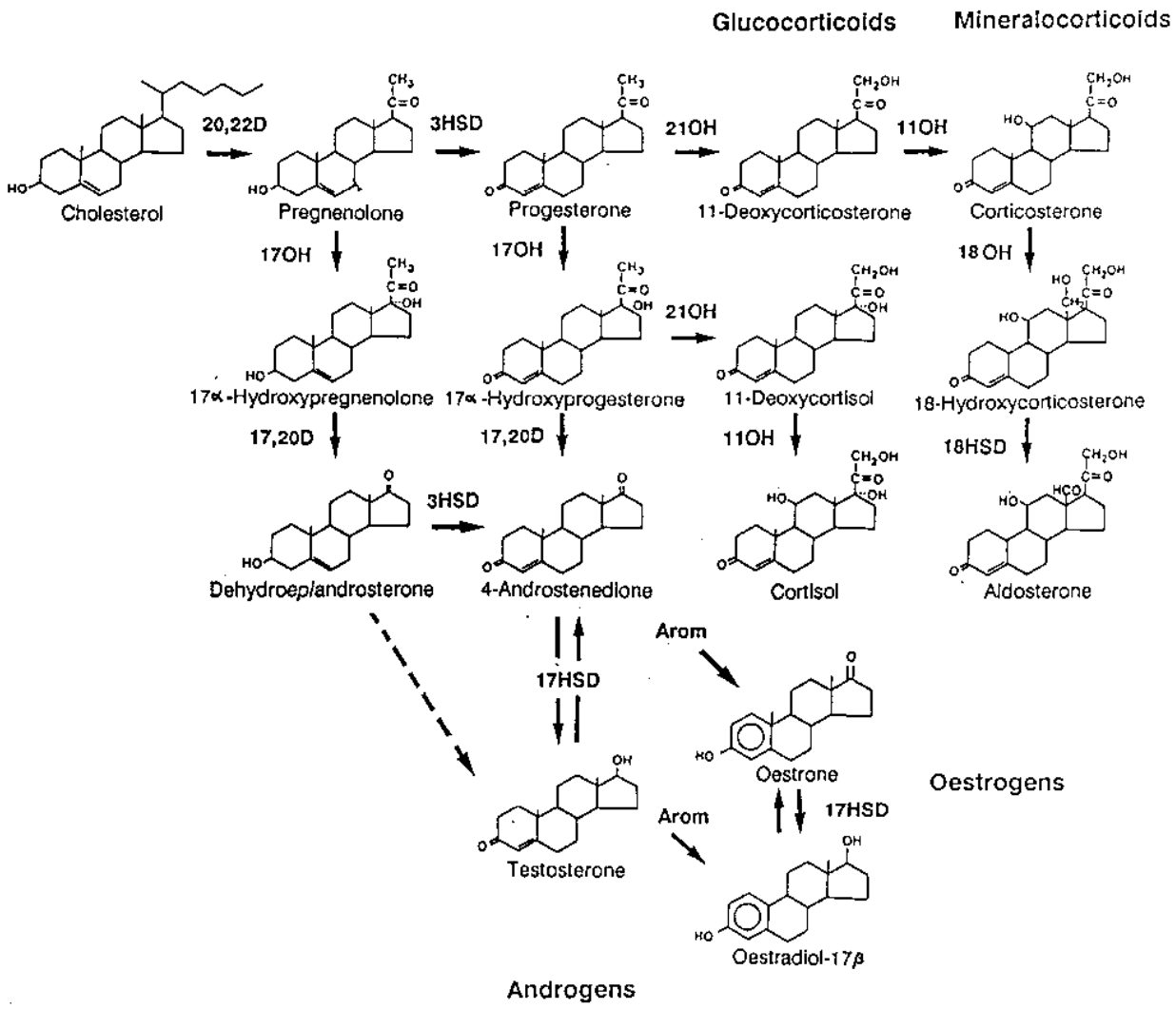


Figure 1-3. Major pathways for steroid synthesis within the body.
http://www.gfmer.ch/Books/Reproductive_health/Steroid_hormone_metabolism_Fig2.html

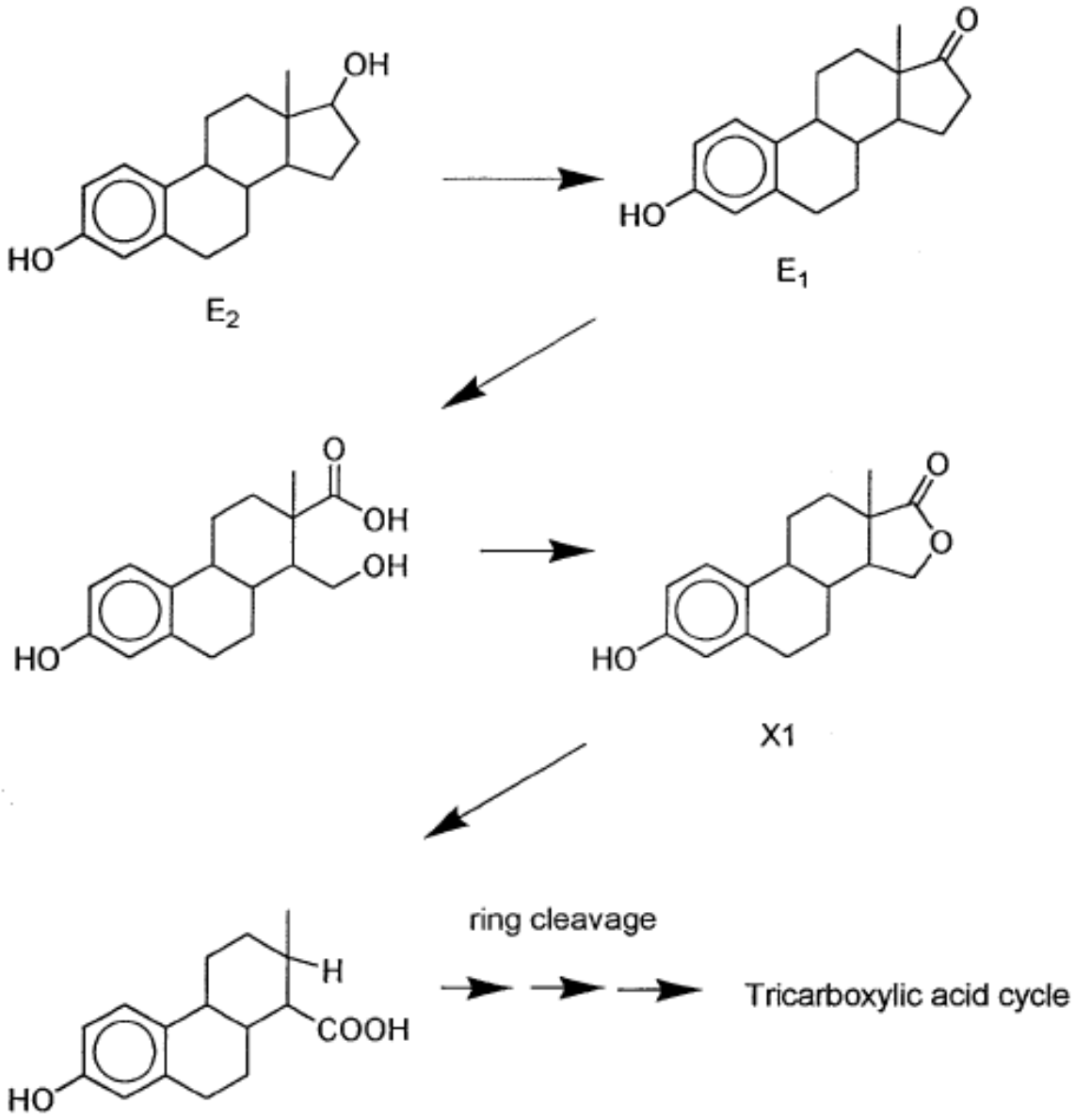


Figure 1-4. Metabolic pathway for 17β-estradiol by sewage bacteria. From Lee & Liu 2002. E2=17β-estradiol, E1=estrone, X1=unnamed observed metabolite.

Microorganisms capable of oxidizing 17β-estradiol to estrone under oxic conditions are ubiquitous in the environment and require no prior adaptation [Colucci et al. 2001]. 17β-estradiol undergoes conversion to estrone by microorganisms in dairy waste solids [Raman et al. 2001], soils [Lee et al. 2003, Colucci et al. 2001, Casey et al. 2003, Das et al. 2004], river water [Jurgens et al. 2002], and wastewater [Joss et al. 2004] over timescales ranging from hours to days. 17β-estradiol can also be oxidized to estrone under anoxic conditions, but with slower transformation than under oxic conditions [Czajka et al. 2009, Jurgens et al. 2002, Yang et al. 2010, Zheng et al, 2012].

The transformation of 17α-estradiol by microorganisms has not been as well studied as that of 17β-estradiol, but increases in estrone concentration have been observed as 17α-

estradiol disappeared from dairy waste [Zheng et al. 2008] and dairy waste lagoons [Zheng et al. 2012]. Although data are very limited, 17α-estradiol seems to be converted to estrone at a slower rate than 17β-estradiol, with half-lives in anoxic dairy lagoon water that were 25 times longer than those of 17β-estradiol [Zheng et al. 2012]. There is also evidence of inter-conversion between the two epimers through estrone under anoxic conditions [Czajka et al. 2009, Zheng et al. 2012].

Cleavage of the cyclopentane ring on estrone appears to be more difficult than oxidation of the alcohol on estradiol. As a result, estrone is transformed much more slowly than estradiol. For example, 17β-estradiol was transformed into estrone in several soils within hours, but estrone persisted for up to 3 months [Colucci et al. 2001]. Estrone and its metabolites are slowly mineralized by microorganisms. In oxic soils, mineralization accounted for 5-17% of the initial 17β-estradiol after 3 months incubation [Colucci et al. 2001, Stumpe & Marschner 2007].

Analogous to estradiol, the alcohol moiety on testosterone is readily oxidized to form androstenedione under oxic conditions in soil [Das et al. 2004, Lee et al. 2003, Lorenzen et al. 2005, Stumpe & Marschner 2007] and manure [Yang et al. 2010], with half-lives also on the timescale of hours to days. Androstenedione is less stable than estrone, however, with mineralization rates that are about ten times faster than those of 17β-estradiol in soils [Stumpe & Marschner 2007, Fan et al. 2007] and twice as fast in biosolids [Layton et al. 2003].

Very little is known about progesterone transformation by microorganisms in the environment. Progesterone concentrations decreased below detection limits in less than 1 day in microcosms simulating river water with various types of organic matter [Lim et al. 2008], and in microcosms made with pig manure [Yang et al. 2010]. Oxygen concentrations were not reported, but in both cases, progesterone transformation rates were similar to the rates for testosterone under oxic conditions but no stable intermediates were identified. Progesterone is removed from municipal wastewater to a similar degree as testosterone [Chang et al. 2011, Kolodziej et al. 2003]. On the basis of these limited data, it may be assumed that progesterone is transformed with half-lives on the timescale of hours to days in animal manure under oxic conditions.

Despite numerous reports suggesting rapid biotransformation of steroid hormones in the presence of microorganisms, several researchers have observed little or no transformation of the compounds in the presence of manure. For example, Schlenker et al. (1998) observed nearly constant levels of estrogenic activity in bovine manure during 9 weeks of incubation at 23° C, but did not report oxygenation conditions [Schlenker et al. 1998]. Other researchers have reported that storage of cattle manure for 7 days at room temperature had no effect on estradiol concentrations [Bamberg et al.1986]. Under similar conditions, a half-life of 25 days was reported for bovine fecal progestins [Masunda et al. 1999].

Even in soil, the addition of organic matter from animal manure, animal urine, and wastewater has been shown to significantly decrease the rate of steroid transformation [Stumpe & Marschner 2007, Stanford et al. 2010, Stumpe & Marschner 2010, Lucas et al. 2009, Zitnick et al. 2011, Caron et al. 2012]. For example, the addition of colloidal and dissolved organic matter derived from swine manure to soil caused 17β-estradiol to persist throughout the 14-day experiment while it was completely transformed into estrone within 7 days in the absence of the manure-derived organic matter [Zitnick et al. 2011]. Thus, when animal waste is present, or when steroids come from animal waste, it is possible that steroids may persist for weeks or months.

The increased persistence of the steroid hormones in the presence of manure has several possible explanations. First, manure consists almost entirely of organic matter. As discussed

earlier, steroid hormones exhibit a strong affinity for organic matter. Steroids that are absorbed by particulate or colloidal organic matter may be less bioavailable to microorganisms because only dissolved compounds can undergo enzymatic transformation. Decreased bioavailability in the presence of organic matter in soil has been demonstrated for more hydrophobic pollutants such as PAHs [Salloum et al. 2002, Carmichael & Pfaender 1997, Johnsen et al. 2005], DDT [Di Toro et al. 1991], and pesticides [Di Toro et al. 1991]. These compounds have log K_{ow} values ranging from 4 to 7 compared with a range of 2.8 to 4 for the steroid hormones. Therefore, the usual, small amount of organic matter in the soil is unlikely to affect biotransformation rates of steroid hormones. However, the very high concentrations of organic matter in manure and manure-amended soils could result in decreased bioavailability for moderately hydrophobic compounds, such as the steroid hormones.

Second, increased microbial activity caused by the breakdown of labile organic compounds in manure could deplete oxygen and cause anoxic conditions. As discussed previously, anoxic conditions slow the rate of microbial transformation reactions of steroid hormones [Czajka et al. 2006, Hashimoto et al. 2009].

Third, many feedlots are located in regions that experience periods of very low rainfall such as California or the southwestern U.S. Often, the surface of the feedlots consists of a hardpan of clay soil. When manure is deposited on the hardpan it quickly dessicates and is broken apart. Below field capacity, microbial activity [Miller & Johnson 1964] and steroid transformation rates [Colucci et al. 2001, Khan et al. 2010] decrease with decreasing moisture content. Therefore, dry conditions could prolong the lifetime of steroids on feedlots.

1.4 Motivation and Research Objectives

1.4.1 Motivation

Elevated concentrations of steroid hormones have been detected in surface waters impacted by animal agriculture, but the factors that determine the fraction of excreted hormones that reach surface waters are unknown. Laboratory studies and simple transport models suggest that steroid hormones should remain associated with the soil and manure solids of the CAFO site where they will eventually be broken down by microorganisms. However, field observations suggest that steroid hormones are transported to surface waters, and can persist in soil and manure for extended periods. To reconcile these inconsistencies, research is needed to better describe the field-scale observations. There is also a need to better understand the factors that control steroid hormone sorption and transformation. Such information can provide a basis for evaluating treatment practices designed to reduce the release of steroids hormones to the aquatic environment. The following sections describe experiments designed to address these issues as presented in subsequent chapters.

1.4.2 Chapter 2: *Quantify the transport and transformation of steroid hormones in runoff and soil under well-controlled field conditions*

Plot-scale experiments on a research feedlot were used to evaluate the effect of rainfall rate and aging time on concentrations of steroid hormones in runoff and soil. After application of a known volume of simulated rainfall, concentrations of filterable and particle-associated steroid hormones were quantified throughout the hydrograph. In addition, steroid

concentrations in animal waste and soil were measured before and after rainfall to assess the effects of loading rates and aging on the transport and transformation of steroids. A mass balance was used to quantify the steroid hormones transport and transformation.

1.4.3 Chapter 3: *Determine the effect of manure on rates of transformation and equilibrium partitioning of steroid hormones*

Results from plot-scale simulated rainfall experiments suggested that several steroid hormones were more stable than expected. The presence of manure can stabilize steroids by reducing microbial activity and decreasing bioavailability of the compounds. To assess the role of manure in steroid hormone stabilization, microcosms were constructed using steer manure and soil from the research feedlot to simulate conditions encountered in feedlots. Steroid stability was evaluated under varying conditions over a period of 7 days. Treatments included killed controls, varying doses of manure, and varying equilibration times.

1.4.4 Chapter 4: *Determine the efficacy of multi-step treatment trains on steroid hormones*

Commercial feedlots often employ multi-step treatment trains to remove nutrients and pathogens from runoff. The ability of these treatment systems to remove steroids is unknown. To address this deficiency, steroid hormone concentrations were measured in runoff and soil samples from a commercial feedlot that employed a solids settling basin, a vegetated infiltration basin, and a vegetated treatment area. Runoff samples were collected over a five-month period to assess the efficacy of the processes. In addition, steroid hormones were quantified in runoff samples from commercial feedlots after storms to verify data from the experimental feedlots. To assess steroid hormone transport in groundwater, tile drain discharge samples were analyzed from two locations where manure was applied to fields.

Chapter 2. Fate of Endogenous Steroid Hormones in Steer Feedlots Under Simulated Rainfall-Induced Runoff

Reprinted with permission from Mansell, D. S.; Bryson, R. J.; Harter T.; Webster, J. P.; Kolodziej, E. P.; Sedlak, D. L. Fate of Endogenous Steroid Hormones in Steer Feedlots Under Simulated Rainfall-Induced Runoff. *Environmental Science & Technology* **2011**, *45* (20), 8811-8818. (Slightly modified for formatting)

2.1 Introduction

During the past decade, estrogens, androgens, and progestogens have been detected in surface waters at concentrations high enough to affect fish and other aquatic organisms. In particular, attention has been focused on the feminization of fish in surface waters receiving discharges of steroid-containing municipal wastewater effluent [Purdom et al. 1994, Routledge et al. 1998, Jobling et al. 1998]. Exposure of sensitive species of fish to estrogenic hormone concentrations as low as 1-10 ng/L can result in biological changes, decreased reproductive success and alterations in species composition in receiving waters [Purdom et al. 1994, Kidd et al. 2007]. Androgens and progestogens have also been detected in surface waters subjected to municipal wastewater effluent discharges [Chang et al. 2011, Kolok et al. 2007, Kolodziej et al. 2003] at concentrations known to elicit behavioral and biochemical responses in fish [Kolodziej et al. 2003, Stanko & Angus 2007, Sorenson & Stacey 1999].

In addition to wastewater effluent, animal husbandry activities in which large numbers of animals are maintained in a small area may be important sources of steroid hormones in some watersheds. Concentrated animal feeding operations [Kolok et al. 2007, Lee et al. 2007, Matthiessen et al. 2006], grazing cattle [Kolodziej et al. 2007], dairies [Kolodziej et al. 2004], and aquaculture facilities [Kolodziej et al. 2004] all have the potential to release steroid hormones to surface waters. In watersheds where runoff from feedlots, pastures or land to which manure has been applied is not sufficiently treated or diluted, steroid hormones could reach concentrations that pose risks to aquatic ecosystems. However, the diffuse or intermittent nature of agricultural runoff coupled with the transport of hormones in association with particles and organic matter make it difficult to predict steroid hormone concentrations in surface waters impacted by agriculture.

Previous studies have demonstrated the presence of steroid hormones in runoff and waste storage lagoons at dairies and beef cattle feedlots at concentrations up to several orders of magnitude higher than reported thresholds for biological responses [Kolodziej et al. 2004, Durhan et al. 2006, Zheng et al. 2008]. However, these wastes often receive some form of treatment before reaching surface waters. The most common forms of treatment include application to cropland and pastures, infiltration through soil, composting, and treatment in ponds or wetlands. Irrespective of whether or not runoff is treated prior to release, management decisions require an understanding of the fraction of the steroid hormones excreted by animals that is released to surface waters.

In cattle feedlots, steroid hormones in runoff only account for a small fraction of the mass of the excreted steroids [Lange et al. 2002, Arts et al. 1991]. The steroid hormones that are not transported in runoff are assumed to be transformed by microorganisms in the soil and waste. Results from lab experiments in which manure and/or soil were stored or incubated under moist, oxic conditions indicate that most endogenous steroids are rapidly transformed to other biologically active steroids or compounds with little affinity for steroid receptors with half-lives on the order of hours to days [Lee et al. 2007, Lee et al. 2003, Colucci et al. 2001, Raman et al. 2001, Casey et al. 2003]. However, some studies indicate that the steroid hormones can persist for several weeks to several months during manure storage [Lange et al. 2002]. In addition, most steroids exhibit a relatively high affinity for soils, retarding their movement in runoff and groundwater and increasing the importance of transport of particle-associated steroids in surface waters [Lee et al. 2003, Casey et al. 2003, Casey et al. 2005].

As a result of the important role of natural attenuation and particle-associated transport in the release of steroid hormones to surface waters, it is important to understand the role of factors such as soil moisture, aging, and phase partitioning on the transformation and transport of steroids in feedlots. Because some transformation products exhibit biological activity, it is also important to identify steroids formed in animal wastes after excretion. To gain insight into these processes, field studies were conducted using rainfall simulators in beef cattle feedlot pens under conditions representative of commercial animal feeding operations.

2.2 Methods

2.2.1 Experimental design

Experiments were conducted during the summer and fall of 2009 at the University of California's Animal Science Feedlot in Davis, CA. A weather station (MK2Q3) at the Davis/Woodland/Winters airport approximately 8 km northwest of the feedlot reported a high, low, and average temperature during that period of 39° C, 12° C, and 24° C, respectively. Average humidity was 50%. Average wind speed was 8 km/h. No precipitation fell during the period when these experiments were conducted. Fourteen steers were housed in two, 190-m^2 pens with 33% shade cover for 14 days prior to initiating the experiments. The pens were scraped to the clay layer and graded at 3% slope prior to the introduction of animals. The animals were implanted with Revalor S (Intervet Inc. Millsboro, DE), an implant which contains 120 mg of trenbolone acetate and 24 mg estradiol, either 15 or 8 days before they were placed in the pens, providing a steady release of steroid hormones to the animals.

After 14 days, the cattle were removed from the pens where a new soil layer of mostly dried manure solids (mixed with soil particles from the dense clay layer below) had developed. Rainfall was simulated on eight, 3-m^2 subplots within each pen using a rainfall simulator designed for use on plot-scale runoff experiments on feedlots and pastures [Miller 1987, Humphrey et al. 2002]. Briefly, a ½-inch nozzle (Spraying Systems Co, Carol Stream, IL) held on an aluminum frame 3 m above the ground provided uniform rainfall over a 2 m x 1.5 m plot (Figure 2-1). A solenoid valve and timer were used to control the intensity of the rainfall. The simulator provided a drop size, velocity, and uniformity close to that of rainfall [Miller 1987, Humphrey et al. 2002]. Reverse osmosis water (Applied Membranes Inc., Vista, CA) used in the simulator was equilibrated overnight in an open 500-gallon (1890 L) container prior to rainfall simulation. The simulator was operated at a rainfall intensity of either 15 mm/hr (low rate) or 25 mm/hr (high rate) until runoff began, and then continued for one hour. Four plots received the low rainfall rate and four received the high rate within each pen. Experiments in which simulated rainfall was applied immediately after the cattle were removed from the pen were repeated twice. After these two experiments, an additional experiment was conducted on the same pens in which the wastes were aged an additional 7 days after removing the cattle before simulating rainfall. The pens were scraped to the packed clay layer between each experiment.

Prior to rainfall application, an aluminum frame was placed in the ground around each plot to capture runoff and channel it into an aluminum pipe where samples were collected (Figure 2-2). Runoff samples were collected 5, 20, 40, and 60 minutes following initiation of runoff. The runoff flow rate was measured by timing flow into a 250-mL container. The samples at each time point from four replicate plots were composited. This process was repeated at both rainfall rates in both pens.

For each experiment, 10-12, 100-g (wet weight) grab samples of the topsoil and the clay layer were collected in plastic bags and composited prior to and immediately following simulated rainfall. A grab sample of fresh manure from one of the pens was also collected. Runoff samples were transported on ice to UC Berkeley for analysis. All liquid samples were extracted within 48 hours of collection. Solids samples were frozen, homogenized at UC Davis, and shipped on ice to UC Berkeley where they were kept frozen until extraction, which occurred within 3 months.

Figure 2-1. Building and testing rainfall simulator used in experiments

Figure 2-2. Building apparatus used for collecting runoff.

2.2.2 Chemical analysis

Total suspended solids (TSS) (2540 D), organic matter fraction of the suspended solids (f_{om}) (2540E), moisture content and organic matter fraction of the soil (2540 G), dissolved organic carbon (DOC) (5310 B), chloride (4500-Cl^- F), and nitrate (4500-NO_3^- C) were analyzed using standard methods [APHA, AWWA, WEF 1995]. DOC was analyzed using a Shimadzu TOC 5000A analyzer. Chloride and nitrate concentration were analyzed using a Dionex DX-120 ion chromatograph with an Ionpac AS14A 4 x 250 mm column.

2.2.3 Steroid hormone analysis

Steroid hormones were analyzed using solid phase extraction, Florisil cleanup, derivatization, and gas chromatography tandem mass spectrometry (GC/MS/MS) with isotope dilution. A surrogate standard consisting of 100 ng each of 17α-estradiol-2,4-d_2, 17β-estradiol-2,4,16,17,17-d_5, estrone-2,4,16,16-d_4, 4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7, testosterone-16,16,17-d_3, progesterone-2,2,4,6,6,-17α-21,21,21-d_9 and mesterolone in acetonitrile was added to each runoff sample, suspended solids sample, and soil sample.

Runoff samples (1 L) were centrifuged at 5000 RPM (4620 g) for 10 minutes to remove suspended solids. The pellet was frozen until extraction. The supernatant was filtered through a 1-μm, glass fiber filter (Millipore, Bellerica, MA), the surrogate standard was added, and the sample was extracted on a Supelco ENVI-18 solid phase extraction cartridge (Sigma Aldrich, St. Louis, MO). The cartridge was eluted with 12 mL methanol and dried at 60° C in a vacuum oven. The sample was resuspended in 2 mL 95% dichloromethane/5% methanol and passed through a Supelco LC-Florisil cartridge (Sigma Aldrich, St Louis, MO) to remove polar organic matter. The cartridge was eluted with 20 mL of 95% dichloromethane/5% methanol which was collected, added to the 2 mL of solvent which had already passed through the cartridge, and dried in a vacuum oven. When the solvent remaining was less than 1 mL, the sample was transferred to a 2 mL gas chromatography vial (Grace, Deerfield, IL), dried under vacuum, and resuspended in 200 μL acetonitrile.

The extracts were derivatized using 50 μL heptaflourobutyric anhydride followed by heating at 80° C for 90 min. The samples were then dried under a gentle stream of nitrogen and resuspended in 100 μL isooctane with 1 ng/μL hexachlorobenzene as an internal standard. Steroids were analyzed by GC/MS/MS using the parent and daughter ions listed in Table 2-1. Quantification was done using an external calibration curve ranging between 0.1 and 500 ng/L. Response was calculated by dividing the sum of the area of both target ions for a compound by the sum of the area of the target ions of the deuterated surrogate standard corresponding to that compound. This helped correct for matrix effects and small errors during processing.

For soil-associated steroids, approximately 1-2 g (wet weight) of soil was weighed into a 15 mL centrifuge tube and the surrogate standard was added.10 mL methanol was then added and the tubes were shaken by hand for 20 seconds followed by sonication for 15 minutes. They were then centrifuged at 5000 RPM (3836 g) and the supernatant was decanted. A 10 mL aliquot of methanol was added and the extraction process was repeated two more times. 70 mL of deionized water was added to the 30 mL of methanol and the solution was filtered through an AP40 glass fiber filter. The solution was then extracted on a C-18 SPE cartridge followed by Florisil cleanup, derivatization, and GC/MS/MS analysis as described above. Particle-associated steroids were analyzed in a similar manner except that the whole pellet after centrifugation of

the runoff was extracted in 500-mL centrifuge bottles using three times the amount of methanol and deionized water.

Table 2-1, Parent and daughter ions used for quantification, limits of quantification (LOQ), and limits of detection (LOD) for each of the steroids measured.

Steroid	Parent ion	Daughter ions	LOQ, filtered runoff (ng/L)	LOQ, soil, manure/ suspended solids (ng/g)	LOD, filtered runoff (ng/L)	LOD, soil, manure/ suspended solids (ng/g)
17α-estradiol	664	237, 450	1	1 / 0.5	0.3	0.3/ 0.2
17β-estradiol	664	237, 451	2	0.5 / 0.5	0.3	0.2/ 0.2
Estrone	466	422, 448	1	1/ 0.5	0.3	0.3 0.2
Testosterone	680	665, 320	1	1 / 0.5	0.3	0.3 0.2
Androstenedione	482	467, 269	5	5 / 5	3	3 / 1
Progesterone	510	495, 425	10	10 / 5	3	3 / 3

2.2.4 Quality assurance/quality control

To assure accurate and precise analysis in the complex matrix, blanks and matrix recoveries were included with each set of samples. Steroids were never detected above quantification limits in blanks. For one time point per hydrograph, triplicate samples were collected and analyzed. Two samples were analyzed as duplicates, and the third was used as a matrix spike. Matrix spikes of filtered and suspended phases after separation were amended with 100 ng of 17α-estradiol, 17β-estradiol, estriol, estrone, testosterone, androstenedione, and progesterone in 100 μL acetonitrile prior to analysis. One of every six soil samples also served as matrix spikes. Despite all precautions, the complex matrix and multiple sample handling steps occasionally resulted in incomplete recovery of matrix spikes or altered instrument responses. The mean spike for each compound for each round was calculated from all the individual spikes, and, in approximately 11% of these, exceptionally high or low recoveries were observed, most likely due to the presence of high concentrations of NOM or colloids. To avoid systematic errors, only data from groups of samples where mean spike recoveries between 75 and 140% were observed were used for all compounds except 17α-estradiol. For this compound, the deuterated surrogate standard was not added to any of the runoff samples or the first round of soil samples, so 17β-estradiol-2,4,16,17,17-d_5 was used as a surrogate. As a result, spike recoveries for 17α-estradiol exhibited greater variability and spike recoveries between 75 and 180% were considered to be acceptable.

The relative percent difference (RPD) of the duplicate samples was calculated as the difference between the two samples divided by the mean multiplied by 100. The RPD varied with the measured steroid concentration, with higher RPD values at lower concentrations. Median RPDs were 18%, 21%, and 7% for filtered samples with steroid concentrations above 10 ng/L and suspended solids and soil samples with steroids concentrations above 10 ng/g. However, median RPDs were 17%, 31%, and 21% for filtered, suspended, and soil samples below those concentrations, respectively.

The quantification limit was between 1 and 10 ng/L for filtered runoff, 0.5 and 10 ng/g for suspended solids, and 0.5 and 5 for soil and manure depending on the steroid (Table

2-1). For samples with detectable steroid concentrations below the quantification limits, half the limit of quantification was used in calculations.

2.3 Results

2.3.1 Steroid concentrations in manure and soil

The concentrations of steroid hormones in the fresh manure ranged from below detection limit to 15 ng/g (dry weight) (Figure 2-3). The concentration of testosterone in fresh manure (2 ng/g) agreed with published values for median steroid excretion rates for veal calves (2.8 ng/g) [Arts et al. 1991] which has been used to estimate endogenous steroid excretion by steers [Lange et al. 2002, Johnson et al. 2006]. Progesterone was detected in the fresh manure, but below the limit of quantification. While Arts et al. did not report the presence of progesterone in manure; they did detect the steroid in the urine of veal calves [Arts et al. 1991]. The concentration of 17α-estradiol (15 ng/g) was approximately five times higher than the median reported value in Arts et al. (3 ng/g), presumably because some of the 17β-estradiol in the implants was metabolized to 17α-estradiol by the cattle [Rico 1983, Hanselman et al. 2003]. The water content of the fresh manure was 79%.

The surficial soil sampled at the initiation of the experiment consisted of manure deposited during the 14-day period in which the cattle were present mixed with clay and feed by the action of the animals walking in the pen. The top 3-cm of surficial soil above the packed clay layer had a water content between 3 and 6% and was between 19 and 25% organic matter on a dry weight basis. After aging, the organic matter content was between 25 and 32%. The water content of the soil did not change significantly during the 7-day period after the animals were removed, so all drying must have taken place during the 14-day period in which the cattle were present.

All six steroids were detected in the surficial soil. Concentrations of 17α-estradiol and testosterone were lower in the soil than in the manure, but concentrations of all other hormones were substantially higher (Figure 2-3). In particular, androstenedione and progesterone increased from concentrations near or below detection limits to concentrations up to approximately 60 ng/g. The mean concentration of androstenedione decreased by approximately 70% during the 7-day period after the cattle were removed whereas the mean concentration of progesterone increased by approximately 30% (Figure 2-3).

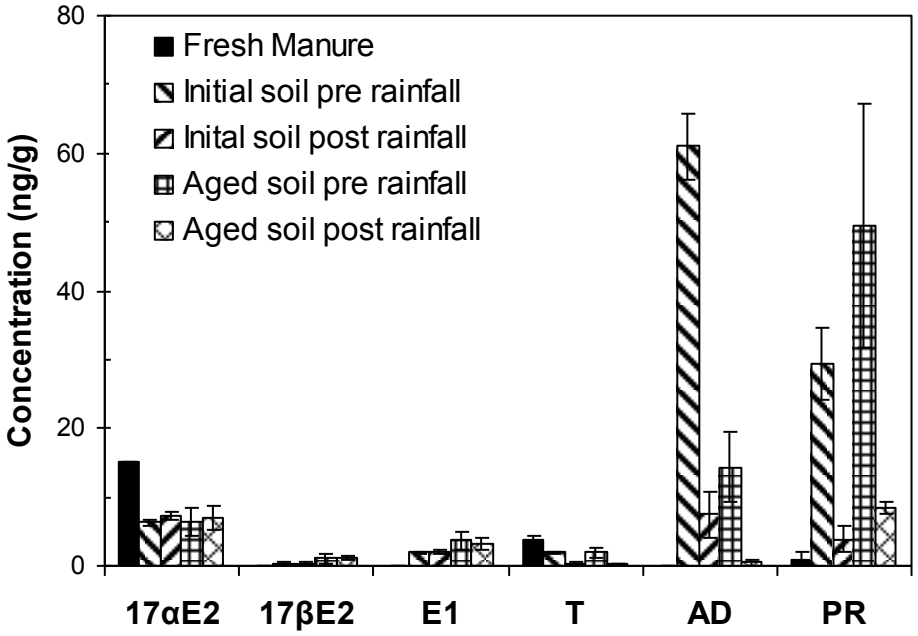


Figure 2-3. Mean steroid hormone concentrations (ng/g dry weight) in fresh manure and surficial soil before and after aging 7 days and after simulated rainfall. 17αE2=17α-estradiol, 17βE2=17β-estradiol, E1=estrone, T=testosterone, AD=androstenedione, and PR=progesterone. Error bars represent ± standard error.

Samples collected from the clay layer had concentrations of steroid hormones near or below the limit of quantification at all times with concentrations never exceeding 1 ng/g with the exception of androstenedione and progesterone, which were detected at concentrations up to 11 ng/g and 6 ng/g, respectively (Table 2-2).

Table 2-2. Steroid hormone concentrations (ng/g dry weight) measured in the clay layer beneath the soil in the pens where cattle were kept for 14 days before and after simulated rainfall.

Sample	17αE2	17βE2	E1	T	AD	PR
Clay layer (pre-rainfall)	1	BDL	1	BDL	11	5
Clay layer (post-rainfall) 1	0.5	BDL	0.5	BQL	7	5
Clay layer (post-rainfall) 2	BQL	BQL	0.8	BQL	6	6

BDL= below detection limits. BQL= below quantification limits17αE2=17α-estradiol, 17βE2=17β-estradiol, E1=estrone, T=testosterone, AD=androstenedione, and PR=progesterone.

Soil samples collected after simulated rainfall showed little or no change in concentration of estrogens (i.e., 17α-estradiol, 17β-estradiol, and estrone) while concentrations of androstenedione, progesterone, and testosterone decreased by approximately 85% after simulated rainfall (Figure 2-3). The water content of the soil increased from 3-6% before rainfall to 44% ± 3% after simulated rainfall.

2.3.2 Water quality parameters of runoff

In the runoff, concentrations of chloride and dissolved organic carbon decreased with runoff duration during the experiments while suspended solids concentrations remained approximately constant (Figure 2-4). The higher rainfall rate resulted in lower chloride and dissolved organic carbon concentrations, but higher suspended solids concentrations (Table 2-3 and Figure 2-4). The organic matter content of the suspended solids was similar to that of the soil (i.e., the suspended solids consisted of 23 to 48% organic matter compared to 19 to 32% for the soil). While the relationship between the suspended solids concentration and runoff rate was weak (r^2=0.32), the suspended solids concentrations were higher from plots receiving the higher rainfall rate (p=0.005). Aging caused a decrease in the average suspended solids concentration in the runoff from approximately 5 g/L to 3 g/L (p=0.007). Aging also increased the concentration of chloride in the runoff at both rainfall rates. However, this may be because the same pens were used for each experiment and excess salts were not removed when the pens were scraped between experiments.

Table 2-3. Mean total suspended solid (TSS), percent organic, dissolved organic carbon (DOC), and chloride concentrations for the runoff collected. Average ± standard error. For changes with time, see Figure 2-4.

	TSS (g/L)	Percent organic in TSS (%)	DOC (mg/L)	Chloride (mg/L)
From initial plots, High rainfall rate	5.9 ± 0.4	34 ± 1	498 ± 48	155 ± 26
From initial plots, Low rainfall rate	4.6 ± 0.3	36 ± 2	636 ± 70	215 ± 36
From aged plots, High rainfall rate	3.3 ±0.2	31 ± 2	509 ± 52	398 ± 45
From aged plots, Low rainfall rate	2.4 ± 0.1	29 ± 1	645 ± 55	464 ± 44

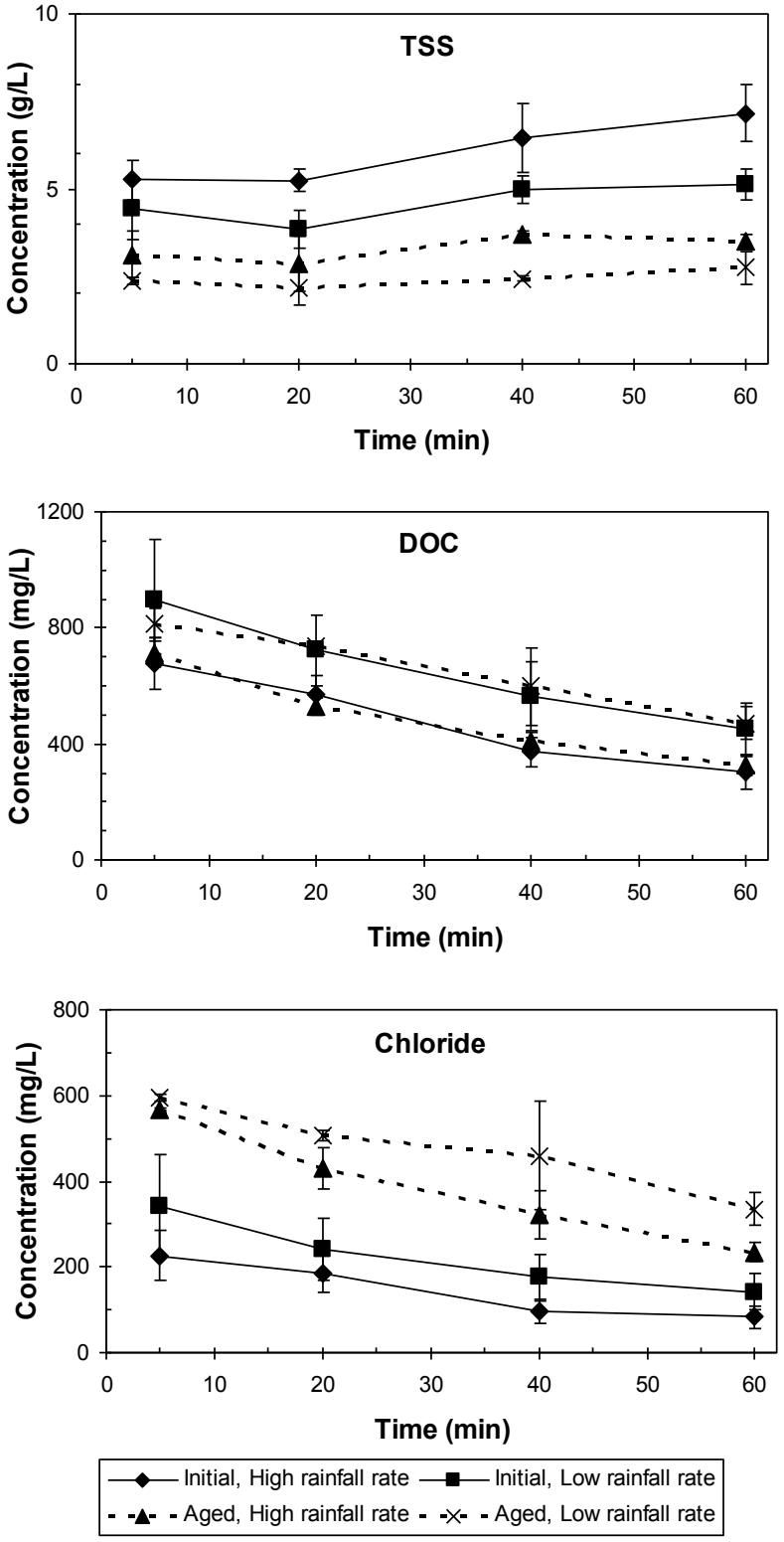


Figure 2-4. Average Total Suspended Solids (TSS), Dissolved Organic Carbon (DOC) and chloride concentrations in runoff with time. Error bars represent ± standard error.

2.3.3 Steroid concentrations in runoff

All six steroids were detected consistently in the runoff with concentrations remaining approximately constant throughout the hydrograph and showing no significant correlation with rainfall rate (Figure 2-6). Therefore, comparisons among treatments were made by averaging all time points in the hydrograph at both rainfall rates (Figure 2-5). Concentrations of 17α-estradiol, androstenedione, and progesterone ranged from 50 to 250 ng/L whereas concentrations of 17β-estradiol, estrone, and testosterone were usually less than 50 ng/L. With the exception of 17β-estradiol, a significant portion of each steroid was detected in both the filtered and particle-associated phases. Aging affected both the concentrations and partitioning of each steroid. For most of the steroids, a higher fraction of the steroids were present in the filtered phase of the runoff from the aged plots where TSS concentrations were approximately 45% lower. The runoff from the aged plots contained less 17α-estradiol and testosterone, but more androstenedione, progesterone, and estrone relative to the unaged plots.

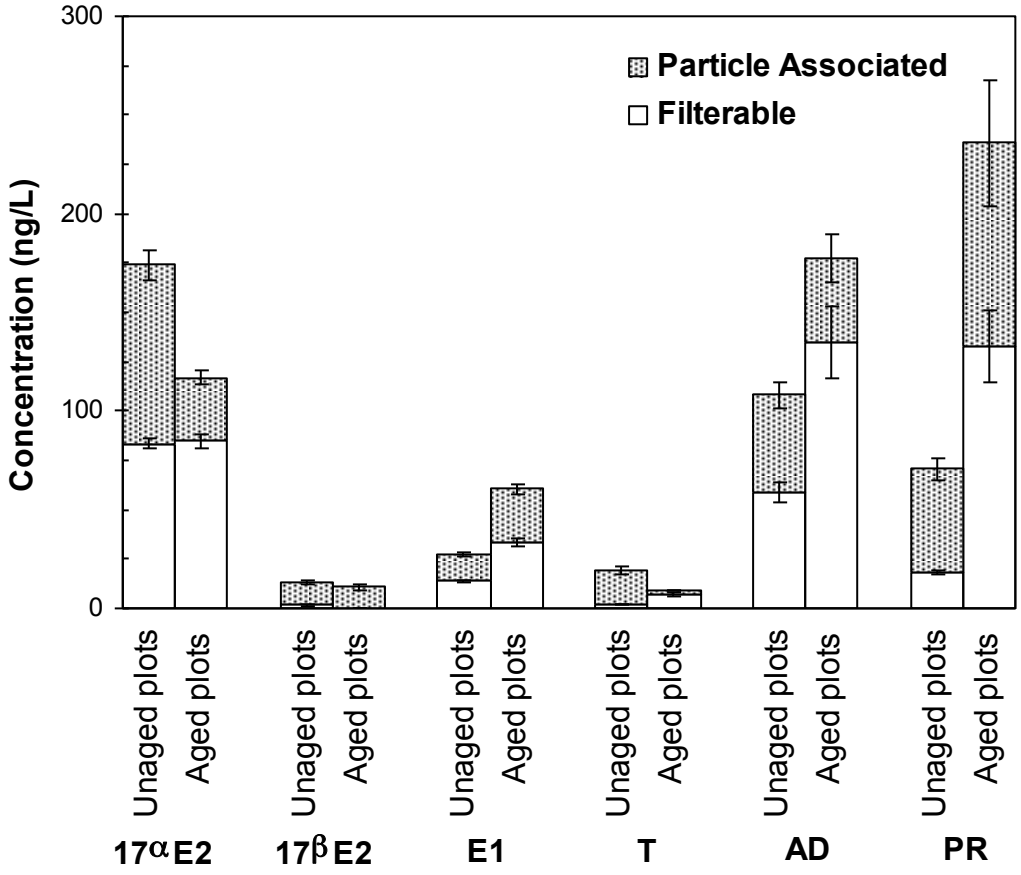


Figure 2-5. Mean steroid hormone concentrations in runoff from unaged and aged plots after simulated rainfall. 17αE2=17α-estradiol, 17βE2=17β-estradiol, E1=estrone, T=testosterone, AD=androstenedione, and PR=progesterone. Error bars represent ± standard error.

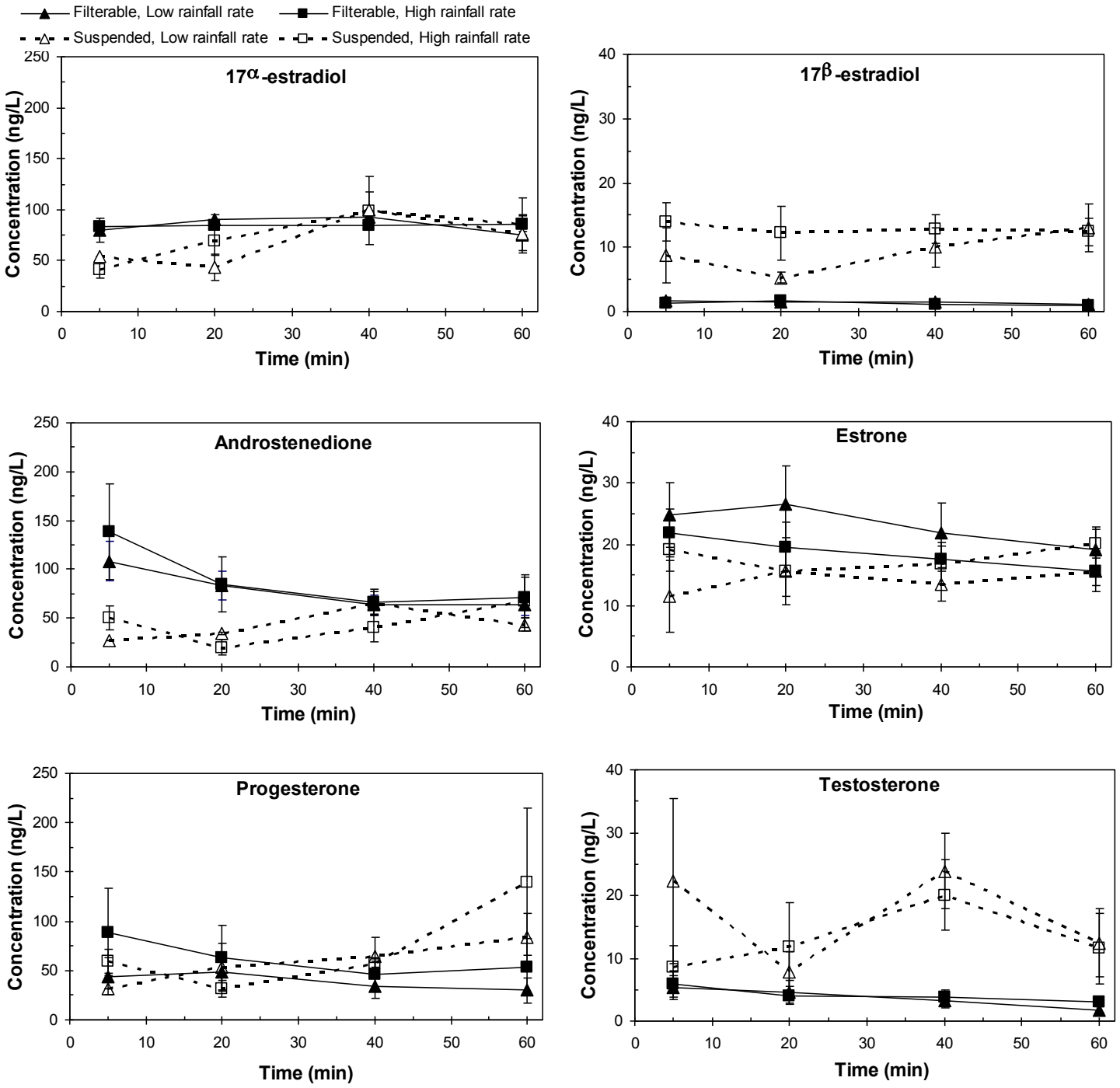


Figure 2-6. Mean steroid concentrations in runoff with time. Error bars represent ± standard error.

2.4 Discussion

2.4.1 Mass balance

A mass balance estimate for the plots was made using published data on hormone excretion rates along with measured hormone concentrations in manure, soil, and runoff. Several assumptions were made in order to construct a mass balance for the steroids. A total dry manure production of 2.7 kg/animal/day and a total urine production of 9 L/animal/day was estimated based on discussions with feedlot operators and published values [Johnson et al. 2006, Khan et al. 2008]. To estimate steroid contributions from urine, values from Arts et al. [Arts et al. 1991] were used, and it was assumed that all conjugated steroids were deconjugated immediately upon excretion. The steroid concentrations in manure reported by Arts et al. agree well with the steroid concentrations we measured, so it is assumed that the urine concentrations are a good estimate for our cattle as well. An initial dry topsoil mass of 20 kg/plot was also assumed.

The total mass for each steroid from manure (M) and urine (U) was calculated as:

$$M = MC * \frac{Plot}{Pen} * MYield*\#animals*\#days*1000$$

$$U = UC * \frac{Plot}{Pen} * UYield*\#animals*\#days*1000$$

respectively, where M is the total mass of steroids applied from manure (ng), U is the total mass of steroids applied from urine (ng), MC is the steroid concentration in the manure (ng/g dry mass), UC is the steroid concentration in the urine (μg/L), Plot is the plot area (3 m^2), Pen is the pen area (190 m^2), MYield is the dry manure yield per animal per day (2.7 kg dry mass/day), UYield is the urine yield per animal per day (L/day), #animals is the number of cattle on the pen (14), and #days is the total time on the cattle were left on the plot (14 days). These two values were summed for the total steroid mass applied to the plot.

The mass in the dry soil was calculated as:

$$DSM = SC * SMass * 1000$$

where DSM is the mass of steroids in dry soil (ng), SC is the measured dry soil steroid concentration (ng/g), and SMass is the total mass of soil on plot (20 kg). A similar calculation was used for the wet soil except that the loss of soil mass in the runoff was accounted for using:

$$WSMass = SMass - \frac{TSS * Flow * Time}{1000}$$

where WSMass is the mass of wet soil, TSS is the average total suspended solids concentration in the runoff (g/L), Flow is average flow rate of the runoff (L/hr), and Time is the total time of runoff occurred (1 hr). The mass of steroids in the runoff was calculated as:

$$RM = RConc * Flow * Time$$

where RM is the mass of steroid in the runoff (ng) and RConc is the steroid concentrations in the runoff (ng/L). The values obtained from this analysis are shown in Figure 2-7.

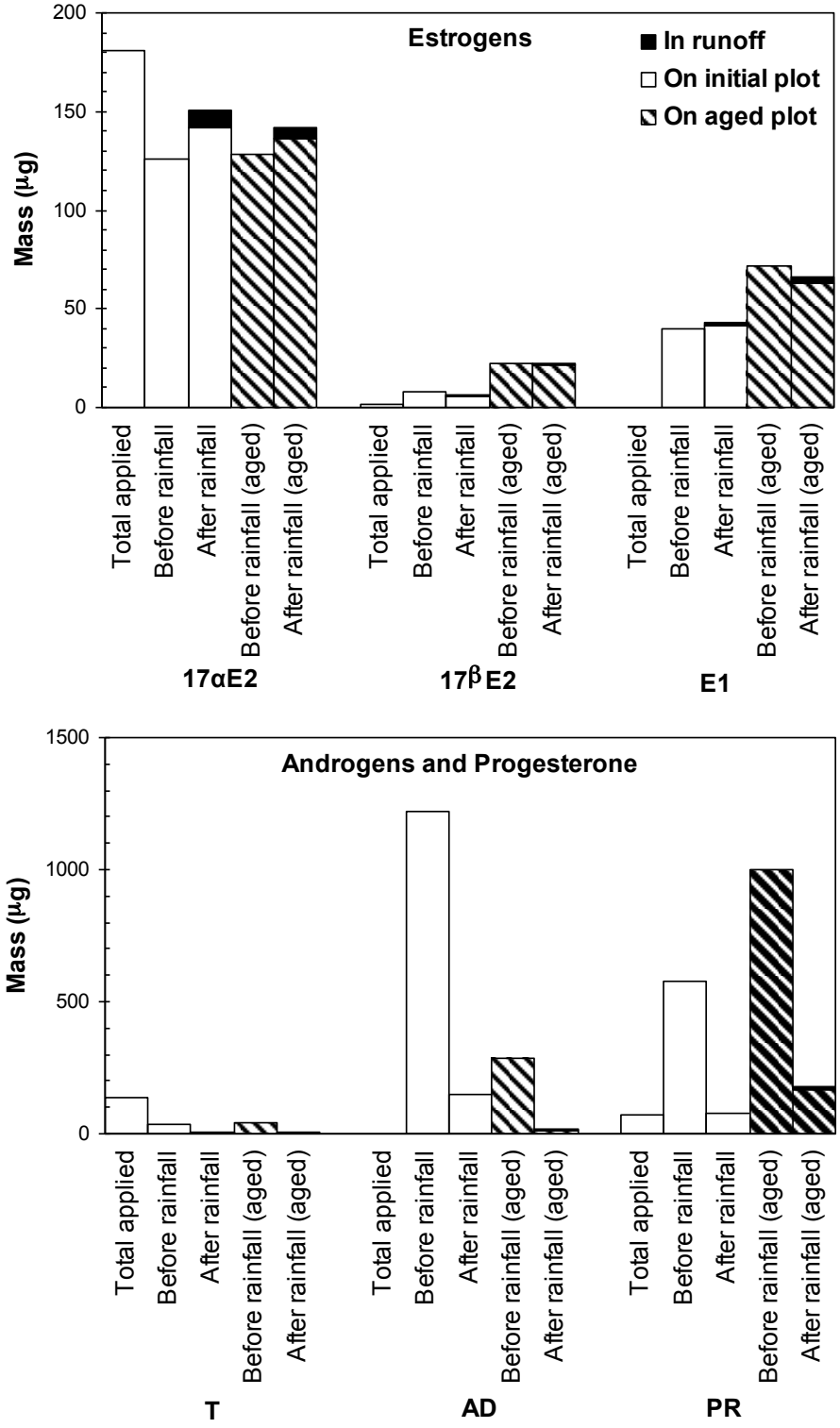


Figure 2-7. Mass balance estimate on steroid hormones. 17αE2=17α-estradiol, 17βE2=17β-estradiol, E1=estrone, T=testosterone, AD=androstenedione, and PR=progesterone.

The mass balance calculation suggests that approximately a quarter of the 17α-estradiol excreted by the cattle was converted into estrone and 17β-estradiol during the 14-day period in which the cattle were present in the pen (Figure 2-7). Aging of soil after the animals were removed resulted in a small increase in the mass of estrone and 17β-estradiol, and simulated rainfall had little effect on the total mass of estrogens in the soil (Figure 2-7). The mass of testosterone decreased by approximately 70% during the 14-day period in which the cattle were present. No further loss was observed during the 7-day period after the animals were removed (Figure 2-7). After rainfall, approximately 5% of the testosterone excreted by the cattle remained in the soil. Removal of testosterone from the plot via runoff accounted for less than 1% of the missing mass (Figure 2-7).

Unlike the estrogens and testosterone, androstenedione and progesterone exhibit a different pattern. Androstenedione was not detected in the fresh manure, but had the highest mass of any of the steroids in the soil after the 14-day period in which the cattle were present. During the 7-day period after the cattle were removed, the mass decreased by approximately 75% (Figure 2-7). The mass of progesterone increased by roughly an order of magnitude while the cattle were present, and increased further during the aging period after the animals were removed (Figure 2-7). Although 83% to 97% of the masses of these two steroids were lost after simulated rainfall, only a small fraction of the masses of these two steroids was recovered in the runoff.

2.4.2 Estrogens

Under the relatively dry conditions of the feedlot soil, the estrogens were surprisingly stable. While there was an initial decrease in the concentration of 17α-estradiol accompanied by an increase in the concentration of estrone and 17β-estradiol during the 14-day period when the cattle were present, the concentrations remained essentially unchanged during the 7-day aging period and in the brief period after water was added in the simulated rainfall (Figure 1). Estrone is a metabolite of 17α-estradiol, and interconversion of 17β-estradiol and 17α-estradiol through estrone has been observed in sediments under anoxic conditions [Czajka & Londry 2006]. It is also possible that the 17β-estradiol was produced when conjugated forms of the steroid in the urine were deconjugated in the soil. The total concentration of estrogens in the soil was approximately constant throughout the 14 to 21-day experimental period (Figure 2-3).

Other researchers have reported rapid transformation of 17α-estradiol and 17β-estradiol to estrone in dairy manure and waste lagoons [Zheng et al. 2008]. 17β-estradiol has also been observed to undergo conversion to estrone in dairy waste solids [Raman et al. 2001], soils [Colucci et al. 2001, Casey et al. 2003], river water [Jurgens et al. 2002], and wastewater treatment plants [Joss et al. 2004] on the time scale of hours to days. However, the storage conditions of the manure appear to play a role in the rate of transformation. For example, Lorenzen et al. observed that under some storage conditions, the estrogenicity of manure remained constant or even increased by several orders of magnitude [Lorenzen et al. 2004]. However, they were unable to identify the conditions responsible for the stability of the compounds.

One possible explanation for the enhanced stability of estrogens in the feedlot soils is decreased microbial activity in the relatively dry soils. Microbial activity in soil typically increases with moisture content until aeration becomes insufficient and is lowest under air-dried conditions [Miller & Johnson 1964]. Transformation rates of 17β-estradiol [Colucci et al.

2001] and trenbolone [Khan & Lee 2010] in soil also have been reported to decrease steadily as soil moisture is reduced, so it is possible that the dry conditions were responsible for the high stability of the estrogens. However, the estrogen concentrations also remained constant upon wetting of the soil by the simulated rainfall while the androgens and progesterone concentrations decreased substantially (Figure 2-3). Also, androstenedione and progesterone concentrations changed substantially during the dry, 7-day aging period suggesting there was some microbial activity in the soil (Figure 2-3). It is possible that other factors, such as location of the steroid within the manure also protected the estrogens from biotransformation. Only a small fraction of the mass of the estrogens was released into the runoff, suggesting that they were not easily removed from the soil (Figure 2-7). This close association of these steroids with the soil could also have limited their bioavailability, increasing their persistence.

2.4.3 Androgens and progesterone

Although the cattle excreted little androstenedione and progesterone, relatively high concentrations of the steroids were observed in the soil after the 14-day period when the cattle were present on the feedlot (Figure 2-3). In a previous experiment, manure from pregnant cattle was aged 0-14 days and applied to pasture plots prior to simulated irrigation and runoff samples were collected and analyzed for steroid hormone concentrations. Progesterone concentrations in that runoff also increased as the manure aged (Figure 2-8). Zheng et al. also observed an increase in progesterone concentrations when dairy manure was aged, with concentrations increasing from below detection limits to approximately 200 ng/g over a 2-week period [Zheng et al. 2008]. Androstenedione and progesterone have also been detected concurrently in streams at relatively high concentrations downstream of grazing cattle with concentrations up to 44 ng/L and 27 ng/L, respectively. While only detected in 5% of the samples, when present, progesterone concentrations were usually higher than those of the other steroids measured [Kolodziej & Sedlak 2007].

Androstenedione is produced from testosterone in soils with half-lives on the order of several hours to a few days depending on the soil [Lee et al. 2003]. However, even if all the testosterone excreted by the cattle were converted to androstenedione, it would only account for approximately 10% of the measured concentration. An alternative explanation is production of androstenedione and progesterone by the transformation of sterols in manure. Soil bacteria and *E. coli* isolated from human feces have been shown to convert cholesterol, sitosterol, and stigmasterol into androstenedione [Marsheck et al. 1972, Nagasawa et al. 1969, Owen et al. 1978, Conner et al. 1976]. Reported yields for these reactions varied from 1% to 39% depending on the sterol, the organism, and the medium used for incubation. Cholesterol and stigmasterol have been measured in cow manure at concentrations of 3770 ± 250 μg/g and 490 ± 20 μg/g (mean ± standard deviation), respectively [Tyagi et al. 2008]. If the steer manure contained similar concentrations of these sterols and the lowest reported yield for conversion of these two sterols into androstenedione (i.e. 1%) is used, the calculated mass of androstenedione would be between 30 and 250 times higher than those measured in the soils under the conditions observed in our experiments. Cholesterol has been shown to form oxidation products in frozen food even while frozen at -20 C [Pie et al. 1991]. Therefore, it is possible that some of the additional androstenedione and progesterone were formed in the frozen soil and manure during storage. However, it is more likely that they were formed at the ambient temperatures on the pen.

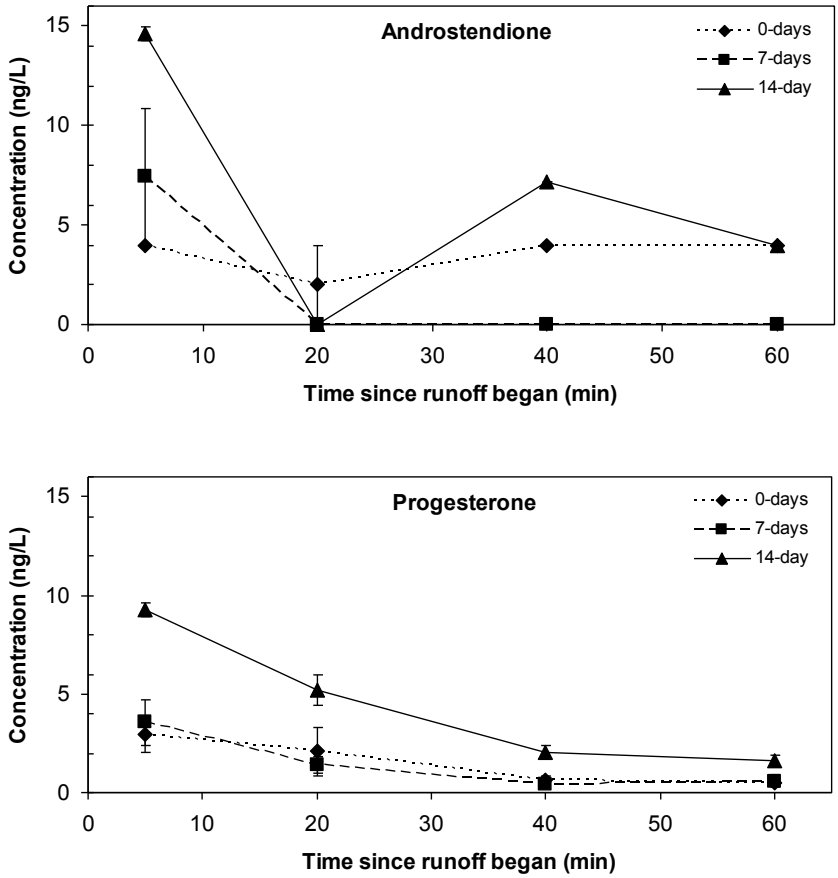


Figure 2-8. Mean steroid concentrations in runoff from irrigated pasture plots dosed with manure from pregnant cows which was aged 0, 7, and 14 days. Error bars represent ± standard error.

Progesterone and androstenedione have also been detected in surface waters subject to discharges from sterol-rich pulp and paper mill effluents where masculinization of fish was observed [Jenkins et al. 2001]. Lab experiments verified the release of progesterone from pine wood used for pulp followed by conversion to androstenedione by soil bacteria [Jenkins et al. 2004, Carson et al. 2008]. While our findings suggest that a similar process may also be occurring in manure-containing soils, additional research is needed to determine which sterols are responsible for progesterone and/or androstenedione formation and to better understand the factors affecting their formation.

When simulated rainfall was applied to the plots, soil concentrations of the androgens and progesterone decreased substantially within a very short period. The loss of steroid mass from the soil was not accounted for by the runoff (Figure 2-7). It is also unlikely that the steroids were leached through the soil; the clay layer under the soil was very dense, and the soil was only wetted to a depth of 2 to 3 cm. Furthermore, concentrations of steroids detected in the clay layer before and after simulated rainfall were near or below quantification limits (Table 2-2). One possible explanation for the rapid loss is stimulation of microbial activity in the brief period between wetting and extraction. For example, in aged feedlot runoff, Havens et al. reported rapid transformation of estrogens and androgens within 3 hours of spiking if the spiked samples

were not acidified [Havens et al. 2010]. The soil samples from our feedlot were placed on ice immediately after collection and were kept frozen until extraction. Runoff samples were also placed immediately on ice after collection and were extracted within 2 days. While a similar rapid disappearance was not observed for the estrogens, previous research has demonstrated that testosterone is more labile than estradiol in soils [Lee et al. 2003, Casey et al. 2004]. While data on the transformation of androstenedione and progesterone in soil or agricultural runoff are limited, Chang et al. reported higher removal efficiencies for these compounds (96-97%) than for the estrogens (67-80%) in municipal wastewater treatment systems, implying they are more labile in the presence of oxic bacteria [Chang et al. 2011]. Further research is needed to determine if wetting the soil could induce rapid degradation of the androgens and progesterone, but not the estrogens.

2.4.4 Partitioning and transport of steroids to surface waters

Because the centrifuged and filtered runoff contained dissolved organic carbon concentrations as high as 1400 mg/L, it is likely that a significant portion of the steroids measured in the filtered fraction of the runoff were not truly dissolved, but were bound to colloidal organic matter. Reported partition coefficients for association of steroids with colloidal organic matter are typically between 10^3 and 10^6 [Neale et al. 2009]. Using these values along with the mean filtered organic carbon concentration of 570 mg/L we estimate that between 37% and 99% of the steroids could have been associated with organic matter that was not removed by centrifugation and filtration. Colloidal organic matter potentially enhances transport [Casey et al. 2003, Stanford et al. 2010], increases the fraction of steroids in the filtered fraction [Yu et al. 2004, Stumpe & Marschner 2007, Bowman et al. 2002], and slows biotransformation of steroids [Stumpe & Marschner 2010]. Management practices, such as settling basins and lagoons, which rely on gravitational settling to remove suspended solids from feedlot runoff will remove steroid hormones associated with large particles, but may have little effect on steroids associated with colloidal organic carbon.

The partitioning of the steroids between the filtered and particulate phases did not fit equilibrium hydrophobic partitioning models, with or without consideration of colloids. There also was no relationship between the fraction of steroids associated with particles and the concentration of suspended solids as would be predicted from equilibrium partitioning. Equilibrium partitioning models assume a matrix where hydrophobic partitioning yields nearly linear partitioning provided sufficient time is allowed for equilibration. The matrix in this runoff was complex and heterogeneous and may not have reached equilibrium prior to sample collection. Equilibration times for 17β-estradiol and estrone to soils and sediments vary greatly with some researchers reporting equilibrium partitioning after 5-24 hours [Casey et al. 2005], while others report a range from 3 to 14 days [Yu et al. 2004, Bowman et al. 2002]. Previous studies have also indicated that steroid sorption to soils was not correlated with log K_{ow} values Das et al. 2004, Lee et al. 2003]. Thus, equilibrium hydrophobic partitioning models may not accurately predict observations for conditions encountered in feedlots.

2.4.5 Potential effects on aquatic life

The concentrations of steroids measured in the runoff exceed reported thresholds for endpoints related to feminization, masculinization, and interference with pheromonal

responses for fish [Purdom et al. 1994, Stanko & Angus 2007, Shappell et al. 2010, Adams et al. 1987]. The estrogenicity of estrone and 17α-estradiol relative to 17β-estradiol depends upon the assay used [Shappell et al. 2010, Liu et al. 2009], but assuming the lowest and highest values reported (0.08 and 0.8 for 17α-estradiol, 0.01 and 1 for estrone relative to 17β-estradiol, the total estrogenicity expressed in 17β-estradiol equivalents in the runoff was between one and two orders or magnitude higher than the 1 ng/L threshold for biological response and was not significantly different after the plots were aged for 7 days. Androstenedione concentrations in the runoff from the initial and aged plots were approximately 3 to 4 times higher than the reported threshold for masculinization of 40 ng/L [Stanko & Angus 2007]. Androstenedione also elicits odorant responses in goldfish at concentrations as low as 0.3 ng/L [Sorenson & Stacey 1999], and testosterone can elicit pheromonal response in Atlantic salmon parr at 0.003 ng/L [Moore & Scott 1991]. Thus the runoff concentrations are 2-3 orders of magnitude above thresholds for odorant responses in fish. While progesterone has been shown to induce vitellogenin expression in crayfish at concentrations of approximately 30 μg/L [Coccia et al. 2010], no data on threshold concentrations for endocrine disruption or pheromonal activity in aquatic life are available. However, other progestogens, such as 17-α,20-β-dihyroxy-4-pregnen-3-one have been shown to elicit pheromonal and odorant responses in goldfish at 3 ng/L and 0.03 ng/L, respectively [Sorenson et al. 1987]. Further research is needed to determine if progesterone also poses a threat to aquatic life at similarly low concentrations.

In applications where treatment or dilution is insufficient, estrogens and androgens could pose threats to aquatic life. Microbial transformation reactions may reduce the concentrations of some steroid hormones, but aging of wastes under dry conditions will have little effect on the mass of estrogens and may even result in a substantial increase in the mass of androgens or progestogens. As a result, caution must be exercised in the management of waste discharges from animal feeding facilities and pastures.

Chapter 3 The Effect of Steer Manure on the Transformation of Endogenous Steroid Hormones in Feedlot Runoff

3.1 Introduction

Steroid hormones pose potential threats to aquatic ecosystems at extremely low concentrations (1-10 ng/L). Reported biological effects on fish include feminization of males by estrogens (Purdom et al. 1994, Kidd et al. 2007, Routledge et al. 1998), masculinization of females by androgens (Stanko & Angus 2007), and decreased fertility and fecundity by progesterone (DeQuattro et al. 2012). In addition, many steroid hormones are potent odorants and pheromones that can cause behavioral responses in fish at extremely low concentrations (Moore & Scott 1991, Sorenson & Stacey 1999, Sorenson et al. 1987).

Wastes from animal agriculture are a potentially important source of steroid hormones in watersheds containing many feedlots or fields fertilized with manure. Steroid hormones can reach surface waters near animal feeding operations through drainage from feedlots (Kolok et al. 2007, Lee et al. 2007, Matthiessen et al. 2006), rainfall- or irrigation-induced runoff from pastures and crops fertilized with manure (Finlay-Moore et al. 2000), excretion into surface waters by animals with direct access to surface waters (Kolodziej & Sedlak 2007), and groundwater discharges from areas impacted by waste lagoons (Arnon et al. 2008), waste-fertilized crops, or pastures (Kjaer et al. 2007). Among these sources, runoff from feedlots and manure-applied fields typically could pose the greatest threats to aquatic life.

Although researchers have been concerned about steroid hormones from animal agriculture for a decade, predictions of hormone releases from feedlots, fields, and pastures are sill imprecise. For example, several studies have documented rapid microbial transformation of steroid hormones in agricultural soils, with half-lives on the order of hours to days (Lee et al. 2003, Colucci et al. 2001, Raman et al. 2001, Casey et al. 2003). However, results from experiments conducted in the presence of manure suggest that steroids can persist for weeks to months (Schlenker et al. 1998, Bamberg et al. 1986, Masunda et al. 1999, Lorenzen et al. 2004, Caron et al. 2012).

In addition, attempts to model the transport of steroids have been complicated by incomplete knowledge about phase partitioning. Under laboratory conditions, steroids strongly partition to soil (Lee et al. 2003, Casey et al. 2003, Casey et al. 2003). However, steroids are often more mobile than predicted by linear partitioning models due to colloid- or organic matter-facilitated transport (Casey et al. 2004, Stanford et al. 2010, Steiner et al. 2010, Fan et al. 2008). Runoff from feedlots or manure-applied fields typically contains high concentrations of suspended solids (>1 g/L) and dissolved organic carbon (>200 mg/L) (Mansell et al. 2011). Under these conditions, transport of steroids on suspended colloids or in association with dissolved organic matter can be significant even if the affinity of the compound for the dissolved organic matter is relatively weak.

The effects of manure and colloids on steroid transformation and transport are especially important in animal feedlots. For example, estrogens, androgens, and progestogens were stable for over 21 days in a cattle feedlot under dry, summer conditions (see Chapter 2). Upon application of simulated rainfall, estrogens were released into runoff, whereas most of the androgens and progesterone were rapidly transformed. While the higher stability of the estrogens was consistent with prior laboratory studies (Stumpe et al. 2007, Fan et al. 2007, Layton et al. 2000, Havens et al. 2010), the observed rates of transformation and extent of transport of the steroids in runoff was unexpected. These observations suggest that the high organic matter content of the feedlot soil and large mass of suspended organic particles in runoff play an important role in steroid transformation and partitioning.

The objective of the research presented in this chapter was to determine the role that manure plays in the transformation and phase partitioning of steroid hormones. To assess the effects of manure under controlled conditions, microcosms were used with varying amounts of steer manure and mineral-rich soil. The effect of the initial form of the steroid on the transformation and partitioning of the compound was evaluated by studying microcosms with and without steroid amendments.

3.2 Methods

3.2.1 Microcosm experiments

Runoff microcosms were constructed using mineral-rich soil and steer manure collected from the University of California research feedlot in Davis, CA. Fresh (i.e., <1 hour since excretion) manure was collected from steers immediately before experiments over a 5-month period. The steers had been implanted with Synovex Choice (Pfizer), an implant that contains 100 mg of trenbolone acetate and 14 mg estradiol benzoate, between 0.5 and 6 months prior to manure collection. Steers were added to and removed from the herd at different times throughout the 5-month period. New animals were implanted prior to introduction to the pen. Therefore, time since implantation varied considerably among the animals.

Mineral-rich (i.e., 3-4% organic matter) soil with a high clay fraction was collected from just outside the pens, in locations where manure was not present. Manure and soil samples were transported to UC Berkeley where microcosms were started within three hours of collection. Microcosms consisted of 250 mL of tap water that was boiled to remove chlorine, 0.5 g soil, and varying amounts of manure in 1-L amber glass bottles that were loosely capped. Soil and manure masses were selected to approximate total suspended solids (TSS) and dissolved organic carbon (DOC) concentrations observed in runoff collected from the pens when simulated rainfall was applied in the experiments presented in Chapter 2. Moisture content and organic matter fraction of the soil and manure were analyzed using standard methods (APHA 1995).

In some experiments, microcosms were amended with 100 ng 17β-estradiol, testosterone, and progesterone, or 100 ng 17α-estradiol, in 100 μL acetonitrile. Abiotic control microcosms were obtained by acidifying to pH 2 with 1 mL of 5 N hydrochloric acid. (Havens et al. 2010). Triplicate microcosms were extracted after 2 hours, 24 hours, 4 days, and 7 days, except for one experiment in which they were extracted after 30 minutes, 2 hours, and 7 days. The bottles were gently agitated approximately three times per day.

Treatments included the addition of varying amounts of manure and steroids, abiotic controls, and experiments in which one of the microcosm components was added 24 hours after the experiment began (Table 3-1). In one of these experiments, an aliquot of manure was added to microcosms that were then acidified and amended with steroid hormones. After 24 hours, the acid was neutralized with 1 mL of 5 N sodium hydroxide, and a second aliquot of manure was added to restore microbial activity. In another experiment, microcosms were amended with steroids 24 hours after the manure was added.

After the appropriate time interval, all of the microcosms were acidified to pH 2 to prevent further transformation of the steroid hormones. Prior to centrifugation, filtration, and extraction, the acid was neutralized with sodium hydroxide.

Table 3-1. Manure doses, steroid amendments, and hydrochloric acid amendments used for

synthetic runoff microcosm experiments.

Table 3-1. Feedlot runoff microcosm experimental conditions.

Experiment Number	Manure (g, dry wt)	Steroids added (100 ng each)	Acidified?	Steroid Notes
1	2.1	-	No	
	2.1	17βE2, T, PR	Yes	
	2.1	17βE2, T, PR	No	
	0.8	17βE2, T, PR	No	
	0.1	17βE2, T, PR	No	
	0	17βE2, T, PR	No	
2	1.8	-	No	
	1.8	-	Yes	
	1.8	17βE2, T, PR	No	
	1.8	17βE2, T, PR	Yes	
3	0.5	-	No	
	0.5	-	Yes	
	0.5	17βE2, T, PR	No	Steroids added while acidified
	0.5	17βE2, T, PR	Yes	Steroids added while acidified
	0.5	17βE2, T, PR	No	Steroids added at neutral pH
	0.5	17βE2, T, PR	Yes	Steroids added at neutral pH
4	0.4	-	No	
	0.4	17βE2, T, PR	No	Steroids added upon construction
	0.4	17βE2, T, PR	Yes	Steroids added upon construction
	0.4	17βE2, T, PR	No	Steroids added after 24 hours
	0.4	17βE2, T, PR	Yes	Steroids added after 24 hours
5	0.7	-	No	
	0.7	-	Yes	
	0.7	17αE2	No	
	0.7	17αE2	Yes	

17βE2=17β-estradiol, 17αE2=17α-estradiol, T=testosterone, PR=progesterone. See text for additional descriptions of experiments 3 and 4.

3.2.2 Steroid hormone analysis

Steroid hormones were analyzed using solid phase extraction, Florisil cleanup, derivatization, and gas chromatography-tandem mass spectrometry (GC/MS/MS) with isotope dilution as described previously in Chapter 2. 4-androstene-3,17-dione-2,3,4-$^{13}C_3$ and progesterone-2,3,4-$^{13}C_3$ were incorporated into the analytical method to improve precision.

Prior to extraction, the entire sample was centrifuged at 5000 RPM (4620 g) for 10 minutes and the supernatant was filtered through a 1-μm, glass fiber-filter. A surrogate standard consisting of 100 ng each of 17α-estradiol-2,4-d_2, 17β-estradiol-2,4,16,17,17-d_5, estrone-2,4,16,16-d_4, 4-androstene-3,17-dione-2,3,4-$^{13}C_3$, testosterone-16,16,17-d_3, progesterone-2,3,4-$^{13}C_3$, and mesterolone in 100 μL acetonitrile was added to both fractions. The particulate fraction was extracted in 30 mL methanol by shaking for 20 seconds, sonicating for 15 minutes, and centrifuging at 5000 RPM (4620 g) for 10 minutes. The methanol was poured off, and the process was repeated two more times. Deionized water (250 mL) was added to the methanolic extracts, and the solution was filtered through a 1-μm, glass fiber filter.

The filtrate from the solvent extracted particulate fraction and the filtrate from the runoff were extracted on a solid-phase extraction cartridge (Supelco, St Louis, MO). After extraction, the cartridge was eluted with methanol and the methanol and the extracts were dried in a vacuum oven. The extracts were resuspended in 2 mL of 95% dichloromethane/5% methanol and passed through a Florisil cartridge (Supelco, St Louis, MO) to remove polar organic matter. The cartridge was eluted with 95% dichloromethane/5% methanol. The purified extract was dried in a vacuum oven.

After drying, the extracts were derivatized using 50 μL heptaflourobutyric anhydride in 200 μL acetonitrile followed by heating at 80° C for 90 min. The derivatized extracts were dried under a stream of nitrogen and resuspended in 100 μL isooctane with 1 ng/μL hexachlorobenzene as an internal standard prior to GC/MS/MS analysis. The method previously described was altered to include 4-androstene-3,17-dione-2,3,4-$^{13}C_3$ and progesterone-2,3,4-$^{13}C_3$. The parent ion used to identify 4-androstene-3,17-dione-2,3,4-$^{13}C_3$ had a m/z ratio of 485, and the two daughter ions had ratios of 470 and 272. The parent ion used to identify progesterone-2,3,4-$^{13}C_3$ had a m/z ratio of 513, and the two daughter ions had ratios of 498 and 428. Steroids were quantified using an 8-point calibration curve between 0 and 500 ng. The quantified masses of steroid hormones from both the suspended solids and filtrate were added to calculate the total steroid mass in each microcosm.

3.2.3 Quality assurance/quality control and statistical tests

To assure accuracy and precision in the complex matrix, blanks and matrix spike recoveries were included with each set of samples. Steroids were never detected above quantification limits in blanks. Mean matrix spike recoveries ranged between 81% and 120% for all experiments and for all compounds except for estrone, which exhibited recoveries were between 68% and 156%. Quantification limits were between 0.2 and 1.2 ng/L for 17β-estradiol, 17α-estradiol, estrone, and testosterone, and between 3 and 5 ng/L for androstenedione and progesterone. When concentrations of steroids were below the limit of quantification, half the limit of quantification was used in calculations.

The two-tailed, unpaired, unequal variance student's t-test was used to determine statistical significance between time points to determine changes with time and between treatments to determine effect of treatments. Statistical significance was assigned for all $P<0.05$.

3.3 Results

Acidification prevented steroid transformation in the abiotic control microcosms.

Recoveries of 17β-estradiol, testosterone, and progesterone ranged between 79 and 113% over 7 days of incubation (Figure 3-1). There were four instances where steroid concentrations at a time point for one treatment were statistically significant from the neighboring time point, but there was no consistent trend with time, indicating that acidification stabilized the steroid hormones (Figure 3-1). In microcosms that did not receive steroid amendments, the concentrations of the steroids in the acidified controls had no statistically significant changes with time suggesting that the steroids in unamended microcosms were also stable when acidified (Figures 3-2 and 3-3).

When not preserved by acidification, progesterone and testosterone were rapidly transformed, with concentrations approaching quantification limits within 24 hours at the two highest concentrations of manure added (Figure 3-4a-b). Higher concentrations of added manure resulted in more rapid disappearance of testosterone and progesterone. For example, testosterone concentrations decreased by approximately 75%, 30%, and 0% in 2 hours for manure concentrations of 8.4 g/L, 3.2 g/L, and 0.4 g/L, respectively (Figure 3-4b). When no manure was added, testosterone and progesterone concentrations still decreased, but much more slowly. After 7 days, concentrations of progesterone and testosterone decreased by 70-85% under all conditions (Figure 3-4a-b). Statistically significant changes between time points were consistently seen for all steroids except 17α-estradiol at all manure doses, but were not consistently seen for other time points at all manure doses. 17α-estradiol only had one significant change between days 4 and 7 at the highest manure dose. 17b-estradiol had a significant change between days 1 and 4 at the 3.2 g/L manure dose, and between days 4 and 7 when no manure was present. Progesterone had a significant change between days 4 and 7 when no manure was present. For all other steroids at all other manure doses at all time points, the only consistently significant difference between neighboring time points was observed between 2 hours and 1 day.

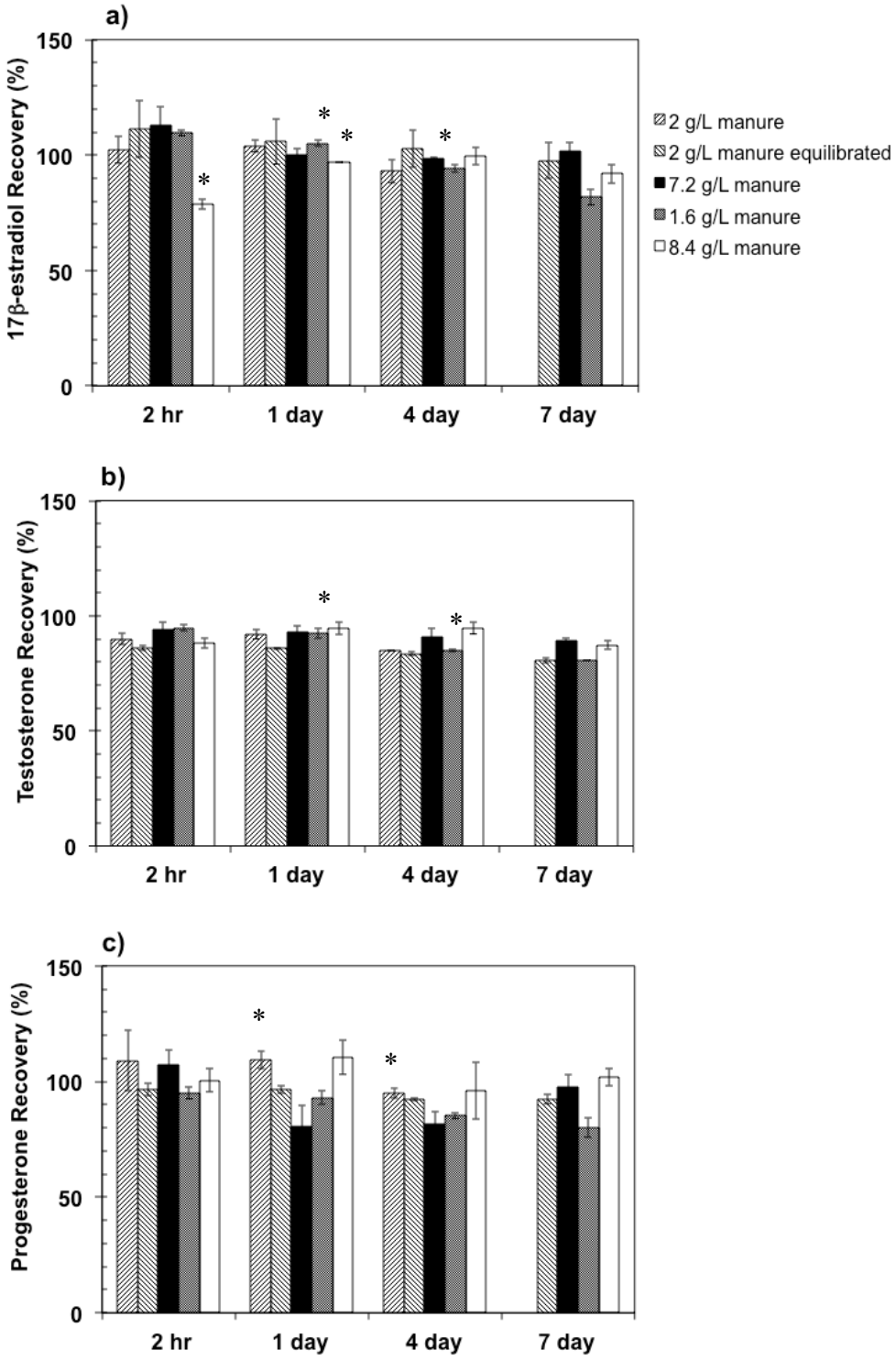


Figure 3-1. Mean steroid recoveries detected in acidified synthetic feedlot runoff microcosms amended with 100 ng 17β-estradiol, testosterone, and progesterone. Error bars represent ± standard error. Manure concentrations are based on dry mass added. * denotes statistical significance ($P<0.05$) from neighboring time point.

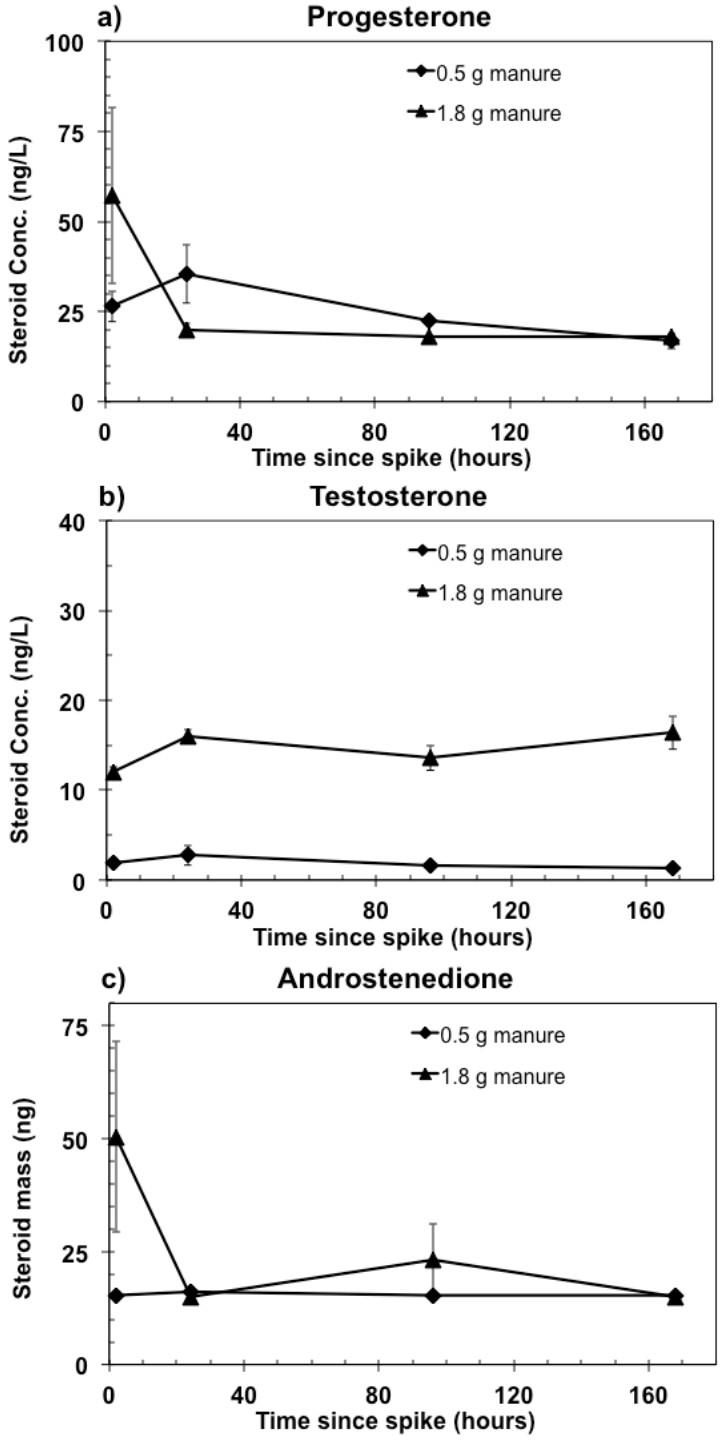


Figure 3-2. Mean androgen and progesterone concentrations detected in acidified synthetic feedlot runoff microcosms not amended with steroids. Error bars represent ± standard error. Manure concentrations are based on dry mass added.

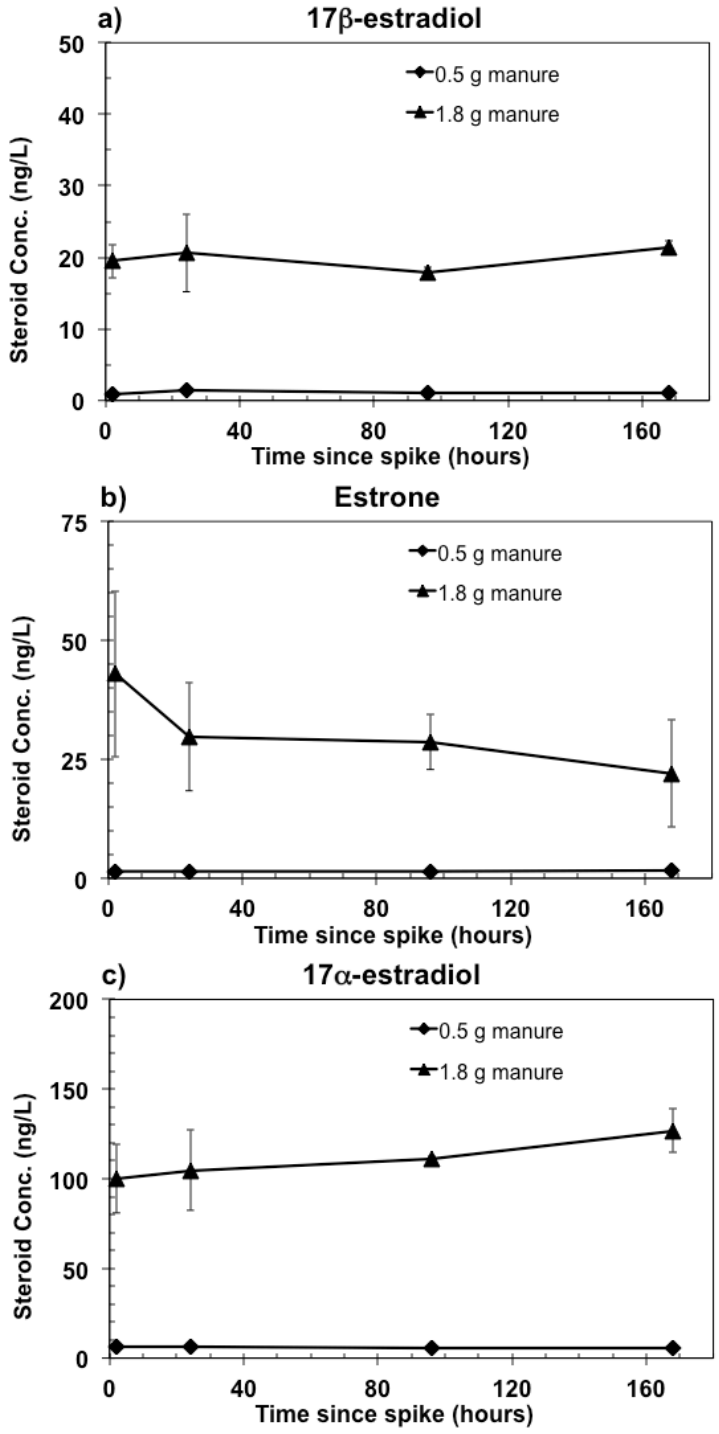


Figure 3-3. Mean estrogen concentrations detected in acidified synthetic feedlot runoff microcosms not amended with steroids. Error bars represent ± standard error. Manure concentrations are based on dry mass added.

Androstenedione, the primary microbial metabolite of testosterone, was detected within 2 hours of incubation. Its concentration decreased to levels below the quantification limits within 24 hours at the two highest manure concentrations (Figure 3-4c). At the lowest manure concentration, it accounted for almost half of the initial testosterone between 24 and 100 hours.

Concentrations of 17β-estradiol also decreased during incubation, but the patterns were different from what was observed for progesterone, testosterone, and androstenedione (Figure 3-5a). After an initial period of rapid transformation, 17β-estradiol concentrations stabilized at the two highest manure concentrations. At the lowest manure concentration, they continued to decrease throughout the duration of the experiment. After 7 days, the highest 17β-estradiol concentrations were observed in the microcosms with the highest manure mass.

In the manure-free microcosm, the concentrations of 17β-estradiol ranged from 10-25 ng/L after 2 hours (Figure 3-5a). The disappearance of 17β-estradiol was not accompanied by an increase in the concentrations of estrone or 17α-estradiol (Figure 3-5b-c). Most of the loss of 17β-estradiol in the manure-containing microcosms could be accounted for by formation of estrone (Figure 3-5b). Increases in estrone concentration accounted for between 70-84% of the losses in 17β-estradiol during the first 24 hours at each treatment in which manure was added (Figure 3-5b). After 24 hours, estrone concentrations remained approximately constant even though 17β-estradiol concentrations continued to decrease in the microcosm with the lowest manure mass. After 7 days, estrone only accounted for approximately 50% of the 17β-estradiol lost in the microcosm with the lowest manure concentration.

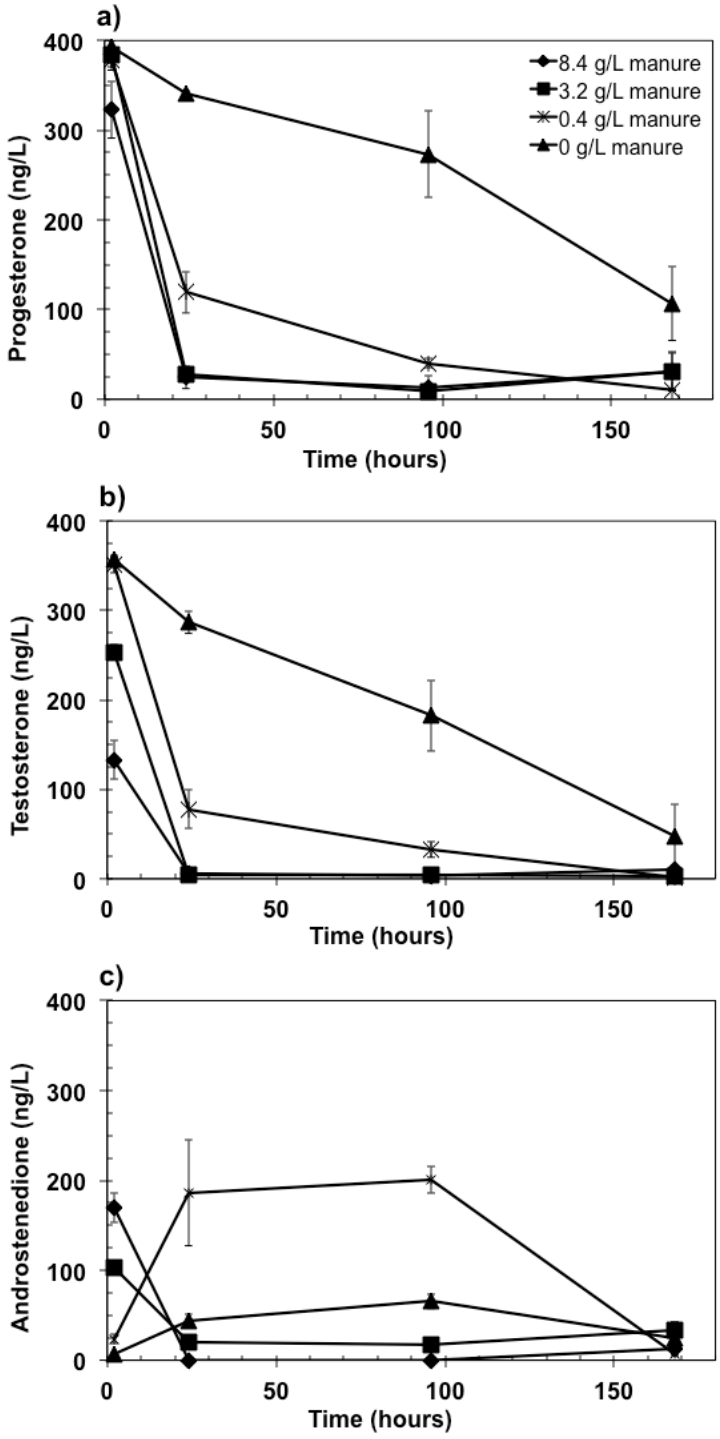


Figure 3-4. Mean androgen and progesterone concentrations detected over a 7-day period in synthetic feedlot runoff microcosms amended with 400 ng/L 17β-estradiol, testosterone, and progesterone. Error bars represent ± standard error. Manure concentrations are based on dry mass added.

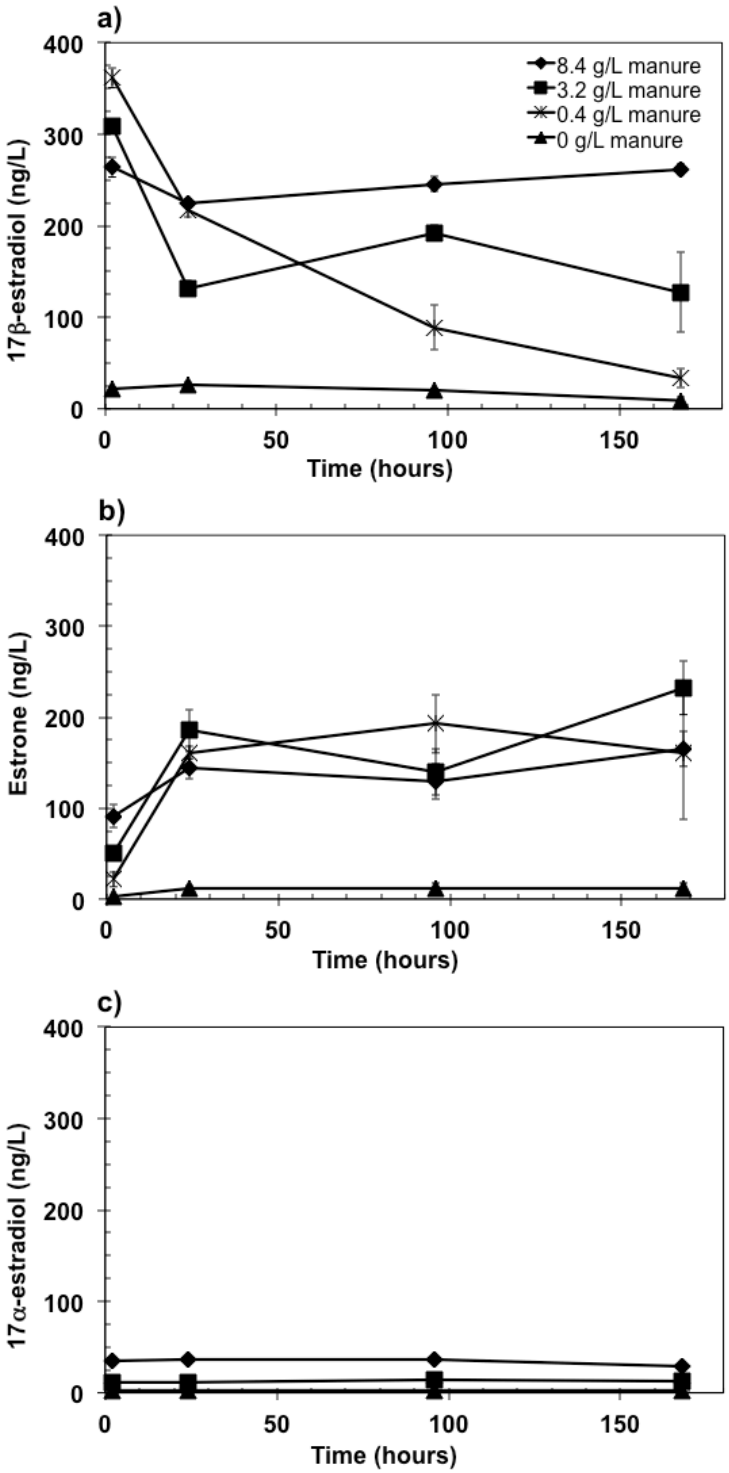


Figure 3-5. Mean estrogen concentrations detected over a 7-day period in synthetic feedlot runoff microcosms amended with 400 ng/L 17β-estradiol, testosterone, and progesterone. Error bars represent ± standard error. Manure concentrations are based on dry mass added.

Loss of estrogens was accompanied by product formation, but the pattern was different between the two forms. When 17β-estradiol was added to the microcosms, a background 17α-estradiol concentration up to 40 ng/L was detected in the manure-amended microcosms (Figure 3-5c). In microcosms amended with 400 ng/L of 17α-estradiol, a loss of approximately 160 ng/L of 17α-estradiol was accompanied by a 110 ng/L and a 50 ng/L increase in the concentrations of estrone and 17β-estradiol, respectively (Figure 3-6a-c). This suggests that approximately 10% of the added 17α-estradiol was converted to 17β-estradiol. While a statistically significant decrease in 17α-estradiol was seen even when acidified, and no statistical difference between the 17α-estradiol and estrone concentrations at 7 days was seen between the acidified and regular microcosms, a statistical difference in 17β-estradiol concentrations was seen between acidified and regular microcosms.

Dissolved oxygen concentrations decreased rapidly in the manure amended microcosms. At the highest manure concentration (i.e., 8.4 g/L), the concentration of dissolved oxygen decreased to less than 1 mg/L within 5 hours and remained below detection limits throughout the experiment. At the two lower manure masses, dissolved oxygen concentrations were below 1 mg/L after 24 hours and remained below detection limits for the duration of the experiment. Dissolved oxygen concentrations in the manure-free microcosms were always greater than 8 mg/L.

The microcosms that were not amended with steroids also contained steroid hormones at concentrations well above the quantification limits (Figures 3-7 & 3-8) with progesterone, androstenedione, and 17α-estradiol detected at the highest concentrations. The steroid concentrations in the unamended microcosms were not related to the mass of manure added (Figures 3-7 & 3-8), indicating that the manure collected on different days for these experiments contained different concentrations of steroids. Steroid excretion has been reported to vary with time since implantation in steers (Webster et al. 2012). The concentration of these steroids was mostly constant with only three instances of statistical differences between time points, but no clear trends with time (Figures 3-7 & 3-8).

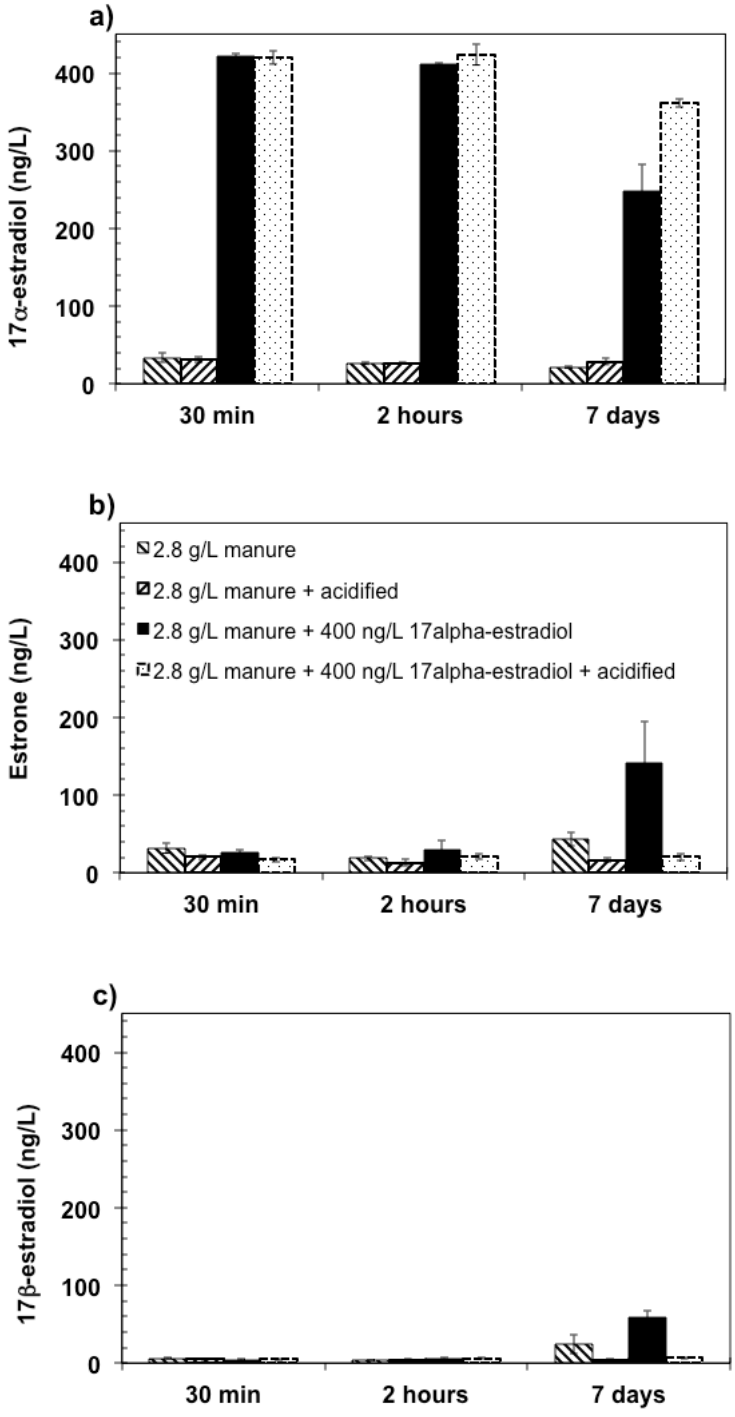


Figure 3-6. Mean estrogen concentrations detected over a 7-day period in synthetic feedlot runoff microcosms amended with 400 ng/L 17α-estradiol. Error bars represent ± standard error. Manure concentrations are based on dry mass added.

The steroids originating from the manure often exhibited different behavior than those in the microcosms that had steroid amendments, especially the estrogens. Concentrations of 17β-estradiol and estrone in the unamended microcosms slowly increased (Figure 3-8a,b), while 17α-estradiol concentrations remained constant throughout the 7-day experiments (Figure 3-8c). Progesterone concentrations in the microcosms that received no steroid amendments decreased rapidly (Figure 3-7a), while initial concentrations of testosterone in the unamended microcosms were low and did not follow any clear temporal trends (Figure 3-7b). Androstenedione concentrations were variable with higher concentrations occurring at the lowest manure mass and no clear temporal trends (Figure 3-7c).

To assess whether association of steroids with organic matter could prevent or slow steroid transformation, an experiment was conducted in which steroids were added to an acidified microcosm and equilibrated for 24 hours prior to raising pH and adding additional manure as a source of live microorganisms. Equilibration of the steroids prior to addition of the live microorganisms had no noticeable effect on steroid transformation rates with the only statistically significant difference between the treatments coming after 1 day for progesterone (Figures 3-9 & 3-10, diamond markers).

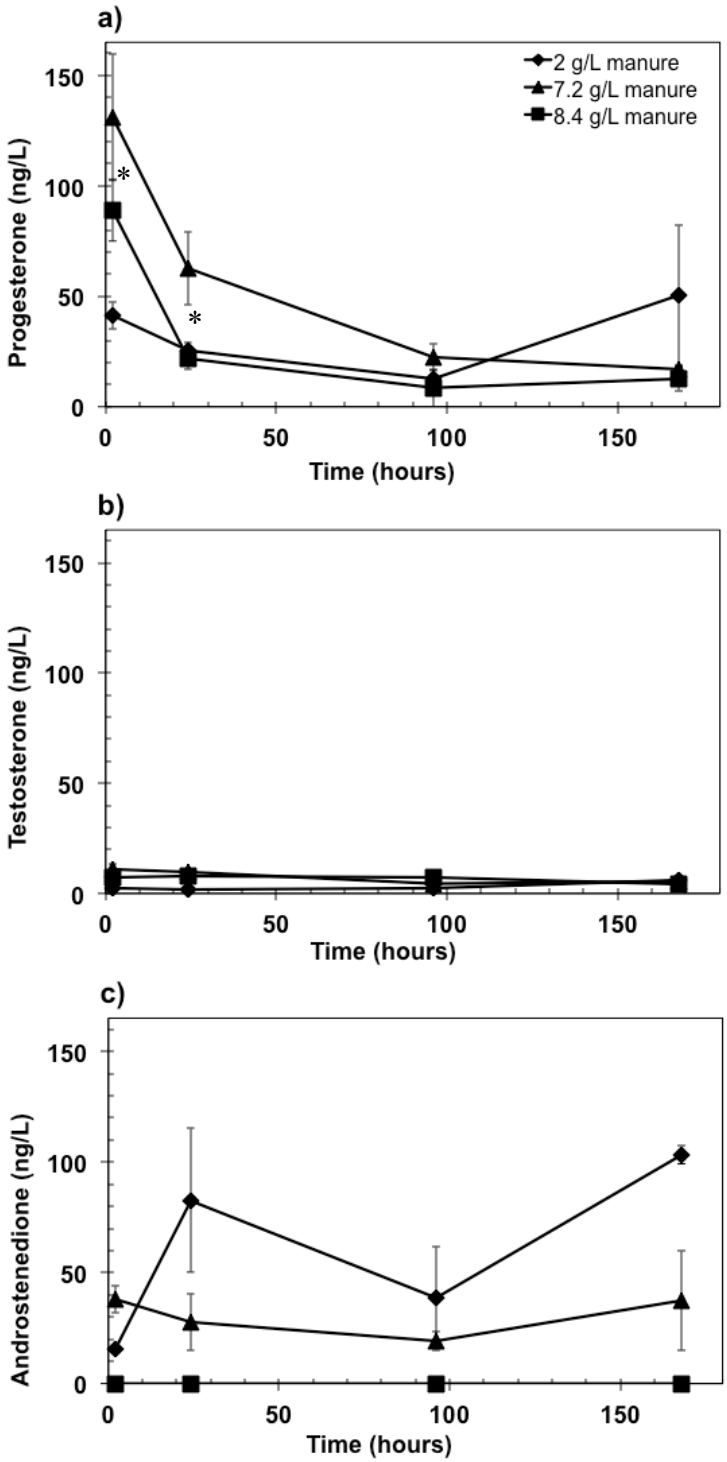


Figure 3-7. Mean androgen and progesterone concentrations detected over a 7-day period in synthetic feedlot runoff microcosms. Error bars represent ± standard error. Manure concentrations are based on dry mass added. * denotes statistical significance ($P<0.05$) from neighboring time point.

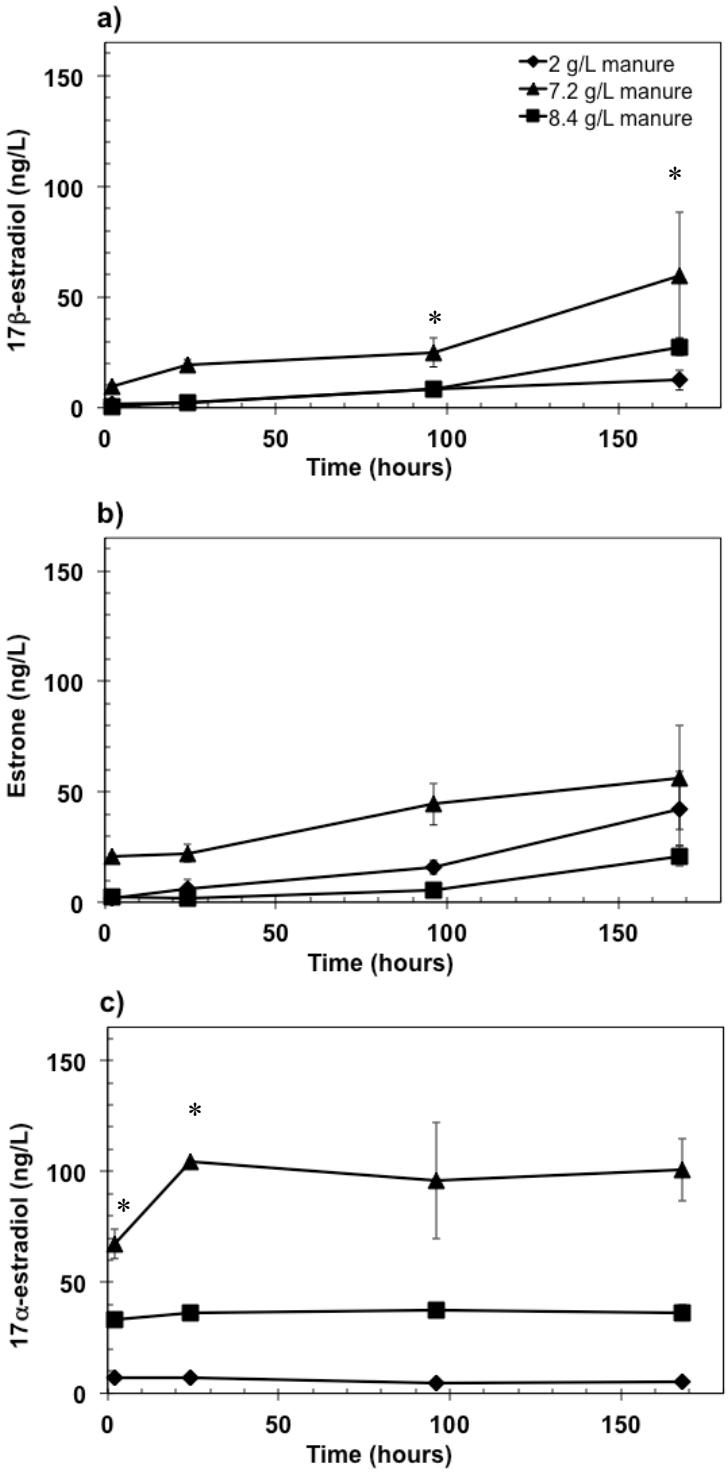


Figure 3-8. Mean estrogen concentrations detected over a 7-day period in synthetic feedlot runoff microcosms. Error bars represent ± standard error. Manure concentrations are based on dry mass added. * denotes statistical significance ($P<0.05$) from neighboring time point.

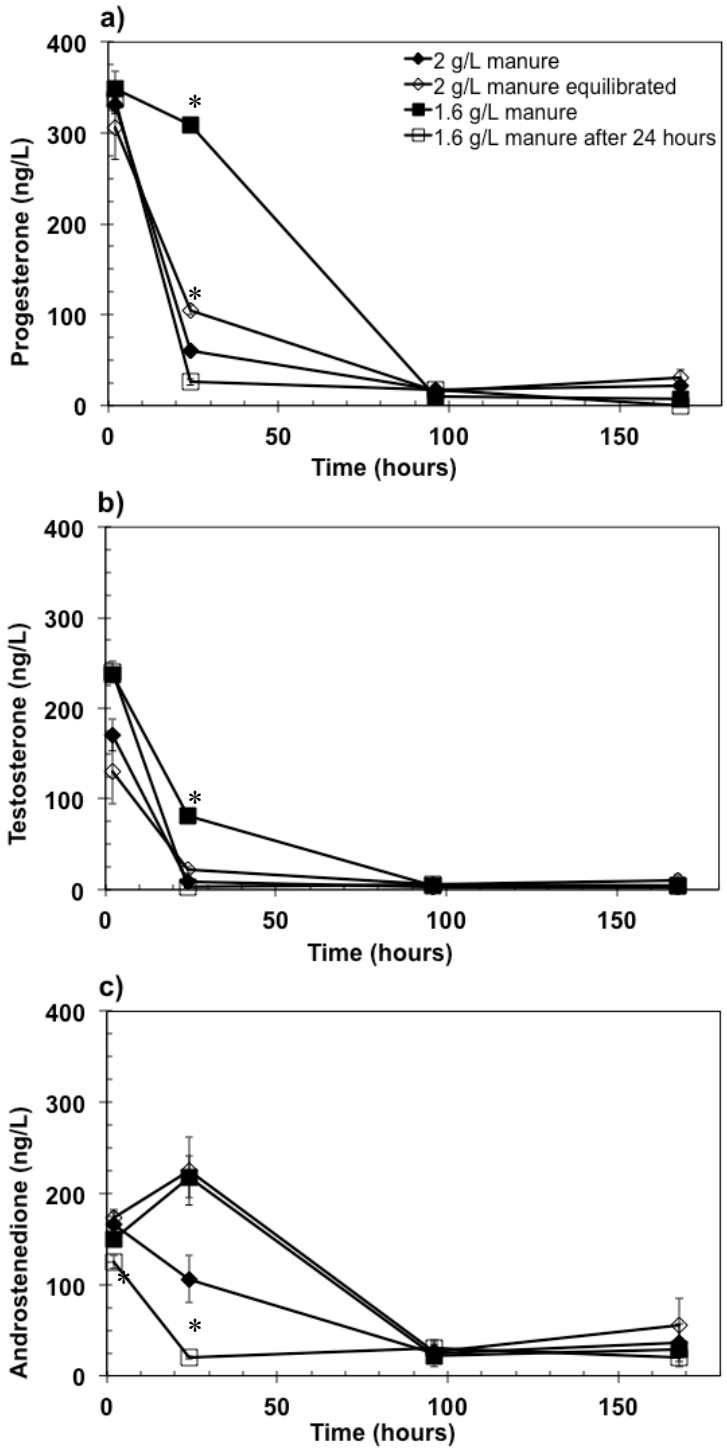


Figure 3-9. Mean androgen and progesterone concentrations detected over a 7-day period in synthetic feedlot runoff microcosms amended with 400 ng/L 17β-estradiol, testosterone, and progesterone. Equilibrated microcosms were held under acidified conditions 24 hours before raising pH and adding more manure. 'After 24 hour' microcosms were amended with steroids 24 hours after creating microcosm. Error bars represent ± standard error. Manure concentrations are based on dry mass added. * denotes statistical significance ($P<0.05$) from other treatment.

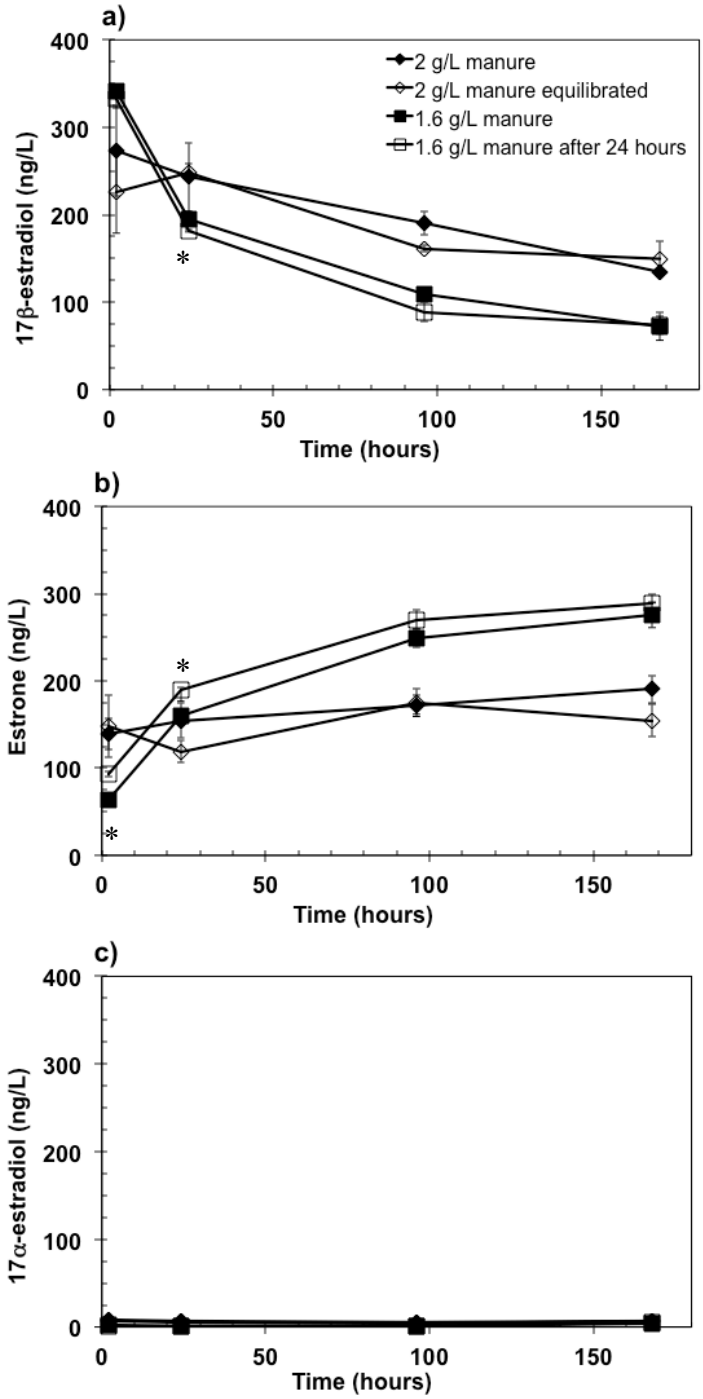


Figure 3-10. Mean estrogen concentrations detected over a 7-day period in synthetic feedlot runoff microcosms amended with 400 ng/L 17β-estradiol, testosterone, and progesterone. Equilibrated microcosms were held under acidified conditions 24 hours before raising pH and adding more manure. 'After 24 hour' microcosms were amended with steroids 24 hours after creating microcosm. Error bars represent ± standard error. Manure concentrations are based on dry mass added. * denotes statistical significance ($P<0.05$) from other treatment.

To assess whether anoxic conditions prevented transformation of the estrogens after 24 hours, experiments were performed in which steroids were added to one set of microcosms 24 hours after the manure was added. Steroid hormone transformation rates were similar in both sets of microcosms with the only consistent statistically significant difference in steroid concentrations between the figures occurring at 1 day (Figures 3-9 &3-10, square markers).

Significant concentrations of steroids were detected in both the particle-associated and filtrate phases in all experiments with a weak relationship between manure mass and the fraction of steroid in the filtrate phase (Figures 3-11 and 3-12). Most of the data fell within a range of predictions for equilibrium partitioning of compounds with organic carbon-normalized partitioning coefficients between 10^3 and 10^4. Addition of the steroids 24 hours after construction of the microcosm resulted in a higher fraction of the steroids in the filtrate for 17β-estradiol, testosterone and progesterone, although the differences were not always statistically significant (Figure 3-13). No other treatments (i.e., acidification, steroid amendments, equilibrating, or time) caused consistent changes in partitioning.

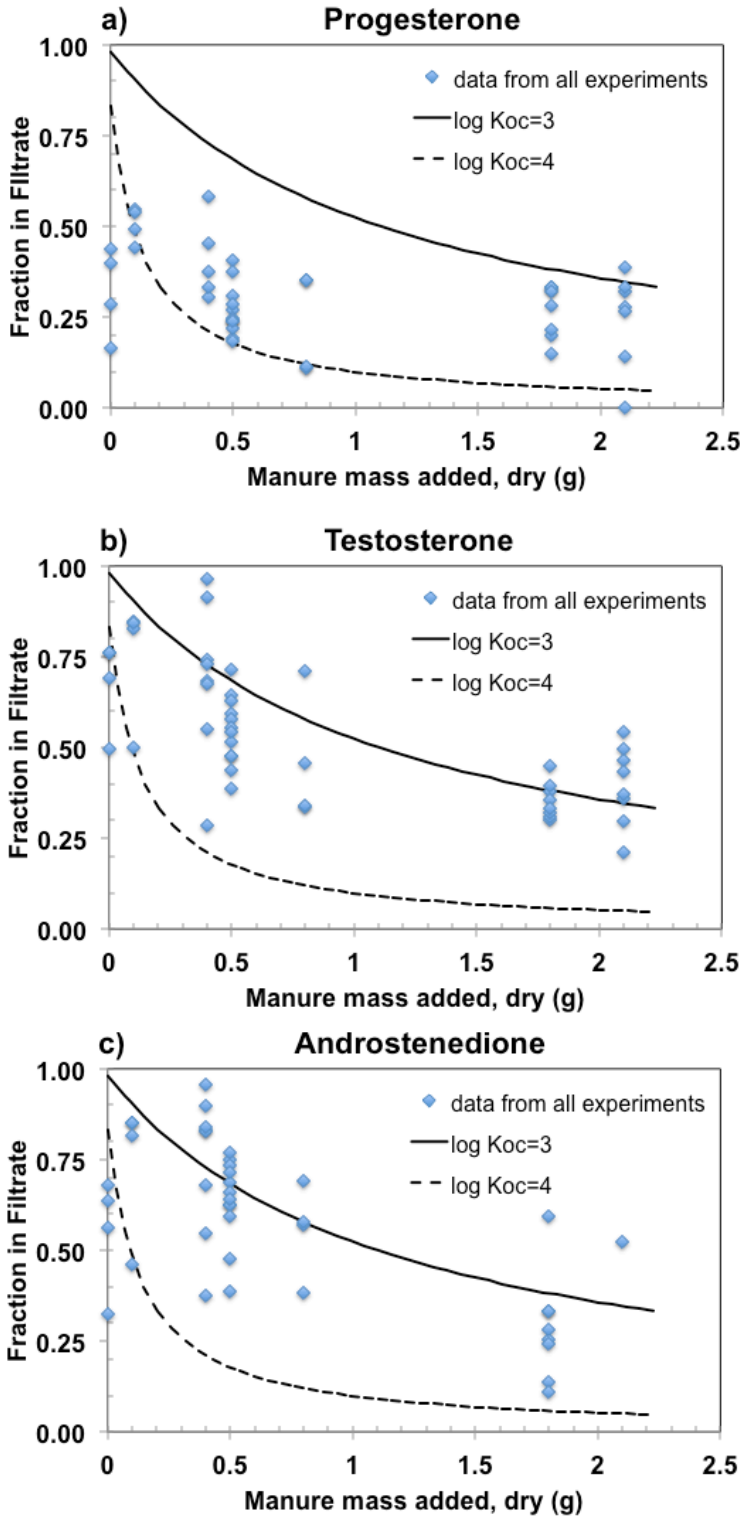


Figure 3-11. Fraction of measured androgens and progesterone in filtrate of 250-mL synthetic feedlot runoff microcosms as a function of manure dose. Model is fit using linear equilibrium partitioning using a partitioning coefficient, K_{oc} a) progesterone, b) testosterone, c) androstenedione.

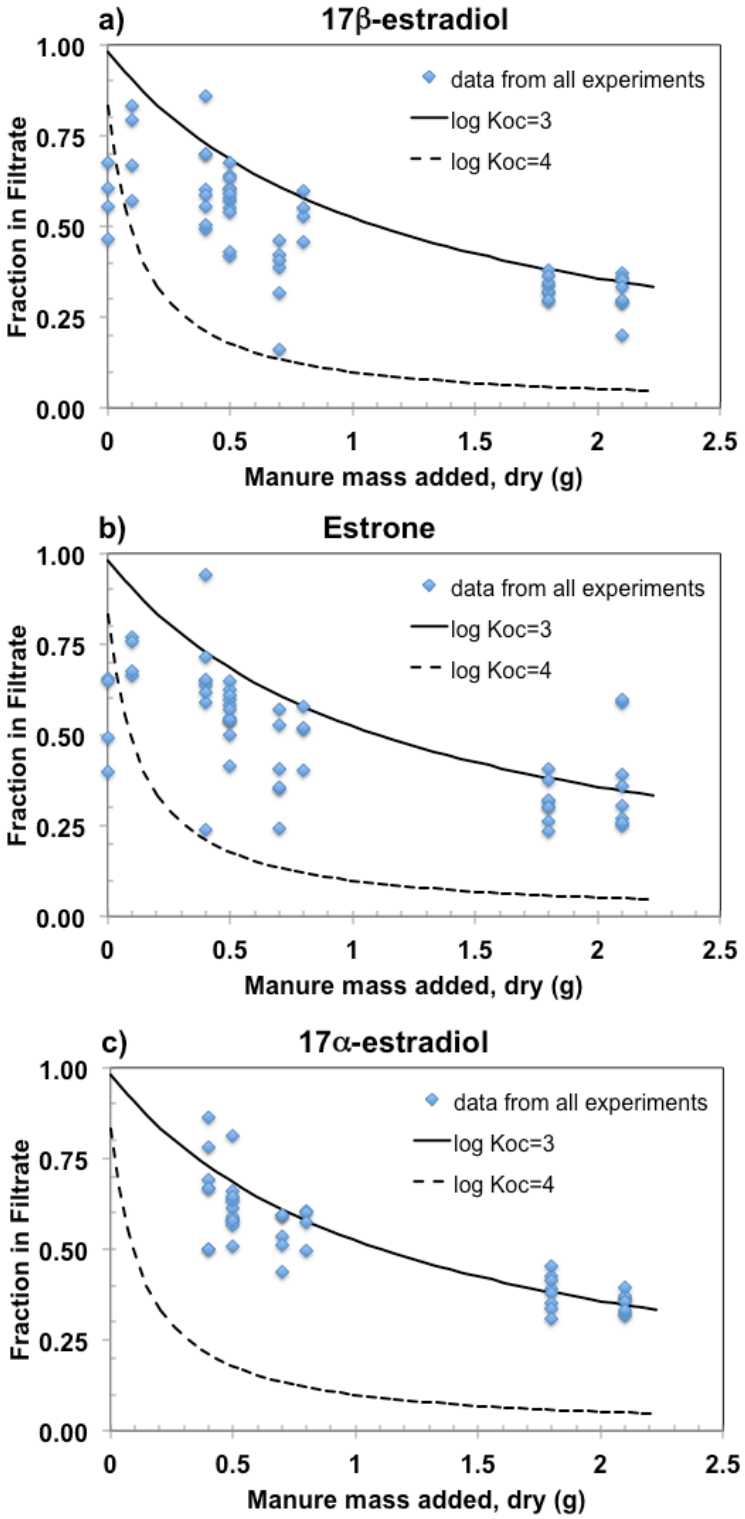


Figure 3-12. Fraction of measured estrogens in filtrate of 250-mL synthetic feedlot runoff microcosms as a function of manure dose. Model is fit using linear equilibrium partitioning using a partitioning coefficient, K_{oc} a) 17β-estradiol, b) estrone, c) 17α-estradiol.

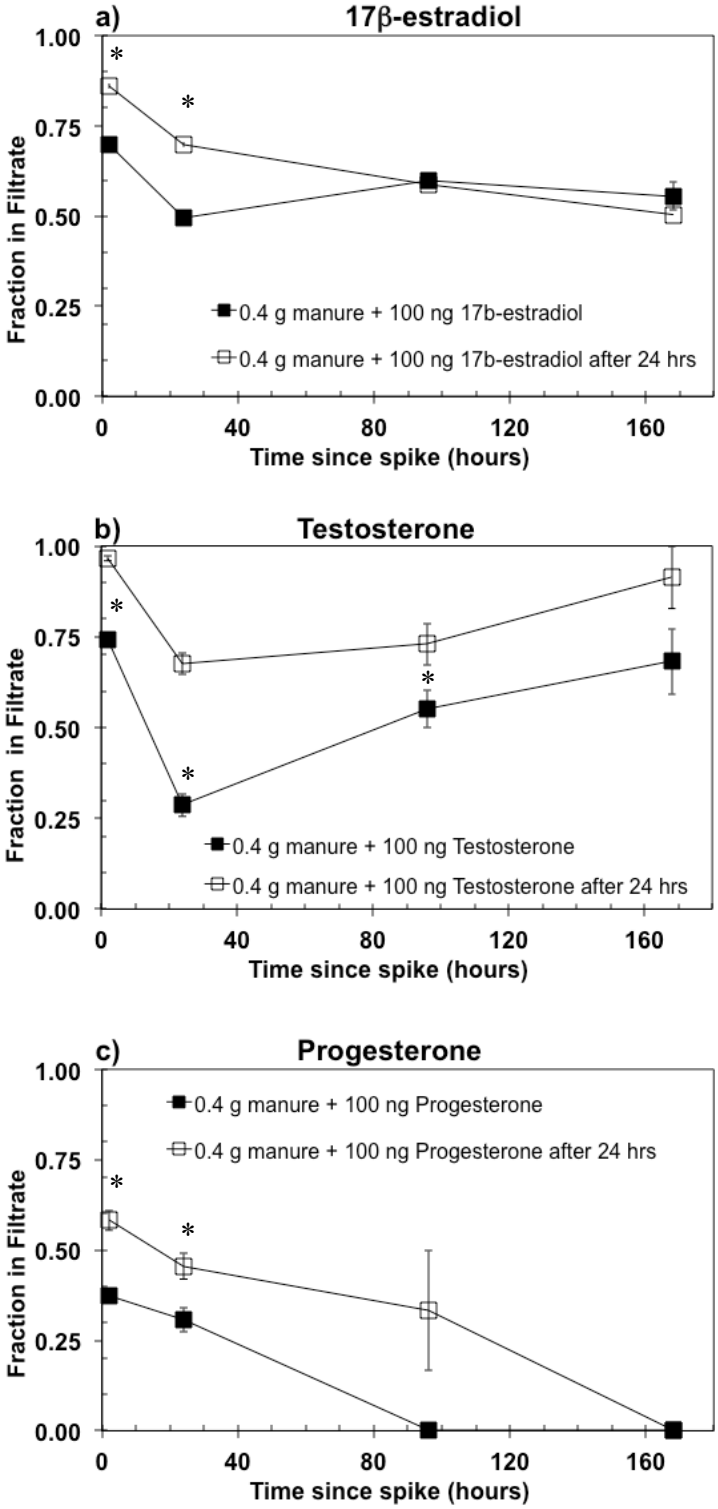


Figure 3-13. Fraction of steroids in filtrate of synthetic feedlot microcosms. 'After 24 hours' refers to the microcosms that were amended with steroids 24 hours after construction. a) 17β-estradiol, b) testosterone, c) progesterone. Error bars represent ± standard error. * denotes statistical significance ($P<0.05$) from other treatment.

3.4 Discussion

The presence of manure can affect steroid transformation rates in runoff in at least three ways. First, manure contains microorganisms capable of transforming steroids. Therefore, addition of manure could increase microbial activity and introduce microorganisms capable of transforming steroids. This effect has been observed for 17β-estradiol and testosterone when animal wastes or municipal wastewater biosolids are added to soil (Jacobsen et al. 2005, Lucas & Jones 2006). Second, steroids can partition into organic matter (e.g., lipid globules) from manure in both the particulate and colloidal phases where they can be protected from microbial transformation. The decrease in rates of transformation of 17β-estradiol and estrone when organic carbon derived from manure or autoclaved domestic septic tank effluent was added to soils was attributed to the stabilizing effect of organic matter (Zitnick et al. 2011, Stumpe & Marschner 2010, Stanford et al. 2010). Finally, respiration by microorganisms in manure could deplete dissolved oxygen, causing a shift to anoxic microbial communities, which tend to exhibit slower transformation rates of steroid hormones (Czajka & Londry 2006, Jurgens et al. 2002, Yang et al. 2010, Zheng et al. 2012, Ying & Kookana 2003).

In our microcosms, all of the steroid hormones were transformed more rapidly at higher manure masses during the first two hours. This effect was presumably due to the increased microbial activity caused by the higher mass of manure and is consistent with previous studies (Jacobsen et al. 2005, Lucas & Jones 2006). However, after the initial 24 hours, transformation of 17β-estradiol and estrone ceased at the two highest manure concentrations. It is unclear if a similar phenomenon occurred for testosterone and progesterone because they were almost entirely transformed within 24 hours. The stabilization of estrogens is significant because it suggests that the steroids could persist much longer than expected in the presence of manure.

While our previous field experiment suggested the occurrence of this phenomenon, this is the first time this finding has been documented under conditions relevant to agricultural runoff. Havens et al. (2010) reported losses of 17β-estradiol and estrone of 90% and 75%, respectively, after 14 days in synthetic runoff containing 16 mg/L of DOC (Havens et al. 2010). Our microcosms with the lowest manure mass, which contained about twice as much DOC as those studied by Havens et al. (2010), also exhibited a slow loss of 17β-estradiol, but the suspended solid and DOC concentrations in the microcosms containing the lowest concentration of manure were substantially lower than those measured in steer feedlot runoff, where DOC concentrations between 200-1400 mg/L are common (Mansell et al. 2011).

Attempts to determine the cause of the observed stabilization of the estrogens after 24 hours were unsuccessful. Adding the steroids 24 hours after the manure would have resulted in less steroid loss in the first 24 hours of incubation if anoxic conditions were responsible for the stabilization (Figs 3-9 & 3-10, square markers). The lack of an effect of pre-equilibration of the steroids prior to addition of active microorganisms suggests that equilibration with the organic matter also was not the cause of stabilization (Fig 3-9 & 3-10, diamond markers).

Most researchers studying the fate of steroid hormones in soil and agricultural runoff add steroids to facilitate quantification of the compounds and to avoid uncertainty associated with production of steroids from precursors in the manure. It is typically assumed that the added steroids behave similarly to those initially present in the manure. However, some researchers have reported differences in behavior between added steroids and those coming from manure (Laegsmand et al. 2009, Schuh et al. 2011). For example, Schuh et al. (2011), observed that

17β-estradiol added to a pig manure slurry disappeared within 24 hours, while the 17β-estradiol initially present in the waste was stable during the 3-day experiment (Schuh et al. 2011). In our experiment, the estrogens originating in the manure were much more stable than those that were added to the microcosms.

The main difference between the added steroids and those originating in the manure is their location at the start of the experiment. In cattle, steroids are excreted mainly in the feces (i.e., 60-70% of the mass) (Hanselman et al. 2003, Lange et al. 2002). Steroids in manure are likely closely associated with lipid-rich fractions of the manure. For these steroids to be transformed by microorganisms, they must first partition out of the organic material. In contrast, steroids added to a manure/soil suspension will partition to the solid, but will not necessarily reach equilibrium within 24 hours. Equilibration times for steroid partitioning to organic material vary widely with a reported range between 1 hour and 14 days (Lee et al. 2003, Lai et al. 2000, Mansell & Drewes 2004, Yu et al. 2004, Schiffer et al. 2004, Bowman et al. 2002, Das et al. 2004). Equilibrium partitioning coefficients for steroid partitioning to particulate organic material range between 10^3 and 10^4 (Lee et al. 2003, Schiffer et al. 2004, Das et al. 2004, Holthaus et al. 2002). Therefore, in runoff with a high concentration of organic solids, the vast majority of the steroids would be expected to remain absorbed to organic matter.

When no manure was present, approximately 95% of the added 17β-estradiol disappeared within 2 hours. The decrease in 17β-estradiol concentration was not accompanied by an increase in estrone or 17α-estradiol concentrations. Possible explanations for these observations include rapid, irreversible sorption of the steroids to the mineral soil or rapid transformation to a product other than estrone. Biotransformation is unlikely given the low microbial activity expected in the mineral soil. Furthermore, testosterone and progesterone were both transformed very slowly in the manure-free microcosms, suggesting that rapid microbial transformation is not a viable explanation. When clays and metal oxides surfaces are not covered by organic matter they can catalyze the abiotic transformation of organic compounds (Lee et al. 2003). Unlike the androgens and progesterone, estrogens contain phenolic moeities that are readily oxidized by manganese oxides (Stone 1987). Further research is needed to determine what mechanism could be responsible for the rapid loss of 17β-estradiol and their potential importance for steroid fate.

In all experiments and at all manure concentrations, the three estrogens were transformed more slowly and to a lesser extent than testosterone, androstenedione, and progesterone. This finding was consistent with observations of estrogens and testosterone in feedlot soil and runoff (Mansell et al. 2011), soil (Casey et al. 2004, Stumpe & Marschner 2007, Fan et al. 2007), biosolids (Layton et al. 2000), and synthetic feedlot runoff (Havens et al. 2010). Progesterone transformation has not been studied in detail in the presence of estrogens, but the compound has been observed to undergo microbial transformation at similar rates to testosterone (Yang et al. 2010, Lim et al. 2008). It is also removed to a similar degree as testosterone in municipal wastewater treatment plants (Kolodziej et al. 2003, Chang et al. 2011).

The main difference in the chemical structures of androgens and estrogens is the presence of the phenolic group on the estrogens. Some researchers have hypothesized that the phenolic moiety increases the affinity of the estrogens for organic matter, thereby protecting them from microbial transformation (Shore et al. 1993). However, the androgens and estrogens exhibit similar affinities for organic matter (Lee et al. 2003, Das et al. 2004, Casey et al. 2004). In our experiments, no significant difference was seen in phase partitioning between the androgens and estrogens. In addition, equilibrating the steroids with manure prior to allowing microbial transformation did not significantly affect transformation rates. Thus, the small differences in

affinity of the estrogens, androgens, and progesterone for dissolved or particulate organic matter are not likely to explain the differences in transformation rates. Further research on microbial communities and their ability to transform steroids might help to explain the differences in transformation rates among the different classes of steroids.

In steroid-amended microcosms, conversion of 17α-estradiol to 17β-estradiol was observed, but not conversion of 17β-estradiol to 17α-estradiol. Conversion of 17α-estradiol to estrone and 17β-estradiol, as well as conversion from 17β-estradiol to estrone and 17α-estradiol has been reported previously under anoxic conditions (Mansell et al. 2011, Czajka & Londry 2006, Zheng et al. 2012, Hutchins et al. 2007). The observation of 17α-estradiol to 17β-estradiol conversion is significant because most of the estrogen excreted by cattle consists of 17α-estradiol (Hanselman et al. 2003), a form that is much less potent than 17β-estradiol with respect to feminization of fish (Shappell et al. 2010). In the manure-amended microcosms, approximately 10% of the added 17α-estradiol was converted to 17β-estradiol. 17β-estradiol is approximately 8-9 times more potent than 17α-estradiol at feminizing fathead minnows (Shappell et al. 2010), so a 10% conversion to 17β-estradiol would increase overall solution estrogenicity by 70-80%.

Chapter 4 Steroid Hormone Removal From Feedlot Runoff by a Multi-Step Treatment Train Employing a Vegetated Treatment System

4.1 Introduction

Wastes generated by concentrated animal feeding operations (CAFOs) have long been a concern with respect to their potential to contaminate surface waters with sediments, nutrients, pathogens, organic matter, and toxic metals. More recently, concerns have been expressed about their role as sources of veterinary pharmaceuticals and steroid hormones (Burkholder et al. 2007). To help mitigate impacts associated with their discharges to surface waters, most CAFOs are required to implement best management practices (BMPs) to either minimize discharge of waste to surface waters or to treat the wastes prior to their discharge to surface waters (Koelsch 2005). However, most BMPs were designed to remove pathogens, nutrients, and sediment, and their efficacy for removing pharmaceuticals and steroid hormones is not well understood.

The most common BMP strategies are collection of CAFO runoff in solids settling basins, which are also called waste lagoons, and application of solid waste and lagoon water onto cropland and pastures (Koelsch 2005). However, solids settling basins do not always prevent the discharge of elevated concentrations of nutrients, pathogens, and steroid hormones to surface and groundwaters (Burkholder et al. 1997, Mallin 2000, Arnon et al. 2008, Zheng et al. 2012, Durhan et al. 2006). Animal wastes applied to crops and pastures also can be important sources of contaminants, including steroid hormones, to surface waters through overland flow (Khaleel et al. 1980, , Dutta et al. 2012, Nichols et al. 1998, Finlay-Moore et al. 2000) or subsurface flow (Evans et al. 1984, Thompson et al. 2009, Peterson et al. 2000) after rainfall or irrigation.

A third potential pathway for CAFO wastes to reach surface waters is through tile drain discharges. Tile drains are perforated pipes buried under agricultural land that are used to increase drainage. Water that reaches tile drains is typically pumped into nearby creeks or canals. Discharges from tile drains where wastes have been added often contain elevated concentrations of nutrients and pesticides (Kladivko et al. 1991). Some data suggest that tile drains may also be sources of steroid hormones to surface waters (Kjaer et al. 2007, Gall et al. 2011). However, prior studies were conducted on fields with pronounced macropore flow, which may have increased steroid hormone transport to tile drains.

To help mitigate potential threats to water quality from CAFOs, many CAFO operators are starting to use vegetated treatment systems to help control runoff and treat discharges from CAFOs and manure-applied fields (Koelsch et al. 2006). Vegetated treatment systems include vegetated treatment areas and vegetated infiltration basins (a.k.a. vegetated swales). Vegetated treatment areas contain tall grasses or other vegetation, such as legumes or forbs. When runoff or CAFO effluent is applied to these systems as overland flow, the water is filtered through the vegetation as it flows across the surface on the gentle slope which decreases flow and intercepts particles and contaminants. Pollutants are removed through several mechanisms, including sedimentation or interception of particle-associated contaminants, sorption of dissolved contaminants to soil and plants, transformation by microorganisms, and uptake by plants (USDA 1988, Dickey et al. 1981). Vegetated infiltration basins are similar to vegetated treatment areas except that they are built in depressions in order to retain the water and allow it to infiltrate through the soil. Sizes of these systems varies depending on the volume of water, the retention time needed, and land space limitations.

Vegetated treatment systems can remove nutrients, sediments, oxygen-consuming organic matter, and pathogens from feedlot runoff (Dillaha et al. 1988, Dickey et al., 1981, Koelsch et al. 2006) and manure-applied fields (Coyne et al. 1998, Chaubey et al. 1995). For example, a vegetated treatment system removed as much as 95% of the nutrients and

biological oxygen demand (BOD) from feedlot runoff (Dickey et al. 1981). Vegetated buffer strips, a treatment option for runoff from manure-fertilized crops or pastures, removed up to 94% of the17β-estradiol from runoff from grassland where poultry litter was applied (Nichols et al. 1998). Based on this evidence, it is likely that vegetated treatment systems will be effective for removing steroid hormones from feedlot runoff. However, little data are currently available on systems employed for cattle feedlots.

The purposes of this study were to determine the extent to which vegetated treatment systems remove steroid hormones from feedlot runoff, and to examine the potential contribution of steroid hormones from tile drains and commercial feedlot runoff. To accomplish these objectives, discharge and soil samples were collected from each step of a treatment system including a solids settling basin, vegetated infiltration basin, and a vegetated treatment area in series that received runoff from a cattle feedlot. Commercial feedlot runoff and tile drain discharge samples were also analyzed for steroid hormone concentrations.

4.2 Methods

4.2.1 Runoff sample collection from feedlot in Northern California

Over a period of three years, a total of 21 samples of runoff from a Northern California feedlot were collected after each winter rainstorm that was large enough to cause runoff. The approximately 22 acre feedlot contained 87 animals including steers, bulls, heifers, calves, and cows at various stages of development. Runoff was sampled as it was leaving the pens and as it pooled in the retention basin (Figures 4-1 and 4-2). 1 L grab samples (and replicates) were collected in 1 L amber glass bottles and shipped on ice overnight to UC Berkeley where they were extracted on the day that they were received.

Figure 4-1. Runoff from a feedlot in Northern California that was sampled and analyzed for

steroid hormones. (February 15, 2009)

Figure 4-2. Runoff from a feedlot draining into a retention basin in Northern California that was sampled and analyzed for steroid hormones. (January 2, 2010)

4.2.2. Samples from tile drains under dairies and manure-applied fields in Northern California

Discharges from four tile drains in Colusa County, California were collected on four occasions between April and May 2009. Sampling was timed to capture the water discharged during the first period that the fields were irrigated after manure application (Figure 4-3). Each drain collected water from an area of roughly 4 square kilometers that contained dairies, manure-applied fields, manure compost piles, and waste lagoons. Grab samples were collected in 1-L amber glass bottles and were stored on ice during transport to UC Berkeley where they were extracted the same day.

Figure 4-3. Tile drain discharging leachate drained from an area containing dairies, manure applied fields, and manure storage near Modesto, CA. (April 30, 2009)

4.2.3 Runoff and soil samples from a cattle feedlot and treatment system in Iowa

Runoff and soil samples were collected from a feedlot and treatment system that received runoff from an 2.64-acre feedlot near Ames, Iowa between June and November, 2009. The feedlot contained roughly 500 cattle of various types that had been in the feedlot for 120 days prior to sample collection. The treatment system consisted of a 473 m^3 solids settling basin, a vegetated infiltration basin, and a vegetated treatment area connected in series. The solids settling basin contains round hay bales which create a porous dam (Figure 4-4). The outlet consists of a 12" PVC pipe which releases into the vegetated infiltration basin. The vegetated infiltration basin consists of a 0.83 acre basin planted with reed canary grass and brome grass with a 4" perforated pipe 4 feet below the surface which discharge to the vegetated treatment area (Figure 4-5). The discharge is distributed evenly along the vegetated treatment area by using a gated pipe Figure 4-6). The vegetated treatment area is a 0.5 acre area also planted with reed canary grass and brome grass. Soil samples from the feedlot, vegetated infiltration basin, and vegetated treatment area and sediment samples from the solids settling basin were collected before and after each storm. Runoff from the feedlot was collected as it discharged into the solids settling basin, and water from the solids settling basin, vegetated infiltration basin, and vegetated treatment area were collected at their discharge points. Flow rates varied depending on storm size. The discharge ranged from 0.1 to 0.9 m^3/hr.

Water samples were collected using an ISCO autosampler throughout the entire hydrograph for a total of 1 L. Samples were collected in polyethylene 1-L autosampler bottles to which 10 mL formaldehyde had been added to inhibit microbial activity. Samples were decanted into a 1-L amber glass bottle and shipped along with soil samples on ice overnight to UC Berkeley where they were extracted the day that they were received.

Figure 4-4. Solids settling basin collecting runoff at the Iowa feedlot.

Figure 4-5. Vegetated infiltration basin receiving discharge from the solids settling basin and filtering it through the soil profile where it is collected in perforated pipes and discharged to vegetated treatment area.

Figure 4-6. Vegetated treatment area showing effluent being applied from VIB through gated pipes.

4.2.4 Steroid hormone and chemical analysis

Chemical analysis of runoff samples was conducted using the metyhods described in Chapter 2. Briefly, total suspended solids (TSS) (2540 D), organic matter fraction of the suspended solids (f_{om}) (2540E), gravimetric moisture content and organic matter fraction of the soil (2540 G), dissolved organic carbon (DOC) (5310 B), chloride (4500-Cl^- F), and nitrate (4500-NO_3^- C) were analyzed using standard methods [APHA, AWWA, WEF 1995]. The concentration of dissolved organic carbon was not analyzed in samples from the treatment system in Iowa because the formaldehyde preservative prevented accurate measurement of DOC concentrations.

Steroid hormones were analyzed using solid phase extraction, Florisil cleanup, derivatization, and gas chromatography tandem mass spectrometry (GC/MS/MS) with isotope dilution as described in Chapter 2. Briefly, runoff samples were centrifuged and filtered, the surrogate standard was added, and the sample was extracted on a solid phase extraction cartridge. The cartridge was eluted with methanol, dried, and resuspended in 2 mL 95% dichloromethane/5% methanol and passed through a Florisil cartridge. The cartridge was eluted, dried, and transferred to a GC vial where it was resuspended in acetonitrile. Extracts were derivatized, dried, reconstituted in isooctane, and analyzed by GC/MS/MS using the parent

and daughter ions listed in Table 2-1. Soils and suspended solids were extracted by sonicating a 1-2 gram aliquot in 30 mL pure methanol three times. After sonicating, 250 mL water was added to the methanol, and the solution was filtered through a 1 μm glass fiber filter. Samples were extracted on a C-18 SPE cartridge followed by Florisil cleanup, derivatization, and GC/MS/MS analysis as described above. Tile drain samples contained virtually no suspended solids. Therefore, the suspended solids were not analyzed for steroid hormones. Suspended solids from the feedlot runoff samples from Northern California collected during 2008 were not extracted because a suitable method had not yet been developed at the time when those samples were collected. Limits of quantification were between 1 and 10 ng/L for androstenedione and progesterone and 0.1-1 ng/L for all other steroids in both the filtrate and suspended solids.

4.2.5 Quality assurance/ quality control and statistical methods

To ensure quality of the data, blanks, duplicates, and matrix recovery spikes were included with each round of samples collected. Steroids were never detected above quantification limits in blanks. Matrix recovery spikes ranged between 84% and 153% for the tile drain samples, 58% and 180% for the Northern California feedlot samples, and 71% and 136% for the Iowa feedlot samples. Relative percent differences averaged 26% for the Northern California feedlot samples and 34% for the Iowa feedlot samples and were generally higher at lower concentrations, as expected.

The two-tailed, unpaired, unequal variance student's t-test was used to determine statistical significance sampling points on the vegetated treatment train to determine effect of treatments. Statistical significance was assigned for all $P<0.05$.

4.3 Results

4.3.1 Steroid concentrations in runoff from a commercial feedlot in Northern California

Steroid hormone concentrations in runoff from the feedlot in Northern California exhibited considerable variability (Table 4-1). Concentrations ranged from below detection limits to approximately 3,000 ng/L. 17α-estradiol, estrone, and testosterone were the most frequently detected steroids. Androstenedione, estrone, 17α-estradiol, and progesterone had the highest concentrations. Mean concentrations of the steroids and water quality parameters were similar to those detected from simulated runoff from small plots on a feedlot in Chapter 2, with concentrations of chloride and TSS > 1 g/L, and DOC concentrations averaging over 300 mg/L (Table 4-1).

Table 4-1 Steroid concentrations and other water quality parameters in feedlot runoff from storms over three rainy seasons. N is the number of samples taken where the compound was analyzed.

	Steroid hormones (ng/L)									
	17αE2	T	17βE2	AD	E1	PR	Cl^- (mg/L)	NO_3^- (mg N/L)	DOC (mg/L)	TSS (g/L)
N	15	6	14	13	10	11	15	15	15	12
% detections	87	83	50	54	100	73	100	7	100	100
Max	540	6	120	3000	600	450	3400	1	890	7
Mean	51	3	12	310	120	120	1000	0	350	2

17αE2=17α-estradiol, 17βE2=17β-estradiol, E1=estrone, T=testosterone, AD=androstenedione, and PR=progesterone, Cl^-=chloride, NO_3^-=nitrate, DOC=dissolved organic carbon, TSS=total suspended solids.

4.3.2 Steroid concentrations in tile drain discharges from dairies and manure-applied fields

In contrast to the relatively high concentrations of steroid hormones detected in runoff, steroids concentrations were below detection limits in discharges from the four tile drains with one exception (Table 4-2). On the first date that samples were collected from tile drain 1N, androstenedione and estrone were detected at concentrations just above detection limits. The water also contained suspended solids at concentrations that were 10-20 times higher than other samples from the tile drains. Subsequent samples from tile drain 1N contained approximately 1 ng/L estrone. Discharges from the tile drains contained roughly two orders of magnitude less chloride, DOC, and TSS, than the feedlot runoff, but contained much higher concentrations of nitrate.

Table 4-2. Steroid concentrations and other water quality parameters in tile drain discharges from areas containing dairies, manure-applied fields, and manure storage.

	N	AD		E1		Cl^- (mg/L)	NO_3^- (mg N/L)	DOC (mg/L)	TSS (mg/L)
		%	Max (ng/L)	%	Max (ng/L)				
All others	8	0	0	25	1	16-96	25-60	3-13	<1-15
Tile Drain 1N	1		1		5	30	33	4	186

Note: 17α-estradiol, 17β-estradiol, testosterone, progesterone were also analyzed, but never detected in any samples
AD=androstenedione, E1=estrone, Cl^-=chloride, NO_3^-=nitrate, DOC=dissolved organic carbon, TSS=total suspended solids.

4.3.3 Steroid concentrations in runoff and soil from a commercial feedlot in Iowa

Runoff from the feedlot in Iowa contained widely varying concentrations of steroid hormones. No correlation was observed between the steroid concentrations in feedlot runoff and precipitation. Therefore, steroid concentrations were averaged for all storm events at each point along the treatment train (Figure 4-7). Similar to the runoff from the feedlot in Northern California, 17α-estradiol, estrone, androstenedione, and progesterone were the steroids detected at the highest concentrations. Steroid concentrations in the feedlot runoff spanned a similar range as those in the runoff from the feedlot in Northern California. Except for the occasional detections of androstenedione at concentrations exceeding 1000 ng/L, the concentrations of steroid hormones from these commercial feedlots were in a similar range as the concentrations observed in the runoff from the plot scale system described in Chapter 2.

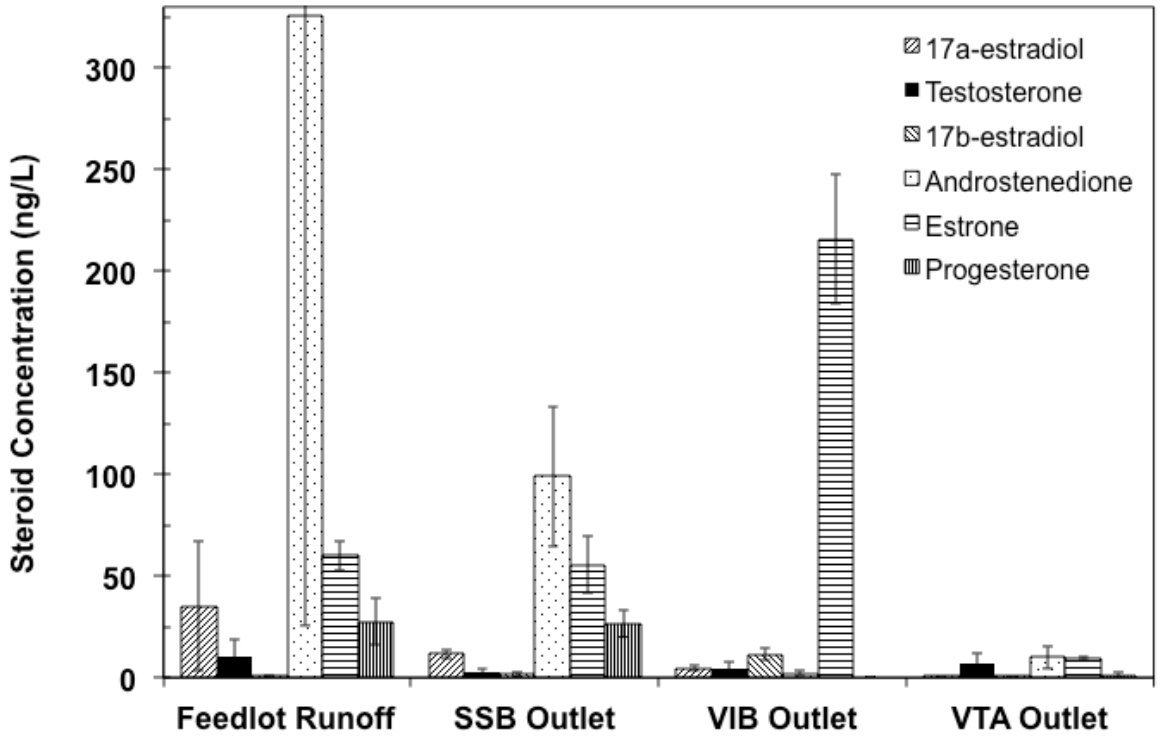


Figure 4-7. Mean steroid concentrations at each step of the feedlot runoff treatment train at the Iowa facility. SSB=solids settling basin, VIB=vegetated infiltration basin, VTA=vegetated treatment area. Error bars represent ± standard error.

Concentrations of steroid hormones in the feedlot runoff generally decreased during treatment. The first step in the treatment process, the solids settling basin, reduced the average concentration of all of the steroids by approximately 70% except for progesterone and estrone which were not affected (Figure 4-7). However, none of the changes were statistically

significant, so the effect of the SSB cannot be determined with high confidence. The vegetated infiltration basin further reduced the average concentration of 17α-estradiol, androstenedione, and progesterone, each by a statistically significant amount. The concentration of testosterone was not statistically changed by the vegetated infiltration basin. The concentration of 17β-estradiol and estrone increased by ten-fold and four-fold, respectively, during passage through the vegetated infiltration basin, both with statistically significant changes. The next step, the vegetated treatment area, further lowered steroid concentrations by statistically significant amounts for all steroids except testosterone, androstenedione, and progesterone which were not statistically affected. While testosterone and androstenedione were sporadically detected in the outlet from the vegetated treatment area at concentrations near 10 ng/L, in most samples, their concentrations were below 1 ng/L. Estrone was the only steroid detected consistently in the outlet from the vegetated treatment area at an average concentration of 9 ng/L.

The concentration of steroid hormones detected in the soils of the feedlot, vegetated infiltration, vegetated treatment area, and the sediments of the solids setting basin did not exhibit much temporal variability. Therefore, the steroid concentrations from each location was averaged (Figure 4-8). Feedlot soils were dominated by estrone, androstenedione, and progesterone, with mean concentrations of approximately 10 ng/g (dry weight of soil) (Figure 4-8). The sediments from the solids settling basin had a fraction organic carbon approximately twice as high as the feedlot soils, and steroid concentrations that were approximately 6 times higher with all of the increases being statistically significant. The increased steroid concentrations in the sediments coupled with the somewhat decreased steroid concentration in the outlet from the solids settling basin suggests that particle-associated steroids were removed by settling out of the water column. Steroid concentrations in both the vegetated infiltration basin and vegetated treatment area soils were much lower than in the solids settling basin, by statistically significant margins, suggesting that processes other than sorption removed steroids after the initial treatment step.

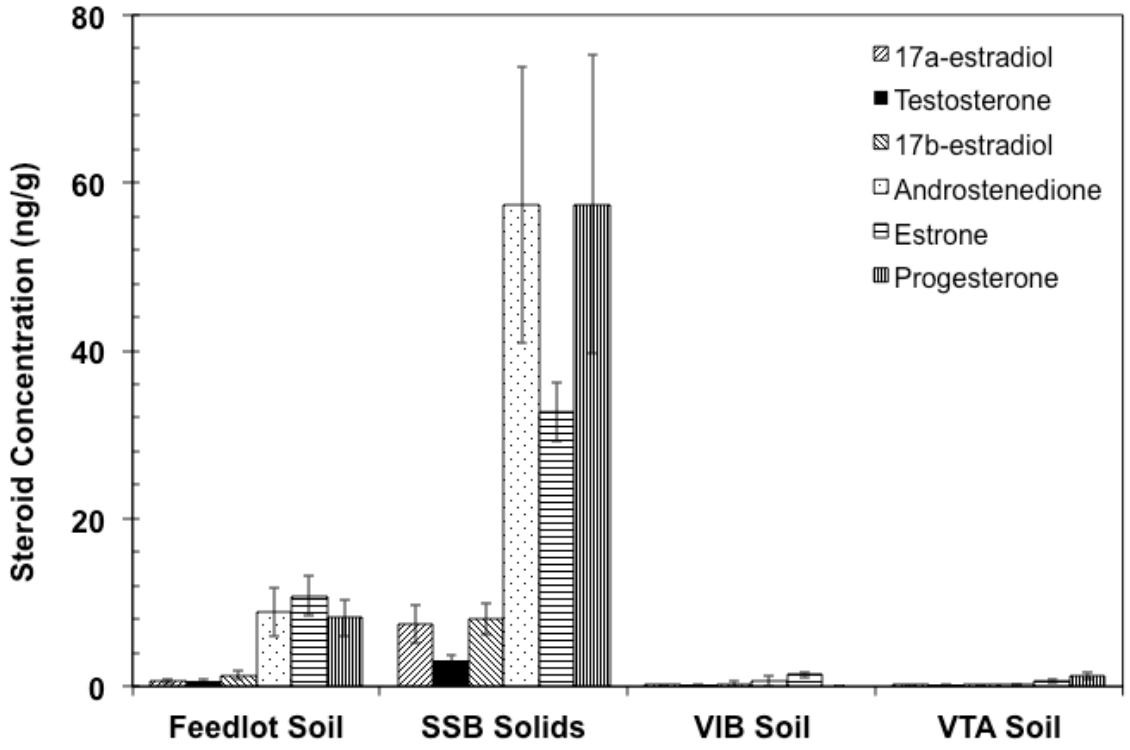


Figure 4-8. Steroid concentrations in soil and sediments from steps along a feedlot runoff treatment train. SSB=solids settling basin, VIB=vegetated infiltration basin, VTA=vegetated treatment area. Error bars represent ± standard error.

4.4 Discussion

4.4.1 Potential environmental risks associated with steroid hormones in runoff and tile drain discharges

Runoff from feedlots is much more likely to contribute steroid hormones to the environment than tile drain discharges. Steroid hormone concentrations in runoff from cattle feedlots were several orders of magnitude above thresholds for biological response (e.g., see Table 1-1) at both the commercial feedlots and the plot-scale system described in Chapter 2. If untreated, this runoff would require dilution factors of up to 10,000 to assure the safety of sensitive aquatic life from endpoints like endocrine disruption and olfactory interference. In contrast, steroid hormone concentrations in tile drain discharges were never high enough to cause endocrine disruption, and only exceeded thresholds for olfactory interference in one sample.

Table 4-3. Thresholds for biological response in sensitive aquatic species.

Steroid	17α–E2	17β-E2	E1	T	AD	PR
Conc. (ng/L)	~1 (endocrine disruption)	9 (endocrine disruption)	5 (endocrine disruption)	0.003 (olfactory)	0.3 (olfactory)	10 (endocrine disruption)

17β-E2=17β-estradiol. 17α-E2=17α-estradiol, E1=estrone, T=testosterone, AD=androstenedione, PR=progesterone

The low concentrations of steroid hormones observed in tile drain discharges suggests that manure application to crops and pastures may be an effective treatment method for steroid hormone removal in some applications if runoff can be controlled. Steroids are effectively removed during transport through soil (Lee et al. 2003, Das et al. 2004, Schiffer et al. 2004), but in soils prone to macropore flow, steroids can be transported more rapidly than expected. For example, in a field with significant macropore flow, Kjaer et al. (2007) observed concentrations of estrone as high as 66 ng/L in tile drain discharges up to 3 months after manure application (Kjaer et al. 2007). Gall et al. (2012) observed concentrations up to 50 ng/L, 40 ng/L and 50 ng/L for 17α-estradiol, estrone, and androstenedione in tile drain discharges from a field prone to macropore flow (Gall et al. 2012). In contrast, Kolodziej et al. (2005) and Shappell et al. (2011) did not observe steroid concentrations greater than 1 ng/L in tile drain discharges from soils that did not contain macropores (Kolodziej et al. 2004, Shappell et al. 2011). The tile drains sampled in this study drained soils that did not contain significant macropores (Thomas Harter, personal communication). Therefore, the apparent discrepancy between the results of this study and observations of high concentrations of steroids in other tile drain discharges is likely related to the absence of macropores in the soils sampled in California.

Steroid hormone concentrations in the feedlot runoff were highly variable, spanning three orders of magnitude with no trends with rainfall rate or soil concentration. Similar variability has also been observed in waterbodies impacted by feedlot runoff (Soto et al. 2004, Matthiessen et al. 2006) and in runoff from manure-applied fields (Finlay-Moore et al. 2000). This variability makes predicting steroid hormone concentrations in runoff very difficult and necessitates multiple measurements over extended time periods. The fact that the concentrations were not correlated with rainfall rates or soil concentrations suggests that other factors were important to steroid release. The factors could be related to the location of steroids within the manure-soil matrix, to the microbial community present, or to other unknown factors.

4.4.2 Efficacy of vegetated treatment systems for steroid removal from feedlot runoff

Solids settling basins alone are not likely to decrease steroid hormone concentrations in feedlot runoff to concentrations below levels of concern. While most steroid concentrations decreased by roughly 70% in the solids settling basin, the changes were not statistically significant. Steroid hormone concentrations in the discharge from the solids settling basin were still approximately three orders of magnitude above thresholds for biological responses in sensitive aquatic life. These data were similar to results from previous studies on discharges from solids settling basins treating cattle feedlot effluent (Kolodziej et al. 2004, Hutchins et al. 2007, Zheng et al. 2007). For example, Zheng et al., (2007) reported total estrogen concentrations of 500 ng/L in a solids settling basin treating dairy runoff (Zheng et al. 2007), while Kolodziej et al. (2004) observed testosterone, androstenedione, and progesterone concentrations of

approximately 100 ng/L in a solids settling basin treating dairy runoff (Kolodziej et al. 2004).

As expected, the solids settling basin removed a considerable fraction of the steroid hormones through gravitational settling. The elevated concentration of steroids in the sediments of the settling basin relative to the feedlot soil suggests that particle-associated steroids have accumulated over time. The fraction of steroids in the feedlot runoff that were associated with particles varied, but was generally between 50 and 90%. Therefore, the average removal rate of 70% for most of the steroids was most likely mainly due to settling of particles. However, the fact that estrone and progesterone concentrations did not decrease suggests that microbial transformation occurred because these compounds also tend to partition onto particles. Because the sediment water interface of lagoons is anoxic, rates of microbial transformation should be slow. For example, Zheng et al. (2012) observed only 15%, 22%, and 23% transformation of 17α-estradiol, 17β-estradiol, and estrone, respectively, after 52 days in an anoxic waste lagoon (Zheng et al. 2012).

After passage through the vegetated infiltration basin, the concentration of most of the steroids were below thresholds for biological response in aquatic life. While no other data were available on steroid removal from feedlot runoff using vegetated infiltration basins, an infiltration basin treating municipal wastewater effluent removed 90% of the estrogenicity (Conroy et al. 2006). The steroid concentrations in the soil were also very low, suggesting that the steroids removed during passage through the vegetated infiltration basin were removed by microbial transformation or uptake into plants.

In contrast to the other steroids, the concentrations of 17β-estradiol and estrone increased during passage through the vegetated infiltration basin. Both increased by much more than could be accounted for by the decrease in the concentration of 17α-estradiol. Temporal variability is unlikely to explain the consistently higher concentrations of these compounds over the period where samples were collected. Based on the research described in Chapter 3, estrogens can be more stable than the other steroids in feedlot runoff, but increased stability would not explain the increase in 17β-estradiol and estrone while 17α-estradiol decreased. Further research is needed to determine the possible cause for the increase in 17β-estradiol and estrone during vegetated infiltration basin treatment.

The vegetated treatment area, in combination with the vegetated infiltration basin, decreased all steroid concentrations to concentrations below thresholds for biological response in sensitive aquatic species. 17β-estradiol and estrone concentrations decreased to below quantification limits and 9 ng/L, respectively. All other steroids were already present at very low concentrations after the vegetated infiltration basin treatment. As a result, the effect of the vegetated treatment area cannot be determined for these compounds.

While this study showed that a vegetated treatment system was effective at removing steroid hormones from cattle feedlot runoff, additional research is needed to make these findings more broadly applicable. Mechanistic studies are needed to better determine the removal mechanisms in each of the treatment systems and support the design of systems that maximize these removal mechanisms. Manure from other types of livestock contains different concentrations of steroid hormones along with different types of organic matter. The efficacy of vegetated treatment systems for these types of feedlot effluents requires additional study. Bench scale and plot scale studies are needed to study the effect of unit processes on steroid hormone removal.

Chapter 5: Conclusions

The research described in this dissertation examined the occurrence, transformation, and partitioning of steroid hormones in runoff from cattle feedlots at a laboratory, plot, and field scale. The relative contribution of steroid hormones to surface waters in runoff from feedlots and tile drains under feedlots and manure-applied fields were also examined. Additionally, the efficacy of a three-step treatment train for removing steroid hormones from feedlot runoff was determined.

5.1 Fate of steroid hormones in cattle feedlot soil and runoff

Previous research had demonstrated that steroid hormones were often present in waterbodies near cattle feedlots. However, the fate of steroid hormones after excretion, and the mechanism of their transport to surface waters was not understood. The research presented in Chapter 2 demonstrated that endogenous steroids were present in feedlot runoff at concentrations that were several orders of magnitude above thresholds for biological response. Therefore, runoff is likely the dominant transport mechanism for steroids to nearby water bodies.

This research also showed that significant concentrations of steroids in runoff were transported in both the particle-associated and filterable phases. Given the high concentrations of dissolved organic carbon, it is likely that most of the steroids in the filterable phase were associated with organic colloids. Therefore, treatment methods designed to remove particle-associated steroids, such as lagoons, are likely to remove roughly half of the steroids in feedlot runoff. Association with colloids could increase transport and decrease transformation rates for the steroids in runoff, enabling them to reach surrounding water bodies.

Although steroids were present at high concentrations in feedlot runoff, only a small fraction of the steroids produced on the feedlot were detected in the runoff. In contrast to incubation studies, the steroids were very stable in dry, feedlot soils for up to three weeks. Upon wetting by rainfall, the estrogens remained associated with the soil, and the androgens and progesterone were rapidly transformed upon wetting of the soil by rainfall. Therefore, androgens and progesterone are likely to be less of a concern except on short timescales, while estrogens could leach from soils over longer periods of time.

Finally, research described in Chapter 2 demonstrated the importance of microbial transformations of steroids hormones in the soil after excretion. Significantly more androstenedione and progesterone were present in the soil than could be accounted for by measured concentrations in manure. This suggests that precursors, such as sterols, in manure can be transformed into steroids after excretion. This phenomenon could increase steroids loads to nearby water bodies well above what would be predicted based on excretion rates. In addition, 17α-estradiol, the less bioactive isomer that is the dominant estrogen excreted by cattle, was partially converted into 17β-estradiol, the more bioactive isomer. This transformation could increase the overall estrogenicity of runoff from feedlots.

5.2 Effect of steer manure on transformation of steroid hormones in cattle feedlot runoff

The results of the research in Chapter 2 suggested that the steroids were stable in dry soil, but that androgens and progesterone were rapidly transformed after simulated rainfall. The research presented in Chapter 3 further explored this phenomenon using runoff microcosms. Results showed that the transformation rates of steroid hormones were strongly affected by the presence of manure. Higher manure concentrations in the runoff caused the steroids to be

transformed more rapidly in the first 24 hours. After the first 24 hours, higher manure concentrations slowed the rate at which estrogens were transformed. Experiments suggested that neither interactions with the organic matter in the manure nor anoxic conditions caused by the increased microbial activity were responsible for the observed change in stability after 24 hours. This effect of manure could explain why steroids, especially the estrogens, are so much more stable in feedlot soils and runoff than expected based on incubation studies that show them to be rapidly transformed.

The estrogens initially present in the manure were more stable than those that were added to microcosms, suggesting that estrogens may be even more stable than predicted by experiments using steroid amendments. In addition, approximately 10% of the added 17α-estradiol was converted to 17β-estradiol, a finding that confirms data from feedlot soils reported in Chapter 2. Because 17β-estradiol is 8-9 times more potent with respect to feminization of fish, this transformation could increase the estrogenicity of feedlot runoff above what would be predicted based on excreted steroids. In conditions encountered in feedlot runoff and in runoff from manure-applied fields, estrogens are likely to persist, while androgens and progesterone are likely to be transformed.

5.3 Efficacy of a vegetated treatment system and soil for steroid hormone removal from feedlot runoff

Most feedlots are required to treat their runoff prior to discharge to reduce pathogen and nutrient release to receiving waters. The most common form of treatment is a solids settling basin. Recently, many feedlot operators have begun to use vegetated treatment systems. Almost all feedlots apply manure to fields, and many of these fields have tile drains. The research presented in Chapter 4 examined the relative contribution of steroid hormones to surface waters of tile drains and feedlot runoff. It also determined the efficacy of a vegetated treatment system for removing steroid hormones from feedlot runoff. Results indicate that runoff is likely to contribute several orders of magnitude more steroid hormones than tile drains. Therefore, treatment efforts for steroid hormones should be directed primarily at controlling runoff from feedlots and manure-applied fields. However, in situations where tile-drained fields contain significant macropores, tile drain discharges could contain significant concentrations of steroid hormones. Therefore, application of manure to fields could be an effective treatment method if soils are not prone to macropores, and runoff from the fields is contained.

A multi-step vegetated treatment system containing a solids settling basin, a vegetated infiltration basin, and a vegetated treatment area in series effective decreased steroid hormone concentrations in feedlot runoff below levels of concern. The solids settling basin alone did not sufficiently decrease steroid hormone concentrations below thresholds for biological response.

5.4 Research needs

Predicting steroid hormone concentrations in cattle feedlot runoff is complicated due to the complexity and heterogeneity of the matrix, the low concentrations of the steroid hormones, and the lack of understanding about the interactions of steroids hormones with soil, manure, and microorganisms. The research in this dissertation provided insight into the behavior of steroid hormones in feedlot soil and runoff, the effect of manure on their microbial transformation,

and the efficacy of treatment systems at removing them. However, the mechanisms controlling their interactions with soil and manure, other factors affecting their transformation in soils and runoff, and the mechanisms of removal in vegetated treatment systems are still not well understood.

Additional research is needed to understand the factors controlling steroid partitioning under conditions encountered in feedlot runoff. Manure is a complex mixture of different kinds of organic matter that has varying affinities for steroid hormones and is in particles of various sizes. A better understanding of how steroids partition to the types of organic matter in soil and manure could help to better predict their partitioning and transport in both runoff and the subsurface. In particular, studies to determine specific mechanisms of sorption are needed so as to create better models to predict how steroids will interact with soil and manure on timescales typical of rainstorms and irrigation events. This could be done by determining the individual components of manure, and conducting experiments looking at interactions of steroids with individual components.

While this research showed that many of the steroids were much more stable than expected based on laboratory incubation studies, our attempts to determine the cause of the observed stability were inconclusive. Further research is needed to determine the reasons for the observed stabilization of the estrogens. A better understanding of the microbial community in manure-soil systems, and the enzymes responsible for transformation of different classes of steroids may be helpful in gaining insight into this phenomenon. Exposing different classes of steroids to individual microorganisms or enzymes known to be present in manure could help determine why different classes of steroids are transformed so differently. Exposure to these microorganisms in the presence of various components of manure, and under various environmental conditions, could help determine the factors that control their transformation rates.

Androstenedione and progesterone were produced after excretion in feedlot soil, but not in feedlot runoff. Among the steroids studied, these two compounds exhibited the most variability between replicates. While it is known that precursors of these compounds exist in manure, and that microorganisms capable of transforming these precursors exist, further research is required to determine the factors that control their formation and transformation under conditions encountered in agricultural wastes. This kind of information could be useful in predicting steroid concentrations in soils and runoff.

This research documented that a vegetated treatment system effectively removed steroid hormones from cattle feedlot runoff, and that a solids settling basin alone did not. The mechanisms of removal and the factors controlling it are still not well understood. Further research is needed to determine how steroids behave in vegetated treatment systems to identify approaches for improving passive treatment systems.

5.5 Concluding remarks

The question of how steroid hormones from feedlots reach surface waters despite their strong affinity for soil and rapid transformation by microorganisms has been unclear for the last decade. This research made a significant contribution towards understanding how steroids from feedlots reach surface waters and the factors controlling their stability and transport. We determined that the steroids were much more stable in the presence of manure, and that their sorption does not follow simple equilibrium partitioning. This increased stability and

transport is likely what enables them to reach surface waters. The results of this research will be useful to researchers in designing management practices that can minimize the amount of steroid hormones that reach surface waters.

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