DISSERTATION

DEGRADATION AND TRANSPORT PATHWAYS OF STEROID HORMONES FROM HUMAN AND ANIMAL WASTE

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ABSTRACT OF DISSERTATION

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Steroid hormones have been widely detected in various environmental matrices, including soils, groundwater, surface water, and sediments. Agricultural operations where manure and biosolids are applied as fertilizers and soil amendments are potential sources of steroid hormones to the environment. The aim of this research is to assess the potential for surface runoff and to elucidate biodegradation pathways of steroid hormones from human and animal waste, respectively.

A field-scale study was conducted to assess the potential for runoff of seventeen different steroid hormones from an agricultural field applied with biosolids at an agronomic rate and the major runoff mechanisms. Steroid hormones were present in runoff from the biosolids amended agricultural field, and high concentrations of androgens and progesterone were observed in the runoff even after multiple rainfall events and up to one month after biosolids application. The observed correlation between rainfall amount and hormone mass flux suggests that intense rainfall promotes hormone runoff. Hormones were found to be transported primarily in the aqueous phase or by particles smaller than 0.7 μm.

The potential for biodegradation of testosterone, 17β-estradiol and progesterone by swine (*Sus scrofa*) manure-borne bacteria was examined, and the impact of different environmental factors on testosterone degradation kinetics was determined. Testosterone, 17β-estradiol and progesterone were rapidly degraded under aerobic conditions, and testosterone has the potential for degradation by manure-borne bacteria under a wide range of environmentally relevant conditions.

Finally, a study was conducted to enrich manure-borne bacteria capable of testosterone degradation and to elucidate the testosterone mineralization pathway by the enriched bacteria under aerobic conditions. Six DNA sequences of bacteria from the Proteobacteria phylum were identified in a testosterone-degrading enriched culture, suggesting that Proteobacteria may play an important environmental role in the degradation of testosterone and other similar structural compounds. The microbial enrichment caused 48% of the added ^{14}C-testosterone to be mineralized to $^{14}CO_2$ within 8 days of incubation.

The findings in this dissertation contribute important information that will help improve our current understanding of the environmental fate of steroid hormones as well as assist in the development of best management practices for biosolids and manure.

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CHAPTER 1

INTRODUCTION

Steroid Hormones − In General

In general, steroids are characterized by a carbon skeleton consisting of four fused rings (a cyclopentan-o-perhydrophenanthrene ring; Figure 1.1) (*1*).

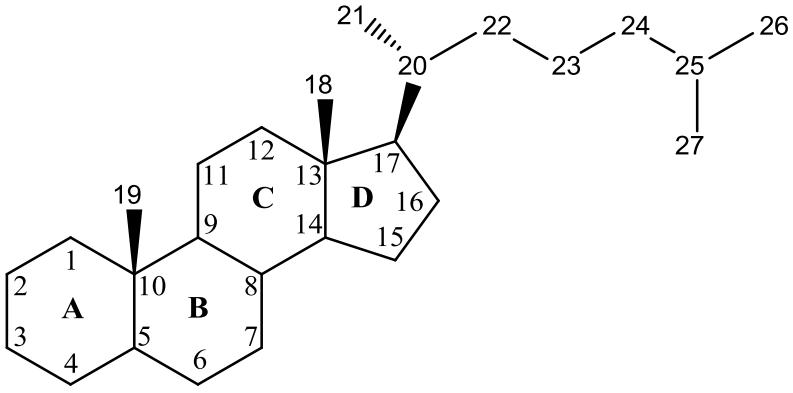

Figure 1.1. Basic steroid hormone structure. The steroid skeleton is characterized by four fused rings, labeled from A to D. Each carbon is labeled from 1 to 27.

Differences among steroids arise from variations in the number and location of double bonds, and the type and stereochemical arrangements of functional groups along the

carbon skeleton (*2*). Most steroid hormones are slightly soluble in water, moderately hydrophobic, and are weak acids with low volatility (e.g., 17β-estradiol (βE2) has a pK_a of 10.23; the vapor pressure of βE2 is approximately 3×10^{-8} Pa) (*1, 3, 4*). There are three classes of steroid hormones: estrogens, androgens, and progestagens. In vertebrates, estrogens (e.g., estrone (E1), βE2, and estriol (E3)) are predominantly female hormones, which are important for development of female traits, ovulation, reproduction, mating and breeding behavior, and somatic cell function (*5*). Androgens (e.g., testosterone, androstenedione, 11-ketotestosterone) play a key role in male traits, reproduction, mating and breeding behavior, and muscle growth (*6*). Progestagens (e.g., progesterone) can be thought of as a hormonal balancer and helps to maintain pregnancy (*7*). Natural and synthetic steroid hormones are administered to humans and livestock for a variety of pharmaceutical purposes. In humans, βE2, 17α-ethinylestradiol (EE2; synthetic estrogen), mestranol (synthetic estrogen), equilin (equine-derived estrogen), testosterone, progesterone, and norethindrone (synthetic progestagen) are used for a wide variety of purposes such as contraception, cancer treatment, and hormone replacement therapy (*8, 9*). In livestock, testosterone, trenbolone (synthetic androgen), βE2, progesterone, and melengestrol (synthetic progestagen) are used for growth promotion and reproductive control (*9-11*).

The quantity of steroid hormones excreted by humans and animals varies as a function of gender, physiological and developmental state, and, for animals, their species (*12, 13*). Human females excrete a large quantity of estrogens daily in their water-soluble

glucuronide- and sulfate-conjugated forms, mainly via urine (95%) (Table 1.1). The highest levels of estrogen excretion are recorded during pregnancy, the concentrations of which depend on the stage of pregnancy. Human males excrete about 2 to 20 mg d^{-1} of androgens (primarily testosterone and androstenedione). Lange et al. (*12*) estimated the yearly steroid hormone excretion by farm animals, for the year 2000 in the USA and the European Union, based on existing data and considering the amount of excreta produced per animal along with the number of animals (Table 1.2).

Table 1.1 Human production and excretion of estrogens (modified from ref. (*14*)).

Steroid hormone	**Amount excreted in urine ($\mu g\ d^{-1}$)**	**Amount produced ($\mu g\ d^{-1}$)**	**Gender (reproductive status)**
Estrone	0.3−5	82−695	Female (cycling)
Estrone		13	Female (pre-pubertal)
Estrone	1.5	48	Male
Estrone		6.5	Male (pre-pubertal)
17 β-estradiol	3−65		Female (pregnant)
17 β-estradiol	2−20	110−497	Female (cycling)
17 β-estradiol		41	Female (pre-pubertal)
17 β-estradiol	3	88	Male
Estriol		35	Male (pre-pubertal)
Androgens	2100−23100	6500 (testosterone)	Male
Androgens	800−10500	240 (testosterone)	Female

Table 1.2 Estimated yearly steroid hormone excretion by farm animals in the European Union and the United States−Year 2000 (modified from ref. (*12*)).

Species	European Union				USA			
	Million head	**Estrogens (tons)**	**Androgens (tons)**	**Gestagens (tons)**	**Million head**	**Estrogens (tons)**	**Androgens (tons)**	**Gestagens (tons)**
Cattle	82	26	4.5	185	98	45	1.9	253
Pigs	122	3.0	1.0	79	59	0.83	0.35	22
Sheep	112	1.3		58	7.7	0.092		3.9
Chickens	1002	2.8	1.6		1816	2.7	2.1	
Total	1318	33	7.1	322	1981	49	4.4	279

Steroid Hormones in the Environment

Potential Impact on Humans, Fishes, Frogs, Alligators and other Wildlife

There have been many strong retrospective observations regarding adverse effects of endocrine disrupting compounds (EDCs) such as steroid hormones on human and wildlife health (*15-20*). Effects in humans for which links with exposure to endocrine disrupters have been suggested include increased incidence of testicular, prostate, female breast cancer, polycystic ovaries in women and altered physical and mental development in children (*21, 22*). Adverse effects have also been identified in a wide range of wildlife species. Adverse effects of endocrine disruption in fishes include masculinization of females, feminization of males, altered sex ratios, intersexuality, and reduced fertility and fecundity (*5, 23, 24*). More notably, estrogens such as E1, βE2, and EE2 have been observed to cause feminization of male fish at concentrations as low as a few ng L^{-1} (*25-27*). Androgens such as androstenedione also have been linked to reproductive abnormalities in fishes and frogs at similarly low concentrations (*28-33*). Sorensen et al. (*32*) reported that androstenedione as a male pheromone in goldfish may serve an inhibitory physiological role amongst males that need to conserve milt. Intersex white suckers (*Catostomus commersoni*) comprised 18 to 22% of a population at a wastewater effluent influenced site (*24*). Exposure of fish to estrogens during early life, notably during the period of gonadal sexual differentiation, has been shown to result in skewed sex ratios (*34*), induction of intersex (*35*) and reductions in functional reproductive capacity later in life (*36, 37*). A partial life-cycle exposure of juvenile zebrafish (*Danio*

rerio) to EE2 concentrations ranging from 1 to 25 ng L^{-1} resulted in a dose-dependent increase in vitellogenin concentrations (*38*).

Life-long exposure of breeding populations of zebrafish and other species to EE2 at very low, environmentally relevant concentrations has been shown to exert severe deleterious effects on their reproductive success (*34, 35, 39*). A seven-year, whole-lake study at the Experimental Lakes Area in northwestern Ontario, Canada, assessed the subcellular-level through population-level effects of EE2 on fathead minnow (*Pimephales promelas*) (*40*). The study showed that exposure of fathead minnow to low concentrations (i.e., 5 to 6 ng L^{-1}) of EE2 led to feminization of males and altered oogenesis in females. These observations demonstrated that the concentrations of estrogens and their mimics observed in freshwaters can impact the sustainability of wild fish populations. In a recent study, Coe et al. (*41*) investigated the impact of a transient exposure to EE2 over the period of sexual differentiation (from 20 to 60 days post fertilization) on the subsequent reproductive behavior in both male and female zebrafish. Reproductive success in males exposed to 2.76 ng L^{-1} of EE2 was increased in competitive spawning scenarios. In contrast, exposure of females to 9.86 ng L^{-1} EE2 during early life reduced their subsequent reproductive success in competitive spawning.

Li et al. (*42*) used the sandwich enzyme-linked immunosorbant assay (ELIS) to measure bullfrog (*Rana catesbeiana*) vitellogenin (VTG) levels in males induced by βE2. After two weeks of βE2 exposure, the induced bullfrog-VTG level was significantly higher than control female in bullfrogs ($p < 0.05$). When the injection period reached four

weeks, the reproductive cells became more loosely arranged from the cyst of the seminiferous tubes, indicating that males would have decreased sperm count or gradually lose reproductive ability. One of the most extensively studied and best documented cases of endocrine disruption was reported by Guilette et al. (*43*) who examined the steroidogenic activity removed from juvenile alligators (*Alligator mississippiensis*) obtained from contaminated or control lakes in central Florida. Cultures of tests obtained from control lake males exhibited very low βE2 relative to testosterone, whereas tests obtained from alligators living in the contaminated lake had significantly elevated βE2 relative to testosterone due to elevated βE2 synthesis. When ovaries from alligators hatched from the contaminated and control lakes were compared, a significantly different *in vitro* synthesis of βE2 was observed. On the basis of these observations, it was suggested that the changes in the reproductive and endocrine systems could be due, in part, to modification of gonadal steroidogenic activity and synthesis of plasma sex steroid binding proteins. A similar correlation between hormone exposure and abnormal reproductive function has also been observed in other studies (*44, 45*)

Major Sources and Presence of Steroid Hormones in the Environment

There are many potential sources for steroid hormones, including sewage treatment plants (STPs), septic systems, concentrated animal feeding operations (CAFOs), and agricultural operations (*12, 19, 46, 47*). Natural and synthetic steroid hormones are contributed to the environment predominantly through human and livestock wastes, including hormones used for medical treatment. The hormones in wastewater,

biosolids and animal manure that are most likely to influence freshwaters include estrogens (E1, E2, and E3), androgens (testosterone), and progestagens (progesterone) (*48*).

A recent review paper showed that the total estrogen concentration (i.e., sum of E1, αE2, βE2, and E3) detected in different waste storage structures varied from 47 to 34326 ng L^{-1} for liquid swine waste and from 600 to 6800 µg g^{-1} dry weight (d.w.) in solid swine waste (*13*). The corresponding values for cattle waste varied from 46 to 8960 ng L^{-1} and 130 to 2104 µg kg^{-1} d.w. for liquid and solid waste, respectively. For lagoons receiving poultry waste, the reported estrogen concentrations ranged from 41 to 7818 ng L^{-1}. Another study examined lagoon samples from swine, poultry and cattle operations, and found that total estrogen levels (i.e., sum of E1, αE2, βE2, and E3) in primary swine lagoons were up to 21 µg L^{-1} (*49*). Estrogen concentrations in dairy and swine waste treatment and storage structures were also quantified by Raman et al. (*50*). In solid dairy manure which stemmed from fresh manure stocked in a dry area for two weeks, the concentrations reported for E1, αE2, βE2, and progesterone were 697, 172, 37, and 196 µg kg^{-1}, respectively (*51*). Other studies have detected steroid hormones at ng L^{-1} concentrations in groundwater, drainage water, and surface waters near animal feeding operations (*19, 52-54*). A one year study conducted by Zhao et al. (*55*) detected very low concentrations (<1 ng L^{-1}) of αE2 and βE2 in three headwater streams within a dairy CAFO certified as organic (no growth promoters are administrated) that had implemented many Whole Farm Planning practices (e.g., 12-month-capacity waste storage lagoons).

The low concentrations of estrogens in stream water were probably the result of the long residence time (8 months) of the manure in the lagoons where most of the estrogens were degraded during storage.

Studies throughout the world have examined receiving waters for the presence of steroid hormones (*56-60*). In a national reconnaissance study conducted by the U.S. Geological Survey (USGS) from 1999 to 2000, steroid hormones were observed at various concentrations and frequencies in water samples from 139 stream sites across the United States (*61*). A similar study demonstrated that estrogenic hormones and their glucuronides were present in surface water in the Netherlands at low parts per trillion levels (ng L^{-1}) concentrations (*62*).

Numerous studies have also reported the presence of natural and synthetic hormones in STPs (*63-65*). The concentrations of hormones in raw sewage from various STPs around the world ranged from less than 0.5 to 670 ng L^{-1} for E1, less than 0.5 to 125 ng L^{-1} for βE2, and 2 to 660 ng L^{-1} for E3. The synthetic estrogen EE2 varied from less than 0.2 to 70 ng L^{-1} (*13, 66, 67*). Biosolids, the treated form of municipal waste, have been widely used on agricultural soils; for example, approximately 50, 30, and 60% of biosolids are land applied in the USA, Canada, and France, respectively (*13, 68*). During a survey of organic wastewater contaminants in nine different biosolids products, E1 was detected in one biosolids product at a concentration of 150 µg kg^{-1} (*69*). Nieto et al. (*70*) evaluated the presence of hormones and their conjugates in biosolids from two STPs in Spain. The hormone with the highest concentration was E3 (406 µg kg^{-1} d.w.).

Estradiol-3 sulfate and E1 were observed in all samples with levels ranging from below the limit of quantification to 189 μg kg^{-1} d.w. while E2-acetate was not found in any samples. A 2009 survey of targeted national biosolids reported by the U.S. Environmental Protection Agency (EPA) showed that concentrations of estrogens (E1, αE2, βE2, E3, and equilin), androgens (testosterone, androsterone, and androstenedione), and progestagens (progesterone, norethindrone and norgestrel) in various biosolids samples ranged from 7.56 to 965 μg kg^{-1}, 21.3 to 2040 μg kg^{-1}, and 21 to 1360 μg kg^{-1}, respectively (*71*). Additionally, estrogenic and androgenic activities have been detected in municipal biosolids, and it was reported that aerobically treated biosolids exhibited lower estrogenic and androgenic activities than biosolids treated anaerobically (*72*). When measuring hormone activity by estrogen and androgen receptor gene transcription, a significantly higher estrogenic and (for all but one site) androgenic response gene was systematically obtained from municipal biosolids that had undergone anaerobic treatment vs. aerobic treatment (*72*). Holbrook et al. (*73*) determined the estrogenic activity associated with the processed biosolids constituted between 5 and 10% of the influent estrogenic activity. In contrast, treated liquid effluent contained between 26 and 43% of the estrogenic activity, and 51 to 67% of the estrogenic activity was biodegraded during the wastewater or biosolids treatment processes. Another study examined the presence and concentration of twelve natural and synthetic estrogens (i.e., E1, αE2, βE2, E3, EE2, equilin, 17α-dihydroequilin, norgestrel, medrogestone, and trimegestone) in three STPs (*64*). The concentration of estrogens in the influent and effluent of the three STPs ranged from 1.2 to 259 ng L^{-1} and 0.5 to 49 ng L^{-1}, respectively. The estrogen found in the

highest concentration was E1 (ranging from 57 to 83 ng L^{-1} and 6.3 to 49 ng L^{-1} for influent and effluent, respectively) and it was detected at all of the STPs. 17β-estradiol (βE2) and E3 were detected at two STPs; and EE2 was detected at one STP.

Ultimately, land application of biosolids and livestock wastes may result in the loading of hormones and their metabolites to agricultural fields.

Runoff and Leaching of Steroid Hormones from Agricultural Operations

Multiple reports have indicated that surface runoff and leaching from agricultural operations can carry steroid hormones to receiving surface and ground waters (*52, 53, 74-76*). Transport of steroid hormones via runoff or leaching has been observed for estrogens (E1, αE2, βE2, and EE2), androgens (testosterone and androstenedione), and progestagens (medroxyprogesterone). Transport of these substances is influenced by their sorption behavior and the presence of organic matter and manure or biosolids in the soil matrix. Lysimeter soil monoliths were treated with pig slurry containing estrogenic hormones (i.e., E1, αE2, and βE2) to investigate leaching of estrogenic hormones (*77*). Estrogens from pig slurry-treated soil monoliths were transported to a depths of 1 m in loamy soil and sandy soil, and the concentrations in the leachate were at a level (i.e., sum of E1, αE2, and βE2 ranged up to 10 ng L^{-1}) known to affect the endocrine system of aquatic organisms (*77*). Swartz et al. (*75*) found that E1 and βE2 had leached from wastewater tanks through glacial deposits of sand and gravel and were present in concentrations up to 120 and 45 ng L^{-1} in groundwater at depths of 3.2 to 3.5 m, respectively. Peterson et al. (*78*) measured concentrations of βE2 ranging from 6 to 66 ng

L^{-1} in water from five springs in mantled karst aquifers, indicating that animal waste can contribute βE2 to ground water. Arnon et al. (*53*) measured the deep vertical distribution of estrogen and testosterone in unsaturated sediments below a CAFO waste lagoon and they observed testosterone and estrogen in sediments to depths of 45 and 32 m, respectively. Based on the elevated concentrations of testosterone and estrogen compared to the reference site, they concluded that ground water samples were directly impacted by the dairy farm. A one year study examined the transport of E1 and βE2 from manure to tile drainage systems in loamy soil. The estrogens leached from the root zone to tile drainage water in concentrations exceeding the lowest observable effect level (LOEL) for as long as 3 months after application, with the maximum recorded concentration of E1 and E2 being 68.1 and 2.5 ng L^{-1}, respectively, during a major precipitation event (*52*). Transport of estrogens from the soil to the aquatic environment was governed by pronounced macro-pore flow and consequent rapid movement of the estrogens to the tile drains. These findings suggest that the application of manure to structured soils poses a potential contamination risk to the aquatic environment with estrogens, particularly when manure is applied to areas where the majority of stream water derives from drainage water (*52*).

Several studies have shown that surface applied animal manures can result in significantly elevated concentrations of estrogens and androgens in adjacent surface waters (*79-82*). Runoff from poultry litter amended fields contained 38.7 to 196.3 ng L^{-1} of βE2 and 3.3 to 7.4 ng L^{-1} of testosterone following a rainfall event in four different cropped watersheds (*79*). Another study found runoff concentrations of βE2 and

testosterone following poultry litter application to tilled and no-till crop land ranging from 23 to 389 ng L^{-1} and 6 to 19 ng L^{-1}, respectively (*80*). Bushee et al. (*83*) investigated (flow-weighted mean) runoff concentrations of βE2 from plots amended with horse stall bedding or municipal biosolids and observed 600 ng L^{-1} (mass transport = 61 mg ha^{-1}) and 80 ng L^{-1} (mass transport = 12 mg ha^{-1}) of βE2, respectively. Alum addition to either material did not significantly reduce βE2 runoff concentrations which is in contrast to the findings of Nichols et al. (*82*) who reported that amending poultry litter with alum reduced mean βE2 concentrations by 42% and mass loss by 46% in first-storm runoff probably due to the alum-induced decreases in pH and soluble organic compounds. The contrasting observations of alum effects might be due to the different types of matrices, mixing procedures, and reaction time used in these studies.

Agricultural management practices such as tillage and rate of manure application may affect the eventual exports of steroid hormones in runoff. For instance, βE2 runoff concentrations increased with increasing application rate of poultry litter to pasture, reaching a maximum of 1.28 µg L^{-1} from the plot receiving a d.w. fresh litter application rate of 7.05 Mg ha^{-1} (*82*). In another study, Finlay-Moore et al. (*81*) measured runoff concentrations of βE2 and testosterone ranging from 20 to 2530 ng L^{-1} and 10 to 1830 ng L^{-1} from soil amended with broiler litter, respectively, depending on broiler litter application rates and time between application and runoff. Soil concentrations of βE2 and testosterone were reported to be 675 and 165 ng kg^{-1}, respectively (*81*). In contrast, a recent study conducted by Dutta et al. (*84*) found that higher litter application rates did

not yield higher amounts of βE2. The authors suggested that sorption of estrogens may play a significant role in controlling the concentration of hormones in runoff water. Compared with raw litter and reduced tillage, no-tillage and pelletized litter treatments yielded much lower exports and concentrations of estrogens, indicating that pelletized litter and no-tillage could be used as best management practices to reduce estrogen exports from agricultural fields (*84*). The past history of conventional-tillage on the reduced-tillage plots could have resulted in the development of impeding layers, which could have reduced infiltration and enhanced runoff (*85*). Jenkins et al. (*80*) also observed higher exports of βE2 in runoff from conventional-tillage (1300 μg ha^{-1}) versus no tillage (600 μg ha^{-1}) with poultry litter plots.

These studies clearly show the potential for both leaching and runoff of hormones from manure amended soils and that there is a limited amount of information regarding transport of hormones from biosolids amended soils.

Steroid Hormones – Sorption and Degradation Processes

Once steroid hormones enter the environment, they are subject to a variety of transport and removal processes, including sorption to soils and sediments, microbial degradation, and abiotic degradation. In the following sections information on sorption and degradation of steroid hormones in the environment and to a limited extent in STPs is reviewed.

Sorption

Sorption of steroid hormones by soils and sediments has the potential to affect the fate and transport of steroid hormones in the environment in various ways. As a result, many studies have examined sorption rates, the distribution and partitioning of steroid hormones between water and soils or sediments, and sorption to clay minerals, organic colloids, and river sediments (*86-91*). For the most part, either batch equilibrium or column displacement techniques have been used. Lee et al. (*92*) employed batch equilibration techniques to examine sorption and transformation of testosterone, βE2, and EE2 in Midwestern soils. Measured sorption isotherms for the three parent chemicals and their principal transformation products were generally linear. Equilibrium and kinetic batch sorption experiments and column experiments were used to elucidate the fate and transport of βE2 and E1 in natural soil (*93*) and to identify the fate and transport of radiolabeled [^{14}C] testosterone in agricultural soils (*94*). The results indicated that the testosterone sorption affinity was lower than βE2, and testosterone has a greater potential for migration than βE2, even though it has a higher rate of transformation (*94*). Another study examined the soil sorption and transport characteristics for βE2 and testosterone in large undisturbed soil columns and suggested that hormones adsorbed to soil surfaces may contaminate groundwater under conditions of preferential flow (*88*). A recent study conducted by Caron et al. (*95*) compared the sorption of E1, βE2, and E3 in a wide range of Alberta soils. The soil sorption coefficient (K_d) and the sorption coefficient per unit organic carbon (K_{oc}) values were determined in 121 soil samples, and both values followed the order E1 (33 L kg^{-1} soil and 1557 L kg^{-1}, respectively) > βE2 (23 L kg^{-1} soil

and 1082 L kg^{-1}, respectively) = E3 (23 L kg^{-1} soil and 1059 L kg^{-1}, respectively). A significant positive correlation (R^2 ranging from 0.50 to 0.75, p<0.001) between soil organic carbon (SOC) content and K_d values was observed. Ying and Kokana (*96*) observed a different order of estrogen sorption on soils: βE2 (mean K_d and K_{oc} values are 65 L kg^{-1} and 3714 L kg^{-1}, respectively) > E1 (54 L kg^{-1} and 2961 L kg^{-1}, respectively) > E3 (28 L kg^{-1} and 1404 L kg^{-1}, respectively). The slightly different observations may be due to different matrices or experimental approaches.

A few studies have examined sorption of steroid hormones to colloids and its potential impact on subsurface transport of hormones (*86, 91*). Cross-flow ultrafiltration was used for the isolation of natural colloids and determining the binding coefficients of EDCs to natural colloids (*86*). A lack of significant correlation between the (colloidal) organic carbon normalized partition coefficient (K_{coc}) values and the octanol-water partition (K_{ow}) values for bisphenol A (BPA), E1, βE2, and EE2 was observed. Similar results were obtained by Yamamoto et al. (*97*) and Holbrook et al. (*91*), who also observed poor linear relationships between K_{coc} and K_{ow} for various hormones (including E1, βE2, E3, and EE2) suggesting that there are likely to be important sorption mechanisms other than hydrophobic partitioning between hormones and colloids.

Sorption behavior is influenced by the properties of the soil and sediment, including pH, organic carbon content, and metal oxide content. Lai et al. (*4*) estimated kinetics of sorption (influence of binding sites, total organic carbon (TOC), and salinity) of E1, βE2, E3, EE2, and mestranol onto sediments from the United Kingdom and found that sorption increased with increasing TOC content. Additionally, sorption increased

with increasing compound hydrophobicity, as indicated by log K_{ow} partition values: mestranol > EE2 > E2 > E1 > E3. The sorption of estrogens (i.e., βE2, E3, and EE2) onto a wide variety of organic colloidal substances at different pH levels was investigated with batch experiments (*98*). The results suggested decreasing sorption with increasing in pH from 4.5 to 9.5. Since βE2 (pK_a = 10.23) is mostly in a nonionic form in the investigated pH range and thus not significantly affected by pH, the most likely reason for this observation is that the high pH can change the structure of humic or folic acid molecules resulting in deprotonation of phenolic or carboxylic groups. Sun et al. (*99*) investigated sorption of EE2 and BPA by organic matter in soils and sediments. The BPA adsorption capacity was higher than that of EE2 on nonhydrolyzable carbon (NHC), and there was obvious difference in isotherm nonlinearity between these two compounds. The results suggested that BPA may have more access to the pore sites of NHC than EE2 due to its small molecular size. And the π-π bond formed between BPA and NHC could be stronger than that between EE2 and NHC due to the fact that BPA has one more benzene ring than EE2. The authors concluded that the contribution to the overall sorption depended on both the type of solutes and adsorbents.

Many previous studies have shown that partitioning of hydrophobic organic compounds such as steroid hormones in the environment is primarily controlled by the physicochemical properties of those compounds, in particular K_{ow}. However, there is also evidence that processes other than hydrophobic partitioning to organic carbon contribute to sorption of steroid hormones as the organic carbon content of soil or sediment decreases. The adsorption mechanism of iron oxides and clay minerals is attributed to ion

exchange interactions between the oxide's surface hydroxyl groups and charged or polar solutes (e.g., estrogens have polar phenolic groups). Van Emmerik et al. (*100*) investigated sorption of βE2 onto goethite and the clay minerals kaolinite, illite, and montmorillonite. They found that the clay minerals sorbed more βE2 than goethite, suggesting that βE2 is adsorbed at the surface of goethite, kaolinite, and illite, but uptake of βE2 by montmorillonite is principally by intercalation into the interlayer region of swelling clays. The interlayer spacing in wet montmorillonite can expand to 3 nm or more depending on the conditions, while kaolinite and illite are nonexpanding clays. Thus, the interlayer spacing in montmorillonite would fit βE2 (ca. 1.1 × 0.6 × 0.4 nm) easily resulting in much higher sorption capacity than kaolinite and illite. The nonpolar βE2 sorbed only slightly to goethite, which does not have a layered structure, and was totally removed by methanol, suggesting that βE2 interacts only weakly with the surface functional groups. In addition, the impact of pH on sorption showed that sorption to clay minerals was independent of pH; conversely, there was a sorption maximum between pH 7 and 7.5 in the goethite system, indicating that βE2 binds primarily to uncharged surface hydroxyl groups. The decreased sorption at lower and higher pH may result from the increasing polarity of the goethite surface as more of the surface hydroxyls become protonated. In addition, progressive ionization of the phenolic hydroxide of βE2 (pK_a = 10.23) above the point of zero charge (PZC) of goethite (PZC = 7 to 9) would result in electrostatic repulsion between E2 and the surface, respectively. Another study conducted by Shareef et al. (*101*) reported that uptake of E1, EE2 and BPA, by goethite and kaolinite suspensions was low (<20%) and only slightly affected by pH. Sorption by

montmorillonite was greater (ranging from 20 to 60%), and steadily increased from about pH 7. These studies suggest that estrogen sorption is substantially influenced by mineral type, and that sorption on mineral surfaces is generally rapid, weak, and reversible.

Abiotic Degradation of Steroid Hormones

Photodegradation is an important abiotic degradation pathway in natural waters (*102*). Photodegradation of estrogens (E1, βE2, E3, and EE2) has been studied in dilute aqueous solution (*103*) and river water (*104*). The half-lives of estrogens were 2 to 3 h and 5 to 42 h in river water and air-saturated purified water, respectively, indicating that the accelerated photolysis rates were attributed to photosensitization by dissolved organic matter (DOM) in river water (*104*). Another study examined the influences of different factors on the TiO_2-assisted photodegradation of E1 and βE2 in aqueous solutions, and pseudo-first-order kinetics based half-lives of 0.3 to 0.8 h were observed (*105*). The degradation rate was increased with pH values up to 7.6, beyond which the degradation rate started to decrease. The presence of humic acid enhanced the degradation of E1 and βE2 (*105*). Photo-Fenton degradation involving UV-VIS/Fe(III)/H_2O_2 resulted in 98.4 % degradation of E1 after 60 min irradiation. The relative degradability among different estrogens was E2 > EE2> E1 (*106*). In a recent study, the degradation of testosterone under UV irradiation was studied in phosphate buffers and in natural waters at various excitation wavelengths, and the major transformation product formed was identified (*107*). Testosterone was rapidly transformed in laboratory conditions and the pH of water did not affect the rate of phototransformation. Several photoproducts were also obtained

during photodegradation; the major one was a hydroxylated derivative of testosterone coming from the photohydratation of the enone group.

Manganese oxides, with a high redox potential in soils and sediments, have been used as important oxidizing or catalyzing agents of organic and inorganic compounds (*108-110*). For example, de Rudder et al. (*108*) explored the use of manganese oxide (MnO_2) as an oxidative removal substrate and found that at an initial concentration of approximately 15 μg L^{-1} of EE2, the MnO_2 reactors were capable of removing 81.7% of estrogenic activity. Batch experiments have also been conducted to assess the oxidative transformation of βE2 in aqueous solution by MnO_2 and the probable reaction pathway (*109*). More than 90% of the βE2 was oxidized by MnO_2 within 8 h of reaction. The initial reaction rate of βE2 oxidation increased with increasing MnO_2 dose and lower pH. For instance, the rate of βE2 decreased more than 90% after reaction for 0.25 h at pH 4.0, while the rate was decreased by 80% at pH 6.8 and by 75% at pH 9.0 even after 1 h of reaction. The strong pH dependence of the reaction rate can be attributed to the effect of pH on adsorption of βE2 to the oxide surface and the electron-transfer reaction. Two oxidation products of βE2 were determined to be E1 and 2-hydroxyestradiol. More detailed research was conducted to determine the reaction kinetics and estrogenic activity removal of βE2 by MnO_2 under different environmental conditions (*110*). This study demonstrated that the oxidative transformation of βE2 by MnO_2 did not exhibit pseudo-first-order kinetics at time periods exceeding 30 min, while it did follow pseudo-first-order kinetics during the first 30 min (R^2 = 0.97). Similar complex reaction kinetics were also observed in another study (*111*), and it has been suggested that the reactivity of the

mineral surface changed due to the accumulation of reaction products and deactivation of active surface sites. Additionally, the presence of metal ions and carboxylic acids had an inhibitory effect on the initial reaction rate as a result of the decreased number of active surface sites in the presence of co-solutes.

Microbial Deconjugation and Degradation of Steroid Hormones

Natural and synthetic steroid hormones are mainly excreted from humans and livestock as a variety of inactive glucuronide or sulfonide conjugates, which are cleaved during STP, manure storage and treatment processes (*47, 112*). Due to the presence in the STP of high population densities of microorganisms, it is often assumed that common fecal microorganisms such as *Eschericia coli (E. coli)* are capable of hydrolyzing conjugates via glucuronidase and sulfatase enzymes to unconjugated forms (*62, 113, 114*). A laboratory biodegradation test confirmed that glucuronated estrogens are readily deconjugated in wastewater, presumably due to the large amount of the β-glucuronidase enzymes produced by fecal bacteria (*E. coli)* (*115*). Multiple studies have investigated the degree and rate of estrogen deconjugation during the sewage treatment process (*116-118*). Panter et al. (*118*) found that the E2-3-glucuronide conjugate of E2 was very readily converted to the active hormone E2 and suggested that no glucuronide conjugates would survive after the sewage treatment. Moreover, a recent study has shown a strong correlation (r^2 = 0.994) between increasing temperature and estrogen deconjugation (*119*). At 4°C, 38% of the spiked EE2-3-glucuronide remained in the aqueous phase, whereas this compound was completely deconjugated to the free EE2 form at 22°C.

Increased temperature also resulted in faster deconjugation of E2 and it was suggested that this was due to increased hydrolysis resulting from a positive impact on microorganisms producing glucuronide and aryl-sulfatase enzymes (*112*).

Hormone removal efficiencies by STPs in different countries have been estimated to vary from 49 to 99% for E1, 80 to 98% for βE2, 95 to 100% for E3, and 69 to 94% for EE2 (*58, 112, 113, 117, 121*). The fate of seven steroid hormones (i.e., E1, E2, E3, EE2, testosterone, androstenedione, and progesterone) was determined in various processes of two pilot-scale STPs operated under conventional loading conditions (*65*). Mass removal efficiencies of 69, 90, and 88% were observed for E1, E2, and EE2, respectively, that had undergone aerobic digestion. The mass removal efficiencies observed for estrogens that had undergone anaerobic digestion ranged from 60 to 77%. Carballa et al. (*121*) recorded 85% removal of the sum of E1, βE2, and EE2 during continuous anaerobic treatment in pilot reactors containing sewage sludge under mesophilic (37°C) conditions. In contrast, 70% of the E1 and 80% of βE2 detected were present in the conjugated form in an up-flow anaerobic sludge biological (UASB) septic tank effluent, demonstrating that deconjugation did not take place under anaerobic conditions (*122*). Similar results were observed in an experiment with a duration of three years with biosolids and sediments under anaerobic conditions (*123*). The high variability of hormone removal efficiencies could be due to differences between treatment facilities and within sampling protocols.

The contribution of conjugated estrogens to the total estrogen loads in animal wastes has been assessed by Hutchins et al. (*49*). The conjugated estrogens were present

in dairy, poultry, and pig lagoons. The major conjugated forms detected were the sulfate forms of E1 (2 to 91 ng L^{-1}), αE2 (141 to 182 ng L^{-1}), and βE2 (8 to 44 ng L^{-1}), and the highest concentrations were measured in a dairy lagoon. The fraction of conjugated forms in the total load of estrogens, determined after enzymatic treatment, corresponded to 27 to 35% for the swine nursery, beef feedlot, and poultry primary lagoons, 57% for the dairy lagoon, 95% for the poultry tertiary lagoon. These results underline the importance of considering conjugated estrogens in animal wastes since they can be deconjugated by microorganisms into active free estrogens.

Removal of steroid hormones from the environment is expected to be largely the result of microbial degradation. Many of the biodegradation studies to date have focused on estrogens; however, information about the factors influencing the degradation rates and pathways are limited. Degradation of steroid hormones (i.e., βE2 and testosterone) may be affected by environmental conditions such as temperature, pH, dissolved oxygen and the presence of specific degrading bacteria. In general, studies have shown that degradation of hormones occurs more readily under aerobic than anaerobic conditions (*124*), is facilitated under higher (up to 30°C) temperatures (*125*-*127*), and is correlated to soil water content (*128*). For example, Fan et al. (*124*) reported that the first-order degradation rate constants (k) of βE2 and testosterone in native soil under aerobic and anaerobic conditions were 0.0006 and 0.0001 h^{-1}, and 0.012 and 0.004 h^{-1}, respectively. Under aerobic conditions at 22 to 25°C, Layton et al. (*129*) found that βE2 (k = 0.252 h^{-1}) and testosterone (k = 0.912 h^{-1}) were rapidly mineralized to CO_2 in municipal biosolids. The difference in k values between the Fan et al. (*124*) and Layton et al. (*129*) studies

may have been caused by potentially higher microbial population densities in the biosolids. The effect of temperature on the biodegradation of βE2 was investigated in batch experiments (*130*). The biodegradation of βE2 by *Pseudomonas aeruginosa* TJ1, isolated from aerobic activated sludge, followed first-order reaction kinetics with a *k* of 2.63, 3.49, and 3.98 h^{-1} at 10, 20, and 30°C, respectively. Li et al. (*131*) performed semicontinuous batch degradation experiments under aerobic conditions with and without the presence of glucose and found that the addition of the easily biodegradable substrate (glucose) seemed to be inhibiting the degradation of βE2 and E1.

Numerous studies have assessed the biodegradation of steroid hormones, in particular estrogens, in agricultural soils (*132*), soil that has been amended with manure or biosolids (*125*), sewage sludge (*129*, *131*), river water and sediments (*133*, *134*), and pure culture media (*135*). Rapid biodegradation of natural estrogens in Japanese river water was observed by Matsuoka et al. (*136*). 17β-estradiol (βE2) and E1 were completely degraded by bacteria in river water within five days in the summer, and within seven days in the winter. However, the synthetic estrogen EE2 was observed to be less degradable (two weeks or more) than natural estrogens, and complete degradation was not observed. Similar observations were reported for English rivers by Jürgens et al. (*134*). In a recent paper, significant mineralization of βE2, E1, and testosterone were found in aerobic studies containing river sediment, with biodegradation rates of testosterone consistently greater than that of estrogens. This study clearly illustrates the importance of considering microbial processes in sediments with respect to transformation of these relatively hydrophobic compounds that tend to partition into the sediment phase in rivers (*133*).

Several species of bacteria, gram-positive bacteria (*Bacillus, Nocardia, Rhodococcus, Mycobacterium, Nocardia*) and gram-negative bacteria (*Comamonas* and *Pseudomonas*) are known for their ability to degrade androgens, estrogens, and progestogens (*135, 137, 138*). Biodegradation of βE2 and its related metabolite (i.e., E1) were reported by sewage bacteria under both aerobic and anaerobic conditions (*139*). *Novosphingobium* sp, isolated from activated biosolids has been found to degrade E2 (*137*). Two sewage bacterial strains were identified as *Rhodococcus zopfii* and *Rhodococcus equi*, and they were able to degrade βE2 from an initial concentration of 100 mg L^{-1} to 1 mg L^{-1} within 24 h (*135*). Shi et al. (*140*) found that both nitrifying activated biosolids and the ammonia-oxidizing bacterium *Nitrosomonas europaea* were capable of degrading E1, E2, and E3. More recently, βE2-degrading bacteria (strains KC1-14) isolated from activated biosolids were capable of converting βE2 to E1, and a few of them (strains KC6, KC7, and KC8) showed the ability to degrade E1 (*138*). Another recent study conducted by Roh et al. (*141*) reported that the βE2-utilizing bacterium, *Sphingomonas* strain KC8, was capable of degrading testosterone and further utilize testosterone as a growth substrate (*141*). In most of the reported studies the transformation of hormones was investigated under aerobic conditions. One study focused on the transformation of testosterone by the gammaproteobacterium *Steroidobacter denitrificans* strain FS^T under denitrifying conditions, and ten transformation products were observed and determined to be 3β-hydroxy-5α-androstan-17-one, 5α-androstan-3,17-dione, dehydrotestosterone (17β-hydroxy-androstane-1,4-dione-3-one; DHT), 4-androstene-3,17-dione (AD), and 1,4-androstadiene-3,17-dione (ADD) (*142*).

Research on the biodegradation of steroid hormones in manure during storage and after being land applied is limited. Hakk et al. (*143*) measured the decrease in the water-soluble fraction of βE2 and testosterone in composting chicken manure over 139 d. The decrease in βE2 and testosterone followed first-order kinetics, with rate constants of 0.01 d^{-1} and 0.015 d^{-1}, respectively. Slow mineralization of E1, βE2, and testosterone in breeder and broiler litters were observed by Hemmings and Hartel (*126*). In addition, two studies have measured dissipation and mineralization rates in soils amended with different types of manure (*125, 144*). Lucas and Jones (*144*) found that addition of sheep and cattle wastes to soils increased the rate of βE2 degradation. The half-lives of E1 and βE2 degraded in manure amended soils ranged from 1 to 9 d, in contrast to 5 to 25 d in unamended soil, with mineralization being largely independent of manure age and type (*144*). Incubation of moist soils amended with swine manure and biosolids in laboratory microcosms showed increased conversion of βE2 and testosterone to less hormonally active transformation products, namely βE2 to E1, and testosterone to AD, 5a-androstan-3,17-dione, and ADD (*125*). However, the mineralization was slowed down by the addition of manure and biosolids, perhaps by inhibiting the soil microorganisms or by enhancing sorption. In natural environments, such as rivers, many bacterial species have also been reported to transform cholesterol and plant sterols (both often found in high concentrations) into steroid hormones, such as conversion of plant sterols to progesterone and androstenedione (*145*). Thus, many steroid hormone sources, sorption mechanisms, and degradation pathways need to be considered to enable a prediction of their environmental fate.

Research Objectives

The **overarching goal of this dissertation** was to assess the potential for surface runoff and to elucidate biodegradation pathways of steroid hormones from human and animal wastes, respectively.

The **first objective** was to determine the potential for runoff of steroid hormones from a biosolids amended agricultural field and the major transport mechanisms using a rainfall simulator. While numerous studies have investigated the transport of hormones from manure amended soils (*81, 82, 84*), no detailed studies have been conducted with biosolids amended soil. Chapter 2 describes the transport behavior of different classes of steroid hormones during a series of rainfall events.

To date, most research has been focused on the environmental fate (e.g., biodegradation) of estrogens; however, detailed information about testosterone biodegradation kinetics and pathways is scarce. Additionally, the factors influencing the degradation rates and pathways of steroid hormones are poorly understood. Thus, the **second objective** was to reveal the potential for biodegradation of selected steroid hormones (i.e., testosterone, 17β-estradiol, and progesterone) by manure-borne bacteria under different environmental conditions (i.e., temperature, pH, glucose amendments, and dissolved oxygen) with special emphasis on testosterone degradation kinetics and products (see Chapter 3).

While several species of bacteria have been described as being capable of utilizing testosterone and various steroids as sole carbon and energy sources (*141, 142,*

146), little is known about the bacteria responsible for testosterone degradation in animal manure. Thus, the **third objective** for this dissertation, which is addressed in Chapter 4, was to identify potential swine manure-borne bacterial species involved in testosterone degradation by enrichment followed by DNA sequencing and to elucidate the testosterone degradation or mineralization pathways by the enriched culture.

Most of the dissertation work is either planned for submission to or already published in peer-reviewed journals. Chapter 2 (Yang et al.) is being prepared for submission to the Journal of Environmental Science and Technology. Chapter 3 has been published in the Journal of Environmental Quality (Yang et al., 2010, vol. 39, issue 4, pp. 1153-1160). Chapter 4 (Yang et al.) is currently in preparation for submission to a peer-reviewed journal to be determined. Parts of the dissertation have also been presented at several national and international conferences, i.e., at the 2nd International Conference on Occurrence, Fate, Effects, and Analysis of Emerging Contaminants in the Environment (EmCon) in Fort Collins, CO (2009), at 236th ACS National Meetings in Philadelphia, PA (2008), the Society of Environmental Toxicology and Chemistry (SETAC) North America 29th Annual Meetings in Tampa, FL (2008), the 2008 GSA-ASA-CSSA-SSSA Joint Annual Metting in Houston, TX, Colorado State University Global Water Colloquium in Fort Collins, CO (2008), the 2009 U.S. Geological Survey Science Day in Denver, CO, and the 2009 Rocky Montain Reuse Workshop in Golden, CO, and we have been invited to present our research at the ASA-CSSA-SSSA International Annual Meetings, Oct 31-Nov 3, **2010,** Long Beach, CA.

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CHAPTER 2

STEROID HORMONE RUNOFF FROM AN AGRICULTURAL FIELD APPLIED WITH BIOSOLIDS

Introduction

The presence of endocrine-disrupting chemicals (EDCs) in the environment, including natural and synthetic hormones, has become a growing concern because low part per trillion concentrations of these chemicals have caused adverse impacts on aquatic organisms (*1*, *2*). Possible sources of hormones to the environment include discharges from wastewater treatment plants (WWTPs), use of reclaimed water for irrigation, domestic septic systems, effluents from concentrated animal feeding operations (CAFOs), and runoff from agricultural fields where manure and biosolids are applied as fertilizers and soil amendments (*3*-*6*).

Numerous studies have investigated the persistence and degradation pathways of hormones in manure (*7*), WWTP affected streams and groundwater (*8*, *9*), and biosolids (*10*). However, only a limited amount of research has investigated the fate and transport of hormones from manure and especially biosolid amended soils under field conditions. Jacobsen et al. (*11*) investigated the impact of biosolids amendments to soils on

testosterone degradation in laboratory microcosms. The observed inhibition of [^{14}C] testosterone mineralization in a loam soil heavily amended with biosolids was suggested to result from inhibition of microbial activity or increased sorption. Stumpe and Marschner (*12*) conducted laboratory incubation experiments to determine the mineralization potential of 17β-estradiol (βE2) and testosterone in soils with long-term biosolids application and wastewater irrigation. The mineralization rate of βE2 was about an order of magnitude lower than that of testosterone in all test soils, reaching 5-7% and 50-59%, respectively. The Freundlich sorption coefficient (K_F; 1.2 to 1.6-fold higher) and log K_{oc} were higher for βE2 than testosterone and indicated that βE2 had a higher sorption affinity in all soils. The results show that long-term application of biosolids had no effects on the mineralization of hormones during the 21-day incubation period; however, long-term irrigation with wastewater had a negative effect on hormone mineralization in the soils likely due to sorption to soluble sorbents (e.g., colloidal and dissolved organic matter) resulting in decreased bioavailability. Interestingly, a recent study did not find a correlation between soil organic carbon (SOC) and estrogen mineralization rates. In fact, the mineralization of the estrogens was enhanced by up to 147% or depressed by up to 50%, depending on site and organic waste type (i.e., manure, biosolids, wastewater) but not related to changes in sorption parameters (*13*). It is likely that organic waste amendments may result in an improved nutrient or substrate availability or a change in microbial community favoring hormone mineralization in certain soils and increased sorption resulting in decreased hormone bioavailability in other soils.

Other studies have indicated that land application of manure can result in hormone leaching and runoff. Peterson et al. (*14*) reported that concentrations of βE2 in ground water adjacent to fields amended with poultry litter and cattle manure ranged from 6 to 66 ng L^{-1}. Nichols et al. (*6*) determined the effects of alum amendments on runoff concentrations and mass losses of βE2 from poultry litter applied to pasture. They demonstrated that field-applied poultry litter can contribute βE2 to runoff, and amending poultry litter with alum reduced mean βE2 concentrations by 42% and mass loss by 46% in first-storm runoff, which were probably due to the alum-induced decreases in pH and solubility of organic compounds. Finlay-Moore et al. (*15*) measured the βE2 and testosterone concentrations in runoff water and soil from broiler litter-amended grasslands. After broiler litter application, surface runoff water concentrations of βE2 and testosterone ranged between 20 to 2530 ng L^{-1} and 10 to 1830 ng L^{-1}, respectively. In field soil (the soil series on the site were Aquic Hapludults, Typic Kanhapludults, Aquic Hapludults, Aquultic Hapludalfs), the highest observed concentration of βE2 and testosterone was 675 and 165 ng kg^{-1}, respectively. In these studies, runoff concentration appeared strongly dependent on the litter application rate and frequency (*6*, *15*). Another study by Shore et al. (*16*) measured testosterone and estrogen (E2 + estrone (E1)) concentrations at 15 sites for two consecutive rainy seasons in the Upper Jordan Valley in Israel, which included small farms, cattle pastures, and fish ponds. The first rainy season was the first above average season after a 3-year period of well below average rainfall. In the rainy season of 2001/2002 following a rain sequence of 131 mm/week there was an initial large increase in the concentration of testosterone (max. 6 ng L^{-1}) accompanied by

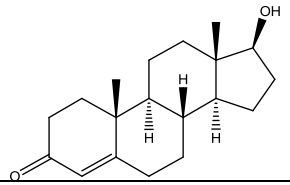
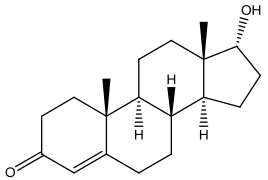
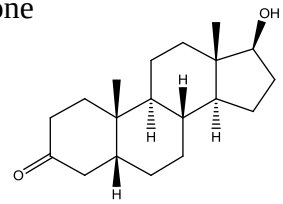
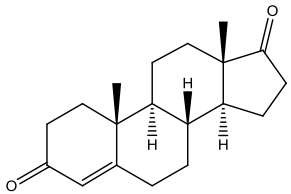
a similarly high estrogen concentration, which then gradually declined to non-detectable levels (< 0.3 ng L^{-1}) over a period of 3 months. These peaks originated from runoff from a cattle pasture and fish pond effluent. Later peaks consisted only of testosterone that was moderately associated with sulfate and somewhat associated with phosphorus indicating that the origin was leaching from the sulfurous peat soil. In the following rainy season, no testosterone peaks above 1 ng L^{-1} were seen. They concluded that the testosterone accumulated in the Upper Jordan Valley was washed out in two stages, first as surface runoff from cattle pasture and then as discharge from the soil.

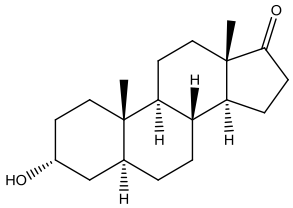
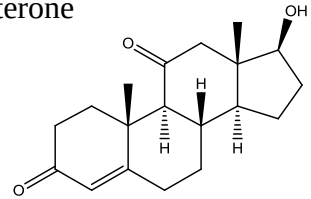
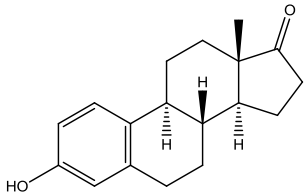
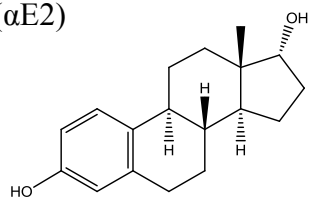
Xu et al. (*17*) examined estrogenic compounds (i.e., E1, 17α-estradiol (αE2), βE2, and 17α-ethynylestradiol (EE2)) in runoff from potato fields in southern California that were irrigated with treated wastewater. The concentrations of E1 and EE2 in runoff samples were 75±36 ng L^{-1} and 17±12 ng L^{-1}, respectively, from a potato field treated with polyacrylamide (PAM) application. Polyacrylamide works by stabilizing the soil surface structure and pore continuity. However, untreated plots resulted in runoff concentrations of 39±21 (E1) and 55±15 (EE2) ng L^{-1} indicating that PAM only limited the runoff concentration of EE2.

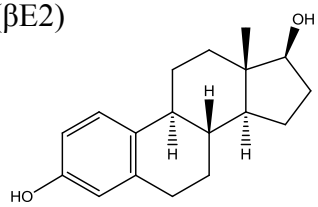
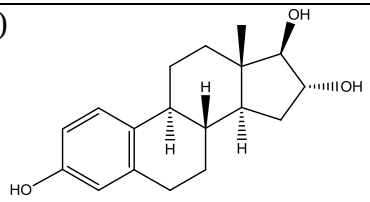
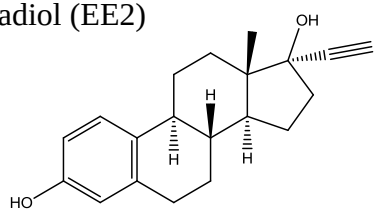
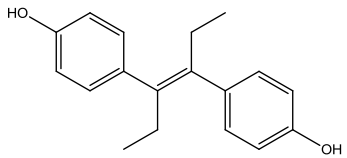
In the United States, it is estimated that the average WWTP produces 240 kg dry weight of solids per million liters of wastewater treated, resulting in approximately 8 million tons (dry weight basis) of biosolids produced per year in the U.S., of which about 50% are land applied (*18*). Surprisingly, little is known about the potential for hormone runoff after land application of biosolids. Thus, the main objectives of this study were to

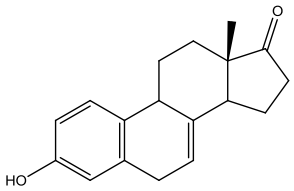
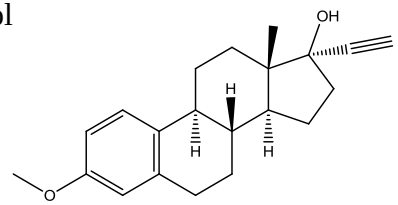
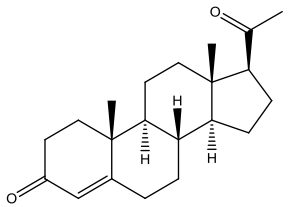
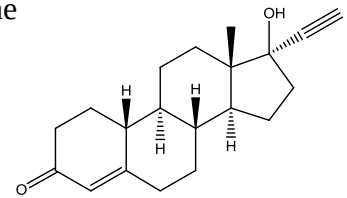
evaluate the potential for hormone (compounds listed in Table 2.1) runoff from an agricultural field applied with biosolids and to elucidate the major transport mechanism.

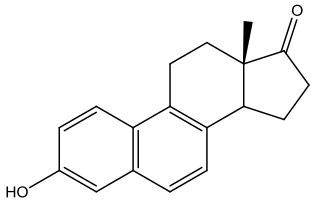
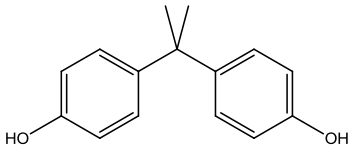
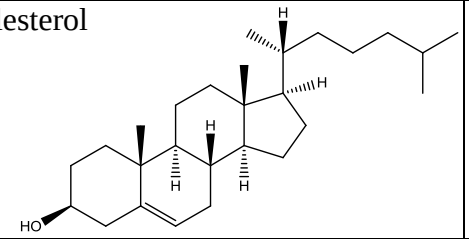
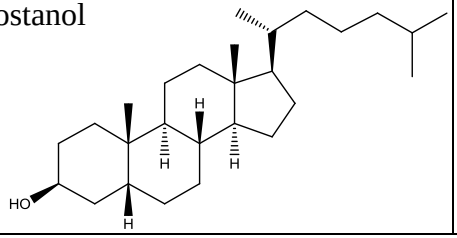
Table 2.1. Chemical structures and physical and chemical properties, including molecular weights (MW), octanol-water partition coefficients (K_{ow}) and water solubilities (S_w) of target compounds.

ANDROGENS	**chemical data**	**log K_{ow}**	**S_w (mg/L)**	**source**	**references**
testosterone 	$C_{19}H_{28}O_2$ MW: 288.43 CAS:58-22-0	3.27 3.32	 18-25 23.2±1.6 23.4	KOWWIN™ computer model Literature value Literature value Experimental (pH 6.8; 23ºC; n=6) Literature value (25ºC)	(*19*) (*20*) (*21*)
epi-testosterone 	$C_{19}H_{28}O_2$ MW: 288.43 CAS:481-30-1	3.27 3.32	 23.4	KOWWIN™ computer model Literature value Literature value (25ºC)	(*19*) (*21*)
dihydrotestosterone 	$C_{19}H_{30}O_2$ MW: 290.45 CAS:521-18-6	3.07 3.55	 5.25±0.05	KOWWIN™ computer model Literature value Literature value (25ºC)	(*19*) (*22*)
androstenedione 	$C_{19}H_{26}O_2$ MW: 286.42 CAS:63-05-8	2.76 2.75	 37-41 50.5±2.1 57.8	KOWWIN™ computer model Literature value Literature value (37ºC) Experimental (pH 6.8; 23ºC; n=6) Literature value (25ºC)	(*19*) (*20*) (*22*)

ANDROGENS	**chemical data**	**log K_{ow}**	**S_w (mg/L)**	**source**	**references**
cis-androsterone 	$C_{19}H_{30}O_2$ MW: 290.45 CAS:53-41-8	3.07 3.69	 12 20.2	KOWWIN™ computer model Literature value Literature value (23ºC) Literature value (23ºC)	(*19*) (*22*) (*22*)
11-ketotestosterone 	$C_{19}H_{26}O_3$ MW: 302.41 CAS:53187-97-7				
ESTROGENS					
estrone (E1) 	$C_{18}H_{22}O_2$ MW: 270.37 CAS:53-16-7	3.43 3.13	 0.8-12.4 1.3±0.08 2.1±0.03	KOWWIN™ computer model Literature value Literature value Experimental (pH 7; 25±0.5ºC; n=6) Experimental (pH 6.8; 23ºC; n=6)	(*19*) (*23*) (*24*)
17α-estradiol (αE2) 	$C_{18}H_{24}O_2$ MW: 272.39 CAS:57-91-0	3.94 4.01	 3.6 3.9	KOWWIN™ computer model Literature value Literature value (27ºC) Literature value (25ºC)	(*19*) (*22*) (*22*)

ESTROGENS	**chemical data**	**log K_{ow}**	**S_w (mg/L)**	**source**	**references**
17β-estradiol (βE2) 	$C_{18}H_{24}O_2$ MW: 272.39 CAS:50-28-2	3.94 4.01	 1.54±0.04 3.1±0.02 3.1-12.96 3.6 3.9	KOWWIN™ computer model Literature value Experimental (pH 7; 25±0.5ºC; n=6) Experimental (pH 6.8; 23ºC; n=6) Literature value Literature value (27ºC) Literature value (25ºC)	(*19*) (*23*) (*24*) (*22*) (*22*)
estriol (E3) 	$C_{18}H_{24}O_3$ MW: 288.39 CAS:50-27-1	2.81 2.45 3.67	 13	KOWWIN™ computer model Literature value Literature value Literature value	(*19*) (*25*)
ethinylestradiol (EE2) 	$C_{20}H_{24}O_2$ MW: 296.41 CAS:57-63-6	4.12 3.67	 3.1-19.1 3.1±0.03 9.2±0.09 11.3	KOWWIN™ computer model Literature value Literature value Experimental (pH 6.8; 23ºC; n=6) Experimental (pH 7; 25±0.5ºC; n=6) Literature value (27ºC)	(*19*) (*23*) (*24*)
diethylstilbestrol 	$C_{18}H_{20}O_2$ MW: 268.36 CAS:56-53-1	5.64 5.07	 12	KOWWIN™ computer model Literature value Literature value (25ºC)	(*19*) (*22*)

ESTROGENS	**chemical data**	**log K_{ow}**	**S_w (mg/L)**	**source**	**references**
equilin 	$C_{18}H_{20}O_2$ MW: 268.36 CAS:474-86-2	3.35	1.41	KOWWIN™ computer model Literature value (25ºC)	(*19*) (*22*)
mestranol 	$C_{21}H_{26}O_2$ MW: 310.44 CAS:72-33-3	4.68	3.498	KOWWIN™ computer model Literature value (25ºC)	(*19*)
PROGESTAGENS	**chemical data**	**log K_{ow}**	**S_w (mg/L)**	**source**	**references**
progesterone 	$C_{21}H_{30}O_2$ MW: 314.47 CAS:57-83-0	3.67 3.87	8.81	KOWWIN™ computer model Literature value Literature value (25ºC)	(*19*) (*22*)
norethindrone 	$C_{20}H_{26}O_2$ MW: 298.43 CAS:68-22-4	2.99 2.97	7.04	KOWWIN™ computer model Literature value Literature value (25ºC)	(*19*) (*22*)

OTHER	**chemical data**	**log K_{ow}**	**S_w (mg/L)**	**source**	**references**
equilenin 	$C_{18}H_{18}O_2$ MW: 266.34 CAS:517-09-9	3.93	1.52	KOWWIN™ computer model Literature value (25ºC)	(*19*) (*22*)
bisphenol A 	$C_{15}H_{16}O_2$ MW: 228.29 CAS:80-05-7	3.64 3.32	120	KOWWIN™ computer model Literature value Literature value (25ºC)	(*19*) (*26*)
cholesterol 	$C_{27}H_{46}O_1$ MW: 386.67 CAS:57-88-5	8.74	0.095	KOWWIN™ computer model Literature value (30ºC)	(*19*) (*22*)
coprostanol 	$C_{27}H_{48}O_1$ MW: 388.68 CAS:360-68-9	8.82	0.0003391	KOWWIN™ computer model Literature value (25ºC)	(*19*)

Experimental Section

Experimental Site and Sample Collection

During April to June, 2008, three experimental plots (6 m^2; 2m wide by 3 m long) in northern Colorado (Roggen; latitude 40°06'08"N, longitude 104°12'43"W) were established, and rainfall simulations replicating a 100-year rainfall event (approx. 65 mm/hr) were performed 5 days before, and 1, 8, and 35 days after, routine biosolids application. Biosolids had not been previously applied to this site. The actual application rate was about 3.5 dry metric tons of biosolids per hectare. After application, the biosolids were partially incorporated to the soil to a depth of about 15 cm. The plots were established parallel to the slope (~3%) and row direction, and the soil at the lowest position (plot 1) had the finest texture and most gentle slope (Table 2.2). The soil type on the experimental site was loamy sand (Aridic Haplustalfs).

Artificial rainfall was applied to each 6-m^2 plot with an oscillating-nozzle rainfall simulator that used a TeeJet^{TM}1/2HH-SS50WSQ nozzle (median drop size = 2.3 mm). The simulator was placed approximated 10 feet above each plot, and well water was used in all experiments (pH = 6.5 to 7, EC = 0.04 to 2 dsm^{-1}). Calibration cans were placed at three sides of the plot to collect rainfall in order to determine the uniformity of the event the plot. Runoff and sediment yields from each 6-m^2 plot were measured during each experiment. Rainfall was applied until runoff flow rate approached steady state (30 to 60 min per test). Twelve runoff samples were collected in 1-L amber glass bottles from each plot of each simulated rainfall event, and then placed on ice in the dark and transported to the laboratory. In the laboratory, the water collected from each experiment was split into

3 composite samples, representing the early, middle, or late sampling time during the rainfall event. Each composite runoff sample was then split into two portions. One aliquot was filtered through a 0.7-µm glass-fiber filter (GFF, Whatman), and the other was stored without filtration. Isotope-dilution standards (IDSs) were added to each sample, and samples were stored at -60°C in high-density polyethylene (HDPE) containers until analysis.

Soil Analysis

Major physical and chemical properties of each soil sample were measured at the Soil, Water, and Plant Testing Laboratory at Colorado State University. Soil characterization methods are detailed in references (Table 2.2) (*27-29*).

Table 2.2. Physical and chemical properties of soils.

plot number	slope (deg)	clay (%)	silt (%)	sand (%)	texture	OM (%)	pH	EC (mmhos/cm)	CEC (meq/100 g)
1	2.6	6	12	82	Sandy Loam	ND	ND	ND	ND
2	3	5	9	86	Loamy Sand	ND	ND	ND	ND
3	2.1	4	8	88	Loamy Sand	ND	ND	ND	ND
1-3*	ND	12	12	76	Sandy Loam	0.3	5.8	0.2	2.7
references					(*27*)	(*28*)	(*29*)	(*28*)	(*28*)

*Data obtained before biosolids application and the data represent the average of the three plots; ND, no detect.

Sample Analysis

All solvents (HPLC grade) were purchased from Burdick & Jackson (Muskegon, MI). Organic free reagent water (18 MΩ –cm.) was generated with a Solution 2000 system (Aqua-Solutions, Jasper, GA). All steroids were obtained at >98% chemical purity, while all labeled isotope-dilution standards were obtained at >97% isotopic purity. Stock solutions of hormones were prepared in methanol (MeOH) at desired concentration and stored at -25°C.

Prior to use, all glassware was silanized. Hormones were isolated from filtered and unfiltered runoff samples using C_{18} solid-phase extraction (SPE) disks and cleaned up using Florisil cartridges (1g; Biotage) and evaporated to dryness. Suspended solids from filters and biosolids samples were isolated using pressurized solvent extraction (PSE), and interferences were removed by extraction on OASIS HLB extraction cartridges (Waters Inc.) and elution across Florisil cartridges (2g; Biotage). The solid-packed cells were extracted with a mixture of water and isopropyl alcohol (v/v, 50:50) at 120ºC and water and isopropyl alcohol (v/v, 20:80) at 200ºC for 3 static extractions (40 min total) at each temperature at a pressure of 2000 psi. Cartridges were dried with nitrogen gas; retained compounds were eluted with 5% MeOH in dichloromethane solution, and concentrated. Both SPE and PSE extracts were derivatized with activated N-methyl-(N-trimethylsilyl)trifluoroacetamide (MSTFA, Sigma-Aldrich) for 1 h at 65°C prior to gas chromatography-tandem mass spectrometry (GC-MS/MS) analyses. Target hormones were separated using an Agilent 6890 gas chromatograph, and identified and

quantified with a 7673B auto sampler coupled with a quadrupole-hexapole-quadrupole mass spectrometer (Quattro Micro, Waters), using a Restesk Rti-XLB column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas, with a column flow rate of 1 ml min^{-1}. Injector temperature was 275°C. The GC interface and the ion source temperature were set at 300 and 230°C, respectively. The mass spectrometer was operated in the selected ion monitoring mode with electron ionization voltage of 70 eV. The injection volume was 2 μL. Results were reported only if they met qualitative GC/MS/MS criteria (retention time, mass spectrometric ion-abundance ratios, and mass spectra) before being quantitated based on a 5 to 8 point calibration curve. For more details on isolation and analysis, see APPENDIX.

Quality Assurance/Quality Control (QA/QC)

Laboratory QA/QC including sets of spikes and blanks provided additional insights and qualification of method performance and subsequent data reporting for samples analyzed during this study. At least one fortified laboratory spike and one laboratory blank were analyzed with each set of 10 environmental samples. All methods had isotope-dilution standards added to samples prior to extraction to monitor method performance. The response data were adjusted according to the relative ratios of the responses to the surrogates in the sample and internal standard.

Results

Steroid Hormone Runoff during a Series of Rainfall Events

Twelve hormones were detected in the biosolid samples, representing a wide range of concentrations (Table 2.3). Cis-androsterone was found in the highest concentration (5417 - 8158 ng g^{-1}) in biosolids, followed by progesterone (290 - 840 ng g^{-1}) and androstenedione (230 - 630 ng g^{-1}). These three compounds were at least two-fold greater in concentration than other hormones.

Fifteen of the seventeen hormones, coprostanol, cholesterol, and bisphenol A were detected in at least one runoff sample during the experimental period. Two synthetic hormones, norethindrone and mestranol, were not detected in any of the samples. The average concentrations of coprostanol and cholesterol in runoff were circa 39 and 54 µg L^{-1}, respectively, and bisphenol A was approximately 1.3 µg L^{-1}. The hormones that were detected were present at parts-per-trillion concentrations (ng L^{-1}). Only two hormones were detected in whole-water runoff samples prior to biosolids application, specifically estrone (<0.8 to 2.23 ng L^{-1}), and androstenedione (<0.8 to 1.54 ng L^{-1}). However, runoff samples collected one day after biosolids application contained substantially higher concentrations of these and other hormones. Overall, summed estrogen (E1, αE2, βE2, and estriol (E3)), androgen (testosterone, epitestosterone, 11-ketotestosterone, cis-androsterone, and androstenedione), and progestogen (progesterone) concentrations one day after biosolids application ranged from <0.8 to 15.3 ng L^{-1}, <2 to 216.14 ng L^{-1}, and 17.4 to 98.9 ng L^{-1}, respectively (Table A1 and A2).

Table 2.3. Concentrations of hormones in biosolids.

compound name	**biosolids (ng/g)**	**biosolids (ng/g)**
bisphenol A	8416	23974
diethylstilbestrol	<RL	<RL
cis-androsterone	8158	5417
epitestosterone	<161	<182
17-alpha-estradiol	10	18
dihydrotestosterone	150	91
androstenedione	229	632
estrone	68	143
17-beta-estradiol	10	12
testosterone	<21	61
equilin	<RL	296
11-ketotestosterone	<RL	<RL
norethindrone	<RL	<RL
mestranol	<RL	<RL
equilenin	<RL	<RL
ethinyl estradiol	<RL	3
estriol	<RL	13
progesterone	292	842
coprostanol	1190859	2313063
cholesterol	437559	801586

<RL, less than reporting level.

Among the hormones, androstenedione was found in the highest concentration in runoff, followed by cis-androsterone and progesterone (Figure 2.1 to 2.3). One day after biosolids application, the concentration of androstenedione was between one and two orders of magnitude higher than E1 or βE2. In general, the highest concentrations of androgens in runoff occurred on the first day after biosolids application (day 1) and for all three experimental plots. For example, in plot 1, the runoff concentrations of testosterone, dihydrotestosterone, androstenedione, and cis-androsterone decreased by 53 to 75% from day 1 to day 8, and decreased again by more than 40% from day 8 to day 35 (Figure 2.1). Similar trends were observed for runoff concentrations of coprostanol and cholesterol. In plot 1 on day 1, the highest concentrations of coprostanol and cholesterol observed were 399 and 275 μg L^{-1}, respectively. Conversely, in plot 1, the runoff concentrations of E1 and βE2 increased by more than 30% from day 1 to day 8, but decreased by more than 40% from day 8 to day 35. Finally, the runoff concentrations of progesterone were dramatically reduced at least 95% at day 8, and then increased to a similar level as day 1 on day 35. Similar trends were observed in plots 2 and 3 (Figure 2.2 and 2.3). In addition to the observed changes in hormone concentrations, the number of different hormones observed as a function of time decreased (i.e., 10, 7, and 7-8 compounds were observed at day 1, 8, and 35, respectively).

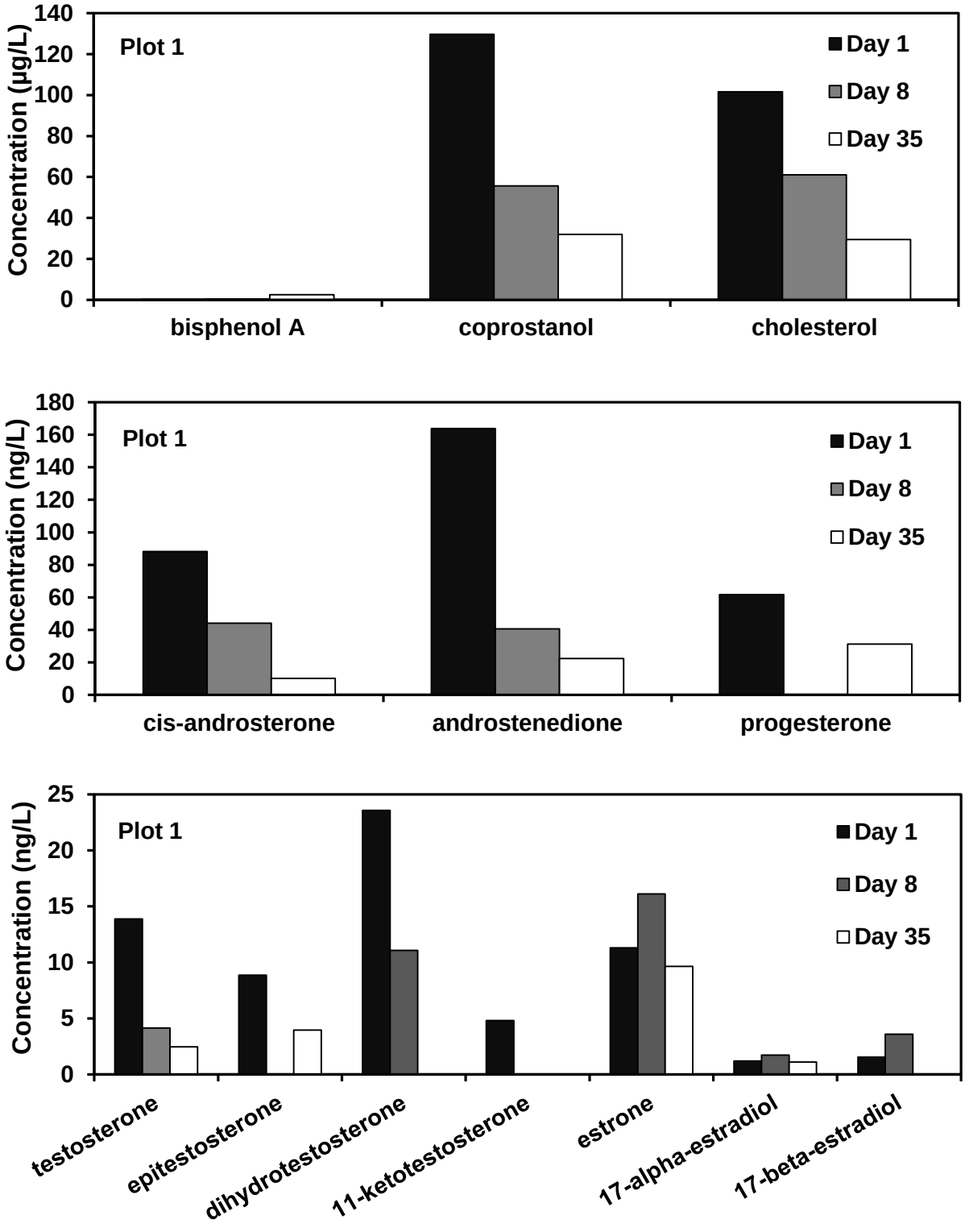


Figure 2.1. Mean (three sampling times) hormone concentrations in whole water runoff samples collected from plot 1 at day 1, 8, and 35 after biosolids application.

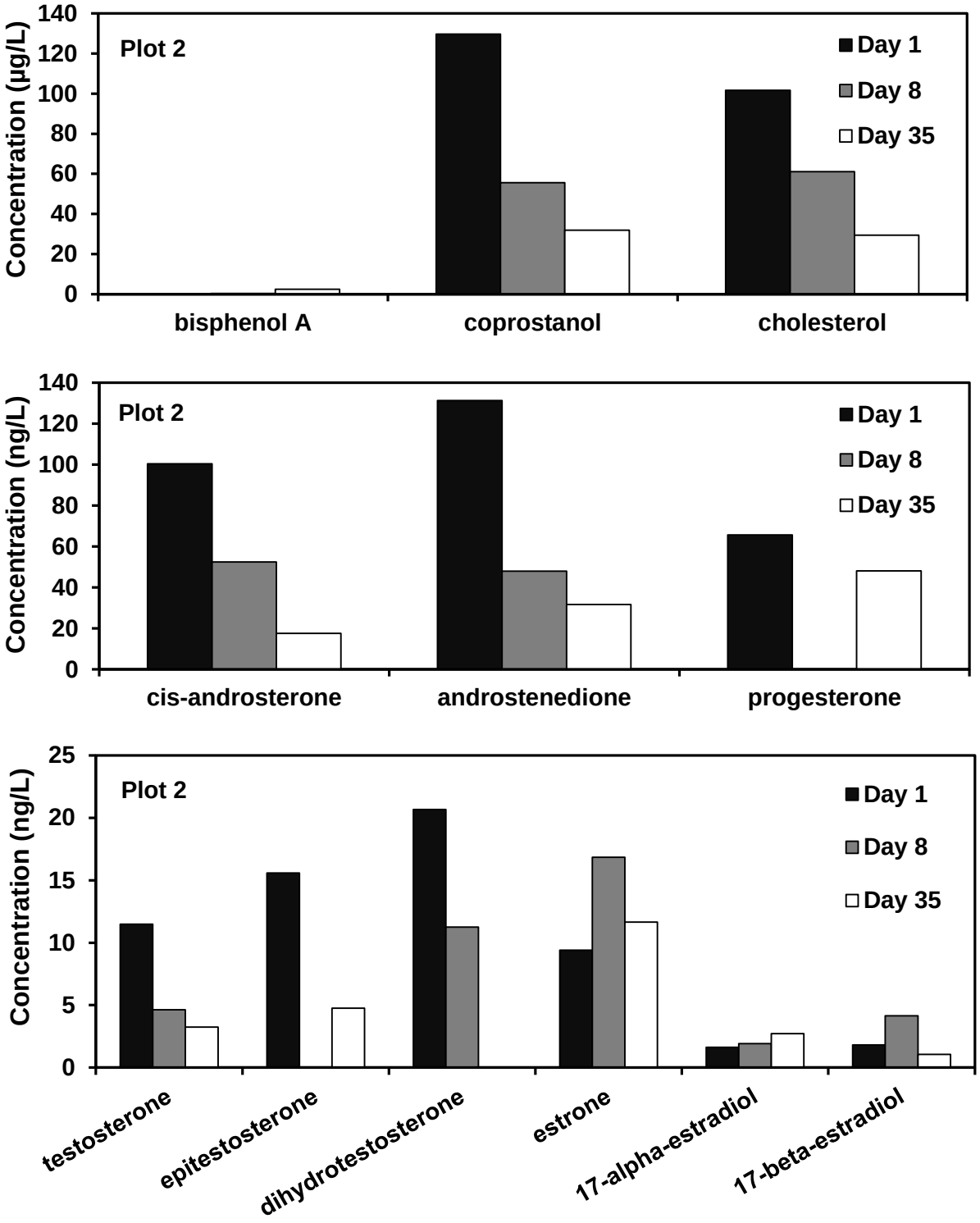


Figure 2.2. Mean (three sampling times) hormone concentrations in whole water runoff samples collected from plot 2 at day 1, 8, and 35 after biosolids application.

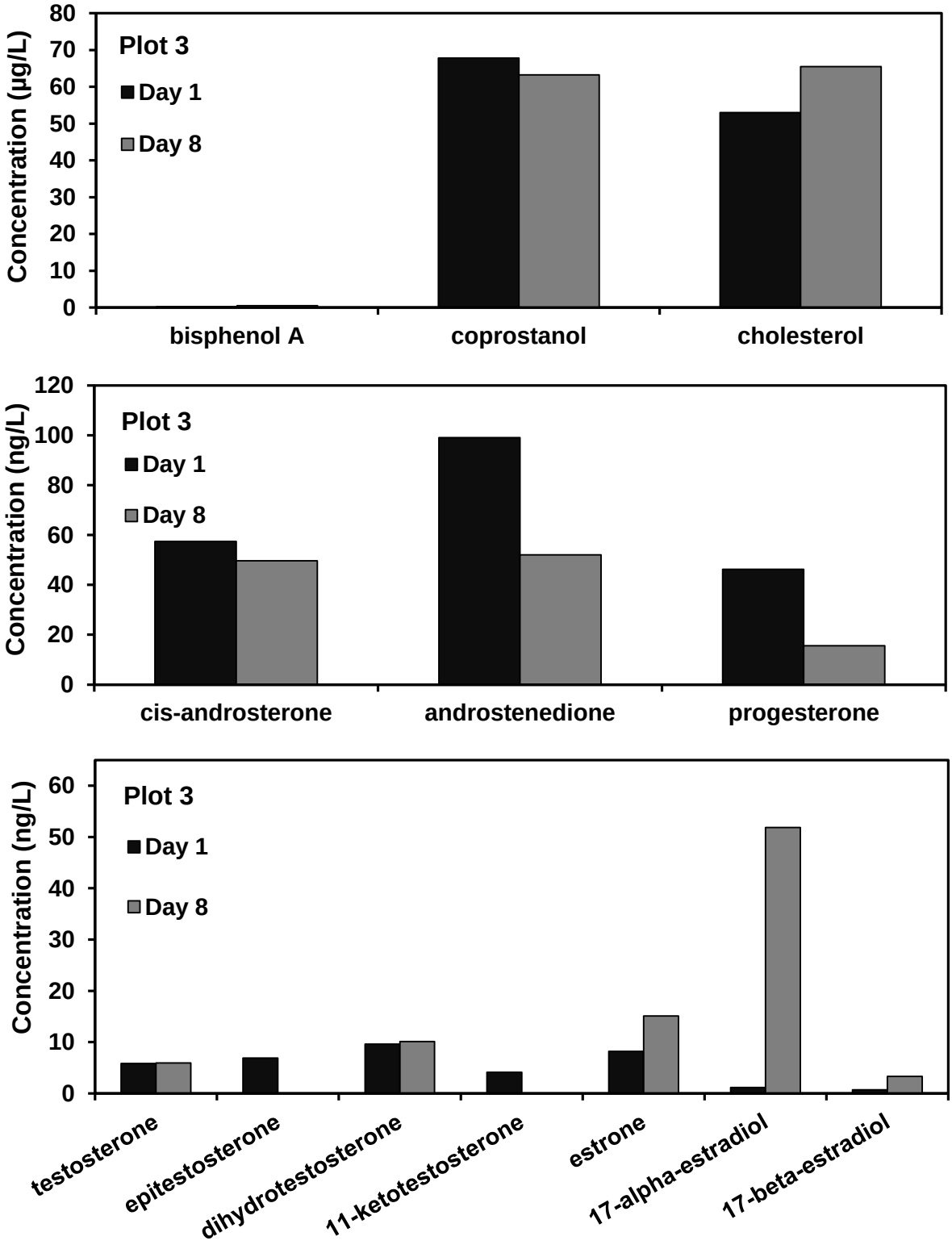


Figure 2.3. Mean (three sampling times) hormone concentrations in whole water runoff samples collected from plot 3 at day 1 and 8 after biosolids application.

The runoff mass fluxes of hormones and runoff rates for plots 1, 2, and 3 on day 1, 8, and 35 are shown in Figures 2.4 to 2.7. The mass flux was calculated in the following way:

$$\text{Mass flux (ng min}^{-1}\text{)} = \text{Runoff rate (L min}^{-1}\text{)} \times \text{Runoff concentration (ng L}^{-1}\text{)}$$

The results are provided as mass flux in nanograms per minute for each targeted compound. The target compound concentration was multiplied by the runoff rate that was determined closest to the compound sampling time. The overall trend of mass flux changes is consistent between different rainfall events, and the highest mass flux of hormones correlated with the rainfall amount.

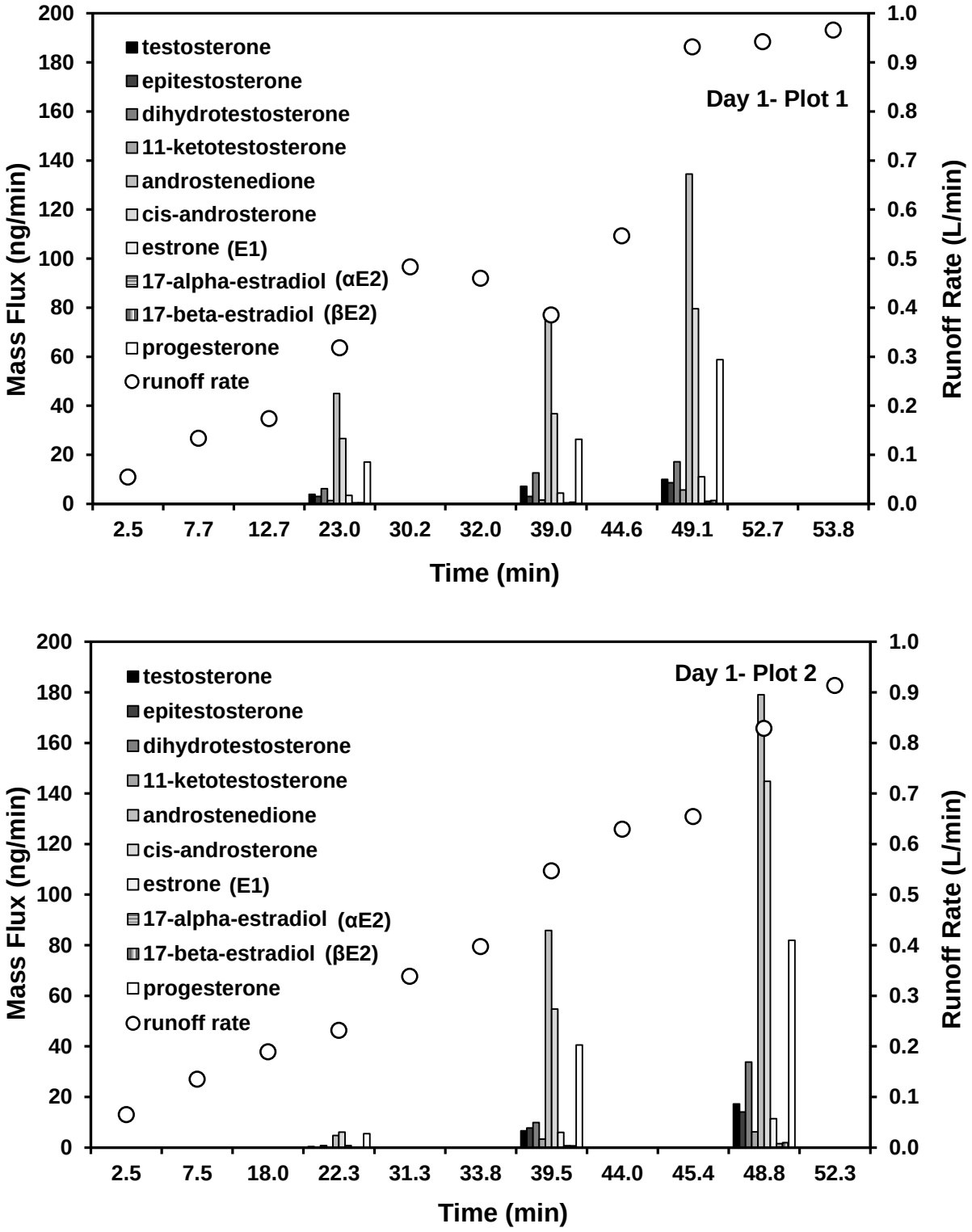


Figure 2.4. Steroid hormone mass flux and runoff rate from plots 1 and 2 during the simulated rainfall events 1 day after biosolids application. Note x-axis is not linear.

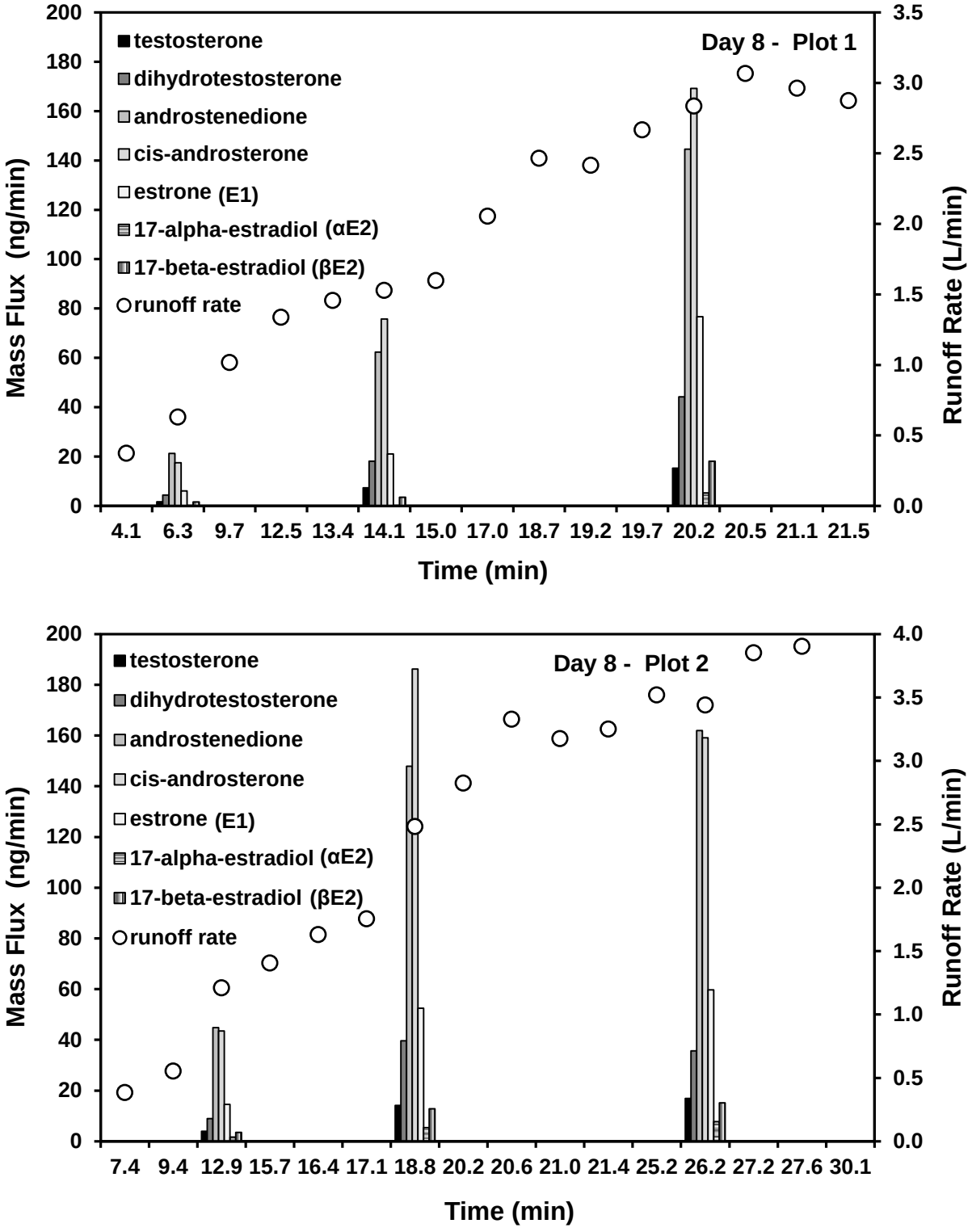


Figure 2.5. Steroid hormone mass flux and runoff rate from plots 1 and 2 during the simulated rainfall events 8 days after biosolids application. Note x-axis is not linear.

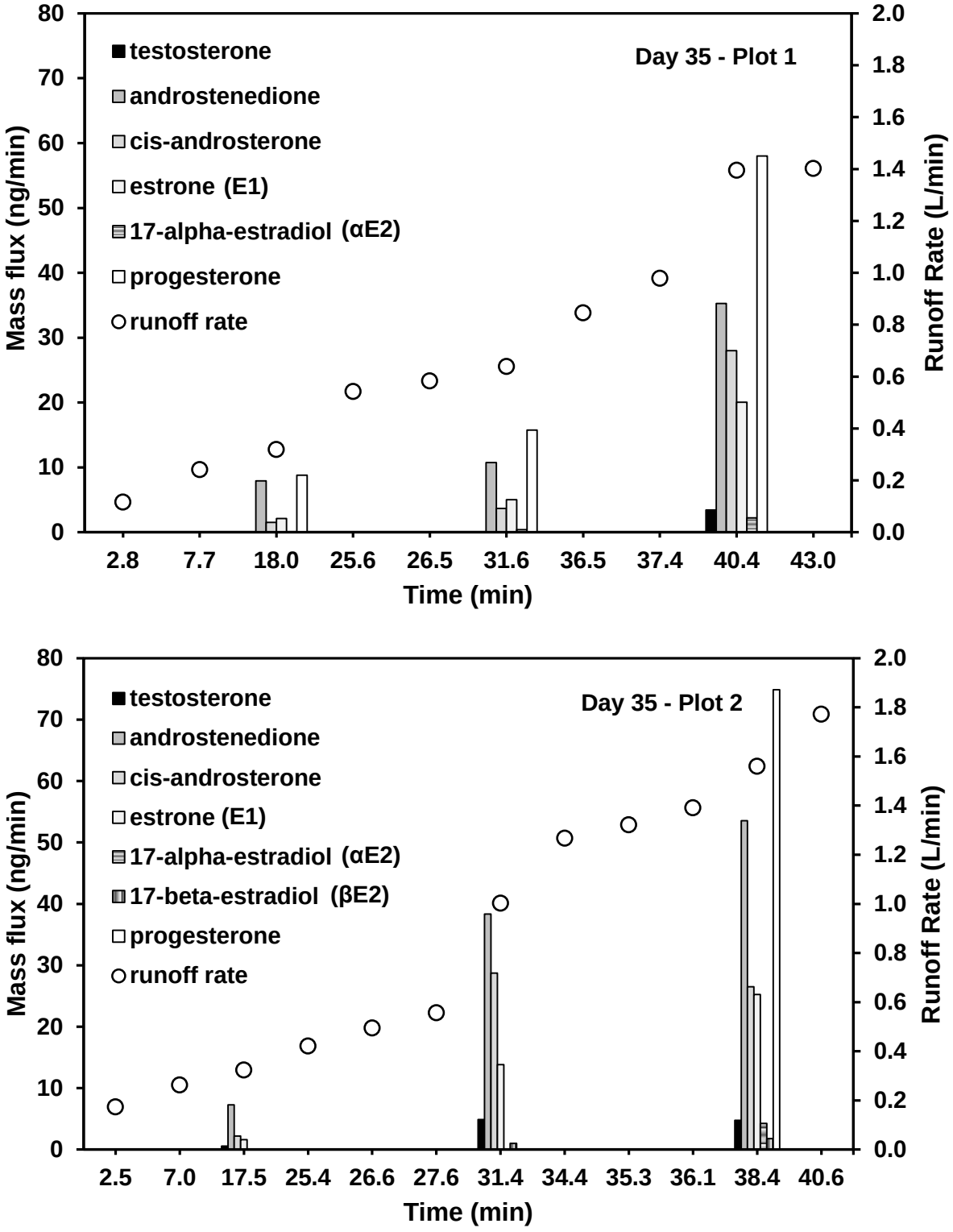


Figure 2.6. Steroid hormone mass flux and runoff rate from plots 1and 2 during the simulated rainfall events 35 days after biosolids application. Note x-axis is not linear.

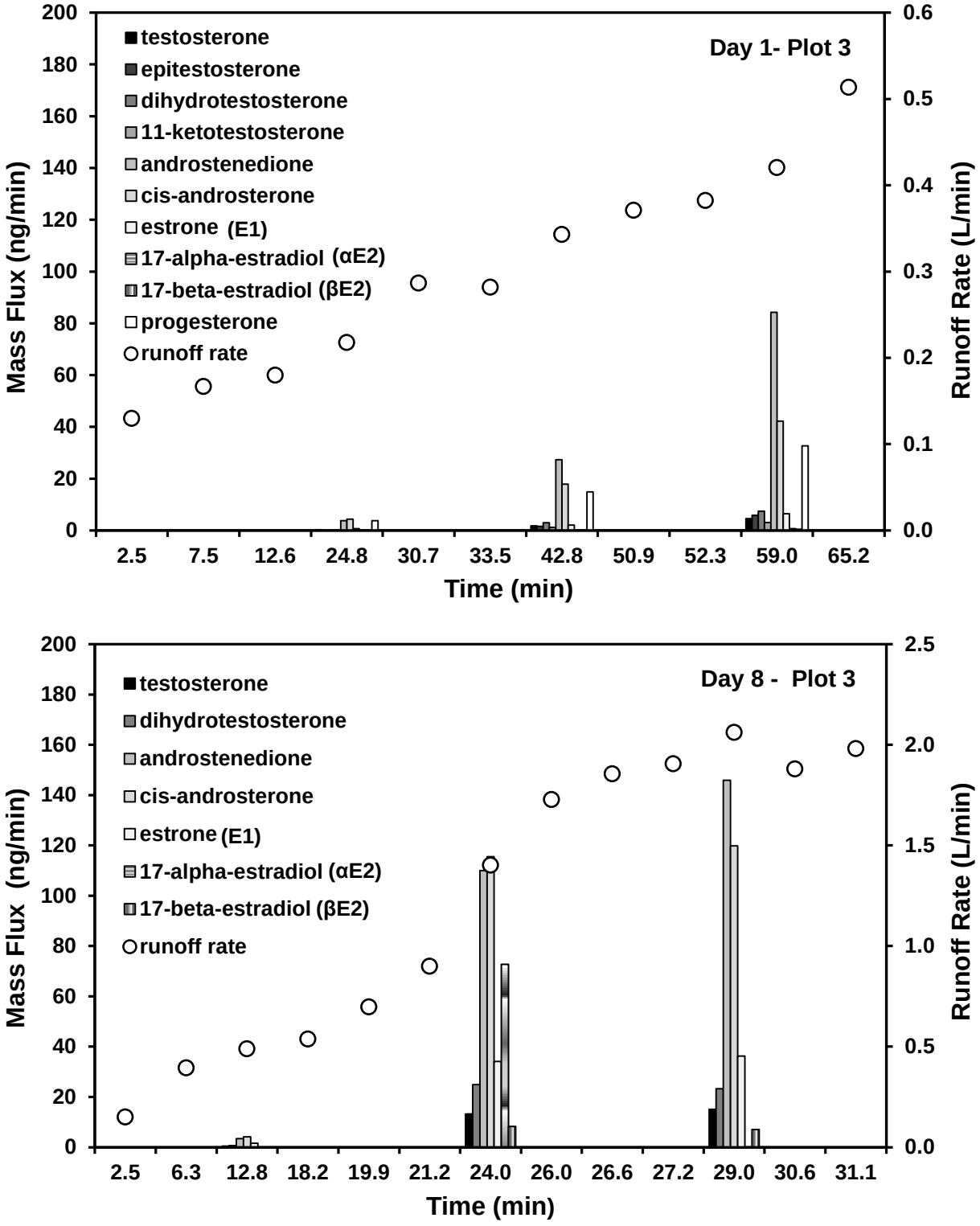


Figure 2.7. Steroid hormone mass flux and runoff rate from plot 3 during the simulated rainfall events 1 and 8 days after biosolids application. Note x-axis is not linear.

Steroid Hormone Fractionation between the Particulate and Aqueous Phases

For this study, aqueous-phase hormones are operationally defined as the fraction of hormones in water that passes through a 0.7 μm glass-fiber filter. Likewise, particle-bound hormones are defined as the fraction of hormones that are retained on the same filter. To investigate the major hormone transport mechanism, the hormone (mass) concentrations (per liter of suspension or per liter of whole water) in the particle phase were compared to the concentrations in the aqueous phase (Figure 2.8). Coprostanol and cholesterol partition strongly to the suspended particles (i.e., particles >0.7 μm), while testosterone, androstenedione, cis-androsterone, E1, E2, and progesterone remain primarily in the aqueous phase. The percentage of particle-bound hormones varied from <0.8 to 22% for testosterone, 8 to 17% androstenedione, 8 to 16% for cis-androsterone, 21 to 31% for E1, and 22 to 64% for E2 based on the results from three experimental plots, 1 day after biosolids application. However, progesterone was only observed in the aqueous phase.

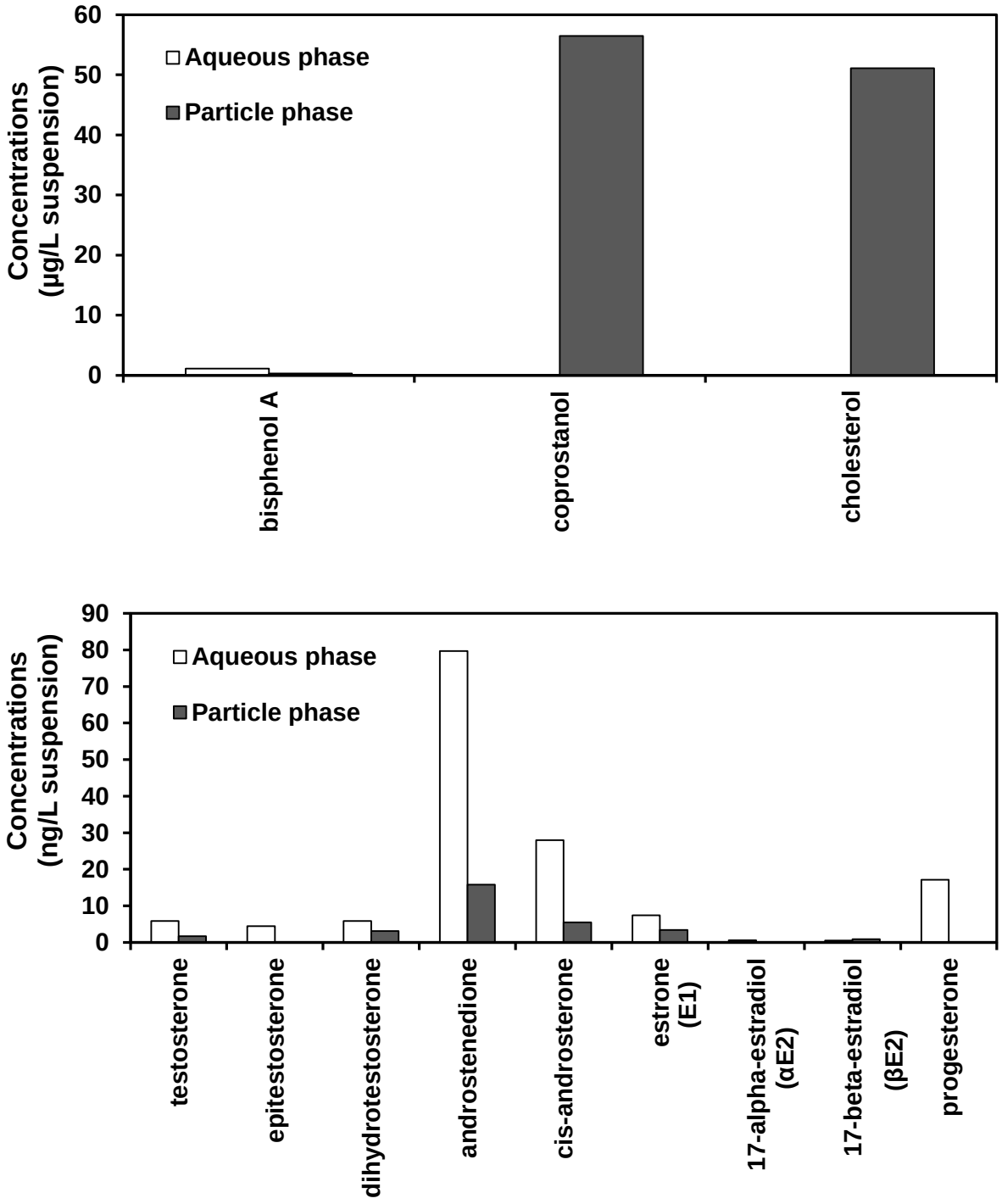


Figure 2.8. Mean bisphenol A, coprostanol, cholesterol, and hormone concentrations in the aqueous (including <0.7 µm particles) and particle phase in runoff samples collected from plot 1 during the rainfall events 1 day after biosolids application.

Discussion

Steroid Hormone Runoff during a Series of Rainfall Events

Only two hormones were observed in runoff prior to biosolids application, and both were observed at low concentration. In contrast, substantial concentrations of hormones were observed in runoff samples after biosolids application, demonstrating that biosolids-amended agricultural soils can result in hormone runoff under the current experimental conditions. This suggests that runoff could be an important source of hormones to receiving waters, especially in areas where application of biosolids is common, and a substantial portion of surface water flow derives from agricultural runoff to small streams (*2*).

The runoff patterns observed for each of the hormones during the experimental period is most likely associated with differences in partitioning behavior, (bio)transformations, and (de)conjugation of the individual compounds. Concentrations of androgens detected in biosolids are in the following order: cis-androsterone >> androstenedione > dihydrotestosterone > testosterone. However, the runoff concentrations of androgens observed at day 1 after biosolids application followed the order androstenedione > cis-androsterone > dihydrotestosterone > testosterone. This suggests that both the initial hormone concentration in biosolids and physicochemical properties affect the observed runoff concentrations. For example, the concentration of androstenedione in biosolids was more than an order of magnitude lower than cis-androsterone, but the observed runoff concentration was in general higher, which can likely be attributed to the much higher water solubility of androstenedione (Table 2.1).

In addition, the concentrations of androgens in runoff decreased during the series of rainfall events, which is in agreement with previous studies showing that androgens can be transported in agricultural soils (*30*). Previous batch, column and field studies have also shown that the sorption affinity of testosterone is lower, while the dissipation/transformation rate and potential for migration is higher than for βE2 (*15*, *30*, *31*).

An increase in estrogen (i.e., E1, αE2, and βE2) concentrations from day 1 to day 8 after biosolids application was observed in this study. Statistical analysis has indicated that the most predominant factors contributing to the fate and transport of βE2 and testosterone in the field were soil-water status (i.e., soil saturation percentages), organic matter content, and colloid-facilitated transport (*32*). However, to elucidate the mechanism responsible for the observed increase in estrogen concentration was beyond the scope of this dissertation. The concentrations of E1 were found to be consistently greater than αE2, and βE2 for all simulated rainfall events. While both αE2 and βE2 are observed in very low concentrations (< ~2 ng L^{-1}), at day 35 E1 is present in the runoff at much higher concentrations (>10 ng L^{-1}). The greater E1 concentrations are most likely due to the higher initial concentration in the biosolids and to a smaller extent biodegradation of E2 to E1 based on the fact that the concentration profile of E2 followed the profile of E1 (Table 2.3; Figure 2.1 to 2.3) (*3*, *33*). Interestingly, a recent study found that E1 had a higher average soil sorption coefficient (K_d = 33 L Kg^{-1} soil) and a higher average soil sorption coefficient per unit organic carbon (K_{oc} = 1557 L Kg^{-1}) than βE2 (K_d = 23 L Kg^{-1} soil and K_{oc} = 1082 L Kg^{-1}) based on investigations of 121 surface (0-15 cm) soils (*34*).

Thus stronger sorption of βE2 is not a likely explanation for this observation. However, it is likely that some αE2, and βE2 was degraded to E1 in the biosolids-amended soil and may, in part, have contributed to the observed concentration of E1 on day 8 and day 35 (*3*, *35*). The runoff concentrations of progesterone were high on day 1, the concentrations decreased dramatically for the subsequent rainfall event, and then increased again on day 35. It is noted that there was no precipitation between the day biosolids were applied to the field and one day after biosolids application. Two precipitation events between days 1 and 8 after biosolids application and day 8 and 35 after biosolids application resulted in about 25.4 and 10.4 mm, respectively. The intermittent wetting and drying of the soil throughout the entire study could influence the transport and biodegradation of hormones, but this possibility was not studied in detail. The absence of progesterone (<8 ng L^{-1}) at day 8 and the reappearance at day 35 suggests either a slow desorption from the biosolids into the soil solution or microbial formation.

There was a general trend of increasing hormone mass flux with increasing rainfall amount resulting in the highest mass flux toward the end of each rainfall simulation. This observation is in agreement with a recent study that reported significant correlations between rainfall amounts and mass exports of estrogens from poultry litter amended soil and that suggest mass exports and concentrations of estrogens do not necessarily monotonically decrease with successive rainfall events (*36*). Collectively, these results suggest that intense rainfall may promote runoff with high hormone concentrations from both manure- and biosolids-amended soils. Additionally, Kjær et al. (*37*) assessed leaching of estrogens from manure-treated structured soils and found that

leaching appears to be influenced by preferential transport, fast solute transport, and drainage water dynamics. Additional study is needed to address the leaching potential of hormones from biosolids-amended soils.

Steroid Hormone Fractionation between the Particulate and Aqueous Phases

In this study, coprostanol and cholesterol were shown to partition to suspended particles to a greater extent (>99% was found in this fraction) than estrogens, androgens, and progesterone, likely due to their hydrophobic properties (Table 2.1). In general, the mean percentage of particle-bound hormones observed in this study followed the order estrogens > androgens >> progesterone. This is in partial agreement with previous studies and may be due to the lower water solubility of estrogens (S_w = ~1 to 4 mg L^{-1}) than androgens (S_w = ~20 to 50 mg L^{-1}) and progesterone (S_w = 8.81 mg L^{-1}) (Table 2.1). The log K_{ow} for progesterone (3.87) is higher than the log K_{ow} values (2.75 to 3.69) for the androgens but similar to or lower than the log K_{ow} (3.13 to 4.01) of E1 and E2 (Table 2.1). It is also likely that the dipole effects would be stronger for estrogens than the other compounds (*38*). The fraction of biosolids in the soil is relatively low so there could be some polar interactions occurring with clays such as electron donor-acceptor (EDA) complexation (*39*).

Interestingly, we did not detect progesterone in the particulate fraction (Figure 2.7). Esperanza et al. (*40*) assessed the fate of sex hormones in two pilot-scale municipal wastewater treatment plants and found that testosterone, androstenedione, and progesterone tended to remain in the aqueous phase (filtrate; passing through 1-µm filters) and did not partition significantly to the solids. It is noted that the aqueous and

particulate phases were separated by a standard 0.7-µm glass-fiber filter in the present study. Thus, it is likely that the aqueous fraction contained small particles and/or colloids containing sorbed hormones. Holbrook et al. (*41*) observed that up to 60% of E2 and EE2 in wastewater is associated with aqueous colloidal material, but the log K_{oc} values and log K_{ow} values are not well-correlated with estrogen sorption to colloids. Similar observations were obtained by Liu (*42*), who also observed poor linear relationships between log K_{ow} and log K_{oc} for bisphenol A, E1, βE2, and EE2. Collectively, these findings suggest that aquatic colloids may play an important role in the environmental behavior of hormones. Additional research is needed to evaluate the physicochemical properties of colloids that influence transport of hormones.

Environmental Implications

The present study assessed the runoff potential for estrogens, androgens, and progestogens from an agricultural field applied with biosolids. Two factors need to be considered when evaluating this dataset 1) the data represent a worst case scenario (100-year rainfall event or 65 mm h^{-1}) for our selected field site and 2) the hormone runoff concentrations reported would likely be diluted by the receiving waters. In addition, it is likely that a soil with a lower sand content (<86%) or higher soil organic matter concentration (>0.3%) than used in this study will behave very differently. Nevertheless, our findings demonstrate that hormones can be present in runoff from biosolids-amended agricultural fields, and that relatively high concentrations of androgens and progesterone are likely to be found in the runoff even after multiple rainfall events and more than one

month after application of biosolids. The rainfall amount correlated with increased hormone mass flux, emphasizing that a heavy rainstorm event will likely promote a pulse of hormones in the runoff rather than dilute the hormones. This suggests that biosolids could be an important source of steroid hormones to surface waters. The adverse effects on fish due to part-per-trillion (ng L^{-1}) estrogen exposure of the aquatic environment have frequently been reported elsewhere (*43-48*). The concentrations of estrogens and androgens, in particular androstenedione, detected in this study are higher than concentrations that have been shown to alter biochemistry and behavior in susceptible fish. Additional research is required to evaluate the potential for hormone transport from biosolids-amended soils with different soil composition, means of biosolids application, and climate conditions (e.g., precipitation rate).

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CHAPTER 3

DEGRADATION KINETICS OF TESTOSTERONE BY MANURE-BORNE BACTERIA: INFLUENCE OF TEMPERATURE, pH, GLUCOSE AMENDMENTS, AND DISSOLVED OXYGEN

Introduction

Environmental steroid hormones are endocrine-disrupting compounds (EDCs), which have the potential to adversely affect wildlife development and reproduction (*1, 2*). The retention and removal of steroid hormones in the environment is expected to be largely the result of a combination of sorption and biotic and abiotic degradation. Biodegradation of steroid hormones has previously been studied in agricultural soils (*1*), biosolids (*3, 4*), river water and sediments (*5*), and pure culture media (*6, 7*).

Jürgens et al. (*5*) showed that microorganisms in a river water sample were capable of transforming 17β-estradiol (βE2) to estrone (E1) with a half-life ($t_{1/2}$) of 0.2 to 9 d, and degrading E1 with a half-life of 0.1 to 11 d. A gram-positive bacterium (i.e. *Bacillus* sp.) isolated from soil was found to be capable of transforming progesterone to 6β- and 14α-hydroxyprogesterones, but no kinetic data were obtained (*8*). Lee et al. (*9*) measured testosterone first-order half-lives ($t_{1/2}$) in aerobic soil-water slurries that ranged

from 0.3 to 7.3 d, and Casey et al. (*10*) observed first-order testosterone degradation rate constants (k) of 0.4 to 0.6 h^{-1} in agricultural soils.

Several studies have examined the degradation of estrogens in soil and soil that has been amended with manure, and the impact of pH, carbon source and temperature on the degradation kinetics (*11-13*). However, little work has been published on the degradation of androgenic steroid hormones by manure-borne microorganisms and the impact of environmental factors, such as temperature, moisture, pH, organic carbon and redox conditions, on the degradation kinetics. Jacobsen et al. (*14*) investigated the impact of swine manure amendments to three different soil types on testosterone degradation at various temperatures. Under all conditions testosterone and its transformation products were dissipated within a few days. Addition of swine manure slurry to soil hastened the transformation of testosterone (4-androsten-17β-ol-3-one) to androstenedione (4-androsten-3,17-dione). Two other testosterone transformation products, 5α-androstanedione (5α-androstan-3,17-dione) and androstadienedione (1,4-androstadien-3,17-dione), were also detected. Experiments with sterilized soil and sterilized swine manure slurry suggested that the transformation of ^{14}C-labeled hormonal parent compounds was mainly caused by microorganisms in the manure slurry, while mineralization of the hormones to $^{14}CO_2$ required viable soil microorganisms. In addition, Lorenzen et al. (*15*) investigated the degradation of testosterone in three different soils, and found that 50% dissipated in 8.5 h (loam soil) to 21 h (silt loam soil) at 30°C, but that testosterone dissipated progressively more slowly at 12 and 4°C. They found only a minor impact of soil moisture (7–39%) on testosterone dissipation rates.

While several studies have investigated the degradation of estrogens in sludge, soils and manures, little is known about the potential for biodegradation of testosterone and progesterone by manure-borne bacteria and their degradation kinetics and pathways. Thus, the main objectives of this study were to reveal the potential for biodegradation of testosterone by swine manure-borne bacteria and to determine the impact of temperature, pH, glucose amendments, and the presence of molecular oxygen on testosterone degradation kinetics. In addition, selected experiments were conducted with E2 and progesterone for comparison.

Experimental Section

Chemicals

Testosterone, E2 (99.6%) and progesterone (98%) were purchased from Pfaltz & Bauer (Waterbury, CT), Calbiochem (La Jolla, CA), and Acros Organics (Morris Plains, NJ), respectively. Dehydrotestosterone (DHT), AD, ADD, and epitestosterone were purchased from Steraloids, Inc. (Newport, RI). Chemicals used to prepare the phosphate buffer solution (Na_2HPO_4, KH_2PO_4, NaCl, and NH_4Cl) and minimal growth media (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $MgSO_4$-$7H_2O$, $CaCl_2$-$2H_2O$, and $C_6H_{12}O_6$) were all of ACS grade and purchased from Fisher Scientific (Fair Lawn, NJ). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were purchased from MP Biomedicals (Solon, OH), and prepared according to the manufacturer's instructions. HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ) and Honeywell Burdick

& Jackson (Muskegon, MI). Formic acid (88% A.C.S.) and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO) and Mallinckrodt Baker (Phillipsburg, NJ). LC-MS grade water was purchased from Honeywell Burdick & Jackson (Muskegon, MI). Deionized water was obtained using a Milli-Q reagent water purification system (Millipore, Bedford, MA).

Manure Collection

Fresh swine feces from stud boars was collected from the Colorado State University Agricultural Research, Development and Education Center (ARDEC) swine barn. All samples were collected in Ziploc (SC Johnson, Racine, WI) plastic bags, and transported on ice to the laboratory within 2 h of collection. Fecal samples were kept frozen at –22°C until used.

Biodegradation Experiments

To study the degradation of testosterone, βE2, and progesterone by manure-borne bacteria, batch incubation experiments were conducted in minimal growth media with swine manure (*system 1*), and in a pre-enriched culture of swine manure-borne bacteria (*system 2*). All glassware was sterilized in an autoclave for 15 min at 121°C and 20 psi before use.

Biodegradation Experiments-*System 1*

In *system 1*, 0.5 g of sterilized (autoclaved for 15 min at 121°C and 20 psi) or unsterilized swine manure was mixed in 250-mL Erlenmeyer flasks with 100 mL of minimal growth media (pH 7) and an initial steroid hormone concentration of 3 mg L^{-1}.

The minimal growth media (pH 7) was composed of 2 mmol L^{-1} $MgSO_4$-$7H_2O$, 3 mmol L^{-1} glucose, 0.1 mmol L^{-1} $CaCl_2$-2 H_2O, 48 mmol L^{-1} Na_2HPO_4, 22 mmol L^{-1} KH_2PO_4, 9 mmol L^{-1} NaCl, and 19 mmol L^{-1} NH_4Cl. The sterilized swine manure was used as an abiotic control, and to estimate the extent of testosterone sorption during the batch incubation experiments. Blanks were prepared with testosterone in minimal media, but no manure, and all treatments were prepared in triplicate. Incubation was conducted in the dark at 22°C on a rotary shaker at 250 rpm. Samples were collected at regular intervals, and immediately filtered through 0.2 μm filters (0.2 μm, Spartan 13/A, regenerated cellulose, Schleicher & Schuell MicroScience, Inc., FL) into 2 mL amber glass vials for analysis. No more than 4% of any steroid hormone was retained on these filters.

Biodegradation Experiments-*System 2*

In *system 2*, the pre-enriched culture of swine manure-borne bacteria was prepared by mixing 1 g of swine manure with 100 mL of TSB in 250-mL Erlenmeyer flasks. The enrichment culture was incubated at 22°C on a rotary shaker at 250 rpm under oxic conditions. An Agilent 8453 UV-visible spectrophotometer was used to measure the optical density at 600 nm (OD_{600}) of samples collected from the enrichment culture, and the OD_{600} measurements were correlated with biomass concentration (colony-forming units [CFU] mL^{-1}). The TSB and TSA were used for preparation of serial dilutions and plate counts to determine the growth curve. When the culture reached the late log phase (14 h; OD_{600} = 3.8; ~10^8 CFU mL^{-1}), the cell suspension was centrifuged at 3,000 g for 10 min, and resuspended in 100 mL of phosphate buffer solution (pH 7). Cells were centrifuged a second time, and resuspended in minimal growth media.

Next, a 1 mL portion of the cell suspension was inoculated into 250-mL Erlenmeyer flasks containing 99 mL of minimal growth media and either testosterone, E2, or progesterone, resulting in an initial cell density of approximately 10^6 CFU mL^{-1} and an initial steroid hormone concentration of 3 mg L^{-1}. To determine the impact of temperature, pH, glucose amendments, and the presence of molecular oxygen on testosterone degradation kinetics, triplicate incubations of the following treatments were also used: (A) 22 and 37°C; (B) pH 6, 7 and 7.5 ; (C) 0, 3, and 22 mmol L^{-1} glucose; and (D) aerobic vs. anaerobic conditions. For anaerobic conditions, the solutions used for the phosphate buffer and minimal growth media were boiled and purged with N_2 for 45 min and sampled periodically in an anaerobic (O_2−free) glovebag. The flasks were incubated in the dark at 22°C on a rotary shaker operated at 250 rpm. Samples were collected at regular intervals, and immediately filtered through 0.2 μm regenerated cellulose filters into 2 mL amber glass vials for analysis.

Analytical Methods

To determine the degradation kinetics of testosterone, E2, and progesterone, samples were analyzed using an Agilent 1200 Series high performance liquid chromatography (HPLC) system with a diode array detector (DAD). The UV chromatograms were quantified at 220 nm for E2, 245 nm for progesterone, and 254 nm for testosterone. The analysis was performed using a Zorbax Eclipse XDB-C18 column (150 mm by 4.6 mm i.d., 5 μm particle size, Agilent, Santa Clara, CA), preceded by a guard column of the same packing material. For the androgens and E2, an isocratic analysis was performed with a mobile phase consisting of acetonitrile (45% for

androgens; 40% for βE2) and water (55% for androgens; 60% for βE2), and a flow rate of 1 mL min^{-1}. The total run time was 20 min. For progesterone, a gradient method was used, with a binary mobile phase consisting of acetonitrile and water, and the mobile composition was adjusted as follows: held at 40% acetonitrile for 9 min, increased linearly to 100% acetonitrile over 5 min, held at 100% acetonitrile for 5 min, decreased linearly to 40% acetonitrile over 1 min, and held at 40% acetonitrile for 5 min. The flow rate was 1 mL min^{-1}, and the total run time, including conditioning, was 25 min. The injection volume of each sample was 20 μL. Testosterone, progesterone and βE2 samples were quantified by reference to a linear calibration, using least squares regression, of six external steroid hormone standards in methanol (0.15, 0.25, 0.5, 1, 2 and 3 mg L^{-1}). The reporting limit was based on the lowest calibration point.

Testosterone's degradation products in *system 2* were identified using the HPLC-DAD analysis described above, and confirmed by LC/TOF-MS using an Agilent 1200 series HPLC system interfaced to an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an Agilent 6510 quadrupole time-of-flight mass spectrometer. For the HPLC analysis, a Luna C18 column (150 mm by 4.6 mm i.d., 5 μm particle size, Phenomenex, Torrance, CA) and a gradient method were used, with a binary mobile phase consisting of methanol and 2.5 mmol L^{-1} ammonium acetate in water. The flow rate was 800 μL min^{-1}, and the mobile-phase composition was adjusted as follows: held at 10% methanol for 0.5 min, increased to 65% methanol at 0.51 min, and then increased linearly to 100% methanol over 17.5 min. Afterwards, the column was flushed with 100% methanol for 2 min at 1.5 mL min^{-1}, and equilibrated with 10% methanol for 4 min

at 800 μL min^{-1}. The mass spectrometer was operated in ESI^+ mode using the following ion source parameters: capillary voltage at 4.5 kV, fragmentor voltage at 200 V, skimmer voltage at 65 V, nebulizer pressure at 20 psig, and drying gas temperature at 325°C. Nitrogen was used as the drying gas with a flow of 5 L min^{-1}. The injection volume of each sample was 20 μL.

Results

Steroid Hormone Degradation by Swine Manure-Borne Bacteria–*System 1* and *2*

The normalized concentration profiles of testosterone, βE2, and progesterone spiked separately into *systems 1* and *2* are illustrated in Figure 3.1and 3.2. No degradation of testosterone, βE2, or progesterone was observed in minimal media in the absence of manure. Sterilization of manure by autoclaving was performed to elucidate the role of sorption and the potential for abiotic degradation.

In *system 1*, some sorption of steroid hormones to swine manure was observed in sterilized controls (i.e. 7% of testosterone, 15% of βE2, and 29% of progesterone) within the first hour of reaction. Steroid hormones in sterilized controls did not exhibit a significant loss after the first hour of incubation (Figure 3.1). Testosterone, βE2, and progesterone were observed to degrade in *system 1* within 4 to 12 h after a lag phase of approximately 5 to 9 h. The degradation of βE2 appears to be faster than progesterone and testosterone. Specifically, no βE2, progesterone, or testosterone was observed in *system 1* after 9, 17, and 21 h of reaction initiation, respectively (Figure 3.1).

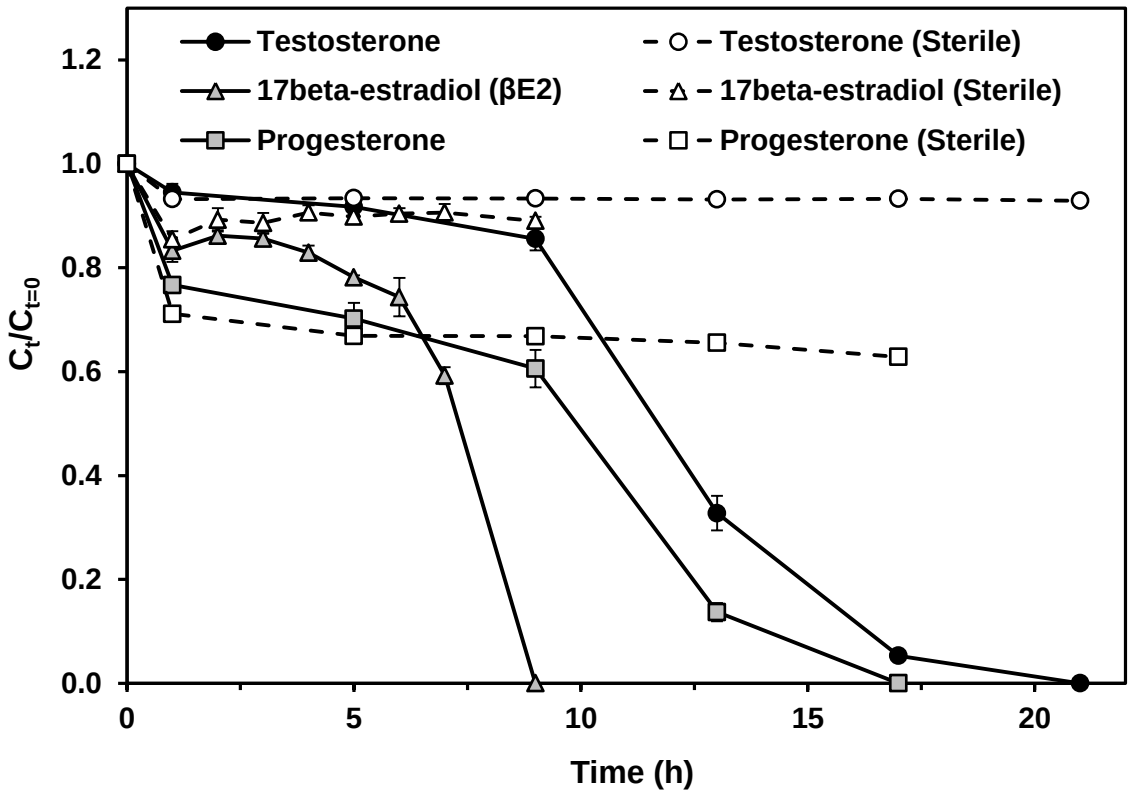

Figure 3.1. Degradation of testosterone, 17β-estradiol, and progesterone under aerobic conditions in *system 1* (with swine manure). Error bars represent the standard deviation of triplicate samples.

In *system 2*, no degradation or sorption of steroid hormones was observed in sterilized controls. Conversely, steroid hormones were observed to degrade in the TSB pre-enriched biologically active systems (Figure 3.2; *system 2*). Testosterone, βE2, and progesterone degradation in *system 2* were initiated without a lag phase. Testosterone and progesterone were transformed in a similar fashion, and followed pseudo first-order reaction kinetics. The degradation of βE2 followed a zero-order reaction kinetics model during the observed time period. To compare the degradation rates for the three steroid

hormones, their rate constants (k) and half-lives ($t_{1/2}$) were calculated based on an initial rate method for the first 8 h (Table 3.1), as described previously (*16, 17*). Degradation rates and associated 95% confidence intervals were estimated with nonlinear regression analysis using the Statistical Analysis System's (SAS 9.2) exponential decay model. Multiple comparisons were conducted using ANOVA at $\alpha = 0.025$, and a p value < 0.05 was considered to indicate significance. The degradation rates followed the order progesterone > testosterone >> E2 at pH 7 and 22°C (Table 3.1; $R^2 > 0.99$).

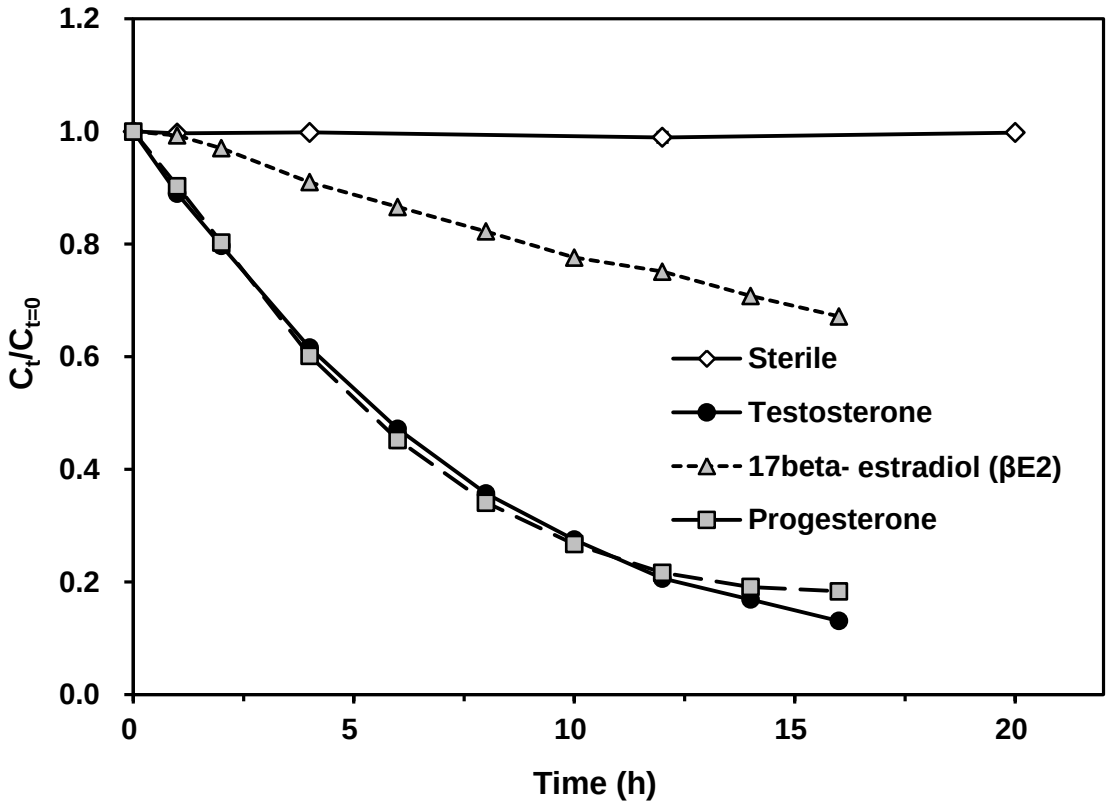

Figure 3.2. Degradation of testosterone, 17β-estradiol, and progesterone under aerobic conditions in *system 2* (TSB pre-enriched culture) at 22°C. Sterile represents sterilized system. Error bars represent the standard deviation of triplicate samples.

Table 3.1. First-order rate constants based on the first 8 h of reaction (*k*; standard deviation in parenthesis), and corresponding half-lives ($t_{1/2}$; normalized to biomass [CFU mL^{-1}] in parenthesis) calculated for the degradation of testosterone, 17β-estradiol, and progesterone in *system 2*.

Compound	**Conditions**	***k*** **(h^{-1})**	**$t_{1/2}$ (h)**
	Aerobic; pH 7; 22°C; 3 mmol L^{-1} glucose		
Progesterone		0.137 (± 0.003)	5.06 (4.63)
17β-estradiol		0.025 (± 0.001)	26.9 (24.6)
Testosterone		0.120 (± 0.003)	5.78 (5.29)
Testosterone	Anaerobic; pH 7; 22°C; 3 mmol L^{-1} glucose	0.026 (± 0.002)	27.1 (27.1)
	Aerobic; pH 7; 3 mmol L^{-1} glucose		
	22°C	0.150 (± 0.004)	4.61 (4.61)
	37°C	0.181 (± 0.008)	3.83 (3.83)
	Aerobic; 22°C; 3 mmol L^{-1} glucose		
	pH 6	0.200 (±0.002)	3.46 (4.88)
	pH 7	0.224 (±0.002)	3.10 (4.36)
	pH 7.5	0.210 (±0.002)	3.30 (4.65)
	0 mmol L^{-1} glucose	0.140 (±0.003)	4.95 (4.95)
	3 mmol L^{-1} glucose	0.150 (± 0.004)	4.61 (4.61)
	22 mmol L^{-1} glucose	0.135 (± 0.004)	5.14 (5.14)

Aerobic versus Anaerobic Degradation of Testosterone–*System 2*

An anaerobic treatment was setup to investigate the influence of molecular oxygen on the degradation rate of testosterone (Figure 3.3). During the observed time period, the degradation of testosterone under anaerobic conditions followed a zero-order reaction kinetics model, in contrast to pseudo first-order reaction kinetics under aerobic conditions. The testosterone concentration decreased by 58% within 6 h of reaction time under aerobic conditions, in contrast to a decrease of only 15% under anaerobic conditions. The half-life of testosterone under anaerobic conditions was observed to be five to six times longer than under aerobic conditions (Table 3.1).

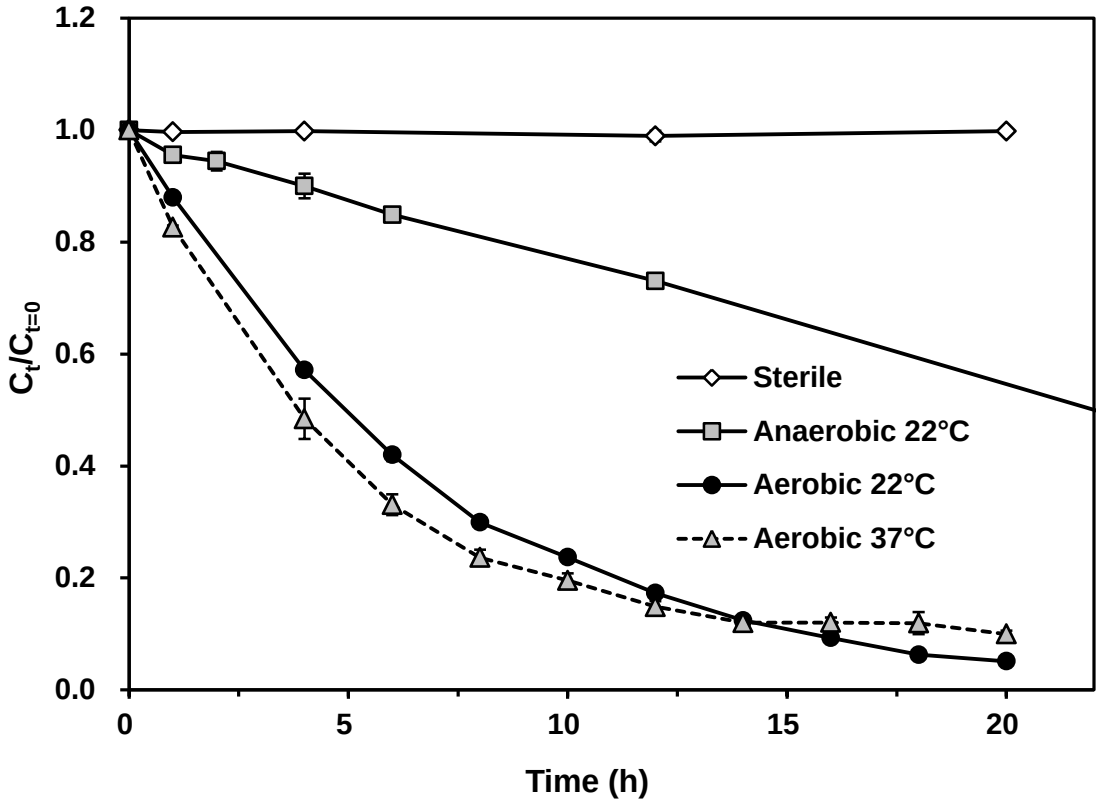

Figure 3.3. Influence of temperature and molecular oxygen at pH 7. Error bars represent the standard deviation of triplicate samples.

Influence of Temperature on Testosterone Degradation–*System 2*

The degradation kinetics of testosterone (*system 2*) were investigated at 37 and 22°C to simulate conditions optimal for fecal bacteria and a temperature relevant for the conditions that swine feces is exposed to within the first 24 h of excretion. The degradation rate was significantly (p value < 0.025) slower (17%) at 22°C than 37°C based on the initial rate calculation (Table 3.1; Figure 3.3).

Influence of pH on Testosterone Degradation–*System 2*

Fresh swine feces (i.e., pH 6.8 for this study), fertile agricultural soils, and waters (e.g., rivers) often vary in pH from approximately 6 to 7.5. Therefore, this pH range was chosen to investigate the impact of pH on testosterone degradation by manure-borne bacteria. The normalized concentration profiles of testosterone obtained for experiments conducted at pH 6, 7, and 7.5 indicated that pH within the investigated range had only a minor impact on the degradation rate. The fastest degradation rate was observed at pH 7, and the degradation rate was approximately 11 and 6% slower in experiments conducted at pH 6 and 7.5, respectively (Table 3.1; Figure 3.4). No significant difference (p value > 0.05) was found between the degradation rates of testosterone at pH 6 and 7.5, whereas a significant difference was observed between pH 6 and 7 and pH 7 and 7.5 (p value < 0.025) .

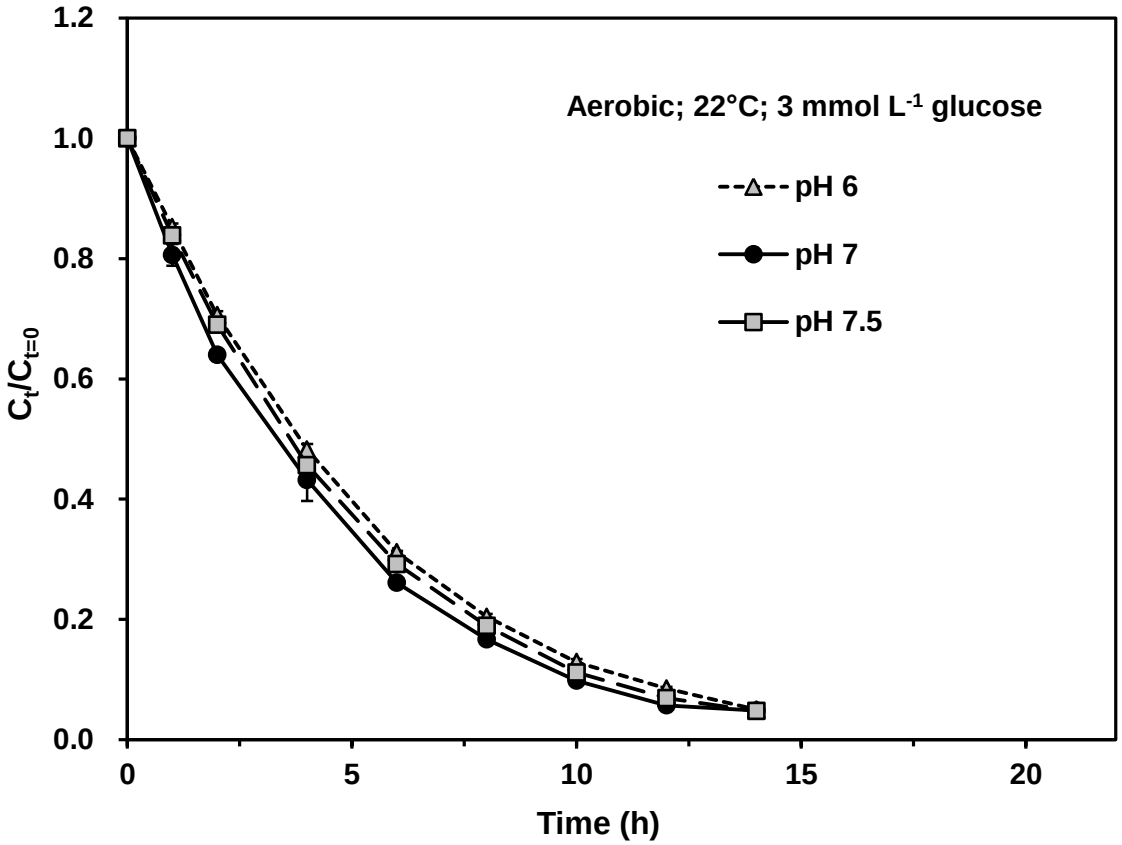

Figure 3.4. Influence of pH on testosterone biodegradation in *system 2*. Error bars represent the standard deviation of triplicate samples.

Influence of Glucose Amendments on Testosterone Degradation–*System 2*

The impact of glucose amendments was investigated to provide insight into the microbial mechanism responsible for testosterone degradation. Glucose amendments (i.e., 0, 3, and 22 mmol L^{-1}) were found to have minor influence on testosterone degradation and metabolite formation within the 18-h time period investigated (Table 3.1; Figure 3.5). Similar results were also observed in *system 1* (data not shown). The difference between the observed rate constant at 0 and 22 mmol L^{-1} glucose amendment was only 4%, but

this difference was not significant (*p* value > 0.05). A significant difference (p value < 0.025) was observed between 3 and 22 mmol L^{-1} glucose amendment.

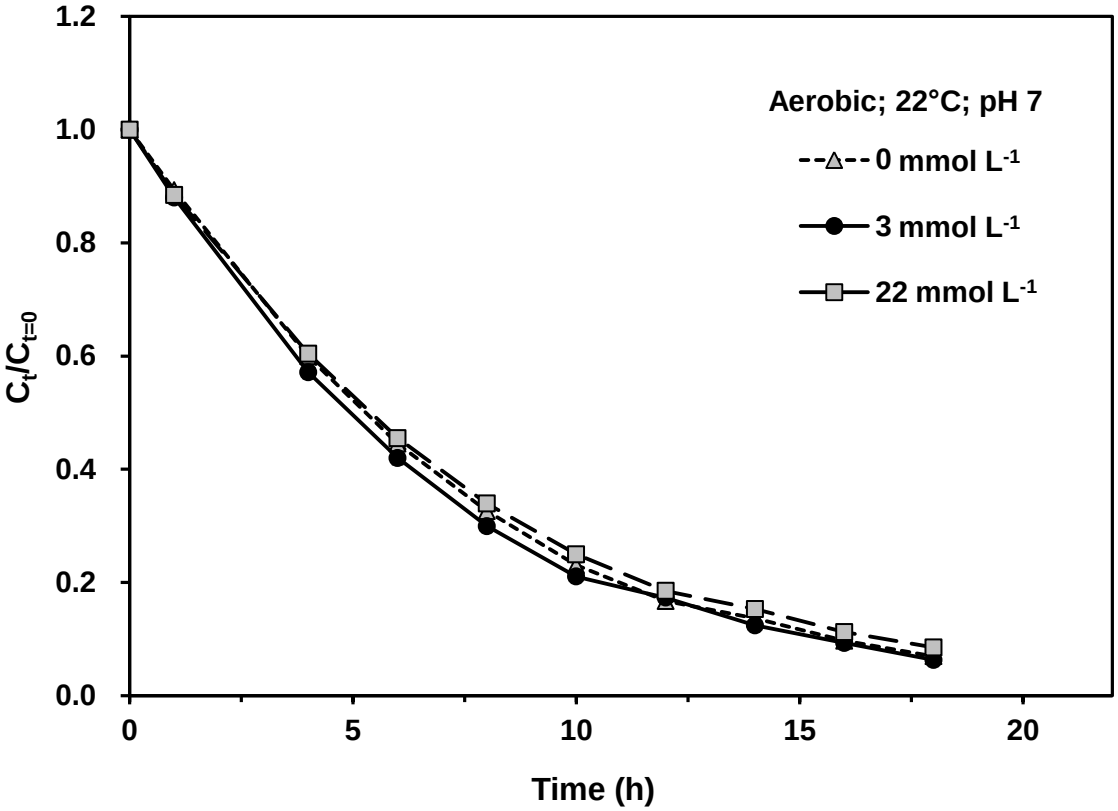

Figure 3.5. Influence of glucose amendments on testosterone degradation in *system 2*. Error bars represent the standard deviation of triplicate samples.

Testosterone Degradation Products–*System 2 (Aerobic conditions)*

In *system 2* under aerobic conditions, HPLC-DAD and LC/TOF-MS analysis revealed three degradation products of testosterone (Figures 3.6 and 3.7). The degradation products were identified through HPLC-DAD analysis by comparing their retention times (t_R) to the retention times of chemical standards, and confirmed by TOF-MS analysis (absolute mass error < 5 ppm). The degradation products were identified as

DHT (absolute mass error 2.1 ppm; major degradation product), ADD (absolute mass error 1.68 ppm), and AD (absolute mass error 0.24 ppm; minor degradation product). Aerobic testosterone degradation pathway by manure-borne bacteria based on metabolites observed within 24 h of incubation are illustrated in Figure 3.8. Estrone (E1) was the only degradation product observed (but not quantified) from βE2, and one unidentified product was observed from progesterone in both systems (Figure 3.9).

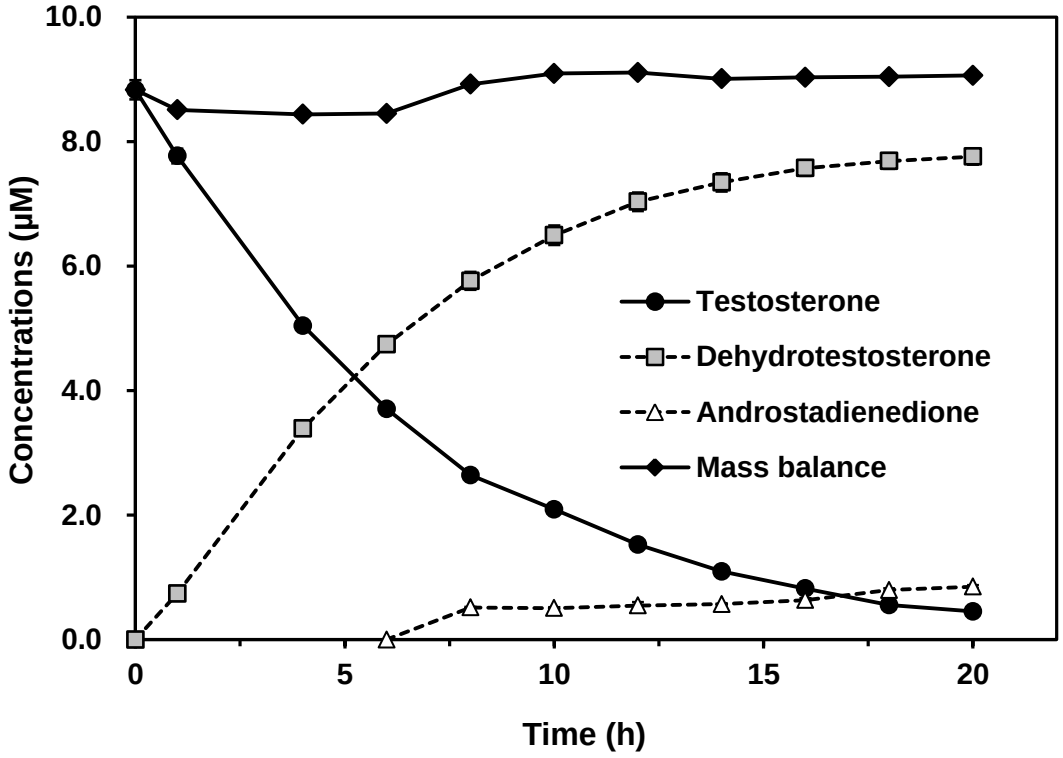

Figure 3.6. Degradation of testosterone and formation of degradation products under aerobic conditions in *system 2* at 22°C and pH 7. Error bars represent the standard deviation of triplicate samples.

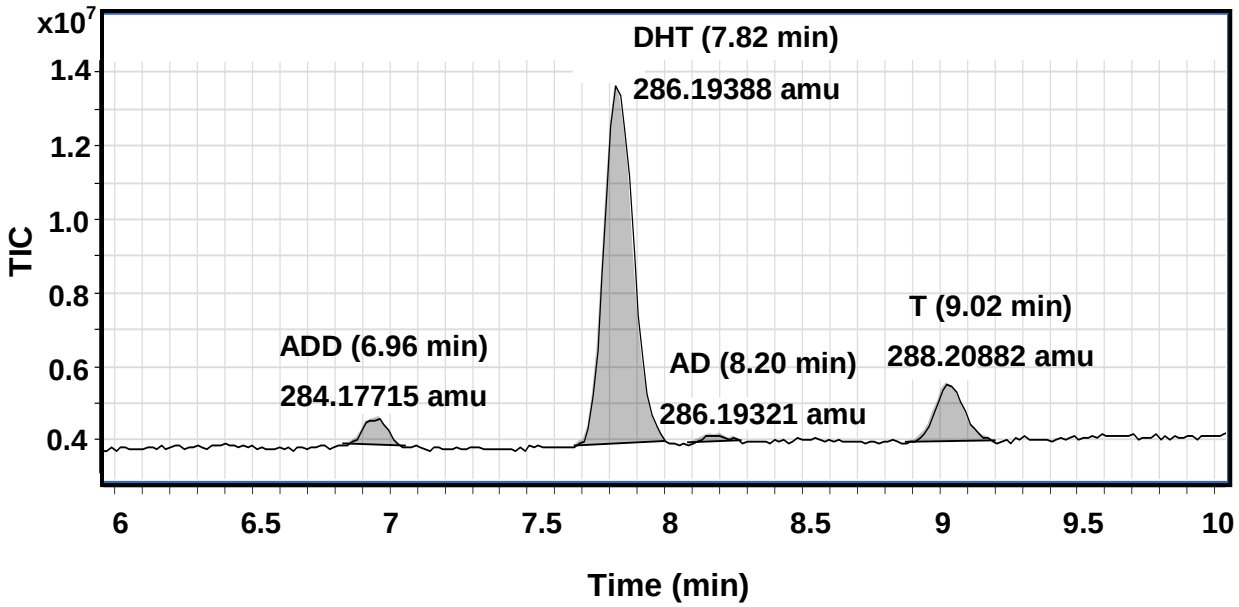

Figure 3.7. Total ion current (TIC) chromatogram obtained by LC/TOF-MS analysis after 12 h of testosterone biodegradation (*system 2*) showing the presence of androstadienedione (ADD), dehydrotestosterone (DHT), androstenedione (AD), and testosterone (T). The molecular mass (amu) and retention time are shown for each compound.

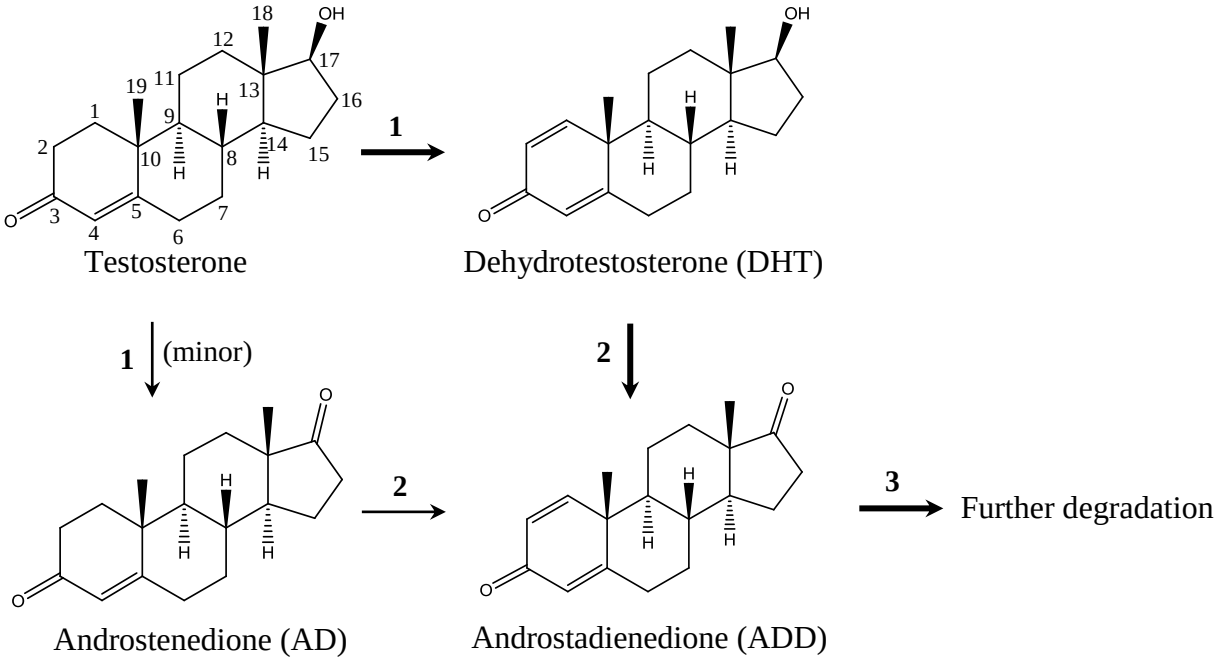

Figure 3.8. Proposed pathways of testosterone degradation by manure-borne bacteria under aerobic conditions based on metabolites observed within 24 h of incubation.

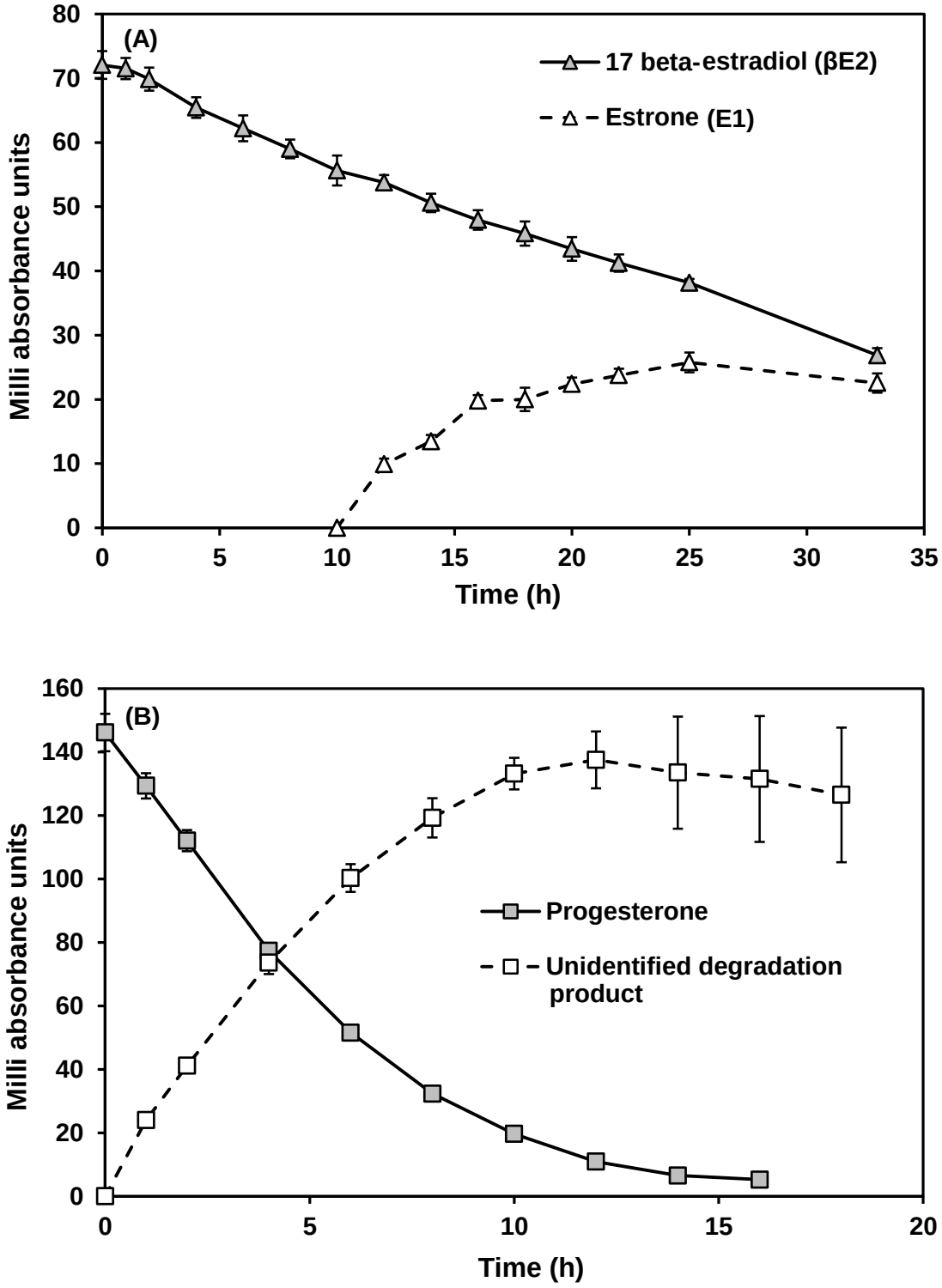

Figure 3.9. Degradation of (A) 17β-estradiol and (B) progesterone and the two observed of degradation products (by HPLC-DAD) under aerobic conditions in *system 2* at 22°C and pH 7. Error bars represent the standard deviation of triplicate samples.

Discussion

Steroid Hormone Degradation by Manure-Borne Bacteria–*System 1* and *2*

Microorganisms such as bacteria common in feces (i.e., swine manure) can transform testosterone, βE2, and progesterone to other potential endocrine active compounds under a range of physical (e.g., temperature) and chemical (e.g., pH and redox) conditions relevant for natural environments.

No sorption (i.e., <2%) in manure-free systems, and no degradation of steroid hormones in sterilized controls, were observed. In contrast, steroid hormones were rapidly degraded in biologically active systems, indicating that manure-borne bacteria were most likely responsible for the observed degradation. The observed lag phase in *system 1* indicates that it takes approximately 5 to 9 h before the bacteria have adapted to the minimal media or produced a sufficient amount of enzymes for steroid hormone degradation. In addition, bacterial enumeration was performed as a part of one of the testosterone studies, and indicated an inverse relationship between the number of bacteria and testosterone concentration (data not shown). In contrast, the absence of a lag phase in *system 2* (pre-enriched) indicates that steroid hormone degradation was not limited by induction or proliferation of steroid hormone-degrading microorganisms, which is in agreement with previous laboratory studies of testosterone degradation in agricultural soils (*15*). 17β-estradiol (βE2) was degraded to below the detection limit within approximately 9 h in systems directly inoculated with swine manure (*system 1*), but only 20% of the βE2 was degraded within the same incubation period in systems inoculated with TSB pre-enriched microbial cultures (*system 2*) despite the higher cell density.

The most likely reason for the slower and different (zero- vs. pseudo first-order kinetics) degradation pattern in *system 2* is that the TSB pre-enrichment disfavored the bacteria that were responsible for the rapid degradation in *system 1* (*15*). The relative degradation rates of βE2, testosterone and progesterone observed in *system 1* (βE2 > progesterone > testosterone) are in contrast to results obtained from studies conducted with soils, biosolids and swine manure applied soils, broiler litter, and composted chicken (*Gallus gallus*) manure (*9, 10, 14, 18-20*). This may, in part, be due to stronger sorption of βE2 (which contains an aromatic ring) than testosterone to soil and undiluted manure and compost, resulting in a lower bioavailability (*9, 10, 21*). Alternatively, the bacteria in soil, composted chicken manure, and similar media might be unable to degrade βE2 as efficiently, perhaps due to the difficulty of degrading the aromatic A-ring of estradiol (*19*). Interestingly, the pseudo first-order rate constant obtained for βE2 in *system 2* (0.025 h^{-1} at 22°C) is similar to a previously reported pseudo first-order rate constant for E2 dissipation in a loam soil (0.02 h^{-1} at 19°C; (22)). Overall, testosterone, βE2, and progesterone were all observed to degrade in the presence of manure-borne bacteria, with half-lives from approximately 5 to 27 hours.

Anaerobic versus Aerobic Degradation of Testosterone–*System 2*

Swine manure-borne bacteria were found to significantly degrade testosterone under both aerobic and anaerobic conditions (Figure 3.3). More than 80% of the testosterone was transformed under aerobic conditions within 12 h, in contrast to 27% under anaerobic conditions. The most plausible explanation for this observation is that manure-borne facultative anaerobic bacteria are more efficient at transforming

testosterone under aerobic conditions than anaerobic conditions (obligate aerobic bacteria are not likely to be found in manure because the gastrointestinal tract is very anaerobic). In this study, the bacterial growth conditions excluded the presence of strict anaerobic bacteria, which might otherwise have contributed to the transformation of testosterone under anaerobic conditions. Despite less efficient degradation under anaerobic conditions, the findings support previous observations of estrogen and androgen removal in anaerobic swine manure lagoons, anaerobic digesters, and anoxic soils (*18*, *23-25*). The degradation of testosterone under anaerobic conditions followed a zero-order reaction kinetics model during the observed time period, suggesting that the degradation mechanisms under aerobic and anaerobic conditions may be different. A study by (*26*) indicated co-metabolic degradation of 17α-ethynylestradiol (EE2) by nitrifying bacteria. On the other hand, both gram-positive bacteria, including *Nocardia, Arthrobacter*, *Mycobacterium*, *Rhodococcus*, and gram-negative bacteria, such as *Comamonas* and *Pseudomonas*, have been described as being capable of using testosterone and other steroids as sole carbon and energy sources (*27*). It was beyond the scope of this study to characterize the microbial communities and the mechanisms responsible for testosterone degradation.

Influence of Temperature and pH on Testosterone Degradation–*System 2*

Testosterone was degraded approximately 17% faster at 37°C than at room temperature (i.e., 22°C), which is likely due to the fact that many fecal-derived enzymes have optimal activity at physiological temperature. Lorenzen et al. (*15*) observed indistinguishable testosterone dissipation rates (i.e., testosterone was below detection

within 25 h) at 23 and 30°C, but progressively more slow rates at 12 and 4°C. Previous studies of temperature on dissipation of E2 and EE2 in soil (*28, 29*) showed a pattern similar to the study by Lorenzen et al. (*15*). The negligible impact of temperatures above approximately 20°C on steroid hormone degradation in soils might be due to the fact that microbial communities in soils produce enzymes with similar activities at higher temperatures.

The impact of pH values relevant for a majority of agricultural soils and rivers (i.e., pH 6.0−7.5) indicated that the microbial activity was only slightly different within this pH range. However, testosterone was observed to degrade significantly faster at pH 7 (i.e., 6−11%) than at both pH 6 and 7.5 (Table 3.1). To the best of our knowledge this is the first study of pH impact on testosterone degradation by manure-borne bacteria. The results of this study suggest that steroid hormones are likely to biodegrade under a wide range of temperature and pH conditions in the environment.

Influence of Labile Carbon on Testosterone Degradation–*System 2*

Several studies have documented that the presence of a labile organic carbon source can influence the degradation of estrogens, indicating a co-metabolic process (*30*). Li et al. (*11*) performed semi-continuous aerobic batch experiments to investigate the impact of a coexisting organic carbon source (glucose) on the biodegradation of E2 and E1. When the initial glucose concentration was varied from 0 to 100 mg L^{-1}, the apparent disappearance rates of E2 and E1 ranged from 0.84 to 4.31 h^{-1} and 0.15 to 0.84 h^{-1}, respectively, assuming first-order kinetics (*11*). Another study examined the effects of glucose concentration on βE2 and EE2 mineralization in different soils and found that

glucose induced faster βE2 and EE2 degradation (*30*). The present study indicated no significant impact of glucose on the testosterone degradation. Glucose most likely did not have a substantial impact on testosterone degradation due to a large initial concentration of exogenous enzymes in the investigated system thus eliminating the need for co-metabolic processes involving glucose in short-term (< 24 h) incubations (*31*). Alternatively, steroid hormones have been observed to mineralize (*32, 33*), which indicates that some bacteria have the potential to use steroid hormones as their sole carbon source.

Testosterone Degradation Products–*System 2*

Three degradation products (i.e., DHT, AD, and ADD) of testosterone were observed under aerobic conditions (Figures 3.6 and 3.7). The main degradation product in this study was observed to be DHT. As far as the authors are aware, this is the first time that DHT has been reported as a degradation product of testosterone by manure-borne bacteria. However, DHT has previously been reported in sewage effluent (*34*). The formation of DHT (major degradation product) is likely a result of 1(2)-dehydrogenase catalyzed testosterone transformation, while AD (minor degradation product) is likely formed by enzyme catalyzed 17β-dehydrogenation of testosterone (Figure 3.8) (*35*). Actinobacteria such as *Mycobacterium and Nocardia* have been described as being capable of introducing 1(2)-dehydrogenation to 3-keto steroids, such as testosterone (e.g., conversion of T to DHT, or AD to ADD) and *Mycobacterium* have also been observed to oxidize steroids at position 17 (e.g., conversion of T to AD, or DHT to ADD) (*35*). Actinobacteria are not common in swine manure but similar transformation processes

appear to be occurring in *system 2*. Previous studies have primarily reported that degradation of testosterone results in the initial formation of androstenedione (*1, 9, 14, 15*). Jacobsen et al. (*14*) reported that microorganisms in a swine manure slurry were able to convert testosterone to 4-AD, 5α-AD, and ADD. The same three degradation products were observed in unmanured agricultural soils (*15*). Interestingly, an unidentified testosterone metabolite was also observed in previous soil column studies (*1*), but it was unclear if the compound was produced directly from testosterone. Lee et al., (*9*) also observed a testosterone degradation product that they were unable to characterize, and hypothesized it to be androst-4-ene-3-one-16,17-diol (no confirmation was made). It was beyond the scope of this study to determine the degradation products of E2 and progesterone, although current studies in our laboratory are trying to elucidate the degradation pathways.

Conclusions

Testosterone, βE2, and progesterone were rapidly (i.e., within 27 h) degraded by swine manure-borne bacteria under aerobic conditions. Testosterone was degraded significantly faster under aerobic ($t_{1/2} \approx 4$ h) than anaerobic ($t_{1/2} \approx 27$ h) conditions in tryptic soy broth pre-enriched systems. The biodegradation rate of testosterone was influenced to a smaller extent ($t_{1/2}$ ranged from 3.8−5.1 h) by different temperatures (22 and 37°C), pH (6, 7 and 7.5), and glucose (0, 3 and 22 mmol L^{-1}) amendments, indicating that testosterone has the potential for degradation by manure-borne bacteria under a wide range of environmentally relevant conditions. However, the formed degradation products

(e.g. DHT, AD, and ADD) are still of potential concern due to their endocrine disrupting potential. Thus, future work (see chapter 4) needs to carefully elucidate the complete degradation pathways and mechanisms of testosterone, βE2, and progesterone, to help advance our current understanding of the extent to which these hormones and their degradation products contribute to endocrine disruption in terrestrial and aquatic environments.

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CHAPTER 4

IDENTIFICATION OF BACTERIA IN A TESTOSTERONE-MINERALIZATING CULTURE ENRICHED FROM SWINE MANURE

Introduction

Steroid hormones have been an increasing public concern because they are able to act as endocrine disruptors and thus adversely affect wildlife reproduction (*1, 2*). One major source of steroid hormone contaminants to the environment is the land application of animal manures as fertilizer or amendment. Livestock manure often contains a large amount of natural and synthetic chemicals including hormones (*3, 4*). Previous studies (*5-9*) have reported that steroid hormones could potentially contaminate the aquatic environment via surface runoff or leaching from agricultural fields amended with manure. Androgenic hormones including testosterone and its metabolites have been detected in water bodies receiving feedlot effluent (*7*). Orlando et al. (*10*) collected wild fathead minnows exposed to cattle feedlot effluent, and observed that the minnows exhibited significant altered reproductive biology. Male fish were demasculinized (having lower testicular testosterone synthesis, altered head morphometrics, and smaller testis size). Defeminization of females, as evidenced by a decreased estrogen:androgen

ratio of in vitro steroid hormone synthesis, was also documented. The authors hypothesized that androgenic substances were at least in part responsible based on detected potent androgenic responses to the feedlot effluent using cells transfected with the human androgen receptor.

Biodegradation has been suggested as the most important steroid hormone removal mechanism in the environment (*3, 11*) . Microbial transformation of testosterone (4-androsten-17β-ol-3-one) has been observed in several environmental matrices such as soils (*12, 13*), soils amended with manure or biosolids (*14, 15*), stream sediments (*16*), and wastewater treatment plants (WWTPs) (*17*). Jacobsen et al. (*18*) observed rapid conversion of testosterone to androstenedione (4-androstene-3,17-dione ; AD) and other metabolites, and mineralization of ^{14}C-testosterone within a swine manured soil. However, these effects did not occur in soil amended with sterilized manure, suggesting that microbial activity plays an important role in testosterone degradation.

Several species of *Arthrobacter, Mycobacterium, Nocardia, Rhodococcus, Comamonas,* and *Sphingomonas* have been found to degrade testosterone and other steroids such as βE2 and AD (*19-22*). Four strains of *Rhodococcus* isolated from WWTPs were found capable of degrading estrone (E1), 17β-estradiol (βE2), estriol (E3) and EE2 (*22*). *Rhodococcus erythropolis* and *Rhodococcus equi* were demonstrated to degrade EE2, removing up to 47 and 39% of the initial 1.4 mg L^{-1} (EE2) in 13 and 65 h, respectively, in the presence of a co-substrate (i.e., adipic acid or glucose) (*23*). The results also indicated that the presence of an easily degradable carbon source plays an important role in the removal of EE2 when exposed to microorganisms. A gram-negative

bacterium, *Comamonas testosteroni*, is known for its ability to metabolize testosterone as a sole carbon and energy source (*24*). Degradation of testosterone in *C. testosteroni* is considered to be initiated by dehydrogenation of the 17β-hydroxyl group to AD, which is then converted to androstadienedione (1,4-androstadiene-3,17-dione; ADD), and proceeds via aromatization of the A-ring to complete mineralization (*24-26*). A recent study showed a βE2-utilizing bacterium, *Sphingomonas* strain KC8 isolated from a WWTP, that was capable of degrading and further utilizing testosterone as a growth substrate (*27*). The gammaproteobacterium *Steroidobacter denitrificans* strain FS^T, isolated and enriched from anoxic digested biosolids (*28*), was found capable of transforming testosterone under anoxic conditions (*29*). Ten transformation products including 3β-hydroxy-5α-androstan-17-one, 5α-androstan-3,17-dione, dehydrotestosterone (17β-hydroxy-androstane-1,4-dione-3-one; DHT), AD, and ADD were characterized in their study.

While several species of bacteria have been described as being capable of utilizing testosterone and other steroids as sole carbon and energy sources (*19, 22*), little is known about the manure-borne bacteria responsible for testosterone mineralization and their degradation pathways under aerobic conditions. This type of information is vital for development of best management practices for optimal hormone removal in, for instance, manure lagoons and soils where manure is applied. In our previous study (Chapter 3), we demonstrated that testosterone is rapidly degraded by manure-borne bacteria under a wide range of environmentally relevant conditions, and three degradation products (i.e., DHT, AD, and ADD) were observed under aerobic conditions within 24 h of incubation (*30*).

The specific objectives in this study were (i) to enrich manure-borne bacteria capable of using testosterone as their sole carbon source under aerobic conditions, (ii) to DNA sequence the enriched microbial culture, and (iii) to elucidate the testosterone degradation/mineralization pathway by the enriched bacteria.

Experimental Section

Chemicals

Testosterone was purchased from Pfaltz & Bauer (Waterbury, CT). Dehydrotestosterone (DHT), androstenedione (AD), androstadienedione (ADD), and epitestosterone were purchased from Steraloids, Inc. (Newport, RI). Chemicals used to prepare the phosphate buffer solution (Na_2HPO_4, KH_2PO_4, NaCl, and NH_4Cl) and minimal growth media (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $MgSO_4$-$7H_2O$, $CaCl_2$-$2H_2O$, and $C_6H_{12}O_6$) were all of ACS grade and purchased from Fisher Scientific (Fair Lawn, NJ). Tryptic soy agar (TSA) was purchased from MP Biomedicals (Solon, OH), and prepared according to the manufacturer's instructions. [4-^{14}C]-testosterone was obtained from American Radiolabeled Chemicals (St. Louis, MO). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ) and Honeywell Burdick & Jackson (Muskegon, MI). Formic acid (88% A.C.S.) and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO) and Mallinckrodt Baker (Phillipsburg, NJ). LC-MS grade water was purchased from Honeywell Burdick & Jackson (Muskegon, MI). Deionized water was obtained using a Milli-Q reagent water purification system (Millipore, Bedford, MA).

Manure Collection

Fresh swine feces from stud boars was collected from the Colorado State University Agricultural Research, Development and Education Center (ARDEC) swine barn. All samples were collected in Ziploc (SC Johnson, Racine, WI) plastic bags, and transported on ice to the laboratory within 2 h of collection. Fecal samples were kept frozen at –22°C until used.

Enrichment Culture of Testosterone–Degrading Bacteria

An enrichment culture was used to obtain testosterone-degrading bacteria in swine manure. To study degradation of testosterone by manure-borne bacteria, batch incubation experiments were conducted. A half gram of sterilized (autoclaved for 15 min at 121°C and 20 psi) or unsterilized swine manure was mixed in 250-mL Erlenmeyer flasks with 100 mL of minimal growth medium (pH 7) and an initial testosterone concentration of 3 mg L^{-1}. The minimal growth medium (pH 7) was composed of 2 mmol L^{-1} $MgSO_4$-$7H_2O$, 0.1 mmol L^{-1} $CaCl_2$-2 H_2O, 48 mmol L^{-1} Na_2HPO_4, 22 mmol L^{-1} KH_2PO_4, 9 mmol L^{-1} NaCl, and 19 mmol L^{-1} NH_4Cl. The sterilized swine manure was used as an abiotic control. Incubation was conducted in the dark at 22°C on a rotary shaker at 250 rpm, and all treatments were prepared in triplicate. Samples were collected at regular intervals and immediately filtered through 0.2-µm filters (0.2-µm, Spartan 31/A, regenerated cellulous, Schleicher & Schuell MicroScience, Inc., FL) into 2 mL amber glass vials for analysis. To further enrich the testosterone-degrading bacteria present in swine manure, several transfers were conducted. When testosterone degradation was in the late phase of biodegradation, a 1 mL aliquot of the cell suspension

from the sterilized- or unsterilized-manure systems was transferred into 250-mL Erlenmeyer flasks containing 99 mL of fresh minimal growth medium and an initial testosterone concentration of 3 mg L^{-1}. Incubation was conducted in the dark at 22°C on a rotary shaker at 250 rpm. Tryptic soy agar (TSA) was used for preparation of plate counts to determine the growth curve during the degradation process. The culture suspension from the fifth transfer was transferred into a 50 mL sterilized plastic centrifuge tube (Thermo Fisher Scientific Inc.) and stored at –80°C prior to DNA extraction. The following experiments were all conducted with enriched cultures from the fifth transfer (approximately 4 to 5 weeks from the first transfer).

^{14}C Mineralization Laboratory Assays

A 0.5 mL portion of the cell suspension from the enrichment culture was added into 49.5 mL of fresh minimal growth medium containing 3 mg L^{-1} testosterone. In order to determine the amount of CO_2 produced during mineralization of testosterone, approximately 25 million dpm of [4-^{14}C]-testosterone (Figure 4.1) was added to 125-mL Erlenmeyer flasks containing 50 mL medium. All $^{14}CO_2$ was trapped in these experiments by purging air through a Teflon tube into the microbial medium via a rubber stopper, and the air from the outlet of the test flasks was passed through a Teflon tube that was connected to a scintillation vial containing a scintillation cocktail that can trap any produced $^{14}CO_2$. An abiotic control containing a sterilized cell suspension was also set up to elucidate whether the conversion of testosterone to $^{14}CO_2$ was biologically facilitated. Six additional samples, including sterilized controls and enriched cultures, were setup without air sparging to allow for measurements of ^{14}C in the aqueous phase.

The flasks were incubated in the dark on a rotary shaker at 250 rpm, and all treatments were prepared in triplicate. Trapped $^{14}CO_2$ and aqueous ^{14}C was subsequently counted on a scintillation counter (Packard 2500R, PerkinElmer, USA) after samples were taken, and the scintillation cocktail (OX-161, R.J. Harvey Instrument CO., USA) used in the $^{14}CO_2$-trap was replaced every time to prevent saturation/evaporation.

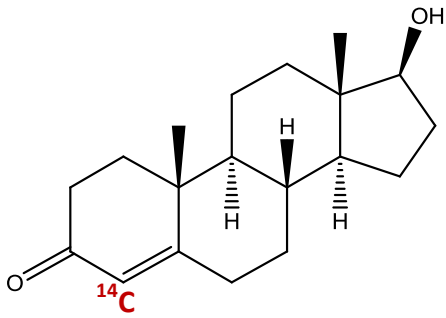

Figure 4.1. Molecular structure of [4-^{14}C]-testosterone. The radiolabeled carbon was located in carbon position 4.

DNA Extraction

DNA was extracted from cell pellets of the testosterone-degrading culture from the enrichment culture using the UltraClean microbial DNA isolation kit (Mo Bio, Carlsbad, CA) according to the manufacturer's instructions. DNA quality was verified by agarose gel electrophoresis. The extracted DNA was stored at –80°C for subsequent studies.

16S rRNA Gene Polymerase Chain Reaction (PCR) Assays

The DNA extract was diluted 1:10 with sterile water and used as template for 16S

rRNA gene PCR assays. The PCR amplification was performed in a Mastercycler® pro thermal cycler (Eppendorf, Ontario, Canada). Primers U341F (primer 1 of Muyzer et al. (*31*)) and 1492R (*32*) were used to amplify ~ 1150 bp of the 16S rRNA gene in a 25 μL reaction mixture containing 10 mM deoxynucleoside triphosphates (dNTPs), 5 μM each primer, 1X TaqMaster PCR enhancer (5 Prime, Hamburg, Germany), 1X reaction buffer (5 Prime), 1.75 U *Taq* DNA polymerase (5 Prime), 1 μL of DNA template, 0.25 μL formamide, and 12.4 μL deionized water. The PCR thermal cycle was as follows: initial denaturing at 94ºC for 180 sec, followed by 35 cycles of 94ºC for 30 sec, 50ºC for 30 sec, 72ºC for 90 sec, and final extension step for 7 min at 70ºC. Presence of PCR product of the expected size was determined by electrophoresis on 1.2% (w/v) agarose gels.

Cloning and Sequence Analysis

The U341F-1492R PCR products were cloned and transformed into competent *E. coli* cells with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The inserts were PCR amplified directly from the colonies using the vector-specific M13F/M13R PCR primers. The reaction mixture included 10 mM dNTPs, 20 μM each primer, 1X TaqMaster PCR enhancer (5 Prime), 1X GenScript buffer (GenScript, Piscataway, NJ), 1.75 U GenScript *Taq* DNA polymerase, 0.25 μL formamide, and 15.9 μL deionized water to bring the volume to 25 μL. The amplification conditions were 3 min at 94ºC, followed by 35 cycles of 20 min at 94ºC, 30 min at 55ºC, 70 sec at 72ºC, and final extension step for 7 min at 70ºC.

Amplified ribosomal DNA restriction analysis (ARDRA) was performed on the M13 PCR products with the restriction enzyme *Msp1*. M13 PCR products from all the different *Msp1* restriction digest patterns were sequenced at the CSU Proteomics and Metabolomics facility. Rarefaction curves of the ARDRA patterns were determined by plotting the number of unique restriction patterns versus the total number M13 PCR products digested with *Msp1*. The DNA sequences obtained from cloning were aligned to the sequences of the closest identified microorganisms by the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). The Tree Builder tool from the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) website was used to build a phylogenetic tree with the DNA sequences from the samples and reference 16S rRNA gene sequences from the RDP database using the Weighbor weighted neighbor-joining tree building algorithm.

Analytical Methods

To determine the concentrations of testosterone, filtrate samples were analyzed using an Agilent 1200 Series high performance liquid chromatography (HPLC) system with a diode array detector (DAD). The UV chromatograms were quantified at 254 nm for testosterone. The analysis was performed using a Zorbax Eclipse XDB-C18 column (150 by 4.6 mm i.d., 5µm particle size, Agilent, Santa Clara, CA), preceded by a guard column of the same packing material. An isocratic analysis was performed with a mobile phase consisting 45% of acetonitrile and 55% of water, and a flow rate of 1 mL min^{-1}. The injection volume of each sample was 20 µL, and the total run time was 20 min.

Testosterone was quantified by reference to a linear calibration, using least squares regression, of six external testosterone standards in methanol (0.15, 0.25, 0.5, 1, 2, and 3 mg L^{-1}). The reporting limit was based on the lowest calibration point.

Testosterone's degradation products were identified using the HPLC-DAD analysis described above, and confirmed by LC/TOF-MS using an Agilent 1200 series HPLC system interfaced to an HTC-PAL autosampler (CTC analytics, Zwingen, Switzerland) and an Agilent 6510 quadrupole time-of-flight mass spectrometer. For the HPLC analysis, a Luna C18 column (150 mm by 4.6 mm i.d., 5 μm particle size, Phenomenex, Torrance, CA) and a gradient method were used, with a binary mobile phase consisting of methanol and 2.5 mmol L^{-1} ammonium acetate in water. The flow rate was 800 μL min^{-1}, and the mobile-phase composition was adjusted as follows: held at 10% methanol for 0.5 min, increased to 65% methanol at 0.51 min, and then increased linearly to 100% methanol over 17.5 min. Afterwards, the column was flushed with 100% methanol for 2 min at 1.5 mL min^{-1}, and equilibrated with 10% methanol for 4 min at 800 μL min^{-1}. The mass spectrometer was operated in ESI^+ mode using the following ion source parameters: capillary voltage at 4.5 kV, fragmentor voltage at 200 V, skimmer voltage at 65 V, nebulizer pressure at 20 psig, and drying gas temperature at 325°C. Nitrogen was used as the drying gas with a flow of 5 L min^{-1}. The injection volume of each sample was 20 μL.

Results

Characterization of Microbial Community in Testosterone–Degrading Culture Enriched from Swine Manure

Amplified ribosomal DNA restriction analysis (ARDRA) of 60 M13 PCR products with the restriction enzyme *MspI* produced 10 different restriction patterns (Figure 4.2). The rarefaction curve based on ARDRA patterns approached saturation, indicating that nearly all the diversity of the sample had been covered (Figure 4.3). The DNA sequences of 30 M13 PCR products, representing all 10 restriction patterns, were determined to identify the bacterial species (Figure 4.4; Table 4.1). The DNA sequencing results revealed that the microorganisms in the sample were distributed among six different genera - *Acinetobacter*, *Brevundimonas*, *Comamonas*, *Sphingomonas*, *Stenotrophomonas, and Rhodobacter* - of three classes: Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria (Figure 4.5). With over 46% of the total bacterial sequences, the *Sphingomonas* sp. JEM-1 represented the dominant DNA sequence in the microbial enrichment. The second most abundant DNA sequence corresponded to *Rhodobacter* sp. M2T8B7 (~25%). Approximately 15% of the sequences corresponded to *Comamonas testosteroni* strain TDKW (Figure 4.4; Table 4.1).

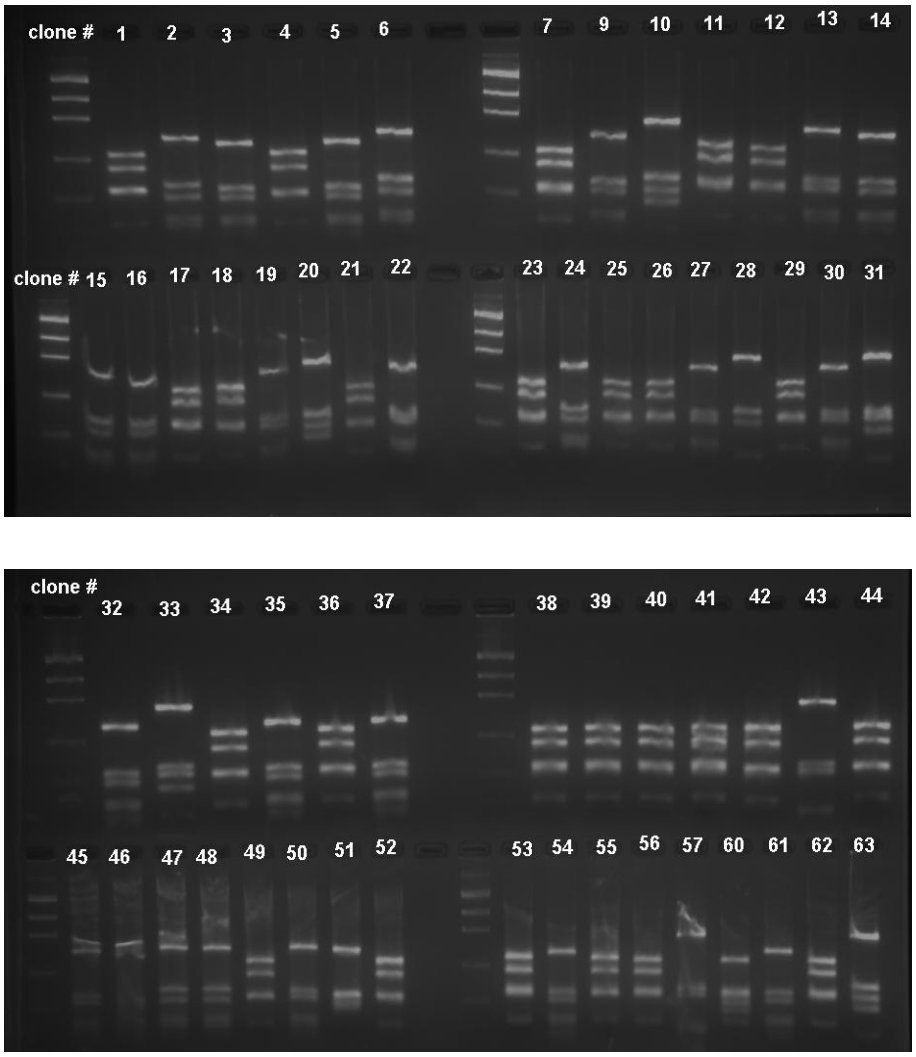

Figure 4.2. Agarose gel (1.2% w/v) of ARDRA patterns of 60 partial 16S rRNA gene sequences digested with the restriction enzyme *Msp1*. Numbers on top of each restriction pattern indicate the M13 PCR product from which the pattern originated. Low DNA mass™ ladder (Invitrogen) was used to aid in the comparison of the patterns.

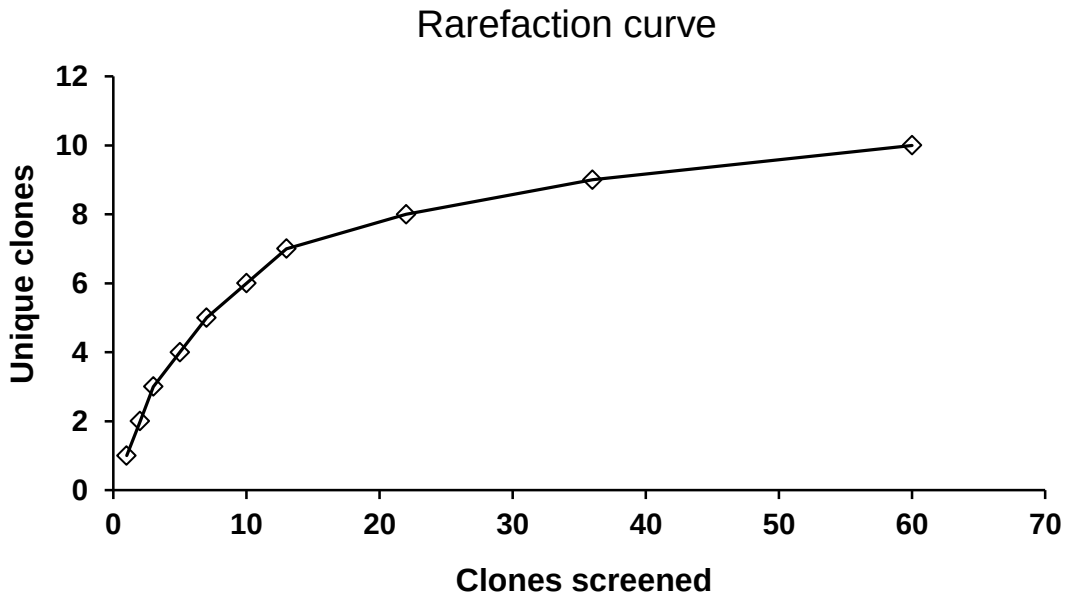

Figure 4.3. Rarefaction curve calculated for the different ARDRA patterns from partial 16S rRNA gene sequences digested with the restriction enzyme *MSP1*.

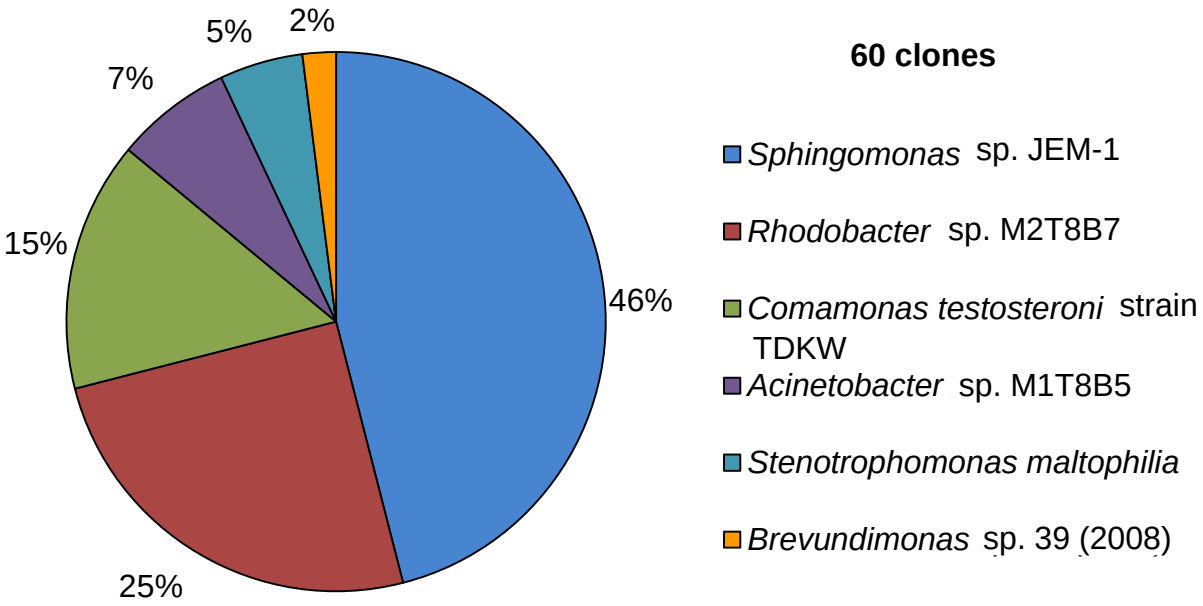

Figure 4.4. Summary of bacteria identified and their relative frequency of observation as determined by cloning of 16S rRNA gene followed by ARDRA.

Table 4.1. DNA sequencing results of clones of PCR products obtained with the 16S rRNA gene primers U341F and 1492R.

Accession number	% Screened clones	ID	Clones	% Identity
AB219359.1	46	*Sphingomonas* sp. JEM-1	25	98.86
			56	98.8
			11	98.79
			18	98.61
			7	98.19
			41	98.11
			52	98.11
			12	97.95
			29	97.92
			36	97.78
			60	96.45*
			1	95.5*
GQ246710.1	25	*Rhodobacter* sp. M2T8B7	37	98.56
			54	98.38
			46	97.98
			32	97.86
			5	97.58

* could be a different bacterial species (based on a 97% threshold for positive ID).

Table 4.1. Continued.

Accession number	% Screened clones	ID	Clones	% Identity
GQ259481.1	15	*Comamonas testosteroni* strain TDKW	48	98.97
			13	98.46
			15	98.46
			24	98.46
			45	98.38
			2	97.98
GQ246681.1	7	*Acinetobacter* sp. M1T8B5	10	98.59
			63	98.5
			20	98.38
			31	97.75
AB294556.1	5	*Stenotrophomonas maltophilia*	43	98.37
			57	98.1
FJ197848.1	2	*Brevundimonas* sp. 39 (2008)	51	99.36

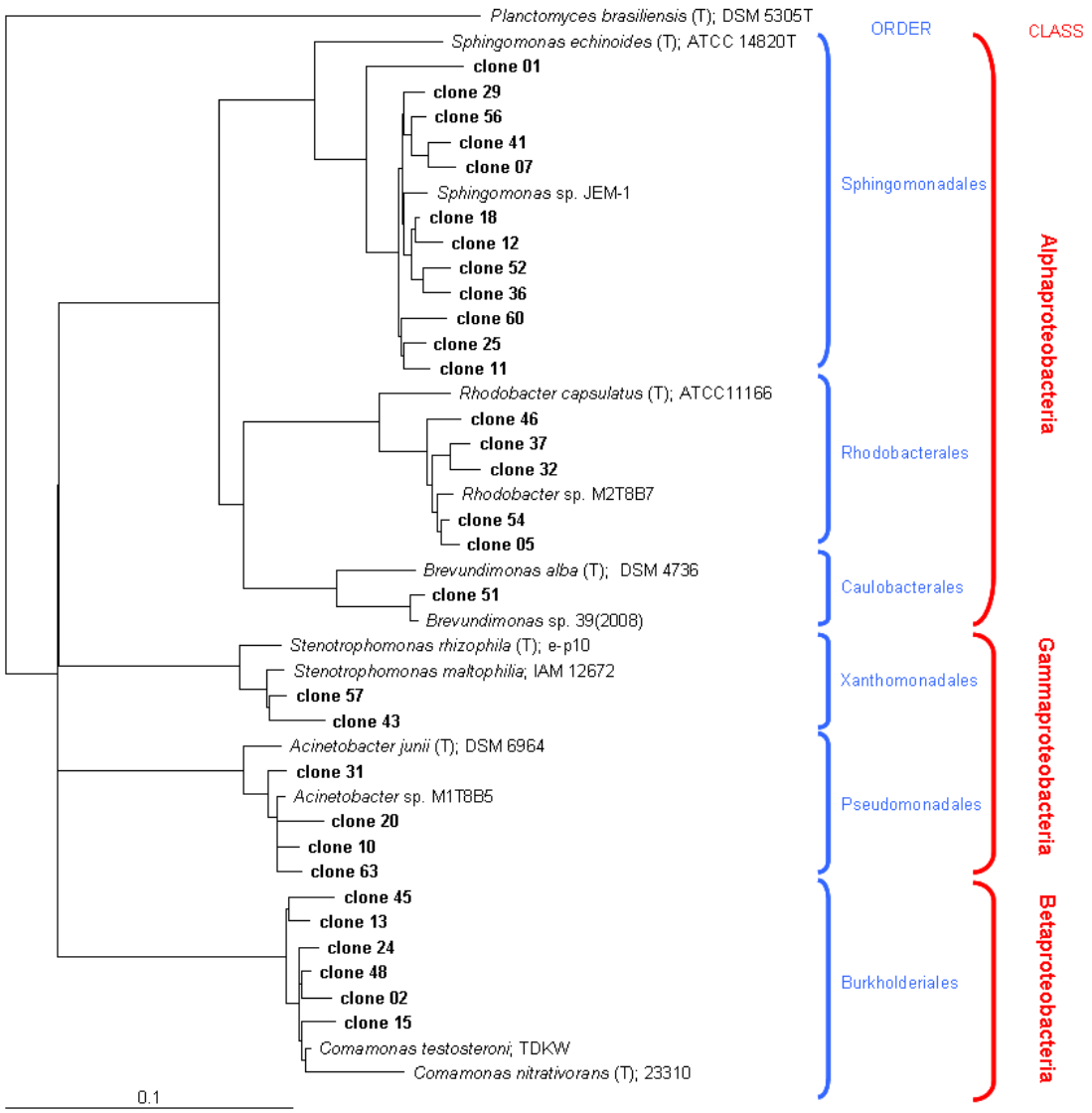

Figure 4.5. Phylogenetic tree constructed by using the neighbor-joining method based on 16S rDNA sequences from a testosterone-degrading culture enriched from swine manure. The sequence of *Planctomyces brasiliensis* was used as an out group for tree calculations. The bar indicates a genetic distance of 0.1.

Testosterone Degradation by Swine Manure–Borne Bacteria

Within enriched cultures under aerobic conditions, testosterone was observed to degrade within 29 h after a lag phase of approximately 22 h (Figure 4.6). No testosterone was observed after 51 h of incubation. In contrast, no degradation of testosterone was observed in sterile controls. The extent of microbial growth was determined at the beginning of the cultivation period and after 24, 48, and 96 h incubation. The number of colony-forming units [CFU] mL^{-1} was ~ 2×10^4 for 0 h, 6×10^4 for 24 h, 1.2×10^6 for 48 h, and 5×10^6 for 96 h, respectively, as determined by TSA plating.

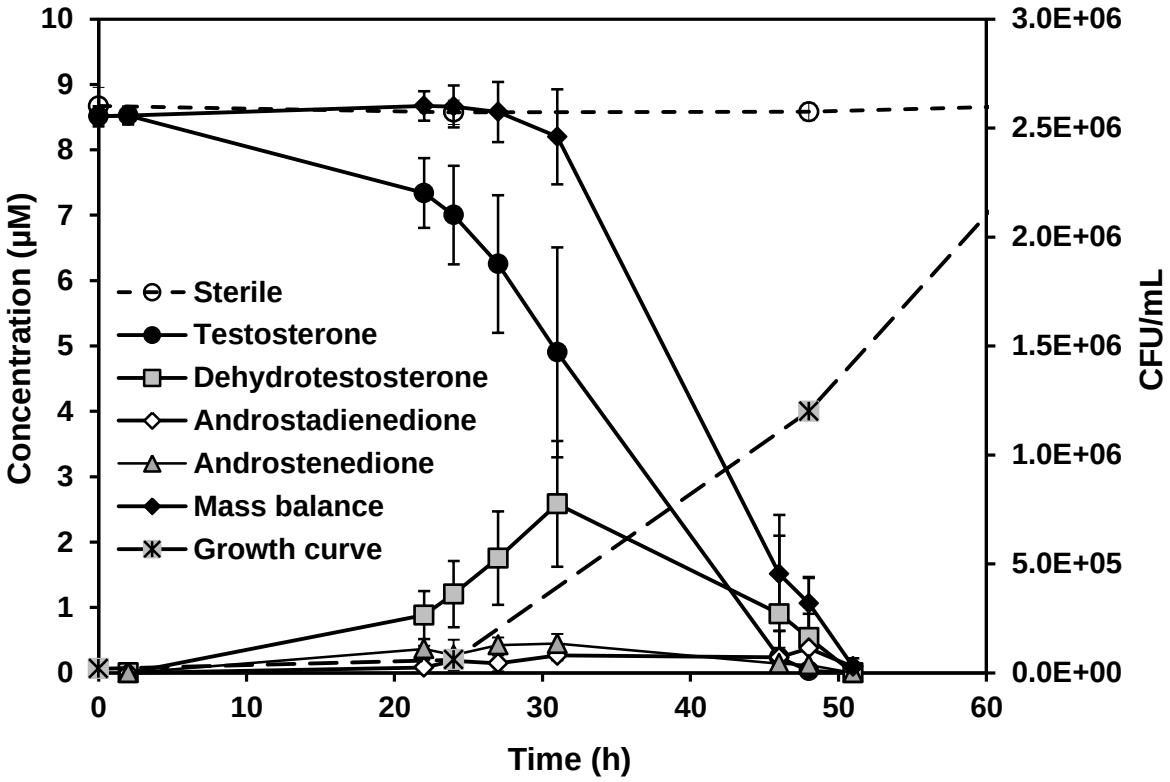

Figure 4.6. Degradation of testosterone and formation of degradation products in enriched cultures by HPLC-DAD. Error bars represent the standard deviation of triplicate samples.

After 48 h incubation, HPLC-DAD and LC/TOF-MS analysis revealed six degradation products of testosterone within the enriched cultures (Figure 4.6, 4.7, and Table 4.2). Based on the proposed formula and molecular mass for the compounds and by considering the known patterns of testosterone degradation (*26*, *33*, *34*), the degradation products were tentatively identified as 9α-hydroxytestosterone (9α-OH-T), ADD, 9α-hydroxyandrostadienedione (9α-OH-ADD), 9α-hydroxyandrostenedione (9α-OH-AD), DHT, and AD. Among the six degradation products, DHT was observed to be the major testosterone degradation product within the first 48 h. For this reason, the degradation product tentatively identified as 9α-OH-AD might instead be a hydroxylated form of DHT. The identities of three of the degradation products (i.e., DHT, ADD, and AD) were confirmed by comparing their retention times (t_R) to the retention times of chemical standards.

The mass balance determined using HPLC-DAD started to decrease substantially after 27 h of incubation (Figure 4.6). This could be due to nondetectable degradation products or the conversion of testosterone to CO_2. In order to verify whether testosterone was completely mineralized to CO_2, a mineralization experiment was performed. Within 2 d, 35−60% of the added ^{14}C-testosterone had been mineralized to $^{14}CO_2$. At the end of an 8 d incubation period, using the microbial enrichment, testosterone mineralization reached a maximum of 49−68%. In contrast, no mineralization was observed in sterilized controls (Figure 4.8). The mineralization data were well described ($R^2 > 0.986$) using first-order kinetics based on the initial rate method for the first 24 h, as described previously (*35*).

First-order rate constants *k* were determined for the removal of ^{14}C-testosterone from the aqueous phase and removal by mineralization. The rate expression

$$\frac{dC}{dt} = kC \qquad (i)$$

where C is the concentration of the ^{14}C-testosterone and t is time (h), was assumed to model the removal of testosterone. The rate expression can be integrated and written in the linear form

$$\ln (C_0\text{-}C(^{14}CO_2) = kt \qquad (ii)$$

where C_0 is the initial concentration of the ^{14}C-testosterone, and $C(^{14}CO_2)$ is the concentration of ^{14}C labeled CO_2. The value of the rate constant *k* for mineralization was estimated by plotting the data in the manner suggested by eq (*ii*) and finding the value of *k* that gave the best fit of a straight line to the data.

A first-order mineralization rate was determined for the removal of testosterone by mineralization to $^{14}CO_2$ with rate constants *k* and half-lives ($t_{1/2}$) ranging from 0.005−0.072 h^{-1}, and 10−143 h, respectively. The recovery of ^{14}C at the end of the experiment, as determined by measuring the radioactivity remaining in the aqueous phase and adding these values to the amount of $^{14}CO_2$ trapped, were >96% and 79−83% for sterilized controls and enriched cultures, respectively. Based on the degradation products observed by LC/TOF-MS and the evidence of mineralization, the proposed conversion pathway of testosterone by enriched swine manure-borne bacteria is shown in Figure 4.9.

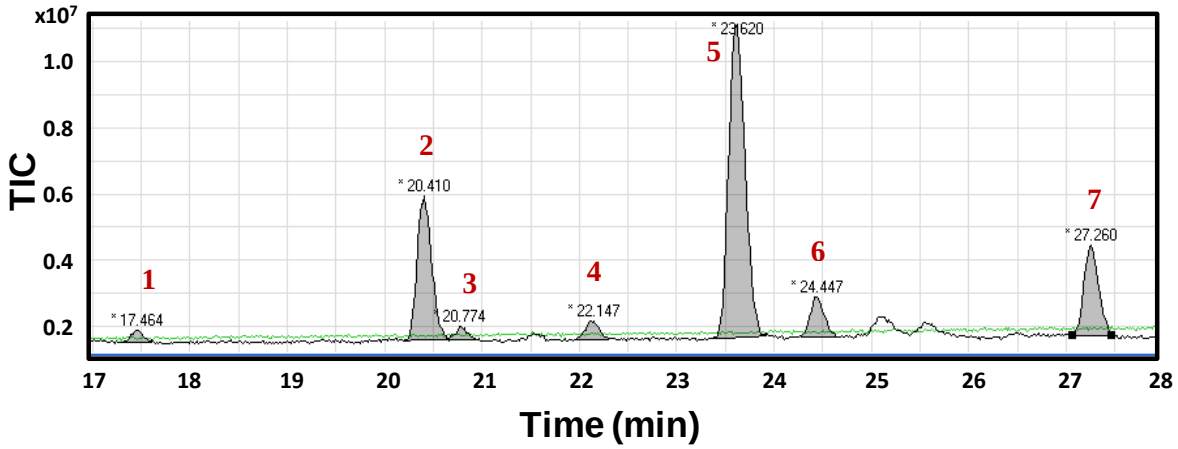

Figure 4.7. Total ion current (TIC) chromatogram obtained by LC/TOF-MS analysis after 48 h of testosterone biodegradation showing the presence of (1) 9α-hydroxytestosterone (9α-OH-T), (2) Androstadienedione (ADD), (3) 9α-hydroxyandrostenedione (9α-OH-ADD), (4) 9α-hydroxyandrostenedione (9α-OH-AD)/ 9α-hydroxydehydrotestosterone (9α-OH-DHT), (5) dehydrotestosterone (DHT), (6) Androstenedione (AD), and (7) testosterone (T). The retention time is shown for each compound above the peak.

Table 4.2. Proposed identities of products from testosterone degradation by a microbial enrichment from swine manure after 48 h of incubation, determined by LC/TOF-MS analysis, including proposed formulas, accurate masses, and associated mass errors.

Peak	t_R (min)	Proposed formula	Proposed ID	Mass	Mass Error (ppm)
1	17.46	$C_{19}H_{28}O_3$	9α-hydroxytestosterone	304.20384	0.48 (MH^+) 0.65 (MNa^+)
2	20.41	$C_{19}H_{24}O_2$	Androstadienedione	284.17763	0.15 (MH^+) 1.07 (MNa^+)
3	20.77	$C_{19}H_{24}O_3$	9α-hydroxyandrostadienedione	300.17254	1.59 (MH^+) 0.09 (MNH_4^+) 0.35 (MNa^+)
4	22.15	$C_{19}H_{26}O_3$	9α-hydroxyandrostenedione (9α-hydroxydehydrotestosterone?)	302.18819	0.6 (MH^+) 0.91 (MNH_4^+) 0.65 (MNa^+)
5	23.62	$C_{19}H_{26}O_2$	Dehydrotestosterone	286.19328	0.57 (MH^+) 1.54 (MNa^+)
6	24.45	$C_{19}H_{26}O_2$	Androstenedione	286.19328	0.05 (MH^+) 1.21 (MNa^+)
7	27.26	$C_{19}H_{28}O_2$	Testosterone	288.20893	1.26 (MH^+) 0.49 (MNa^+)

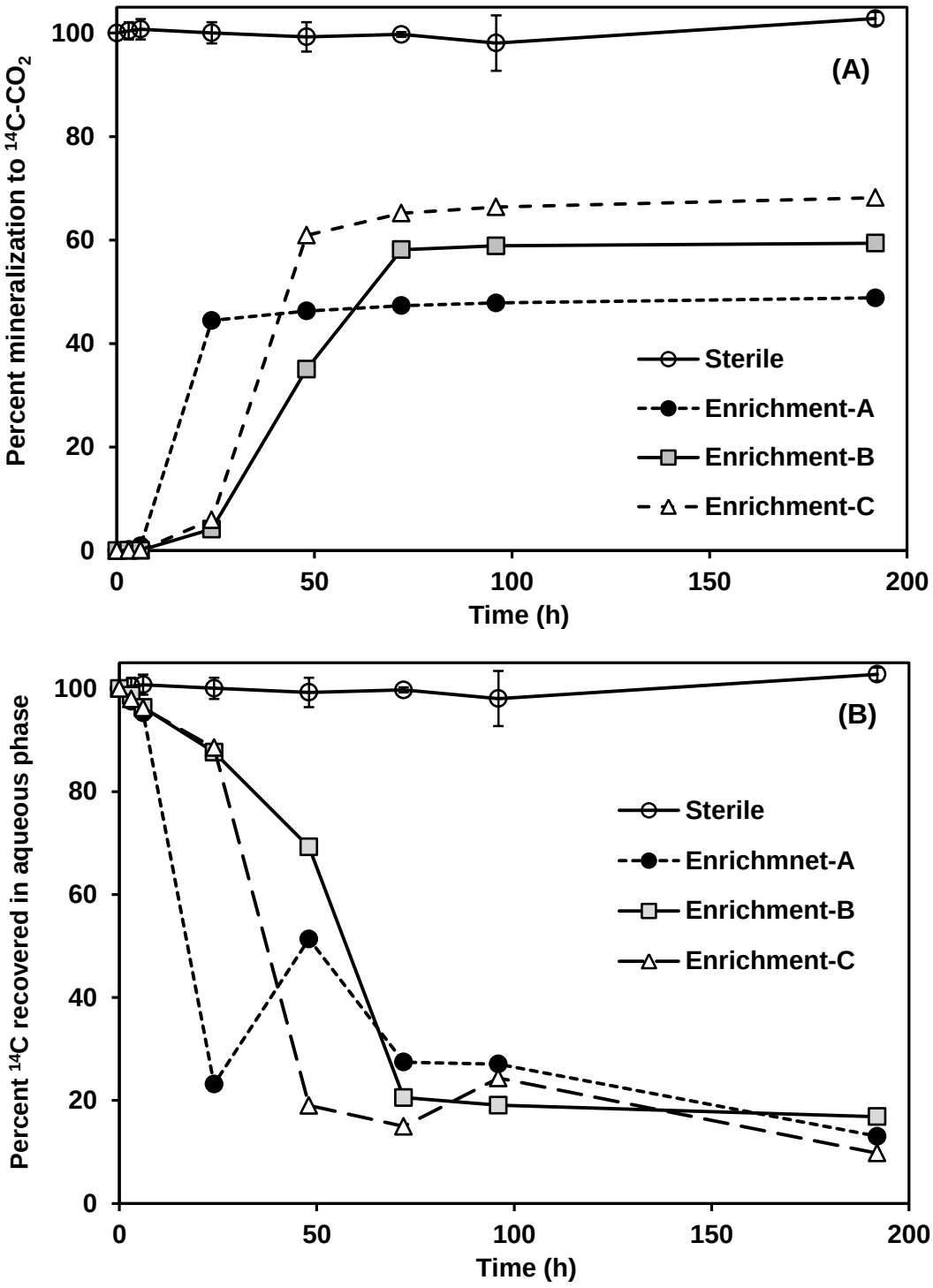

Figure 4.8. Percent of (A) ^{14}C-testosterone mineralization to $^{14}CO_2$ by a microbial culture enriched from swine manure and (B) the ^{14}C recovered in the aqueous phase. Enrichment A, B, and C represent triplicate enrichment samples.

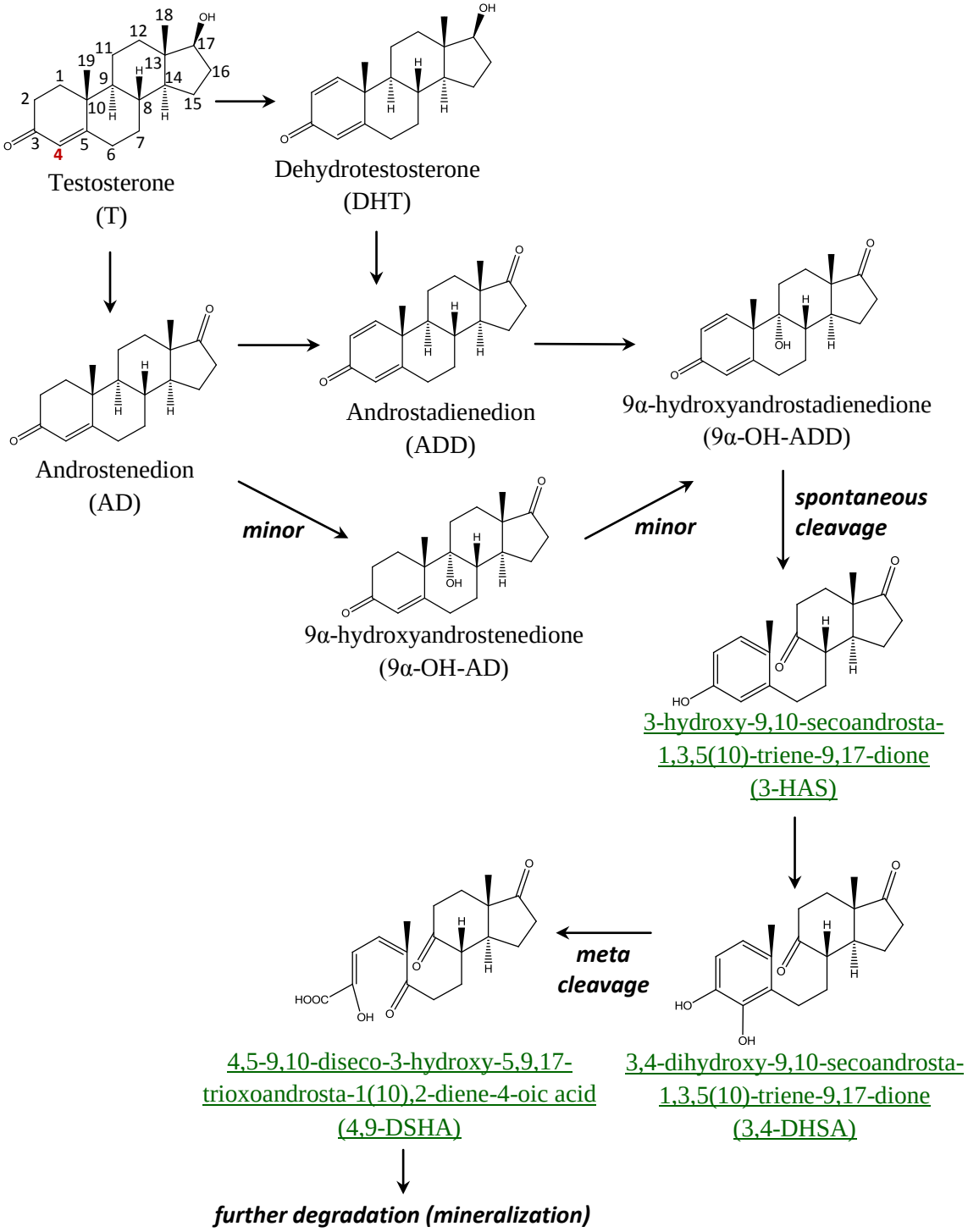

Figure 4.9. Proposed conversion pathway of testosterone by the microbial culture enriched from swine manure. Compounds not observed in this study are indicated in green and underlined (modified from ref (*26*)).

Discussion

Characterization of Microbial Community in Testosterone–Degrading Culture Enriched from Swine Manure

In this study, partial 16S rRNA gene sequences corresponding to *Acinetobacter* sp. M1T8B5, *Brevundimonas* sp. 39, *Comamonas testosteroni* strain TDKW, *Rhodobacter* sp. M2T8B7, *Sphingomonas* sp. JEM-1, and *Stenotrophomonas maltophilia* were found in a testosterone-degrading culture enriched from swine manure. At the phylum level, all of the 16S rRNA gene sequences derived from the swine manure were assigned to Proteobacteria, which is in agreement with previous studies (*36, 37*).

In one study involving aerated pig slurry, 16S rRNA gene sequences (n=48 clones) belonging to the phylum Proteobacteria (i.e., ~17% of Alpha-, 10% of Beta-, and 8% of Gamma- Proteobacteria) were among the most abundant. In addition, among Proteobacteria, some of the predominant bacterial sequences found were associated with the genera *Acinetobacter*, *Comamonas, Sphingomonas,* and *Stenotrophomonas* (*36*). Another study of the microbial community in piggery wastewater sampled from an anaerobic digester reported that the following bacterial lineages were dominant*:* Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Fimicutes with > 93% identity with sequences in the NCBI database (*37*). Similar results were reported in studies that investigated bacterial populations in dairy waste where the majority of the operational taxonomic units (OTUs) from the circulated dairy wastewater (*38*) and aerobic reactor effluent (*39*) were associated with the phylum Proteobacteria. A wide array of diversity in isolates, including *Brevibacterium, Acinetobacter,* and *Comamonas,*

was found in a study investigating the diversity of tetracycline resistance genes among bacteria isolated from a swine lagoon (*40*).

The genus *Acinetobacter, Novosphingobium, Nitrosomonas, and Sphingomonas* belonging to the phylum Proteobacteria were shown elsewhere to degrade several aromatic compounds (*41-46*). A βE2–degrading bacterium isolated form a WWTP in Japan was suggested to be *Novosphingobium* spp. (*43*). An ammonia-oxidizing bacterium *Nitrosomonas europaea* was found to significantly degrade E1, βE2, E3, and EE2 (*44*). Additionally, sequences corresponding to the microorganism *Sphingomonas* sp. JEM-1 found in the present study have also been isolated from soil and activated sludge and described as being capable of utilizing 7-ketocholesterol as a sole carbon and energy source, resulting in its mineralization (*45*).

Some of the microorganisms identified through DNA sequencing in the testosterone-degrading enrichment culture from swine manure are also found in soil and WWTPs, and are known degraders of estrogens and androgens. For example, members of the genera *Comamonas* and *Sphingomonas* are well known for their broad catabolic potential and ability to degrade sterols and steroids such as cholesterol, estrogens, and androgens (*24, 25, 27, 47, 48*). Fourteen phylogenetically diverse βE2-degrading bacteria, distributed among eight different genera (*Aminobacter, Brevundimonas, Escherichia, Flavobacterium, Microbacterium, Nocardioides, Rhodococcus,* and *Sphingomonas*) and three phyla (Proteobacteria, Actinobacteria, and Bacteroidetes), were isolated from activated sludge by Yu et al. (*47*). A further study conducted by Roh et al. (*27*) reported that a βE2-utilizing bacterium, *Sphingomonas* strain KC8, can degrade and further utilize

testosterone as a growth substrate. The maximum specific substrate utilization rates reported were 0.50, 0.37, and 0.17 mg-substrate/mg-protein/d for E1, βE2, and testosterone, respectively. Taken together, it is reasonable to expect some species of the genus *Sphingomonas* and *Comamonas* may possess an enzyme system that degrades testosterone or other similar structural compounds. To the best of our knowledge, *Rhodobacter* has never been described as capable of steroid hormone degradation.

Testosterone Degradation by Swine Manure–Borne Bacteria

The results of testosterone degradation showed that enriched swine manure-borne bacteria can degrade testosterone without a readily available carbon source (e.g., glucose). When inoculated into fresh medium, a lag phase of 22 h was observed (no observed microbial growth) before the onset of testosterone-degradation (Figure 4.6). No detectable testosterone was observed after 29 h in the enriched culture. However, the cell growth was not inhibited at 96 h, suggesting that the enriched microbial culture can grow not only on testosterone but also its degradation products.

The six testosterone degradation products (i.e., 9α-OH-T, ADD, 9α-OH-ADD, 9α-OH-AD/9α-OH-DHT, DHT, and AD) observed in this study have also previously been reported as intermediates in the testosterone degradation pathway. A study conducted by Jacobsen et al. (*15*) reported that microorganisms in swine manure were able to convert testosterone to AD, 5α-androstan-3,17-dione (5α-AD), and ADD; however, the authors did not attempt to enrich the testosterone degrading culture. The same three degradation products were also observed in unmanured agricultural soil (*13*).

The conversion of testosterone to DHT (major degradation product), AD, and ADD (Figure 4.5) by the enriched culture was also observed in Chapter 3 in which the swine-manure borne bacteria had been pre-enriched or grown in TSB indicating a similar biodegradation pathway for the two systems (*30*). A degradation pathway of testosterone by *Comamonas testosterone* has previously been proposed (*25, 49*). The bacterial testosterone degradation process was reported to be initiated by (i) 17α-dehydrogenation to AD, which then undergoes Δ^1-dehydrogenation to ADD, or (ii) Δ^1-dehydrogenation to DHT, which then undergoes 17β-dehydrogenation to ADD (*19*). Kim et al. (*34*) reported degradation of testosterone to AD and 9α-OH-T, followed by degradation to 9α-OH-AD and ADD by *Rhodococcus equi* ATCC 14887. An EE2-degrading bacterium isolated from a WWTP, *Sphingobacterium* sp. JCR5, was found capable of growing on EE2 as sole source of carbon and energy, and metabolized up to 87% of the substrate added (30 mg L^{-1}) within 10 d at 30 degrees C. In addition to EE2, the strain could be cultivated on steroidal estrogens like E1, E2, E3 and mestranol (MeEE2). Mass spectrum analysis of the EE2 degradation showed that in the first step it is oxygenized to E1, 2-hydroxy-2,4-dienevaleric acid and 2-hydroxy-2,4-diene-1,6-dioic acid, which are the main catabolic intermediates. The former was analogous to the pathway of a previously reported testosterone-degrading bacterium *Comamonas testosteroni* TA441 and the latter is a metabolite with a different cleavage position of 3-hydroxy-4,5-9,10-disecoestrane-1(10),2-diene-5,9,17-trione-4-oic acid from the former (*50*).

Testosterone Mineralization

The oxidation of ^{14}C-testosterone to $^{14}CO_2$ requires ring cleavage and thus complete inactivation of testosterone. In enriched cultures, the mineralization observed was > 48 % within 8 d incubations. The high percentage of ^{14}C-testosterone converted to $^{14}CO_2$ suggests testosterone served as an energy source. Although the trend in testosterone mineralization was similar in all replicates, mineralization rates varied among them, probably due to biological variability. In contrast, the sterilized controls showed that no testosterone was converted to $^{14}CO_2$ (Figure 4.8), clearly indicating that the microbial enrichment was responsible for testosterone mineralization. These findings were similar to Jacobsen et al. (*15*), who reported 47% and 36% of testosterone was mineralized to $^{14}CO_2$ in manured and unmanured treatments, respectively, following a 6 d incubation under aerobic conditions. Another study also observed that approximately 50% of the applied ^{14}C-testosterone in agricultural soil was mineralized to $^{14}CO_2$ after 120 h (*13*). The mineralization of ^{14}C-testosterone, E1, and βE2 in breeder and broiler litters under different conditions was determined by Hemmings and Hartel (*51*), and they reported that after 23 wks, an average of 27% of the ^{14}C-testosterone applied to breeder litter was mineralized to $^{14}CO_2$ at 25°C.

The mineralization of ^{14}C-testosterone in our study followed pseudo first-order reaction kinetics. Layton et al. (*14*) reported that mineralization of testosterone, βE2, and EE2 by biosolids from WWTP resulted in 55-65% conversion of ^{14}C-testosterone to $^{14}CO_2$ under aerobic conditions within 1 d. First-order mineralization reaction kinetics

with *k* values of 0.0152±0.0021 min^{-1} and 0.0042±0.0002 min^{-1} were reported for ^{14}C-testosterone and ^{14}C-βE2, respectively. It was unclear why larger amounts of ^{14}C-testosterone, rather than ^{14}C-βE2 were mineralized to $^{14}CO_2$ in the industrial biosolids (*14*). The *k* values determined in this study (0.005−0.072 h^{-1}) are similar to the observations from Fan et al. (*35*), who found the first-order mineralization rate constant for testosterone in native soil to be 0.012 h^{-1}.

The percent of ^{14}C-testosterone mineralized to $^{14}CO_2$ was inhibited after 72 h of incubation. The total recovery of ^{14}C at the end of the experiment was between 79 and 83 %. We believe that inadequate trapping of $^{14}CO_2$ during the experiments is the most likely reason for the lack of a full recovery of ^{14}C. Taken together, these results suggest that testosterone can be degraded by swine manure-borne bacteria, in the absence of a readily available carbon source, and be further mineralized to $^{14}CO_2$.

Environmental Significance

In this study, the microorganisms in a testosterone-degrading culture originally inoculated with swine manure were identified using 16S rRNA gene-based methods, and the degradation pathway was examined. One important finding of this study is that swine manure-borne bacteria are capable of mineralizing testosterone to CO_2 under aerobic conditions. This suggests that aerobic biodegradation of testosterone can be an environmentally important mechanism for removing hormones such as testosterone from

aerated manure treatment systems. Relatively little information is available on biodegradation of hormones in aerated lagoons, or other manure treatment systems. A recent study investigated the removal of estrogens (i.e., E1, 17α-estradiol (αE2), and βE2) and estrogenic activity in dairy shed effluent within two systems, including anaerobic and aerobic stages (*52*). In both systems, the greatest reduction in estrogenic activity occurred in aerobic ponds, suggesting that active aeration is likely to provide the most cost-effective and acceptable solution to dairy farms. Our previous study (Chapter 3) support these findings since we also observed that testosterone was degraded significantly faster under aerobic ($t_{1/2}$ ≈4 h) than anaerobic ($t_{1/2}$ ≈27 h) conditions (*30*). The use of aerated lagoons or aerated caps to provide an oxygenated zone on the surface layer of anaerobic lagoons would be a potentially viable option for more effectively treating hormones in manure wastewater treatment systems. Moreover, the findings in this study might also be useful in optimizing methods of manure land application (e.g., surface application vs. injection) for optimal hormone removal.

In this study, six DNA sequences of bacteria from the Proteobacteria phylum were identified in the testosterone-degrading enriched culture suggesting that Proteobacteria may play an important environmental role in the degradation of testosterone and other similar structural compounds.

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CHAPTER 5

SUMMARY

The research performed in this dissertation was designed to assess the potential for surface runoff and to elucidate biodegradation pathways of steroid hormones from human and animal wastes, respectively. Four important aspects for the comprehensive understanding of steroid sex hormones behavior under environmentally relevant conditions were investigated: (I) runoff, to reveal the potential for surface transport of hormones after biosolids application (Chapter 2); (II) biodegradation, to gain fundamental knowledge of the impact of swine manure-borne bacteria on testosterone, 17β-estradiol, and progesterone degradation under various environmentally relevant conditions (Chapters 3 and 4); (III) microbial enrichment, to enrich manure-borne bacteria capable of using testosterone as their sole carbon source under aerobic conditions (Chapter 4); (IV) mass spectrometry, to reveal degradation pathways of testosterone (Chapters 3 and 4). The objectives listed in the Introduction (Chapter 1) were addressed in three separate but interrelated research projects, which are summarized in the three main chapters of this dissertation. This chapter summarizes the key findings and implications, discusses research limitations, and suggests future research needs.

The potential endocrine-disrupting effects of steroid sex hormones in biosolids used as agricultural fertilizers have become an environmental concern. A field-scale

study was conducted to assess the potential for runoff of seventeen different hormones, including androgens and estrogens from an agricultural field applied with biosolids at an agronomic rate and the major mechanisms controlling hormone transport during simulated rainfall events (Chapter 2). Samples were isolated by solid phase extraction (water samples) and pressurized solvent extraction (solid samples), derivatized, and analyzed by gas chromatography-tandem mass spectrometry. Few hormones were observed at low concentrations in whole water runoff samples prior to biosolids application (estrone <0.8 to 2.23 ng L^{-1} and androstenedione <0.8 to 1.54 ng L^{-1}). In contrast, substantially higher concentrations of multiple estrogens (<0.8 to 25.02 ng L^{-1}), androgens (<2 to 216.14 ng L^{-1}), and progesterone (17.4 to 98.9 ng L^{-1}) were observed in runoff samples taken 1, 8 and 35 days after biosolids application. Androgen runoff concentrations declined from day 1 to day 35 after biosolids application but the concentrations, in particular for androstenedione, observed 35 days after biosolids application were still higher than concentrations known to affect the endocrine system of aquatic organisms. Overall, these results indicate that rainfall can mobilize steroid hormones from biosolids amended agricultural fields, and they could be transported directly to surface waters via runoff. However, the data represent a worst case scenario and the concentrations reported would likely be diluted by the receiving waters and which must be considered when evaluating this dataset.

Based on a good correlation between rainfall amount and hormone mass fluxes, this study also suggests that intense rainfall promotes runoff rather than dilution of hormones. Hormones in runoff were primarily present in the aqueous phase (<0.7um GF

filter), and to a smaller extent in the particulate phase. The mean percentage of particle-bound hormones followed the order estrogens > androgens >> progesterone, which is most likely due to their physical-chemical properties. Additionally, aquatic colloids may also play an important role in controlling the mobility of hormones. However, current understanding of the mechanisms controlling hormone transport is still limited. The work described herein did not investigate physical and chemical properties, including size and types of colloids in the suspension. The process of separating dissolved, colloidal, and particulate phases is challenging. Various methods have been used to determine the distribution of steroid hormones between dissolved and colloidal phases, and they vary in terms of accuracy, time efficiency, cost, and ease of use. While transport of hormones in the environment has been investigated, there are still a lot of mechanistic details, such as how the physical-chemical properties of colloids influence hormone mobility, which are not fully understood and require further investigation.

Land application of manure may contribute EDCs such as steroid hormones to the environment. Little attention has been paid to the potential for degradation of steroid hormones by manure-borne bacteria and their degradation kinetics and pathways. In a laboratory study, the potential for biodegradation of testosterone, 17β-estradiol and progesterone by swine (*Sus scrofa*) manure-borne bacteria was examined (Chapter 3). In addition, the impact of temperature (22 and 37°C), pH (6, 7, and 7.5), glucose amendments (0, 3, and 22 mmol L^{-1}), and presence of oxygen on testosterone degradation kinetics was determined. Testosterone, 17β-estradiol and progesterone were biodegraded within 25 h of reaction initiation under aerobic conditions. The degradation of

testosterone followed pseudo first-order and zero-order reaction kinetics under aerobic and anaerobic conditions, respectively, in tryptic soy broth (TSB) pre-enriched systems. The half-life ($t_{1/2}$) for the degradation of testosterone under anaerobic conditions was six times longer than aerobic conditions. Testosterone degradation was found to significantly increase (~ 17%) when incubated at 37°C vs. 22°C. The impact of pH ($t_{1/2}$ ranged from 4.4-4.9 h) and glucose amendments ($t_{1/2}$ ranged from 4.6-5.1 h) on the testosterone degradation rate were found to be small. Testosterone was transformed to dehydrotestosterone (DHT) (major degradation product), androstenedione (AD), and androstadienedione (ADD) under aerobic conditions as revealed by liquid chromatography-time-of-flight mass spectrometry. These results indicate that testosterone is rapidly degraded by manure-borne bacteria under a wide range of environmentally relevant conditions. However, the formed degradation products are still of potential concern due to their endocrine disrupting potential.

Several species of bacteria have been described as being capable of utilizing testosterone as sole carbon and energy sources. However, most testosterone-degrading bacteria were isolated from wastewater treatment plants (WWTPs), while little is known about manure-borne bacteria responsible for testosterone degradation and their degradation pathways. Thus, a further study was conducted to enrich manure-borne bacteria capable of testosterone degradation and to elucidate their testosterone degradation/mineralization pathways (Chapter 4). Six DNA sequences of bacteria from the Proteobacteria phylum widely distributed among six different genera - *Acinetobacter, Brevundimonas, Comamonas, Sphingomonas, Stenotrophomonas,* and *Rhodobacter* were

identified in a testosterone-degrading enriched culture suggesting that Proteobacteria may play an important environmental role in the degradation of testosterone and other similar structural compounds. Six degradation products of testosterone were identified as 9α-hydroxytestosterone (9α-OH-T), ADD, 9α-hydroxyandrostadienedione (9α-OH-ADD), 9α-hydroxyandrostenedione (9α-OH-AD), DHT, and AD based on the proposed formula and molecular mass of compounds and by consideration of the known patterns of testosterone degradation. The six enriched swine manure-borne bacteria identified in this study may also have the ability to degrade other hormones or organic compounds with a similar chemical structure. One important observation in this study is that more than 48% of the ^{14}C-testosterone had been mineralized to $^{14}CO_2$ within 8 d of incubation. The high percentage of ^{14}C-testosterone converted to $^{14}CO_2$ suggests that testosterone served as an energy source. The mineralization of ^{14}C-testosterone followed pseudo first-order reaction kinetics in the enriched cultures with $t_{1/2}$ ranging from 10−143 h. Based on these findings, we suggest that the use of aerated lagoons or aerated caps to provide an oxygenated zone on the surface layer of anaerobic lagoons would be a potentially viable option for more effectively treating hormones in manure wastewater treatment systems.

This research contributes to the further understanding of the transport and degradation of hormones in the environment. The data provided herein can also would help to develop effective best management practices for manure and biosolids. There are, however, still gaps in our understanding. Those gaps are outlined in the following paragraphs.

Substantial concentrations of hormones were observed in runoff after biosolids application, and the differing patterns observed for each of the hormones during the experimental period is most likely associated with differences in their physical-chemical properties. However, we do not yet fully understand the mechanisms causing these differences in behavior and their implications in terms of environmental risk. Further research is also required to evaluate the potential for hormone transport from biosolids-amended soils with different soil composition, means of biosolids application, and climate conditions.

Researchers are still focusing on only a small proportion of the hormones in use, mostly estrogens. There are many more classes of hormones in use; therefore, it would be worthwhile to develop an understanding of the fate and effects of other important classes in the environment. Additionally, information on the formation of hormone degradation products and ecotoxicity data on hormone metabolites as well as mixtures of hormones are sparse. Although hormone removal is possible before or after land application, some of these hormones or their degradation products are still likely to enter the aquatic environment. In some instances, these degradation products may also be more ecotoxic, more persistent, and more mobile than the parent compound. Improved understanding of the transformation of hormones would help identify the circumstances under which metabolites deserve more detailed attention during risk assessment.

Although the half-lives of testosterone degradation/mineralization observed in this study are short, the abundance and activity of bacteria in the environment is often unknown. Additionally, the relatively low concentration of hormones relative to other

pollutions in the environment may be insufficient to induce enzymes that are capable of degrading hormones. Thus, it is important to better understand the microbial communities in the environment that are capable of hormone degradation and the factors that influence their activity.

Taken together, to better assess the real contribution of biosolids and livestock manure on hormone inputs to the environment, further studies are needed to better understand how application methods and timing of application influence the contribution of steroid hormones to the aquatic environment. Finally, we believe that more research should focus on development of best management practices for optimal reduction of steroid hormones at the source (e.g., manure lagoon or WWTP) prior to land application rather than after waste application to agricultural fields.

APPENDIX

Supplemental Information for Chapter 2

Soild-Phase Extraction (SPE)

Water samples were poured into stainless-steel extraction tubes fitted with a multigrade GFF positioned over a reverse phase octyldecyl surface-modified-silica embedded glass-fiber filter disk (C_{18} disk). The samples were passed through the combined GFF/C_{18} disk under pressure, as needed. Following compound isolation, the GFF/C_{18} disks were rinsed with 10-mL of 25% MeOH in reagent water to remove polar compounds that interfere with gas chromatographic-tandem mass spectrometry (GC-MS/MS) analysis. Nitrogen gas (N_2) was passed through the GFF/C_{18} disk to remove residual water, and the compounds were eluted with two 20-mL additions of MeOH. The eluents were evaporated to dryness with a gentle N_2 stream while the tubes were submerged in a bath at 16°C. The extracts were reconstituted in 2-mL of a 5% MeOH in dichloromethane solution (5% MeOH/DCM) and allowed to sit for 30 minutes. To remove interferences during the GC analysis, the extracts were cleaned up using 1-g Florisil SPE cartridge and eluted with an addition 20-mL of 5% MeOH/DCM. The clean up eluents were then concentrated to approximately 2 mL under N_2 and transferred quantitatively to a 5-mL reaction vial with 5% MeOH/DCM rinses, and evaporated to dryness. The samples were derivatized by adding 200 μL N-methyl-N-(trimethylsilyl)-

trifluoroacetamide (MSTFA) activated with 2-(trimethylsilyl)ethanethiol, and heated for 1 h at 65°C before injection to the GC-MS/MS system.

Pressurized Solvent Extraction (PSE)

Solid samples were extracted using PSE with 50% water in isopropyl alcohol (50% water/IPA) at 120ºC and 20% water/IPA at 200ºC for 3 static extractions (40 min total) at each temperature at a pressure of 2000 psi. The extracts were cleaned up using Oasis HLB extraction cartridge (20 mL/1g 60 µm, Waters Inc.) and rinsed with 20-mL of elution solvent (5% MeOH/DCM). 50-mL phosphate buffer solution was added to each ASE collection vial twice, and each fraction was added to the reservoir. Once all the solution has passed through the cartridge, the cartridge was dried for 5 min under N_2. To ensure all the compounds have been removed from the glass surfaces, a 5-mL of elution solvent was added to each ASE collection vial. Florisil SPE cartridge (15 mL/2g, Biotage) was placed on the vacuum manifold for each sample, and approximately 2.5 g of burned sodium sulfate was added to each cartridge. The cartridges were rinsed with 25-mL acetone under N_2 and allowed a maximum of 5 min for the N_2 to remove any residual solvent. A 5-mL elution solvent from each ASE collection vial was added to each corresponding reservoir, and another 20-mL elution solvent was added after the solution passed through the SPE chain. The clean up eluents were then concentrated and derivatized as the method presents above.

Table A1. Hormones data for runoff samples from rainfall-runoff simulations on a biosolids-applied field near Roggen, Colorado, 2008. Municipal biosolids were applied to this field April 29, 2008. D-5 = samples obtained 5 d before biosolids application; D1 = samples obtained 1 d after biosolids application.

compound name							bisphenol A	diethylstibestrol	cis-androsterone	epitestosterone	17-alpha-estradiol	dihydrotestosterone	androstenedione	estrone	17-beta-estradiol	testosterone
sample	**date (m-d-y)**	**plot**	**sample aliquot**	**start time (min:sec)**	**end time (min:sec)**	**Ave. time (min:sec)**										
D-5	4/24/08	1	early	05:40	10:56	08:18	1367.88	<RL	<RL	<RL	<RL	<RL	1.54	1.62	<RL	<RL
D-5	4/24/08	1	middle	13:00	25:50	19:25	1349.95	<RL	<RL	<RL	<RL	<RL	<RL	2.23	<RL	<RL
D-5	4/24/08	1	late	37:09	40:40	38:54	176.94	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
D-5	4/24/08	2	early	09:37	17:50	13:44	639.85	<RL	<RL	<4	<RL	<4	<RL	0.95	<RL	<RL
D-5	4/24/08	2	middle	23:29	29:10	26:19	754.4	<RL	<RL	<RL	<RL	<RL	<RL	1.27	<RL	<RL
D-5	4/24/08	2	late	37:22	42:00	39:41	604.15	<RL	<RL	<RL	<RL	<RL	<RL	1.15	<RL	<RL
D-5	4/24/08	3	early	08:32	21:50	15:11	695.18	<RL	<RL	<4	<RL	<RL	<RL	1.35	<RL	<RL
D-5	4/24/08	3	middle	27:15	35:00	31:07	911.96	<RL	<RL	<RL	<RL	<RL	<RL	<0.8	<RL	<RL
D-5	4/24/08	3	late	40:06	47:30	43:48	1131.38	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
D1	4/30/08	1	early	16:09	29:15	22:42	305.87	<RL	83.89	9.37	1.35	19.47	141.4	10.7	1.44	12.34
D1	4/30/08	1	middle	33:53	43:50	38:52	224.43	<RL	95.32	7.91	1.08	32.82	205.62	11.33	1.72	18.54
D1	4/30/08	1	late	45:46	52:21	48:34	233.55	<RL	85.46	9.29	1.13	18.41	144.36	11.85	1.46	10.77
D1	4/30/08	2	early	11:33	30:00	20:46	209.06	<RL	26.21	<RL	<RL	3.28	20.8	3.37	<RL	1.56
D1	4/30/08	2	middle	35:44	43:20	39:32	173.53	<RL	100.04	14.22	1.36	17.98	156.82	11.04	1.27	12.14
D1	4/30/08	2	late	46:28	50:53	48:41	179.75	<RL	174.77	16.95	1.88	40.76	216.14	13.81	2.37	20.78
D1	4/30/08	3	early	16:29	29:18	22:53	332.48	<RL	19.84	<RL	<RL	<RL	17.26	3.11	<RL	1.44
D1	4/30/08	3	middle	36:00	50:00	43:00	229.06	<RL	52.13	4.52	<RL	8.61	79.82	6.11	<RL	5.19
D1	4/30/08	3	late	53:48	64:15	59:01	184.79	<RL	100.4	13.85	1.64	17.78	200.35	15.3	1.09	10.85

All concentrations are in nanograms per liter. <RL, less than reporting level.

Table A1.
continued.

compound name							equilin	11-ketotestosterone	norethindrone	mestanol	equilenin	ethinyl estradiol	estriol	progesterone	coprostanol	cholesterol
sample	**date (m-d-y)**	**plot**	**sample aliquot**	**start time (min:sec)**	**end time (min:sec)**	**Ave. time (min:sec)**										
D-5	4/24/08	1	early	05:40	10:56	08:18	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	41423.7
D-5	4/24/08	1	middle	13:00	25:50	19:25	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	34931.89
D-5	4/24/08	1	late	37:09	40:40	38:54	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	5550.12
D-5	4/24/08	2	early	09:37	17:50	13:44	<RL	<RL	<RL	<RL	<RL	<RL	<2	<RL	<RL	11000.47
D-5	4/24/08	2	middle	23:29	29:10	26:19	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	22159.79
D-5	4/24/08	2	late	37:22	42:00	39:41	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	20780.86
D-5	4/24/08	3	early	08:32	21:50	15:11	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	30743
D-5	4/24/08	3	middle	27:15	35:00	31:07	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	19865.79
D-5	4/24/08	3	late	40:06	47:30	43:48	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	15989.76
D1	4/30/08	1	early	16:09	29:15	22:42	<RL	4.28	<RL	<RL	2.7	<RL	<RL	53.75	298792.82	210113.48
D1	4/30/08	1	middle	33:53	43:50	38:52	<RL	4.21	<RL	<RL	4.53	<RL	<RL	68.28	398805.08	274933.84
D1	4/30/08	1	late	45:46	52:21	48:34	<RL	6.05	<RL	<RL	<RL	<RL	2.51	63.11	88843.56	58511.78
D1	4/30/08	2	early	11:33	30:00	20:46	<RL	<RL	<RL	<RL	<RL	<RL	<RL	23.69	236877.49	174040.01
D1	4/30/08	2	middle	35:44	43:20	39:32	<RL	6.2	<RL	<RL	0.83	<RL	2.3	74.1	36435.51	35277.07
D1	4/30/08	2	late	46:28	50:53	48:41	<RL	7.46	<RL	<RL	2.8	<RL	3.63	98.9	115779.51	95598.86
D1	4/30/08	3	early	16:29	29:18	22:53	<RL	<RL	<RL	<RL	<RL	<RL	<RL	17.4	17278.38	26274.39
D1	4/30/08	3	middle	36:00	50:00	43:00	<RL	3.49	<RL	<RL	0.67	<RL	<RL	43.47	116800.53	85797.55
D1	4/30/08	3	late	53:48	64:15	59:01	<RL	7.4	<RL	<RL	1.5	<RL	3.09	77.79	69433.93	46955.76

All concentrations are in nanograms per liter. <RL, less than reporting level.

Table A2. Hormones data for runoff samples from rainfall-runoff simulations on a biosolids-applied field near Roggen, Colorado, 2008. Municipal biosolids were applied to this field April 29, 2008. D8 = samples obtained 8 d after biosolids application; D35 = samples obtained 35 d after biosolids application.

compound name							bisphenol A	diethylstibestrol	cis-androsterone	epitestosterone	17-alpha-estradiol	dihydrotestosterone	androstenedione	estrone	17-beta-estradiol	testosterone
sample	**date (m-d-y)**	**plot**	**sample aliquot**	**start time (min:sec)**	**end time (min:sec)**	**Ave. time (min:sec)**										
D8	5/08/08	1	early	04:13	09:00	06:36	452.15	<RL	27.75	<RL	<RL	7	33.7	9.6	2.53	2.65
D8	5/08/08	1	middle	12:40	15:25	14:02	493.08	<0.8	49.56	<4	<1.16	11.84	40.76	13.75	2.29	4.75
D8	5/08/08	1	late	19:19	21:33	20:26	466.84	<RL	55.17	<4	1.71	14.41	47.15	25.02	5.9	4.98
D8	5/08/08	2	early	10:35	15:15	12:55	333.46	<RL	36.03	<4	1.35	7.41	37.09	12.02	2.89	3.28
D8	5/08/08	2	middle	17:38	19:54	18:46	395.19	<RL	75.07	<RL	2.16	15.99	59.61	21.14	5.16	5.71
D8	5/08/08	2	late	25:28	27:02	26:15	433.92	<0.8	46.27	<4.75	2.25	10.38	47.09	17.35	4.4	4.92
D8	5/08/08	3	early	08:12	17:10	12:41	547.89	<RL	8.48	<RL	<RL	1.27	7.06	3.32	0.72	0.98
D8	5/08/08	3	middle	22:10	25:39	23:54	586.83	<RL	82.47	<5.72	51.86	17.77	78.46	24.33	5.87	9.44
D8	5/08/08	3	late	27:45	30:17	29:01	473.51	<0.8	58.16	<5.9	<1.95	11.32	70.8	17.57	3.4	7.31
D35	6/03/08	1	early	10:40	25:10	17:55	2818.42	<RL	4.74	<RL	<0.8	<RL	24.79	6.67	<0.8	<2.18
D35	6/03/08	1	middle	27:34	35:29	31:31	2601.06	<RL	5.75	<RL	<RL	<RL	16.83	7.87	<0.8	<1.77
D35	6/03/08	1	late	38:24	42:30	40:27	2261.65	<RL	20.08	5.95	1.59	<4.82	25.29	14.37	<1.29	2.45
D35	6/03/08	2	early	10:05	24:45	17:25	2229.44	<RL	6.78	<RL	<0.8	<4	22.53	4.99	<RL	1.79
D35	6/03/08	2	middle	28:34	33:55	31:14	2901.44	<RL	28.69	6.69	<1.2	6.42	38.25	13.79	0.99	4.89
D35	6/03/08	2	late	36:45	40:11	38:28	2193.92	<RL	17	5.39	2.72	<4	34.34	16.2	1.13	3.05
D35	6/03/08	4	early	11:34	31:20	21:27	2635.82	<RL	60.01	<RL	<RL	9.58	34.14	7.41	1.38	4.12
D35	6/03/08	4	middle	35:07	47:30	41:18	2087.93	<RL	17.85	<RL	1.24	4.12	20.94	8.27	0.73	2.11
D35	6/03/08	4	late	53:10	66:12	59:41	2200.41	<RL	14.94	<RL	1.87	<4.11	17.91	13.82	<0.8	2.52
D35	6/03/08	5	early	11:00	19:36	15:18	3272.36	<RL	303.59	<RL	4.52	66.49	313.62	24.09	7.21	32.71
D35	6/03/08	5	middle	21:37	24:44	23:11	4497.43	<RL	332.27	<21.35	4.14	68.96	617.8	20.77	5.53	38.86
D35	6/03/08	5	late	27:26	29:39	28:33	2576.52	<RL	323.72	<RL	4.11	81.23	491.28	18.94	3.85	38.62

All concentrations are in nanograms per liter. <RL, less than reporting level.

Table A2. continued.

compound name							equilin	11-ketotestosterone	norethindrone	mestanol	equilenin	ethinyl estradiol	estriol	progesterone	coprostanol	cholesterol
sample	**date (m-d-y)**	**plot**	**sample aliquot**	**start time (min:sec)**	**end time (min:sec)**	**Ave. time (min:sec)**										
D8	5/08/08	1	early	04:13	09:00	06:36	<6.65	<RL	<RL	<RL	<RL	<RL	<2	<8	85601.29	92392.67
D8	5/08/08	1	middle	12:40	15:25	14:02	<5.72	<RL	<RL	<RL	<RL	<RL	<2	<8	49306.93	52892.14
D8	5/08/08	1	late	19:19	21:33	20:26	<9.05	<RL	<RL	<RL	<3.95	<RL	<RL	<8	70329.22	73170.62
D8	5/08/08	2	early	10:35	15:15	12:55	<4.37	<RL	<RL	<RL	<2.55	<RL	<RL	<8	29736.05	35928.52
D8	5/08/08	2	middle	17:38	19:54	18:46	<6.96	<RL	<RL	<RL	<3.67	<RL	<2	<8	80389.93	83640.1
D8	5/08/08	2	late	25:28	27:02	26:15	<8.25	<RL	<RL	<RL	<RL	<0.8	<RL	<8	56680.13	63598.97
D8	5/08/08	3	early	08:12	17:10	12:41	<RL	<RL	<RL	<RL	<RL	<RL	<RL	15.61	27415.46	31908.96
D8	5/08/08	3	middle	22:10	25:39	23:54	<16.73	<RL	<RL	<RL	4.8	<RL	2.86	<8	91662.17	94543.06
D8	5/08/08	3	late	27:45	30:17	29:01	<7.4	<RL	<RL	<RL	3.52	<RL	2.47	<8	70557.32	70041.32
D35	6/03/08	1	early	10:40	25:10	17:55	<4	<RL	<RL	<RL	<RL	<RL	<RL	27.57	41893.46	36750.56
D35	6/03/08	1	middle	27:34	35:29	31:31	<4	<RL	<RL	<RL	<RL	<RL	<RL	<24.65	23755.33	22990.16
D35	6/03/08	1	late	38:24	42:30	40:27	<4	<RL	<RL	<RL	<2.76	<RL	<RL	41.6	78238.92	70334.26
D35	6/03/08	2	early	10:05	24:45	17:25	<4	<RL	<RL	<RL	<RL	<RL	<RL	<47.51	45953.83	42480.18
D35	6/03/08	2	middle	28:34	33:55	31:14	<RL	<RL	<RL	<RL	<5.11	<RL	E	<RL	E	E
D35	6/03/08	2	late	36:45	40:11	38:28	<4	<RL	<RL	<RL	3.43	<RL	2.06	48	17874.8	16376.3
D35	6/03/08	4	early	11:34	31:20	21:27	<5.33	<RL	<RL	<RL	<2	<RL	<RL	77.85	92509.26	94054.92
D35	6/03/08	4	middle	35:07	47:30	41:18	<4	<RL	<RL	<RL	<RL	<RL	<RL	<33.15	61015.82	60973.95
D35	6/03/08	4	late	53:10	66:12	59:41	<4	<RL	<RL	<RL	<RL	<RL	<RL	<32.17	52137.06	50565.97
D35	6/03/08	5	early	11:00	19:36	15:18	<RL	<RL	<RL	<RL	18.42	<RL	<RL	218.8	<RL	<RL
D35	6/03/08	5	middle	21:37	24:44	23:11	<RL	<RL	<RL	<RL	15.92	<RL	<2.4	<RL	E	E
D35	6/03/08	5	late	27:26	29:39	28:33	<29.31	<RL	<RL	<RL	<15.64	<RL	<2.01	191.06	53359.64	51291.91

All concentrations are in nanograms per liter. <RL, less than reporting level; E, concentration estimated-IDS recovery 5-10%.