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Occurrence of Steroid Sex Hormones in the Cache la Poudre River, and Pathways for their Removal in the Environment

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Abstract

Some chemicals have the apparent ability to disrupt normal endocrine system functions after exposure to concentrations so small that they are difficult to detect in the environment. In recent years, these so-called "endocrine disruptors" have become the subject of intensive scientific research. In wildlife, most of the evidence for endocrine disruption has come from studies on species living in, or closely associated with, aquatic environments. Reported effects of endocrine disruption include abnormal blood hormone levels, masculinization of females, feminization of males, altered sex ratios, intersexuality, and reduced fertility and fecundity. Among suspected endocrine disruptors, exogenous steroid sex hormones generally have the highest potencies for disrupting normal steroid sex hormone functions. In a national reconnaissance study conducted by the U.S. Geological Survey (USGS) from 1999 to 2000, steroid sex hormones were detected at varying concentrations and frequencies in water samples from 139 stream sites located in 30 states. Other studies have detected steroid sex hormones in surface waters throughout the world, including Colorado. Potential sources of steroid sex hormones in the environment include sewage treatment plants, septic systems, animal feeding operations, rangeland grazing, paper mills, aquaculture, and agricultural operations where manure and biosolids are applied as fertilizers. The objectives of this study were to investigate the presence of steroid sex hormones in northern Colorado's Cache la Poudre River, to determine the potential for steroid sex hormone biodegradation and photodegradation under natural conditions, and to characterize the mobility of selected steroid sex hormones in agricultural fields using a rainfall simulator. The study determined that steroid sex hormones are present in the Cache la Poudre River, at concentrations ranging from 0.6 ng L^{-1} (epitestosterone) to 22.6 ng L^{-1} (estrone). The study also determined that testosterone, progesterone, and 17β-estradiol can be degraded by manure-borne bacteria, and that testosterone degradation is faster under aerobic conditions and at higher temperatures (i.e., 37°C vs. 22°C), but little affected by changes in pH (from 6 to 7.5) or glucose amendments. In ultraviolet light $\lambda > 340$ nm, the study observed direct photodegradation of testosterone and progesterone, and indirect photodegradation of testosterone and 17β-estradiol in the presence of Elliot soil humic acid. On the other hand, in ultraviolet light $\lambda > 310$ nm, direct photodegradation of androstenedione was substantially faster than direct photodegradation of testosterone in ultraviolet light $\lambda > 310$ nm, and no indirect photodegradation observed. The study detected and identified three testosterone biodegradation products (dehydrotestosterone, androstenedione, and androstadienedione), and detected several products of testosterone and androstenedione photodegradation which appear to retain their steroid structure, and possibly their endocrine disrupting potential. Finally, the study generally observed that androgen runoff concentrations follow runoff rates and decrease after successive rainfall events, while runoff concentrations of other analytes (e.g., estrone) peak after the maximum runoff rate and first rainfall event. Sample and data analysis from the study are continuing, and comprehensive finding and recommendations are expected after the date of this report.

Keywords

Steroid hormone, endocrine disruption, EDC, wastewater, effluent, manure, Cache la Poudre River, biodegradation, photodegradation, transformation product

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Abbreviations

ACN – acetonitrile

AD – androstenedione

ADD – androstadienedione

AFO – animal feeding operation

ASE – accelerated solvent extraction

CAFO – concentrated animal feeding operation (AFOs that meet certain EPA criteria)

cps – counts per second

DHT – dehydrotestosterone (a/k/a boldenone)

DOM – dissolved organic matter

E1 – estrone

E2 – 17β-estradiol

E3 – estriol

EE2 – 17α-ethinylestradiol

EDC – endocrine disrupting chemical or "endocrine disruptor"

EPA – U.S. Environmental Protection Agency

ESI^+ – positive electrospray ionization

GC-MS/MS – gas chromatography tandem mass spectrometry

GFF – glass fiber filter

HPLC – high performance liquid chromatography

k – first order (or pseudo-first order) rate constant

k_{ow} – octanol-water partition coefficient

LC-DAD – liquid chromatography with diode array detection

LC-MS/MS – liquid chromatography tandem mass spectrometry

LC-TOF MS – liquid chromatography time-of-flight mass spectrometry

LOD – limit of detection

MeOH – methanol

mg/L – milligrams per liter, or parts-per-million

μg/L – micrograms per liter, or parts-per-billion

MRM – multiple reaction monitoring

NAS – nitrifying activated sludge

Near UV – ultraviolet light with a spectral range from 300 to 400 nm

ng/L – nanograms per liter, or parts-per-trillion

PNEC – predicted no effect concentration

SPE – solid phase extraction

SRFA – Suwannee River fulvic acid

SRHA – Suwannee River humic acid

STP – sewage treatment plant

T – testosterone

t_R – retention time

$t_{½}$ – degradation half-life

TSA – tryptic soy agar

TSB – tryptic soy broth

USGS – U.S. Geological Survey

UV – ultraviolet light

UV-A – ultraviolet A, or ultraviolet light with a spectral range from 320 to 400 nm

UV-B – ultraviolet B, or ultraviolet light with a spectral range from 280 to 320 nm

UV-C – ultraviolet C, or ultraviolet light with a spectral range from 100 to 280 nm

WWTP – wastewater treatment plant

Background

Endocrine Disruption, in General

Some chemicals have the apparent ability to disrupt normal endocrine system functions after exposure to concentrations so small that they are difficult to detect in the environment. In recent years, these so-called "endocrine disruptors" (EDCs) have become the subject of intensive scientific research. In surface waters, suspected endocrine disruptors include natural hormones, synthetic hormones, plant sterols, phytoestrogens (plant compounds that are structurally similar to estrogens), and organic chemicals used in pesticides, detergents, plastics, and other products [1-2].

The endocrine system functions by producing hormones, which travel through the bloodstream in extremely small concentrations to specialized receptors in target organs and tissues, including mammary glands, bone, muscle, the nervous system, and male and female reproductive organs [3]. Hormones bind to the specialized receptors, and the resulting complexes affect gene expression, cell differentiation, hormone secretion, and other bodily processes [4]. In general, hormones operate as chemical signals, enabling the endocrine system to regulate a variety of biological functions such as homeostasis (the body's ability to maintain a state of balance), growth, development, sexual differentiation, and reproduction [3, 5].

The mechanisms of endocrine disruption are complex. Endocrine disruptors appear to operate by mimicking, enhancing, or inhibiting the actions of endogenous (i.e., self-produced) hormones, interfering with hormone synthesis or metabolism, disrupting hormone transport, or altering hormone receptor populations [1, 6-7]. An endocrine disruptor's potency is related to its affinity for binding to hormone receptors, and to the shape of the resulting complex, but its potency can be affected by subsequent interactions and rate-limiting events [8]. The relationship between endocrine disruptor potency and concentration is often nonlinear (e.g., U-shaped), which could reflect different mechanisms of action at different concentrations [9-11]. In addition, mixtures of endocrine disruptors can have additive or even synergistic effects [12-17]. It is difficult to generalize the risk of endocrine disruption across species, because the basic mechanisms of sex differentiation, metabolism, and receptor structure and function differ [18-20]. However, the risk of endocrine disruption generally appears to be highest during critical stages of growth and development [7, 21].

The health effects of endocrine disruption have been extensively reviewed [1, 3, 6-7, 21-24]. Most of the evidence for endocrine disruption in wildlife has come from studies on species living in, or closely associated with, aquatic environments [19]. The observed effects often appear to result from the disruption of steroid sex hormone functions, particularly those of estrogens [19]. Reported effects of endocrine disruption include abnormal blood hormone levels, masculinization of females, feminization of males, altered sex ratios, intersexuality (e.g., the presence of female oocytes in male testicular tissue), and reduced fertility and fecundity [19, 24-26]. One study from 1995 to 1996 examined wild populations of freshwater fish (roach; *Rutilus*

rutilus) throughout the United Kingdom, and reported that the incidence of intersexuality in male fish ranged from 4%, in the laboratory population and at one field control site, to 100%, in two populations downstream from sewage treatment plants (STPs) [25]. Other studies have reported evidence of endocrine disruption in freshwater ecosystems throughout the world, including Boulder Creek near Boulder, Colorado [26-29].

Steroid Sex Hormones as Endocrine Disruptors

Steroid sex hormones are found in a range of vertebrate and invertebrate species [7] (Figure 1). Steroid sex hormones are hydrophobic in nature, and commonly act by diffusing through cell membranes and binding to nuclear hormone receptors, although interactions with transmembrane receptors also occur [1, 8, 10].

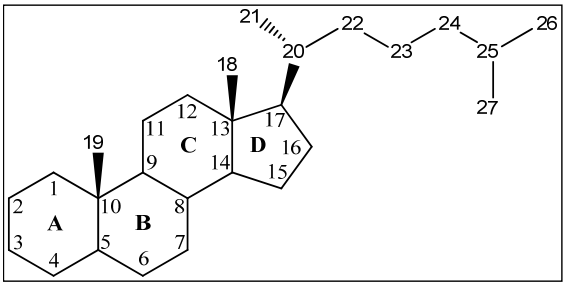


FIGURE 1. Basic steroid structure (27 carbon cholestane). The steroid skeleton is characterized by four fused rings, labeled from A to D. Each carbon is labeled from 1 to 27.

There are three classes of steroid sex hormones: androgens, estrogens, and progestagens [5]. In vertebrates, androgens play a key role in the development of male traits, spermatogenesis, mating and breeding behaviors, reproduction, and muscle growth. [5, 30]. The most common vertebrate androgens are testosterone and 5α-dihydrotestosterone, but 11-ketotestosterone is important among fish [1, 5]. Estrogens are crucial for the development of female traits, ovulation, reproduction, mating and breeding behaviors, and somatic cell function [5, 19, 23]. In egg-laying vertebrates, estrogens also stimulate the liver to produce vitellogenin, a precursor of egg yolk constituents and eggshell proteins [1]. The most common vertebrate estrogens are 17β-estradiol, estrone, and estriol [1]. Finally, progestagens influence water and salt metabolism, and help to maintain pregnancy through various anti-estrogenic and anti-androgenic effects [31]. The most common vertebrate progestagen is progesterone, but 17α, 20β-dihydroxyprogesterone is important among fish [5].

Like vertebrates, the endocrine systems of invertebrates regulate growth, development, and reproduction, but the endocrine systems of invertebrates are more diverse, and less well-documented, than vertebrates [19, 32]. Testosterone, 17β-estradiol, estrone, and progesterone have been reported in many invertebrate groups, but their role is not well understood [19, 32]. Progesterone has even been detected in the dry mature wood, pine bark, and pine needles of loblolly pine (*Pinus taeda* L.) [33].

Among suspected endocrine disruptors, exogenous (i.e., not self-produced) steroid sex hormones generally have the highest affinities for binding to steroid sex hormone receptors, and the highest potencies for disrupting normal steroid sex hormone functions [12, 18, 20, 30, 34]. In laboratory experiments with some fish species, steroid sex hormones have been linked to endocrine disruption after three weeks of exposure to concentrations of 17α-ethinylestradiol as low as 1 ng/L (fathead minnows; *Pimephales promelas*), 17β-estradiol as low as 1-10 ng/L (rainbow trout; *Oncorhynchus mykiss*), and estrone as low as 25-50 ng/L (rainbow trout; *Oncorhynchus mykiss*) [35-36]. In a 7-year, whole-lake experiment in northwestern Ontario, Canada, chronic exposure of fathead minnows to 17α-ethinylestradiol at concentrations ranging from 5 to 6 ng/L adversely affected gonadal development in males and egg production in females, and led to a near extinction of fathead minnows from the lake [37]. After a review of more than 100 studies on the effects of 17α-ethinylestradiol on aquatic organisms, 0.35 ng/L was recommended as the predicted no-effect concentration (PNEC) for 17α-ethinylestradiol in surface water [38]. To put this number into perspective, 1 ng/L is 1 part per trillion, the equivalent of 1 second in more than 31,700 years. In other words, extremely small concentrations are sufficient to create a risk of endocrine disruption.

Potential Sources of Steroid Sex Hormones in the Environment

Steroid sex hormones have many natural sources. They are excreted continuously by vertebrates, and some microbial species have the ability to transform cholesterol and plant sterols into steroid sex hormones (e.g., plant sterols → androstenedione) [39-40]. Potential sources of steroid sex hormones in the environment include STPs, septic systems, animal feeding operations (AFOs), rangeland grazing, paper mills, aquaculture, and agricultural operations where manure and biosolids are applied as fertilizers [33, 41-52].

Humans and animals excrete steroid sex hormones primarily in the form of sulfate or glucuronide conjugates, which are biologically inactive and more water soluble than unconjugated ("free") hormones [51, 53-55]. Studies have suggested that glucuronide conjugates in wastewater are deconjugated by sewage bacteria (e.g., *Escherichia coli*) before they reach STPs [54-56]. Sulfate conjugates are more recalcitrant, and have been detected in STP influent and effluent [54, 57-58]. The types of steroid sex hormones that are excreted, and

the degree of conjugation, varies with species, gender, and stage of reproduction, as reviewed previously [42, 48, 51, 55].

Natural and synthetic steroid sex hormones are also administered to humans and livestock for pharmaceutical purposes. In humans, 17β-estradiol, equine-derived estrogens (e.g., equilin and equilenin), synthetic estrogens (e.g., 17α-ethinylestradiol and mestranol), natural and synthetic progestagens (e.g., progesterone and norethindrone), and testosterone are used for contraception, palliative care during cancer treatment, and hormone replacement therapy for menopause and osteoporosis [22, 59-60]. In livestock, testosterone, trenbolone (synthetic androgen), 17β-estradiol, zeranol (non-steroidal estrogen), progesterone, and melengestrol (synthetic progestagen) are used as growth promoters or for synchronization of ovulation [22, 42, 48, 59-60]. Synthetic steroid sex hormones (e.g., 17α-ethinylestradiol) are specifically designed for increased potency, bioavailability, and degradation resistance, and might be persistent if discharged to the environment [60].

Presence of Steroid Sex Hormones in the Environment

In a national reconnaissance study conducted by the U.S. Geological Survey (USGS) from 1999 to 2000, steroid sex hormones were detected at varying concentrations and frequencies in water samples from 139 stream sites located in 30 states [61]. A similar study demonstrated that steroid sex hormones are ubiquitous in Dutch surface waters at low ng L^{-1} concentrations [56]. Other studies have detected steroid sex hormones in surface waters throughout the world [29, 57-58, 62-66], including Colorado [26].

An early series of U.K. studies detected 17β-estradiol (1-50 ng L^{-1}), estrone (1-80 ng L^{-1}), and 17α-ethinylestradiol (up to 7 ng L^{-1}) in STP effluents after anglers casually observed the occurrence of intersex fish in STP lagoons [41, 67] . Since then, many studies have detected steroid sex hormones in STP effluents [26, 29, 56, 68-70]. Steroid sex hormone concentrations in STP effluent are influenced by several factors, including the composition of STP influents, and the treatment processes used [29, 55, 62, 64, 71-73].

Livestock wastes are commonly applied to agricultural fields as fertilizers and soil amendments. In AFOs, solid wastes are commonly separated, dewatered, and collected for application as fertilizers, and liquid wastes are collected in lagoons, diluted with irrigation water, and applied as fertilizers [45, 74-75]. AFO lagoons typically function as holding reservoirs or anaerobic digesters, where biodegradation can occur [75-76]. However, no additional treatment is required before land application [75-76]. One study examined whole lagoon effluents from swine, poultry and cattle operations, and found free (unconjugated) estrogens at concentrations of up to 21,000 ng/L (primary lagoon, swine operation) [76]. Other studies have detected steroid sex hormones at ng/L concentrations in groundwater, drainage water, and surface waters near animal feeding operations, or evidence of endocrine disruption [45, 77-80].

Biosolids (treated sewage sludge), like livestock wastes, are commonly applied to agricultural fields as fertilizers and soil amendments. Estrogen sorption to activated and inactivated sewage sludge has been observed, and both androgenic and estrogenic activities have been detected in municipal biosolids [81-83]. Like STP effluent, steroid sex hormone concentrations in biosolids are influenced by the treatment processes used. According to one study, hormone activities were substantially higher after anaerobic digestion (mean estrogen: 1,233 ng g^{-1} dry weight; mean androgen: 543 ng g^{-1} dry weight) than after aerobic digestion (mean estrogen: 11.3 ng g^{-1} dry weight; androgen: < LOD) [83].

When manure and biosolids are land applied to fields, some risk of steroid sex hormone leaching and runoff to ground and surface waters is created [77, 84-85].

Steroid Sex Hormone Fate in the Environment

Once steroid sex hormones enter the environment, their fate is influenced by a variety of physical and transformation processes, including sorption to soils and sediments, microbial degradation, and abiotic processes such as photodegradation [86-87].

Sorption

Sorption has been defined as "the accumulation of a substance or material at an interface between the solid surface and the bathing solution" [88]. Sorption can occur through a variety of mechanisms, including hydrophobic partitioning, hydrogen bonding, and nonspecific van der Waals interactions [89]. The actual mechanism is influenced by physical and chemical properties of both the sorbate (the substance that is being sorbed) and the sorbent (the surface where sorption occurs). For example, a finely divided solid, such as a clay particle (diameter < 2 μm), will have a high sorption capacity because its surface area is large relative to its volume [90]. The sorption of neutral hydrophobic contaminants to soil also has been positively correlated with soil organic matter content [91-92].

Many studies have examined sorption rates, the distribution of steroid sex hormones between water and soils or sediments, and sorption to various materials, including clay minerals, organic colloids, and river sediments [93-95]. Environmental conditions, such as pH, can affect the rate or extent of sorption [96]. For example, one study found that steroid sex hormone sorption to organic matter is stronger at acidic pH [97].

Sorption has the potential to affect the environmental fate and transport of steroid sex hormones in various ways. Sorption to immobile soil components can inhibit leaching, and reduce bioavailability to microorganisms. On the other hand, sorption to mobile soil particles, such as clay or dissolved organic matter, can enhance steroid transport via runoff or leaching, and enhance bioavailability to solid phase bacteria (e.g., bacteria in biofilms) [90, 98-101]. Similarly, photodegradation can be inhibited if steroids diffuse into unreactive micropores in soil

particles or organic matter, but higher concentrations of singlet oxygen (a reactive oxygen species associated with indirect photodegradation) have been observed near the surface of dissolved organic matter, where sorption would be expected to occur [102-104].

Biodegradation

Bacteria can use steroids in redox (reduction/oxidation) reactions to gain energy, or metabolize the steroids completely as a carbon source for cell growth [105-106]. In addition, several microbial species (including species of *Mycobacterium*, *Arthrobacter*, *Bacillus, Pseudomonas*, *Escherichia*, and *Micrococcus*) are known to transform cholesterol or plant sterols into steroid sex hormones, or transform steroid sex hormones into different steroid sex hormones [39-40, 107-108].

Biodegradation of steroid sex hormones has been studied in sewage sludge [109-110], river water and sediments [99, 111], and pure culture media [112-113]. Other studies have examined the degradation of steroid sex hormones in soil and soil that has been amended with manure, and the impact of oxygen (e.g., aerobic vs. anaerobic conditions), pH, carbon source and temperature on the degradation kinetics [114-122]. To date, estrogen biodegradation has been studied more extensively, and is better understood, than androgen or progestagen biodegradation. In addition, the products and pathways of steroid biodegradation are not well understood.

Photodegradation

Photodegradation is an important abiotic degradation pathway in natural waters [123]. Direct photodegradation occurs when an organic contaminant absorbs light, becomes electronically excited, and changes chemically from this excited state [60, 123-124]. Indirect (or sensitized) photodegradation occurs when another compound absorbs light, becomes electronically excited, and initiates a reaction that changes an organic contaminant chemically [60, 123-124].

Unsaturated organic molecules (e.g., ketones, olefins, and aromatic hydrocarbons) absorb light in the photochemical region from 200-700 nm [124]. As sunlight passes through the Earth's atmosphere, wavelengths < 280 nm are absorbed completely by atmospheric oxygen (O_2) and ozone (O_3), and wavelengths < 315 nm are absorbed efficiently, but not completely, by O_3 [125]. Therefore, the photochemical region in natural waters is generally restricted to approximately 295 to 700 nm, which includes visible, UV-A, and UV-B light [126]. Many steroid sex hormones absorb light in this range (Figure 2), and natural waters contain many other unsaturated organic molecules (e.g., dissolved organic matter) and inorganic species (e.g., nitrate

and nitrite) that absorb light in this range [127-129]. For this reason, natural water bodies have been described as "large photochemical reactor systems" [127].

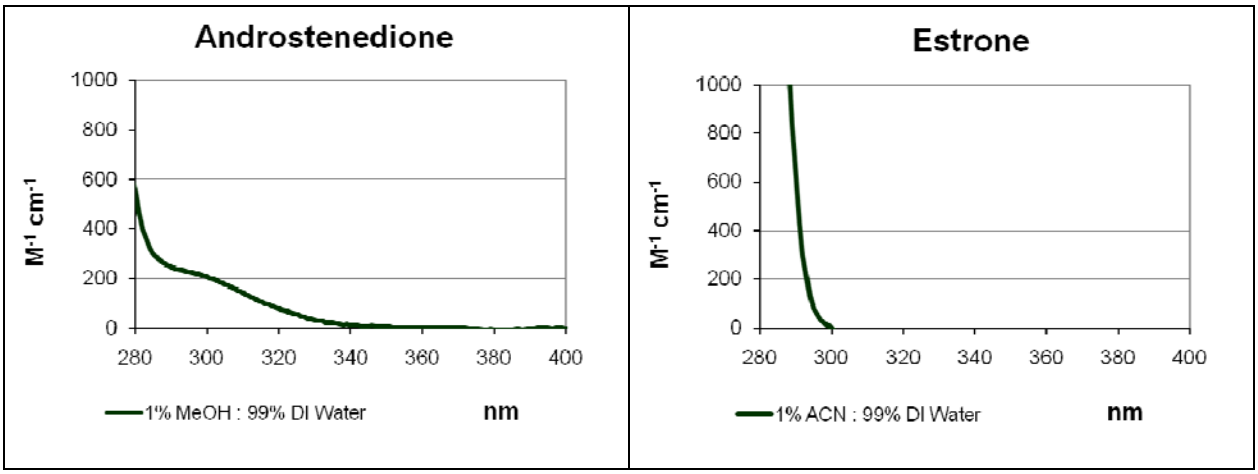


FIGURE 2. Absorption spectra of androstenedione and estrone in UV-A and UV-B spectral range (as measured by a Perkin Elmer Lambda 45 dual beam spectrophotometer using a 5 cm quartz cell, a 1 nm slit width, and a 240 nm/min scan rate).

However, as sunlight passes through a water body, its intensity decreases and its spectral distribution changes [126]. In inland surface waters, the intensity of sunlight is attenuated primarily through absorption by dissolved organic matter (DOM), and attenuation generally increases with decreasing wavelength [126]. Therefore, photodegradation rates, and even photodegradation mechanisms (e.g., direct vs. indirect photodegradation), can change with depth according to the water's attenuation coefficient, the organic contaminant's molar absorptivity, and other environmental factors.

Several studies have examined direct and indirect photodegradation of estrogens in UV light [130-140]. Depending on the types of lamps that were used (e.g., xenon vs. mercury), 17α-ethinylestradiol, 17β-estradiol, and estrone have been observed to undergo direct photodegradation [139-141]. In addition, 17α-ethinylestradiol, 17β-estradiol, estrone, and estriol have been observed to undergo indirect photodegradation in the presence of river water or humic acid, and several photoproducts of 17β-estradiol have been identified [131, 138-140]. Less is known about the photodegradation of androgens and progestagens under environmental conditions.

Project Objectives

Because steroid sex hormones and other endocrine disruptors have been detected in locations around the world at concentrations that could have adverse biological and ecological effects, it is important to understand their sources, the processes that transform them, the factors that influence their transport, and ultimately, their fate in the environment. With this knowledge, management practices can be developed for producers and water managers to minimize the risks of endocrine disruption from wastewater, runoff from agricultural fields, and other potential sources of exogenous steroid sex hormones.

The overall hypothesis of this study was:

> ***Steroid sex hormones are present in sewage treatment plant effluents, wastewater from animal feeding operations, and the Cache la Poudre River, and can be degraded by photolysis and bacteria.***

The specific objectives of this study were to:

1) Identify hormones and their major metabolites in animal wastewater, sewage treatment plant effluents, and the Cache la Poudre River Basin of Colorado.

2) Determine 17β-estradiol, testosterone, progesterone, and trenbolone acetate degradation rates and products, formed in batch incubations with *Mycobacterium* (common in soil, sediment and sludge), *Escherichia coli* (common in manure and human/animal intestines), and a manure/STP inoculum as a function of dissolved organic matter, temperature, redox-potential, pH, iron, and nitrate.

3) Assess the impact of photoinduced hormone degradation as a function of potential photo-sensitizers (specifically, dissolved organic matter, manure/sewage, Fe, and nitrate).

4) Characterize the mobility of hormones and their degradation products in field studies using a rainfall simulator, and spatially relative to AFO and STP locations in the Cache La Poudre River Basin of Colorado.

5) Correlate data from objectives 1-4 to assemble a comprehensive view of the influence of microbial, chemical and physical processes on the behavior of hormones in natural and engineered water systems, and make these data available to water managers and the public through a CSU based homepage.

Project Accomplishments

1) *Identify hormones and their major metabolites in animal wastewater, sewage treatment plant effluents, and the Cache la Poudre River Basin of Colorado.*

During the period from November, 2007 to March, 2008, the study detected 6 steroid sex hormones (cis-androsterone, epitestosterone, androstenedione, 17β-estradiol, estriol, and estrone) in water samples from the Cache la Poudre River, at maximum concentrations ranging from 0.6 ng/L (epitestosterone) to 22.6 ng/L (estrone). In March, 2008, the study also detected 3 steroid sex hormones (17β-estradiol, estriol, and estrone) in STP effluent samples discharged into the Cache la Poudre River, at concentrations ranging from 6.0 ng/L (17β-estradiol) to 73.5 ng/L (estrone). To date, the study has not analyzed wastewater samples obtained directly from animal feeding operations. In general, the study has determined that steroid sex hormones are present in the Cache la Poudre River, as hypothesized, at concentrations sufficient to create some risk of endocrine disruption.

2) *Determine 17β-estradiol, testosterone, progesterone, and trenbolone acetate degradation rates and products, formed in batch incubations with Mycobacterium (common in soil, sediment and sludge), Escherichia coli (common in manure and human/animal intestines), and a manure/STP inoculum as a function of dissolved organic matter, temperature, redox-potential, pH, iron, and nitrate.*

The study has determined rates and half-lives for the degradation of testosterone, 17β-estradiol, and progesterone by manure-borne bacteria in batch incubation experiments using two media: (a) swine manure mixed with minimal growth media, and (b) a pre-enriched culture of swine manure-borne bacteria. In the pre-enriched culture, where degradation rates and half-lives were not influenced by lag phases or sorption, half-lives ranged from 4.63 h (progesterone) to 24.6 h (17β-estradiol). The study also examined testosterone degradation in the pre-enriched culture to determine the effects of oxygen (aerobic vs. anaerobic conditions), pH, temperature, and glucose amendments. The half-life of testosterone was observed to be 5-6 times longer under aerobic conditions (27.1 h) than under anaerobic conditions (5.29), and the degradation rate was 17% slower at 22°C than at 37°C. Glucose amendments and pH had little effect under the conditions studied. The study also detected and identified 3 products of testosterone degradation in the pre-enriched culture (dehydrotestosterone, androstenedione, and androstadienedione) which might have, or are already known to have, endocrine disrupting potential. To date, the study has not examined the effects of iron or nitrate, and has not analyzed trenbolone acetate or pure bacteria cultures. In general, the study has determined that steroid sex hormones can be degraded by manure-borne bacteria, as hypothesized.

3) *Assess the impact of photoinduced hormone degradation as a function of potential photo-sensitizers (specifically, dissolved organic matter, manure/sewage, Fe, and nitrate).*

The study has determined rates and half-lives for the degradation of testosterone, 17β-estradiol, and progesterone upon exposure to UV-A light ($\lambda > 340$ nm). In buffered water (pH 5.5), where no photosensitizers were present, 17β-estradiol was not degraded, testosterone had a half-life of 50 h, and progesterone had a half-life of 79 h. The study also examined photodegradation in the presence of various photosensitizers (nitrate, Elliot soil humic acid, and a combination of both). The half-life of progesterone was unaffected, the half-life of testosterone increased (17 h in buffered humic acid solution), and 17β-estradiol had the shortest half-life (2.7 h in buffered humic acid and nitrate solution). Additional experiments have been conducted with testosterone and androstenedione, in sunlight and UV-A light ($\lambda > 310$ nm), under different pH conditions (pH 8.0) and in the presence of different photosensitizers (Suwannee River humic and fulvic acids). In buffered water, possibly because of differences in the lamps used, direct photodegradation of androstenedione occurred substantially faster ($t_{½} = 1.7$ h) than direct photodegradation of testosterone in the initial experiments ($t_{½} = 50$ h), and indirect photodegradation was not observed. Numerous transformation products of androstenedione and testosterone were detected and tentatively identified, including numerous isomers and photoaddition products that appear to retain their steroid structures, and possibly their endocrine disrupting potential. Overall, the study has determined that steroid sex hormones can be transformed in the environment through photochemistry, as hypothesized.

4) *Characterize the mobility of hormones and their degradation products in field studies using a rainfall simulator, and spatially relative to AFO and STP locations in the Cache La Poudre River Basin of Colorado.*

The study has determined steroid sex hormone concentrations in whole-water (unfiltered) runoff samples during a single rainfall simulation event, and during a series of simulated rainfall events over approximately a one-month period. During the single rainfall simulation event, androgen runoff concentrations generally followed runoff rates, peaking at the maximum runoff rate, while estrone runoff concentrations appeared to increase after the maximum runoff rate occurred. During the series of rainfall simulation events, androgen runoff concentrations generally decreased with each simulated rainfall event, while estrogen runoff concentrations (excluding estriol) generally peaked during the second rainfall event after biosolids application. The study also determined steroid sex hormone concentrations in filtered runoff samples (0.7 μm GFF), in order to determine the distribution of steroid sex hormones between the filtrate (including particles < 0.7 μm) and the particle fraction (particles > 0.7 μm). In general, the distribution varied by analyte. Sample and data analysis are continuing, but overall the data suggest that the mobility of steroid sex hormones and their degradation products will vary considerably depending on the compound's identity and a variety of environmental factors and conditions.

5) *Correlate data from objectives 1-4 to assemble a comprehensive view of the influence of microbial, chemical and physical processes on the behavior of hormones in natural and engineered water systems, and make these data available to water managers and the public through a CSU based homepage*.

Work on the study is continuing. Data and sample analysis from the runoff study is ongoing. Water samples are being collected from the Cache la Poudre River on a regular basis to determine whether additional steroid sex hormones are present, and to monitor trends in steroid sex hormone concentrations over time. Additional biodegradation experiments are being conducted to examine the long-term degradation pathway of steroid sex hormones by manure-borne bacteria under aerobic and anaerobic conditions, and to determine the major bacterial species involved in such degradation. Also, additional photodegradation experiments are being conducted to reveal the identities and environmental fates of the observed photodegradation products, to examine the effects of pH and other environmental conditions on photodegradation rates, and to quantify such rates in natural sunlight and natural waters.

Nevertheless, as of the date of this report, partial results from the study have been disseminated in the following publications, which are accessible through the homepage of Dr. Thomas Borch (http://soilcrop.colostate.edu/borch/home.html):

- Borch, T., Young, R.B., Gray, J.L., Foreman, W.T., Yang, Y.-Y. Presence and Fate of Steroid Hormones in a Colorado River. Division of Environmental Chemistry - *Abstract of Papers of the American Chemical Society (preprint),* **2008**, 8 (2), 689-694.

- Jones, J.M.; Borch, T.; Young, R.B.; Davis, J.G.; Simpson, C.R. Photolysis of testosterone, progesterone, and 17β-estradiol by UVA light In *Emerging Contaminants of Concern in the Environment: Issues, Investigations, and Solutions*; Drewes, J. E., Battaglin, W. A., Kolpin, D. W., Eds.; American Water Resources Association, Middleburg, Virginia,: Vail, Colorado, **2007**; Vol. Proceedings of the AWRA 2007 summer specialty conference, TPS-07-2, CD-ROM (5 pages).

- Yang, Y.-Y.; Borch, T.; Young, R.B.; Goodridge, L.D.; Davis, J.G. Degradation Kinetics of Testosterone by Manure-Borne Bacteria: Influence of Temperature, pH, Glucose Amendments, and Dissolved Oxygen. *J. Environ. Qual.* **2009,** *In Press.*

- Young, R.B.; Borch, T.; Yang, Y.-Y.; Davis, J.G. Occurrence and Fate of Steroid Hormones in Sewage Treatment Plant Effluent, Animal Feeding Operation Wastewater and the Cache la Poudre River of Colorado. *Colorado Water - Newsletter of the Water Center of Colorado State University* **2008,** *25*, (3), 10-14.

- Young, R.B.; Borch, T. Sources, Presence, Analysis, and Fate of Steroid Sex Hormones In Freshwater Ecosystems - A Review. In *Aquatic Ecosystem Research Trends*, Nairne, G. H., Ed. Nova Science Publishers, Inc. : Hauppauge, New York, **2009**; pp 103-164.

Review of Methods Used

The study was divided into four primary research tasks:

1) River Study: Analysis of water samples from the Cache la Poudre River in northern Colorado, to determine the presence of selected steroid sex hormones in locations near various sources.

2) Biodegradation Experiments: Investigate the biodegradation of selected steroid sex hormones to determine the rates and mechanisms of degradation, the parameters that influence degradation, and the resulting degradation products.

3) Photodegradation Experiments: Investigate the photochemical degradation of selected steroid sex hormones to determine the rates and mechanisms of degradation, the parameters that influence degradation, and the resulting degradation products.

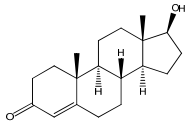
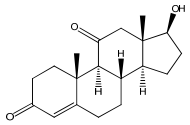
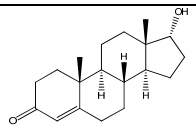
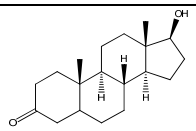
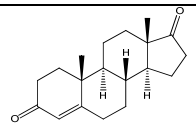
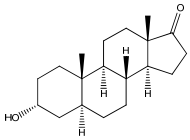
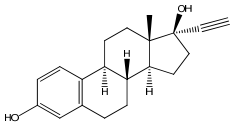
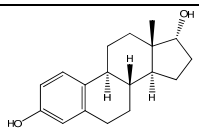
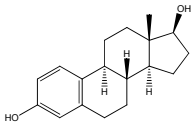
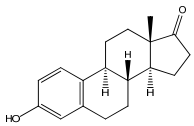
4) Runoff Study: Characterize the mobility of selected steroid sex hormones in field studies using a rainfall simulator.

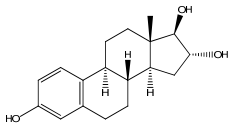
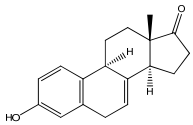
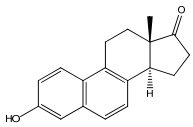
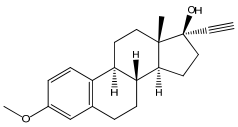
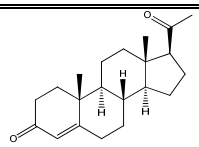
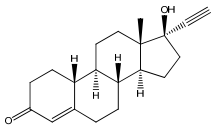
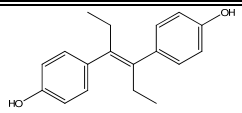
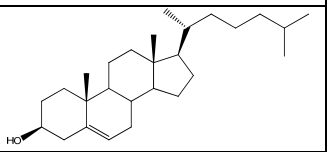
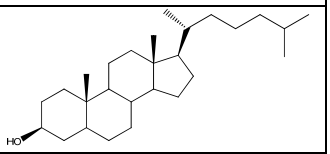
The following review of methods is organized by research task.

River Study

In November 2007, January 2008 and March 2008, 500 to 1000 mL grab samples were collected from flowing water near the shore at up to 6 sites along the Cache la Poudre River. The sampling sites ranged in character from locations relatively unimpacted by urban or agricultural development to locations influenced by intensive agriculture, confined animal feeding operations, and the cities of Fort Collins and Greeley, Colorado. Site 4, in particular, was located immediately downstream from a STP in Fort Collins. The water samples were transported on ice, and frozen until laboratory analysis. Reversed-phase (C-18) solid-phase extraction (SPE) disks were used to extract 16 steroid hormones, one stilbene, and two sterols from the unfiltered water samples (Table 1). After the SPE disks were eluted with methanol, the eluate was evaporated to dryness under nitrogen gas, and redissolved in dichloromethane. Polar interferences were removed using Florisil columns, and target analytes were derivatized to trimethylsilyl-substituted analogs. The trimethylsilyl derivatives were analyzed by gas chromatography–tandem mass spectrometry (GC-MS/MS) operated in electron impact ionization mode. Multiple-reaction monitoring (MRM) and isotope dilution procedures were used for improved sensitivity and reliable compound quantification. In addition, laboratory reagent-water spike and process blank samples were analyzed for quality control purposes.

TABLE 1. List of river study analytes.

	Chemical Data	*Function*	*Structure*
Androgens			
Testosterone	$C_{19}H_{28}O_2$ MW: 288.42 CAS: 58-22-0	Sex hormone	
11-ketotestosterone	$C_{19}H_{26}O_3$ MW: 302.41 CAS: 53187-98-7	Sex hormone (fish)	
Epitestosterone	$C_{19}H_{28}O_2$ MW: 288.42 CAS: 481-30-1	Sex hormone	
Dihydrotestosterone	$C_{19}H_{30}O_2$ MW: 290.44 CAS: 521-18-6	Sex hormone (testosterone metabolite)	
Androstenedione	$C_{19}H_{26}O_2$ MW: 286.42 CAS: 63-05-8	Sex hormone (testosterone metabolite)	
Cis-androsterone	$C_{19}H_{30}O_2$ MW: 290.44 CAS: 53-41-8	Sex hormone	
Estrogens			
17α-ethinylestradiol	$C_{20}H_{24}O_2$ MW: 296.41 CAS: 57-63-6	Ovulation inhibitor (human use)	
17α-estradiol	$C_{18}H_{24}O_2$ MW: 272.39 CAS: 57-91-0	Sex hormone	
17β-estradiol	$C_{18}H_{24}O_2$ MW: 272.39 CAS: 50-28-2	Sex hormone	
Estrone	$C_{18}H_{22}O_2$ MW: 270.37 CAS: 53-16-7	Sex hormone; (17β-estradiol metabolite)	

	Chemical Data	*Function*	*Structure*
Estriol	$C_{18}H_{24}O_3$ MW: 288.39 CAS: 50-27-1	Sex hormone (17β-estradiol metabolite)	
Equilin	$C_{18}H_{20}O_2$ MW: 268.35 CAS: 474-86-2	Sex hormone (horses); estrogen replacement (human use)	
Equilenin	$C_{18}H_{18}O_2$ MW: 266.33 CAS: 517-09-9	Sex hormone (horses); estrogen replacement (human use)	
Mestranol	$C_{21}H_{26}O_2$ MW: 310.44 CAS: 72-33-3	Ovulation inhibitor (human use)	
Progestagens			
Progesterone	$C_{21}H_{30}O_2$ MW: 314.47 CAS: 57-83-0	Sex hormone	
Norethindrone	$C_{20}H_{26}O_2$ MW: 298.43 CAS: 68-22-4	Ovulation inhibitor (human use)	
Other			
Diethylstilbestrol	$C_{18}H_{20}O_2$ MW: 268.36 CAS: 56-53-1	Synthetic non-steroidal estrogen; teratogen	
Cholesterol	$C_{27}H_{46}O$ MW: 386.65 CAS: 57-88-5	Cell membrane component; sex hormone precursor	
Coprostanol	$C_{27}H_{48}O$ MW: 388.67 CAS: 360-68-9	Fecal steroid (cholesterol metabolite)	

Biodegradation Experiments

To study the degradation of testosterone, 17β-estradiol, and progesterone by manure-borne bacteria, batch incubation experiments were conducted in two media: (a) swine manure mixed with minimal growth media ("*system 1*"), and (b) a pre-enriched culture of swine manure-

borne bacteria ("*system 2*"). Fresh swine feces from unsterilized stud boars was collected in Ziploc® plastic bags from the Colorado State University Agricultural Research, Development and Education Center (ARDEC) swine barn, transported on ice to the laboratory within 2 h of collection, and kept frozen at -22°C until used.

In *system 1* (the swine manure system), 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 sex hormone concentration of 3 mg L^{-1}. The minimal growth media was composed of 2 mM $MgSO_4$-$7H_2O$, 3 mM glucose, 0.1 mM $CaCl_2$-2 H_2O, 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, and 19 mM 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 into 2 mL amber glass vials for analysis. No more than 4% of any steroid sex hormone was retained on the filters.

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}). 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, 17β-estradiol, or progesterone, resulting in an initial cell density of approximately 10^6 CFU mL^{-1} and an initial steroid sex 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 mM 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.

To determine biodegradation rates, the samples were analyzed by reversed-phase liquid chromatography with diode array detection (LC-DAD) using the following wavelengths: 244 nm (testosterone), 245 nm (progesterone), and 220 nm (17β-estradiol).

Testosterone's degradation products in the pre-enriched culture were identified by reversed-phase LC-DAD, and confirmed by reversed-phase liquid chromatography-time-of-flight mass spectrometry (LC-TOF MS) using positive electrospray ionization (ESI^+).

Photodegradation Experiments

During initial experiments, testosterone, progesterone and 17β-estradiol solutions were prepared at a concentration of 1 mg/L in deionized water, phosphate buffer solution (10 mM, pH 5.5), and three solutions composed of the phosphate buffer solution and either 10 mg/L nitrate, 5 mg/L Elliot Soil humic acid, or a mixture of both. The buffered and unbuffered aqueous solutions were used to study direct photodegradation, and the buffered nitrate and humic acid solutions were used to study indirect photodegradation. Triplicate 8 mL samples of each amended solution were collected in borosilicate glass culture tubes and placed in a photochemical reactor equipped with a carousel (5 rpm) and UV-A lamps (Rayonet, $\lambda > 340$ nm) (Figure 3). At periodic intervals, a 200 µL sample was collected from each culture tube and analyzed by reversed-phase LC-DAD using the following wavelengths: 244 nm (testosterone), 245 nm (progesterone), and 220 nm (17β-estradiol).

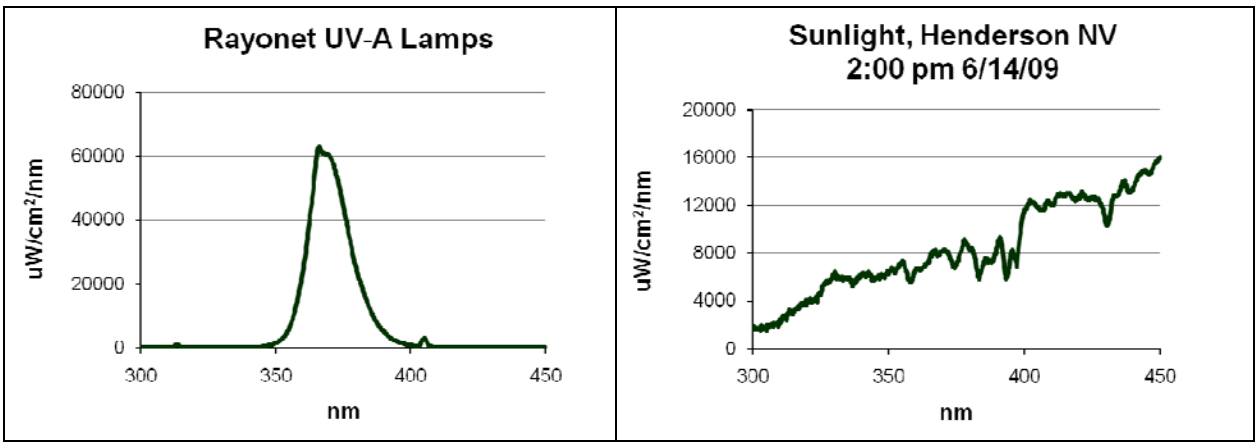


FIGURE 3. Spectral irradiance of Rayonet lamps used in initial experiments, and comparison to solar spectrum (measured by an Ocean Optics USB4000 radiometer equipped with a fiber optic cable and cosine corrector).

In subsequent experiments, 100 µg/L androstenedione solutions were prepared in deionized water, a phosphate buffer solution (5 mM PO_4, pH 8, ~15 mM ionic strength), and buffered and unbuffered solutions of Suwannee River humic and fulvic acids (10 mg/L, 5 mM PO_4, pH 8, ~15 mM ionic strength). Androstenedione and testosterone have similar molecular structures, and similar UV absorption spectra, but androstenedione contains an additional unsaturated bond (a ketone at carbon 17) that is likely to cause different photochemical reactions and products for comparison to testosterone. The buffered and unbuffered aqueous solutions were used to study direct photodegradation, and the buffered fulvic and humic acid solutions were used to study indirect photodegradation. Triplicate 7 mL samples of each amended solution were collected in borosilicate glass culture tubes and placed in the photochemical reactor, equipped with UV-A lamps that were different from those used in the initial experiments (Luzchem, $\lambda > 310$ nm) (Figure 4). At periodic intervals, a 250 µL sample was collected from each culture tube and analyzed by reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) using ESI^+ and MRM.

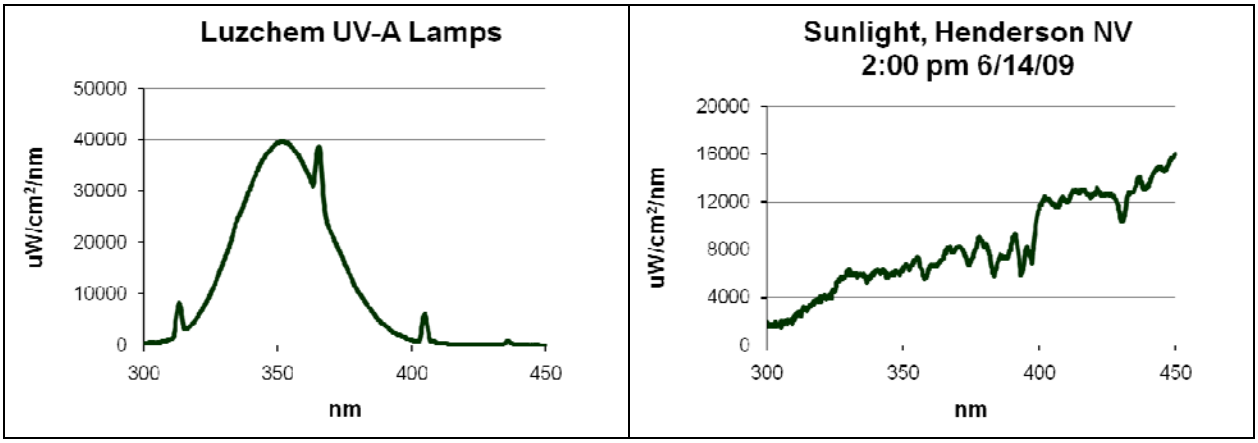


FIGURE 4. Spectral irradiance of Luzchem UVA lamps used in subsequent experiments, and comparison to solar spectrum (measured by an Ocean Optics USB4000 radiometer equipped with a fiber optic cable and cosine corrector).

Additional experiments were conducted in the photochemical reactor, using the lamps described in Figure 4, to examine and compare the photochemical reaction products of androstenedione and testosterone. Specifically, 10 mg/L solutions of androstenedione and testosterone were prepared in deionized water, using methanol stock solutions, and 7 mL samples of each solution were collected in borosilicate glass culture tubes and placed in the photochemical reactor. At periodic intervals, a 500 µL sample was collected from each culture tube and analyzed by reversed-phase LC-TOF MS using ESI^+.

Finally, qualitative experiments were conducted to verify androstenedione's potential for direct and indirect photodegradation in natural sunlight. Specifically, 100 µg/L androstenedione solutions were prepared in deionized water, a phosphate buffer solution (5 mM PO_4, pH 7.5, ~12.5 mM ionic strength), and buffered and unbuffered solutions of Suwannee River humic and fulvic acids (25 mg/L, 5 mM PO_4, pH 7.5, ~12.5 mM ionic strength). During late May 2009, triplicate 7 mL samples of each amended solution were collected in cork-stoppered quartz glass culture tubes, and placed outdoors in Henderson, NV using a customized rack angled approximately 33° from the ground. At periodic intervals, a 250 µL sample was collected from each culture tube and analyzed by reversed-phase LC-MS/MS using ESI^+ and MRM.

Runoff Study

During April to June, 2008, three experimental plots (6 m^2; 2 m wide by 3 m long) in northern Colorado (latitude 40°06'08" longitude 104°12'43") were established, and rainfall simulations (i.e., simulating a 100 year rainfall event) were performed 3 days before, and 1, 8, and 35 days after, biosolids applications (Figure 5). The plots were established parallel to the slope (~3%) and row direction. The soil at the highest position (plot 3) was sandiest and had the steepest slope, and the soil at the lowest position (plot 1) had the finest texture and most gentle slope. Thirty-five days after the biosolids applications, simulated rainfall was conducted on two additional experimental plots (plots 4 and 5) to examine the influence of rainfall frequency and time between the biosolids application and the first simulated rainfall event.

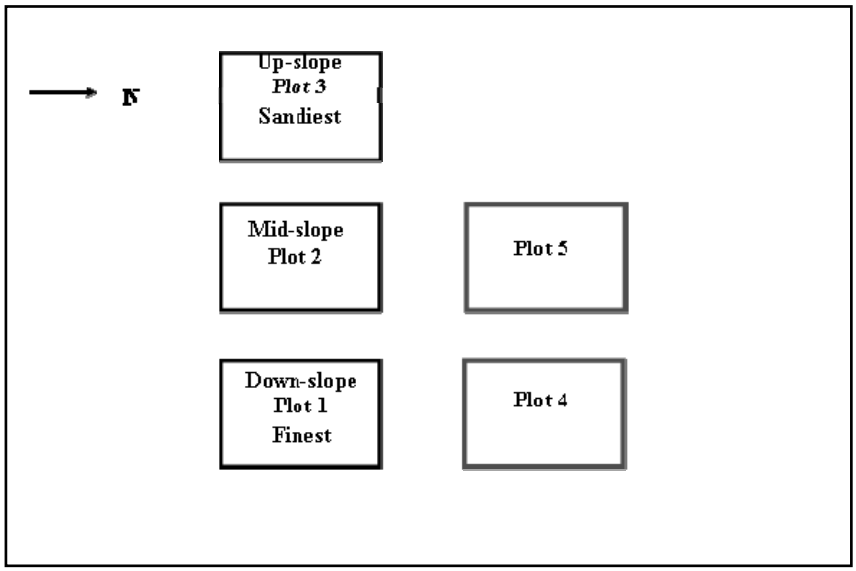


FIGURE 5. Schematic of rainfall simulation plots.

Before simulating rainfall, soil water content was determined gravimetrically from samples taken from three areas outside of each 6-m^2 rainfall simulation plot [142]. Simulated rainfall was applied to each 6-m^2 plot with an oscillating nozzle rainfall simulator (median drop size = 2.3 mm) [143]. The simulator was placed approximately 3 m above each plot, and well water was used in all simulations. Runoff and sediment yields from each 6-m^2 simulator plot were measured gravimetrically during each simulated rainfall event.

Before and after each simulated rainfall event, soil samples were collected in triplicate from locations outside the experimental plots, at depths of 0 to 2, 2 to 6, and 6 to 10 cm. The soil samples were combined into a composite sample to examine leaching potential.

Runoff samples were collected in 0.5 L silanized glass bottles from each plot at the beginning, middle, and end of each simulated rainfall event, and then placed in ice and kept in the dark until transported to the lab. Each sample was split into two portions. The first portion was preserved for analysis as a "whole-water" sample, without filtration. The second portion was preserved for analysis as a "filtrate" sample, after filtration through a 0.7 μm glass fiber filter (GFF). The samples were stored at -60°C until analyzed.

Surrogate standards were added to each whole-water sample prior to storage, and to each filtrate sample after filtration. The whole-water and filtrate samples were enriched by SPE, cleaned of polar interferences, derivatized, and analyzed by GC-MS/MS according to the same methods used for the Cache la Poudre River samples.

Project Results and their Significance

River Study

Estrogens and androgens were detected in samples from multiple sites along the Cache la Poudre River (Table 2). The most commonly detected steroid sex hormone, estrone, is a known degradation product of 17β-estradiol [55]. Estrone and particularly estriol also are excreted by humans and livestock in urine and feces [144]. Androstenedione, the next most commonly detected steroid sex hormone, may be a degradation product of testosterone, but also could result from degradation of cholesterol, progesterone, and the phytosterols stigmasterol and β-sitosterol [44]. Cholesterol, coprostanol, estrone, and estriol were detected in samples from Site 4 (immediately downstream from a STP), and in some samples collected downstream of Site 4, during all sampling periods. 17β-Estradiol was detected in samples from Site 4, and downstream from Site 4 in January 2008 and March 2008.

TABLE 2. Analytes detected in Cache la Poudre River water samples. (All values in ng/L except laboratory spike sample in percent recovered. Non-qualifying, interfering peaks resulted in raised reporting levels.)

		cis-androsterone	**epi-testosterone**	**androstene-dione**	**estrone**	**17β-estradiol**	**estriol**	**coprostanol**	**cholesterol**
Lab Spike		101.0%	96.8%	94.2%	100.2%	98.4%	98.9%	224.0%	215.7%
11/07	Site 1	<0.8	<4	3.7	<0.8	<0.8	<0.8	<4000	<4000
	Site 2	<0.8	<4	<2	<0.8	<0.8	<0.8	<4000	<4000
	Site 3	<0.8	0.6	0.9	0.9	<0.8	<0.8	<4000	<4000
	Site 4	<1.6	<4	3.3	1.4	<0.8	0.8	6140	7470
	Site 5	<0.8	<4	<2	0.8	<0.8	<0.8	<4000	2350
	Site 6	<0.8	<4	0.2	<0.8	<0.8	<0.8	<4000	<4000
1/08	Site 3	<0.8	<4	<2	<0.8	<0.8	<0.8	<4000	<4000
	Site 4	<0.8	<4	<2	3.0	0.5	2.2	4280	7050
	Site 5	1.7	<4	1.0	3.7	1.0	<0.8	<4000	3410
	Site 6	<0.8	<4	1.3	1.8	0.4	<0.8	<4000	2200
3/08	Site 3	< 0.8	< 4	< 2	< 0.8	< 0.8	< 0.8	< 4000	< 4000
	STP effluent	< 0.8	< 4	< 2	73.5	6.0	24.7	22900	35800
	Site 4	< 0.8	< 4	1.9	22.6	3.0	11.1	2770	7740
	Site 5	< 0.8	< 4	< 2	7.8	1.8	2.4	2610	5810

No values in excess of reporting levels (in ng/L) were observed for any of the following hormones: 17α-estradiol (0.8); testosterone (0.8); equilin (4); dihydrotestosterone (0.8); 11-ketotestosterone (0.8); mestranol (0.8); equilenin (4); 17α-ethinyl estradiol (0.8); norethindrone (0.8); and progesterone (no data available). Diethylstilbestrol (0.8) was observed once (0.85) in the WWTP effluent.

Collectively, the data suggest that the STP influences steroid sex hormone concentrations in the Cache la Poudre River. The data also demonstrate that steroid sex hormones are present in the Cache la Poudre River, as hypothesized, at concentrations sufficient to create some risk of endocrine disruption.

Biodegradation Experiments

Biodegradation of Steroid Sex Hormones in Swine Manure (system 1) and Pre-Enriched Culture of Swine Manure-Borne Bacteria (system 2)

In *system 1* (the swine manure system), steroid sex hormones sorbed to the swine manure in sterilized controls within the first hour of incubation (i.e. 7% of testosterone, 15% of 17β-estradiol, and 29% of progesterone), but no additional losses were observed thereafter (Figure 6(A)). In unsterilized manure in *system 1*, testosterone, 17β-estradiol, and progesterone were observed to degrade within 4 to 12 h after a lag phase of approximately 5 to 9 h, and 17β-estradiol was observed to degrade faster than progesterone and testosterone.

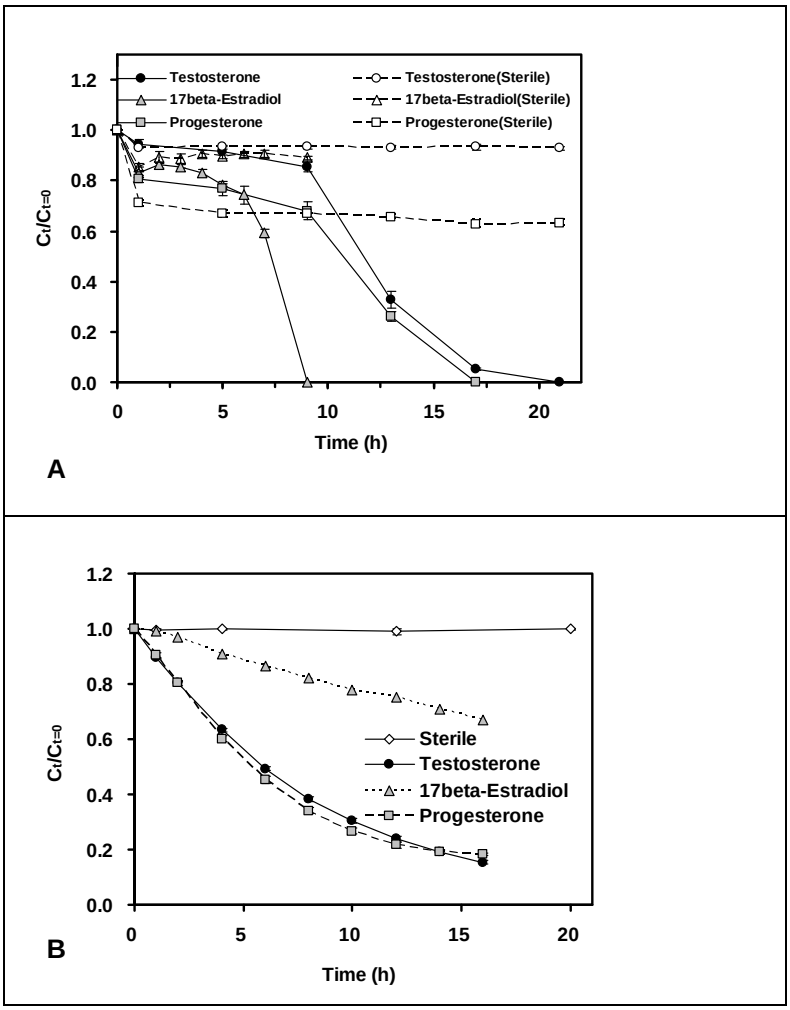


FIGURE 6. Degradation of testosterone, 17β-estradiol, and progesterone under aerobic conditions at 22°C in (A) *system 1* (swine manure system) and (B) *system 2* (pre-enriched culture). Error bars represent the standard deviation of triplicate samples.

In *system 2* (the pre-enriched culture of swine manure-borne bacteria), no degradation or sorption of steroid sex hormones was observed in sterilized controls, and testosterone, 17β-estradiol, and progesterone degradation were initiated without a lag phase (Figure 6(B)). Testosterone and progesterone were transformed in a similar fashion, and followed pseudo first-order reaction kinetics. The degradation of 17β-estradiol followed a zero-order reaction kinetics model during the observed time period. In order to compare the degradation rates for the three steroid sex 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), as described previously [145-146].

TABLE 3. 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 mM 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 mM glucose	0.026 (± 0.002)	27.1 (27.1)
	Aerobic; pH 7; 3 mM 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 mM 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 mM glucose	0.140 (±0.003)	4.95 (4.95)
	3 mM glucose	0.150 (± 0.004)	4.61 (4.61)
	22 mM 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 7(A)). 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 5-6 times longer than under aerobic conditions (Table 3).

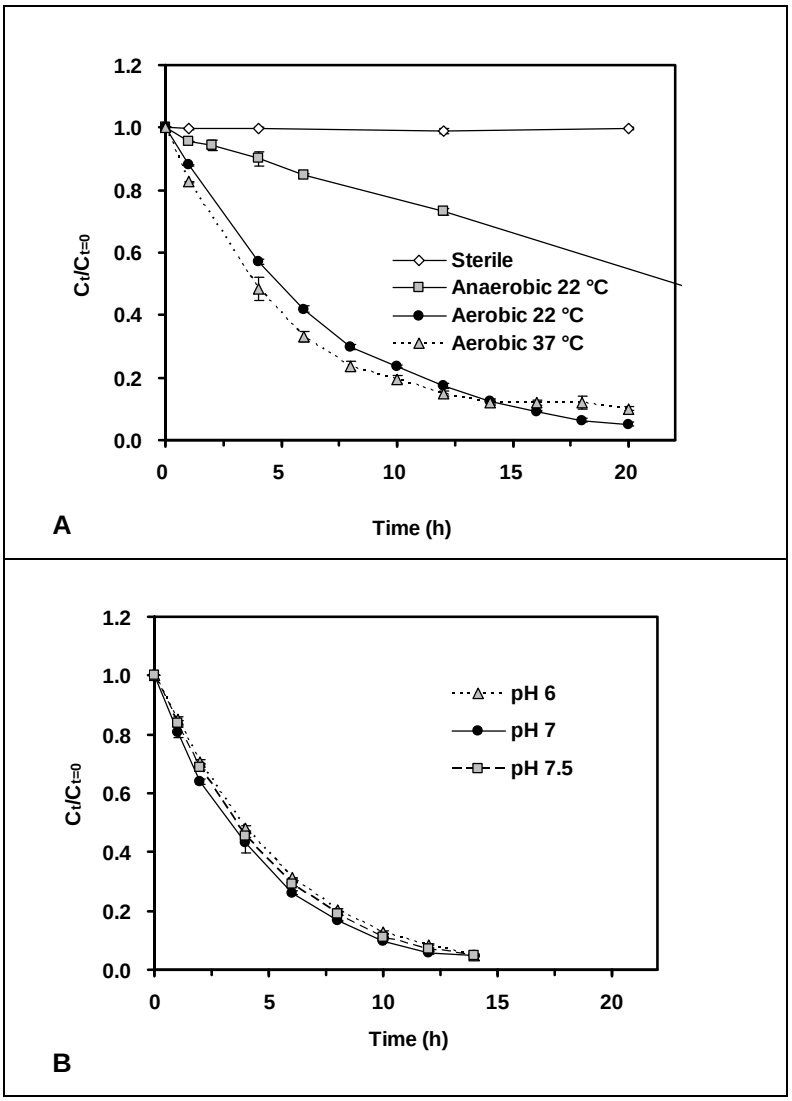


FIGURE 7. (A) Influence of temperature and molecular oxygen at pH 7, and (B) pH on testosterone biodegradation in *system 2*. Error bars represent the standard deviation of triplicate samples.

Influence of Temperature on Testosterone Degradation (System 2)

The kinetics of testosterone degradation (*system 2*) were also 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 17% slower at 22°C than 37°C, based on the initial rate calculation (Table 3; Figure 7(A)).

Influence of pH on Testosterone Degradation (System 2)

Fresh swine feces (i.e., pH 6.8 for this study), fertile agricultural soils, and natural waters often vary in pH, so a pH range of 6 to 7.5 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 (Figure 7(B)). 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; Figure 7(B)). 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) .

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 (e.g., cometabolism). Glucose amendments (i.e., 0, 3, and 22 mM) were found to have minor influence on testosterone degradation and metabolite formation within the 18 h time period investigated (Table 3; Figure 8). Similar results were also observed in *system 1* (data not shown). The difference between the observed rate constant at 0 and 22 mM 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 mM glucose amendment.

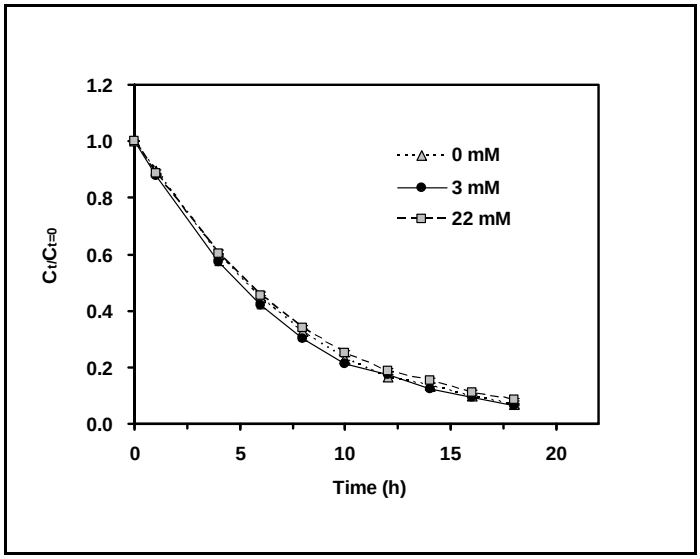


FIGURE 8. Influence of glucose amendments on testosterone degradation in *system 2*. Error bars represent the standard deviation of triplicate samples.

Testosterone Degradation Products (System 2 under Aerobic Conditions)

In *system 2* under aerobic conditions, HPLC-DAD and LC/TOF-MS analysis revealed three degradation products of testosterone: dehydrotestosterone, androstadienedione, and androstenedione (Figure 9). 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). Because the identified degradation products might have, or are already known to have, endocrine disrupting potential, additional biodegradation experiments are being conducted to examine longer term degradation pathways [30, 147-148].

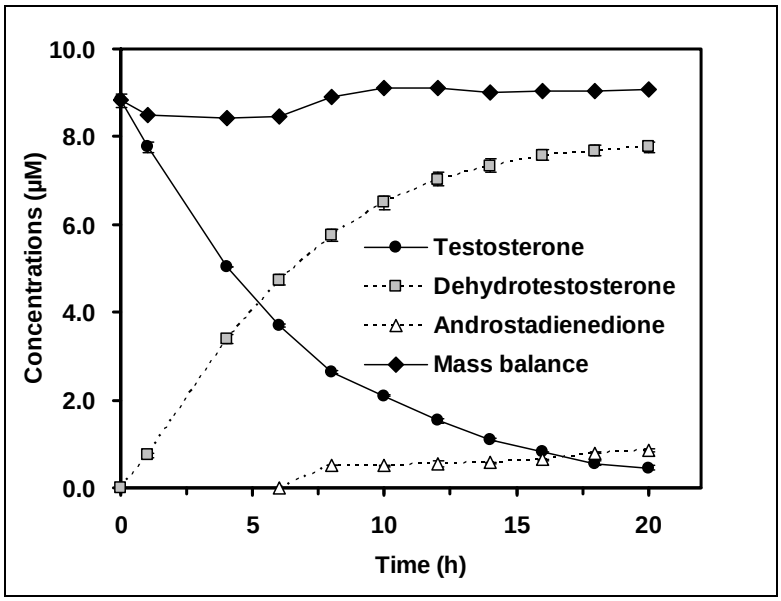


FIGURE 9. Degradation of testosterone and formation of degradation products under aerobic conditions in *system 2* (22°C; pH 7). Error bars represent the standard deviation of triplicate samples.

Collectively, the biodegradation experiments demonstrate that steroid sex hormones can be degraded by manure-borne bacteria, as hypothesized.

Photodegradation Experiments

Photodegradation of Testosterone, 17β-estradiol, and Progesterone in UV-A Light (λ > 340nm)

During the initial experiments, direct photodegradation of testosterone ($t_{½}$ = 50) and progesterone ($t_{½}$ = 79) was observed in buffered water, and indirect photodegradation of testosterone ($t_{½}$ = 17) and 17β-estradiol ($t_{½}$ = 3.3) was observed in the presence of 5 mg/L humic acid (Figure 10). Degradation was not observed in dark controls. Although nitrate is a major source of hydroxyl radicals in natural waters, 10 mg/L nitrate did not appear to affect steroid sex hormone photodegradation [129].

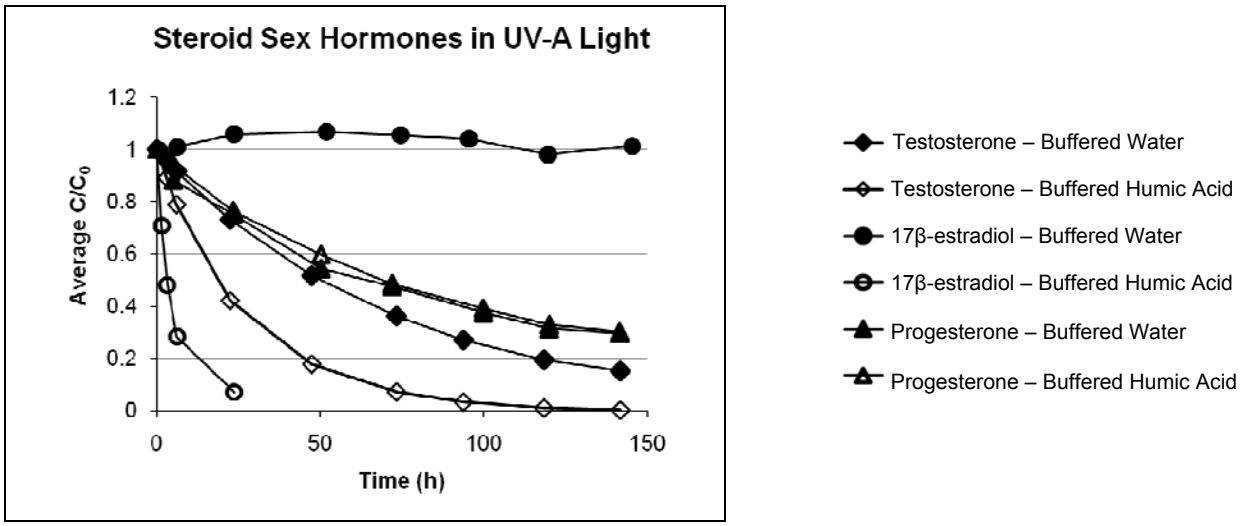


FIGURE 10. Photodegradation of testosterone, 17β-estradiol and progesterone by UV-A light (Rayonet, λ > 340 nm) in buffered water and buffered humic acid.

Photodegradation of Androstenedione in UV-A Light (λ > 310nm)

During the subsequent experiments, direct photodegradation of androstenedione was observed ($t_{½}$ = 1.7 h), but the rate of photodegradation decreased in the presence of SRFA ($t_{½}$ = 2.0 h) and SRHA ($t_{½}$ = 2.1 h). Data from the subsequent experiments did not readily fit a first order kinetics model, as expected, possibly due to interference by photochemical reaction products (Figure 11). Therefore, first order degradation rates and half-lives of androstenedione were estimated with data from the first 5 hours of the experiments [145-146]. Degradation was not observed in dark controls.

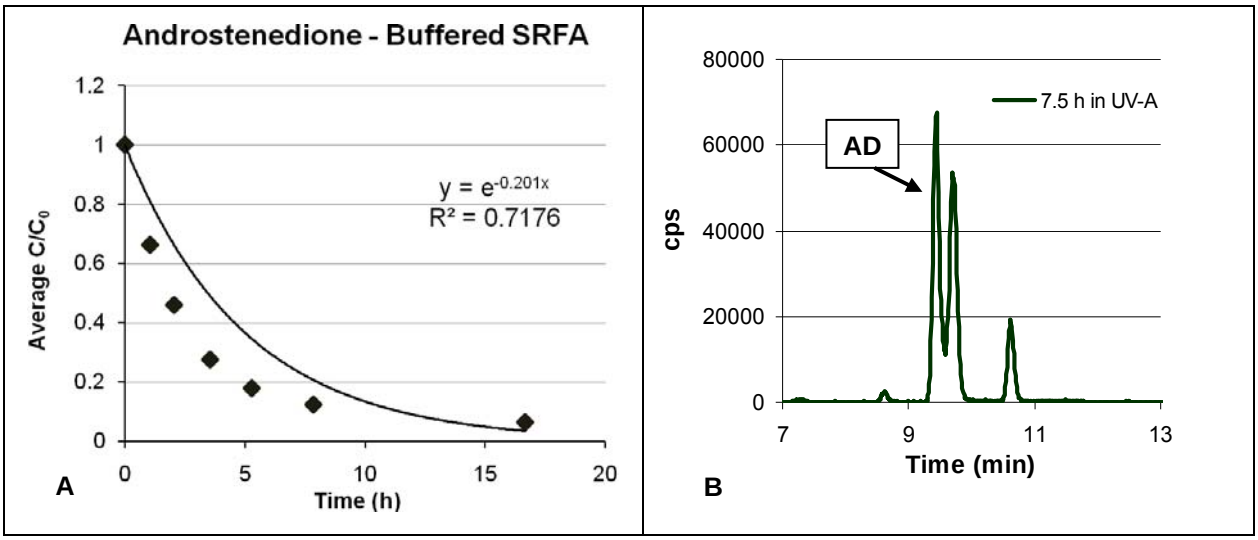


FIGURE 11. (A) Photodegradation of androstenedione (AD) by UV-A light (Luzchem, $\lambda > 310$ nm) in buffered SRFA; (B) chromatogram of primary MRM transition (m/z 287 → m/z 97) used in LC-MS/MS analysis of androstenedione.

In buffered water, direct photodegradation of androstenedione occurred substantially faster ($t_{½}$ = 1.7 h) than direct photodegradation of testosterone in the initial experiments ($t_{½}$ = 50 h). This difference is likely attributable to differences in the lamps that were used, because the UV absorption spectra of androstenedione (Figure 2) and testosterone overlap with the emission spectrum of the Luzchem lamps (Figure 4) much more than the emission spectrum of the Rayonet lamps (Figure 3). Differences in the lamps also might explain why indirect photodegradation of testosterone was observed in the presence of Elliot Soil humic acid, and photodegradation of androstenedione decreased in the presence of SRFA and SRHA. Under the Luzchem lamps, direct photodegradation was substantially faster, and SRFA and SRHA might have inhibited direct photodegradation, through light attenuation, more than they contributed to indirect photodegradation [126, 149-150]. Alternatively, differences in pH (5.5 in the initial experiments vs. 8.0 in the subsequent experiments) might have affected the rate of indirect photodegradation. Although humic substances generally absorb more light with increasing pH, stronger sorption of steroid sex hormones to organic matter has been observed at acidic pH, and higher concentrations of singlet oxygen (a reactive oxygen species associated with indirect photodegradation) have been observed near the surface of dissolved organic matter, where sorption would be expected to occur [97, 102, 130]. Research on the effects of pH, singlet oxygen, and other environmental factors is ongoing.

Subsequent method development has revealed that photochemical reaction products were interfering with androstenedione's quantification during the subsequent experiments, as suspected. After a revised method was created with a more efficient chromatographic column (incorporating smaller diameter, solid core particles), 7 chromatographic peaks were resolved

from the 7.5 h sample that originally yielded 4 chromatographic peaks (Figure 12(B)). After the revised method was optimized to achieve apparent baseline separation of androstenedione (Figure 12(B)), androstenedione was no longer represented by the most prominent peak in the chromatogram, and the data better fit a first order kinetics model (Figure 12(A)).

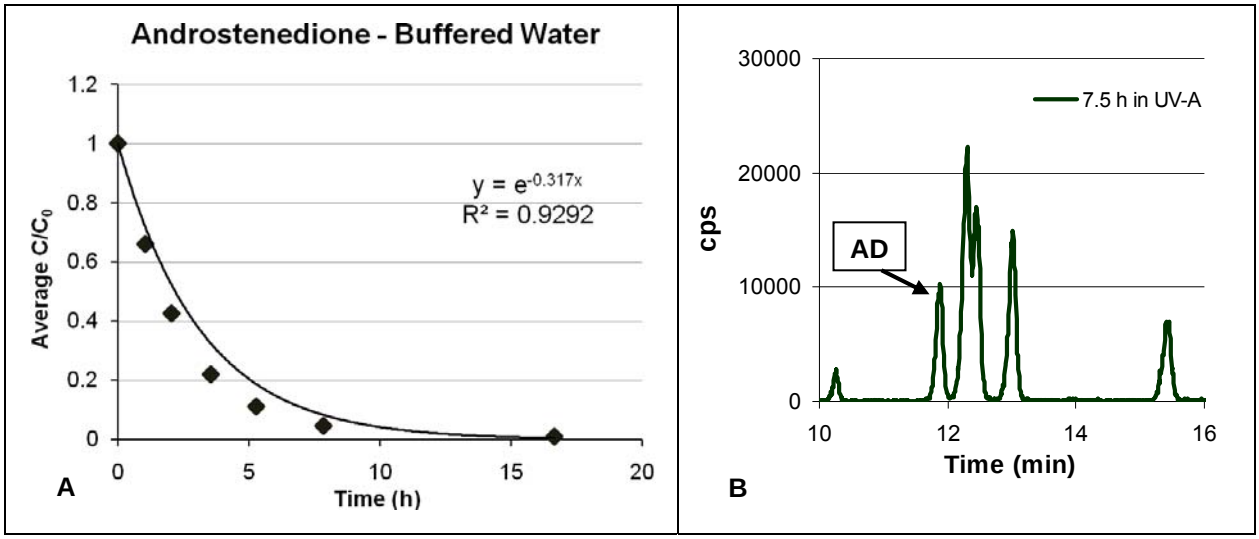
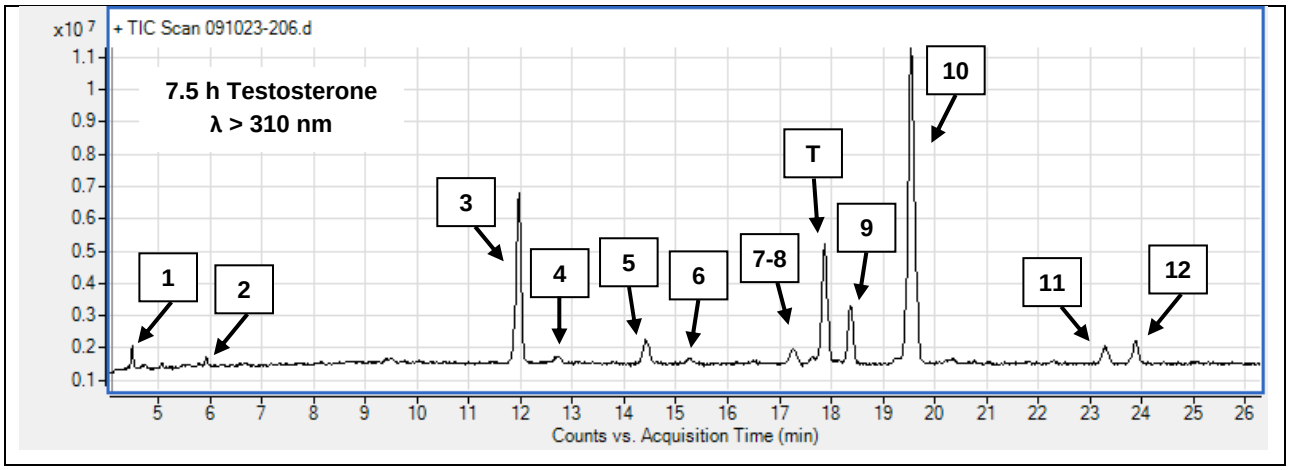


FIGURE 12. (A) Photodegradation of androstenedione (AD) by UV-A light (Luzchem, λ > 310 nm) in buffered SRFA under revised chromatography method; (B) chromatogram of primary MRM transition (m/z 287 → m/z 97) used in LC-MS/MS analysis of androstenedione under revised chromatography method.

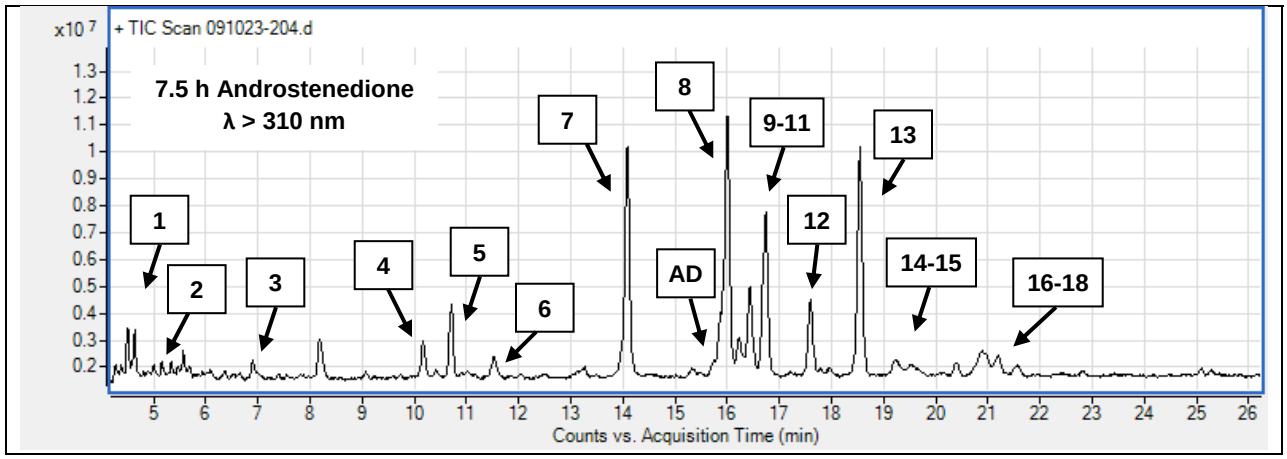
Photodegradation Products of Androstenedione and Testosterone in UV-A Light (λ > 310nm)

Numerous photochemical reaction products were observed after 10 mg/L aqueous solutions of androstenedione and testosterone were exposed to UV-A light for 7.5 h under the Luzchem lamps (λ > 310 nm) (Figures 13 and 14).



Peak	t_R	Proposed Formula	Proposed ID	Ions Detected (Absolute Mass Error in ppm) MH^+	MNH_4^+	MNa^+
Testosterone						
1	4.50	$C_{18}H_{28}O_5$	Unknown	1.16	–	0.70
2	5.92	$C_{18}H_{26}O_4$	Unknown	1.02	–	0.99
3	11.96	$C_{19}H_{30}O_3$	Testosterone + H_2O	–	0.52	0.35
4	12.69	$C_{19}H_{30}O_3$	Testosterone + H_2O	–	2.46	1.62
5	14.41	$C_{19}H_{28}O_4$	Testosterone + O_2	0.39	–	–
		$C_{20}H_{30}O_4$	Testosterone +O_2 + MeOH – H_2O	1.39	–	–
		$C_{20}H_{32}O_5$	Testosterone +O_2 + MeOH	–	–	1.41
6	15.26	$C_{19}H_{30}O_4$	Testosterone + H_2O_2	0.66	–	1.67
7	17.26	$C_{19}H_{30}O_3$	Testosterone + H_2O	0.37	–	0.76
		$C_{19}H_{28}O_4$	Testosterone + O_2	0.54	–	–
		$C_{20}H_{30}O_4$	Testosterone +O_2 + MeOH – H_2O	0.36	–	–
		$C_{20}H_{32}O_5$	Testosterone + O_2 + MeOH	–	–	0.70
8	17.62	$C_{19}H_{26}O_3$	Testosterone + O_2 – H_2O	0.92	–	–
		$C_{20}H_{30}O_4$	Testosterone +O_2 + MeOH – H_2O	0.58	–	0.02
T	17.89	$C_{19}H_{28}O_2$	Testosterone	1.91	–	0.37
9	18.37	$C_{19}H_{30}O_3$	Testosterone + H_2O	0.13	1.06	0.45
10	19.54	$C_{19}H_{28}O_2$	Testosterone Isomer	1.06	–	0.40
11	23.30	$C_{19}H_{28}O_2$	Testosterone Isomer	0.18	0.60	0.32
12	23.88	$C_{19}H_{28}O_2$	Testosterone Isomer	0.09	0.26	0.54

FIGURE 13. LC-TOF MS ESI^+ total ion chromatogram after exposure of testosterone (T) to UV-A light for 7.5 h, and table of associated data.



Peak	t_R	Proposed Formula	Proposed ID	Ions Detected (Absolute Mass Error in ppm) MH^+	MNH_4^+	MNa^+
Androstenedione						
1	4.64	$C_{18}H_{26}O_5$	Unknown	1.28	0.38	2.00
2	5.15	$C_{18}H_{24}O_4$	Unknown	1.26	–	0.80
3	6.89	$C_{19}H_{28}O_3$	Androstenedione + H_2O	0.28	–	1.26
4	10.15	$C_{19}H_{30}O_4$	Androstenedione + 2 H_2O	–	1.16	0.01
5	10.70	$C_{19}H_{26}O_4$	Androstenedione + O_2	–	–	0.01
		$C_{19}H_{28}O_3$	Androstenedione + H_2O	–	0.43	0.24
6	11.51	$C_{19}H_{28}O_3$	Androstenedione + H_2O	0.22	–	1.26
7	14.09	$C_{19}H_{28}O_3$	Androstenedione + H_2O	3.51	0.13	0.29
AD	15.88	$C_{19}H_{26}O_2$	Androstenedione	1.64	–	1.47
8	16.01	$C_{19}H_{26}O_2$	Androstenedione Isomer	1.85	–	0.01
9	16.22	$C_{19}H_{26}O_2$	Androstenedione Isomer	1.11	–	0.73
10	16.44	$C_{19}H_{26}O_2$	Androstenedione Isomer	0.9	–	0.08
11	16.74	$C_{19}H_{26}O_2$	Androstenedione Isomer	1.15	–	0.4
		$C_{20}H_{32}O_4$	Androstenedione + H_2O + MeOH	–	–	1.48
12	17.60	$C_{19}H_{28}O_3$	Androstenedione + H_2O	0.56	0.67	0.88
13	18.54	$C_{19}H_{26}O_2$	Androstenedione Isomer	0.43	–	0.32
14	19.24	$C_{20}H_{30}O_3$	Androstenedione + MeOH	–	–	0.41
15	19.53	$C_{20}H_{30}O_3$	Androstenedione + MeOH	–	–	0.98
16	20.91	$C_{20}H_{30}O_3$	Androstenedione + MeOH	0.35	–	1.18
17	21.20	$C_{20}H_{30}O_3$	Androstenedione + MeOH	–	–	0.54
18	21.55	$C_{20}H_{30}O_3$	Androstenedione + MeOH	0.72	–	0.99

FIGURE 14. LC-TOF MS ESI^+ total ion chromatogram after exposure of androstenedione (AD) to UV-A light for 7.5 h, and table of associated data.

In general, the direct photodegradation products of androstenedione and testosterone were similar. Each produced several isomeric compounds with the same molecular formula, and

each produced several photoaddition products incorporating oxygen and solvent molecules. Androstenedione produced more isomers, which suggests that the ketone at carbon 17 is involved in photochemical isomer formation [151]. Although the molecular structures of the photochemical reaction products have not been identified, the data suggest that the observed direct photodegradation products are the result of rearrangement and photoaddition reactions, and may retain their steroid structure and potential for endocrine disruption (Figure 15) [30, 148]. Over time, however, the observed reaction products diminished upon continued exposure to UV-A light (Luzchem, $\lambda > 310$ nm). Work to identify the molecular structures and photochemical fate of the photodegradation products is continuing.

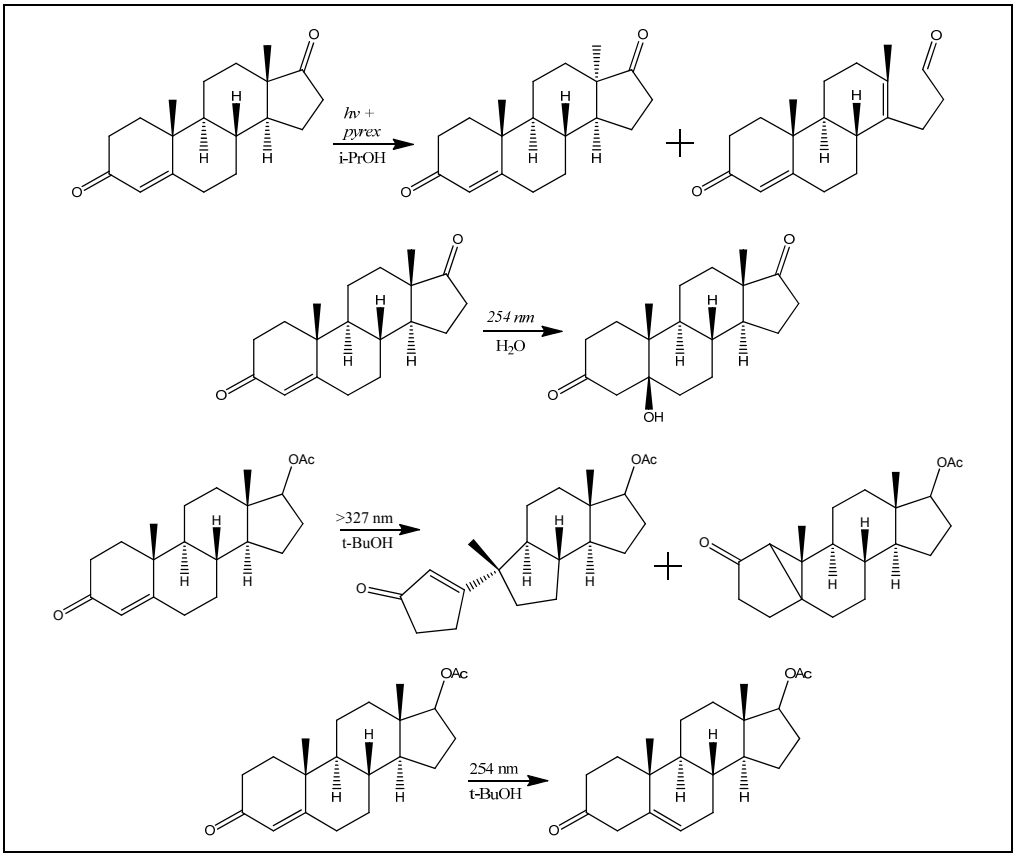


FIGURE 15. Molecular structures of potential androstenedione and testosterone photodegradation products that have been identified in prior published research [151-153].

Photodegradation of Androstenedione in Natural Sunlight

Direct photodegradation of androstenedione was observed qualitatively in natural sunlight. Future experiments will be conducted to quantify the rates of degradation in natural sunlight, after accounting for variations in solar irradiance [154].

Collectively, these data suggest that sunlight can directly or indirectly degrade steroid sex hormones in natural waters, as hypothesized. However, the efficiency of their photodegradation will vary with the water's depth, attenuation coefficient, and other environmental factors.

Runoff Study

Runoff Rates and Concentrations during a Single Simulated Rainfall Event

In general, runoff rates in the experimental plots increased gradually to a steady maximum rate (e.g., after 20 to 25 min in plot 1), and then decreased gradually during the remainder of the simulated rainfall event (Figure 16).

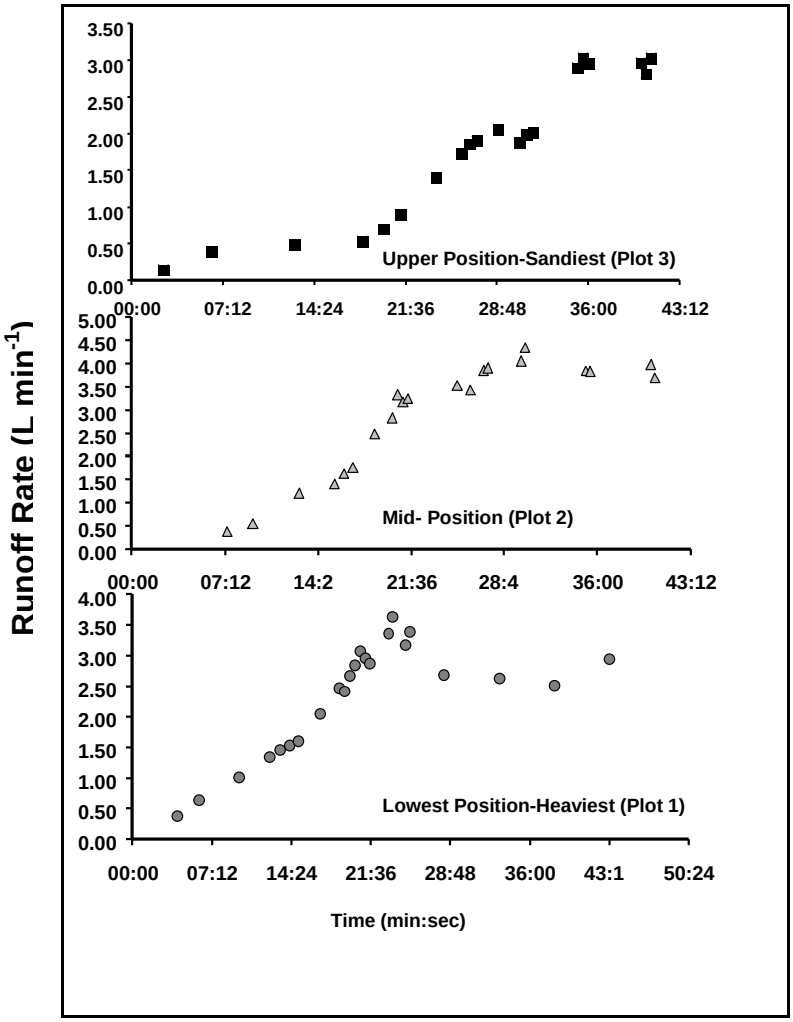


FIGURE 16. Hydrographs for the three investigated field plots obtained during the simulated rainfall event 8 days after biosolids application.

During the simulated rainfall event 1 day after biosolids application, androgen runoff concentrations generally appeared to follow runoff rates, peaking at the maximum runoff rate (Figure 17). In contrast, estrone runoff concentrations tended to increase with time throughout the simulated rainfall event, possibly because of differences in water solubility and sorption behavior (Figure 17(A)). For example, androstenedione, testosterone, and cis-androsterone are more water soluble, and have smaller octanol-water partition coefficients (which often correlates to sorption behavior), than estrone [155].

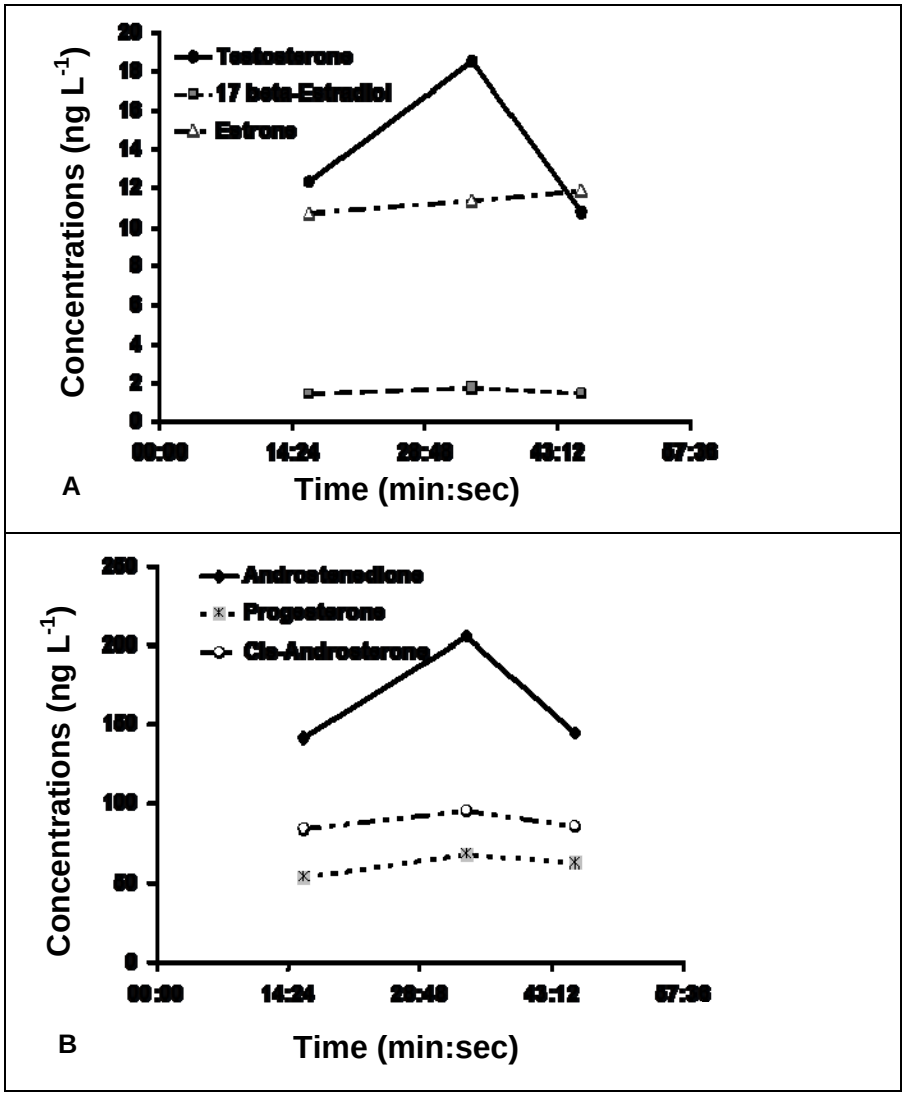


FIGURE 17. Concentrations of steroid sex hormones in whole water runoff samples collected from plot 1 during the simulated rainfall event 1 day after biosolids application.

Runoff Concentrations during a Series of Simulated Rainfall Events

Three compounds were detected in whole-water runoff samples prior to biosolids application: androstenedione (1.5 ng L^{-1}), estrone (2.2 ng L^{-1}), and cholesterol (41 µg L^{-1}) (Table 4).

TABLE 4. Maximum analyte concentrations in whole water runoff samples collected (A) from plots 1, 2, and 3 during the simulated rainfall events 3 days before, and 1 and 8 days after, biosolids application; and (B) from plots 1 and 2 during the simulated rainfall event 35 days after biosolids application. (The maximum analyte concentration collected from plots 4 and 5 during the simulated rainfall event 35 days after biosolids application is in parenthesis).

		Maximum concentration (ng L^{-1})			
Chemical	**RL (ng L^{-1})**	**3 Days before**	**Day 1**	**Day 8**	**Day 35**
Testosterone	0.8	< RL	20.8	9.4	3 (38.6)
Epitestosterone	4	< RL	16.9	< RL	6 (2.9)
Androstenedione	0.8	1.5	216.1	78.5	34.3 (491.3)
Cis-androsterone	0.8	< RL	174.8	82.5	20.1 (323.7)
Dihydrotestosterone	4	< RL	40.8	17.8	< RRL (81.2)
11-Ketotestosterone	2	< RL	7.5	< RL	< RRL (< RRL)
17α-Estradiol	0.8	< RL	1.9	51.9	2.7 (4.5)
17β-Estradiol	0.8	< RL	2.4	5.9	1.1 (7.2)
Estrone	0.8	2.2	13.8	25	16.2 (24.1)
Estriol	2	< RL	3.6	2.9	2.1 (1.7)
Equilin	4	< RL	< RL	9.1	< RRL (<RRL)
Progesterone	8	< RL	98.9	15.6	48 (218.8)
Coprostanol	2000	< RL	3.9×10^5	9.2×10^4	7.8×10^4 (9.3×10^4)
Cholesterol	2000	4.1×10^4	2.7×10^5	9.5×10^4	7×10^4 (9.4×10^4)

After biosolids application, the highest runoff concentrations of androgens and progesterone occurred during the first simulated rainfall event (Table 4; Figure 18). For example, in plot 1, the runoff concentrations of testosterone, dihydrotestosterone, and androstenedione decreased by 53 to 75% during the second simulated rainfall event after biosolids application, and decreased again by more than 41% during the final simulated rainfall event (Figure 18(A)

and (B)). Conversely, in plot 1, the runoff concentrations of estrone and 17β-estradiol increased by more than 30% during the second simulated rainfall event after biosolids application, and then decreased by more than 40% during the final simulated rainfall event. Finally, progesterone runoff concentrations were dramatically reduced from 62 to 3 ng L^{-1} during the second simulated rainfall event after biosolids application, and then increased to 35 ng L^{-1} during the final simulated rainfall event. Similar results were observed in plot 2 (Figure 18(C) and (D)). The delayed maximum in estrogen runoff concentrations generally followed estrone's behavior during the single simulated rainfall event, where estrone's runoff concentration continued to increase after the maximum runoff rate occurred (Figure 17).

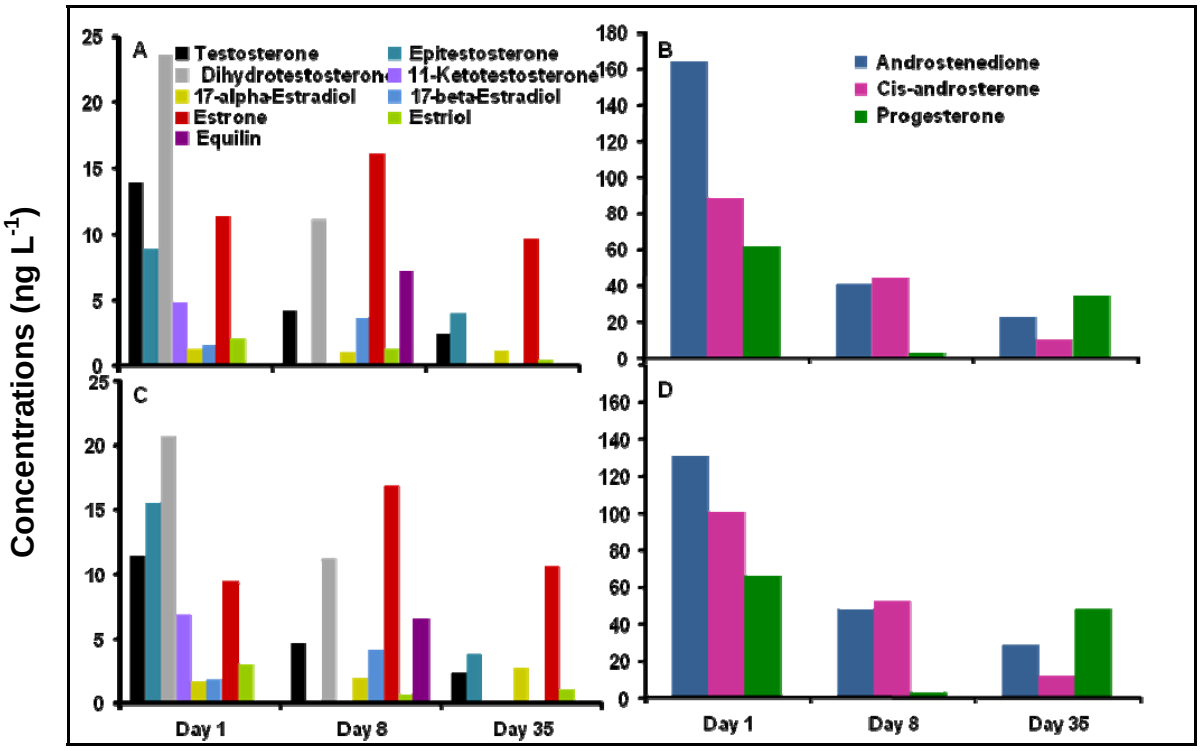


FIGURE 18. Steroid sex hormone concentrations in whole water runoff samples collected from plots 1 (A and B) and 2 (C and D) during the first, second, and third simulated rainfall events after biosolids application. The values represent the average concentration of three samples collected during each simulated rainfall event.

Influence of Rainfall Frequency and Time between the Biosolids Application and the First Simulated Rainfall Event

Runoff samples collected from plot 4 during the simulated rainfall event 35 days after biosolids application (the first simulated rainfall event on plot 4 after biosolids application) had higher concentrations for most hormones (except epitestosterone and 17α-estradiol) than runoff samples collected from plot 1 at the same time (the third simulated rainfall event on plot 1 after biosolids application) (Figure 19).

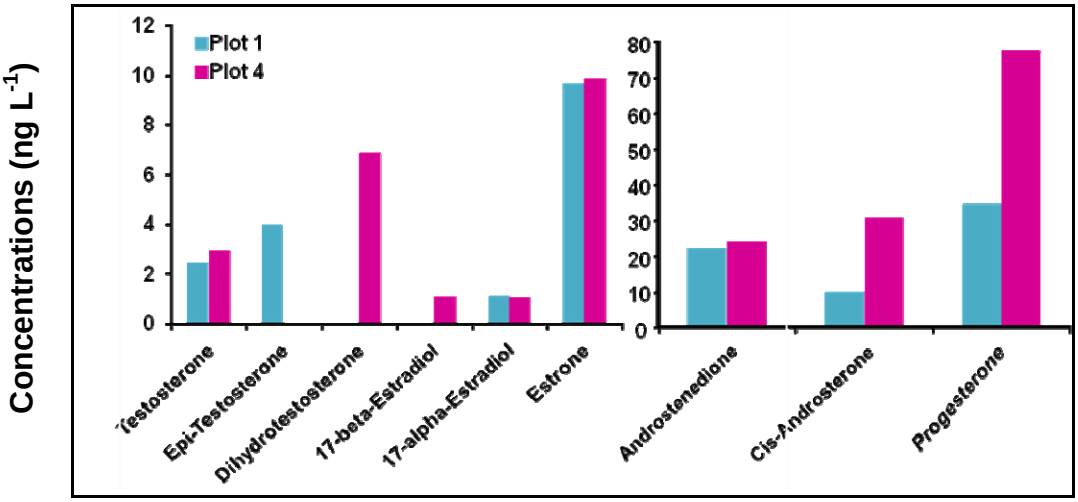


FIGURE 19. Steroid sex hormone concentrations in whole water runoff samples collected from plots 1 and 4 during the simulated rainfall event 35 days after biosolids application. The values represent the average concentration of the three samples collected per simulated rainfall event.

Runoff Concentrations in the Particle and Filtrate Fractions

Steroid sex hormone concentrations in the whole-water (unfiltered) and filtrate fractions were compared to determine the distribution of steroid sex hormones between the particle and filtrate fractions, and the weight percent of the particle fraction (particles > 0.7 µm) in the whole-water runoff samples was determined gravimetrically. Among analytes, distribution between the particle fraction (filtrand) and the filtrate (including particles < 0.7 µm) varied (Figure 20). For example, approximately 40 to 50% of testosterone and androstenedione were detected in the filtrate, while 67 to 72% of progesterone and 17β-estradiol were attributable to the particle fraction.

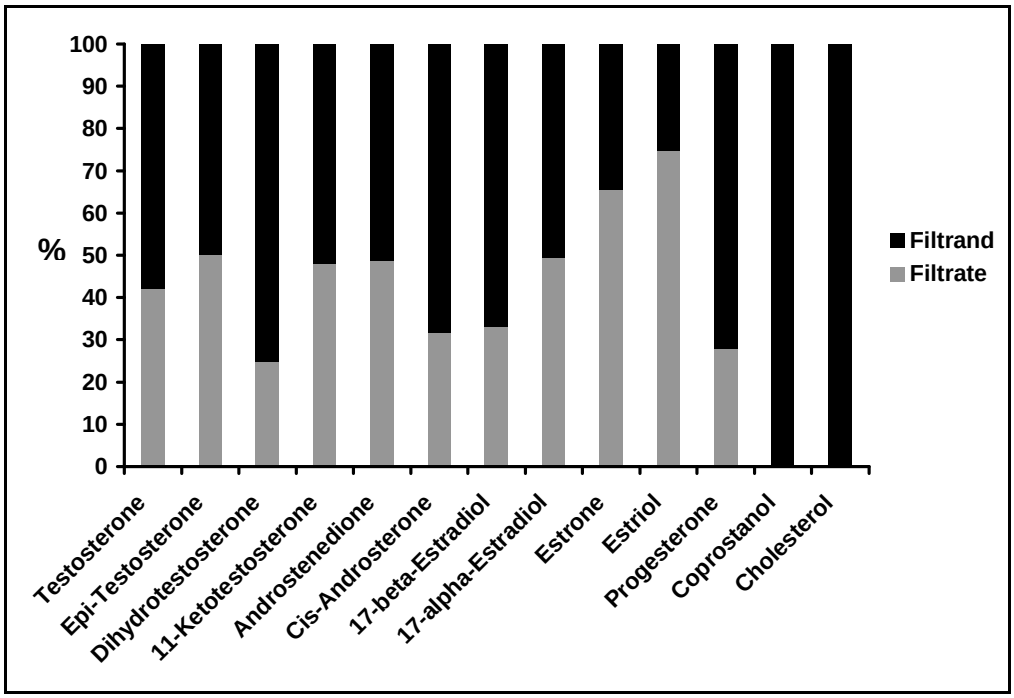


FIGURE 20. Percentages of analytes in the filtrate (including < 0.7 µm particles) and particle fraction (filtrand) in runoff samples collected from plot 1 during the first simulated rainfall event after biosolids application. The weight percent of the particle fraction in the whole-water runoff samples was 0.3 to 2.5%. The values represent the average percentages of three samples collected in the beginning, middle, and end of the simulated rainfall event.

In general, androgen concentrations were higher in the filtrate, which is consistent with their behavior during the simulated rainfall events, where androgen concentrations followed runoff rates (Figure 17) and decreased after each simulated rainfall event (Table 4). However, estrone concentrations were also higher in the filtrate, and estrone appeared to be retained longer during the simulated rainfall events (Figure 17; Table 4).

Sample and data analysis are continuing, but overall the data suggest that the mobility of steroid sex hormones and their degradation products will vary considerably, depending on the compound's identity, soil properties, and other environmental factors.

Principal Findings, Conclusions and Recommendations

River Study

The study has determined that steroid sex hormones are present in the Cache la Poudre River, as hypothesized, at concentrations ranging from 0.6 ng L^{-1} (epitestosterone) to 22.6 ng L^{-1} (estrone), which are sufficient to create some risk of endocrine disruption. Several of the detected compounds (i.e., androstenedione, estrone, and estriol) are known steroid sex hormone degradation products. Additional research is needed to better understand the impact of steroid sex hormones in the Cache la Poudre River. Studies should be conducted to detect the presence and potential sources of other known degradation products (e.g., androstadienedione and dehydrotestosterone) and synthetic hormones commonly used in agricultural operations (i.e., trenbolone and melengestrol). In addition, studies should be conducted to examine the temporal variability of steroid sex hormone concentrations in the Cache la Poudre River, and to look for evidence of endocrine disruption. Currently, this research is ongoing.

Biodegradation Experiments

The study has determined that steroid sex hormones can be degraded by manure-borne bacteria, as hypothesized. Specifically, the study has determined that testosterone, progesterone, and 17β-estradiol can be degraded by manure-borne bacteria, and that testosterone degradation is faster under aerobic conditions and at higher temperatures (i.e., 37°C vs. 22°C), but little affected by changes in pH (from 6 to 7.5) or glucose amendments. In addition, the study has detected and identified three testosterone degradation products (dehydrotestosterone, androstenedione, and androstadienedione). Additional research is needed to identify other steroid sex hormone degradation products, and to characterize the long-term degradation pathways and bacterial species involved in steroid sex hormone biodegradation. Currently, this research is ongoing. Additional research is also needed to determine the waste management practices that will provide optimal conditions for biodegradation by manure-borne bacteria.

Photodegradation Experiments

The study has determined that sunlight can directly or indirectly degrade steroid sex hormones in natural waters, as hypothesized. However, the efficiency of their photodegradation will vary with the water's depth, attenuation coefficient, and other environmental factors. In ultraviolet light $\lambda > 340$ nm, the study observed direct photodegradation of testosterone and progesterone, and indirect photodegradation of testosterone and 17β-estradiol in the presence of Elliot soil humic acid. On the other hand, in ultraviolet light $\lambda > 310$ nm, direct photodegradation of androstenedione was substantially faster than direct photodegradation of testosterone in ultraviolet light $\lambda > 310$ nm, and no indirect photodegradation observed. These

differences are likely attributable to differences in the lamps used, but could also reflect the changes that will occur with depth in the water column, as sunlight's intensity and spectral distribution changes. The study detected several products of testosterone and androstenedione photodegradation which appear to retain their steroid structure, and possibly their endocrine disrupting potential. Additional research is needed to reveal the identities and environmental fates of the observed photodegradation products, to examine the effects of pH and other environmental conditions on steroid sex hormone photodegradation rates, and to quantify such rates in natural sunlight and natural waters. Currently, this research is ongoing. Additional research is also needed to determine if photodegradation can be efficiently incorporated into waste management practices (e.g., by incorporating a shallow, open water section into constructed wetlands).

Runoff Study

During the study, androgen runoff concentrations were generally observed to follow runoff rates and decrease after each simulated rainfall event, but runoff concentrations of other analytes (e.g., estrone) peaked after the maximum runoff rate or first simulated rainfall event following biosolids application, possibly because of differences in water solubility and sorption behavior. Sample and data analysis are continuing, but overall the data suggest that the mobility of steroid sex hormones and their degradation products will vary considerably, depending on the compound's identity, soil properties, and other environmental factors.

Summary

The study did find that steroid sex hormones are present in the Cache la Poudre River, and that steroid sex hormones can be degraded by sunlight and manure-borne bacteria. As a result, it should be possible to develop management practices for producers and water managers to minimize the risks of endocrine disruption from wastewater, runoff from agricultural fields, and other potential sources of exogenous steroid sex hormones. Sample and data analysis from the study are continuing, however, and comprehensive finding and recommendations are expected after the date of this report.

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