University of Nevada, Reno

Environmental Fate of Trenbolone Acetate Used in Animal Agriculture: Mechanistic Studies of Hormone Biodegradation in Various Redox Conditions

A thesis submitted in partial fulfillment of the

requirements for the degree of Master of Science in

Civil and Environmental Engineering

by

Jaewoong Lee

Dr. Edward P. Kolodziej/Thesis Advisor

December, 2011

University of Nevada, Reno
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We recommend that the thesis
prepared under our supervision by

JAEWOONG LEE

entitled

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be accepted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

Edward P. Kolodziej, Advisor

Eric A. Marchand, Committee Member

Glenn C. Miller, Graduate School Representative

Marsha H. Read, Ph. D., Associate Dean, Graduate School

December, 2011

Abstract

Recent studies have been reported that natural and synthetic hormones are major endocrine-disrupting contaminants (EDCs) that can cause contaminant-associated reproductive and developmental alternation on aquatic organisms. In particular, synthetic steroids such as trenbolone acetate (TBA) are widely used as growth promoters in beef cattle. It has been reported that livestock excrete potential endocrine disrupting hormones such as the TBA metabolites (17α-trenbolone, 17β-trenbolone, and trendione) into aquatic environments. Although livestock likely contain much higher levels of steroid contaminants due to the increased use of growth-promoting hormones, relatively little is known about the fate of these synthetic steroids under various aquatic environments. Therefore, this study undertook a series of experiments using biodegradation microcosms to develop a mechanistic understanding of the fate of TBA metabolites in various redox conditions. Redox potentials were controlled by using reagents (titanium(III) citrate, L-cysteine, and dithiothreitol) to develop anaerobic (E_h < -400 mV), anoxic (-400 mV < E_h < -200 mV), and suboxic (-200 mV < E_h < 0 mV) conditions. In aerobic conditions (E_h > 100 mV), although abiotic losses in sterile controls were observed for three androgen steroids, 17α-trenbolone and 17β-trenbolone were degraded by as much as 80% and 40% by biotic processes over 10 days. However, trendione degraded by only 20% biotic processes during 3 days, with slight increased after 3 days. Half-lives of 17α-trenbolone, 17β-trenbolone, and trendione were 0.9, 1.5, and 2.7 days in aerobic experiments, respectively. In anaerobic (-438±149 mV), anoxic (-269±45 mV), and suboxic (-100±22 mV) conditions, losses by abiotic processes were observed for all three conditions. In anaerobic and anoxic conditions, observed losses of 17α-trenbolone, 17β-trenbolone, and

trendione in sterile controls were around 70% and biotic processes were only responsible for about 20% of attenuation. In suboxic conditions, losses of 17α-trenbolone and 17β-trenbolone were nearly 30% in sterile controls, and observed biotic processes may have contributed 20% to degradation. Half-lives of 17α-trenbolone, 17β-trenbolone, and trendione were 19.2, 8.8, and 7.8 days in anaerobic conditions, respectively. In anoxic conditions, half-lives of 17α-trenbolone and 17β-trenbolone were 3.3 days and 3.2 days, and in suboxic conditions half-lives of 17α-trenbolone, 17β-trenbolone, and trendione were 2.7, 2, and 3.6 days. Although uncertain due to the losses in sterile controls, ORP (oxidation-reduction potential) values obviously affect biodegradation rates for all three androgen metabolites. From the data, aerobic conditions seem to be more favorable conditions than suboxic, anoxic, and anaerobic conditions for 17α-trenbolone, 17β-trenbolone, and trendione degradation.

Acknowledgements

Firstly, I would like to acknowledge my advisor while I studied in graduate school at UNR, Dr. Edward Kolodziej. He has not been the best advisor and excellent teacher but also a patient and supportive advisor to me. I learned incredible knowledge from research and classes with you and it was really good experience to develop my research skill and understand water chemistry. Thank you for all things that you did for me. I would also like to thank committee, Dr. Eric Marchand and Dr. Glenn Miller, that you gave me excellent advice and support to my thesis.

I would like to appreciate my research group member, Gerrad, Stephanie, Victoriya. Without your wonderful help in lab research, I can't image to finish my thesis today. I would also like to thank other faculty members, graduate students and staff members while I studied in Civil and Environmental Engineering. I hope to see all my UNR collaborators in the future as long as we serve in environmental research areas.

I would like to acknowledge my family, my spouse, Sukhyun Lee and my lovely two sons, Geonho Lee and Eunho Lee for their full support and love while my graduate studies. Last, I would like to acknowledge my parents, Eulhee Lee and Heesoon Lee for your love and faith throughout all my life. All glories to my God, Jesus...

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Chapter 1: Introduction

1. Introduction

1.1. Environmental Endocrine Disrupting Contaminants (EDCs)

There are many chemicals present in aquatic environments capable of causing contaminant-associated reproductive and developmental impacts on wildlife. These contaminants are often called endocrine disrupting contaminants (EDCs) (Lee et al., 2002, Sone et al., 2005). Generally, an environmental endocrine disruptor is defined as "an exogenous agent that interferes with the synthesis, storage/release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (www.epa.gov/endocrine/, Schiffer et al., 2004). These compounds originate from various sources, and they include synthetic compounds such as organochlorine pesticides, alkylphenolic compounds, and natural and synthetic hormones (Robinson et al., 2009 and Esperanza et al., 2007). EDCs have many different transport pathways to water bodies but studies demonstrate that the primary EDC sources are municipal wastewater treatment effluents and runoff from farmlands with animal agriculture (Esperanza et al., 2007). The first studies about environmental endocrine disrupting pollutants were conducted in the United Kingdom, where it was reported that EDCs caused feminization of both feral and caged fish. Masculinization of fish because of androgenic compounds in paper and pulp effluents has also been reported (Howell et al., 1980, Larsson et al., 2000, Orlando et al., 2004). Several studies suggested that estrogenic hormones present in sewage treatment effluent can disrupt endocrine function, leading to observations such as vitellogenin production in male fish (Jobling et al., 1998). Also, it has been reported that

livestock excrete potential endocrine disrupting hormones such as estrogens, androgens and progestins in urine and feces (Lange et al., 2002), which are regarded as a potentially significant source (Matthiessen et al., 2006, Johnson et al., 2006). Studies report that some EDCs, especially synthetic hormones originating from animal manure, have persistent tendencies in the aquatic environment, increasing their risk to aquatic organisms (Lee et al., 2001). Thus, there are newly emerging EDCs in the aquatic environment from human and animal sources, and they may potentially have effects on natural ecosystem.

1.2. Growth-Promoting Compounds Uses

Growth-promoting hormones are used to increase the profitability of the cattle industry as they can induce the rapid growth of cattle (Sone et al., 2005). In the U.S.A, three synthetic hormones have been approved by the U.S. Food and Drug Administration for growth promotion in cattle and sheep, and most steers and heifers are implanted with synthetic or natural hormones. Generally, growth-promoting implants were formulations of the following natural and synthetic hormones: estradiol (E2), progesterone (P), testosterone (T) propionate, trenbolone acetate (TBA), melengestrol acetate (MGA) or zeranol (Kolok et al., 2008) (Table 1). Among these compounds, there are three synthetic hormones used widely for meat production in Unites States: the androgenic trenbolone acetate (TBA), the estrogenic zeranol, and the progestin melengestrol acetate (MGA) (Orlando et al., 2004).

Recently, many countries using growth-promoting compounds have become concerned about the potential for environmental exposures of synthetic growth-promoting hormones. Due to this reason, these compounds have been banned for use in livestock in the European Union since 1988 (Khan et al., 2008, Lange et al., 2002). However, according to the lobbying association "Cattlemen on the Hill", about 90 % of cattle were implanted with growth-promoters in the United States. Therefore, anabolic steroids such as growth-promoting compounds as well as natural estrogen hormones excreted from animal wastes would be present in aquatic environments potentially affecting aquatic organisms (Soto et al., 2004).

1.3. Biological Effects of TBA Metabolism

Some studies have reported that TBA metabolites can affect various endocrine processes in fish. Also, studies demonstrate the potential adverse effects of androgen exposure on aquatic organisms (Soto et al., 2004, Jensen et al., 2006). Jensen et al., (2006) indicated that the 17α-trenbolone and 17β-trenbolone could cause adverse effects on fish such as masculinization of female fish and decreased reproductive capacity of fathead minnow at exposure 100 ng/L and 27 ng/L, respectively. Orlando et al., (2003) observed significant alternation in their reproductive function after exposure to diluted CAFO effluent, such as demasculinization in male fathead minnows and defeminization of female fathead minnows. The feedlot effluent likely contained a mixture of natural endogenous steroids in fecal material and androgenic growth promoters used as growth implants. Also, Seki et al., (2006) reported that exposure to 1-10 µg/L of 17β-trenbolone

masculinized female mosquitofish, and exposure ≥ 40 ng/L of 17β-trenbolone can cause the reduction of plasma vitellogenin in female medaka, fathead minnow, and zebra fish. This evidence suggests that androgenic hormones such as TBA metabolites can affect reproductive and endocrine systems of aquatic organisms at low concentration. Therefore, we can assume that a large mass of synthetic or natural hormones can be released from animal wastes and transported into aquatic environments (Kolok et al., 2008), and these reproductive endocrine contaminants can threaten ecosystem health.

Table 1. Commercially available implant formulations currently used by the beef cattle industry (Kolok et al., 2008)

Compounds	Quantity in implant (mg)	Potency
Single compound implants		
Estradiol (E2)	24	Mild estrogen
TBA	140-200	Strong androgen
Zeranol	36-72	Mild, strong estrogen based on dose
Combination implants		
Estradiol benzonate/progesterone	10-20/100-200	Mild, strong estrogen based on dose Mild androgen
Estradiol benzonate/ Testosterone proprionate	20 200	Mild, strong androgen based on dose
Estradiol/TBA	8-28/40-200	

1.4. The Environmental Fate of Steroid Hormones

1.4.1. Sources and Levels of Steroid Hormones in Environment

The major source of steroid hormones to the environment is animal agriculture (Shore et al., 2003). For example, 32% of the land in the United States is used for

rangeland grazing, and grazing animals often have direct access to rivers, ponds, and creeks where they potentially can release high concentrations of steroids into these receiving waters (Kolodziej et al., 2007). Cattle excrete steroid hormones mainly in their fecal matter (58%) while swine and poultry excrete estrogens mostly in urine (96% and 69%, respectively) (Lucas et al., 2006). Excretion of steroid hormones in animal wastes is a process that is dependent on animal age, gender and reproductive state. Typical estrogen mass excretion range from 45-540 µg/d for cattle and 23-25 µg/d for sheep (Lange et al., 2002). The excretion of androgens by urinary testosterone in male veal calves and bulls is approximately 330 µg/d and 110 µg/d, respectively, while progesterone fecal excretion concentration can be as high as 95 µg/kg during the proliferative phase and 285 µg/kg during the secretory phase. Thus, endogenous hormones originating from livestock likely exist in surface water, and studies report that a variety of endogenous hormones are detected at low concentration (1 ng/L) in receiving waters impacted by animal agriculture (Shore et al., 2003, Lucas et al., 2006, Kolodziej et al., 2007).

The rapid growth of CAFO (concentrated animal feeding operations) based agriculture in United States has likely increased the mass transport of steroid hormones into aquatic environments (Shore et al., 2003). In the case of growth-promoting compounds, generally a combination of 20 mg of estrogens and 200 mg of androgens or progesterone is used in the implants. Therefore, if we assume that 8 % of the applied hormone dose reaches the aqueous environment, we can estimate that at least 1,000 kg of androgens (or progesterone) is excreted into the environment annually near animal

agriculture operations(Lange et al., 2002). Durhan et al., (2006) reported the metabolites of trenbolone acetate (17α-trenbolone, 17β-trenbolone) were present in receiving water near CAFO feedlot discharges at concentration ranging from <10 to 120 ng/L, from 10 to 20 ng/L, respectively. Soto et al., (2004) also reported the anabolic steroids 17α-trenbolone, 17β-trenbolone, and trendione, were detected in runoff water downstream of feedlots at concentration ranging from < 0.4 to 1.5 pg/L, from 1.6 to 5.4 pg/L, and from 1.9 to 7.6 pg/L, respectively. Thus estrogenic steroids and androgenic steroids may be excreted from feedlot discharges where animals were treated with steroid hormones into nearby receiving waters (Soto et al., 2004) where they may be possibly affect wildlife.

1.4.2. Transformations and Transport of Synthetic Hormones

Several research studies have highlighted the concerns about synthetic hormones because they have high physiological activities at low concentration. Concentration and persistence of hormones in receiving waters is dependent on excretion, breakdown to intermediate metabolites, and resistance to hydrolytic and oxidative biodegradation. Most importantly, synthetic hormones are more resistant to microbial degradation compared with natural hormones, but little information is known about the fate of many synthetic hormones originating from feedlots in aquatic environments (Lange et al., 2002). Hasse et al., (1982) and Rumsey et al., (1977) demonstrated that synthetic hormones were stable in liquid manure stored under anaerobic conditions. Similarly, Shiffer et al., (2001) also reported that the metabolites of TBA were quite stable in manure piles over 260 days.

Generally, growth-promoters can undergo several attenuation pathways after they are excreted into the environment (Table 2). These pathways include microbial transformation and mineralization in soil, abiotic transformation, sorption to solid phases, uptake in plants, photo transformation, and mobilization after heavy rainfall (Lange et al., 2002, Schiffer et al., 2004). Therefore, the expected exposure concentration of synthetic hormones to aquatic organisms would be lower than expected based solely on mass excreted due to the complex attenuation pathways (Lange et al., 2002). Soil processes may likely be the dominant attenuation pathways as animal waste disposal to agricultural lands is widespread. Although growth-promoters excreted from animal manure would undergo various attenuation pathways, they were still detected in receiving waters from feedlots, suggesting that these synthetic hormones may be persistent and accumulate in aquatic environments (Schiffer et al., 2001). But still, the relative contribution of different attenuation pathways to the fate of synthetic hormones in the environment is not fully understood (Lucas et al., 2006).

Table 2. The primary compounds excreted from beef cattle administered growth-promoting compounds (Kolok et al., 2008)

Administered compound	Primary excreted compounds
Estradiol	17-α Estradiol, estrone
Progresterone	Progesterone (urine)
	3α-Hydroxy-5β-pregnan-20β-one (fecal)
	5β-Pregnane-3-20β-diol (fecal)
Trenbolone acetate	17α-Trenbolone, 17β-Trenbolone
Melengestrol acetate	Melengestrol acetate

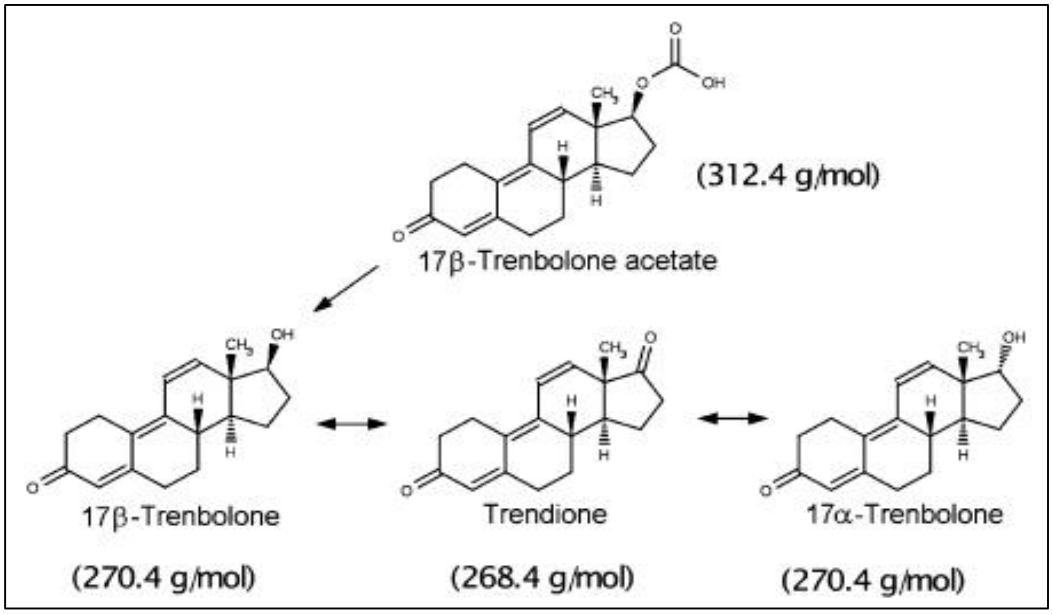


Figure 1. Trenbolone acetate (TBA) metabolism (Khan et al., 2008).

1.4.3. The fate of TBA in Environment

Among the various synthetic growth promoters, trenbolone acetate (TBA), approved as an androgenic and anabolic steroid, is most often implanted into cattle (Sone et al., 2005). Trenbolone acetate is rapidly hydrolyzed to produce the active form 17β-trenbolone, followed by oxidation to trendione and then reduction to 17α-trenbolone, with these last two steps hypothesized as reversible in the environment (Fig. 1) (Khan et al., 2008). Recent studies suggest that the metabolites of TBA, 17α-trenbolone and 17β-trenbolone, can be very stable in storage of liquid manure over 267 days (Schiffer et al., 2001) and these compounds were detected in discharge or runoff from a feedlot and in surface water (Durhan et al., 2006). Whereas the use of TBA was widespread, little information about the fate of metabolites of TBA in environment is known.

Degradation. Few studies have investigated the degradation of the synthetic steroid hormones trenbolone acetate (TBA) (Fig. 1) and melengestrol acetate (MGA) (Khan et al., 2008). For example, Schiffer et al., (2001) studied the residues and degradation of TBA and MGA in solid dung and liquid manure of TBA treated heifers during a 5.5-month storage period. During the liquid manure storage, the concentrations of 17α-trenbolone and 17β-trenbolone decreased from 1,700 to 1,100 pg/g and from 160 pg/g to 100 pg/g, respectively, with half lives of 267 days and 257 days. Meanwhile, the concentration of TBA and MGA in solid dung decreased from 5 - 75 ng/g to 10 ng/g for TbOH and from 0.3 to 8 ng/g for MGA. The data also suggest that these synthetic hormones were detected in soil due to their strong adsorption to soil particles, which immobilized them for several months until subsequent degradation by soil bacteria and/or

sun light (Lange et al., 2002). Khan et al., (2008) also studied the degradation of 17α-, 17β-trenbolone and trendione, in agricultural soils. They found that these synthetic androgens were degraded well in aerobic soil conditions, within 2 days, which was in contrast to the results of Schiffer et al., (2001). One explanation for this discrepancy is that the latter study experienced anaerobic conditions but the Khan et al., (2008) study environment was aerobic, indicating that the redox conditions may have played an important factor in synthetic hormone degradation. These studies also reported that higher concentrations of synthetic androgens decreased the degradation rates, similar to the results of Marcus et al., (1995), who demonstrated that higher concentrations of testosterone and related androgens reduced degradation rates.

Sorption. The partitioning and distribution of synthetic hormones in the environment is dependent on physicochemical properties such as soil type and specific hormone properties (Lucas et al., 2006, Ying et al., 2002). Manure from animals treated with growth-promoting compounds is often applied in agriculture land as a fertilizer, risking subsequent transport into receiving waters in proportion to the affinity of hormones with soil (Khan et al., 2009, 2010). Shore et al., (1993) and Ying et al., (2002) explained hydrophobic sorption mechanisms as dependent upon phenolic or ketone functinoal groups (Khan et al., 2009) in hormones, and high K_{oc} (or K_{ow}) (Table 3) values increased sorption to soil. In soil column studies by Schiffer et al., (2004), growth promoters exhibited substantial mobility in soils, but the binding of both hormones with organic matter in soil also was strong. Similarly, Khan et al., (2009) and Khan et al., (2010) demonstrated a strong relationship between K_d (L/kg) and % OC (organic carbon)

in soil (R^2 values > 0.97), and also that temperature and moisture content in soil affected both sorption and degradation.

Table 3. Summary of log K_{oc} and log K_{ow} for TBA (Khan et al., 2009)

Metabolite	Average ± SD[a]	
	log K_{oc}[b]	log K_{ow}[c]
17α-trenbolone	2.77 ± 0.12	2.72 ± 0.02
17β-trenbolone	3.08 ± 0.10	3.08 ± 0.03
Trendione	3.38 ± 0.19	2.63 ± 0.05

[a] Standard deviation. [b] Organic-carbon normalized sorption coefficient. [c] Octanol-water partition coefficient.

1.5. Key Research Questions

Significant amounts of natural and synthetic hormones are excreted from animals treated with growth promoters used to increase cattle production. Although a large mass of growth-promoting compounds would be present in the environment, little information about the fate of synthetic hormones is known. Studies need to investigate if synthetic hormones excreted from animal manure can remain in soil or be transported into ground water and surface water. Once synthetic hormones reach the aquatic environment, the fate of synthetic hormones is dependent upon photolysis, biodegradation, sorption and abiotic chemical transformation in receiving water. However, these processes are limited by certain agricultural systems which are likely to be anoxic or anaerobic. These aquatic environments may critically affect biodegradation rate of synthetic hormones because

redox states are unfavorable conditions for aquatic microbes. This is likely to reduce biotransformation rates and/or slow degradation of synthetic hormones in agricultural environments. Therefore, we should assess the specific effect of various redox states on environmental persistence of synthetic hormones.

1.6. Objectives

The purpose of this study is to evaluate the biodegradation rates of TBA metabolites under various redox conditions because redox conditions can affect the environmental fate of steroids across a range of environments (Jurgens et al., 2002, Kolodziej et al., 2004). The redox condition is one of the most important quantitative parameters in predicting the rate of TBA biodegradation because microbial activity may be strongly influenced by the redox environment. Therefore, this study consist of a series of batch degradation experiments determining the biodegradation rates of 17α-trenbolone, 17β-trenbolone, and trendione in a variety of aerobic and anaerobic conditions. These mesocosms will be controlled by adding redox-stabilizing chemicals (titanium (III) citrate, L-cysteine, and dithiothreitol(DTT)). From these biodegradation experiments, we can obtain the degradation rates of TBA metabolites under different redox conditions; also we can understand the role of redox conditions in biotic or abiotic processes in degradation of TBA. Biotic and abiotic degradation processes will be evaluated by comparison with control samples that are both non-sterile and autoclave sterilized.

Chapter 2: Manuscript draft

Environmental Fate of Trenbolone Acetate Used in Animal Agriculture: Mechanistic Studies of Hormone Biodegradation in Various Redox Conditions

Jaewoong Lee and Edward P. Kolodziej*

Department of Civil and Environmental Engineering, University of Nevada, Reno Mail Stop MS 0258, Reno, Nevada 89557

*corresponding author; telephone: (775) 782-5553; fax: (775) 784-1390; email: koloj@unr.edu

Current Address:

Department of Civil and Environmental Engineering

MS 0258

Reno, NV 89557

Abstract

Recent studies have been reported that natural and synthetic hormones are major endocrine-disrupting contaminants (EDCs) that can cause contaminant-associated reproductive and developmental alternation on aquatic organisms. In particular, synthetic steroids such as trenbolone acetate (TBA) are widely used as growth promoters in beef cattle. It has been reported that livestock excrete potential endocrine disrupting hormones such as the TBA metabolites (17α-trenbolone, 17β-trenbolone, and trendione) into aquatic environments. Although livestock likely contain much higher levels of steroid contaminants due to the increased use of growth-promoting hormones, relatively little is known about the fate of these synthetic steroids under various aquatic environments. Therefore, this study undertook a series of experiments using biodegradation microcosms to develop a mechanistic understanding of the fate of TBA metabolites in various redox conditions. Redox potentials were controlled by using reagents (titanium(III) citrate, L-cysteine, and dithiothreitol) to develop anaerobic (E_h < -400 mV), anoxic (-400 mV < E_h <-200 mV), and suboxic (-200 mV < E_h < 0 mV) conditions. In aerobic conditions (E_h > 100 mV), although abiotic losses in sterile controls were observed for three androgen steroids, 17α-trenbolone and 17β-trenbolone were degraded by as much as 80% and 40% by biotic processes over 10 days. However, trendione degraded by only 20% biotic processes during 3 days, with slight increased after 3 days. Half-lives of 17α-trenbolone, 17β-trenbolone, and trendione were 0.9, 1.5, and 2.7 days in aerobic experiments, respectively. In anaerobic (-438±149 mV), anoxic (-269±45 mV), and suboxic (-100±22 mV) conditions, losses by abiotic processes were observed for all three conditions. In anaerobic and anoxic conditions, observed losses of 17α-trenbolone, 17β-trenbolone, and

trendione in sterile controls were around 70% and biotic processes were only responsible for about 20% of attenuation. In suboxic conditions, losses of 17α-trenbolone and 17β-trenbolone were nearly 30% in sterile controls, and observed biotic processes may have contributed 20% to degradation. Half-lives of 17α-trenbolone, 17β-trenbolone, and trendione were 19.2, 8.8, and 7.8 days in anaerobic conditions, respectively. In anoxic conditions, half-lives of 17α-trenbolone and 17β-trenbolone were 3.3 days and 3.2 days, and in suboxic conditions half-lives of 17α-trenbolone, 17β-trenbolone, and trendione were 2.7, 2, and 3.6 days. Although uncertain due to the losses in sterile controls, ORP (oxidation-reduction potential) values obviously affect biodegradation rates for all three androgen metabolites. From the data, aerobic conditions seem to be more favorable conditions than suboxic, anoxic, and anaerobic conditions for 17α-trenbolone, 17β-trenbolone, and trendione degradation.

Introduction

Various chemical contaminants having endocrine disrupting properties can threaten ecosystem health following exposure due to problems such as reproductive and developmental alteration (1-3). The first reported study about the impact of EDCs on fish was performed in the UK in 1994, suggesting that EDCs affected fish by feminization of both feral and caged fish (1, 3). Also, effluents from many industrial facilities can cause the masculinization of fish because of the presence of androgenic compounds in sources such as paper and pulp mill effluents (1, 4-6). Animal agriculture facilities along with municipal waste water and industrial effluents often are regarded as the largest sources of EDCs to the environment (4, 7) because they can introduce potentially large masses of natural and synthetic hormones into the environment (5, 12).

Studies suggest that natural and synthetic estrogens are the major EDC classes that can disrupt endocrine function, leading to observations such as vitellogenin production in male fish (8). The available data suggests that synthetic estrogens may be more persistent than natural estrogens, more likely to accumulate in ecosystems and adversely affect aquatic organisms (4,9,11). Recent studies have demonstrated the negative effects of synthetic hormones on biological processes, indicating that exposure to 17β-trenbolone and 17α-trenbolone can impact various endocrine processes in fish (1, 13, 15, 19). Additionally, other studies reported that masculinization of female fish and decreased reproductive capacity of fathead minnow occurred after exposing 17α-trenbolone and 17β-trenbolone to fish at concentrations of 0.1 µg/L and 0.027 µg/L, respectively (3). It was also reported that exposure to 1-10 µg/L (17β-trenbolone) has

masculinized female mosquitofish and exposure ≥ 40 ng/L of 17β-trenbolone can cause the reduction of plasma vitellogenin in female medaka, fathead minnow, and zebra fish (19). In male fathead minnows, significant alternation in reproductive function was observed after exposure to CAFO effluent. Effects were likely due to a mixture of natural androgens in manure and androgenic pharmaceuticals used in growth implants (6). Therefore, we can predict that a large mass of synthetic or natural hormones can originate from feedlots and transport into aquatic environments, and these endocrine contaminants may adversely affect aquatic organisms (2, 13).

Significant amounts of natural and synthetic hormones are excreted from animals treated with growth promoters used to increase cattle production (1, 12-13). Although cattle feedlots likely contain much higher contaminant concentrations due to higher use of growth-promoting compounds, little information about the environmental fate of these growth promoting compounds has been reported (13). The most used growth-promoting hormones are trenbolone acetate, a synthetic androgen, often administrated as an ear implant (14). Post-implantation, TBA is hydrolyzed to the metabolite 17β-trenbolone, followed by oxidation to trendione, then further reduced to 17α-trenbolone (Figure 1). 17α-trenbolone comprises about 95% of the TBA metabolite mass excreted by cattle (11). According to recent studies, 17α-trenbolone and 17β-trenbolone have been detected at concentrations ranging from <10 ng/L to about 120 ng/L, and from 10 - 20 ng/L, respectively, in feedlot effluents (11). During a 5.5-month storage period of liquid manure, 17α-trenbolone decreased from 1,700 to 1,100 pg/g suggesting a half-life of 267 days for this compound under anaerobic conditions (16). Furthermore, Khan et al., (15,

17) examined the degradation of TBA in agricultural soils. This study reported that both trenbolone isomers (17α- and 17β- trenbolone) were degraded rapidly in aerobic agricultural soils, with half-lives ($t_{1/2}$) of a few hours to 0.5 days at application concentrations of less than 1 mg/kg (17α- and 17β- trenbolone). Trendione was more persistent than 17α- and 17β-trenbolone, with observed half-lives of 1 to 4 days in the same soils.

Manure from animals treated with growth-promoting compounds is often applied in agriculture land as a fertilizer, risking subsequent transport into receiving waters in proportion to the affinity of hormones with soil. The sorption of hormones is highly related with organic matter in soil (17, 22). Schiffer et al., (21) reported that TbOH and MGA passed quickly in soil column, but both hormones exhibited a high affinity with organic matter in upper layers in soil. Khan et al., (17, 22) also demonstrated that there was a strong relationship between K_d (L/kg) and % OC (organic carbon) in soil (R^2 values > 0.97).

Based on the recent studies, a comprehensive mechanistic understanding of the fate of TBA in the aquatic environment is lacking. To fully understand the fate of TBA, it is necessary to consider a variety of environments associated with animal agriculture as well as transformation pathways. Once TBA is released from animal wastes into receiving waters, TBA degradation will be assessed by a variety of agriculturally-impacted receiving waters such as waste lagoons, shallow ground water under rangelands, sediments environments, and surface water environments that are likely to be anoxic/anaerobic, or aerobic conditions, with a various range of redox potentials. Recent

studies observed that redox states play a key role in biodegradation (11, 15) because the redox conditions may have a large effect on biodegradation rates. In this study, we will determine the biodegradation rates and half lives for 17α-trenbolone, 17β-trenbolone, and trendione by evaluating aerobic and anaerobic degradation in range of environmental systems. In addition, sorption studies will evaluate role of abiotic processes in the fate of 17α-trenbolone, 17β-trenbolone, and trendione. These sorption experiments may be able to account for significant losses of synthetic hormones in sterile controls.

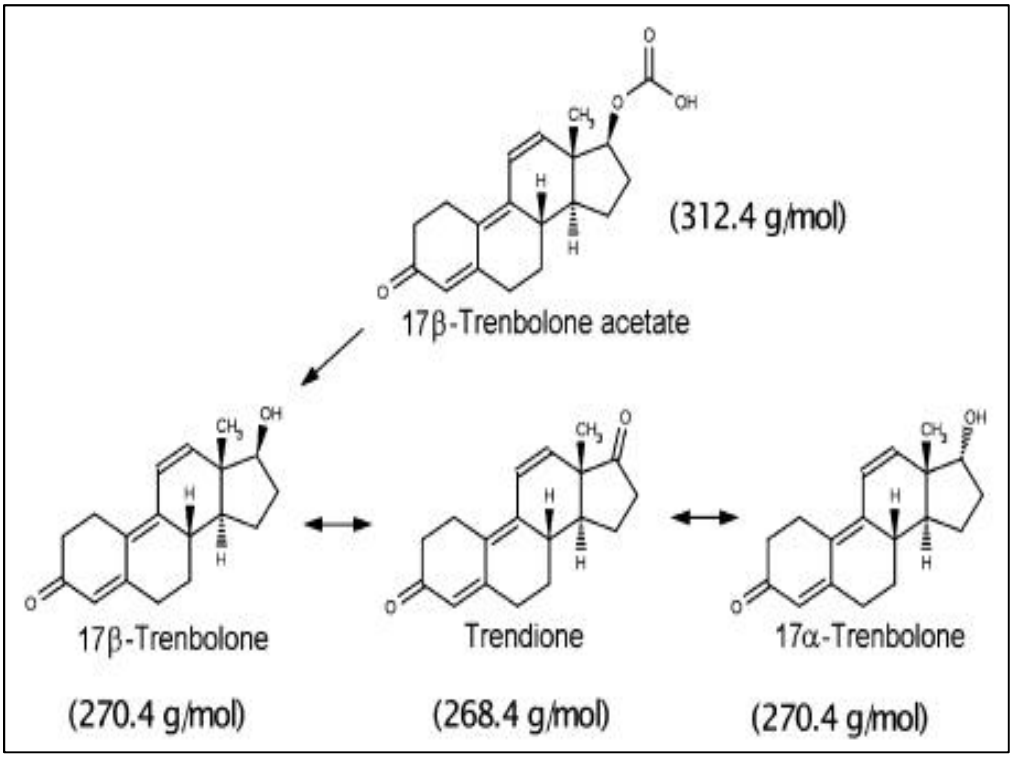


Figure 1. Metabolites of TBA. Figure from Khan et al., (2008a) (15)

Material and Methods

Chemicals. 17α-trenbolone, 17β-trenbolone (Steraloids Inc. RI, USA) and trendione synthesized from 17β-trenbolone (15) were used in these studies. d3-17β-trenbolone was purchased from BDG Synthesis (Lower Hut, New Zealand). N-methyl-N-(trimethylsiyl)trifluoro-acetamine and iodine (99.999% pure) for derivatization were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Reagent solvents, HPLC grade methanol, acetone, and dichloromethane, were obtained from Fisher Scientific (Pittsburg, PA, USA). Reducing chemicals, titanium(III) citrate, dithiothreitol (DTT), and L-cysteine, were purchased from Sigma Aldrich (St. Louis. MO, USA). Titanium (III) citrate was prepared according to Zehnder and Wuhrmann (1976) (26). The fluorescein diacetate (3'6'-diacetyl-fluorescein, Sigma-Aldrich Co.) and fluorescein sodium salt (Sigma-Aldrich Co.), were used for measuring microbial activity by hydrolysis in samples. Sodium azide for sterilizing samples was obtained from Fisher Scientific (New Jersey, USA).

Sample Collection and Culture Conditions. Aerobic sludge and anaerobic sludge were used as inocula for biodegradation and sorption studies and were collected from the Truckee Meadows Water Reclamation Facility, Sparks, NV, and transported to the laboratory for immediate use. Each experiment used these inocula in experimental microcosms with a final inocula:water ratio of 1:40 (v/v). All degradation microcosms employed in an acclimation nutrient medium: (a) KH_2PO_4 8.5 g/L; K_2HPO_4 21.75 g/L; $NaHPO_4{\cdot}2H_2O$ 33.4 g/L; NH_4Cl 0.5 g/L, 10 mL/L (b) $CaCl_2$ 27.5 g/L, 1 mL/L (c)

$MgSO_4 \cdot 7H_2O$ 22.5 g/L, 1mL/L (d) $FeCl_3 \cdot 6H_2O$ g/L, 1mL/L. Glucose (0.5 mM) was used as the carbon source to insure microorganism activity (22).

Oxidation-Reduction Potential Measurement. The redox level (E_h, mV) of microcosms as measured using a Pt (Ag/AgCl) combination electrode connected to a AP 10 ORP meter (Fisher Scientific, New Jersey, USA) (23-25). To evaluate the effect of redox state on biodegradation rates, four target redox condition ranges were simulated for each water sample: aerobic (E_h > 100 mV), suboxic (-200 < E_h < 0), anoxic (-400 < E_h < -200) and anaerobic (E_h < -400 mV). Redox states were controlled by introducing the redox-stabilizing chemical compounds (titanium(III) citrate, dithiothreitol, and L-cysteine) that have been used as redox buffers for biodegradation studies and control of redox microbial activity (23-27). All glassware used in experiments was washed and then baked at 400℃ for 2 hrs prior to use. All suboxic, anoxic, and anaerobic microcosm samples were immediately placed in a glove box filled with N_2 gas (28), and oxidation-reduction potential measurements were conducted in triplicate using an ORP meter. The concentration of titanium(III) citrate, dithiothreitol (DTT), and L-cysteine used in this study were 8 mM, 1 mM, and 20 mM, respectively, which were determined by lab studies that poised redox conditions at suboxic, anoxic, and anaerobic levels.

Microbial Activity Measurement and Cell Counting. Among the various methods used to measure microbial activity, FDA (fluorescein diacetate) hydrolysis has been used widely in a range of samples such as soils, activated sludge, and sediments (29). Compared to other methods, FDA hydrolysis measurement is simple, sensitive, and inexpensive (29). FDA is hydrolyzed by both free enzymes and membrane bound

enzymes and the final hydrolysis product fluorescein can be measured in the visible wavelength (490 nm) by UV-spectrometry (29-31). To quantify microbial activity, a stock solution (0.1 ml of 1000 ug FDA/mL) was added to the samples, then samples were shaken for 30 min at room temperature. After 30 min, the reaction of FDA in samples was stopped by formaldehyde quenching (14% final concentration). The samples were then filtered through a 0.20-µm polyamide membrane (Chromafil, Germany) and measured at 490 nm using a UV-spectrophotometer (Varian Cary-300 Bio). A second, complementary method applied to quantify microbial population is DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) staining, which has stable fluorescence and is widely used to count total bacteria in water after staining (32-33). Aliquots of samples (0.2 mL) were placed in sterilized tubes and were mixed with sodium pyrophosphate (0.2 mL, 0.2 % concentration (v/v)) and shaken (500 rpm) for 30 min, followed by sonication (5 min). Then DAPI (0.4 mL, 10 µg/mL) and Triton X-100 (0.2 mL, 0.1%) were added into the samples. All samples were vortexed (20 sec.) and left in the dark for 10 min (33). The stained samples were filtered through black polycarbonate filters (pore size 0.2µm, Sigma-Aldrich). Filters were then measured under UV light (EX330-380, BA420) using an eclipse microscope (Nikon, USA) with an oil immersion objective lens (UPlan-FI 100× oil) (32). The cells were counted in 10 fields, and the total cell counts were expressed as cells/mL using an Image J software program (32).

Sample Extraction and Derivitization. After filtration, aqueous samples were extracted by C-18 solid-phase extraction discs that were conditioned before use. Conditioning consisted of three wash steps of 5 mL of methanol each, followed by three

rinses with 5 mL of distilled water (34-35). After conditioning of extraction cartridges, samples, typically 40 mL in volume, were spiked with 40 µL of 100 ppb d_3-17β-trenbolone internal standard stock solution, and extracted under vacuum. Extraction was performed in an extraction manifold at a flow of 1 to 5 mL/min. C-18 solid phase extraction cartridges were immediately stored in the refrigerator until they were eluted. Elution of C-18 solid phase extraction cartridges was accomplished by 9 mL of a 95:5 (v/v) methanol:water solution, and then completely drying extracts in the vacuum oven. After vacuum dry down, each sample was re-suspended with 1 mL of dichloromethane and then samples were dried down again under N_2 before derivitization. Derivitization of extracts was performed by adding 50 µL of MSTFA-I_2 (1.44 mg I_2/ml MSTFA) into samples and vortexing; then derivatized extracts were dried down under a gentle N_2 gas. Next, 100 µL MSTFA was used to re-suspend extracts in GC vials, followed by an 60 ℃ incubation for 40 min. Extracts were then analyzed by GC/MS/MS.

GC/MS/MS Analysis. The GC/MS/MS instrument used to analyze the steroid derivatives was an Agilent 6890N gas chromatograph coupled to a Waters Quattro Micro mass spectrometer. Chromatographic separation employed a 5Sil MS fused silica capillary column (30m length × 0.25 mm diameter) (Supelco, Bellefonte, PA, USA). UHP helium was used as the carrier gas at 1.0 mL/min. The GC temperature program was used as following: an initial temperature of 120 ℃ (hold for 2min), increased at 45 ℃/min to 260 ℃/min (hold for 1 min), then 5 ℃/min until 270 ℃ (hold for 8min), increased at 45℃/min to 285 (hold for 6 min), finally increased to 300 ℃ (hold for 2

min). The 70 eV ionization energy was used in the mass spectrometer, and the ion source and GC interface were operated at 180 ℃ and 250 ℃, respectively.

Batch Biodegradation Rates. Biodegradation of 17α-trenbolone, 17β-trenbolone, and trendione was evaluated at various redox states. In these experiments, activated sludge and anaerobic sludge were used as microcosm inocula for aerobic and suboxic/anaerobic study, respectively. Biodegradation potential was monitored by measuring the concentration of the three hormones in aerobic conditions and suboxic, anoxic and anaerobic conditions by 0, 1, 2, 3, 6, and 10 days. All experiments were performed in triplicate. Control samples were sterilized by the moist autoclaving method described by Trevors which was at 121 ℃ and 1.1 atm (1.137kg/cm^2) for 1 hr (37).

Aerobic study. Activated sludge to water (1:40, v/v) were mixed into 50 mL glass bottles and control samples were sterilized by moist autoclaving. 17α-trenbolone, 17β-trenbolone, and trendione solution were spiked at the selected concentration (40 µL of 100 ppb solution in methanol) into tubes. All tubes were incubated while shaken (100 rpm) in a temperature-controlled dark room (20±2℃) over 10 days. All experiments were performed in triplicate, and aerobic conditions were maintained.

Anaerobic study. Anaerobic sludge to water (1:40, v/v) were mixed into 50 mL glass serum bottles and control samples were processed as for the aerobic study. 17α-trenbolone, 17β-trenbolone, and trendione solution were spiked at the selected concentration (40 µL of 100 ppb solution in methanol) into tubes. All tubes were incubated while shaken (100 rpm) in a temperature-controlled dark room (20±2℃) over 10 days. All experiments were performed in triplicate, and the desired redox states were

achieved by adding redox-stabilizing chemicals as described. Biodegradation rates of the applied hormones (k_a, d^{-1}) will be obtained by fitting data to first-order exponential decay model.

For the recovery analysis of sample, 17β-trenbolone-d_3 (40µl of 100 µg/L concentration) was added as an internal surrogate standard to all samples. 17α-trenbolone and 17β-trenbolone was recovered at 160% and 70% in nonsterile conditions, whereas 17α-trenbolone and 17β-trenbolone was recovered at 90% and 60% in controls, respectively. Trendione was recovered at 490% in nonsterile conditions and recovered at 180% in controls due to the lower sensitivity of 17β-trenbolone-d_3.

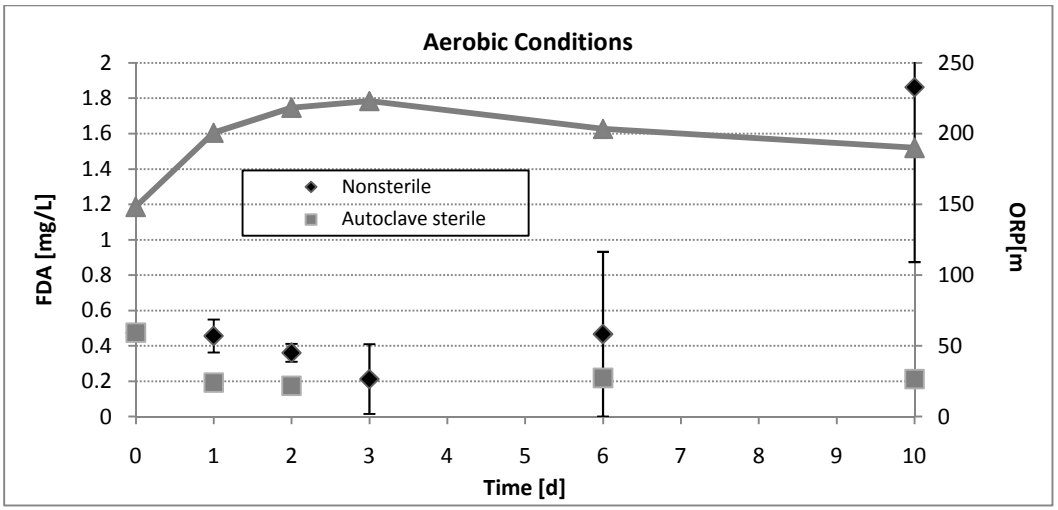
Results and Discussion

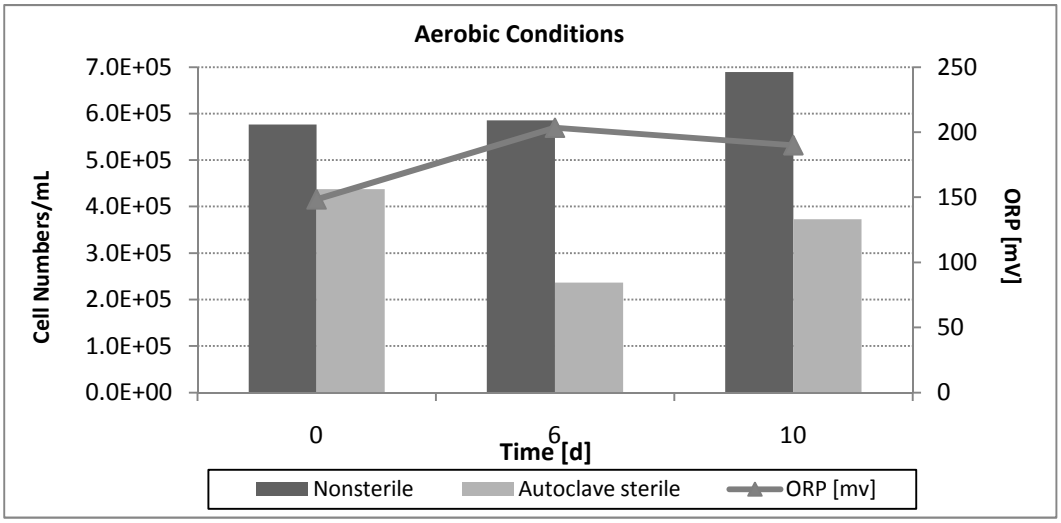
Redox States and Microbial Activity. Results of microbial activity measurements under various redox conditions are presented in Figure 2. Redox potential values initially started at +150 mV and increased up to +200 mV in aerobic conditions over 10 days. To assess microbial activity, we measured the FDA hydrolysis in aerobic conditions. The FDA hydrolysis was little changed over the initial 3 days, but increased exponentially between 3 days and 10 days. Battin et al., (1997) (39) reported that FDA hydrolysis was well represented by a sigmoidal model for microbial growth and Schnurer et al., (1982) (29) demonstrated that there was a lag phase in FDA hydrolysis. FDA hydrolysis in our batch aerobic condition experiments was little changed from 0.5 mg/L to 0.2 mg/L, over the 3 days that seemed to be the microbial lag phase. After 3 days, FDA hydrolysis

increased exponentially from 0.2 mg/L to 1.9 mg/L at 10 days a similar trend to the Battin's results (39). Along with FDA hydrolysis for measuring microbial activity, cell counts by DAPI fluorescence straining was used to measure microbial population size in suboxic, anoxic, and anaerobic conditions. Cell counts in various redox conditions by DAPI fluorescence is shown in Figure 1. Cell counts in aerobic conditions were similar with the results of FDA hydrolysis (Fig 2. (a)). Cells in non-sterile conditions increased from 5.8×10^5 to 6.9×10^5 bacteria cells per mL during 10 days, but autoclave sterilized cells decreased in numbers. Both FDA hydrolysis and microbial counts suggest that microbial activity increased in aerobic conditions.

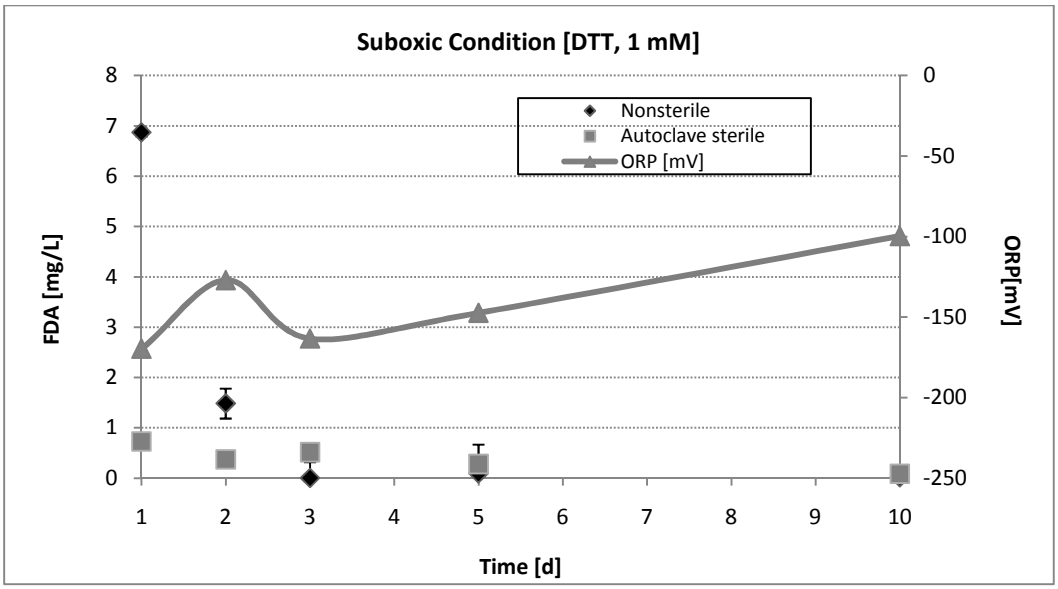
In anaerobic, anoxic, and suboxic conditions poised by titanium(III) citrate, L-cysteine and DTT buffering, redox potentials averaged -438±149, -269±45, and -100±22 mV over 10 days respectively. Variations suggest that it is difficult to maintain the initial redox potentials over the entire course of the experiment because of the complexity of the medium (24). FDA hydrolysis (Fig. 2 (b)-(d)) in suboxic, anoxic, and anaerobic conditions decreased over 10 days suggesting limited microbial activities in these conditions. Kirakosyan et al., (2004) (40) demonstrated that the low redox potentials induce a longer lag phase and slower growth rates. FDA hydrolysis in the anaerobic conditions poised by titanium(III) citrate slowly decreased, compared to L-cysteine and DTT trials. Thus, anaerobic conditions poised by titanium(III) citrate may be favorable conditions for some bacteria even though under anaerobic conditions (26, 42). In the case of L-cysteine and DTT, sharply decreased trends of FDA hydrolysis were observed during the 10 days compared to titanium(III) citrate (Fig. 2 (b)-(c)). Chen et al., (2010)

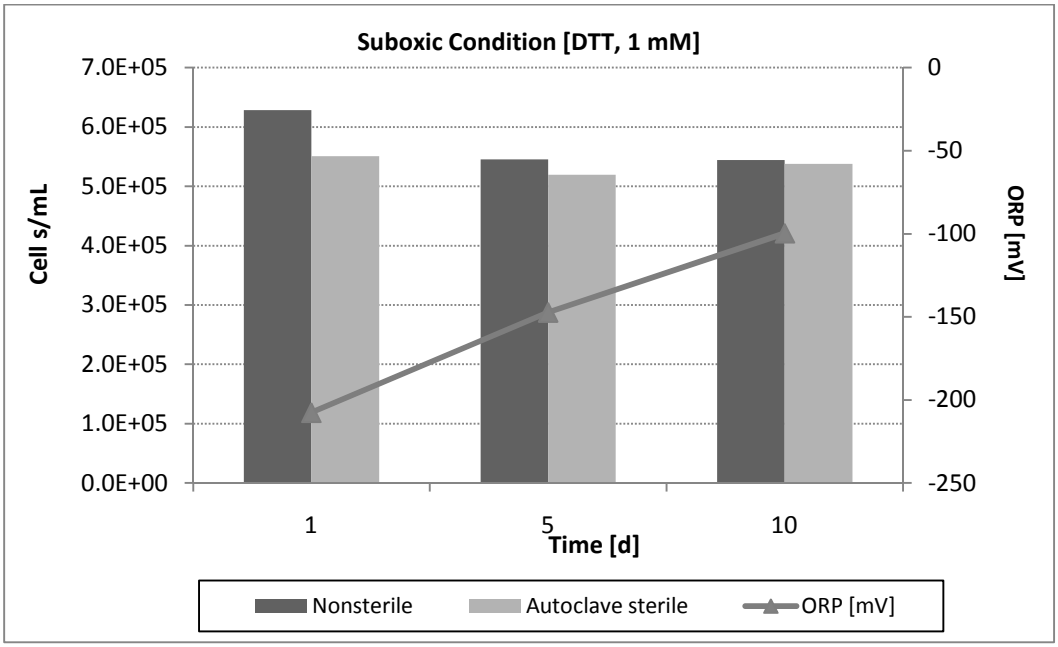
(41) reported that high concentrations of L-cysteine can produce an anaerobic environment to maintain low ORP, though concentrations of L-cysteine over 500 mg/L (4 mM) could affect microbial growth. Kirakosyan, et al (2004) and Chen, et al (2010) (40, 41) demonstrated that the presence of thiol groups in DTT and L-cysteine may affect the transport systems and membrane-associated enzymes in microbes that caused the inhibited cell growth. In suboxic and anoxic conditions poised by DTT and L-cysteine, there was little change in cell growth in both nonsterile and sterile controls over 10 days, similar to the results of FDA hydrolysis (Fig. 2 (b)-(c)). Anaerobic conditions poised by titanium(III) citrate caused cell numbers to decrease in nonsterile samples over 10 days, but cells in sterile controls showed a slight increase after 6 days that suggests some microbes survived in the sterile controls (Fig. 2. (d)). Jin, et al. (1997) (24) reported that the anaerobic conditions poised with 5 mM of titanium(III) citrate were biologically inert for several bacterial species. However, they also demonstrated the adverse effect of titanium(III) citrate on the specific growth rate of E. coli and K-12 when the concentration exceeded 1.25 mM (ORP, -100 mV) of titanium(III) citrate. Thus, we can assume that anaerobic, anoxic, and suboxic conditions as well as the unique structure of reagents may play a key role to control microbial growth and affect the biotic processes governing the fate of 17α-trenbolone, 17β-trenbolone, and trendione in aquatic environments.



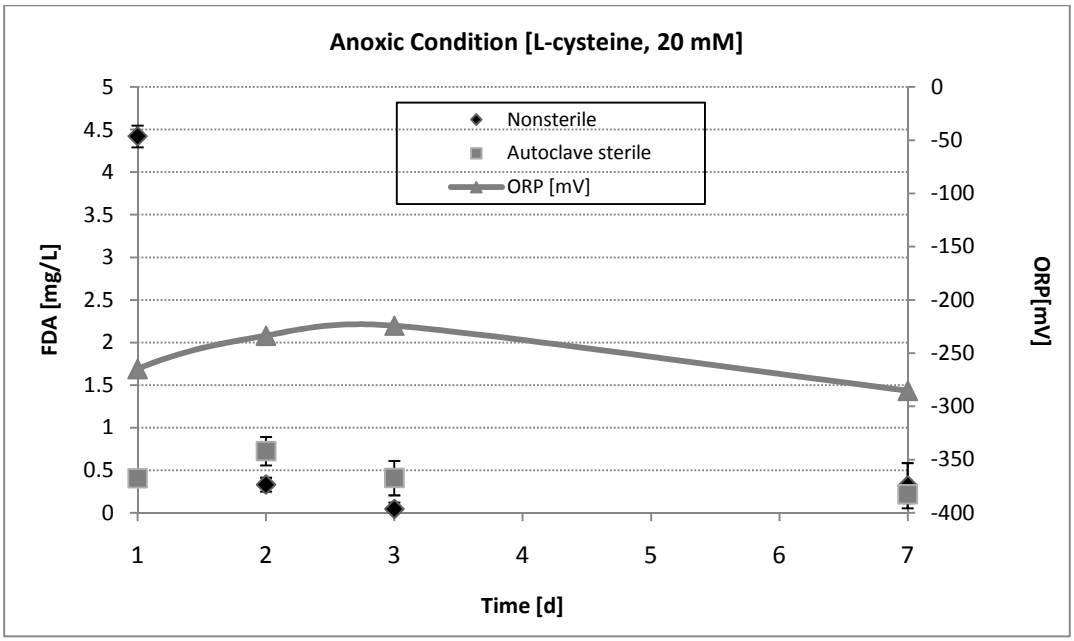


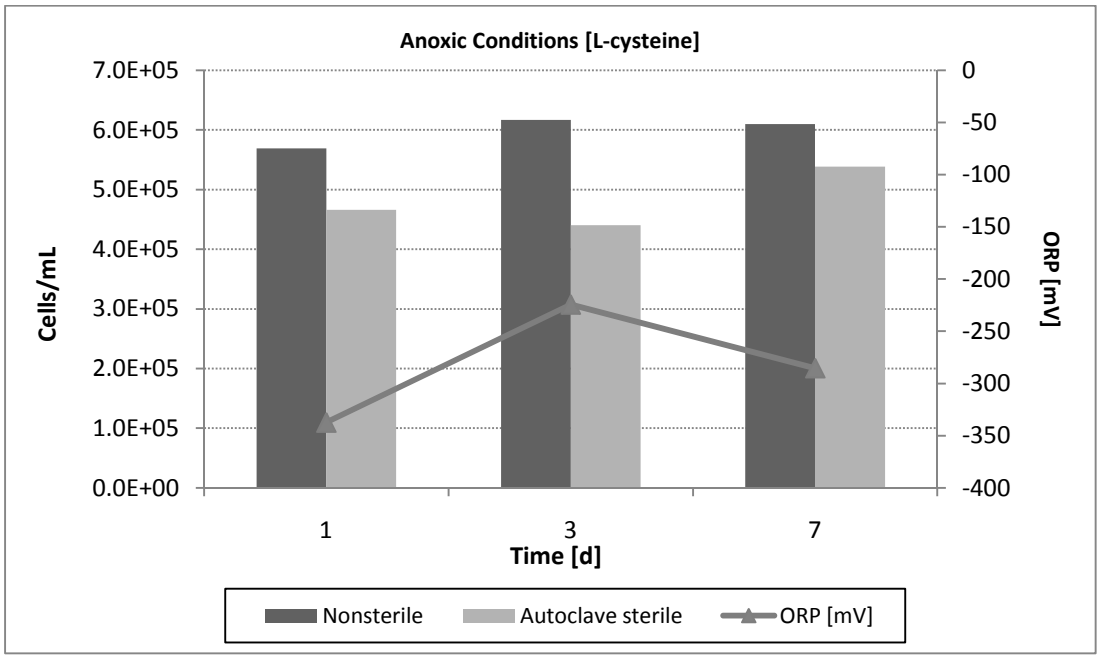
(a) aerobic condition



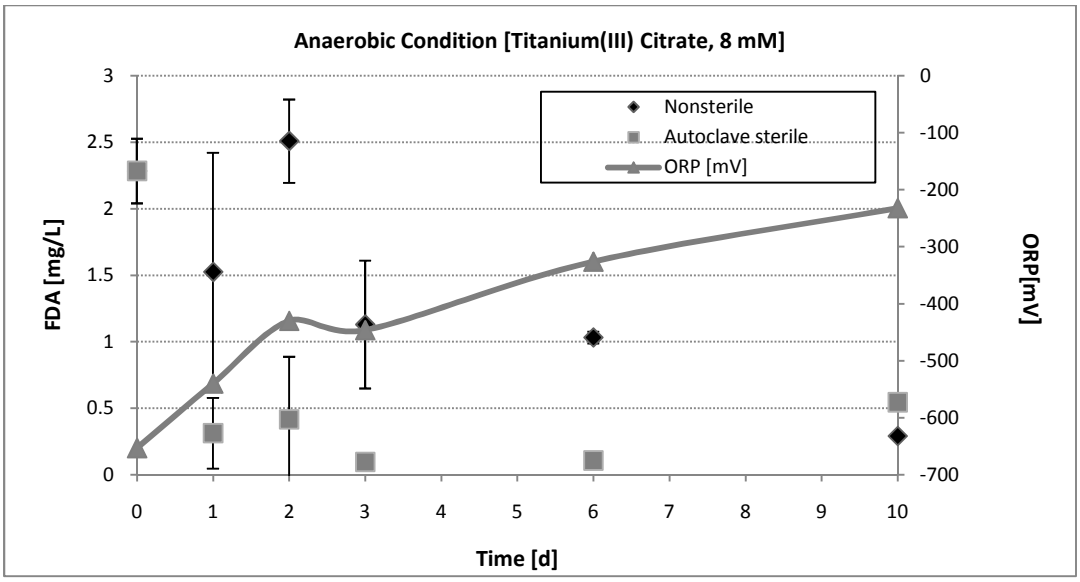


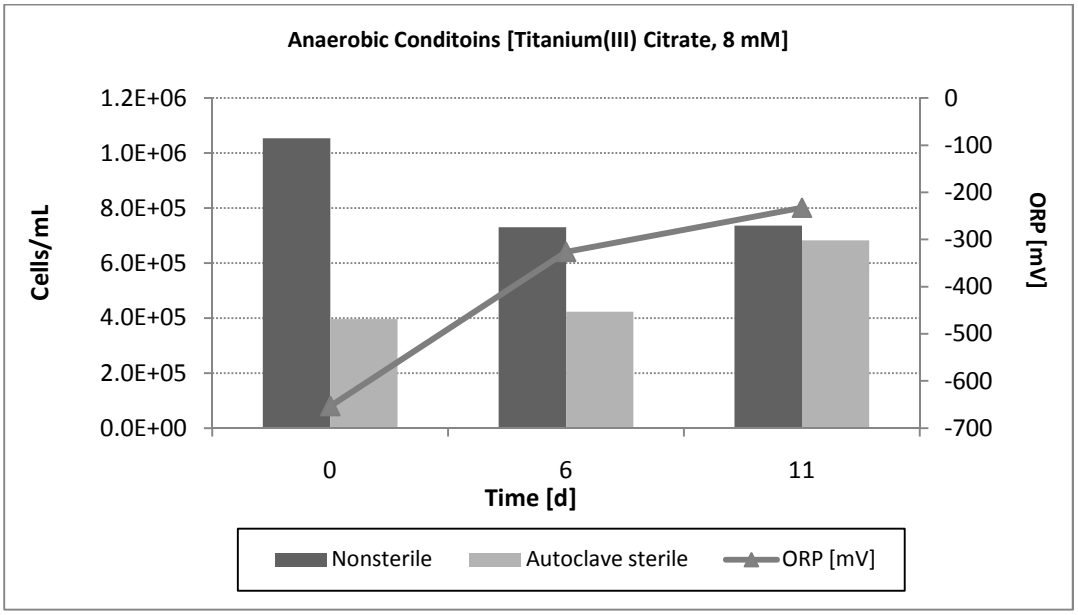
(b) Suboxic Conditon





(c) Anoxic Condition





(d) Anaerobic Condition

Figure 2. Effect of the ORP [mV] on FDA hydrolysis and microbial population size (n=10 for each type of condition) in (a) aerobic, (b) suboxic, (c) anoxic, and (d) anaerobic conditions.

Degradation under in Aerobic conditions. Figure 3 demonstrates the biodegradation of 17α-trenbolone, 17β-trenbolone, and trendione under aerobic conditions with activated sludge inocula over 10 days (all data were corrected by internal standard). During batch aerobic experiments, ORP [mV] values were monitored over time, and averaged 197±27 mV over the 10 day trial (Table 1). The 17α-trenbolone and 17β-trenbolone degraded exponentially over 10 days. However, trendione exhibited similar decreases to 17α-, 17β-trenbolone during the first days of the trials but showed an unexpected increase after 3 days. After accounting for loss in sterile controls, the losses of 17α-, 17β-trenbolone and trendione were observed to be approximately 20%, 50%, and50 %, respectively over the 10 days. Losses were observed for both 17β-trenbolone and trendione in sterile controls, but there was little observed loss for 17α-trenbolone in controls. The loss of 17β-trenbolone and trendione in controls may be caused by abiotic processes such as sorption, because the data showed there were nearly parallel trends over experiments. However, 17β-trenbolone and trendione had similar degradation pattern over 3 days, suggesting that the sorption process may affect the degradation of 17β-trenbolone and trendione more than 17α-trenbolone. Schiffer, et al. (11, 21) reported that there were strong correlations between 17β-trenbolone sorption and organic carbon content in soil and hydrophobic characteristics were primarily responsible for the sorption to organic matter (11, 17). Khan, et al. (17) also demonstrated the sorption strength among 17α-, 17β-trenbolone and trendione in several types of soil, indicating that trendione sorbed most strongly into soil and 17β-trenbolone sorption was higher than 17α-trenbolone sorption. 17α-trenbolone was quickly degraded by nearly 80% during 3 days compared to limited losses of 17α-trenbolone in sterile controls. In contrast, 17β-trenbolone and trendione were degraded

by biotic processes as much as 40% and 20%, respectively, but much loss also was observed in sterile controls. In the case of trendione degradation, trendione is likely the most persistent TBA metabolite, and can be expected to form from 17α- and 17β-trenbolone as a degradation product (22). In our study, 17α- and 17β-trenbolone were well degraded in aerobic conditions, but 17β-trenbolone degraded slightly slower than17α-trenbolone. These results are similar to results reported for other synthetic steroids. For example, Ying, et al. (38) reported that E2 and EE2 were degraded rapidly in marine sediment under aerobic conditions, but EE2 was much more persistent under anaerobic conditions. They also proposed that aerobic conditions were much more favorable for the degradation of E2, EE2 in marine sediments, and the rapid observed loss of some EDC compounds was likely due to abiotic processes in these systems.

These data can be used to derive degradation rates (k_a) and half-lives ($t_{1/2}$) for 17α-, 17β-trenbolone, and trendione in aerobic conditions (Table 4). Degradation rates and half-lives of 17α-, 17β-trenbolone and trendione by biotic process were determined using a first-order decay model:

$$C_t = C_0 e^{-k_a t}$$

where t is time [d], C_0 and C_t is concentration at time 0 and t; k_a is the degradation rate constant [d^{-1}].

And half-lives can be estimated with the following expression.

$$t_{1/2} = -\ln (0.5)/k_a$$

Khan, et al. (15) reported 17α- and 17β-trenbolone half-lives of 4-11 hr and 4.4-38 hr at 0.1 to 10 mg/kg applied concentrations of 17α- and 17β-trenbolone in aerobic agriculture soils. The half-lives of 17α-, 17β-trenbolone, and trendione were calculated by fitting to first-order exponential conditions, yielding 0.9 d and 1.5 d, and 2.7 d half lives, respectively, in the aerobic system. This study observed longer half-lives than previous studies (15), but this study only considered biodegradation of 17α-, 17β-trenbolone and trendione by microorganisms, not including losses in sterile controls.

Table 4. Observed Biodegradation Rates and Half Lives of TBA Metabolites in Aerobic Conditions

Compounds	17α-trenbolone	17β-trenbolone	trendione
ORP [mV]	$197^{a}\pm27^{b}$		
k_a [d^{-1}]	0.904	0.479	0.261
$t_{1/2}$ [d]	0.9	1.5	2.7

[a]Average value and [b]standard deviation.

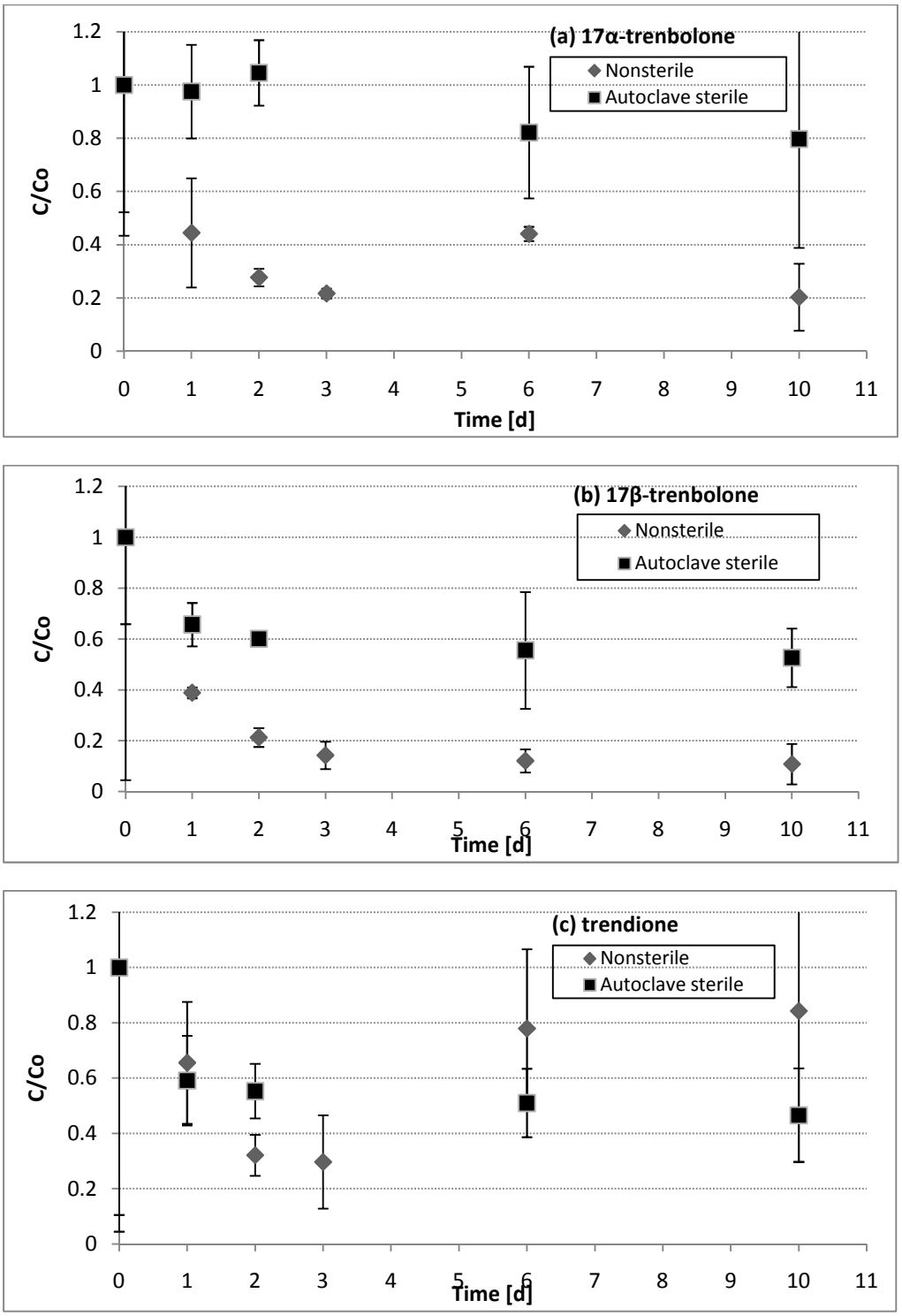


Figure 3. Aerobic degradation of (a) 17α-trenbolone, (b) 17β-trenbolone, and (c) trendione in batch experiments over 10 days at 20±2 ℃. Error bars are the standard deviation in triplicate samples

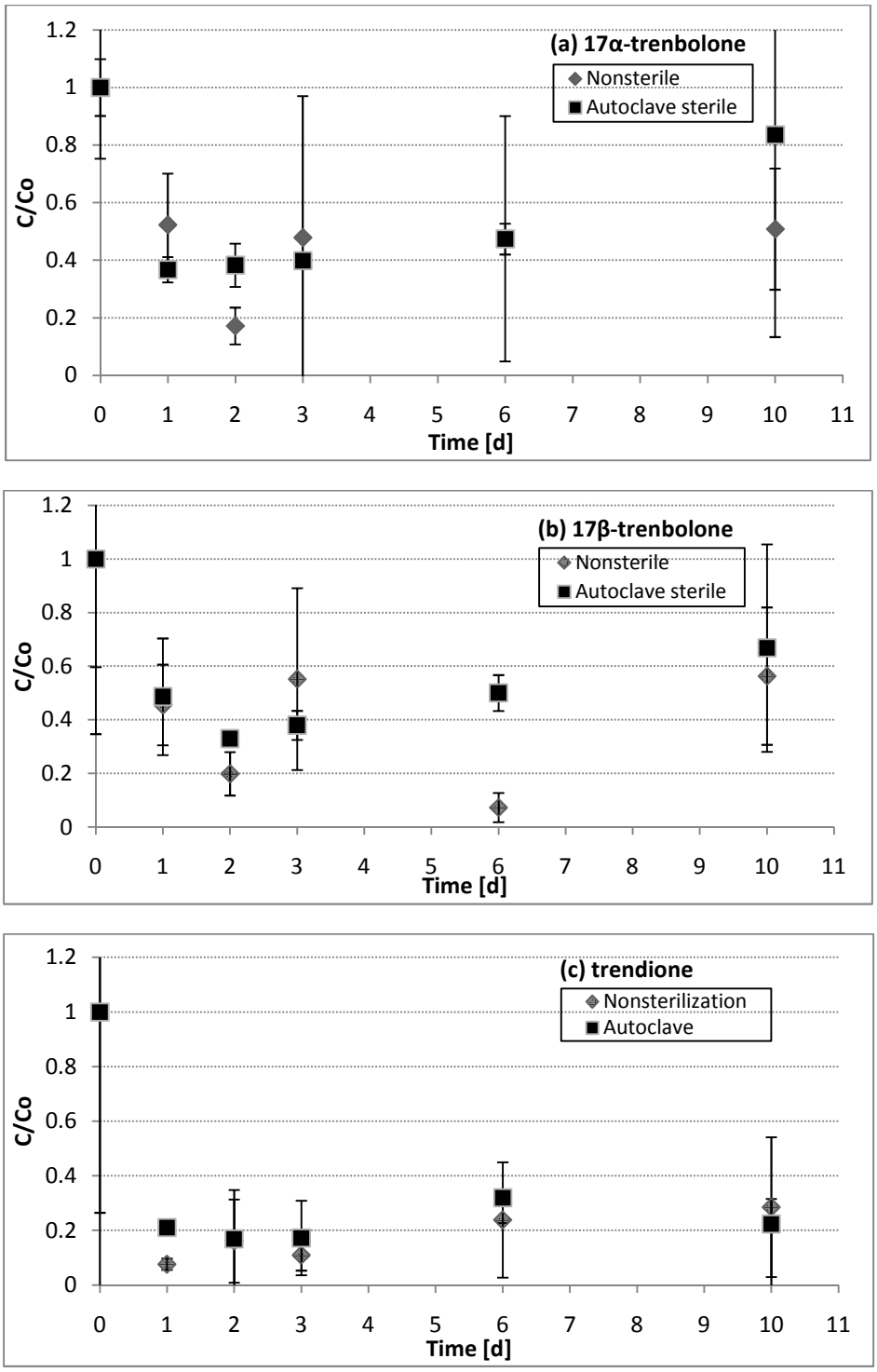


Figure 4. The degradation of (a) 17α-trenbolone, (b) 17β-trenbolone, and (c) trendione in anaerobic conditions [titanium(III) citrate, 8 mM] over 10 days at 20±2 ℃. Error bars are the standard deviation in triplicate samples.

Degradation in anaerobic conditions. To create stable anaerobic conditions, we used three organic chemicals, titanium(III) citrate (8 mM), L-cysteine (20 mM), and dithiothreitol (1 mM) as redox buffers for anaerobic systems. Measured ORP values were -438±149, -269±45.3, and -100±21.7 mV for the representative anaerobic, anoxic, and suboxic redox conditions, respectively.

In the anaerobic system, employing titanium(III) citrate, degradation of 17α-, 17β-trenbolone, and trendione was observed (Figure 4). No significant biodegradation was observed for the three androgens relative to sterile controls, but loss of 17α-, 17β-trenbolone, and trendione was observed in all microcosms. Abiotic processes such as sorption may likely explain these observations because data between nonsterile and sterile experiments had very similar attenuation trends. Observed losses of 17α-,17β-trenbolone, and trendione in controls were around 70%, whereas the potential degradation rates of 17α-, 17β-trenbolone, and trendione were less than 20% over the 10 days, suggesting limited bioactivity in anaerobic conditions. Though uncertain, the half-lives of 17α-, 17β-trenbolone, and trendione in anaerobic conditions poised by titanium (III) citrate were approximately 19.2, 8.8, and 7.8 days, respectively (Table 5).

In anoxic conditions poised by L-cysteine buffering (20 mM), the degradation pattern was similar to results for titanium(III) citrate (Figure 5). Losses of 70% of 17α- and 17β-trenbolone were observed in controls, with little biotic degradation (around 22% and 25%) in the system, respectively. The trendione data followed similar pattern with anaerobic experiments by titanium (III) citrate, but there was an unexpected increase in concentration during the experiment. The degradation rates of trendione in L-cysteine

were higher than those in anaerobic conditions poised by titanium(III) citrate. 17α- and 17β-trenbolone also were persistent in anoxic conditions, but microorganisms in anaerobic sludge could degrade 17α- and 17β-trenbolone, with half-lives of 3.3 and 3.2 days, respectively. Due to the data uncertainty, the half-lives of trendione in anoxic conditions cannot be estimated.

Figure 6 shows degradation of 17α-, 17β-trenbolone, and trendione in suboxic conditions poised by DTT redox buffering (1 mM). Degradation trends were similar to anaerobic and anoxic conditions. For 17α-trenbolone, nearly 30% of loss was likely abotic and a further loss of 20% likely due to biodegradation was observed in suboxic conditions. 17β-trenbolone concentration decreased over 2 days, but little loss due to biodegradation was evident compared to sterile controls. Additionally, trendione was attenuated mostly by abiotic processes (about 60%) with little evidence of biodegradation. The half-lives of 17α-, 17β-trenbolone, and trendione were 2.7, 2.0, and 3.6 days, somewhat shorter than values observed for anaerobic and anoxic conditions.

Biodegradation rates in various ORP values. In current study, 17α-trenbolone and 17β-trenbolone were rapidly degraded in aerobic condition with near complete degradation within 2 days (15). Ying, et al. (38) reported biodegradation of E2 and EE2 in aerobic and anaerobic conditions, with E2 degrading very quickly (half-life of 4.4 days) and EE2 exhibiting slower degradation (half-life >20 days). They also demonstrated that E2 was degraded very slowly with half-lives of 67 days in anaerobic condition. Similar to results for estrogens, Schiffer, et al. (11) reported the half-lives of

17α-, 17β-trenbolone were over 250 days in anaerobic manure storage. In this study, 17α-, 17β-trenbolone, and trendione were degraded rapidly in the aerobic conditions with half-lives of 0.9, 2.5, and 5.3 days. Degradation rates were obtained at various ORP values (Figure 7). Values of k_a for 17α-trenbolone ranged from 0.215 day^{-1} in anaerobic conditions to 0.904 day^{-1} in aerobic conditions, demonstrating a 4-fold increase in rates. The k_a values of 17β-trenbolone ranged from 0.210 day^{-1} in anaerobic conditions to 0.479 day^{-1} in aerobic conditions, a nearly 2-fold increase in rates. For trendione, rates ranged from 0.101 day^{-1} in anaerobic condition to 0.261 day^{-1} in aerobic conditions, demonstrating a 2-fold increase in rates. From these data, we can estimate that 17α-trenbolone would be degraded better in aerobic conditions compared to anaerobic conditions. Similarly, 17β-trenbolone was readily degraded in suboxic and aerobic conditions. But trendione seemed to be more persistent under the same conditions compared to 17α- and 17β-trenbolone. However, trendione also was more rapidly degraded in aerobic and suboxic conditions compared to anaerobic conditions. Results from the current studies suggest that because of their hydrophobic characteristics, all androgen hormones likely showed high abiotic losses by sorption onto suspended solids. 17α-, 17β-trenbolone, and trendione are moderately hydrophobic compounds (log K_{ow}: 2.72±0.02, 3.08±0.03, and 2.63±0.05, respectively) that have strong sorption affinity to macromolecules such as protein and particle (11, 17). Hydrophobic characteristics and different sorption strength of 17α-, 17β-trenbolone, and trendione (15, 17) also could support the high sorption losses onto suspended solids in the system. Khan, et al. (17) reported that trendione sorbed the most, and 17β-trenbolone was twice as likely to sorb compared to 17α-trenbolone in agricultural soil. In our results, trendione demonstrated

the highest potential sorption compared to 17α-, 17β-trenbolone, but sorption rates between 17α-trenbolone and 17β-trenbolone were similar. Comparing sorption rates between aerobic and anaerobic conditions, sorption in anaerobic system was much higher than aerobic system that may be from the higher different organic matter concentration in suspended solids. Khan, et al. (17) also demonstrated that the sorption of 17α-, 17β-trenbolone, and trendione was related to the soil organic carbon contents. In our study, abiotic processes are likely most responsible for the high loss of 17α-, 17β-trenbolone, and trendione in all systems.

Therefore, biotic processes as well as abiotic processes are jointly responsible for the attenuation of 17α-, 17β-trenbolone, and trendione in aerobic conditions. However there is little observed potential for biotic processes to account for the degradation of the three androgen hormones in anaerobic, anoxic, and suboxic systems. Also we found that abiotic processes such as sorption as well as biodegradation may account for significant loss and/or degradation. Though uncertain due to losses in controls, ORP values affected biodegradation rates for all three androgen metabolites. As expected, the observed effect of redox conditions on biodegradation was that the aerobic conditions were more favorable conditions than anaerobic, anoxic, and suboxic conditions for 17α-, 17β-trenbolone, and trendione degradation.

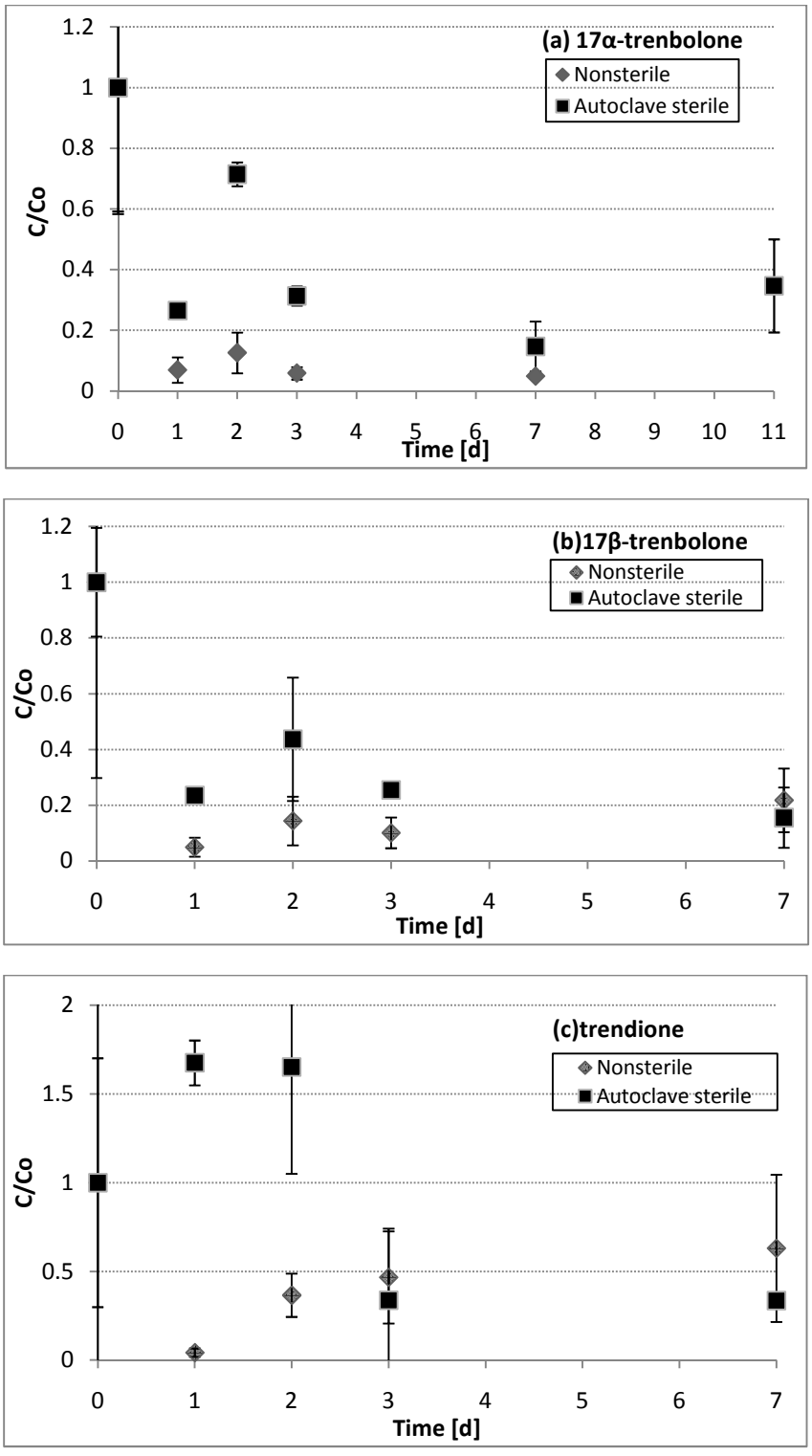


Figure 5. The degradation of (a) 17α-trenbolone, (b) 17β-trenbolone, and (c) trendione in anoxic conditions [Cysteine, 20 mM] over 10 days at 20±2 ℃. Error bars are the standard deviation in triplicate samples.

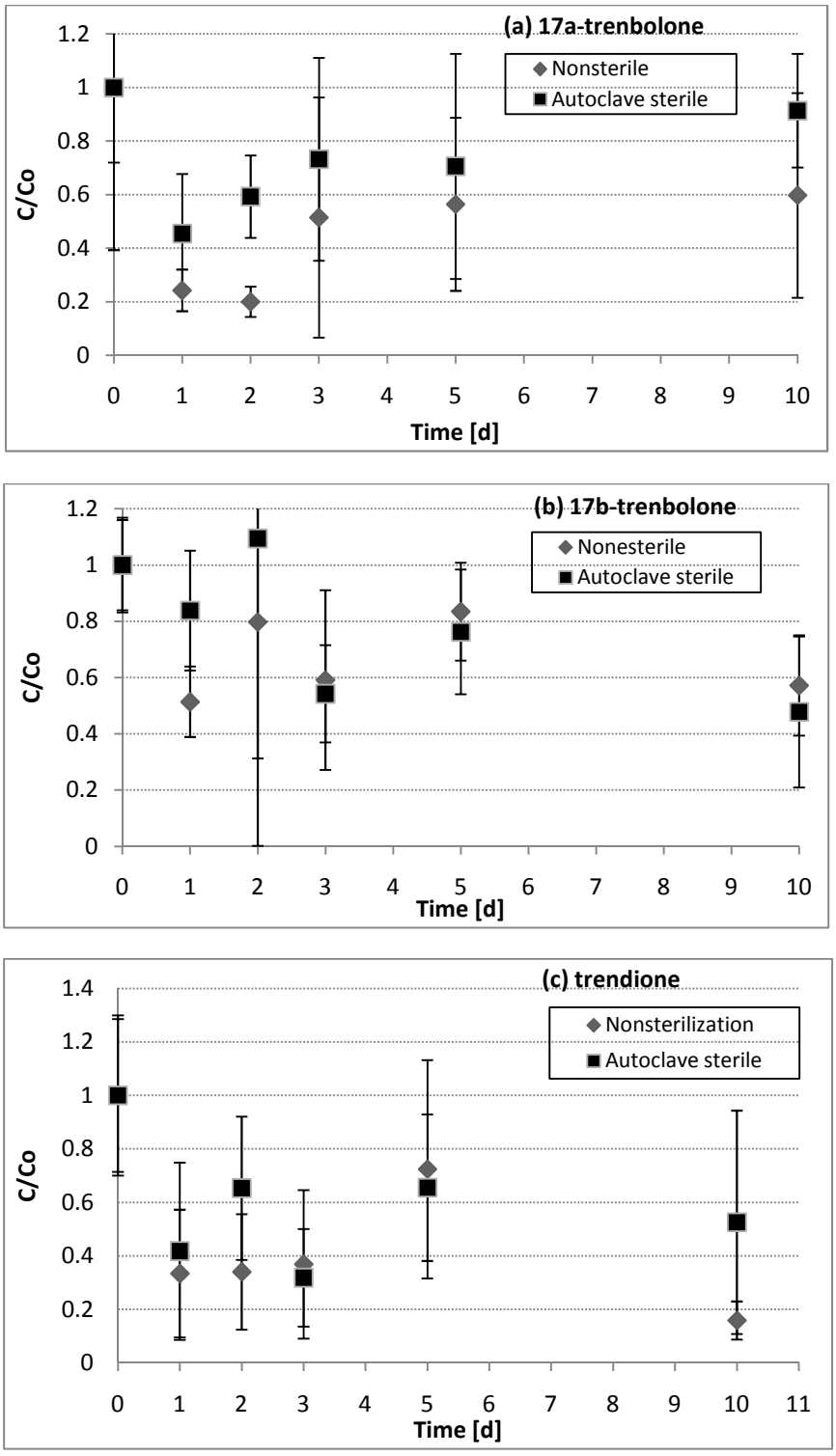


Figure 6. The degradation of (a) 17α-trenbolone, (b) 17β-trenbolone, and (c) trendione in suboxic conditions [DTT, 1 mM] over 10 days at 20±2 ℃. Error bars are the standard deviation in triplicate samples.

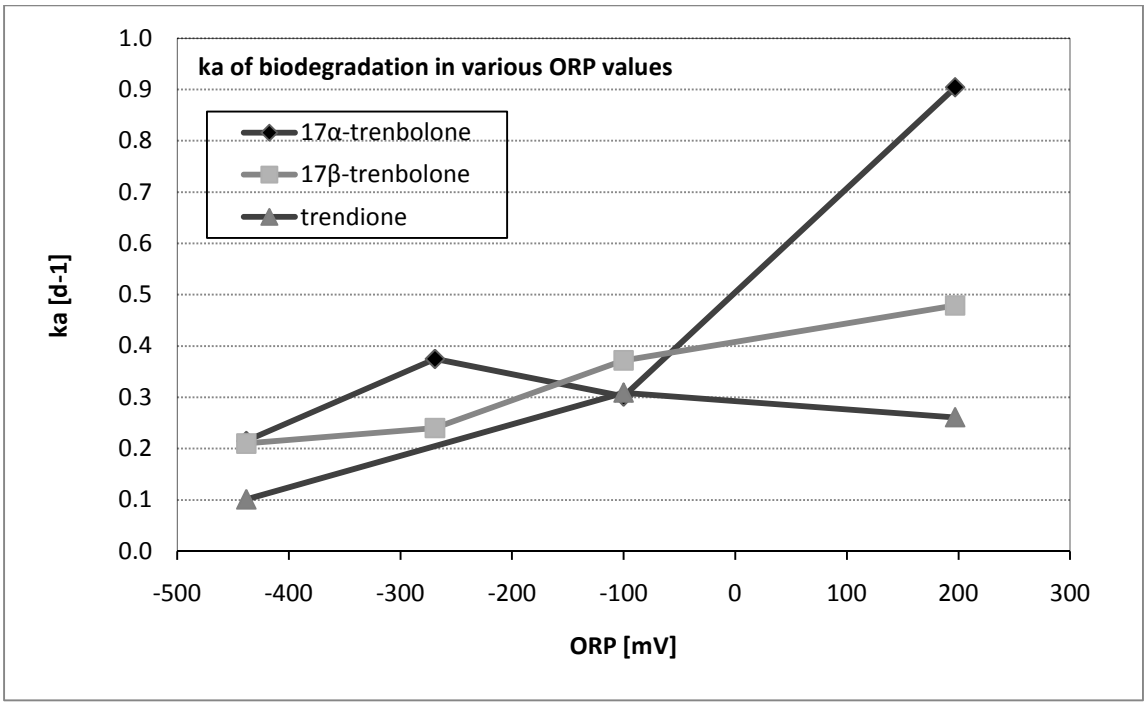


Figure 7. The degradation rates (k_a, d^{-1}) of 17α-trenbolone, 17β-trenbolone, and trendione by biodegradation in various redox conditions.

Table 5. Degradation Rates and Half Lives of TBA Metabolites in Anaerobic Conditions

DTT, 1mM			
Compounds	17α-trenbolone	17β-trenbolone	Trendione
ORP [mV]	-100[a]±22[b]		
k_a [d^{-1}]	0.302	0.372	0.309
$t_{1/2}$ [d]	2.7	2.0	3.6
Cysteine, 10 mM			
Compounds	17α-trenbolone	17β-trenbolone	Trendione
ORP [mV]	-269[a]±45[b]		
k_a [d^{-1}]	0.375	0.24	-
$t_{1/2}$ [d]	3.3	3.2	-
Titanium(III) Citrate, 8 mM			
Compounds	17α-trenbolone	17β-trenbolone	Trendione
ORP [mV]	-438[a]±149[b]		
k_a [d^{-1}]	0.215	0.210	0.101
$t_{1/2}$ [d]	19.2	8.8	7.8

[a]Average values and [b]Standard deviation

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Chapter 3: Summary and Conclusions

Summary

In the current study, we undertook a series of experiments designed to develop an understanding of the environmental fate of agriculturally derived TBA metabolites. These compounds are synthetic steroids approved for use as growth promoters in animal agriculture. These steroid hormones originated from animal manure, and are transformed through environmental degradation processes such as photolysis, biodegradation, sorption, and hydrolysis. However, these steroids are often transported by runoff to receiving waters with certain agricultural systems such as waste lagoons and sediments with a variety range of redox potentials. TBA metabolites were occationally detected in some receiving waters and these steroids were persistent in anaerobic environments (Schiffer et al., 2001). Therefore, the objective of this study was to predict the fate of TBA metabolites by microorganisms, and the specific effects of different redox conditions.

To monitor microbes in various redox conditions, DAPI fluorescence and FDA hydrolysis were used to estimate microbial activities. Cells in non-sterile conditions increased from 5.8×10^5 to 6.9×10^5 cells/mL by DAPI fluorescence and FDA hydrolysis also increased from 0.2 mg/L to 1.9 mg/L over 10 days in aerobic conditions (E_h > 100 mV). However, cell growth in non-sterile conditions appeared to be inhibited in anaerobic (E_h < -400), anoxic (-400 mV < Eh < -200 mV), and suboxic (-100 mV < E_h < 100 mV) buffering conditions where we observed decreasing FDA hydrolysis and microbial population decrease over 10 days.

Half-lives and biodegradation rates of TBA metabolites were measured in different redox conditions over 10 days. In aerobic conditions (E_h > 100 mV), 17α-trenbolone, 17β-trenbolone, trendione degraded nearly 80%, 40%, and 20% by biotic processes over 10 days, but trendione showed an increase after 3 days. We observed that the losses of 17α-trenbolone, 17β-trenbolone, trendione in aerobic conditions were about 20%, 50%, and 50% in sterile controls, respectively. We also obtained half-lives of 17α- and 17β-trenbolone, trendione, yielding 0.9 d, 1.5 d, and 2.7 d, respectively. In suboxic conditions (-200 mV < E_h < 0 mV) poised by DTT redox buffering, 17α-trenbolone was observed to decrease about 30% by abiotic processes in sterile controls, and a further 20% by biodegradation, but 17β-trenbolone showed little loss due to biodegradation compared to sterile controls. In the same system, trendione was attenuated about 70% by abiotic processes with little biodegradation. The observed half-lives of 17α- and 17β-trenbolone, and trendione were 2.7, 2.0, and 3.6 days. In anoxic conditions (-400 mV < Eh < -200 mV) poised by L-cysteine, there were losses of 70% of 17α- and 17β-trenbolone in sterile controls, with little biodegradation. Trendione degraded somewhat, but it's concentration unexpectedly increased in aerobic, anoxic, and anaerobic system. The half-lives of 17α- and 17β-trenbolone were 3.3 and 3.2 days. In anaerobic conditions (E_h < -400 mV) poised by titanium(III) citrate, there was little biodegradation (less than 20%) but nearly 70% losses from abiotic processes were observed for 17α-, 17β-trenbolone, and trendione. The half-lives of 17α-, 17β-trenbolone, and trendione were 19.2, 8.8, and 7.8 days, respectively. From the data, we observed that abiotic processes such as sorption may play a key role in attenuation under all redox conditions because of the moderately hydrophobic characteristics of all three androgen hormones.

Additionally, we measured some correlation between biodegradation rates and ORP values: 17α-trenbolone degraded more rapidly in aerobic conditions compared to anaerobic conditions. Similarly, 17β-trenbolone was degraded easily in suboxic and aerobic conditions compared to anoxic and anaerobic conditions. However, trendione hardly degraded compared to 17α-, 17β-trenbolone in all conditions but trendione also degraded more easily in aerobic conditions compared to anaerobic conditions.

Conclusions

TBA metabolites (17α-, 17β-trenbolone, and trendione) were degraded through biotic processes as well as abiotic processes in various redox conditions. However, little attenuation due to biotic processes was observed in suboxic, anoxic, and anaerobic conditions compared to aerobic conditions. These differences in redox state contributed to different half-lives and biodegradation rates in the systems. ORP values obviously affected biodegradation rates, and aerobic conditions were more favorable conditions compared to suboxic, anoxic, and anaerobic conditions for 17α-, 17β-trenbolone, and trendione. However, significant losses of TBA metabolites in sterile controls suggested that abiotic processes may play the other key a key role in the fate of TBA metabolites in aquatic environments. In future studies, abiotic process studies such as sorption should be assessed for anticipating the fate of TBA metabolites in aquatic environments.