DISSERTATION

PHOTODEGRADATION OF SELECTED ENDOCRINE AND PHARMACEUTICALLY ACTIVE COMPOUNDS UNDER ENVIRONMENTALLY RELEVANT CONDITIONS – PROCESSES AND PRODUCTS

Submitted by

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ABSTRACT

PHOTODEGRADATION OF SELECTED ENDOCRINE AND PHARMACEUTICALLY ACTIVE COMPOUNDS UNDER ENVIRONMENTALLY RELEVANT CONDITIONS – PROCESSES AND PRODUCTS

Within the past few decades, books such as *Silent Spring* and *Our Stolen Future* have done much to raise the public's awareness of the threat to human and ecosystem health from chronic exposure to chemical pollution, and considerable effort has been made to identify priority pollutants, understand their toxicological effects, and examine their presence and fate within the environment. Two classes of chemical pollutant that can induce biological effects at extremely low concentrations are pharmaceuticals and steroid hormones. Each has been linked to adverse health effects in humans or wildlife, and each has been detected in surface waters throughout the world, from sources including wastewater treatment plants and agricultural operations.

Once present in surface waters, pharmaceuticals and steroid hormones are subject to various transformation and removal processes, including biodegradation, photodegradation, and sorption to colloids or sediments. Because biodegradation and sorption are the primary removal processes during wastewater treatment, photodegradation might be especially important in surface waters, at least for those pharmaceuticals and steroid hormones that have survived wastewater treatment. This dissertation examines the photodegradation of selected pharmaceuticals and steroid hormones (androstenedione, testosterone, and lamotrigine) under environmentally relevant conditions, including natural or simulated sunlight, aqueous solutions at different pH, and the presence of dissolved organic matter.

Chapter 1 of the dissertation provides a brief introduction to surface water pollution and environmental photochemistry. In addition, chapter 1 lists the dissertation objectives and the publications, presentations, and outreach efforts derived from it.

Chapter 2 of the dissertation reviews existing literature on the presence of steroid hormones in freshwater ecosystems, their sources, and their potential fate. Chapter 2 compiles information about the physical and chemical properties of selected steroid hormones, and considers how biodegradation, photodegradation, and sorption influence steroid hormone fate in freshwater ecosystems. In addition, chapter 2 offers suggestions for future research.

Chapter 3 of the dissertation examines the direct photodegradation of lamotrigine (LTG), an antiepileptic and mood stabilizing drug that has been detected in wastewater, groundwater, surface water and drinking water. Because LTG is a weak base (pK_a = 5.7) that appears in two protonation states in natural waters, chapter 3 examines the photodegradation of LTG under simulated sunlight in buffered aqueous solutions at pH 3.3 (99.6% protonated), pH 5.3 (71.5% protonated) and pH 7.7 (1% protonated). Lamotrigine's half-life varied little (100 ± 3 to 112 ± 2 h) with solution pH, but its specific light absorption rate was 12 times higher, and its reaction quantum yield was 13 times lower, at pH 7.7 versus pH 3.3. Using these reaction quantum yields and a spectral radiation model (SMARTS v 2.9.5), LTG's estimated photodegradation rate in the estimated midday, midsummer sunlight of Denver, CO, USA (latitude 39.8617 °N) was more than twice as fast at pH 7.7 versus pH 3.3, demonstrating that solution pH can have a substantial effect on LTG's photodegradation in natural waters, depending on lighting and other environmental conditions. Lamotrigine's photoproducts were detected by liquid chromatography-UV diode array detection and time-of-flight mass spectrometry. Solution pH was shown to affect both the identities and relative abundances of

LTG's photoproducts. As a result, different reaction mechanisms were proposed. Finally, LTG's reaction quantum yield ($2.51 \pm 0.07 \times 10^{-5}$ mol einstein^{-1} at pH 7.7) and other results suggested that LTG and three photoproducts are approximately as resistant to direct photodegradation as carbamazepine, a frequently detected and relatively recalcitrant pharmaceutical in surface waters.

Chapter 4 of the dissertation compares the direct photodegradation of dilute (<10 μg L^{-1}) aqueous solutions of androstenedione (AD) and testosterone (T), two commonly observed male sex hormones, and evaluates the endocrine-disrupting potential of the resulting solutions. In addition, Chapter 4 examines the effect of dissolved organic matter (DOM) on AD photodegradation. During spring and summer at Henderson, NV, USA (latitude 36.04 °N), AD and T underwent direct photodegradation, with half-lives ranging from 3.7 to 10.8 h. In three model DOM solutions (Suwannee River fulvic acid, Suwannee River humic acid, and Nordic Reservoir natural organic matter), AD's half-life increased by 11 to 35%. Using screening factors to eliminate DOM's inner filter effect, quantum yield calculations suggested that light screening was primarily responsible for AD's increased half-life, and that physical quenching further inhibited AD's photodegradation in two out of three DOM solutions (Suwannee River fulvic acid and Suwannee River humic acid). *In vitro* androgenic activity of the AD and T solutions decreased approximately as fast as AD and T were removed, suggesting that solar photodegradation reduces the risk of endocrine disruption in surface waters impacted by AD or T, subject to continuing inputs. Finally, the reduced *in vitro* androgenic activity appeared to be related to steroid ring cleavage and the formation of highly oxidized photoproducts.

Chapter 5 of the dissertation presents broad conclusions from the research described in the dissertation, and suggests future work to advance the understanding of pollutant persistence and environmental photodegradation processes.

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The phrase “long and winding road” has new meaning to me now. The transition from business lawyer to environmental scientist was more difficult than expected, but it has been rewarding, and I have many people to thank.

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Others who have encouraged me most include my brother, Bryan Young, my friends, Jens Blotevogel and Mike Massey, and my advisor, Dr. Thomas Borch, who gave me the opportunity to pursue this path with almost no scientific experience. More than anybody, Thomas made the change from business lawyer to environmental scientist possible for me.

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CHAPTER 1- INTRODUCTION

Chronic Exposure to Chemical Pollution

The exponential growth of the human population has created increasing demands on planetary resources, including fresh water [1], and increasing impacts on ecosystems and natural resources, including habitat fragmentation and chemical pollution [2, 3]. The adverse health effects of acute exposure to toxic chemicals are easy to observe, and dramatic in effect – including fish kills from chemical spills [4], and casualties from industrial accidents [5]. In comparison, the adverse health effects of chronic exposure to low concentrations of toxic chemicals are less obvious, and accumulate over time, making the causal connection between exposure and its consequences difficult to establish. In fact, the science of determining appropriate measures for adverse health effects, and "safe" levels of chronic exposure, is itself extremely complex [6-8]. Nevertheless, considerable evidence of such adverse health effects exists [9], and books such as *Silent Spring* [10] and *Our Stolen Future* [11] have done much to raise the public's awareness of the threat to human and ecosystem health from chronic exposure to chemical pollution. As a result, in recent decades, considerable effort has been made to identify priority pollutants [12-14], understand their toxicological effects, and examine their presence and fate within the environment [1, 15-17].

Numerous factors influence the risk to human and ecosystem health from chronic exposure to toxic chemicals. Some chemical pollutants are extremely persistent. For example, there are 209 possible polychlorinated biphenyl isomers and congeners (PCBs), and the environmental half-lives of selected PCBs have been estimated to range from approximately 3 to 500 days in air, 60 days to 27 years in water, and 3 to 38 years in soils and sediments [18]. Many POPs are hydrophobic, and partition strongly to organic matter and sediments [19]. Because

sorption is an equilibrium process, which includes fast and slow sorption processes [20], these persistent organic pollutants (POPs) can repartition among air, water and soil as their relative concentrations change, and disperse over large geographical areas [19]. Hydrophobic POPs are also lipophilic, and become stored in fatty tissues, facilitating their transport through the food chain [19, 21, 22]. In comparison, hydrophilic POPs persist in water (e.g., perfluorinated chemicals) [23], and have the potential to contaminate public drinking water supplies [24-26].

Other chemical pollutants are extremely potent, and have the ability to cause adverse health effects at extremely low concentrations, particularly near their point of origin [27-31]. One early study of the adverse health effects of wastewater treatment plant (WWTP) effluent on fish began after fisherman observed hermaphrodite fish in WWTP lagoons [27]. Similar adverse health effects on fish have been linked to potent endocrine-active chemicals (e.g., steroid hormones) in wastewater-dominated waters [28, 32], and to low concentration, ambient levels of endocrine-active chemicals present in typical British rivers [33]. During a 7-year whole-lake experiment, one study found that the chronic exposure of fathead minnows to 5 to 6 ng L^{-1} concentrations of 17α-ethinylestradiol (EE2), a synthetic hormone and birth control medication, caused the fish population to collapse near extinction [34]. Subsequently, the predicted no effect concentration for EE2 was determined to equal only 0.35 ng L^{-1} [35].

Once present in the environment, many environmental pollutants undergo chemical, biochemical, and photochemical changes to produce a variety of transformation products. Some transformation products are known to be more abundant than their precursors, but most presumably have never been identified [3]. In addition, while many transformation products are more polar, more mobile, and less toxic than their parent compounds, some can be more toxic

and more persistent [3]. Finally, the effect of mixtures of toxic compounds, including parent compounds and their transformation products, is generally poorly understood [36, 37].

Concurrent with the public's increasing awareness of the threat to human and ecosystem health from chronic exposure to chemical pollution, considerable progress has been made in the field of analytical chemistry, enabling more chemicals to be detected in environmental samples at extremely low or "trace" (i.e., $\leq$ ng L^{-1}) concentrations [38]. At the same time, some of the focus of environmental pollution research has shifted from agrochemical and industrial POPs to household products, pharmaceuticals, and other chemicals consumed by the general public [1, 2]. Because many of these chemicals occur in the environment at trace concentrations, they have been termed "micropollutants" [39].

Pharmaceuticals as Micropollutants

Pharmaceuticals are administered to prevent, treat and cure human and animal diseases, and designed to induce biological effects at low concentrations [40]. As a consequence, pharmaceutically active chemicals tend to be extremely potent.

During the ten-year period from 2003 to 2012, global pharmaceutical sales grew from $502.2 billion to $962.1 billion [41]. In the United States during 2012, pharmaceutical sales were estimated to exceed $325 billion [42], and over 4 billion prescriptions were dispensed [43]. At the same time, sales of nonprescription over-the-counter (OTC) medicines in the United States topped $29 billion [44]. Similarly, during 2011, the global sales of veterinary healthcare products, which include pharmaceuticals, biologicals, and medical feed additives, exceeded $21 billion [45].

One consequence of such widespread use is the potential for biologically active pharmaceuticals and metabolites to enter the environment after use or disposal [46]. In fact, trace

quantities of pharmaceuticals and their metabolites have been detected in surface waters, groundwater, drinking water, soils, and biota throughout the world [15, 16, 47, 48]. A recent study synthesized 155 studies involving the detection of 203 pharmaceuticals in surface waters across 41 countries, and reported occurrence data for the 61 most studied compounds [15]. Excluding four antibiotics (ciprofloxacin, enrofloxacin, norfloxacin, and oxytetracycline), the median reported concentrations for the 61 most studied compounds were less than 1 μg L^{-1} [15]. Such concentrations are not generally assumed to represent a serious threat to drinking water quality [38, 46, 49, 50], but the impact of chronic low-level doses or mixtures of pharmaceuticals on specific individuals and ecosystems is not well-understood [38, 39, 51]. For example, within approximately only 15 years, the Oriental white-backed vulture population in Asia collapsed by 99.9%, from tens of millions of birds, after the vultures consumed the carcasses of livestock treated with diclofenac, a human and veterinary anti-inflammatory medication [52, 53]. Subsequent research has determined that the vultures are unable to metabolize diclofenac and other anti-inflammatory medications efficiently, presumably due to genetic differences in their metabolic enzymes [53].

Pharmaceuticals are believed to enter the environment primarily through wastewater treatment systems, after pharmaceuticals are consumed and excreted [15, 54]. This pathway includes overflows from combined sewers [55, 56], effluents from onsite wastewater treatment systems [57], and discharges from centralized wastewater treatment plants (WWTPs), where removal efficiencies vary widely [15, 58, 59]. However, many other pathways are possible, including direct releases from aquaculture and confined animal feeding operations (CAFOs), and leaching or runoff from manure and biosolids applied to land as fertilizer [60-62].

Steroid Hormones as Micropollutants

The endocrine system uses hormones to regulate a wide variety of biological activities, including sexual development, reproduction, metabolism, homeostasis, and immune system functions [63]. Steroid hormones are synthesized by endocrine glands and secreted into the bloodstream, where they travel to target cells, and bind to specific hormone receptors. Once "activated", the receptors dimerize, bind to hormone response elements in DNA, and activate gene expression [64-66]. Hormones are extremely potent, as only picomolar concentrations (< 1 ng L^{-1}) are needed to activate a biological response [67].

Numerous environmental chemicals have the demonstrated ability to disrupt normal endocrine system functions in humans and wildlife (collectively, endocrine disrupting chemicals, or EDCs) [9, 68]. In wildlife, the evidence for endocrine disruption comes primarily from studies of species living in or closely associated with aquatic ecosystems [27, 32], and the reported effects include abnormal blood hormone levels, masculinization of females, feminization of males, altered sex ratios, intersexuality, and reduced fertility [28, 33].

The mechanisms of endocrine disruption vary. Some EDCs (agonists) mimic the body's own steroid hormones, and others (antagonists) inhibit their action [63, 65]. Hormone agonists and antagonists are believed to bind to different receptor sites, and to induce different hormone receptor conformations [64]. Each binds to DNA, but the antagonist-induced conformations fail to activate target genes [64]. Whether natural or synthetic, environmental (exogenous) hormones generally have same binding affinities and potencies as the body's own (endogenous) hormones [69, 70]. Other hormone agonists are less potent, and generally active only in the nanomolar to micromolar range [65, 67].

Environmental steroid hormones originate from many of the same sources as pharmaceuticals, including wastewater treatment plants, concentrated animal feeding operations, and agricultural fields where manure and biosolids are applied as fertilizers [55, 71-77].

Fate of Environmental Micropollutants

Once present in surface waters, environmental micropollutants are subject to various transformation and removal processes, including biodegradation, photodegradation, and sorption to colloids or sediments [78-90]. The relative importance of each removal process will depend on the micropollutant's physical and chemical properties. For example, hydrophobic contaminants are expected to partition strongly to organic matter and sediments [19], where they would generally be less available for biodegradation [20, 91] and photodegradation [92]. Furthermore, to undergo direct photodegradation, micropollutants must possess aromatic rings, heteroatoms, or other functional groups that can absorb light [93].

Biodegradation involves a variety of chemical processes, including dehalogenation, dealkylation, hydrolysis, oxidation, reduction, ring cleavage, conjugation, and methylation, which are catalyzed by various enzymes (e.g., monooxygenase, laccase, dioxygenase, cytochrome P450, dehydrogenase, lignin peroxidase, esterase, and dehalogenase) [94]. For example, cytochrome P450 (CYP) enzymes commonly catalyze monooxygenase reactions, which oxidize a coenzyme and reduce molecular oxygen to produce water and hydroxylate the target compound [95]. Because environmental micropollutants are present at trace concentrations, they are generally believed to be degraded by microbial enzymes through co-metabolism [96].

Cytochrome P450 enzymes are found throughout nature. Humans have approximately 53 CYP enzymes, the tuberculosis bacterium (*Mycobacterium tuberculosis*) has approximately 20,

and one plant (*Arabidopsis thaliana*) has up to 286 [95]. These enzymes perform a variety of functions, including drug metabolism in mammals [95]. In fact, when pharmaceuticals are developed, a major objective is to identify molecules that resist metabolic degradation and persist long enough to produce the desired biological effect at the desired anatomical site [97]. This property can cause a portion of the pharmaceutical to be excreted from the body unmetabolized after administration, and might explain the ability of some pharmaceuticals to survive wastewater treatment.

The primary removal processes during wastewater treatment are biodegradation and sorption [98]. As a consequence, photodegradation could be especially important in surface waters, at least for micropollutants that have survived the wastewater treatment process [93, 99].

Principles of Environmental Photochemistry

Environmental photodegradation is commonly described in terms of direct and indirect photodegradation. Direct photodegradation occurs when a micropollutant absorbs light, becomes electronically excited, and undergoes chemical transformation. Indirect photodegradation, on the other hand, is chemical transformation initiated after a co-solute, like dissolved organic matter (DOM), absorbs light and becomes electronically excited. The excited state co-solute can: (i) transfer energy to the micropollutant (ET, or sensitization), which becomes electronically excited and undergoes chemical transformation; or (ii) generate various reactive species, including hydroxyl radicals ($^{\bullet}OH$), singlet oxygen ($^1O_2$), superoxide radical ions ($O_2^{\bullet-}$), and excited triplet state dissolved organic matter ($^3DOM^*$) [100-103], which then react with the micropollutant (Figure 1-1).

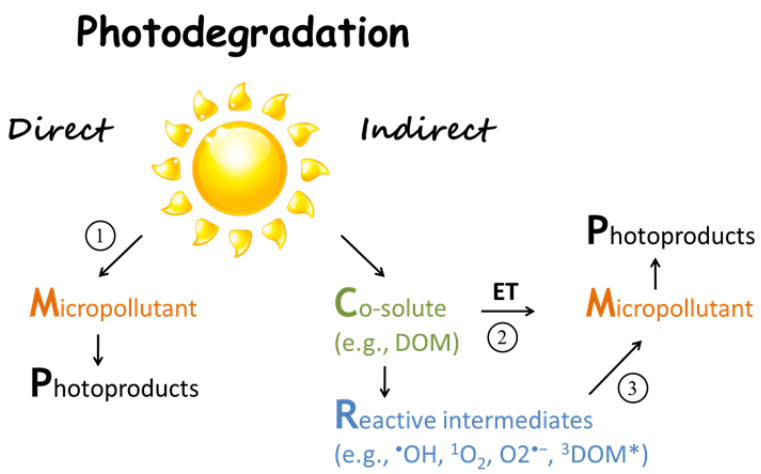

FIGURE 1-1. Direct vs. indirect photodegradation. Schematic illustrating the direct (1) and indirect (2 and 3) photodegradation of a micropollutant, where indirect photodegradation includes energy transfer to the micropollutant (2), or the generation of reactive intermediates (3).

The photochemical spectrum in natural waters ranges from 290 to 700 nm [104]. For a micropollutant, the rate of direct photodegradation can usually be expressed as a first order rate equation (Equation I), where the rate constant (k_{dM}) is determined by the spectrum and intensity of available light, the micropollutant's ability to absorb such light (i.e., molar absorptivity), and the efficiency at which the absorbed light is converted to chemical change (i.e., the reaction quantum yield) (Equation II).

$$-\frac{d[P]}{dt} = k_{dM}[P] \quad \text{(I)}$$

$$k_{dM} = 2.303\, \ell\, \phi_M \sum I_{0\lambda}\varepsilon_{\lambda M} \quad \text{(II)}$$

where: [P] is the micropollutant's concentration; k_{dM} is the first order rate constant for the micropollutant's direct photodegradation (t^{-1}); Φ_M is the micropollutant's reaction quantum yield (mol einstein^{-1}); $I_{0\lambda}$ is the irradiance at each incident wavelength λ (einstein L^{-1} t^{-1}); $\varepsilon_{\lambda M}$ is the micropollutant's molar absorptivity at each incident wavelength λ (L mol^{-1} cm^{-1}), and ℓ is the pathlength of the incident light (cm).

Put differently, in a completely mixed water body, the average rate of direct photodegradation is directly proportional to the rate of light absorption by the micropollutant, and the micropollutant's reaction quantum yield [105].

When the amount of light absorbed by a micropollutant and other water constituents is less than 5% (e.g., near the surface), the rate of light absorption by the micropollutant is approximately independent of other light absorption (e.g., by DOM) [105]. If attenuation exceeds 5%, the fraction of remaining incident light can be quantified with the following light screening factor (S_λ, Equation III) [101]:

$$S_\lambda = \frac{(1 - 10^{-\alpha_\lambda l})}{2.303\ \alpha_\lambda l} \qquad \text{(III)}$$

where: α_λ is the measured attenuation coefficient at each incident wavelength λ (cm^{-1}), and ℓ is the pathlength of the incident light (cm).

As an example, when the pathlength is 1 cm and the water body's measured attenuation increases from 0.01 to 0.1, S_λ decreases from 99% to 89%.

The micropollutant's reaction quantum yield can be expressed as a ratio of the amount of photodegradation to the amount of light absorbed (Equation IV):

$$\phi_M = \frac{moles\ of\ micropollutant\ eliminated}{moles\ of\ photons\ absorbed} \qquad \text{(IV)}$$

As a result, a reaction quantum yield of 0.001 (or 0.1%) indicates that 1 in every 1000 ***absorbed*** photons will eliminate 1 micropollutant molecule. Because upper excited states rapidly decay to lowest excited singlet or triplet states in solution, reaction quantum yields in natural waters are generally assumed to be wavelength-independent [106, 107]. This means that reaction quantum

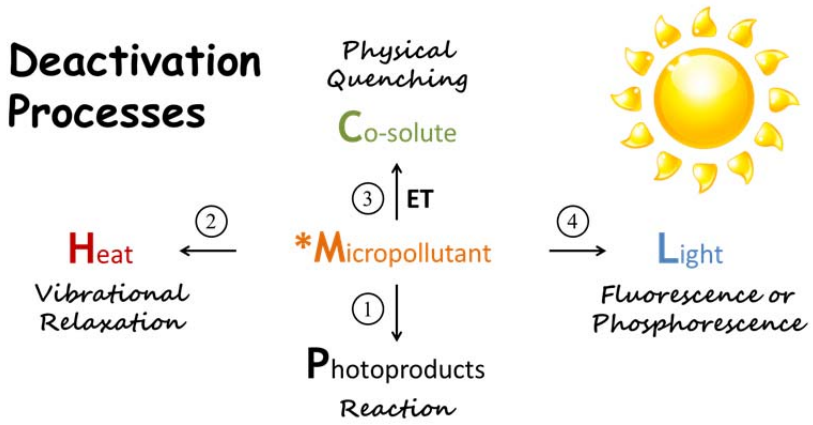

FIGURE 1-2. Excited state deactivation processes. Schematic illustrating selected processes for deactivating a micropollutant's electronically excited state (*M), including (1) photodegradation, (2) vibrational relaxation, (3) physical quenching by a co-solute, and (4) luminescence (i.e., fluorescence or phosphorescence).

yields can be used to predict direct photodegradation rates across various lighting conditions, assuming that the spectrum and intensity of the incident light can be measured or modeled.

A reaction quantum yield below 1 indicates that radiative and non-radiative processes other than photodegradation (e.g., luminescence, vibrational relaxation, and physical quenching by co-solutes) are also deactivating the micropollutant's electronically excited state (Figure 1-2). Each process has its own quantum yield, and the sum of quantum yields for all deactivation processes is expected to equal 1 [105]. However, secondary thermal reactions, such as free radical chain reactions, can cause the aggregate observed quantum yield to exceed 1, because some micropollutant molecules are consumed in non-photochemical reactions. Similarly, abiotic back-transformation reactions can cause the aggregate observed quantum yield to be less than 1, because some micropollutant molecules are being regenerated [105, 108, 109].

In the presence of co-solutes, some micropollutants can undergo ***both*** direct and indirect photodegradation. In this case, the observed reaction quantum yield will be a composite of the direct photodegradation reaction quantum yield and all interactions with the co-solutes and their photoproducts (e.g., $^{\bullet}OH$ and $^{1}O_2$).

In the "typical" photochemical reaction, a micropollutant absorbs light, and becomes electronically excited. The micropollutant's electronically excited state often can be characterized as (i) a half-filled, electrophilic, lower-energy molecular orbital, and a half-filled, nucleophilic higher-energy molecular orbital, or (ii) two degenerate half-filled molecular orbitals that can be either electrophilic or nucleophilic [100]. The micropollutant's excited state undergoes "primary" photochemical reactions (e.g., hydrogen abstraction, homolytic cleavage, proton transfer or electron transfer), in competition with the other deactivation processes, to produce diradical or zwitterionic intermediates, and these intermediates undergo secondary, thermal reactions to produce stable photoproducts (Figure 1-3) [100]. The mechanisms of photochemical reactions can be complex, but the key point is that they are initiated by the separation of two previously paired electrons, often producing a highly reactive intermediate (e.g., a diradical intermediate that undergoes radical-type reactions).

Other important photochemical reactions include electrocyclic reactions (e.g., where a double bond is converted to a new ring-forming bond) and sigmatropic rearrangements (i.e., where a bonded atom or functional group moves to a new location, accompanied by a rearrangement of the molecule's existing double bonds). The fastest direct photochemical

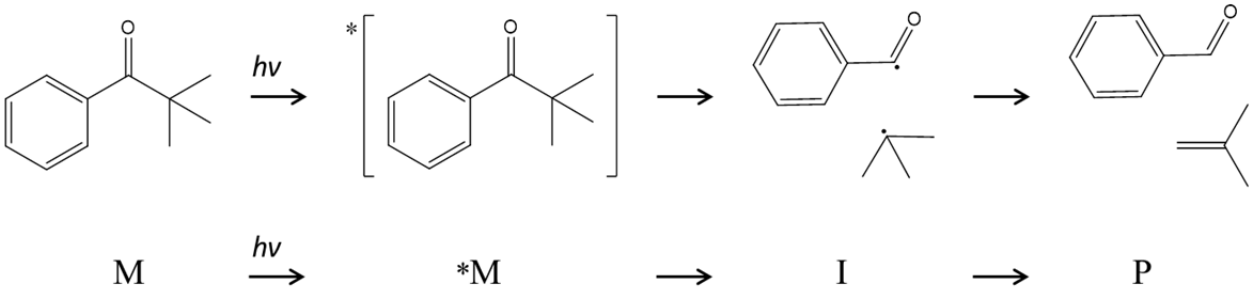

FIGURE 1-3. Representative photochemical reaction. Exemplar illustrating the photodegradation of phenyl tert-butyl ketone. The parent molecule undergoes α-cleavage (the primary reaction) from its excited triplet state to produce a triplet geminate radical pair (the diradical intermediate). The radical pair then undergoes a secondary, disproportionation reaction to produce two photoproducts. (Adapted from ref. 100.)

reactions (~10^{14} s^{-1}) are limited by rates of vibrational motion and electron transfer, and the slowest (~10^{-2} s^{-1}) are limited by slow phosphorescence, a competing deactivation process [100, 110]. In comparison, the fastest indirect photochemical reactions are limited by diffusion rates in water (~10^{10} M^{-1} s^{-1} at 25 °C) [100-103, 111]. As an example, steady state concentrations of $^{\bullet}OH$ in natural waters are reported to range from only 10^{-15} to 10^{-18} M [112], and $^{\bullet}OH$ is known to react with organic molecules at approximately diffusion-controlled rates. Accordingly, environmental micropollutants are expected to react with $^{\bullet}OH$ in natural waters at rates in the range of 10^{-5} to 10^{-8} s^{-1}. These reactions are not competitive with the fastest direct photochemical reactions, but $^{\bullet}OH$ would also be expected to react with their photoproducts.

In summary, photodegradation produces a wide variety of chemical reactions, including reactions that serve only to rearrange the structure of the parent molecule. These reactions are complicated in natural waters by co-solutes such as DOM, Fe^{3+}, CO_3^{2-}, and O_2, which can attenuate the available light, and enhance or inhibit photodegradation. In fact, DOM might produce all three effects, because DOM is, in reality, a complex, heterogeneous mixture of organic compounds, derived primarily from microorganisms and plant material decomposition [113, 114]. Understanding the mechanisms, products, and efficiency of environmental photodegradation can provide new insights into the persistence of micropollutants in natural waters, and new insights into the types of transformation products formed. Significantly, many of these transformation products may already exist, unidentified and undetected, in surface waters.

Dissertation Research Objectives

The overarching goal of this dissertation was to examine the photodegradation of selected endocrine and pharmaceutically active chemicals under environmental relevant conditions. The selected micropollutants (androstenedione, testosterone, and lamotrigine) are potent, biologically active chemicals with a demonstrated presence in surface waters [115-123]. Further, each of them absorbs light from the photochemical spectrum within natural waters (290 to 700 nm).

The first objective of this dissertation was to examine the potential of androstenedione (AD), testosterone (T), and lamotrigine (LTG) to undergo direct photodegradation at environmentally relevant wavelengths, and to determine the associated reaction quantum yields. Many photochemistry studies compare the relative photodegradation rates of different experimental treatments under similar conditions, but fail to determine reaction quantum yields that can be used to predict degradation rates or compare results under different lighting conditions [124-126]. Reaction quantum yields also provide the benchmark for determining the roles of indirect photodegradation, light screening, and physical quenching in natural waters [127].

The second objective of this dissertation was to determine the influence of certain environmentally relevant conditions on the direct photodegradation of AD, T, and LTG. Lamotrigine is a weak base (pKa = 5.7), expected to occur in surface waters in more than one protonation state. Because pH is known to influence a chemical's ability to absorb light and undergo photochemical reactions [128], the direct photodegradation of LTG was examined at various pH. Androstenedione and T are not expected to be affected by pH changes in natural waters, but some steroid hormones have been observed to undergo enhanced photodegradation in the presence of DOM [87, 129, 130]. As a result, the photodegradation of AD and T was examined in the presence of several DOM fractions.

The third objective of this dissertation was to determine the products resulting from direct photodegradation of AD, T and LTG, and the influence of photodegradation on their potency or persistence. For example, LTG was observed to degrade slowly ($t_{½}$ > 95 h) during preliminary experiments in a solar simulator, so an experiment was conducted to examine the identity and persistence of LTG and its photoproducts over a 28-day irradiation period, which extended beyond LTG's effective removal. Similarly, the irradiated AD and T solutions were analyzed to detect and identify photoproducts, and examined with a yeast-based androgen receptor assay to determine the effect of direct photodegradation on their endocrine disrupting potential.

Organization and Publication of Dissertation and Related Work

The research on AD and T photodegradation in this dissertation was part of a larger project to examine the presence, fate and transport of male sex hormones in the environment, supported by a grant from the Colorado Water Institute. Chapter 2 of this dissertation contains a review of the sources, presence, analysis and fate of steroid sex hormones in freshwater ecosystems, which was published by Nova Science Publishers, Inc. in an edited book titled *Aquatic Ecosystem Research Trends* [131]. Chapter 4 of this dissertation contains original research on AD and T photodegradation, which was published by the American Chemical Society in the peer-reviewed journal of "Environmental Science and Technology" [127]. In addition, Appendix A and Appendix B contain original research to which the author contributed on the biodegradation of male sex hormones, published respectively by the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America in the peer-reviewed "Journal of Environmental Quality" [80], and by the American Chemical Society in the peer-reviewed journal of "Environmental Science and Technology" [79].

The original research on AD and T photodegradation was also supported by the Applied Research and Development Center of the Southern Nevada Water Authority (Henderson, NV, USA), where the author worked as a graduate student intern from January 2009 to August, 2012. During that time, the author contributed to original research on other environmental micropollutants, outside the scope of this dissertation, including research on the presence of the artificial sweetener sucralose in U.S. drinking water systems (Appendix C), and research on synthesis of the perbromate ion (Appendix D), published by the American Chemical Society in the peer-reviewed journals of "Environmental Science and Technology" [132] and "Inorganic Chemistry" [133], respectively.

Finally, chapter 3 of this dissertation contains original research on LTG photodegradation, which was published by the Royal Society of Chemistry in an invited, themed issue of the peer-reviewed journal "Environmental Science: Processes & Impacts" titled "Advances in Aquatic Photochemistry" [110]. This research was part of a larger, ongoing project to examine the environmental fate of anti-epileptic drugs and their metabolites, supported by a grant from the United States – Israel Binational Agricultural Research and Development Fund (US-4551-12).

Presentations and Outreach

Parts of this dissertation have been presented at regional, national and international conferences, including Pittcon 2011 in Atlanta, Georgia (2011), the 2nd International Conference on Occurrence, Fate, Effects, and Analysis of Emerging Contaminants in the Environment (EmCon) in Fort Collins, CO (2009), the 236th ACS National Meeting in Philadelphia, PA (2008), and the ACS Rocky Mountain Regional Meeting in Denver, CO (2007).

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CHAPTER 2- SOURCES, PRESENCE, ANALYSIS, AND FATE OF STEROID SEX HORMONES IN FRESHWATER ECOSYSTEMS – A REVIEW[1]

Introduction

The endocrine system produces hormones, which travel through the bloodstream in extremely small concentrations (~10^{-9} g L^{-1} to 10^{-12} g L^{-1}) to specialized receptors in target organs and tissues, including mammary glands, bone, muscle, the nervous system, and male and female reproductive organs [1]. Hormones bind to hormone receptors, and the resulting complexes help to regulate gene expression, cell differentiation, hormone secretion, and other bodily processes [2]. Broadly speaking, the endocrine system uses hormones as chemical signals to regulate various important biological functions, including homeostasis (the body's ability to maintain a state of balance), growth, development, sexual differentiation, and reproduction [1, 3].

In recent years, scientists have become concerned about the exposure of humans and wildlife to chemicals in the environment that can disrupt the normal function of their endocrine systems, especially during critical stages of growth and development [4, 5]. Suspected endocrine disruptors in aquatic environments 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 [6, 7].

The mechanisms of endocrine disruption are complex. Endocrine disruptors 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

[1] Reprinted in part from 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, NY, USA, 2009; pp 103-164 with permission from Nova Science Publishers, Inc.

hormone receptor populations [5, 6, 8]. An endocrine disruptor's potency appears to be related primarily 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 [9]. 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 [10-12]. In addition, mixtures of endocrine disruptors can have additive or even synergistic effects [13-18]. When attempting to assess the environmental risks of endocrine disruption, it is difficult to generalize across species, because the basic mechanisms of sex differentiation, metabolism, and receptor structure and function differ across species [19-21].

The health effects of endocrine disruption have been extensively reviewed [1, 4-6, 8, 22-24]. Most of the evidence for endocrine disruption in wildlife has come from studies on species living in, or closely associated with, aquatic environments [20]. Many of the observed effects appear to result from disruption of the functions of steroid sex hormones, and particularly those of estrogens [20]. Adverse effects of endocrine disruption include abnormal blood hormone levels, masculinization of females, feminization of males, altered sex ratios, intersexuality, and reduced fertility and fecundity [20, 24, 25]. In fish, for example, intersexuality is characterized by the presence of oocytes (female gametophytes) within testicular tissue, or disruption of reproductive duct development [25, 26]. One study from 1995 to 1996 examined wild populations of freshwater fish (roach; *Rutilus rutilus*), and reported a high incidence of intersexuality across the United Kingdom [26]. Other studies also have reported evidence of endocrine disruption in freshwater ecosystems [25, 27-29].

Among suspected endocrine disruptors, exogenous steroid sex hormones (i.e., not self-produced) generally have the highest affinities for binding to steroid sex hormone receptors, and

the highest potencies for disrupting steroid sex hormone functions [13, 19, 21, 30, 31]. 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^{-1} (fathead minnows; *Pimephales promelas*), 17β-estradiol as low as 1-10 ng L^{-1} (rainbow trout; *Oncorhynchus mykiss*), and estrone as low as 25-50 ng L^{-1} (rainbow trout; *Oncorhynchus mykiss*) [32, 33]. 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^{-1} adversely affected gonadal development in males and egg production in females, and led to a near extinction of fathead minnows from the lake [34]. After a review of more than 100 studies on the effects of 17α-ethinylestradiol on aquatic organisms, 0.35 ng L^{-1} has been recommended as the predicted no-effect concentration (PNEC) for 17α-ethinylestradiol in surface water [35]. 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, and their ultimate fate in the environment.

Therefore, the objective of this article is to survey and critically review existing literature regarding the presence of steroid sex hormones in freshwater ecosystems, their sources, and potential fate. In particular, the article will examine discharges from wastewater treatment plants, and transport from agricultural operations. The article also will consider how biodegradation, photodegradation, and sorption to sediments influence the fate of steroid sex hormones in freshwater ecosystems. Finally, the article will compile relevant information about the physical and chemical properties of selected steroid sex hormones, and conclude with suggestions for future research.

Steroid Sex Hormones – In General

Steroid sex hormones and their receptors are found in a range of vertebrate and invertebrate species [5]. 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 [6, 9, 11].

There are three classes of steroid sex hormones: androgens, estrogens, and progestagens [3]. In vertebrates, androgens play a key role in the development of male traits, spermatogenesis, mating and breeding behaviors, reproduction, and muscle growth [3, 31]. The most common androgens among vertebrates are testosterone and 5α-dihydrotestosterone, although 11-ketotestosterone is common among fish [3, 6]. In vertebrates, estrogens are crucial for the development of female traits, ovulation, reproduction, mating and breeding behaviors, and somatic cell function [3, 20, 23]. In egg-laying vertebrates, estrogens also stimulate the liver to produce vitellogenin, a precursor of egg yolk constituents and eggshell proteins [6]. The most common estrogens among vertebrates are 17β-estradiol, estrone, and estriol [6]. In vertebrates, progestagens influence water and salt metabolism, and help to maintain pregnancy through various anti-estrogenic and anti-androgenic effects [36]. The most common progestagen among vertebrates is progesterone, although 17α, 20β-dihydroxyprogesterone is important among fish [3]. 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 [20, 37]. Testosterone, 17β-estradiol, estrone, and progesterone have been reported in many invertebrate groups, but their role is not well understood [20, 37]. In addition, progesterone has been detected in the dry mature wood, pine bark, and pine needles of loblolly pine (*Pinus taeda* L.) [38].

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 hormones [39-42]. Studies have suggested that glucuronide conjugates are deconjugated by sewage bacteria (e.g., *Escherichia coli*) before they reach WWTPs [40, 41, 43]. Sulfate conjugates are more recalcitrant, and have been detected in WWTP influent and effluent [40, 44, 45]. The types of natural steroid sex hormones that are excreted, and the degree of conjugation, varies with species, gender, and stage of reproduction, as reviewed previously [41, 42, 46, 47].

Exogenous natural and synthetic steroid hormones are administered to humans and livestock for a variety of 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, 48, 49]. In livestock, testosterone, trenbolone (synthetic androgen), 17β-estradiol, zeranol (non-steroidal estrogen), progesterone, and melengestrol (synthetic progestagen) are used as growth promoters [22, 46-49]. 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 [49].

Steroid Sex Hormones – Physical and chemical properties

The physical and chemical properties of steroid sex hormones can influence their ability to bind with steroid sex hormone receptors, and their distribution, bioavailability, and persistence in freshwater ecosystems [50].

The molecular structure of a representative steroid (a 27 carbon cholestane) is set forth in Figure 2-1. In general, steroids are characterized by a carbon skeleton consisting of four fused rings (a cyclopentan-*o*-perhydrophenanthrene ring) [51]. Differences among steroids arise from variations in the number and location of double bonds, and the type and stereochemical arrangements of functional groups along the carbon skeleton [52]. The steroid skeleton causes steroids to be rigid and hydrophobic, and variations in double bonds and functional groups along the steroid skeleton determine the intermolecular interactions that are possible (e.g., hydrogen bonding, dipole-induced dipole interactions, etc.) [31]. For example, the phenolic A-ring common to estrogens is polarizable, and can accept or donate hydrogen bonds.

Selected physical and chemical properties of a representative group of steroid sex hormones are set forth in Table 2-1. The representative group includes all three classes of steroid sex hormones (androgens, estrogens, and progestagens), and many of the steroid sex

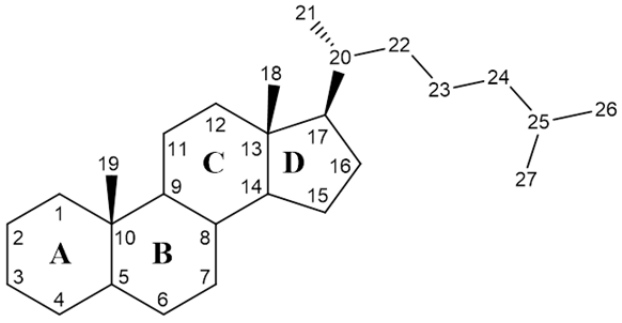


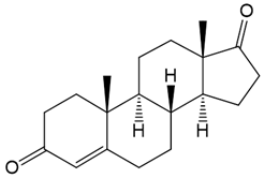
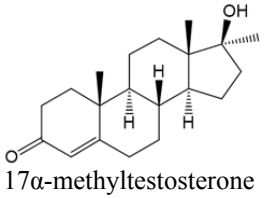
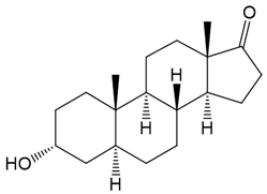
FIGURE 2-1. Basic steroid hormone structure (27 carbon cholestane). The steroid skeleton is characterized by four fused rings, labeled from A to D. Each carbon is labeled from 1 to 27.

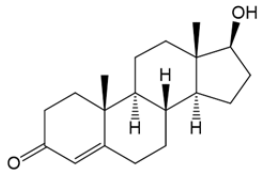
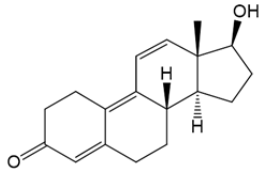
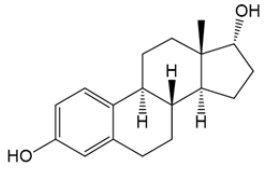
hormones that are common among vertebrates. The physical and chemical properties include molecular structures, molecular weights, water solubilities (S_w), octanol-water partition coefficients (K_{ow}), and relative binding affinities (RBAs) for androgen and estrogen receptors. Acid dissociation constants (pK_a) are not given, because the reported pK_a values for steroid sex hormones substantially exceed expected pH values in freshwater ecosystems (e.g., 17β-estradiol has a pK_a of 10.23) [53, 54]. Likewise, vapor pressures are not given, because steroid sex hormones generally are not volatile, and their vapor pressures are very low (e.g., the vapor pressure of 17β-estradiol is approximately 3×10^{-8} Pa) [51, 55].

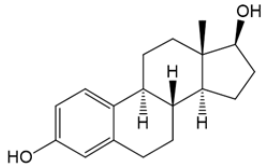
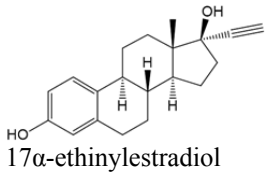
Water solubility data in the scientific literature can be highly variable. For example, some reported estrogen water solubilities vary by a factor of approximately 15 (17β-estradiol = 3.1 to 12.96 mg L^{-1}; 17α-ethynylestradiol = 3.1 to 19.1 mg L^{-1}; and estrone = 0.8 to 12.4 mg L^{-1}) [50]. Experimental conditions such as temperature, pH, and ionic strength can affect water solubilities, and probably represent the source of variability [50, 53, 56].

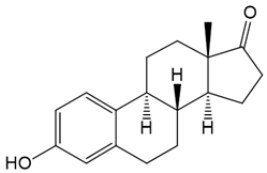
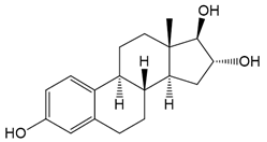
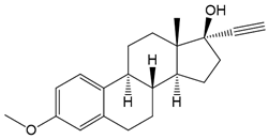
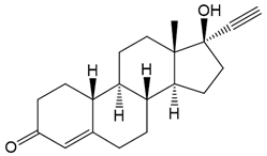
K_{ow} measures a chemical's distribution at equilibrium between water and 1-octanol (an organic solvent), in order to determine the chemical's relative hydrophobicity [57]. Hydrophobicity is important for many biological endpoints, including a chemical's ability to bind to steroid sex hormone receptors [31, 58]. Because measured values range over 12 orders of magnitude (10^{-4} to 10^{8}), K_{ow} values are usually expressed as logarithms (log K_{ow} or log P). Experimental log K_{ow} values, and modeled log K_{ow} values calculated by KOWWIN™ computer software, are set forth in Table 2-1 [59]. A prior version of the same program, which uses an atom/fragment contribution method to estimate log K_{ow} values, predicted log K_{ow} values within ±0.8 log units for over 96% of an experimental dataset of 8,406 compounds [57].

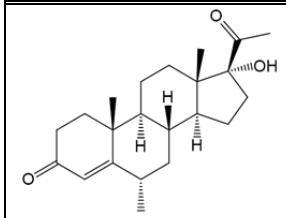
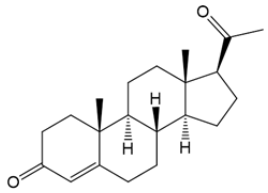
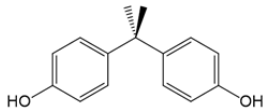
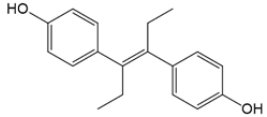
TABLE 2-1. Steroid hormone physical and chemical properties. Steroid hormone physical and chemical properties, including molecular structures, molecular weights (MW), water solubilities (S_w), octanol-water partition coefficients (K_{ow}), and relative binding affinities (RBA) for androgen and estrogen receptors (determined using *in vitro* competitive binding assays). NA = not available; SB = slight binder (< 50% inhibition); NB = non-binder; Ref. = reference.

	Chemical Data	Function	S_w (mg L^{-1})	log K_{ow}	Androgen log RBA	Estrogen log RBA	Source	Ref.
ANDROGENS  androstenedione	$C_{19}H_{26}O_2$ MW: 286.42 CAS: 63-05-8	reproductive hormone	37-41 50.5 ± 2.1				Literature values (37 °C) Experimental (pH 6.8; 23 °C; n=6)	56
			57.8	 2.75 2.76			Literature value (25 °C) KOWWIN™ computer model, v. 1.67 Literature value	59
					-0.62	NA	Experimental (competitive binding assay)	31
 17α-methyltestosterone	$C_{20}H_{30}O_2$ MW: 302.46 CAS: 58-18-4	androgen replacement (human use)	33.9	 3.72 3.36			Literature value (25 °C) KOWWIN™ computer model, v. 1.67 Literature value	59
					1.28	NA	Experimental (competitive binding assay)	31
 cis-androsterone	$C_{19}H_{30}O_2$ MW: 290.45 CAS: 53-41-8	reproductive hormone	12 20.2	 3.07 3.69			Literature value (23 °C) Literature value (23 °C) KOWWIN™ computer model, v. 1.67 Literature value	59
					−2.12	NA	Experimental (competitive binding assay)	31

	Chemical Data	Function	S_w (mg L^{-1})	log K_{ow}	Androgen log RBA	Estrogen log RBA	Source	Ref.
 testosterone	$C_{19}H_{28}O_2$ MW: 288.43 CAS: 58-22-0	reproductive hormone	18-25				Literature values	56
			23.2 ± 1.6				Experimental (pH 6.8; 23 °C; n=6)	
			23.4				Literature value (25 °C)	59
				3.27			KOWWIN™ computer model, v. 1.67	
				3.32			Literature value	
					1.28		Experimental (competitive binding assay)	31
						NB	Experimental (competitive binding assay)	30
 17β-trenbolone	$C_{18}H_{22}O_2$ MW: 270.37 CAS: 10161-33-8	growth promoter (animal use)		2.65			KOWWIN™ computer model, v. 1.67	59
					2.05	NA	Experimental (competitive binding assay)	31
ESTROGENS  17α-estradiol	$C_{18}H_{24}O_2$ MW: 272.39 CAS: 57-91-0	reproductive hormone	3.6				Literature value (25 °C)	59
			3.9				Literature value (27 °C)	
				3.94			KOWWIN™ computer model, v. 1.67	
				4.01			Literature value	
					−2.40		Experimental (competitive binding assay)	31
						0.49	Experimental (competitive binding assay)	30

	Chemical Data	Function	S_w (mg L^{-1})	log K_{ow}	Androgen log RBA	Estrogen log RBA	Source	Ref.
 17β-estradiol	$C_{18}H_{24}O_2$ MW: 272.39 CAS: 50-28-2	reproductive hormone	3.1-12.96				Literature values	50
			1.51 ± 0.04				Experimental (pH 7; 25 ± 0.5 °C; n=6)	
			3.1 ± 0.02				Experimental (pH 6.8; 23 °C; n=6)	53
			3.6				Literature value (25 °C)	59
			3.9				Literature value (27 °C)	
				3.94			KOWWIN™ computer model, v. 1.67	
				4.01			Literature value	
					−0.12		Experimental (competitive binding assay)	31
						2.00	Experimental (competitive binding assay)	30
 17α-ethinylestradiol	$C_{20}H_{24}O_2$ MW: 296.41 CAS: 57-63-6	ovulation inhibitor (human use)	3.1-19.1				Literature values	50
			9.20 ± 0.09				Experimental (pH 7; 25 ± 0.5 °C; n=6)	
			3.1 ± 0.03				Experimental (pH 6.8; 23 °C; n=6)	53
			11.3				Literature value (27 °C)	59
				4.12			KOWWIN™ computer model, v. 1.67	
				3.67			Literature value	
					−1.42		Experimental (competitive binding assay)	31
						2.28	Experimental (competitive binding assay)	30

	Chemical Data	Function	S_w (mg L^{-1})	log K_{ow}	Androgen log RBA	Estrogen log RBA	Source	Ref.
 estrone	$C_{18}H_{22}O_2$ MW: 270.37 CAS: 53-16-7	reproductive hormone; (17β-estradiol metabolite)	0.8-12.4				Literature values	50
			1.30 ± 0.08				Experimental (pH 7; 25 ± 0.5 °C; n=6)	
			2.1 ± 0.03				Experimental (pH 6.8; 23 °C; n=6)	53
				3.43			KOWWIN™ computer model, v. 1.67	59
				3.13			Literature value	
					SB		Experimental (competitive binding assay)	31
						0.86	Experimental (competitive binding assay)	30
 estriol	$C_{18}H_{24}O_3$ MW: 288.39 CAS: 50-27-1	reproductive hormone (17β-estradiol metabolite)	13				Literature value	55
				3.67			Literature value	
				2.81			KOWWIN™ computer model, v. 1.67	59
				2.45			Literature value	
					−3.15		Experimental (competitive binding assay)	31
						0.99	Experimental (competitive binding assay)	30
 mestranol	$C_{21}H_{26}O_2$ MW: 310.44 CAS: 72-33-3	ovulation inhibitor (human use)	0.3				Literature value	55
				4.10			Literature value	
				3.68			KOWWIN™ computer model, v. 1.67	59
					NA	0.35	Experimental: competitive binding assay	30
PROGESTAGENS  norethindrone	$C_{20}H_{26}O_2$ MW: 298.43 CAS: 68-22-4	ovulation inhibitor (human use)	7.04				Literature value (25 °C)	59
				2.99			KOWWIN™ computer model, v. 1.67	
				2.97			Literature value	
					0.41	NA	Experimental (competitive binding assay)	31

	Chemical Data	Function	S_w (mg L^{-1})	log K_{ow}	Androgen log RBA	Estrogen log RBA	Source	Ref.
 medroxyprogesterone	$C_{22}H_{32}O_3$ MW: 344.50 CAS: 520-85-4	ovulation inhibitor (human use); estrus regulator (animal use)		3.50			KOWWIN™ computer model, v. 1.67	59
					−0.41	NA	Experimental (competitive binding assay)	31
 progesterone	$C_{21}H_{30}O_2$ MW: 314.47 CAS: 57-83-0	reproductive hormone	8.81	3.67 3.87			Literature value (25 °C) KOWWIN™ computer model, v. 1.67 Literature value	59
					− 0.70		Experimental (competitive binding assay)	31
						NB	Experimental (competitive binding assay)	30
OTHER  bisphenol-A	$C_{15}H_{16}O_2$ MW: 228.29 CAS: 80-05-7	plasticizer	120	3.64 3.32			Literature value (25 °C) KOWWIN™ computer model, v. 1.67 Literature value	59
					−2.39		Experimental (competitive binding assay)	31
						−2.11	Experimental (competitive binding assay)	30
 diethylstilbestrol	$C_{18}H_{20}O_2$ MW: 268.36 CAS: 56-53-1	synthetic non-steroidal estrogen (use banned)	12	5.64 5.07			Literature value (25 °C) KOWWIN™ computer model, v. 1.67 Literature value	59
					−1.66		Experimental (competitive binding assay)	31
						2.60	Experimental (competitive binding assay)	30

The steroid sex hormones' RBAs for androgen and estrogen receptors were determined using *in vitro* competitive binding assays [31, 58]. *In vitro* assays generally fall into three categories: (a) competitive binding assays that measure a chemical's relative binding affinity for steroid sex hormone receptors; (b) reporter gene assays that measure receptor binding-dependent biological activities; and (c) cell proliferation assays that measure cell proliferation upon exposure to steroid sex hormones (e.g., human breast cancer MCF-7 cells exposed to estrogens) [2, 60]. Each assay type measures different end points at different levels of biological complexity, but relative receptor activities tend to be consistent across different assay methods and species, at least with respect to estrogen receptors [2]. Estrogen receptor binding appears to be the major determinant across all three levels of biological activity, but structural and functional differences among receptors do exist across species [2, 21].

To calculate a test chemical's RBA, the test chemical is combined with hormone receptor proteins and a radiolabeled hormone standard to determine the test chemical amount that is required to prevent 50% of the hormone standard from binding to hormone receptors (inhibitory concentration or IC_{50}) [61]. The test chemical's RBA is then calculated according to the following formula [31, 58]:

$$RBA = \frac{IC_{50}(steroid\ hormone\ standard)}{IC_{50}(test\ chemical\ )} \times 100 \quad (1)$$

17β-estradiol is commonly used as the hormone standard for estrogen receptor studies, and 17α-methyltrienolone (a potent androgen) has been used as the hormone standard for androgen receptor studies [31, 58]. Because measured values can range over several orders of magnitude, RBAs are commonly expressed as logarithms (log RBAs). By definition, a hormone standard has an RBA of 100, and a log RBA of 2. Greater numbers indicate that the test

chemical has a hormone receptor binding affinity that is stronger than the hormone standard, and lesser numbers indicate a weaker binding affinity. For purposes of comparison, test chemicals can be divided into five categories: (a) strong binders (log RBA > 0); (b) moderate binders (−2 < log RBA < 0); (c) weak binders (log RBA < −2); (d) nonbinders that fail to compete with the steroid sex hormone standard (NBs); and (e) slight binders that fail to prevent 50% of the steroid sex hormone standard from binding (SBs) [31, 58].

Because chemicals with similar biological activities commonly share structural features, structure-activity relationships (SARs) have been studied to identify the molecular features that determine a chemical's biological activity. In an SAR analysis of estrogen activity, several structural features were found to be important for estrogen activity, using 17β-estradiol as a template: (a) hydrophobicity, because significant portions of the estrogen receptor are hydrophobic; (b) the presence of a hydrogen bond donor to mimic the phenolic A-ring common to estrogens; (c) the presence of a second hydrogen bond donor to mimic the alcohol group in the β position on carbon 17; (d) a distance between the hydrogen bond donors of approximately 9.7-12.3 Å (1 Å = 10^{-10} m); (e) a ring structure to increase rigidity; and (f) the presence of steric hydrophobic centers at the 7α and 11β positions, which must be able to fit in available cavities on the estrogen receptor [58]. For example, the molecular structure of estrone is identical to the molecular structure of 17β-estradiol, except that estrone has a ketone at carbon 17, instead of an alcohol group (Table 2-1). Ketones accept, but do not donate, hydrogen bonds. As a result, the RBA of estrone (7.3%) is approximately 14 times weaker than the RBA of 17β-estradiol (100%) [58]. Similar structural features, including the ability to form hydrogen bonds at carbons 3 and 17, also are important for androgen activity [31]. Among other things, information from SAR

studies can be used to predict endocrine disruption risks posed by steroid sex hormone degradation products.

Analysis of Steroid Hormones in Environmental Matrices

Analyzing environmental samples for trace levels of target compounds (e.g., ng L^{-1}) is challenging, because small amounts of target compounds must be extracted from large volumes or masses of sample material, and because environmental samples contain numerous potential interferences that can hide the presence of target compounds, mimic the presence of target compounds (generating "false positive" results), or cause overestimation or underestimation of target compound amounts. When target compounds are extracted and concentrated for analysis, potential interferences (e.g., natural organic matter) can be extracted and concentrated with them [40, 62]. For example, natural organic substances often remain after solid phase extraction (SPE) of natural waters, and give strong spectra during mass spectrometry (MS) across the 100-500 m/z range, where steroid sex hormones typically appear [63]. Therefore, considerable time is often spent developing extraction and cleanup methods to maximize the yields of target compounds, and eliminate the presence of interfering compounds [46].

After the sample has been prepared for analysis, some analytical instruments can target specific compounds to improve measurement sensitivity and selectivity, and thereby improve detection limits. For example, when mass spectrometry (MS) is used for analysis, selected ion monitoring (SIM) can improve the instrument's sensitivity and selectivity by focusing the instrument's resources on a narrow m/z range. When tandem mass spectrometry (MS/MS) is used, multiple reaction monitoring (MRM) can further improve selectivity by fragmenting m/z selected ions into characteristic fragments, which can be used to distinguish target compounds

from interferences in the same m/z range [64-66]. In fact, many analytical instruments and methods are available to improve detection limits, but potentially useful information about non-target compounds is often lost when selective methods are employed to lower detection limits [63, 67].

Time-of-flight mass spectrometry (TOF-MS) and quadrupole orthogonal acceleration time-of-flight mass spectrometry (QTOF-MS/MS) provide full-scan data over a broad m/z range, and accurate mass measurements that can distinguish isobaric ions with the same nominal m/z value (e.g., a nominal m/z value of 156, but different exact mass m/z values of 156.0119 and 156.0773) [63, 68]. As a result, TOF-MS and QTOF-MS provide distinctive information about both target and non-target compounds, and help to identify new and unknown compounds [63, 69].

Any study on the presence of steroid sex hormones in the environment should be considered in the context of the analytical techniques that were used, and the compounds that were targeted.

Steroid Sex Hormones – Sources and Presence in the Environment

Sources and "Hotspots"

Steroid sex hormones have many natural sources. They are excreted continuously by vertebrates, and have been reported in many invertebrate groups and loblolly pine [37]. In addition, many microbial species can transform cholesterol and plant sterols into steroid sex hormones (e.g., plant sterols → androstenedione) [70, 71]. Other potential sources of steroid sex hormones include WWTPs, septic systems, concentrated animal feeding operations (CAFOs), agricultural operations, rangeland grazing, paper mills, and aquaculture [38, 42, 46, 47, 52, 72-78]. Once steroid sex hormones enter the environment, they are subject to a variety of transport and removal processes (e.g., sorption, dilution, biodegradation, and photodegradation). Therefore, detectable and

biologically relevant concentrations are likely to diminish with time and space, unless continuous inputs act to create steady-state conditions.

For these reasons, it is important to identify the "hotspots" where steroid sex hormones occur at biologically relevant concentrations, and to understand what happens over time as transport and removal processes take place [28, 76, 77, 79-83]. It is also important to understand the factors that may contribute to hormone hotspots, including such diverse factors as manure management and pharmaceutical disposal practices.

WWTPs

In a series of studies conducted in the U.K. from 1986 to 1989, after anglers casually observed the occurrence of intersex fish in WWTP lagoons, caged rainbow trout were placed in WWTP effluent, and subsequent measurements revealed increased vitellogenin concentrations in their plasma (500 to 100,000 times higher than fish maintained in spring or tap water) [84]. Increased vitellogenin concentrations were also observed in carp, but to a lesser extent. A separate study of seven U.K. WWTPs was conducted to identify and quantify the causes of estrogenic activity in treated wastewater [85]. Various analytical techniques (including a reporter gene assay, SPE, and HPLC) were used to isolate the estrogenic compounds from the effluent, and the resulting fractions were analyzed by GC-MS. The most active fraction (> 80% of the total effluent activity) was found to contain 17β-estradiol (1-50 ng L^{-1}), estrone (1-80 ng L^{-1}), and 17α-ethinylestradiol (up to 7 ng L^{-1}). Because the effluent came from urban sewage treatment plants, the estrogens were assumed to originate from humans.

Additional examples of steroid sex hormone concentrations in WWTP effluents are set forth in Table 2-2. The concentrations of steroid sex hormones in WWTP effluent are influenced

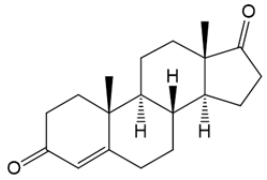
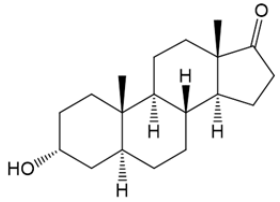
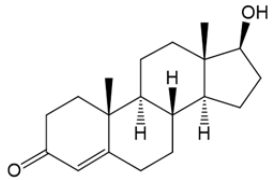
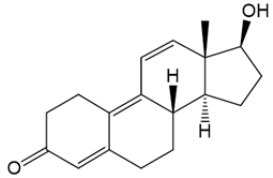
by several factors, including the composition of WWTP influents, and the treatment processes used [29, 41, 82, 86-89].

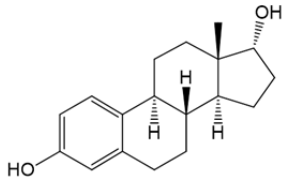
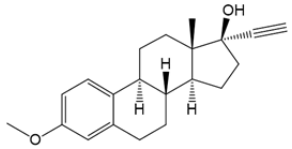
Steroid Sex Hormones – CAFOs

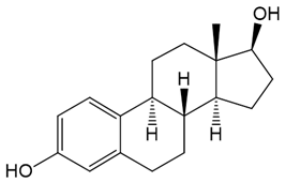
Endocrine disruption has been observed in fathead minnows (*Pimephales promelas*) exposed to feedlot effluent, and female painted turtles (*Chrysemys picta*) in ponds near livestock pastures [90, 91]. In concentrated animal feeding operations (CAFOs), solid wastes are commonly separated, dewatered, and collected for application as fertilizers, and liquid wastes are collected in lagoons, diluted with irrigation water, and applied as fertilizers [74, 92, 93]. Lagoons function as holding reservoirs or anaerobic digesters, and livestock wastes are typically applied to land without additional treatment [93, 94].

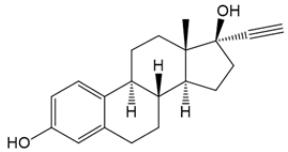
In one study, whole lagoon effluents from swine, cattle, and poultry CAFOs were analyzed to determine concentrations of free estrogens and estrogen conjugates [94]. Lagoon samples were centrifuged, and separated into liquid and solid components. The liquid components were filtered through 1.2 μm glass fiber filters, and split into two components for separate analyses of four free estrogens (17α-estradiol, 17β-estradiol, estrone, and estriol) and 13-16 estrogen sulfate and glucuronide conjugates. The conjugate samples were treated by enzyme hydrolysis, and all liquid samples were preserved with formaldehyde. The free-estrogen samples were extracted with SPE, derivatized, and analyzed by GC-MS/MS, and MDLs ranged from 4 ng L^{-1} (17α-estradiol) to 20 ng L^{-1} (17β-estradiol). The conjugate samples were extracted with SPE, and analyzed by LC-MS/MS, and the LOD was determined from the lowest quantitation standard (1 ng L^{-1}). The solid components were freeze-dried, extracted (liquid extraction, sonication, and SPE), derivatized, and analyzed by GC-MS/MS.

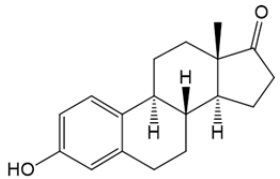
TABLE 2-2. Steroid Hormone Occurrence Data. Selected steroid hormone occurrence data, organized by steroid hormone and country, including median or **mean** (bold) concentrations (conc.), maximum concentrations (max.), numbers of samples (n), limits of detection (LOD), recovery percentages (recov.), sample types, forms of analysis, and references (Refs.; specified data is from first reference). NA = not available.

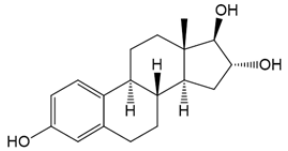
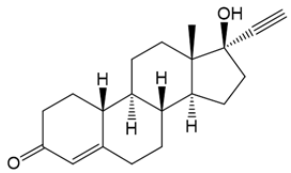
	Conc. (ng L^{-1})	Max. (ng L^{-1})	n	LOD (ng L^{-1})	Recov. (%)	Sample Type	Form of Analysis	Refs.
ANDROGENS								
 androstenedione						**JAPAN**		95, 96
	5.2	NA	2	1.2	91 ± 8.1	WWTP effluent	SPE (HLB, silica); UPLC-MS/MS	
	0.38	NA	4	0.06	85 ± 3.6	Surface water		95
 cis-androsterone						**JAPAN**		95, 96
	< LOD	NA	2	10	86 ± 7.2	WWTP effluent	SPE (HLB, silica); UPLC-MS/MS	
	< LOD	NA	4	5.0	82 ± 5.8	Surface water		95
	17	214	70	5	148.5 (d_3-testosterone)	**USA** Surface water	CLLE; GC-MS	77, 97, 98 97, 98
 testosterone						**JAPAN**		95, 96
	< LOD	NA	2	0.12	87 ± 5.6	WWTP effluent	SPE (HLB, silica); UPLC-MS/MS	
	< LOD	NA	4	0.06	81 ± 4.1	Surface water		95
	116	214	70	5	148.5 (d_3-testosterone)	**USA** Surface water	CLLE; GC-MS	77, 97, 98 97, 98
 17β-trenbolone						**JAPAN**		
	< LOD	NA	2	0.30	88 ± 2.4	WWTP effluent	SPE (HLB, silica); UPLC-MS/MS	
	< LOD	NA	4	0.10	84 ± 5.4	Surface water		95

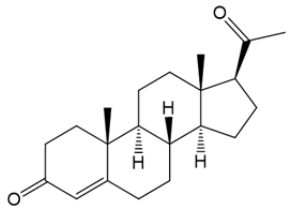
	Conc. (ng L^{-1})	Max. (ng L^{-1})	n	LOD (ng L^{-1})	Recov. (%)	Sample Type	Form of Analysis	Refs.
ESTROGENS						**NETHERLANDS**		43, 99
	< LOD	5.0	4	0.1-1.2	88 ± 12	WWTP effluent	SPE (SVB, C-18, amino); HPLC fractionation; GC-MS/MS	
	< LOD	3.0	6	0.1-0.3		Surface water		43
 17α-estradiol						**GERMANY**		
	0.5	4.5	16	0.15	NA	WWTP effluent	SPE (EVB); HRGC-MS	100
	0.4	2.0	31	0.15	NA	Surface water		
						USA		77, 97, 98
	30	74	70	5	128.8 (d_4-estradiol)	Surface water	CLLE; GC-MS	97, 98
						CHINA		
	1	2	3	0.5	NA	Surface water (low-flow)	SPE (C-18); GC-MS	101
						GERMANY		102
	< LOD	4	16	1	74 ± 1	WWTP effluent	SPE (C-18); silica gel cleanup; GC-MS/MS	
	< LOD	< LOD	15	0.5	90 ± 1	Surface water		102
 mestranol						**USA**		77, 97, 98
	17	407	70	5	128.8 (d_4-estradiol)	Surface water	CLLE; GC-MS	97, 98
						CHINA		
	< LOD	< LOD	1	1	105 ± 19	Surface water (low-flow)	SPE (C-18); GC-MS	101

	Conc. (ng L^{-1})	Max. (ng L^{-1})	n	LOD (ng L^{-1})	Recov. (%)	Sample Type	Form of Analysis	Refs.
 17β-estradiol						**NETHERLANDS**		43, 99
	0.9	12	6	0.5-2.4	88 ± 9	WWTP effluent	SPE (SVB, C-18, amino); HPLC fractionation; GC-MS/MS	
	< LOD	5.5		0.3-0.6		Surface water		43
						GERMANY		100, 102, 103
	< LOD	3	16	1	76 ± 14	WWTP effluent	SPE (C-18); silica gel cleanup; GC-MS/MS	
	< LOD	< LOD	15	0.5	77 ± 14	Surface water		102
						FRANCE		104, 105
	2.1 ± 0.6	NA	6	NA	84-98% (all estrogens)	Upstream (1 km)	SPE (C-18); GC-MS	
	6.6 ± 1.4					WWTP effluent (Colombes)		
	3.2 ± 0.3					Downstream		104
	1.4 ± 0.6					Upstream (1 km)		
	8.6 ± 0.9					WWTP effluent (Achères)		
	3.0 ± 0.6					Downstream (1 km)		
						SPAIN		29, 106, 107
	< LOD	NA	6	5.0	93	WWTP effluent (Calaf)	SPE (C-18); LC-DAD-MS	
	< LOD – 7.6					WWTP effluent (Igualada)		
	< LOD					WWTP effluent (Piera)		29, 106
	< LOD					WWTP effluent (Manresa)		
	< LOD					Surface water		
						USA		77, 97, 98
	9	93	70	5	128.8 (d_4-estradiol)	Surface water	CLLE; GC-MS	97, 98
						CHINA		
	1	2	2	1	113 ± 5	Surface water (low-flow)	SPE (C-18); GC-MS	101

	Conc. ($ng\ L^{-1}$)	Max. ($ng\ L^{-1}$)	n	LOD ($ng\ L^{-1}$)	Recov. (%)	Sample Type	Form of Analysis	Refs.
 17α-ethinylestradiol						**NETHERLANDS**		43, 99
	< LOD	7.5	6	0.3-1.8	96 ± 8	WWTP effluent	SPE (SVB, C-18, amino); HPLC fractionation; GC-MS/MS	
	< LOD	4.3		0.1-0.3		Surface water		43
						GERMANY		100, 102, 103
	1	15	16	1	76 ± 0	WWTP effluent	SPE (C-18); silica gel cleanup; GC-MS/MS	
	< LOD	< LOD	15	0.5	85 ± 0	Surface water		102
	1.1 ± 0.1 **2.7 ± 0.8** **2.3 ± 0.7** **1.5 ± 0.5** **4.5 ± 0.8** **2.9 ± 0.6**	NA	6	NA	84-98 (all estrogens)	**FRANCE** Upstream (1 km) WWTP effluent (Colombes) Downstream Upstream (1 km) WWTP effluent (Achères) Downstream (1 km)	SPE (C-18); GC-MS	104, 105 104
	< LOD	NA	6	5.0	94	**SPAIN** WWTP effluent (Calaf) WWTP effluent (Igualada) WWTP effluent (Piera) WWTP effluent (Manresa) Surface water	SPE (C-18); LC-DAD-MS	29, 106
						USA		77, 97, 98
	94	273	70	5	128.8 (d_4-estradiol)	Surface water	CLLE; GC-MS	97, 98
						CHINA		
	0	1	1	1	98 ± 13	Surface water (low-flow)	SPE (C-18); GC-MS	101

	Conc. (ng L^{-1})	Max. (ng L^{-1})	n	LOD (ng L^{-1})	Recov. (%)	Sample Type	Form of Analysis	Refs.
 estrone						**NETHERLANDS**		43, 99
	4.5	47	6	0.3-1	98 ± 14	WWTP effluent	SPE (SVB, C-18, amino); HPLC fractionation; GC-MS/MS	
	0.3	3.4		0.2-0.3		Surface water		43
						GERMANY		100, 102, 103
	9	70	16	1	82 ± 2	WWTP effluent	SPE (C-18); silica gel cleanup; GC-MS/MS	
	< LOD	1.6	15	0.5	90 ± 2	Surface water		102
						FRANCE		104, 105
	1.2 ± 0.2 **4.3 ± 0.6** **2.2 ± 0.3**	NA	6	NA	84-98% (all estrogens)	Upstream (1 km) WWTP effluent (Colombes) Downstream	SPE (C-18); GC-MS	
	1.1 ± 0.3 **6.2 ± 0.8** **3.0 ± 0.9**					Upstream (1 km) WWTP effluent (Achères) Downstream (1 km)		104
						SPAIN		
	< LOD – 8.1 < LOD – 2.7 < LOD < LOD – 7.2	NA	6	2.5	93	WWTP effluent (Calaf) WWTP effluent (Igualada) WWTP effluent (Piera) WWTP effluent (Manresa)	SPE (C-18); LC-DAD-MS	29, 106
	8.0					Surface water		
						USA		77, 97, 98
	27	112	70	5	128.8 (d_4-estradiol)	Surface water	CLLE; GC-MS	97, 98
						CHINA		
	34	65	8	0.5	118 ± 15	Surface water (low-flow)	SPE (C-18); GC-MS	101

	Conc. ($ng\ L^{-1}$)	Max. ($ng\ L^{-1}$)	n	LOD ($ng\ L^{-1}$)	Recov. (%)	Sample Type	Form of Analysis	Refs.
 estriol	**1.0 ± 0.4** **5.7 ± 1.6** **2.1 ± 0.7** **1.5 ± 0.5** **6.8 ± 0.6** **2.5 ± 0.6**	NA	6	NA	84-98% (all estrogens)	**FRANCE** Upstream (1 km) WWTP effluent (Colombes) Downstream Upstream (1 km) WWTP effluent (Achères) Downstream (1 km)	SPE (C-18); GC-MS	104, 105 104
	4.8 – 18.9 < LOD – 4.1 1.7 – 5.8 10.3 – 21.5 **6.3**	NA	6	0.25	85	**SPAIN** WWTP effluent (Calaf) WWTP effluent (Igualada) WWTP effluent (Piera) WWTP effluent (Manresa) Surface water	SPE (C-18); LC DAD-MS	29, 106
	19	51	70	5	128.8 (d_4-estradiol)	**USA** Surface water	CLLE; GC-MS	77, 97, 98 97, 98
	0	1	1	1	68 ± 6	**CHINA** Surface water (low-flow)	SPE (C-18); GC-MS	101
PROGESTAGENS  norethindrone	< LOD < LOD	NA NA	2 4	0.60 0.30	82 ± 3.6 79 ± 7.6	**JAPAN** WWTP effluent Surface water	SPE (HLB, silica); UPLC-MS/MS	95, 96 95
	48	872	70	5	148.5 (d_3-testosterone) 116.9 (d_7-cholesterol)	**USA** Surface water	CLLE; GC-MS	77, 97, 98 97, 98

	Conc. (ng L^{-1})	Max. (ng L^{-1})	n	LOD (ng L^{-1})	Recov. (%)	Sample Type	Form of Analysis	Refs.
 progesterone						**JAPAN**		95, 96
	0.34	NA	2	0.26	100 ± 12	WWTP effluent	SPE (HLB, silica); UPLC-MS/MS	95
	0.07	NA	4	0.02	83 ± 10	Surface water		
	< LOD < LOD – 1.1 < LOD 0.3 – 1.5 **4.3**	NA	6	0.20	113	**SPAIN** WWTP effluent (Calaf) WWTP effluent (Igualada) WWTP effluent (Piera) WWTP effluent (Manresa) Surface water	SPE (C-18); LC-DAD-MS	29, 106
	110	199	70	5	148.5 (d_3-testosterone) 116.9 (d_7-cholesterol)	**USA** Surface water	CLLE; GC-MS	77, 97, 98 97, 98

In the swine and poultry primary lagoons, free estrogens were distributed as follows: estrone > estriol > 17α-estradiol > 17β-estradiol. By comparison, in the dairy operation (secondary lagoon, 10,000 cows), free estrogens were distributed as follows: 17α-estradiol > 17β-estradiol > estrone > estriol. Generally, swine and poultry excrete more 17β-estradiol than 17α-estradiol, but fecal bacteria can oxidize 17β-estradiol to estrone, and reduce estrone back to 17α-estradiol and 17β-estradiol (17β-estradiol ↔ estrone ↔ 17α-estradiol) [108]. Microbial interconversion also has been observed with 17β-trenbolone (17β-trenbolone ↔ trendione ↔ 17α-trenbolone) [109, 110]. Estrogen sulfate conjugates were detected in the swine sow, poultry, and dairy lagoons, but not in the swine finisher, swine nursery, and beef feedlot lagoons.

Primary lagoon estrogen concentrations appear to be directly related to the number of animals housed in the CAFO. In the swine sow primary lagoon, which housed 662 swine sows, mean estrogen concentrations were 9,940 ng L^{-1} (estrone), 1,200 ng L^{-1} (17α-estradiol), 194 ng L^{-1} (17β-estradiol), and 6,290 ng L^{-1} (estriol). In a large swine sow facility that housed more animals (19,920 gestating sows, 4,980 farrowing sows, and 100 boars), a related study observed mean estrogen concentrations equal to 17,400 ng L^{-1} (estrone), 2,460 ng L^{-1} (17β-estradiol), and 7,830 ng L^{-1} (estriol) [111]. In the primary lagoon of a swine farrowing facility that housed fewer animals (100 sows and 15 boars), a separate study found mean estrogen concentrations equal to 81 ng L^{-1} (estrone), 3 ng L^{-1} (17β-estradiol), and 9.2 ng L^{-1} (estriol) [112].

Another study examined the transport of steroid sex hormones from dairy CAFOs. Groundwater samples were collected from two dairy farms immediately prior to the irrigation season (June – August), and one month after the end of the irrigation system [74]. The groundwater samples were collected from 13 shallow groundwater monitoring wells (7-10 m), including 3 wells located downgradient from dairy waste lagoons, 4 wells located within a

feedlot, 5 wells located downgradient from fields receiving regular manure applications, and 1 well located outside a dairy's influence (to serve as a control). Sandy to loamy sand soils caused high percolation rates, and groundwater ages ranged from a few days to 1-2 years, based on the depth where the water entered the well screen. Additional samples were collected from a deep aquifer well (> 25 m), a dairy waste lagoon, and surface water sites likely to be affected by agricultural operations, including sites upstream and downstream from dairy farms and irrigation canal discharge points, near tile drain pump discharge points, and in irrigation canals. The samples were prepared by various analytical techniques (including filtration, centrifugation, and SPE), derivatized, and analyzed by GC-MS/MS for 7 steroid sex hormones: 17β-estradiol, estrone, estriol, progesterone, medroxyprogesterone, testosterone, and androstenedione. For every 10 samples, one sample was spiked with 10 ng L^{-1} of the steroid sex hormones, and recoveries of the steroid sex hormones were correlated with recoveries of 100 ng L^{-1} mesterolone (used as a surrogate standard). Recoveries of the spiked analytes ranged from 56-85%, and method detection limits ranged from 0.1-0.2 ng L^{-1}.

Steroid sex hormone concentrations in the lagoon samples varied considerably between the two sampling dates. Estrone was the only steroid sex hormone detected on both sampling dates, and estrone concentrations varied by an order of magnitude (~65 ng L^{-1} to 650 ng L^{-1}). Steroid hormones were detected in 7 of the 26 shallow groundwater samples, at concentrations significantly below levels detected in the lagoons (6 detections < 10 ng L^{-1}; all 7 detections < 20 ng L^{-1}). Estriol, androstenedione, and progesterone were not detected in any groundwater samples. Among the groundwater samples, there was no consistency in the hormones detected, or the wells where they were detected. In addition, no steroids were detected in the deep aquifer well, or in samples from the fields' tile drainage system, which are believed to represent a

composite average of shallow groundwater throughout the dairy. In the surface water samples, estrone was most frequently detected (12 of 26 samples), with a maximum concentration of 17 ng L^{-1}. Testosterone was second most frequently detected (6 of 26 samples), with a maximum concentration of 1.9 ng L^{-1}. Estriol, androstenedione, and progesterone were not detected in any surface water samples.

It was suggested that steroid sex hormones are generally adsorbed or degraded over distances of 10-100 m when dairy wastewater infiltrates groundwater, because detections were sporadic and hormones were not detected in the tile drain samples. The sporadic detections were believed to result from preferential flow paths in the subsurface. Finally, runoff was suggested to be more important than leaching, because concentrations of steroid sex hormones in irrigation canal and river samples exceeded tile drain concentrations (e.g., estrone was detected in 13 of 26 surface water samples, and no tile drain samples) [74].

In another study, the transport of steroid sex hormones from a dairy waste lagoon to groundwater was studied at an Israeli dairy farm with approximately 60 dairy cows and 30 calves [113]. The facility used an unlined, earthen waste lagoon with an average depth of 0.5 m, and excess wastewater flowed directly into a dry creek. Two boreholes were drilled for sediment sampling and groundwater monitoring. The first borehole was drilled directly under the waste lagoon, after a portion of the lagoon was dried. The second borehole was drilled upgradient, in an agricultural field used for growing barley. Manure was not used to fertilize the agricultural field. Groundwater levels ranged from 42 m (agricultural field) to 47 m (waste lagoon) below the surface. Three types of sediment were found below the waste lagoon: (i) a layer from 0-6 m characterized by a high content of clay materials and organic matter; (ii) a transition layer of sandy loam from 6-8 m; and (iii) a layer > 8 m of sand with high calcareous content and low

organic matter. Groundwater and sediment samples were prepared by various analytical techniques (including filtration, centrifugation, liquid extraction and SPE), and analyzed by radioimmunoassay for androgens (testosterone, and possibly dihydrotestosterone) and estrogens (17β-estradiol and estrone). The reported detection limit was 0.3 ng L^{-1}, and the reported hormone recoveries were 90% if sample concentrations were above 1.0 ng L^{-1}, and < 50% if sample concentrations were below 0.5 ng L^{-1}. Radioimmunoassays have been criticized for lack of specificity, but the estrogen antibody was reported to exhibit negligible cross-reactions with other steroids [114, 115]. Testosterone and estrogens were found deep in the sediment profile under the waste lagoon, and were generally absent from the agricultural field. Testosterone was detected down to the groundwater surface (47 m), and estrogen was detectable to a depth of 32 m. The results suggested that steroid sex hormones from the dairy farm could be transported throughout the vadose zone, and that a clay lagoon lining may be insufficient to protect groundwater from dairy farm leachates.

Another study examined the transport of 17β-estradiol and estrone from swine manure to tile drainage systems at two Danish field sites on structured loamy soil, in part to account for effects from preferential transport in structured soils [116]. At both sites, the uppermost meter of soil was heavily fractured and bioturbated, and the water table was located approximately 1-3 m below ground surface. The tile drains were located at an average depth of 1 m, and laterally spaced 12-18 m apart. The average soil temperature of the plow layer ranged from 7 °C in April to 17-18 °C in July. The swine manure slurry was applied in accordance with Danish regulations on dose and application methods. Drainage water samples were collected during storm flow events over the 12 month period following manure application. Water samples were immediately adjusted to pH 3, and frozen until analysis. The water samples were prepared for

analysis by various techniques (including filtration, SPE, and silica gel cleanup), derivatized, and analyzed by GC-MS/MS. Manure samples were freeze-dried and frozen until analysis. The manure samples were prepared for analysis by various techniques (including mechanical homogenization, pressurized liquid extraction, centrifugation, SPE, and silica gel cleanup), derivatized, and analyzed by GC-MS/MS.

Estrone and 17β-estradiol leached from the root zone into tile drains at both field sites. Approximately one-half of the 27 samples had detectable estrone concentrations (maximum = 68.1 ng L^{-1}). At one site (Estrup), rapid leaching of estrone and 17β-estradiol occurred during the first storm event (2 days at 23 and 16 mm d^{-1}) at concentrations as high as 0.9 ng L^{-1} (estrone) and 0.2 ng L^{-1} (17β-estradiol). Thereafter, 17β-estradiol leached on a few occasions, and estrone was detected up to 11 months after the manure applications. At the other site (Silstrup), a storm event (26 mm d^{-1}) induced estrone leaching at a concentration of 68.1 ng L^{-1}, and subsequent events did not cause major estrone leaching. 17β-estradiol leached on one occasion at a concentration of 1.8 ng L^{-1}. Among other things, the study demonstrated that preferential transport and soil temperatures may significantly influence steroid sex hormone leaching to groundwater.

Steroid Sex Hormones – Biosolids

Biosolids (treated sewage sludge) are also applied to agricultural fields as fertilizers and soil amendments. Biosolids improve soil physical properties, and add important nutrients including nitrogen and phosphorous [117]. Estrogen sorption to activated and inactivated sewage sludge has been observed, and both androgenic and estrogenic activities have been detected in municipal biosolids [118-120]. In general, 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) [120].

During a survey of organic wastewater contaminants in nine biosolids products destined for land application, estrone was detected in one product at a concentration of 150 µg kg^{-1} [117] (GC-MS; full scan). No MDL was reported for estrone, but the reported MDLs (n = 4) for other steroids ranged from 168 µg L^{-1} (cholesterol) to 367 µg L^{-1} (stigmastanol), while the reported MDLs (n = 19) for non-steroidal pharmaceuticals ranged from 0.76 µg L^{-1} (acetaminophen) to 5.5 µg L^{-1} (gemfibrozil). Estrone might have been detected more frequently, and other steroid sex hormones might have been detected, if the method had been targeted to steroid sex hormones.

Steroid sex hormones probably will be transported like non-steroidal pharmaceuticals, which have been detected in drainage and runoff after land applications of biosolids (no steroids were tested) [121, 122].

Runoff and Leaching from Agricultural Operations – Other Studies

Other studies have determined that runoff and leaching from agricultural operations can be an important source of steroid sex hormones in ground and surface waters [76, 123-125]. Many factors appear to influence steroid sex hormone runoff and leaching from agricultural operations, including soil type, soil structure, precipitation amounts, soil temperature, sorption to colloids, and irrigation with wastewater [126-128]. Studies that fail to take these factors into account are unlikely to accurately describe steroid sex hormone transport.

Presence

Studies throughout the world have examined receiving waters for the presence of steroid sex hormones [29, 44, 45, 86, 89, 102, 129, 130]. Data from selected studies are set forth in Table 2-2 to illustrate the ubiquitous presence of steroid sex hormones around the world.

In one such study, water samples were collected from 5 WWTPs and 11 coastal and freshwater locations throughout the Netherlands [43]. The surface water locations were part of a water quality monitoring program, including river sample locations downstream from densely populated and heavily industrialized areas, and coastal sample locations in areas dominated by agriculture. Various analytical procedures (including SPE, HPLC fractionation, and GC-MS/MS) were used to determine the presence of 17β-estradiol, 17α-estradiol, estrone, 17α-ethinylestradiol, and their glucuronide conjugates. Glucuronide conjugates are not amenable to GC because of their low volatility and thermal instability [45]. Therefore, duplicate samples were taken at the WWTPs and three of the surface water locations, and deconjugated overnight at 37 °C with β-glucuronidase enzyme. The glucuronide conjugates were determined indirectly by comparing untreated samples with enzyme-treated duplicates. Data from the study are set forth in Table 2-2. Generally, steroid sex hormone recoveries ranged from 88-98%. Separate tests indicated that the enzyme reaction was complete, but the recovery of estradiol-17-glucuronide was still only 59%. Limits of detection ranged from 0.1-2.4 ng L^{-1}. 17β-estradiol (median 0.9 ng L^{-1}) and estrone (median 4.5 ng L^{-1}) were detected in most effluent samples, and the highest concentration detected in any sample was 47 ng L^{-1} (estrone). Hormone levels in the untreated samples and the enzyme-treated duplicates generally matched, suggesting that no glucuronide conjugates were present, even though humans excrete steroid sex hormones primarily as conjugates. In the surface water samples, estrone (median 0.3 ng L^{-1}) was detected

most frequently, and the highest concentration detected in any sample was 5.5 ng L^{-1} (17β-estradiol). In general, the study demonstrated that steroid sex hormones are ubiquitous in Dutch surface waters at low ng L^{-1} concentrations.

In a national reconnaissance study conducted by the U.S. Geological Survey (USGS) from 1999 to 2000, water samples from 139 stream sites across the United States were analyzed to determine the concentrations of 95 selected organic wastewater contaminants, including 14 steroid compounds [97, 98]. Each stream site was sampled once, using standard width and depth integrating techniques to obtain representative samples of the stream waters. Five different analytical methods were used, based on the type of compound being analyzed (i.e., antibiotics, hormones, etc.). In the steroid method, steroid compounds were extracted from the water samples by CLLE, and the extracts were concentrated, derivatized, and analyzed by GC-MS. Data from the national reconnaissance study are set forth in Table 2-2. Cholesterol (84.3%), coprostanol (85.7%), and estriol (21.4%) were among the thirty most frequently detected compounds. In addition, cis-androsterone (14.3%), 17β-estradiol (10.0%), and norethindrone (12.8%) were detected in at least ten percent of the stream sites. Among the steroid compounds analyzed with the steroid method, cholesterol (a lipid commonly associated with animals, but synthesized by all eukaryotes) and coprostanol (a metabolite formed from the hydrogenation of cholesterol) were detected at the highest median concentrations (cholesterol: 0.83 μg L^{-1}; coprostanol: 0.088 μg L^{-1}). Stigmastanol (a plant steroid) experienced poor average recoveries (< 60%) under a different method, but its estimated median concentration was 2 μg L^{-1}. The median concentrations of all other steroid compounds ranged from 9 ng L^{-1} (17β-estradiol) to 147 ng L^{-1} (equilin). In general, the reconnaissance study demonstrated that, like the

Netherlands, steroid compounds are ubiquitous in U.S. surface waters at low ng L^{-1} concentrations.

Steroid Sex Hormones – Sorption and Transformation Processes

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 transformation processes including photodegradation [55, 131]. Individually, these processes are complex, and not well understood. Collectively, these processes influence each other, complicating attempts to understand the environmental fate of steroid sex hormones.

Sorption - In General

Sorption has been defined as "the accumulation of a substance or material at an interface between the solid surface and the bathing solution" [132]. Sorption can occur through a variety of mechanisms, including hydrophobic partitioning, hydrogen bonding, and nonspecific van der Waals interactions [133]. The actual mechanism is influenced by physical and chemical properties of the sorbate (e.g., a steroid sex hormone) and the solid-phase sorbent. 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 [134]. Often, sorption to sand (50 μm < diameter < 2,000 μm) is relatively insignificant, and the sand fraction can be treated as diluting the sorptive capacity of clay (diameter < 2 μm) and silt (2 μM < diameter < 50 μm) [135]. In addition to particle size, the sorption of neutral hydrophobic contaminants to soil has been positively correlated with soil organic matter content [135, 136].

Sediments have been characterized as largely eroded soils that have been subjected to redispersion and particle-size fractionation by runoff and other water processes [135]. Because the properties of the parent soil and the dynamics of the specific stream, river, lake, or pond differ, the sediment within a given water compartment may contain a narrow range of particle sizes [135]. For example, suspended sediment in a river might be mostly clay, sediment from the middle of the river might be mostly sand, and sediment from the edge of the river might be mostly silt [135]. As a result, sorption will vary according to water compartment.

Sorption has the potential to affect the fate and transport of steroid sex hormones in the environment 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) [134, 137-140]. Also, photodegradation can be inhibited if steroids diffuse into unreactive micropores and other microenvironments in soil particles or organic matter [141].

Methods for determining chemical sorption to soils and sediments include batch equilibrium experiments and column displacement studies [142]. In batch equilibrium experiments, multiple chemical concentrations are used to measure the effect of concentration on sorption at apparent equilibrium [142]. In column displacement studies, sorption is studied by introducing a chemical into a glass column that is packed with soil, sediment, or another solid-phase sorbent, flowing water through the column, and measuring the chemical's concentration at the column exit as a function of time or relative pore volume (the amount of water in the packed column when saturated) [143, 144].

Frequently, sorption to soils and sediments can be described by the Freundlich equation, which describes a nonlinear relationship between the sorbed chemical amount and the dissolved chemical amount at apparent equilibrium:

$$S = K_f C^{1/n} \tag{2}$$

where: S = amount of chemical sorbed to soil (mg kg^{-1})
K_f = Freundlich sorption coefficient
C = equilibrium solution concentration (mg L^{-1})
1/n = index of nonlinearity

When n = 1, the Freundlich equation simplifies to a linear function, which indicates that the sorbed chemical amount is proportional to its solution concentration at apparent equilibrium:

$$S = K_d C \tag{3}$$

where: S = amount of chemical sorbed to soil (mg kg^{-1})
K_d = sorption coefficient
C = equilibrium solution concentration (mg L^{-1})

Generally, the sorption coefficient (K_f or K_d) for a chemical will vary with the soil's physical and chemical properties. However, because soil organic matter acts as a quasi-solvent for many hydrophobic contaminants, the carbon normalized partition coefficient (K_{oc}) can be reasonably constant across soil and sediment types [136, 142, 145]:

$$K_{oc} = K_d / f_{oc} \tag{4}$$

where: K_{oc} = carbon normalized sorption coefficient
K_d = sorption coefficient
f_{oc} = fraction of total mass attributable to organic carbon

The K_{oc} values of hydrophobic contaminants often correlate well with their octanol-water distribution coefficients (K_{ow}) [57, 135]. Although water solubility is a good estimator of K_{oc} values

for slightly soluble organic compounds, the K_{oc} values of hydrophobic contaminants do not always correlate well with their water solubility values (S_w) [135, 146]. This result might be explained by differences between the partitioning and dissolution processes, but also might be explained by differences in the experimental conditions used to determine water solubility [50, 135].

As the organic carbon content of a soil or sediment decreases, other mechanisms, besides partitioning into soil organic matter, can have increasing influence on sorption. Such other mechanisms include sorption to mineral surfaces, and diffusion or intercalation into clay mineral structures [147].

Sorption often occurs in stages. Generally, rapid sorption occurs first, followed by slower sorption that might last days, weeks, or even months before equilibrium is reached [147, 148]. The different rates of sorption have been attributed to separate sorption mechanisms. For example, rapid sorption has been attributed to hydrophobic partitioning and sorption to external mineral surfaces, and slower sorption has been attributed to diffusion into clay mineral structures and condensed, organic matter [147-149]. The fraction that sorbs slowly tends to be inversely dependent on initial concentration, which suggests that slow sorption is more important at lower concentrations [148]. As a result, sorption experiments that presume rapid sorption may underestimate actual sorption [148].

Sorption to Minerals

Some studies have examined the sorption of estrogens to selected minerals. One study used batch and continuous mode experiments to examine the sorption of 17β-estradiol to the iron oxyhydroxide goethite (BET surface area: 49.6 ± 0.1 $m^2\ g^{-1}$), and the clay minerals kaolinite (BET surface area: 14.7 ± 0.1 $m^2\ g^{-1}$), illite (BET surface area: 123.3 ± 0.3 $m^2\ g^{-1}$),

K-montmorillonite (BET surface area: 31.8 ± 0.1 m^2 g^{-1}) and Ca-montmorillonite (BET surface area: 31.8 ± 0.1 m^2 g^{-1}) [147]. To conduct the experiments, suspensions were created in glass reaction vessels for each mineral by adding the mineral to a 10 mM aqueous KNO_3 solution to obtain a surface area equivalent of 100 m^2 L^{-1} of suspension, and by pre-equilibrating the suspension under nitrogen for 18-25 h at 25.0 ± 0.5 °C. The suspensions were spiked with 17β-estradiol in methanol to an initial concentration of 4.17 μM (~ 1.14 mg L^{-1}). Batch experiments were conducted by transferring aliquots of the suspension to 50 mL polyethylene centrifuge tubes, purging with nitrogen, and mixing at 25 ± 2 °C for the required time. Continuous experiments were conducted in the original reaction vessel by maintaining a temperature of 25.0 ± 0.5 °C, purging continuously with nitrogen, and sampling the suspension periodically. Kinetic experiments were performed in both modes at pH 4.5, 6.5, and 8.0 by sampling periodically for 72 h. Desorption experiments were conducted by equilibrating for 1 week, centrifuging the suspension, and resuspending the mineral paste in 10 mM aqueous KNO_3 solution or methanol. For each sample, aqueous 17β-estradiol was determined by centrifuging the sample and analyzing the supernatant by HPLC.

Initially, 17β-estradiol sorbed rapidly to all substrates. Sorption to goethite ceased after 30 minutes, but illite and kaolinite continued to sorb 17β-estradiol slowly over a three-day period, until 10-15% of the 17β-estradiol was sorbed. The montmorillonite samples sorbed 60% of the 17β-estradiol over the same period. Degradation at the mineral surface was ruled out because there was no evidence of breakdown products, so the slow sorption period was attributed to diffusion-controlled sorption into clay mineral structures. The montmorillonite samples were believed to have higher sorption capacities because their interlayers are more accessible, and X-ray diffraction studies of the montmorillonite samples confirmed that interlayer spacing

(c-axis spacing) changed significantly when 17β-estradiol was present. The rapid sorption period was attributed to sorption on external surfaces. In methanol, 17β-estradiol desorbed completely from goethite, and desorption from kaolinite was greater than illite. In 10 mM aqueous KNO_3, the 17β-estradiol fraction desorbed from kaolinite was greater than illite and goethite. There was no evidence of desorption from the montmorillonite samples after 3 weeks. Sorption to goethite was believed to occur through weak interactions with uncharged surface hydroxyls, because sorption to goethite was greatest at mid pH (7.0), and desorption was complete in methanol. Sorption to the minerals' external surfaces was pH independent, and attributed to hydrophobic interactions (e.g., Van der Waals forces).

Another study examined the sorption of bisphenol A, 17α-ethinylestradiol, and estrone to goethite, kaolinite, and montmorillonite [150]. Like the prior study, suspensions were prepared by adding minerals to 10 mM aqueous KNO_3 solution to obtain a surface area equivalent of 100 $m^2 L^{-1}$ of suspension, and the suspensions were spiked with estrogens in methanol to an initial concentration of 3μM (817 $\mu g L^{-1}$). Continuous mode experiments were conducted in a nitrogen-purged glass reactor vessel pre-equilibrated for 20 h at 25.0 ± 0.5 °C, and batch experiments were conducted in nitrogen-purged glass centrifuge tubes mixed for the appropriate time at 25.0 ± 0.5 °C. Desorption experiments were conducted with 17α-ethinylestradiol, and kinetic experiments were performed in both modes at pH 4, 7, and 10. Separate experiments were also conducted to determine the effect of the flocculation state of suspended montmorillonite particles on sorption, because montmorillonite particles are believed to flocculate face-to-face at high pH, and edge-to-face at low pH.

Like 17β-estradiol in the prior study, 17α-esthinylestradiol and estrone sorbed rapidly to all substrates. Sorption to montmorillonite was slower, and occurred in two stages over a

48-hour period. The estrogens had a much higher affinity for montmorillonite than for goethite or kaolinite. In addition, sorption to montmorillonite increased steadily above pH 7, while sorption to goethite and kaolinite was pH independent. When pre-loaded goethite and kaolinite were resuspended in 10 mM aqueous KNO_3, approximately 80% of the 17α-esthinylestradiol desorbed within 30 minutes. A smaller fraction of the 17α-esthinylestradiol desorbed from montmorillonite at pH 4, and virtually none desorbed at pH 10 after 96 h. Like the prior study, the slow sorption period was attributed to diffusion-controlled sorption into clay mineral structures. Because sorption to goethite and kaolinite was pH independent and substantial desorption occurred in 10 mM aqueous KNO_3 solution, sorption to goethite and kaolinite was attributed to weak interactions with their external surfaces, and it was suggested that surface charge plays no significant role in the sorption process. Edge-to-face interactions of montmorillonite particles at lower pH were believed to reduce access to interlayer sites, inhibit sorption, and enhance desorption relative to montmorillonite at higher pH.

The influence of particle size (i.e., sand, silt, or clay) and mineral type (e.g., clay mineral or goethite) on estrogen sorption has been confirmed elsewhere [54]. It is clear that organic matter is not necessary for estrogen sorption, and that expanding clay minerals (e.g., montmorillonite) have a significant effect on the extent of sorption [54]. However, 17β-estradiol, estrone and, to a lesser extent, 17α-ethinylestradiol sorption has been shown to have a linear relationship with soil organic matter content [54]. Presumably, as organic matter content increases, the mineral influence on estrogen sorption and desorption will decrease. According to a study on pesticide sorption, clay and silt are primarily responsible for pesticide sorption when the soil organic matter content is less than 0.01%, organic matter is primarily responsible for pesticide sorption when the soil organic matter content is greater than 6-8%, and both are involved in the middle range [151]. The

same rule may apply to other steroid sex hormones, even if the actual percentage amounts are different due to the hormone's individual physical and chemical properties.

Together, the preceding studies suggest that estrogen sorption is substantially influenced by particle size and mineral type, and that sorption to mineral surfaces is generally rapid, weak, and reversible. The studies also suggest that diffusion-controlled sorption into clay mineral structures is slow, and difficult to reverse. The same principles will likely apply to androgens and progestagens.

Sorption to Colloids

Colloids are commonly defined as 1 nm to 1 μm sized particles, but larger particles also move with water through worm holes, plant root channels, and other soil macropores and fractures [134, 152]. To distinguish it from organic colloids, *dissolved* organic carbon can be defined as organic carbon that can pass through a molecular weight cutoff ultrafilter of a specified size (e.g., 1 kDa) [127]. Colloids are produced through microbiological processes and weathering, and commonly consist of clay particles, metal oxides, and organic material from manure, plant matter decomposition, and other biological sources [128, 134]. Relatively insoluble contaminants can precipitate as colloids, but sorption to organic or mineral colloids appears to be the most important method of colloidal transport [134]. Colloids have a high surface area in relation to their volume, and sorb contaminants through various mechanisms including hydrophobic partitioning, hydrogen bonding, London dispersion and other intermolecular forces [128, 133]. Physical and chemical properties, including size, determine whether colloids remain in suspension, or coagulate and settle as sediment [153].

Humic substances represent a significant part of organic colloids [154]. Humic substances are heterogeneous mixtures of various organic compounds, and are ubiquitous components of soils, sediments and surface waters [155]. Ordinarily, humic substances are divided into three groups based on their solubility in acids and bases [156]. Fulvic acids are smaller molecular weight molecules (~2 kDa), which are soluble in acids and bases. Humic acids are larger molecular weight molecules (~5-100 kDa), which are soluble only in bases. Humin describes the largest molecular weight molecules (~300 kDa), which are insoluble in acids and bases.

Several studies have examined steroid hormone sorption to colloidal material. However, the process of separating dissolved, colloidal, and particulate phases is challenging. In fact, the "conventional" dissolved phase, after filtration, often includes a mixture of dissolved and colloidal phases. Various methods have been used to determine the distribution of steroid hormones between dissolved and colloidal phases, and they vary in terms of accuracy, time efficiency, cost, and ease of use.

In one study, batch equilibrium experiments were used to investigate the sorption of 17β-estradiol, 17α-ethinylestradiol, estriol, and other suspected endocrine disruptors to seven representative organic colloids [137]. Aldrich humic acid, Suwannee River humic acid, Suwannee River fulvic acid, and Nordic fulvic acid were selected because humic acid and fulvic acid have been estimated to comprise 6-8% and 54-72%, respectively, of the total organic carbon in typical rivers and streams. To model aquatic humic acids better, the peat-based Aldrich humic acid was filtered with a 10 kDa dialysis membrane to eliminate the high molecular fraction, which is believed to cause excess sorption relative to aquatic humic acids. Alginic acid and dextran were selected as representative polysaccharides (a major component of microbial biofilms), which have been estimated to comprise 6-12% of the total organic carbon in typical rivers and streams.

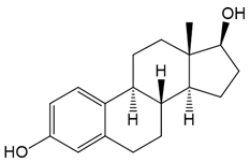
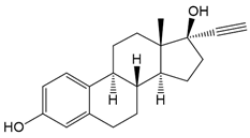
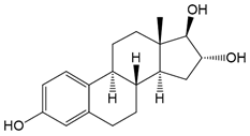
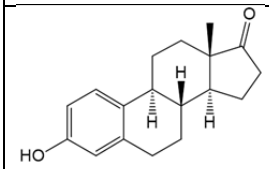
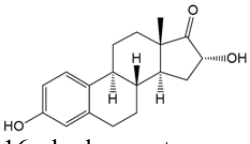
Finally, tannic acid (gallotannin) was selected to model plant residues. The batch equilibrium experiments were conducted in glass centrifuge tubes using four concentrations of the organic colloids, which ranged from 2-10 mg C L^{-1} in accordance with natural organic carbon levels. The initial estrogen concentration in each centrifuge tube was 700 μg L^{-1}, a concentration significantly below each estrogen's reported water solubility limit (Table 2-3). Each solution was adjusted to a pH of 7 and an ionic strength of 0.2 M, and mixed for approximately 24 hours at room temperature until equilibrium was reached. Fluorescence quenching was used to measure estrogen sorption to the organic colloids, but only for estrogens that would not fluoresce when sorbed or fail to fluoresce when aqueous in the presence of dissolved oxygen (a potential "quencher"). When the fluorescence quenching technique was used, organic carbon normalized partition coefficients (K_{oc}) were calculated according to the following formula:

$$F_o/F = 1 + K_{oc}\,[colloids] \tag{5}$$

where: F_0 = fluorescence without organic colloids
F = fluorescence with organic colloids
$[colloids]$ = the colloid concentration

Because 17β-estradiol and *p*-nonylphenol did fluoresce when sorbed to alginic acid and dextran, a more complex solubility enhancement technique was used to measure their aqueous concentrations. After analyzing selected compounds in Suwannee River humic acid and tannic acid under both techniques, the fluorescence quenching technique was believed to overestimate K_{oc} values (Table 2-3), but deemed acceptable for providing easy K_{oc} estimates.

TABLE 2-3. Steroid hormone organic carbon normalized sorption coefficients. Organic carbon normalized sorption coefficients (K_{oc}) for the sorption of selected steroid sex hormones to various colloids, the forms of analysis used (FQ = fluorescence quenching; SE = solubility enhancement; CFF = cross-flow ultrafiltration), and octanol-water partition coefficients (K_{ow}) for comparison; Ref. = reference.

	log Kow	log Koc	Colloid Type (Form of Analysis)	Organic Carbon	Ref.
ESTROGENS					
 17β-estradiol	4.01 (3.94)	4.94	Aldrich humic acid (FQ)	100%	137
		4.92	Suwannee River humic acid (FQ)		
		4.56	Suwannee River humic acid (SE)		
		4.57	Suwannee River fulvic acid (FQ)		
		4.61	Nordic fulvic acid (FQ)		
		3.75	Alginic acid (SE)		
		2.76	Dextran (SE)		
		5.28	Tannic acid (FQ)		
		4.94	Tannic acid (SE)		
	3.94, 4.01	3.98	Longford stream colloids (CFF)	2.3 mg L−1	128
		3.94	River Ouse colloids (CFF)	3.0 mg L−1	
		4.04	Horsham STW effluent colloids (CFF)	9.2 mg L−1	
		3.85	River L'Aa colloids (CFF)	2.9 mg L−1	
		4.86	Seawater colloids (CFF)	0.4 mg L−1	
 17α-ethinylestradiol	3.67 (4.12)	4.78	Aldrich humic acid (FQ)	100%	137
		4.80	Suwannee River humic acid (FQ)		
		4.55	Suwannee River fulvic acid (FQ)		
		4.63	Nordic fulvic acid (FQ)		
		3.23	Alginic acid (SE)		
		3.04	Dextran (SE)		
		5.22	Tannic acid (FQ)		
	4.15, 3.67	4.58	Longford stream colloids (CFF)	2.3 mg L−1	128
		4.81	River Ouse colloids (CFF)	3.0 mg L−1	
		4.73	Horsham STW effluent colloids (CFF)	9.2 mg L−1	
		4.85	River L'Aa colloids (CFF)	2.9 mg L−1	
		5.47	Seawater colloids (CFF)	0.4 mg L−1	
 estriol	2.45 (2.81)	4.99	Aldrich humic acid (FQ)	100%	137
		4.96	Suwannee River humic acid (FQ)		
		4.64	Suwannee River fulvic acid (FQ)		
		NA	Nordic fulvic acid (FQ)		
		NA	Alginic acid (SE)		
		NA	Dextran (SE)		
		5.32	Tannic acid (FQ)		
 estrone	3.43, 3.13	4.85	Longford stream colloids (CFF)	2.3 mg L−1	128
		4.83	River Ouse colloids (CFF)	3.0 mg L−1	
		4.30	Horsham STW effluent colloids (CFF)	9.2 mg L−1	
		4.67	River L'Aa colloids (CFF)	2.9 mg L−1	
		5.04	Seawater colloids (CFF)	0.4 mg L−1	
 16α-hydroxyestrone	3.23	4.20	Longford stream colloids (CFF)	2.3 mg L−1	128
		3.75	River Ouse colloids (CFF)	3.0 mg L−1	
		3.88	Horsham STW effluent colloids (CFF)	9.2 mg L−1	
		4.41	River L'Aa colloids (CFF)	2.9 mg L−1	
		4.67	Seawater colloids (CFF)	0.4 mg L−1	

Log K_{oc} values from the batch equilibrium experiments are set forth in Table 2-3. According to the experimental data, estrogens sorbed to the organic colloids in the following order: tannic acid > humic acids > fulvic acids > polysaccharides. The experimental log K_{oc} values for alginic acid were moderately correlated with the estrogens' reported octanol-water partition coefficients (log K_{ow}), which suggested that hydrophobic partitioning is important for estrogen sorption to polysaccharides. Otherwise, no trend was evident between the experimental log K_{oc} values and reported log K_{ow} values, suggesting that other mechanisms were also involved. No trends were evident between the experimental log K_{oc} values and the organic colloids' molecular weight averages, carboxylic group concentrations, or elemental ratios (e.g., H/O or (O+N)/C). However, the experimental log K_{oc} values did correlate with the phenolic group concentrations in the organic colloids, and with the estrogens' ability to absorb ultraviolet light at 272 nm, a wavelength associated with π electrons and aromaticity [157]. Together, these data suggest that π electron interactions play an important role in estrogen sorption to organic colloids. According to the experimental data, estriol sorbed to organic colloids more than any other estrogen, even though estriol was least hydrophobic estrogen tested (smallest K_{ow} value). No mechanism was suggested, but estriol's sorption might be explained by its ability to donate more hydrogen bonds to acceptor sites in the organic colloids, based on the number of hydroxyl functional groups (−OH) in estriol, and the fact that carbonyl groups (C=O) account for a significant fraction of the carbon atoms in organic colloids [158].

In two additional, related studies, cross-flow ultrafiltration and batch equilibrium experiments were used to investigate the distribution of 17β-estradiol, 17α-ethinylestradiol, estrone, 16α-hydroxyestrone, and other compounds between dissolved and colloidal phases in river water, treated effluent, and seawater [128, 152]. During cross-flow ultrafiltration, samples are

passed through an ultrafilter membrane, and suspended colloids with molecular weights exceeding the pore size are concentrated in the retentate (i.e., the sample portion that fails to pass through the ultrafilter membrane). For the batch equilibrium experiments, water samples from five locations were filtered through 0.7 μm glass fiber filters, and then passed through a 1 kDa ultrafilter membrane during cross-flow ultrafiltration. The resulting retentate contained colloids ranging in size from 1 kDa to 0.7 μm, which were concentrated 15-18 times relative to the original samples. Four different colloid concentrations were prepared from each sample by diluting the retentate with the < 1 kDa permeate (i.e., the sample portion that did pass through the ultrafilter membrane), and 5 L test samples were prepared by spiking the colloid solutions with estrogens to an initial concentration of 600 ng L^{-1}. Various mixing times of up to 1 month were used to equilibrate the solutions, and 0.05% sodium azide was added to samples mixed for more than 5 days to inhibit biodegradation. After mixing, cross-flow ultrafiltration was used to separate estrogens in the < 1 kDa permeate from estrogens sorbed to organic colloids in the retentate. Then, SPE and GC-MS were used to isolate and quantify the estrogens in both phases. Partition constants (K_p) were calculated with the following formula, and divided by the colloidal organic carbon fraction (f_{oc}) to determine the organic carbon normalized partition constants (K_{oc}):

$$C_r/C_f = 1 + K_p\,[colloids] \tag{6}$$

where: C_r = steroid hormone concentration in the retentate
C_f = steroid hormone concentration in the permeate
[*colloids*] = the colloid concentration

Log K_{oc} values from the batch equilibrium experiments are set forth in Table 2-3. In general, the ratio of estrogens in the retentate to estrogens in the permeate increased as colloid concentrations increased, suggesting sorption to the colloids. According to the experimental

data, 17α-ethinylestradiol sorbed to the colloids more than any other estrogen. The experimental data suggested that hydrophobic partitioning was not the only sorption mechanism involved, both because the experimental log K_{oc} values for the effluent and river colloids were similar, despite differences in organic carbon content, and because no trend was evident between the experimental log K_{oc} values and reported log K_{ow} values. The experimental log K_{oc} values did correlate with the colloids' ability to absorb ultraviolet light at 280 nm, suggesting again that π electron interactions play a substantial role in estrogen sorption to colloids.

In the same studies, additional experiments were conducted with water samples collected upstream, downstream, and near the outfall of a WWTP in West Sussex, UK, to determine the distribution of 17β-estradiol, 17α-ethinylestradiol, estrone, 16α-hydroxyestrone, and other compounds between dissolved and colloidal phases in field samples. Water samples were collected in 50 mL stainless steel barrels for cross-flow ultrafiltration, and separate samples were collected in 2.5 L glass bottles to analyze the "conventional" dissolved phase (a combination of dissolved and colloidal phases after filtration). Various analytical procedures (including SPE and GC-MS) were used to determine the estrogens' presence in the dissolved, colloidal, and conventional dissolved phases. Upstream, the mean conventional dissolved concentration of 17β-estradiol was approximately 7 ng L^{-1}, and the mean conventional dissolved concentrations of the other estrogens were significantly lower. At the outfall, mean conventional dissolved concentrations of the estrogens were distributed as follows: estrone (26.5 ng L^{-1}) > 17β-estradiol (22.5 ng L^{-1}) > 17α-ethinylestradiol (< 1 ng L^{-1}) ≈ 16α-hydroxyestrone (< 1 ng L^{-1}). Downstream, the mean conventional dissolved concentrations were substantially reduced (e.g., estrone = 3.9 ng L^{-1}). After the dissolved and colloidal phases were separated by cross-flow ultrafiltration, the mean percentages of colloid-bound estrogens (i.e., estrogens sorbed to

colloids, but present in the conventional dissolved phase) were distributed as follows: 16α-hydroxyestrone (20-33%); 17β-estradiol (15-30%); 17α-ethinylestradiol (20-29%); and estrone (4-26%).

Other studies also have investigated steroid hormone sorption to colloids [127, 155]. In general, log K_{oc} values and log K_{ow} values are not well-correlated in studies of estrogen sorption to colloids, which suggests that estrogen sorption to colloids is influenced by factors in addition to hydrophobic partitioning with organic matter. The same principles also may apply to androgens and progestagens.

Sorption to Soils and Sediments

One study characterized sediments as major sinks of steroidal estrogens in river systems [159]. In that study, a yeast-based reporter gene assay was used to measure the estrogenic activities of effluents from two WWTPs in the U.K., and to measure the estrogenic activities of water and sediments from up to 1.5 km upstream and downstream of the WWTPs, along the Rivers Arun and Ouse (England). Various analytical methods (including SPE and liquid extraction) were used to extract estrogens from the waters and sediments. To identify the sources of estrogenic activity, sample extracts were separated into fractions with HPLC and analyzed by GC-MS. The estrogenic activities of the WWTP effluents ranged from 1.4-2.9 ng EEq L^{-1}, and the estrogenic activities of the river waters upstream and downstream from the WWTPs were below the limits of detection (0.04 ng L^{-1}). The estrogenic activities of the sediments were substantially higher than the overlying water, ranging from 21-30 ng EEq kg^{-1}. Estrone and 17β-estradiol were identified as the major active chemicals in the effluents and the sediments. No significant differences in estrogenic activity were observed between the

sediments collected upstream and downstream from the WWTPs. Long distance transport was suggested as the source of estrogenic activity in the upstream sediments, because both upstream sites were located approximately 5 km downstream from other WWTPs. A related study reported 1-7 day biodegradation half-lives for 17β-estradiol and estrone at 20 °C (a common summer water temperature for the area), and direct photodegradation half-lives of not less than 10 days for 17β-estradiol and 17α-ethinylestradiol (using a polychromatic lamp equipped to simulate natural sunlight) [160]. Under average flow conditions, 17β-estradiol and estrone were estimated to travel 10 km in a few hours, so significant concentrations were expected to remain after transport for 5 km.

Other studies have sought to determine the rate and extent of steroid sex hormone sorption to soils and river sediments. One study examined the sorption of 17β-estradiol, estrone, estriol, 17α-ethinylestradiol, and mestranol to surficial bed sediments from one river (Thames) and one estuary (Blackwater) in the U.K. [55]. The total organic carbon content of the sediments ranged from 0.3-3.3%, and particle sizes were distributed as follows: sand (0-1.6%); silt (61-93%); and clay (7-39%). Batch equilibrium experiments were conducted in 250 mL Teflon bottles containing 200 mL of deionized water, 3 g of sediment, and a mixed estrogen standard containing 100 μg L^{-1} of each estrogen. The bottles were mixed until apparent equilibrium, and centrifuged. Then, 1 mL samples were extracted, derivatized, and analyzed by GC-MS. Separate studies were conducted to examine (i) sorption rates, (ii) the effects of changing estrogen concentrations (10-1,000 μg L^{-1}) and sediment amounts (0.6-15 g), and (iii) competition with 100 μg L^{-1} estradiol valerate (log K_{ow} = 6.41).

Data from preliminary rate experiments with one estuarine suggested that estrogen sorption occurred in three stages. Rapid sorption occurred during the first half hour, followed by

slower sorption for up to 1 hour, and then desorption. Based on these experiments, apparent equilibrium was believed to occur after 1 hour. The apparent decrease in sorption was attributed to estrogen sorption to dissolved organic matter released into solution. In general, slow sorption into clay mineral structures and condensed organic matter occurs by diffusion, and requires more than 1 h to occur [147, 150]. Therefore, the batch equilibrium experiments probably fail to take diffusion-controlled slow sorption into account.

According to the data, at apparent equilibrium, no linear relationship existed between estrogens sorbed to the sediments and estrogens remaining in solution. Because estrogen sorption increased when larger amounts of sediment were added, saturation of the available sediment might explain the nonlinear relationship. In the presence of 100 μg L^{-1} estradiol valerate, the sorption of other estrogens was suppressed, and the magnitude of suppression grew with decreasing estrogen hydrophobicity (estriol > estrone > 17β-estradiol > 17α-ethinylestradiol > mestranol), suggesting that competitive sorption does occur among estrogens. Finally, the relationship between sorption and organic matter content was shown to be linear, which suggested that specific interactions were unimportant during the 1 h equilibration period, despite nonlinear isotherms and relatively low organic matter content (0.3-3.3%).

Another study examined 17β-estradiol and 17α-ethinylestradiol sorption to suspended and bed sediments from three rivers (Aire, Calder, and Thames) and two estuaries (Tyne and Tees) in the U.K. [131]. The Thames River sampling sites were located in a rural area, and the other sampling sites were located near urban and industrial areas. Bulk water samples were collected in 1 L bottles at all sites for use in experiments with sediments from the same sites. Midstream bed sediments were collected from the two estuaries and one Thames River site with a mechanical grab, and all other bed sediment samples were collected near river banks by

removing the top 2-5 cm of bed material. A continuous flow centrifuge was used to collect suspended sediment samples from the Aire, Calder, and Thames Rivers. Batch equilibrium experiments were conducted to determine 17β-estradiol and 17α-ethinylestradiol sorption to bed and suspended sediments at apparent equilibrium, and continuous mode experiments were conducted to study the rate of 17β-estradiol sorption to bed sediments. Suspensions were prepared in PTFE centrifuge tubes by mixing filtered river water with air-dried sediment and [^{14}C]-labeled estrogens to reach desired concentrations. For the continuous mode experiments, 15 mL of filtered river water was mixed with 1.0 g of sediment, and spiked with [^{14}C]-labeled 17β-estradiol for an initial concentration of 5 μg L^{-1}. The samples were placed in a 2.5 L anaerobic jar, mixed at room temperature, and sampled after 1, 2, and 6 days. For batch equilibrium experiments with bed sediments, 15 mL of filtered river water was mixed with 1-5 g of sediment (based on expected sorption), and spiked with [^{14}C]-labeled estrogens to initial concentrations of 0.5-10 μg L^{-1}. After 20 h, samples were collected and centrifuged, and the supernatant was prepared for liquid scintillation counter analysis. In some samples, the original river water was replaced with fresh river water, equilibrated for another 20 h, and examined for desorption. For batch equilibrium experiments with suspended sediments, 5 mL suspensions were prepared at 0.9-54 g L^{-1} sediment concentrations (concentration factor of 60-1,550), and spiked with [^{14}C]-labeled estrogens to initial concentrations of 1.5-10 μg L^{-1}. After 1 h, the suspended sediment samples were analyzed like the bed sediment samples. In all cases, sorbed amounts were determined by subtracting the radioactivity remaining in solution from the initial radioactivity.

According to continuous mode experiments with bed sediments from the Thames and Aires Rivers, 80-90% of estradiol sorption occurred within the first day, but apparent equilibrium

did not occur within two days. Because no steps were taken to inhibit biodegradation, it was acknowledged that no "true equilibrium" could be reached, and that any sorption coefficient calculated with reference to [^{14}C]-labeled 17β-estradiol (before biodegradation) would represent the sorption of 17β-estradiol and its metabolites (e.g., estrone).

An apparent equilibrium period of 20 h was chosen to represent a compromise between incomplete sorption and biodegradation. The resulting sorption isotherms were essentially linear (mean $1/n$ = 0.97), and no significant difference was found between sorption and desorption coefficients. However, the chosen 20 h equilibrium period may have eliminated the effects of slow sorption and desorption, which generally give rise to nonlinear isotherms. The sorption coefficients for 17α-ethinylestradiol ranged from 1.6 to 3.1 times higher than 17β-estradiol, indicating that 17α-ethinylestradiol sorbed more than 17β-estradiol. Desorption coefficients were 1.5 to 3.2 times higher than the original sorption coefficients, suggesting either that desorption occurs more slowly than sorption (i.e., due to the requirement for activation), or that some degree of hysteresis was occurring. A weak correlation was observed between sorption coefficients and the bed sediments' organic carbon content (0.1-10% organic carbon), suggesting that estrogen sorption to the bed sediments was influenced by factors in addition to hydrophobic partitioning with organic matter. In addition, higher sorption coefficients were associated with smaller particle sizes (i.e., sorption coefficients were negatively correlated with sand content). As a consequence, sorption would be strongest in water compartments with the lowest flows (e.g., along the banks), where smaller particle sizes are found. In general, the suspended sediments had higher sorption coefficients than the bed sediments, but they were determined to be much less important for steroid sex hormone removal because of their low concentrations (7.6-52 mg L^{-1}).

Additional studies have used batch and continuous mode experiments to determine the distribution of steroid sex hormones between water and various soils and sediments, and to examine sorption rates [53, 54, 56, 143, 144, 161-167]. Differences among sorption coefficients (K_f), indexes of linearity (1/n), and rates of sorption often can be attributed to differences in organic matter content, mineral types, and experimental conditions (e.g., the steroid and sediment concentrations used). For example, one study found that apparent equilibrium occurred in 1-2 weeks when initial hormone concentrations were 10,000 μg L^{-1}, and 2-3 weeks when initial concentrations were 300 μg L^{-1} [56]. These concentrations are extremely high relative to environmental concentrations, but they do suggest that equilibrium times may vary with concentration. The same study also reported that sorption coefficients increased as steroid sex hormone concentrations decreased (i.e., smaller concentrations sorbed more), and that the order of androgen sorption changed with soil type (e.g., testosterone sorbed to agricultural soils more than androstenedione, and androstenedione sorbed to pond and creek sediments more than testosterone) [56].

Biodegradation - In General

Bacteria can use steroids in redox (reduction/oxidation) reactions to gain energy, or metabolize the steroids completely as a carbon source for cell growth [168, 169]. In redox reactions, bacteria need an electron donor as the energy source, and an external electron acceptor to complete the respiration process. Organic carbon sources commonly serve as electron donors, and the available electron acceptor that will yield the most energy to the bacteria commonly serves as the electron acceptor. Under aerobic conditions, where sufficient oxygen is available, oxygen serves as the electron acceptor. Under anaerobic conditions, where sufficient oxygen is

not available, bacteria generally use available electron acceptors in the following order: NO_3^-; Mn(IV); Fe(III); and SO_4^{2-} [168]. As an example, during activated sludge treatment in WWTPs, 17β-estradiol is commonly oxidized to estrone under aerobic and anaerobic conditions [41, 170]. In nitrifying activated sludge, ammonia (NH_4^+) is oxidized to form nitrites and nitrates, and steroids appear to be oxidized co-metabolically [171-173].

Microorganisms are capable of transforming steroid compounds in various ways. For this reason, since the 1950's, the pharmaceutical industry has engaged in research to identify microorganisms and mechanisms that can assist in the production of steroidal drugs and hormones, as previously reviewed [169, 174-177]. Typically, natural plant and animal compounds, including cholesterol and plant sterols (e.g., stigmasterol, β-sitosterol, and campesterol), are used as the starting materials for steroidal drugs and hormones [174]. For example, several microbial species (including species of *Mycobacterium*, *Arthrobacter*, *Rhodococcus*, *Escherichia*, *Nocardia*, *Pseudomonas*, and *Micrococcus*) can be used to produce androstenedione, androstadienedione, and their derivatives from cholesterol and plant sterols [71, 174, 176]. In addition, species of *Bacillus* and *Nectria* have been used to transform progesterone into androstenedione and androstadienedione, and species of *Mycobacterium* and *Lactobacillus* have been used to transform cholesterol into testosterone [70, 174, 176]. Because several of these species have been isolated from soils (e.g., *Mycobacterium*, *Bacillus*, and *Micrococcus* species), it is reasonable to assume that the same reactions can happen in natural systems.

Actinobacteria are Gram-positive bacteria that are common to soil, and include genera such as *Arthrobacter*, *Micrococcus*, *Mycobacterium*, and *Nocardia*. Actinobacteria can introduce hydroxyl groups at numerous positions along the steroid skeleton, and sequential hydroxylations along carbons 22-27 of the sterol side chain have been identified as stages of

degradation by actinobacteria [169]. In addition to hydroxylation, actinobacteria have the ability to transform steroids through double bond hydrogenation, single bond dehydrogenation, steroid alcohol oxidation (e.g., 17β-OH → 17C=O), steroid ketone reduction (e.g., 17C=O → 17β-OH), and double bond isomerization [169]. Many actinobacteria can metabolize steroids completely as a carbon source for cell growth, but the mechanism of degradation will depend on the steroid type and the genera of actinobacteria involved [169]. Estranes (18 carbons) are often degraded by cleavage of the A-ring, and androstanes (19 carbons) and pregnanes (21 carbons) are often degraded by cleavage of the B-ring [169]. Cholestane (27 carbons), on the other hand, are commonly degraded by cleavage both of the B-ring and the sterol side chain [169].

Actinobacteria (e.g., species of *Nocardia* and *Arthrobacter*) often degrade 3-keto-4-ene steroids such as androstenedione, testosterone and progesterone through cleavage of the B-ring, beginning with the introduction of a double bond between carbons 1 and 2, and hydroxylation at carbon 9, which causes aromatization of the A-ring, cleavage of the B-ring, and the formation of a labile metabolite (3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione) [169, 178]. The labile metabolite is further degraded by hydroxylation at carbon 4, and cleavage of the A-ring, prior to mineralization. The entire degradation pathway is illustrated in Figure 2-2.

Proteobacteria and bacteriodetes are Gram-negative bacteria. Proteobacteria (e.g., species of *Comamonas* and *Pseudomonas*) also use the pathway in Figure 2-2 to degrade 3-keto-4-ene steroids, and bacteriodetes (e.g., species of *Sphingobacterium*) use the same pathway to degrade 17α-ethinylestradiol [178-180]. As a result, the pathway in Figure 2-2 has been described as a general pathway of steroid degradation [181]. However, microbes that use this pathway generally seem unable to metabolize estrone [181].

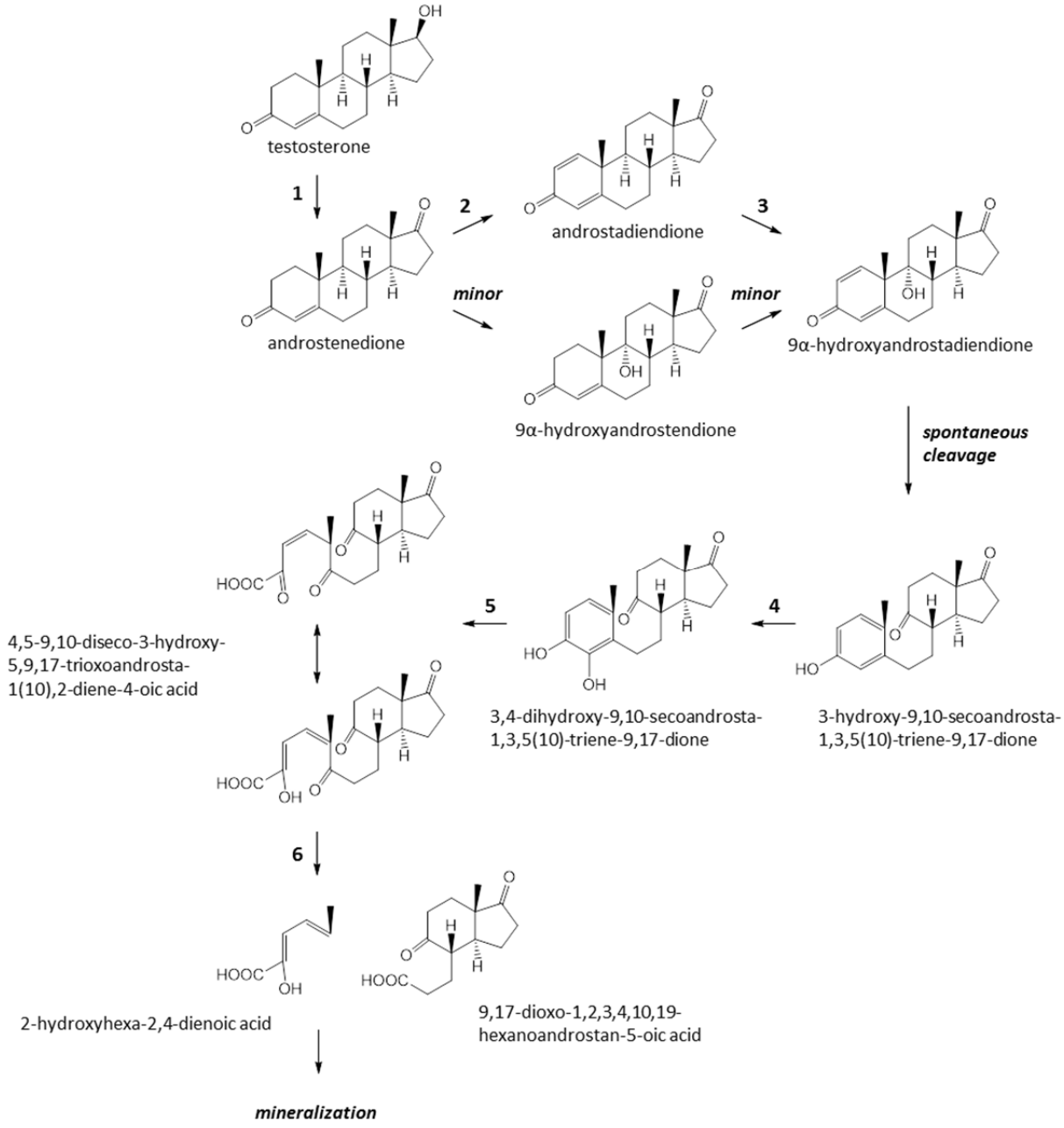


FIGURE 2-2. Biodegradation pathway. General pathway of testosterone biodegradation; reaction steps are numbered (modified from ref. 179).

A separate degradation pathway was proposed for estrone, after incubation for 5 days with a *Nocardia* sp. isolated from soil [181]. Three degradation products were obtained after hydroxylation at carbon 4 and the opening of ring A. One involved a reaction with ammonia,

and the other two involved the opening of ring B, in addition to ring A. Estrone was not mineralized during the 5 day incubation period.

Another 17β-estradiol metabolite was identified after incubation with a 17β-estradiol degrading culture [170]. Two-thirds of the 17β-estradiol (200 μg L^{-1}) was quantitatively oxidized to estrone, with little or no other metabolites, after contact with the culture for 22 h. Almost all of the 17β-estradiol was removed after approximately 3 days, and almost all of the estrone was removed after approximately 14 days. Very early in the process (1-5 h), a labile metabolite was observed, and tentatively identified as a gamma lactone (5 member ring) produced in the D-ring by estrone oxidation. Although some species of *Cylindrocarpon*, *Penicillium*, and *Streptomyces* can introduce a lactone into the D-ring to produce testolactone (a delta lactone with a 6 member ring), they generally do not metabolize the testolactone further [181]. As a result, if the tentative identification is correct, then either the gamma lactone is less stable, or different microbes participated in the gamma lactone degradation.

From the foregoing, it is clear numerous metabolites can form from steroid sex hormone biodegradation by a variety of bacterial species. Microalgae and fungi also have the ability to transform steroids through hydroxylation, steroid ketone reduction, double bond hydrogenation, and single bond dehydrogenation [177, 182-184]. In addition, some microalgae also have the ability to metabolize steroids, including androstenedione and 5α-androstanedione, completely [177, 185].

Biodegradation in Agricultural Soils

Biodegradation in agricultural soils is relevant to aquatic ecosystems because runoff and leaching from agricultural soils represent potential sources of steroid sex hormones in aquatic

ecosystems, and because many of the same microorganisms and degradation pathways may be involved with biodegradation in aquatic ecosystems.

In a series of related studies, the dissipation of testosterone, 17β-estradiol, estrone, and 17α-ethinylestradiol was examined in three agricultural soils: (i) loam (3.2% organic matter; particle sizes: 40% sand, 45% silt, 15% clay); (ii) sandy loam (0.8% organic matter; particles sizes: > 90% sand); and (iii) silt loam (2.9% organic matter; particle sizes: 32% sand, 52% silt, 16% clay) [186-188]. Microcosms were prepared by adding 25-100 g of soil (moist weight) to baby-food jars. Then, each jar was placed in a sealable 1 L mason jar with a vial containing 10 mL of water to maintain moisture, and a second vial containing 5 mL of 1 M NaOH solution to trap $^{14}CO_2$. The microcosms were spiked with ^{3}H-labeled, ^{14}C-labeled, or unlabeled steroid sex hormones to an initial concentration of 1-10 mg kg^{-1} (moist weight), and incubated at 30 °C. Periodically, 5 g soil samples were collected from the microcosms, extracted by liquid extraction (ethyl acetate and acetone), and analyzed by liquid scintillation counter, HPLC-RD (radioactivity detection), HPLC-UV, HPLC-MS, or a yeast-based reporter gene assay. Preliminary studies indicated that hormones were extracted from the soils with the following efficiencies: testosterone (84 ± 2%; n = 9); 17β-estradiol (98.9 ± 10.4%; n = 9); estrone (72.4 ± 4.4%; n = 6); and 17α-ethinylestradiol (80.5 ± 3.3%; n = 5). When necessary, sterile soil was prepared by autoclaving twice (45 min. at 120 °C).

Steroid sex hormone dissipation was determined by the decrease in extractable [^{14}C] material over time. Non-extractable hormones were assumed to be dissipated, and extractable metabolites were distinguished from parent hormones by HPLC-RD. The mineralization of [^{14}C]-labeled hormones was determined by measuring the trapped $^{14}CO_2$ with a liquid scintillation counter. In the testosterone experiments, the mineralization of

1,2,6,7-[^{3}H]-testosterone was determined by collecting two extracts, and evaporating one to dryness under nitrogen. Then, the evaporated extract was redissolved in ethyl acetate, a liquid scintillation counter was used to measure the [^{3}H] material in both extracts, and the difference was assumed to be evaporated $^{3}H_2O$ attributable to testosterone mineralization.

During the 17β-estradiol experiments, most of the [^{14}C] material was not extractable after 3 days of incubation (loam: 90.7% non-extractable; sandy loam: 70.3% non-extractable; silt loam: 56.0% non-extractable). In all cases, the beginning 17β-estradiol concentration was 50% dissipated in less than one-half day, from 5.3 h (sandy loam) to 11.5 h (silt loam). As 17β-estradiol dissipated, estrone accumulated. Throughout the experiment, 17β-estradiol and estrone accounted for approximately 100% of the extractable radioactivity, suggesting that 17β-estradiol was being oxidized to estrone, and that no other transformation product was present. In the loam soil, estrone diminished after 6 h, and was undetectable by the end of the experiment. In the sandy loam and silt loam soils, estrone remained detectable at the end of the experiment, and represented 100% of the extractable [^{14}C] material. Declines in extractable radioactivity closely paralleled declines in estrogenicity, taking into account the relative potencies of 17β-estradiol and estrone. 17β-estradiol was mineralized in all three soils, but only very slowly. After 3 months, only 11.5-17.1% of the 4-[^{14}C]-labeled 17β-estradiol was recovered as $^{14}CO_2$. In the autoclaved soils, 17β-estradiol was oxidized to estrone within 72 h, suggesting that 17β-estradiol was being oxidized abiotically to form estrone. Because extractable radioactivity remained constant in the autoclaved soils as 17β-estradiol was being oxidized to estrone, decreases in extractable radioactivity were associated with the formation of non-extractable residues during estrone degradation. In a similar experiment (described below), 50-67% of the initial radioactivity was not extractable after 5 days, and approximately 90% of the extractable

radioactivity remained 17β-estradiol [156]. Possible explanations for the difference include different soil conditions (e.g., different oxidants), and different experimental conditions.

During the estrone experiments, most of the [^{14}C] material was not extractable after 3 days of incubation (loam: 88.2% non-extractable; sandy loam: 59.4% non-extractable; silt loam: 71.4% non-extractable). In all cases, the beginning estrone concentration was 50% dissipated in 14.7 h (silt loam) to 40.6 h (sandy loam).

17α-ethinylestradiol was 50% dissipated in 59.4 h (sandy loam soil; 12 % moisture) to 97.9 h (silt loam; 15% moisture). The decline in 17α-ethinylestradiol was accompanied by a corresponding decline in total estrogenicity, suggesting that no estrogenic metabolites were formed. 17α-ethinylestradiol was completely appeared to be stable in the autoclaved soil. However, when 17α-ethinylestradiol was oxidized by Mn(III) in another study, a double bond was introduced between carbons 9 and 11 [189]. If this degradation product had been formed, it is unlikely that 17α-ethinylestradiol dissipation would have been detected.

Testosterone was 50% dissipated in 8.5 h (loam soil) to 21 h (silt loam soil), but dissipated more slowly at lower temperatures (4-12 °C). After approximately 120 h, testosterone was 50% mineralized in all soils. Androgenic activity declined faster than extractable [^{14}C] radioactivity, suggesting that the testosterone was transformed into metabolites with less androgenic activity. Three testosterone metabolites were identified: androstenedione; 5α-androstan-3,17-dione; and androstadienedione. None of the [^{3}H] material was recovered as $^{3}H_2O$, but the extractable [^{3}H] radioactivity dissipated rapidly (within 48 h) in moist or saturated soil. In autoclaved soil, extractable [^{3}H] radioactivity dissipated more slowly, and androstenedione formed, suggesting that testosterone was oxidized abiotically to form androstenedione.

Another study examined the biodegradation of 17β-estradiol and testosterone in an agricultural soil under aerobic and anaerobic conditions [156]. Each steroid sex hormone was investigated in four soil microcosms: (i) agricultural soil under aerobic conditions; (ii) agricultural soil under anaerobic conditions; (iii) autoclaved soil under aerobic conditions; and (iv) autoclaved soil under anaerobic conditions. The organic matter content of the agricultural soil was 2.23%, and the particle size distribution follows: 14% clay; 19% silt; and 67% sand. The autoclaved soils were sterilized for 40 min. at 122 °C. The incubation experiments were prepared by mixing radiolabeled hormones with approximately 210 g of soil (not sieved or air-dried) in 250 mL glass flasks. The initial concentrations of 1,500 μg L^{-1} 17β-estradiol and 52 μg L^{-1} testosterone were chosen to represent concentrations found in animal manures applied to agricultural fields. The flasks were covered with aluminum to eliminate photodegradation, and supplied with moist air to maintain aerobic conditions, or humified helium gas to maintain anaerobic conditions. The autoclaved soils were also spiked with 500 mg kg^{-1} $HgCl_2$ to inhibit contamination by airborne bacteria. 200 mL glass flasks containing 3M NaOH were set up to trap $^{14}CO_2$ under all conditions, and a 200 mL flask containing Bray's solution was up to trap $^{14}CH_4$ under anaerobic conditions. Periodic samples were collected over a 132-hour period, and analyzed by liquid scintillation counter. At the end of the experiments, extracts from the soils were examined for radioactivity, thin layer chromatography was used to determine metabolites, and the distribution of non-extractable [^{14}C] among organic fractions (i.e., humic acid, fulvic acid, and humus) was determined with a multi-step fractionation process.

During the testosterone experiments in natural soil under ***aerobic*** conditions, 85.4% of the total [^{14}C] was recovered. Of the recovered amount, 63% was trapped after mineralization to $^{14}CO_2$, 3.4% was extractable from the soil with water or acetone, and 19% was associated with

natural organic matter (3% humic acids, 9% fulvic acids, 7% humin) and non-extractable. Of the extractable [^{14}C] material (3.4%), 17% was testosterone, and 83% was testosterone metabolites. During the 17β-estradiol experiments in natural soil under aerobic conditions, 91% of the total [^{14}C] was recovered. Of the recovered amount, 6% was trapped after mineralization to $^{14}CO_2$, 12% was extractable from the soil with water or acetone, and 73% was associated with natural organic matter (37% humic acids, 17% fulvic acids, 19% humin) and non-extractable. All of the extractable [^{14}C] material (12%) was 17β-estradiol metabolites.

During the testosterone experiments in natural soil under ***anaerobic*** conditions, 89% of the total [^{14}C] was recovered. Of the recovered amount, 46% was trapped after mineralization to $^{14}CO_2$, 2% was trapped after mineralization to $^{14}CH_4$, 16% was extractable from the soil with water or acetone, and 25% was associated with natural organic matter (5% humic acids, 0% fulvic acids, 20% humin) and non-extractable. Of the extractable [^{14}C] material (16%), 13% was testosterone, and 87% was testosterone metabolites. During the 17β-estradiol experiments in natural soil under anaerobic conditions, 89.9% of the total [^{14}C] was recovered. Of the recovered amount, 0.9% was trapped after mineralization to $^{14}CO_2$, 19% was extractable from the soil with water or acetone, and 70% was associated with natural organic matter (37% humic acids, 22% fulvic acids, 11% humin) and non-extractable. Of the extractable [^{14}C] material (19%), 11% was 17β-estradiol, and 89% was 17β-estradiol metabolites.

During the testosterone and 17β-estradiol experiments in the ***autoclaved*** soils under all conditions (aerobic and anaerobic), no $^{14}CO_2$ was recovered in excess of 0.2%, and no $^{14}CH_4$ was recovered. Like the experiments in the natural soils, much of the [^{14}C] material in the autoclaved soil experiments was not extractable with water or acetone (49-67%).

In natural soils, testosterone was mineralized more than 17β-estradiol under all conditions (aerobic and anaerobic). This result was explained by testosterone's greater presence in the dissolved phase, and by noting that more energy is required to cleave the aromatic structure of 17β-estradiol where the 4-[^{14}C]-label was located [126]. The aerobic degradation rates of testosterone ($t_{½}$ = 58 h) and 17β-estradiol ($t_{½}$ = 1,150 h) were faster than their anaerobic degradation rates (testosterone: $t_{½}$ = 173 h; 17β-estradiol: $t_{½}$ = 6,930 h). This difference was explained by the ready availability of electron acceptors under aerobic conditions, and the possible involvement of different microbial species or different degradation pathways.

Biodegradation in Rivers, Lakes and their Sediments

One environmental study examined whether the source of androstenedione in the Fenholloway River (Florida, U.S.A.) might be the microbial transformation of progesterone [71]. In prior studies, androstenedione and progesterone were detected in the water column and sediments of the Fenholloway River, which contains paper mill effluent and masculinized mosquitofish [72, 73]. The study hypothesized that progesterone was being produced by microbial transformation of plant sterols in the mill pulp, and that androstenedione was being produced by microbial transformation of progesterone. To test the hypothesis, progesterone (1 mM) was incubated with *Mycobacterium smegmatis* (a common soil bacterium). Samples of the slurried media were collected periodically over 36 days, and various analytical methods (including liquid extraction and SPE) were used to prepare the samples for HPLC-DAD analysis. 17α-hydroxyestrone, androstenedione, and androstadienedione were identified as transformation products by comparisons with HPLC standards [71]. The concentration of 17α-hydroxyestrone increased from day 0 through day 12, and the highest mean concentration occurred on day 20

(23.0 ± 0.7 μM). The concentration of androstenedione increased from day 0 to day 6 (7.2 ± 0.8 μM), remained constant until day 12, and decreased thereafter. The concentration of androstadienedione mirrored androstenedione, and reached a maximum on day 8 (5.2 ± 0.5 μM). Based on the rates of accumulation of the intermediates during the experiment, it was suggested that the steroid hormones were produced in the following order: progesterone → 17α-hydroxyprogesterone → androstenedione → androstadienedione.

In another study, water and sediment samples were collected from rivers influenced by rural (Thames) and urban or industrial activities (Aire and Calder) to examine the potential for biodegradation of 17β-estradiol and 17α-ethinylestradiol in freshwater ecosystems [160]. Bulk water samples were collected in 1 L glass bottles from the top 0.5 m of the water column during low, medium, and high flow periods between 1997 and 2000. In addition, bed sediments were collected 2-3 m from the banks of the Thames and Calder Rivers by skimming off the top few centimeters with a bucket. River water samples were prepared by adding 50 mL of water to autoclaved 125 mL PTFE flasks, and estrogens were spiked in to a nominal concentration of 100 μg L^{-1}. The water samples were incubated in darkness at 10 or 20 °C, and autoclaved samples of the same water were used as sterile controls. Periodically, water samples were combined for analysis by HPLC-MS or HPLC- DAD. Wet sediment and water from the same site were collected in 100 mL conical flasks, fitted with gas flushing heads, and placed under nitrogen. Autoclaved water and bed sediments were used as sterile controls. Another set of experiments compared aerobic to anaerobic conditions in the bed sediments. To study the potential for mineralization, radiolabeled hormones (4-[^{14}C]-labeled) were added to water samples, and a 50 mM solution of NaOH was used to trap $^{14}CO_2$ as it evolved. Periodically, samples were collected and analyzed by liquid scintillation counter.

In all the non-sterile river water samples, 17β-estradiol dissipated and estrone accumulated. No significant losses were observed in the sterile controls. Faster degradation rates were associated summer water samples, possibly because nutrient concentrations and temperatures are higher during summer. 17α-ethinylestradiol (t½ = 17 d) dissipated much more slowly than the 17β-estradiol (t½ = 1.2 d) under aerobic conditions. In the anaerobic bed sediments, 17β-estradiol was converted to estrone over the 2-day incubation period. The potential for 17α-ethinylestradiol to dissipate in the anaerobic bed sediments was not tested. After approximately 25 days, 24-45% of the [^{14}C] material had evolved as $^{14}CO_2$, suggesting that microorganisms in river waters and sediments can cleave the aromatic A-ring to release the 4-[^{14}C]-radiolabels. Mineralization rates slowed after 25 days, either because nutrient supplies diminished or, if the degradation process is cometabolic, because the cometabolic substrate was exhausted. At the end of the experiment, 18-32% of the original 17β-estradiol concentration was 17β-estradiol, 10-23% was hydrophilic by-products (18-32%); and 5% was sorbed to glassware or charcoal. After 8 days, 17β-estradiol and estrone became undetectable, but sample estrogenicity remained detectable, suggesting that estrone by-products could be slightly estrogenic. After 2 weeks, more than 99% of the initial estrogenicity was lost. Loss of estrogenicity is expected with cleavage of the A-ring, because the A-ring is essential for estrogen receptor binding.

Another study examined the anaerobic biodegradation of 17β-estradiol, estrone, and 17α-ethinylestradiol under methanogenic, sulfate-, iron-, and nitrate-reducing conditions [108]. Cultures were established in 160 mL serum bottles by combining 10 mL of lake sediment, 90 mL of freshwater mineral medium, resazurin (a redox indicator), and estrogens at initial concentrations of 5 mg L^{-1}. Strict anaerobic technique was used, and the following electron

acceptor solutions were added to create reducing conditions: 20 mM $NaNO_3$ (nitrate-reducing conditions); 20 mM Fe^{3+}–nitrilotriacetic acid (iron-reducing conditions); 20 mM Na_2SO_4 (sulfate-reducing conditions); or water (methanogenic conditions). Iron-reducing culture samples were amended with 80 mg L^{-1} ethylenediaminetetraacetic acid (EDTA) to chelate the iron. Aqueous samples were collected with glass syringes, transferred to microcentrifuge tubes containing methanol, and microcentrifuged. The supernatant was analyzed by HPLC-DAD, methane was analyzed by GC-FID, reduced iron was analyzed by ferrozine colorimetric assay, and sulfate and nitrate were analyzed by ion chromatography. Spiked water sample recoveries exceeded 95%, and quantitation limits were 20 μg L^{-1}. Metabolites were identified in separate culture experiments after liquid extraction, derivatization, and GC-MS/MS analysis.

In experiments combining 17α-ethinylestradiol, 17β-estradiol, and lake sediment, 17α-ethinylestradiol was not significantly degraded under anaerobic conditions, even after 35-38 months. By comparison, 17β-estradiol was partially removed under all four anaerobic conditions. 17β-estradiol was oxidized to estrone in each case, but the oxidation rate was slightly faster under nitrate-reducing conditions. HPLC-DAD appeared to detect estriol, but estriol was not confirmed by GC-MS/MS analysis. 17α-estradiol was detected under methanogenic, sulfate-reducing, and iron-reducing conditions, but not nitrate-reducing conditions. Despite the differences in potential energy (e.g., redox potential) available from different electron acceptors, there was no clear correlation between the use of electron acceptors and the rate or extent of 17β-estradiol transformation, suggesting that 17β-estradiol was not oxidized to gain energy. Instead, the oxidation of 17β-estradiol was believed to be used for co-factor regeneration.

The preceding studies demonstrate that steroid sex hormones biodegrade, but may not be mineralized, in rivers, lakes, and their sediments under aerobic or anaerobic conditions. Therefore, in some cases, biodegradation may produce metabolites that reduce, but not eliminate, the biological activities of steroid sex hormones.

Photodegradation - Introduction

Photodegradation is an important abiotic degradation pathway that takes place in natural waters [190]. Direct photodegradation occurs when an organic contaminant absorbs light, becomes excited, and undergoes chemical changes [49, 190, 191]. Indirect (or sensitized) photodegradation occurs when another compound absorbs light, becomes excited, and reacts with the organic contaminant [49, 190, 191]. Saturated organic compounds (i.e., molecules with single bonds) do not absorb light in the 200-700 nm range, which includes all visible light and much of the low energy UV spectrum (UV-A, UV-B, and some UV-C). Unsaturated molecules (e.g., molecules with C=C, C=O, and aromatic groups) can absorb light in this range [191]. The solar spectrum generally ranges from 290-800 nm, and natural waters contain many unsaturated molecules (e.g., dissolved organic matter) and other reactive intermediates (e.g., nitrate and nitrite) that absorb light in this range [192, 193]. As a result, natural water bodies have been described as "large photochemical reactor systems" [192]. Because steroid sex hormones are often unsaturated molecules, they have the potential to undergo direct and indirect photodegradation.

Direct Photodegradation

In laboratory experiments with simulated sunlight, direct photodegradation of 100 $\mu g\ L^{-1}$ solutions of 17α-ethinylestradiol and 17β-estradiol was observed with half-lives of

approximately 10 days [160]. Over a 60 minute period, another laboratory experiment observed direct photodegradation of 17β-estradiol and estrone in UV-C light (λ_{max} = 254 nm), and direct photodegradation of estrone, but not 17β-estradiol, in UV-A plus visible light ($\lambda \geq 365$ nm) [194]. Over a 90 minute period, a related experiment observed direct photodegradation of 17α-ethinylestradiol in UV-C light (λ_{max} = 254 nm), and no direct photodegradation of 17α-ethinylestradiol in UV-A plus visible light ($\lambda \geq 365$ nm) [195]. Because UV-C light generally fails to reach the Earth's surface, estrone is expected to undergo direct photodegradation more easily than 17α-ethinylestradiol, 17β-estradiol, and estriol [193].

In a separate study using UV-A light ($\lambda > 315$ nm), direct photodegradation of testosterone and progesterone were observed [130].

Indirect Photodegradation

In laboratory experiments with simulated sunlight (290nm < λ < 700 nm), estrogens (17α-ethinylestradiol, 17β-estradiol, estrone, and estriol) experienced increased degradation rates in filtered and autoclaved Santa Ana river water (Southern California, USA; 4.6 mg L^{-1} dissolved organic carbon; 22.3 mg L^{-1} nitrate), suggesting indirect photodegradation [193].

In a separate study using UV-A light ($\lambda > 315$ nm) and 10 mg L^{-1} nitrate and 5 mg L^{-1} humic acid as potential reactive intermediates, no indirect photodegradation of progesterone was observed, but indirect photodegradation of testosterone was observed in the presence of 5 mg L^{-1} humic acid [130].

To date, little is known about the mechanisms and products of steroid sex hormone photodegradation.

Steroid Sex Hormones – Fate and Transport

Column studies have been used to study the fate and transport of steroid sex hormones in agricultural soils [138, 143, 144, 166]. Batch equilibrium experiments expose steroid sex hormones to water and soils and sediments, and measure their distribution at apparent equilibrium. Similarly, biodegradation studies incubate microbial cultures in controlled environments, and examine the rates and effects of biodegradation. Column studies, on the other hand, examine the sorption and degradation of steroid sex hormones under non-equilibrium conditions, as water flows through the soils and sediments. Packed columns eliminate preferential flow through soil macropores to simplify the transport analysis, but fail to consider the effects of natural soil structure and macropores on fate and transport [161]. Undisturbed soil columns attempt to preserve these features, in order to better reflect transport through soils under field conditions [161].

In a study of testosterone fate and transport, a 300 mL pulse of 4-[^{14}C]-testosterone (~5.9 μCi) was added to an undisturbed soil column (30 cm height × 15 cm inner diameter), and eluted with approximately 6 pore volumes of a weak salt solution (0.01 M $CaCl_2$) [162]. The soil had a bulk density of 1.54 g cm^{-3}, 0.42 porosity, 2.23% organic matter, 14% clay, 19% silt, and 67% sand. The column was modified to collect [^{14}C]-labeled volatile compounds, and 1 pore volume of 0.75 mg L $HgCl_2$ solution was added after the experiment to stop biological activity. Using the modified column, the pore water velocity was approximately 5 cm h^{-1}. The column effluent was collected in 3.5 minute fractions, and analyzed by liquid scintillation counter. Thin-layer chromatography was used to identify metabolites in the column effluent, and combustion analysis was used to determine the distribution of ^{14}C inside the soil column after the experiment.

Using the modified column, approximately 80% of the [^{14}C] was recovered, and the remainder was deemed lost due to incomplete combustion (i.e., present in the column, but not

recovered). 13.25% of the [^{14}C]-material was recovered in the column effluent, and 23.4% of the [^{14}C]-material was recovered as $^{14}CO_2$ following testosterone mineralization. Of the [^{14}C]-material recovered from the column, 70-74% was found in the top 1-5 cm of the column, possibly because more organic matter was present there. [^{14}C]-concentrations in the column effluent tailed off, and the tailing was attributed to chemical processes (e.g., rate-limited sorption), rather than physical processes (e.g., preferential transport), because no tailing was observed when a chloride ion tracer passed through the column. However, preferential transport is generally expected in undisturbed soil, and likely contributes to the leaching that has been observed in natural systems.

Another undisturbed soil column study was conducted to examine the fate and transport of testosterone and 17β-estradiol [161]. The soil samples were taken from no-till and conventionally tilled agricultural plots. In both plots, total organic carbon was higher in the first 10 cm (0.85-0.88%) than between 20-30 cm (0.38-0.43%). Particle sizes in both plots ranged from 60.7-65.3% sand, 15.3-19.3% silt, and 16.7-24% clay. A 1.1 L pulse containing 0.533 µg 17β-estradiol (17,700 Bq 6,7-[^{3}H]-17β-estradiol plus 0.525 µg unlabeled 17β-estradiol) and 1.21 µg 4-[^{14}C]-testosterone (9,430 Bq) was added to each undisturbed soil column (32 cm height × 15 cm inner diameter), and eluted with 21 L (~10 pore volumes) of weak salt solution (0.01 M $CaNO_3$). The column effluent was collected in ~67 mL fractions. After the experiment, soil cores were collected from the columns, divided into increments by depth, and oxidized with a biological oxidizer to release and capture the ^{3}H and ^{14}C in liquid scintillation cocktails. A liquid scintillation counter was used to measure the soil core cocktails and the column effluent.

17β-estradiol sorbed more strongly than testosterone, as an average of 27% of the 17β-estradiol and 42% of the testosterone leached from the columns. The sorption coefficients of

both hormones decreased with depth, possibly because total organic carbon decreased with depth. Oxidation of the column samples recovered 29% of the 17β-estradiol and 17% of the testosterone, and the majority of sorbed hormones occurred in the top 10 cm of soil. The low recovery rates of 56% 17β-estradiol and 59% testosterone were believed to result from underestimation of sorption by the biological oxidizer method or sorption to column materials. ^{14}C losses also might be attributable to biodegradation. Generally, hormone peak concentrations occurred simultaneously with the chloride tracer, indicating the influence of preferential transport.

Future Research

To date, considerable research has been done to understand the sources, presence, and fate of selected environmental endocrine disruptors, including selected steroid sex hormones, in the environment. In addition, considerable research has been done with certain species of organisms to understand the biological and ecological effects of environmental endocrine disruptors at concentrations already found in the environment. Additional research has addressed the development of treatment technologies, including advanced oxidation processes, and the development of best management practices to limit endocrine disruptor contributions to the environment [93, 112, 196-203].

Thus far, research has shown that the subject of endocrine disruption is complex, and difficult to generalize. Additional research must be done to understand the effects of suspected endocrine disruptors on untested species of organisms, and to understand the influence of untested mechanisms and conditions on the environmental fate of suspected endocrine disruptors. Specific subjects for further research include:

- Biosolids leaching and runoff
- Mechanisms and pathways of biodegradation and photodegradation, including the development of mass balance techniques to identify degradation products that might be missed after the application of selective extraction and detection techniques (e.g., MS/MS in the MRM mode)
- Other mechanisms and pathways of abiotic transformation, including redox reactions involving metal oxides
- Presence and environmental effects of degradation products that have not been analyzed before
- Mixtures of endocrine disruptors, their biological effects, and their influence on environmental fate
- Alternate transport pathways, including atmospheric transport
- Specific sorption mechanisms
- Plant and animal sterols as sources of steroid sex hormones in the environment
- Plant uptake, and the influence of plants on environmental fate
- Isolation and identification of microbial species able to mineralize or transform steroid sex hormones, in order to optimize wastewater treatment, composting, and lagoon processes for steroid sex hormone treatment

As additional progress is made, improved treatment technologies and management practices will help to minimize the risks of endocrine disruption to freshwater ecosystems and the broader environment, even as human populations, agricultural operations, and other possible sources of endocrine disruptors continue to grow.

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CHAPTER 3- DIRECT PHOTODEGRADATION OF LAMOTRIGINE (AN ANTIEPILEPTIC) IN SIMULATED SUNLIGHT – pH INFLUENCED RATES AND PRODUCTS[1]

Introduction

Due to their widespread use, incomplete removal during water treatment, and sensitive analytical techniques, trace quantities of pharmaceuticals and their metabolites have been detected in surface waters, groundwater, drinking water, soils, and biota throughout the world [1-4]. As of the end of 2011, antiepileptics were the tenth most prescribed therapeutic class of pharmaceuticals in the United States (128 million prescriptions), and the twelfth highest selling therapeutic class globally (US $14.1 billion) [5, 6]. A recent study synthesized 155 studies on pharmaceutical occurrence in freshwater ecosystems, covering the detection of 203 pharmaceuticals in surface waters across 41 countries, and determined that one epileptic drug, carbamazepine (CBZ), was the most frequently studied and detected compound in North America and Europe, and third in Asia [1]. Two other antiepileptics (gabapentin and primidone) were among the 61 most frequently studied pharmaceuticals in surface waters, and among those with mean detection frequencies exceeding 75% (12 of 61) [1]. In addition, two antiepileptic drugs (CBZ and phenytoin) have been among the most frequently detected pharmaceuticals in drinking water [2, 7, 8]. Together, these data suggest that antiepileptics are ubiquitous in surface waters, and more recalcitrant than many other therapeutic classes of drugs.

[1] Young, R. B.; Chefetz, B.; Liu, A.; Desyaterik, Y.; Borch, T., Direct photodegradation of lamotrigine (an antiepileptic) in simulated sunlight - pH influenced rates and products. Environmental Science: Processes & Impacts 2014, 16, (4), 848-857, DOI: 10.1039/c3em00581j. Reproduced by permission of The Royal Society of Chemistry.

Lamotrigine [3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine] (LTG) is an antiepileptic and mood stabilizing drug marketed as Lamictal. In 2008, when generic LTG tablets were introduced to the United States, Lamictal was the 19th highest selling brand name pharmaceutical (US $1.5 billion) [9]. As an antiepileptic, LTG appears to be effective at treating primary generalized tonic-clonic seizures and absence seizures, and is at least as effective as CBZ, the standard drug treatment, for treating partial onset seizures [10, 11]. As a mood stabilizer, LTG increases the time between episodes of depression and mania in bipolar disorder, and is particularly effective against depression [11, 12].

Lamotrigine and its primary metabolite, lamotrigine-2-N-glucuronide (LTG-2NG), have recently been detected in wastewater, groundwater, surface water and drinking water [13-15]. In surface water, LTG's detection frequency ranged from 47 to 97% [13-15], and its average concentration ranged from 108 to 455 ng L^{-1} [13, 14]. Also, in a study of the in-stream attenuation of 14 neuroactive pharmaceuticals, LTG, CBZ, and 10,11-dihydro-10,11-hydroxy-carbamazepine (DiOH-CBZ, a metabolite common to CBZ and oxcarbazepine), were determined to be most persistent, with pseudo-first order half-lives ranging from 12 to 21 h [16]. Limited sampling showed that LTG, CBZ and DiOH-CBZ were sequestered into stream biofilm, and the study suggested that interactions with bed sediments and stream biofilm play an important role in their fate and transport.

The predicted no-effect concentration (PNEC) of LTG in drinking water has been estimated to equal 170 μg L^{-1} for children, based upon an exposure duration of 6 years, a drinking water ingestion rate of 1 L d^{-1}, and other factors [17]. This PNEC is lower and more conservative than the PNEC for adults, and orders of magnitude higher than LTG concentrations previously detected in surface and drinking waters. However, PNECs are based on available

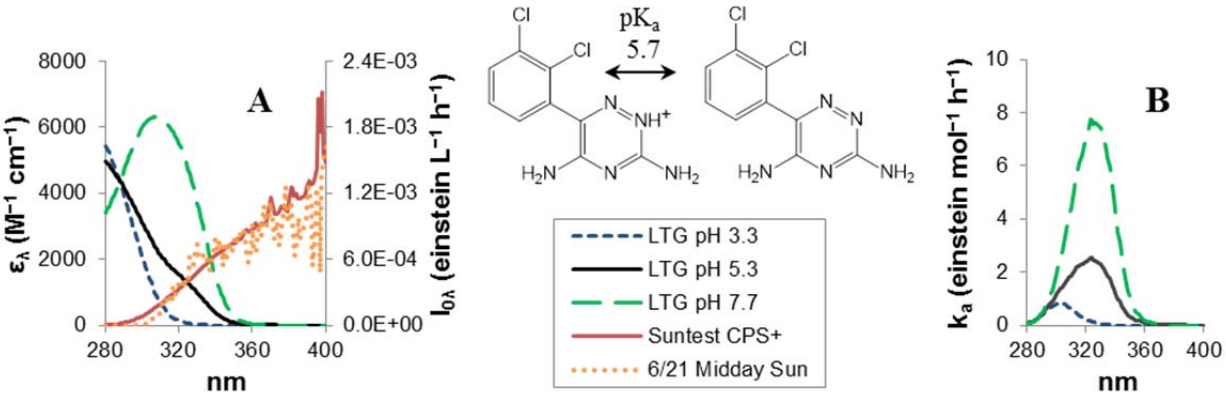

FIGURE 3-1. Molecular structure, absorption and irradiance data of lamotrigine (LTG). Protonated and unprotonated molecular structure of LTG (center), with (A) a comparison of the spectral irradiance ($I_{0\lambda}$) of the Suntest CPS+ solar simulator, the estimated solar irradiance ($I_{0\lambda}$) in Denver, CO, USA (latitude 39.8617 °N) on June 21, 2013 at 1:00 p.m. MDT (SMARTS v 2.9.5), and LTG's molar absorptivity (ε_λ) in buffered aqueous solutions at pH 3.3, 5.3 and 7.7; plus (B) LTG's specific light absorption rate ($k_{a\lambda}$ = 2.303 ℓ $I_{0\lambda}$ ε_λ) in quartz glass culture tubes irradiated by the solar simulator (pathlength, ℓ, = 1 cm).

toxicological information (e.g., no observed adverse effect levels, adjusted for uncertainty factors), and the most sensitive individuals and organisms may not be discovered until after idiosyncratic reactions occur (e.g., the collapse of vulture populations exposed to diclofenac) [18, 19]. In addition, LTG selectively blocks voltage-gated sodium channels, which predate neurons and form the basis of electrical excitability in most vertebrate and invertebrate species [20-23]. This suggests at least a potential for broad ecosystem effects. According to one study, which sought to prioritize the environmental risk of the top 200 prescription drugs from 2009, LTG was one of only 13 pharmaceuticals appearing in the top 20 for at least 6 of the 12 toxic endpoints considered [24].

Because LTG is incompletely removed during wastewater treatment [14-16], and absorbs light at wavelengths present in natural waters (e.g., > 290 nm; Figure 3-1) [25], photodegradation might be expected to affect LTG's fate in sunlit waters more than biodegradation [26]. Direct photodegradation occurs when a molecule absorbs light, becomes electronically excited, and

undergoes chemical transformation. The fastest direct photochemical reactions (~10^{14} s^{-1}) are limited by rates of vibrational motion and electron transfer, and the slowest (~10^{-2} s^{-1}) are limited by slow phosphorescence rates (a competing process) [27]. Indirect photodegradation, by comparison, is chemical transformation initiated after a co-solute (e.g., dissolved organic matter) absorbs light and becomes reactive. Various reactive species can be generated, including hydroxyl radicals ($^{\bullet}OH$), singlet oxygen ($^1O_2$), superoxide radical ions ($O_2^{\bullet-}$), carbonate radicals ($CO_3^{\bullet-}$), and excited triplet state dissolved organic matter ($^3DOM^*$), and the fastest indirect photochemical reactions are limited by diffusion rates in water (~10^{10} M^{-1} s^{-1} at 25 °C) [27-31]. For example, $^{\bullet}OH$ reacts with many compounds, including natural water constituents, at nearly diffusion-controlled rates, but steady state concentrations of $^{\bullet}OH$ in natural waters are reported to range from only 10^{-15} to 10^{-18} M [32]. As a consequence, organic contaminants would generally be expected to react with $^{\bullet}OH$ in natural waters at rates in the range of 10^{-2} to 10^{-5} h^{-1}.

Lamotrigine's direct photodegradation rate will be governed by the spectrum and intensity of available light, LTG's ability to absorb such light (i.e., molar absorptivity), and the efficiency at which the absorbed light is converted to chemical change (i.e., the reaction quantum yield). Because upper excited states rapidly decay to lowest excited singlet or triplet states, reaction quantum yields (Φ) are generally assumed to be wavelength-independent [33, 34]. This means that reaction quantum yields can be used to predict direct photodegradation rates across different lighting conditions. Reaction quantum yields also provide the benchmark for determining the influence of indirect photodegradation, light screening, and physical quenching in natural waters [35]. Therefore, it is appropriate to begin examining LTG's photochemical fate in sunlit waters by determining the rates, efficiency, and products of LTG's direct photodegradation.

Lamotrigine is a water soluble (0.17 mg/mL at 25 °C) weak base (pK_a = 5.7) [36]. The site of LTG's protonation, and its most basic nitrogen, is located within the triazine ring where 2-N-glucuronidation occurs (Figure 3-1) [36, 37]. Because the pH of most natural waters ranges from pH 6 to pH 9 [38], LTG's neutral form will predominate, but its protonated form may also appear. pH is known to influence photochemistry because different protonation states can produce different molar absorptivities, reaction quantum yields, and photodegradation pathways [39-43]. Therefore, pH effects should be considered when examining LTG's direct photochemistry.

The photochemistry of LTG has not been studied in the environmental context, but LTG is known to produce a phototoxic response in some patients, and its photochemical properties have been studied on that basis [44]. The mean half-life of LTG in healthy volunteers and epileptic patients receiving LTG monotherapy is 22.8 to 37.4 h [45]. Accordingly, the phototoxicity study by Bilski et al. (2009) was conducted over relatively short periods (up to 1 h) with high intensity light. Among other things, the study found that irradiation of LTG produced an excited triplet state, generating $^1O_2$ and peroxidizing lineolic acid (a representative fatty acid). The study also detected chloride anions (Cl^-) by electrochemical assay, $O_2^{\bullet-}$ by spin-trapping studies, and a phenolic photoproduct resulting from dechlorination and hydroxylation of LTG's dichlorophenyl ring. Based on these results, the study suggested that $O_2^{\bullet-}$ was formed by electron transfer from LTG to molecular oxygen, and that aryl radicals from photodechlorination might contribute to LTG's phototoxic response. Finally, the study determined that $^1O_2$ photosensitization is the primary mechanism for LTG phototoxicity.

The purpose of this study was to examine the direct photodegradation of LTG in waters exposed to simulated sunlight under different pH conditions, and over time periods appropriate for environmental fate analysis. The results of this study will provide a benchmark for

determining the influence of indirect photodegradation, light screening, and physical quenching in natural waters, and help in predicting photodegradation pathways for LTG and similarly structured pharmaceuticals in surface waters. The results of this study will also help in prioritizing research based on photostability.

Experimental

The following section describes materials and methods used in this study. Unless otherwise indicated, all reported confidence intervals are 95% confidence intervals based on three replicates (n = 3).

Chemicals and Solutions

Ultrapure deionized water (18.2MΩ·cm) was obtained from a Milli-Q reagent water purification system (Millipore, Bedford, MA, USA) fed by distilled water. Lamotrigine was purchased from Sigma-Aldrich (≥ 98%) and Enzo Life Sciences (≥ 98%, Farmingdale, NY, USA). Pyridine (ACS reagent ≥ 99.0%), ammonium acetate (NH_4OAc, ≥ 98%), and formic acid (Fluka brand, ~98%) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), and 4-nitroacetophenone (PNAP, 98%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Finally, acetonitrile (MeCN, ACS/HPLC grade), methanol (MeOH, ACS grade), monobasic sodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, certified ACS), dibasic sodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$, certified ACS), hydrochloric acid (HCl, 12N, certified ACS plus), and ammonium hydroxide (NaOH, 20-22% as NH_3, Optima brand) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Phosphate buffer solutions (5 mM PO_4) were prepared by dissolving $NaH_2PO_4 \bullet H_2O$ or $Na_2HPO_4 \bullet 7H_2O$ into deionized water, and adjusting to pH 3.3 (± 0.2) or pH 7.7 (± 0.1) with HCl or NaOH. The stock solutions of LTG at pH 3.3 (11.4 mg L^{-1}) and pH 7.7 (12.0 mg L^{-1}) were prepared by dissolving LTG directly into the pH 3.3 and pH 7.7 phosphate buffer solutions. The LTG stock solution at pH 5.3 ± 0.4 (11.7 mg L^{-1}) was prepared by mixing the pH 3.3 and pH 7.7 LTG solutions (50/50 v/v), and adjusting the solution pH with HCl or NaOH. All LTG stock solutions were stored at 3 °C in 500 mL amber glass bottles until use. A stock solution of PNAP (1,586 mg L^{-1}) was prepared in MeCN and stored at −14 °C in amber glass vials until use.

Kinetics and Reaction Quantum Yield Experiments

To determine LTG's reaction quantum yield in the phosphate-buffer solutions at pH 3.3, 5.3 and 7.7, a chemical actinometer solution (1.6 mg L^{-1} PNAP, plus 0.05% v/v pyridine) was prepared in deionized water in accordance with published methods for sunlight actinometry [25, 46]. The quantum yield of the PNAP actinometer solution is linear as a function of pyridine concentration (Φ_{PNAP} = 0.0169 [pyridine] up to 0.2 M pyridine in 10 μM PNAP) [25, 46].

During each experiment, triplicate 8.5 mL samples of the LTG and chemical actinometer solutions were placed in quartz glass culture tubes (12 mm o.d. × 100 mm), sealed with Versa Vial PTFE/silicon closures (Supelco, Bellefonte, PA, USA), and positioned at a 35° angle within a Suntest CPS+ solar simulator (Atlas Material Testing Technology LLC, Chicago, IL, USA). The solar simulator's xenon lamp was operated at 765 W m^{-2} irradiance (300 to 800 nm) using a "UV special glass" filter to eliminate wavelengths below approximately 290 nm. The spectrum of the solar simulator was measured with a Maya 2000 Pro spectrometer (Ocean Optics, Inc., Dunedin, FL, USA) using a 400 μm solarization-resistant optical fiber (P400-2-SR) and no

cosine corrector. The spectral irradiance of the solar simulator (Figure 3-1) was estimated with the PNAP actinometer solution, assuming a fixed spectrum and applying a factor (2.2) in accordance with published methods to account for the use of cylindrical tubes [25]. During the experiments, the solar simulator's internal temperature (18.2 ± 0.3 °C, n = 20) was regulated by a Fuji Electric (Tokyo, Japan) PXZ-4 temperature controller (model SR1) and Emerson Quiet Kool (Model 6JC63) air conditioning system. For dark controls, 8.5 mL samples of the LTG and the PNAP actinometer solutions were placed in borosilicate glass culture tubes (13 mm o.d. × 100 mm), sealed with PTFE-lined screw caps, covered with aluminum foil, and positioned within the solar simulator. A 100 µL sample was collected daily from each culture tube over either a 4-day (pH 3.3) or 5-day (pH 5.3 and 7.7) continuous exposure period, and stored at 4 °C in 2 mL amber glass vials until analysis.

Reaction Quantum Yield Calculations

The reaction quantum yields of the phosphate-buffered aqueous solutions of LTG at pH 3.3, 5.3 and 7.7 were calculated using Equation I:

$$\phi_C = \phi_A \times \frac{k_{dC}}{k_{dA}} \times \frac{\sum I_{0\lambda}\varepsilon_{\lambda A}}{\sum I_{0\lambda}\varepsilon_{\lambda C}\, S_\lambda} \qquad \text{(I)}$$

$$S_\lambda = \frac{(1 - 10^{-\alpha_\lambda l})}{2.303\ \alpha_\lambda l} \qquad \text{(II)}$$

where: Φ_x is the contaminant's (x = C) or the actinometer's (x = A) reaction quantum yield (mol einstein^{-1}); k_{dx} is the first order rate constant for direct photodegradation of the contaminant (x = C) or the actinometer (x = A) (t^{-1}); $I_{0\lambda}$ is the irradiance at each incident wavelength λ (einstein L^{-1} t^{-1}); $\varepsilon_{\lambda x}$ is the contaminant's (x = C) or the actinometer's (x = A) molar absorptivity at each incident wavelength λ (L mol^{-1} cm^{-1}); S_λ is the light screening factor determined under Equation II; α_λ is the measured attenuation coefficient for the LTG solution at each incident wavelength λ (cm^{-1}), and ℓ is the pathlength of the incident light (cm).

The procedure to account for light screening using Equation II is described in the literature [28, 46]. In deionized water, when no light screening occurs, the value of S_λ is 1 [47]. The pathlength (l) of the quartz glass culture tubes (12 mm o.d. × 100 mm) was assumed to be 1.0 cm in accordance with published literature values [46].

Photoproduct Experiments

To determine LTG's photoproducts, an 8.5 mL sample of the LTG solution at pH 5.3 was irradiated according to the procedures described for the kinetics and reaction quantum yield experiments. The pH 5.3 solution was chosen for the photoproduct analysis because it contained protonated and neutral LTG and was expected to generate photoproducts from both protonation states. A 500 µL sample was collected every fourth day from each culture tube over a 12-day continuous exposure period, and a 150 µL sample was collected every fourth day thereafter until the total continuous exposure period was 24 days. The photoproduct samples were stored at 4 °C in 2 mL amber glass vials until analysis.

Analytical Methods

Solution pH was measured with an Accumet Excel XL60 pH meter (Thermo Fisher Scientific, Inc.). Absorption spectra were measured with an Agilent 8453 UV-visible spectrophotometer using a 1 cm quartz cuvette, 0.5 s integration time, 1 nm interval, and Agilent UV-visible ChemStation software. To eliminate non-representative solvent effects, all absorption spectra were measured in deionized water except PNAP (0.1% MeCN in deionized water).

LTG was quantified using an Agilent 1200 series HPLC system equipped with a UV-diode array detector (HPLC-UV; Agilent Technologies, Inc., Santa Clara, CA, USA). The chromatographic method used a Kinetex PFP column (100 × 3.1 mm i.d., 2.6 μm particle size, Phenomenex, Torrance, CA), maintained at 40 °C, a constant flow rate of 500 μL min^{-1}, and the following binary gradient: 10% (A) deionized water with 5 mM ammonium acetate and 0.1% v/v formic acid, and 90% (B) acetonitrile, for 4 min; increased to 65% B over 5.5 minutes; stepped to 100% B and held for 4 minutes to flush the column; and equilibrated at 10% B for 4.5 minutes. The injection volume of each sample was 10 μL, and the samples were quantified at 260 nm using a seven-point external calibration curve (method detection limit < 0.1 mg L^{-1}).

4-nitroacetophenone was also quantified using the Agilent HPLC-UV system. The chromatographic method used an XSelect CSH C_{18} XP column (75 × 4.6 mm i.d.; 2.5 μm particle size; Waters Corporation, Taunton, MA, USA), maintained at 40 °C, and the following binary gradient: (A) 60% deionized water, and (B) 40% acetonitrile, at the beginning; increased to 90% B over 5 minutes at 600 μL min^{-1}; stepped to 100% B and held for 1.5 minutes at 750 μL min^{-1} to flush the column; and equilibrated at 40% B for 4 minutes at 600 μL min^{-1}. The injection volume of each sample was 10 μL, and the samples were quantified at 270 nm using a seven-point external calibration curve (method detection limit < 0.1 mg L^{-1}).

LTG's photoproducts were analyzed by liquid chromatography-time of flight mass spectrometry (LC-TOF MS) using an Agilent 1100 series HPLC and Agilent G3250AA MSD TOF system. The chromatographic method was identical to the chromatographic method used to quantify LTG for the kinetics and reaction quantum yield experiments. The mass spectrometer was operated in positive ion mode under the following parameters: 4000 V capillary voltage; 190 V fragmentor voltage; 45 V skimmer voltage; 300 Vpp Oct 1 RF; 45 psig nebulizer pressure;

10 L min^{-1} drying gas (nitrogen) flow; and 325 °C drying gas temperature. The injection volume of each sample was 10 or 50 μL, and internal references masses (purine, $C_5H_4N_4$, m/z 121.05087; and hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine, $C_{18}H_{18}F_{24}N_3O_6P_3$, m/z 922.00980) were infused during each chromatographic run to permit recalibration of extracted mass spectra.

Data Processing

All least squares linear regression analyses, sample means and confidence intervals were calculated using Microsoft Office Excel 2007 for Windows (Microsoft, Inc., Redmond, WA, USA). Agilent ChemStation for LC 3D Systems software was used for all HPLC-UV data processing. Agilent MassHunter Workstation Qualitative Analysis software was used for all LC-TOF MS data processing.

Results

Direct Photodegradation Rates and Reaction Quantum Yields

The following section describes results from experiments in the solar simulator to determine LTG's direct photodegradation rates and reaction quantum yields in buffered aqueous solutions at pH 3.3 ± 0.2 (99 to 100% protonated), pH 5.3 ± 0.4 (50 to 86% protonated), and pH 7.7 ± 0.1 (0.79 to 1.2% protonated). These pH values were chosen to represent LTG in its neutral, protonated, and partially protonated forms, in order to examine the influence of pH on LTG's direct photodegradation.

Figure 3-1.A compares the spectral irradiance of the solar simulator, the estimated midday, midsummer solar irradiance in Denver, CO, USA (latitude 39.8617 °N; SMARTS v 2.9.5), and the molar absorptivities of the three buffered aqueous LTG solutions (pH 3.3, 5.3

and 7.7). Due to protonation of LTG's triazine ring, the absorption maximum of LTG's longest wavelength absorption band shifted from 307 nm (pH 7.7) to 267 nm (pH 3.3). As a consequence, in quartz glass culture tubes irradiated by the solar simulator, the specific light absorption rate (einstein mol^{-1} h^{-1}) of LTG's neutral form (LTG^0) was approximately 12 times greater than its protonated form (LTG^+) (Figure 3-1.B). This difference increased to approximately 28 times in the estimated midday, midsummer Denver sun produced by a spectral radiation model (SMARTS v 2.9.5) [48], primarily because the estimated Denver sunlight did not include the shortest wavelengths in the solar simulator, where LTG^+ absorbed more light (Figure 3-1.A; Figure 3-2).

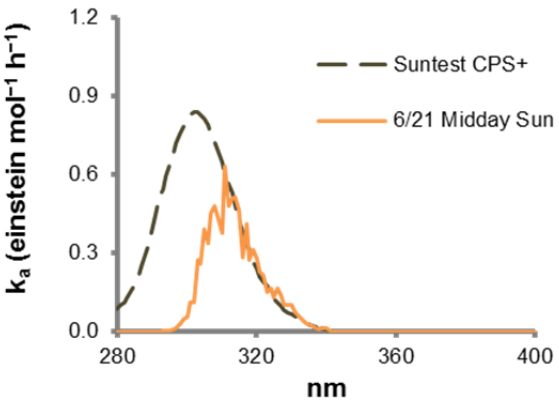

FIGURE 3-2. Solar Simulator versus Solar Irradiance Model. Specific absorption rate of lamotrigine (LTG) in quartz glass culture tubes irradiated by the Suntest CPS+ solar simulator or midday, midsummer sun in Denver, CO, USA (estimated for June 21, 2013 at 1:00 p.m. MDT (SMARTS v 2.9.5)).

Table 3-1 contains the direct photodegradation rates, half-lives, and reaction quantum yields of the LTG solutions after 4 d (pH 3.3) or 5 d (pH 5.3 and 7.7) of continuous irradiation in the solar simulator. Except for one data point (pH 3.3 at 172.7 h), no dark control sample concentration was significantly different ($\alpha = 0.05$, $n = 4$) from the mean starting concentration, and the remaining data point was 96.8% of the mean starting concentration. There was no significant difference between the direct photodegradation rates of LTG^0 and LTG^+, even though LTG^0 absorbed approximately 12 times more simulated sunlight. As reflected in LTG's reaction quantum yields at pH 3.3 and pH 7.7 (Table 3-1), LTG^+ converted the absorbed light to

TABLE 3-1. Direct photodegradation of lamotrigine (LTG). Pseudo-first order degradation rate constants (k), half-lives ($t_{½}$), integrated specific light absorption rates ($\sum k_{a\lambda}$), and reaction quantum yields (ϕ), with 95% confidence intervals ($\alpha = 0.05$), of buffered aqueous solutions of LTG (11.4 to 12.0 mg L^{-1}) after 4 d (pH 3.3) or 5 d (pH 5.3 and 7.7) of continuous irradiation in the solar simulator (n = 3), together with their estimated half-lives at latitude 40°N during summer in a flat water body at a shallow depth (i.e., < 5% attenuation).

pH	k (h^{-1})	$t_{½}$ (h)	$\sum k_{a\lambda}$ (einstein mol^{-1} h^{-1})	Φ (mol einstein^{-1}) [†]	predicted $t_{½}$ (h), summer 40°N[b] [‡]
3.3 ± 0.2	$7.0 \pm 0.2 \times 10^{-3}$	100 ± 3	22	$33 \pm 2 \times 10^{-5}$	500 ± 20
5.3 ± 0.4	$6.2 \pm 0.1 \times 10^{-3}$	112 ± 2	99	$6.3 \pm 0.1 \times 10^{-5}$	273 ± 5
7.7 ± 0.1	$6.7 \pm 0.2 \times 10^{-3}$	103 ± 2	270	$2.51 \pm 0.07 \times 10^{-5}$	230 ± 6

[†] 4-Nitroacetophenone (1.6 mg L^{-1}, 0.05% v/v pyridine) was used as a chemical actinometer, and approximately 83% was removed after 5 d. [‡] Based on estimated solar irradiance in Denver, CO, USA (latitude 39.8617 °N) on June 21, 2013 at 1:00 p.m. MDT (SMARTS v 2.9.5).

photochemical reactions approximately 13 times more efficiently than LTG^0, offsetting the difference in their light absorption rates. When the same reaction quantum yields were used to predict LTG's half-life in a flat water body at a shallow depth (i.e., < 5% attenuation) [49] exposed to the estimated midday, midsummer Denver sun, LTG^0 was estimated to degrade more than twice as fast as LTG^+ (Table 3-1), primarily because LTG^0 was estimated to absorb approximately 28 times more Denver sunlight.

Figure 3-3 shows the photochemical loss of LTG at pH 5.3 after 12 d (290.6 h) of continuous irradiation in the solar simulator. After 12 d, LTG's half-life (105 ± 2) was slightly, but significantly, shorter than the value reported in Table 3-1 after 5 d of continuous irradiation (112 ± 2). Close inspection of Figure 3-3 reveals that LTG's photodegradation rate increased slightly with time (and decreasing LTG concentration), suggesting that some amount of self-screening occurred at the LTG concentration employed (11.7 mg L^{-1}). The reaction quantum yield, integrated specific light absorption rate, and predicted half-life calculations in Table 3-1

were adjusted in accordance with published methods to account for this self-screening effect [28, 35, 46].

In summary, LTG's direct photodegradation half-life varied little (100 ± 3 to 112 ± 2) in the solar simulator as the solution pH changed. However, LTG's molar absorptivities and reaction quantum yields at pH 3.3 and 7.7 were significantly different (Table 3-1), and LTG was estimated to degrade more than twice as fast at pH 7.7 under different lighting conditions. The disparity in reaction quantum yields may suggest that LTG^0 and LTG^+ are following different photodegradation pathways, and possibly producing different photoproducts. Alternatively, LTG's protonation state may influence the relaxation or energy transfer possibilities available to excited state LTG, leading to a lower quantum yield for LTG^0.

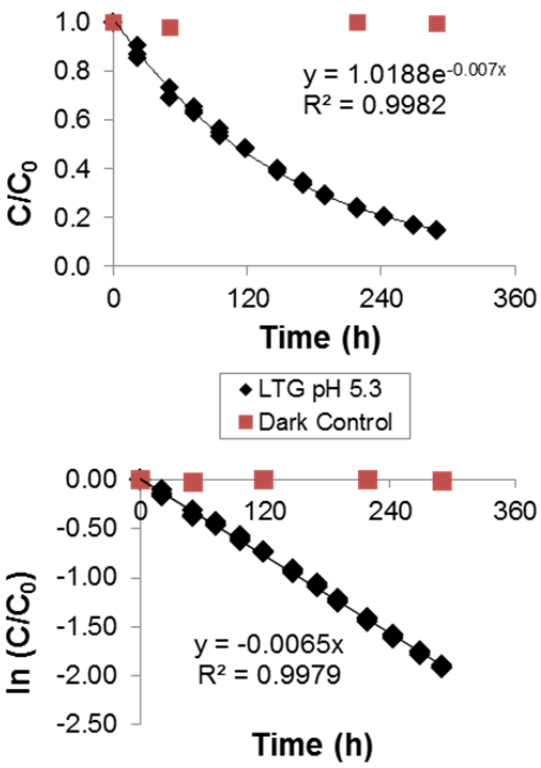

FIGURE 3-3. Photochemical loss of lamotrigine (LTG). Progress of photochemical loss of LTG in the buffered aqueous solution at pH 5.3 during 12 d (290.6 h) of continuous irradiation in the solar simulator (n = 3), with dark control (n = 1).

Photoproducts

The following section describes results from experiments to determine whether LTG produced different photoproducts at different pH. HPLC-UV was utilized to detect photoproduct

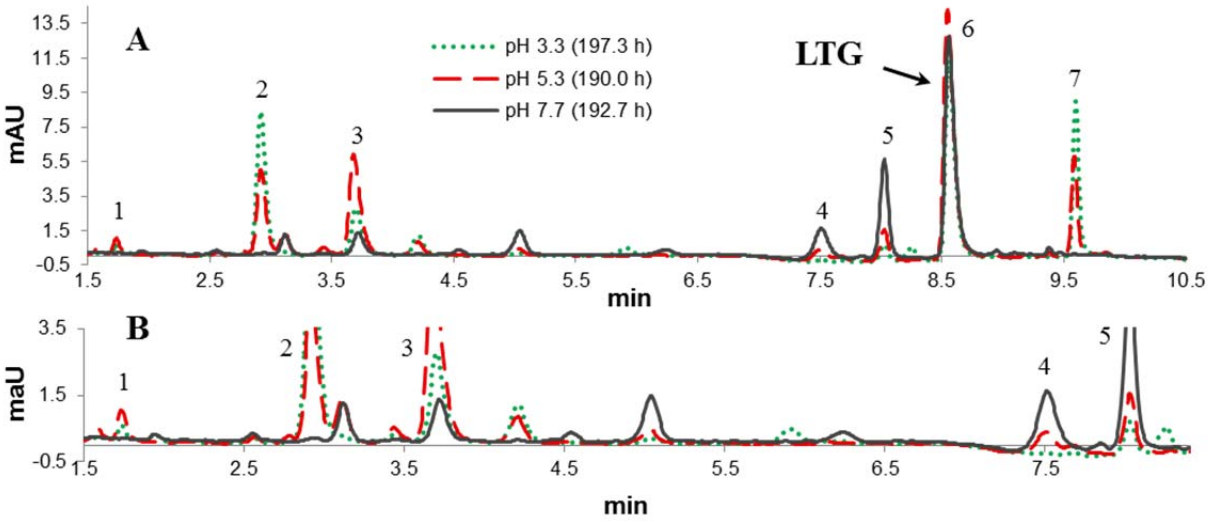

FIGURE 3.4. HPLC-UV chromatograms of lamotrigine (LTG) and its photoproducts. (A) HPLC-UV chromatograms (260 nm) of buffered aqueous solutions of LTG at pH 3.3, 5.3 and 7.7 after 8 d (192 h) of continuous irradiation in the solar simulator. The bottom panel (B) shows a portion (1.5 to 8.4 min) of the top panel (A) in greater detail. This figure is supplemented by Table 3-2, which provides liquid chromatography-time of flight mass spectrometry data for the numbered peaks.

differences, and LC-TOF MS was used to generate accurate mass-to-charge (m/z) measurements and isotopic information to identify the resulting photoproducts.

Figure 3-4 contains overlapping HPLC-UV chromatograms for the LTG solutions at pH 3.3, 5.3, and 7.7 after 8 d of continuous irradiation in the solar simulator. Except for LTG, none of the exhibited peaks was present in any unexposed LTG solution (pre-exposure or dark control). Figure 3-4 is supplemented by Table 3-2, which contains LC-TOF MS data from the pH 5.3 solution for the numbered peaks in Figure 3-4. Preliminary LC-TOF MS experiments for LTG solutions at ~pH 3 and ~pH 8 confirmed that the proposed formulas in Table 3-2 also apply to the numbered peaks in Figure 3-4 from the pH 3.3 and pH 7.7 solutions (data not shown).

Several things stand out in Figure 3-4. First, many photoproducts were detected, compared to the phototoxicity study by Bilski et al. (2009), where one photoproduct was detected over shorter timeframes (up to 1 h) [44]. Second, the initial concentrations of LTG in the

TABLE 3-2. Proposed lamotrigine (LTG) photoproduct identities. Liquid chromatography-time of flight mass spectrometry data for the numbered peaks in Figure 3-4 from the buffered aqueous solution of LTG (11.7 mg L−1) at pH 5.3 after 8 d (190.0 h) of continuous irradiation in the solar simulator.

peak	proposed formula (M)	m/z	ion species	absolute mass error (ppm)	double bond equivalent (DBE)
1	$C_9H_8ClN_5O$	238.04901	$[M+H]^+$	1.00	8
2	$C_9H_9Cl_2N_5O$	274.02569	$[M+H]^+$	1.02	7
3	$C_9H_8Cl_2N_4O$	259.01479	$[M+H]^+$	0.15	7
4	$C_9H_6ClN_5$	220.03845	$[M+H]^+$	0.07	9
5	$C_9H_7Cl_2N_5$	256.01513	$[M+H]^+$	0.02	8
6	$C_9H_7Cl_2N_5$	256.01513	$[M+H]^+$	0.45	8
7	$C_9H_5Cl_2N_5$	253.99948	[M+H]+	0.38	9

pH 3.3, 5.3 and 7.7 solutions were similar (11.4 to 12.0 mg L^{-1}), and the peak area of LTG (peak 6) was approximately the same in all three chromatograms, indicating similar direct photodegradation rates (Table 3-1), and suggesting that differences in photoproduct peak areas (e.g., peak 3) were primarily the result of pH differences. Finally, some photoproduct peaks (e.g., peaks 1, 2 and 7) appeared only in solutions containing LTG^+, and other photoproduct peaks (e.g., peak 4) appeared only in solutions containing LTG^0.

According to the LC-TOF MS data in Table 3-2, peak 1 ($C_9H_8ClN_5O$) corresponds to the photoproduct detected in the phototoxicity study by Bilski et al. (2009) [44], a phenolic photoproduct resulting from dechlorination and hydroxylation of the dichlorophenyl ring of LTG ($C_9H_7Cl_2N_5$). Peak 2 ($C_9H_9Cl_2N_5O$) and peak 3 ($C_9H_8Cl_2N_4O$), by comparison, evidence reactions involving oxygen addition without dechlorination. The proposed molecular formula of peak 2 suggests a photohydration product, and the proposed molecular formula of peak 3 demonstrates that LTG lost one nitrogen and gained one hydrogen atom. Because peak 3 evidences a net increase in hydrogen atoms, peak 3 does not suggest a photoproduct resulting merely from deamination and hydroxylation of LTG's diamino-triazine ring.

The proposed molecular formula for peak 4 ($C_9H_6ClN_5$), which appeared only in solutions containing LTG^0, indicates that LTG lost HCl, and added one double bond equivalent (DBE 8 → 9). Given the aromatic structure of LTG's dichlorophenyl and triazine rings, this suggests a photocycloaddition reaction, and the formation of a third ring. Therefore, peak 4 was tentatively assigned the one chlorine-tricyclic structure in Figure 3-5.

The proposed molecular formula for peak 5 ($C_9H_7Cl_2N_5$) indicates an LTG isomer. Peak 5 was not present at 0 d, and increased in size after 4, 8 and 12 d of continuous irradiation in the solar simulator (Figure 3-6), conclusively establishing that peak 5 is an LTG photoproduct, and not the result of poor chromatography.

Finally, the proposed molecular formula for peak 7 ($C_9H_5Cl_2N_5$), which

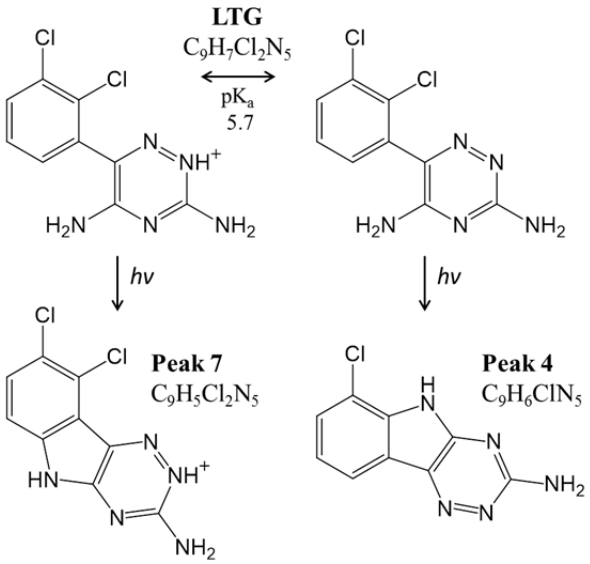

FIGURE 3-5. Selected photoproduct structures. Proposed structures for photoproduct peaks 4 and 7 in Figure 3-4, arising from irradiation of the buffered aqueous solution of lamotrigine (LTG, 11.7 mg L^{-1}) at pH 5.3.

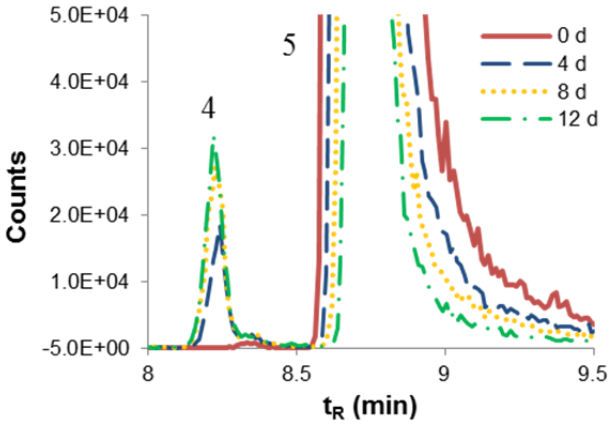

FIGURE 3-6. Evolution of isomeric photoproduct. Extracted ion chromatograms (m/z 256.01513) of phosphate-buffered aqueous solution of lamotrigine (LTG, 11.7 mg L^{-1}; pH 5.3 ± 0.4) after 0 d (0 h), 4 d (96.2 h), 8 d (190.0 h), and 12 d (290.6 h) of continuous irradiation in the Suntest CPS+ solar simulator. LTG is evidenced by peak 5, and its photoisomer is represented by peak 4.

appeared only in solutions containing LTG^+, indicates that LTG lost H_2, and added one double bond equivalent (DBE $8 \rightarrow 9$). In accordance with the reasoning for peak 4, peak 7 was tentatively assigned the two chlorine-tricyclic structure in Figure 3-5.

Figure 3-7 shows changes in the areas of the numbered peaks in Figure 3-4 over a 28 d (670.9 h) extended irradiation period in the solar simulator. Approximately 90% of the initial LTG concentration was eliminated after 12 d (290.2 h). Thereafter, the areas of peaks 2 and 3 declined sharply, the areas of peaks 1 and 4 declined slowly, and the areas of peaks 5 and 7 were relatively stable. There was no relationship between photoproduct stability and pH, because peak 4 only appeared in solutions containing LTG^0, peak 5 appeared at every pH, and peak 7 only appeared in solutions containing LTG^+. According to Figure 3-7, peaks 4, 5 and 7 were somewhat to very stable over the 16 d period after most of the initial LTG concentration had been eliminated, suggesting that the associated photoproducts were even more stable than LTG under simulated sunlight.

In summary, several photoproducts were detected, and their identities, relative abundances and reaction mechanisms were influenced by solution pH. Molecular formulas were proposed for the most prominent peaks, and molecular structures were proposed for peak 4, which appeared only in solutions containing LTG^0, and peak 7, which appeared only in solutions containing LTG^+. Three of the most

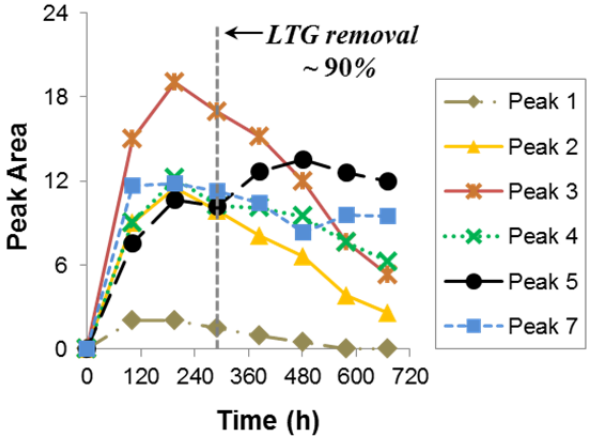

FIGURE 3-7. Evolution of lamotrigine (LTG) photoproducts. Peak areas (detection wavelength = 260 nm) for the numbered peaks in Figure 3-4 over a 28 d (670.9 h) extended irradiation period (n = 1).

prominent peaks appeared to be even more stable than LTG in simulated sunlight, but no relationship between solution pH and photostability was observed.

Discussion

Direct Photodegradation Rates and Reaction Quantum Yields

According to Table 3-1, LTG's reaction quantum yield was 13 times higher at pH 3.3, when LTG was almost 100% protonated, than at pH 7.7, when LTG was approximately 1% protonated. However, LTG^+ absorbs less light than LTG^0 at wavelengths present in natural waters. Therefore, pH influenced LTG's direct photodegradation rate ***both*** by influencing LTG's ability to absorb the available light, and by influencing the efficiency at which such light is converted to photochemical reactions. Because LTG's neutral form is expected to predominate in natural waters, the photochemistry of LTG^0 is expected to exert a greater influence on LTG's fate in sunlit waters.

LTG's reaction quantum yield at pH 5.3 arguably should have been closer to that of pH 3.3 (almost 100% protonated) than that of pH 7.7 (approximately 1% protonated), because LTG is 72% protonated at pH 5.3. In general, when a molecule is present in more than one protonation state, its photochemistry is expected to reflect the photochemistry of all available protonation states in an integrative manner [39, 50]. In fact, LTG's molar absorptivity at pH 5.3 did more closely resemble that of pH 3.3 (Figure 3-1.A). Chlorinated arenes (like LTG's dichlorophenyl ring) have been observed to undergo photochemical reactions through excited state charge transfer complexes [51], and there is at least some potential for LTG^0 and LTG^+ to form ground state complexes via cation-π bond interactions [52], but the LTG concentrations used in this study are low (< 0.05 mM), and LTG concentrations in the environment are even lower.

It is more likely that the reaction quantum yields in Table 3-1 are affected by small errors in determining the solar simulator's spectral irradiance or LTG's molar absorptivity, particularly at pH 3.3 and 5.3 where their spectral overlap is small (Figure 3-1.A). Such errors can cause significant differences in reaction quantum yields [40].

In this study, LTG's reaction quantum yields ranged from 2.51×10^{-5} (pH 7.7) to 33×10^{-5} (pH 3.3) mol einstein^{-1}. These are similar to reaction quantum yields reported for CBZ, which have ranged from 6×10^{-5} to 1.3×10^{-4} mol einstein^{-1} [29, 53-58]. Many pharmaceuticals undergo direct photodegradation with greater efficiency than CBZ and LTG (e.g., naproxen, $\Phi = 0.012$; diclofenac: $\Phi = 0.094$ to 0.13; and sulfamethoxazole, $\Phi = 0.028$ to 0.959 from pH 9.2 to pH 3.2) [26, 40], which might explain why CBZ and LTG seem more recalcitrant than many other drugs in surface waters. However, indirect photodegradation is significantly faster and more efficient than direct photodegradation in some pharmaceuticals [59-61], and further study is needed to examine the effect of indirect photochemistry and biodegradation on LTG's fate in sunlit waters. For example, LTG produces an excited triplet state upon photoexcitation [44], which suggests the possibility of triplet energy transfer to dissolved organic matter (DOM) [62], or triplet state energy transfer from ^{3}DOM* [28]. Similarly, excited state LTG has been reported to undergo electron transfer reactions [44], which suggests the possibility of electron transfer reactions with DOM, $CO_3^{\bullet-}$, and other natural water constituents [31, 63, 64]. The relative importance of each reactant will depend on its steady-state concentration and second-order rate constant for reaction with LTG [31], but LTG's direct photodegradation rates (Table 3-1) do appear consistent with the range of reaction rates described in the Introduction for reactions between organic contaminants and $^{\bullet}$OH.

Photoproducts

As Figure 3-4 demonstrates, pH and degree of protonation affected the identities and relative abundances of most LTG photoproducts. For example, peaks 1, 2 and 7 appeared only in solutions containing LTG^+, and peak 4 appeared only in solutions containing LTG^0.

Figure 3-5 contains proposed molecular structures for peaks 4 and 7, both of which involve the photochemical addition of an amino group from LTG's triazine ring to the adjacent dichlorophenyl ring. In the case of peak 4, dechlorination also occurred. The resulting photoproducts feature tricyclic heteroaromatic systems, and Figure 3-7 suggests that both photoproducts are more stable than LTG in simulated sunlight. Given the apparent similarity of the peak 4 and 7 photoproducts, their mutual exclusivity at different pH is surprising, and suggests that different photodegradation pathways were involved.

Peaks 1 and 7 both evidence the addition of an electron-donating group (OH^- or $R\text{-}NH_2$) to LTG's dichlorophenyl ring. In the case of peak 1, dechlorination also occurred. Each peak appeared only in solutions containing LTG^+, suggesting a similar reaction mechanism. Because the positive charge in LTG^+ is located on the triazine ring, one possibility is that photoexcitation and intersystem crossing to LTG^+'s excited triplet state caused the transfer of an electron from LTG^+'s dichlorophenyl ring to its triazine ring, facilitating the addition of electron-donating groups to LTG^+'s dichlorophenyl ring (Figure 3-8). Bilski et al. (2009) demonstrated that excited triplet state LTG forms upon irradiation [44], and addition reactions have been reported to occur at aromatic rings activated by single-electron oxidation [65] or electron withdrawing groups [66]. Alternatively, LTG^+'s protonated triazine ring might be directing photosubstitution reactions to adjacent (ortho) positions on the dichlorophenyl ring [67], but this mechanism might also be expected to produce the peak 4 photoproduct, which does not appear in solutions

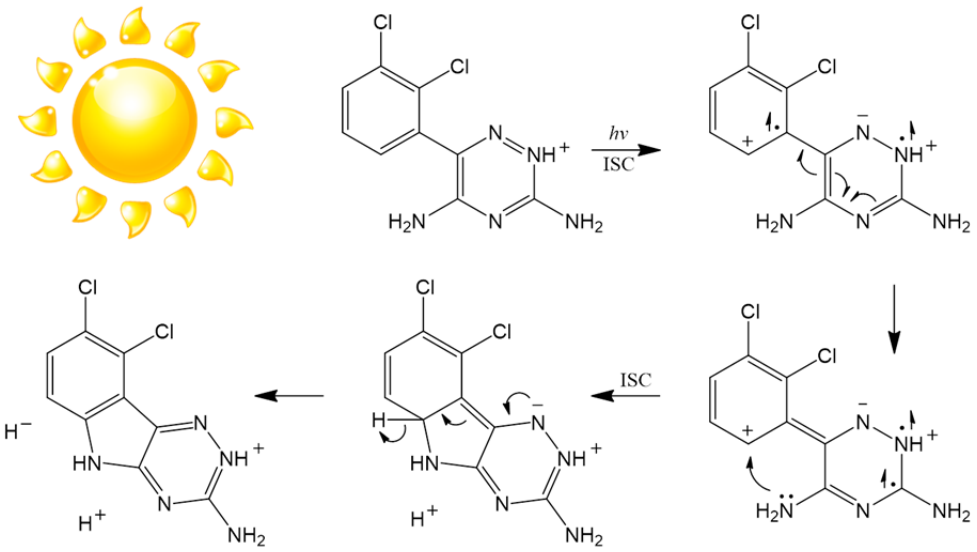

FIGURE 3-8. Proposed pathway to peak 7. Proposed excited triplet state photodegradation pathway from lamotrigine (LTG) to peak 7 (Figure 3-4; Table 3-2).

containing LTG^+. More work is needed to elucidate the mechanisms involved, and the results of such reactions in the presence of natural water constituents like DOM. However, it is significant that the peak 7 photoproduct forms without dechlorination, a common process among other chlorinated arenes [68-71].

Peak 4 appears to evidence electron transfer in the opposite direction, possibly due to the change in LTG's protonation state. Specifically, peak 4 appears to evidence the transfer of an electron from the 5-amino group in LTG's triazine ring to its dichlorophenyl ring, causing dechlorination, and the formation of a new pyrrole ring (Figure 3-9). Triclosan has been observed to undergo a similar reaction, resulting in the formation of a 1,4-dioxin ring [41, 72, 73]. In addition, Bilski et al. (2009) observed $O_2^{\bullet-}$ formation by electron transfer from LTG to molecular oxygen, and "strongly" accelerated Cl^- production in the presence of ascorbic acid (a reducing agent [74]) during LTG photodegradation in polar solvents [44]. Finally, protonation of the electron-donating amino group in 4-chloroaniline has been shown to inhibit heterolytic

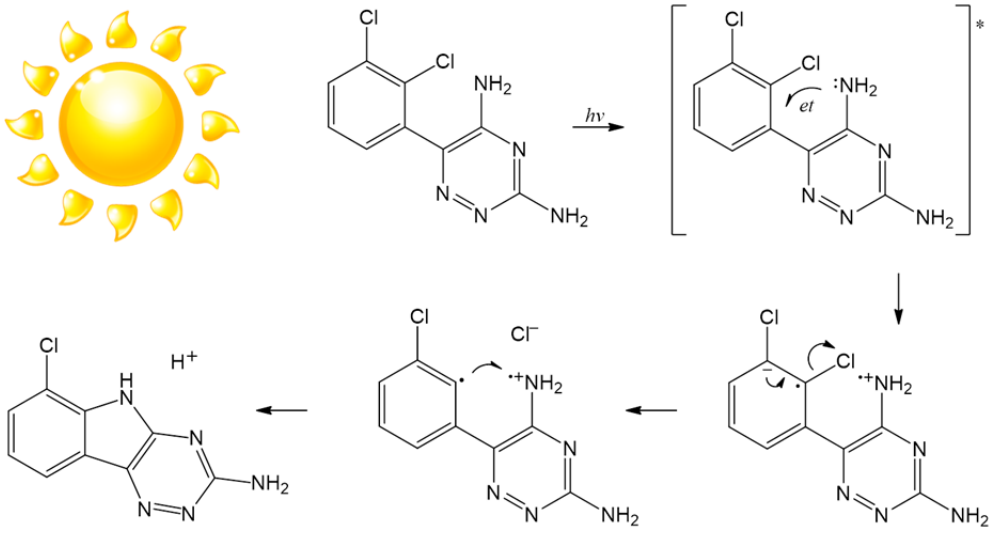

FIGURE 3-9. Proposed pathway to peak 4. Proposed electron transfer (*et*) photodegradation pathway from lamotrigine (LTG) to peak 4 (Figure 3-4; Table 3-2).

photodechlorination [70], and protonation of LTG's triazine ring did inhibit the formation of photoproduct peak 4 in this study. Additional work is needed to elucidate the mechanisms involved, and to explore the effect of such reactions in natural waters, where reducing agents comparable to ascorbic acid (e.g., redox active chromophores in DOM [30]) might enhance LTG's photodegradation through similar electron transfer reactions.

The results of this study suggest that photoinduced substitution and electron transfer reactions could be important pH-influenced photodegradation pathways in natural waters, particularly for LTG and similarly structured pharmaceuticals. Because LTG's neutral form will predominate in natural waters, the electron transfer process could be especially important.

Conclusions

To our knowledge, this is the first study to examine the direct photodegradation of LTG under environmentally relevant conditions, even though LTG has been detected in surface waters

at frequencies of 47 to 97% [13-15], found to be relatively persistent among 14 neuroactive pharmaceuticals during in-stream attenuation [16], and ranked highly across all toxicity endpoints when comparing the environmental risks of top-selling prescription drugs [24]. Three general findings from this study have special significance.

First, LTG undergoes direct photodegradation slowly and inefficiently under environmentally relevant conditions. Under the estimated midday, midsummer sun in Denver, CO, USA (latitude 39.8617 °N; SMARTS v 2.9.5), LTG's half-life was estimated to equal at least 230 ± 6 h of continuous sunlight, and LTG's reaction quantum yield was determined to be no greater than 3.3×10^{-4} mol einstein^{-1}. The reaction quantum yield of CBZ is similar ($\Phi \leq 1.3 \times 10^{-4}$ mol einstein^{-1}) [29, 53-58], and CBZ has been described as the most frequently detected and studied compound in North America and Europe, and third in Asia [1]. Little is known about the biodegradation and indirect photodegradation of LTG in natural waters, but the existing data suggest that LTG is approximately as recalcitrant as CBZ, and commonly present in surface waters like other pharmaceuticals in its therapeutic class.

Second, LTG appears in neutral and protonated forms in natural waters, and this has a significant effect on the direct photodegradation of LTG. Lamotrigine's specific light absorption rate was 12 times higher, and its reaction quantum yield was 13 times lower, at pH 7.7 versus pH 3.3. In addition, certain photoproducts appeared only when neutral LTG was present, and others appeared only when protonated LTG was present. This mutually exclusive behavior suggested different reaction mechanisms, even for photoproducts that appeared structurally similar.

Finally, the pH-dependent reactions observed in this study may be important to LTG's photodegradation in natural waters, and to the photodegradation of structurally similar

compounds. Lamotrigine's pH-dependent reactions suggested a potential for accelerated LTG photodegradation through photoinduced substitution reactions with π electron donors present in DOM, and photoinduced electron transfer reactions with redox active chromophores in DOM. These reactions are not novel [64], but they are not often considered as alternatives to photosensitization and reactive intermediate species (e.g., $^{\bullet}OH$, $^{1}O_2$, and $O_2^{\bullet-}$) in natural waters.

This study was intended to provide insight into the photodegradation of LTG and similarly structured compounds in natural waters. The results of this study will provide a benchmark for determining the influence of indirect photodegradation, light screening, and physical quenching on LTG's photodegradation, and might also help in prioritizing research based on photostability in natural waters.

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CHAPTER 4- DIRECT PHOTODEGRADATION OF ANDROSTENEDIONE AND TESTOSTERONE IN NATURAL SUNLIGHT: INHIBITION BY DISSOLVED ORGANIC MATTER AND REDUCTION OF ENDOCRINE DISRUPTING POTENTIAL[1]

Introduction

Among suspected endocrine disruptors, natural and synthetic exogenous steroid hormones generally have the highest affinities for binding to hormone receptors, and the highest potencies for disrupting normal hormone functions [1-4]. Most of the evidence for endocrine disruption in wildlife comes from studies on species living in, or closely associated with, aquatic environments [5, 6]. Reported effects include abnormal blood hormone levels, masculinization of females, feminization of males, altered sex ratios, intersexuality, and reduced fertility [7, 8].

Steroid hormones originate from many human and animal sources, including wastewater treatment plants, combined sewer overflows, septic systems, concentrated animal feeding operations, and agricultural fields where manure and biosolids are used as fertilizers [9-16]. Once present in surface waters, steroid hormones are subject to various transformation and removal processes, including biodegradation, sorption to colloids or sediments, and direct or indirect photodegradation [17-29].

The rate of direct photodegradation is a function of the intensity of available light, the steroid hormone's ability to absorb such light (i.e., molar absorptivity), and the efficiency at which the absorbed light is converted to photochemical reactions (i.e., reaction quantum yield)

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[1] Reproduced in part with permission from Young, R. B.; Latch, D. E.; Mawhinney, D. B.; Nguyen, T.-H.; Davis, J. C. C.; Borch, T., Direct photodegradation of androstenedione and testosterone in natural sunlight: Inhibition by dissolved organic matter and reduction of endocrine disrupting potential. Environmental Science & Technology 2013, 47, (15), 8416-8424. DOI: 10.1021/es401689j. © 2013 American Chemical Society.

[30]. In natural waters, the intensity of available light is attenuated through absorption by dissolved organic matter (DOM), which generally increases with decreasing wavelength [30]. In addition, DOM can enhance photodegradation, as evidenced by the indirect photodegradation of certain estrogens, or inhibit photodegradation by scavenging or reducing reactive intermediates [31-39]. Due to extremely fast decay from higher excited states, reaction quantum yields are generally assumed to be wavelength-independent, with some notable exceptions (e.g., for molecules with multiple, independent chromophores) [30, 40-43]. Accordingly, reaction quantum yields can be used to predict photodegradation rates across different lighting conditions [30, 40].

Two of the most commonly observed androgenic steroid hormones in surface waters are androstenedione (AD) and testosterone (T). Testosterone has a higher affinity for binding to androgen receptors, and a higher anabolic activity, but AD is often detected in surface waters at higher concentrations (e.g., up to 99 ng L^{-1}) [4, 44-50]. AD and T both absorb light in the wavelength range associated with surface waters (290-700 nm), albeit with the low molar absorptivities associated with n,π* transitions (the excitation of nonbonding electrons from heteroatoms), rather than the higher molar absorptivities associated with π,π* transitions (the excitation of bonding electrons from double bonds, which requires shorter wavelengths) (Figure 4-1) . AD and T are expected to undergo similar photochemical reactions due to the conjugated ketone (α,β-enone) chromophore common to their A-rings. In addition, AD is expected to undergo unique photochemical reactions due to the ketone chromophore in its D-ring [51].

The photochemistry of cyclic ketones and cyclic α,β-enones has been studied extensively under laboratory conditions, but seldom from the environmental perspective (i.e., in solar radiation with water as solvent and molecular oxygen present). Due to extremely fast decay from higher excited states, photochemical reactions in solution generally occur from a chromophore's lowest excited singlet or triplet state [40, 42]. Although exceptions have been observed, excited singlet states of ketones and α,β-enones usually undergo rapid intersystem crossing to longer-lived, triplet states [42, 52, 53]. Based on the foregoing, in natural sunlight, AD and T are expected to form photoproducts characteristic of the lowest excited triplet states of cyclic ketones and cyclic α,β-enones, including various rearrangement and hydration photoproducts [54-59] (Table 4-1).

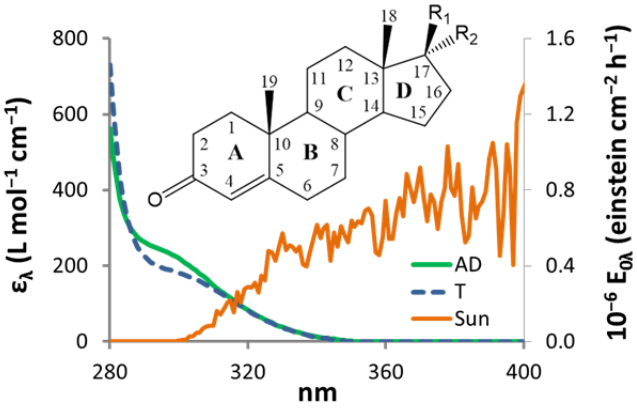
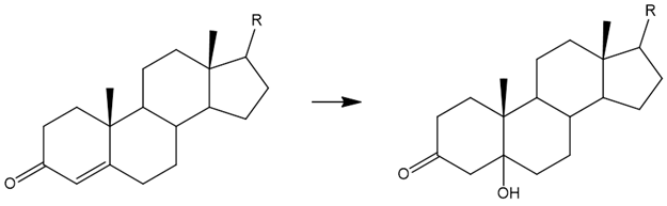
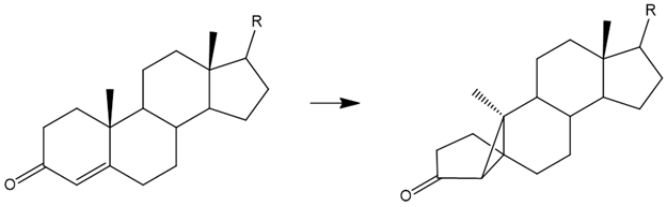
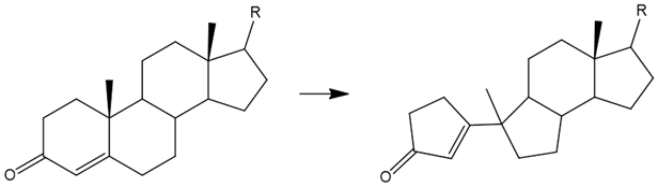
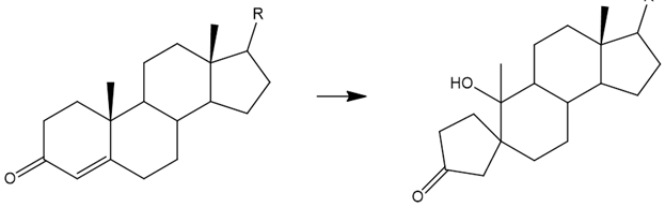
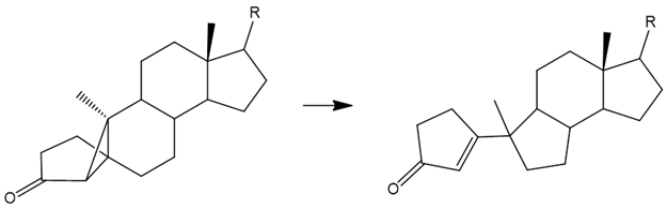
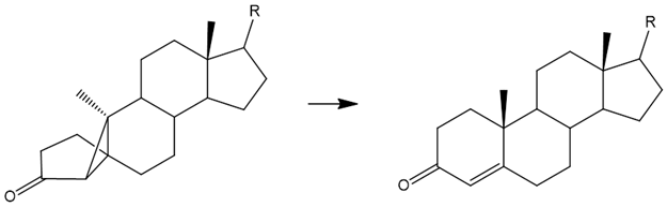
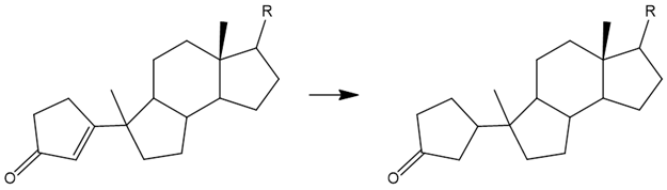
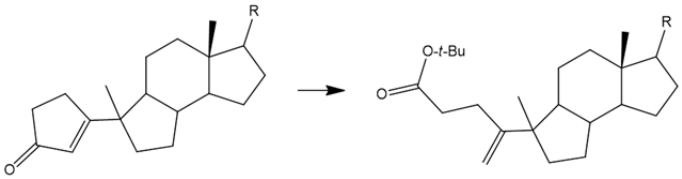
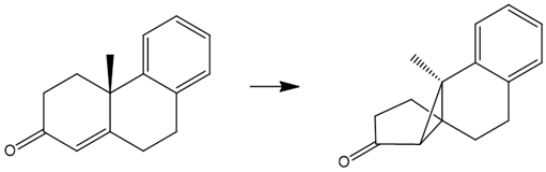


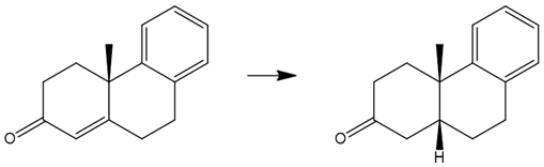
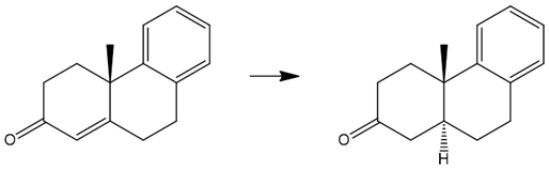
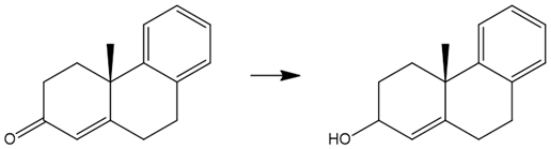
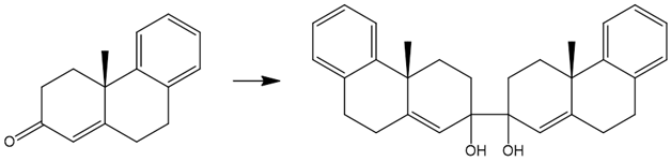
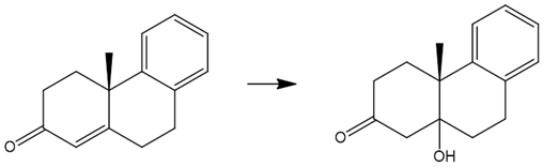
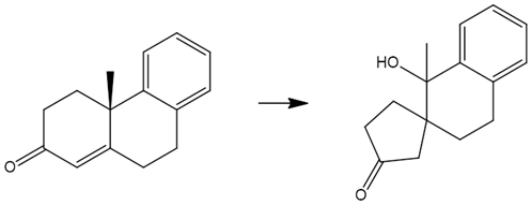
FIGURE 4-1. Molecular structure, absorption and irradiance data of androstenedione (AD) and testosterone (T). Steroid structure and molar absorptivity of AD (R_1, R_2: =O) and T (R_1: β−OH; R_2: H) versus estimated solar irradiance at 12:00 PM (PST), May 16, 2012, Henderson, NV, USA (SMARTS v.2.9.5).

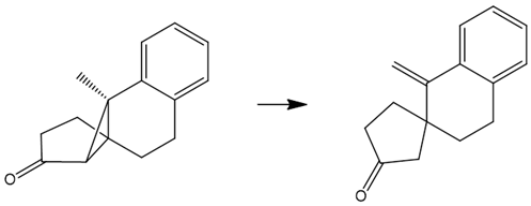
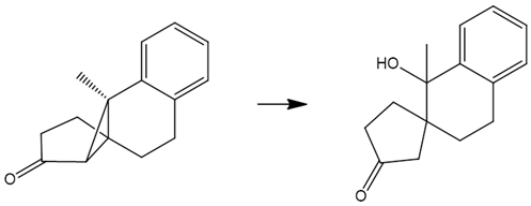
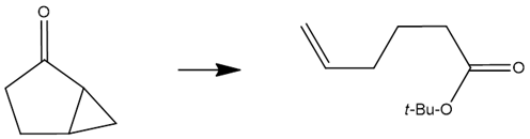
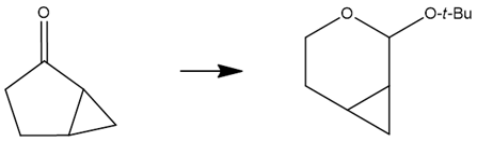
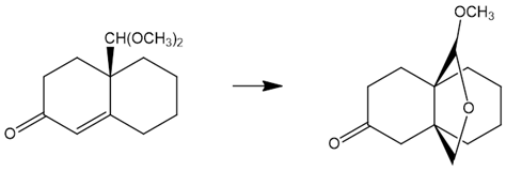
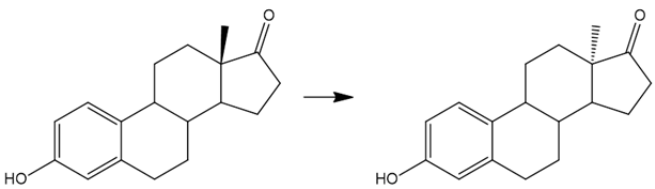
In one study in dilute aqueous and 20% methanol: 80% water (v:v) solutions, 5-hydroxy photoproducts were reported to form when AD and T were exposed to 254 nm monochromatic light [60] (Table 4-1).

TABLE 4-1. Previously identified photoproducts of cyclic ketones and cyclic α,β-enones. Previously identified photoproducts of androstenedione (AD), testosterone (T), and similarly structured compounds, including references (Ref.), wavelength (λ), solvent, and excited state precursor (ND = not determined; T1 = lowest excited triplet state; T2 = second lowest excited triplet state; S2 = second lowest excited singlet state).

	Previously Identified Photoproducts	λ (nm)	Solvent	Excited State	Ref.
P1	 R: =O, β-OH 5-OH hydration product	254	80% H_2O, 20% MeOH	ND	[60]
P2	 R: β-OH [27]; R: β-OAc [55, 58] lumiketone photoproduct	254, 313	H_2O	ND	[27]
		> 327	*t*-BuOH	T_1 (π,π^*)	[58]
		Hg, Pyrex	*t*-BuOH	ND	[55]
P3	 R: β-OH [27]; R: β-OAc [55, 58] cyclopentenone photoproduct	254, 313	H_2O	ND	[27, 61]
		> 327	*t*-BuOH	T_1 (π,π^*)	[58]
		Hg, Pyrex	*t*-BuOH	ND	[55]
P4	 R: β-OH spiro-hydration product	254, 313	H_2O	ND	[27]

	Previously Identified Photoproducts	λ (nm)	Solvent	Excited State	Ref.
	 R: β-OAc	> 280	*t*-BuOH	ND	[58]
	 R: β-OAc parent	> 280	*t*-BuOH	ND	[58]
P5	 R: β-OAc	> 327	*t*-BuOH	ND	[58]
P6	 R: β-OAc	> 280	*t*-BuOH	ND	[58]
P7		350	*i*-PrOH	T_1 (π,π^*)	[56]
		> 290	75% HOAc; *t*-BuOH	ND; T_1	[57]

	Previously Identified Photoproducts	λ (nm)	Solvent	Excited State	Ref.
P8		350	*i*-PrOH	T_1 (π,π^*)	[56]
P9		350	*i*-PrOH	T_2 (n,π^*)	[56]
P10		350	*i*-PrOH	T_2 (n,π^*)	[56]
P11		350	*i*-PrOH	T_2 (n,π^*)	[56]
P12		> 290	75% HOAc	ND	[57]
P13		> 290	75% HOAc	ND	[57]

	Previously Identified Photoproducts	λ (nm)	Solvent	Excited State	Ref.
P14		dark; > 290	75% HOAc; *t*-BuOH	ND	57
		dark	75% HOAc	ND	57
P15		> 250	*t*-BuOH	ND	62
P16		> 250	*t*-BuOH	ND	62
P17		254	*t*-BuOH	S_2 (π,π^*)	42, 53
P18		254	*t*-BuOH	S_2 (π,π^*)	42, 53
P19	 lumiestrone (13α-methyl epimer)	> 300	H_2O	ND	35

In another study, under more realistic environmental conditions, 5 mg L^{-1} T was reported to undergo photodegradation at 20 °C in phosphate-buffered aqueous solutions (pH 4, 7, and 8) and natural waters exposed to 254 or 313 nm monochromatic light [27, 61]. T's photodegradation rate was unchanged in the pH-adjusted and natural waters, suggesting that indirect photodegradation was insignificant. The reaction quantum yields of T at 254 nm (Φ_{254nm} = 0.225) and 313 nm (Φ_{313nm} = 0.0024) were determined using potassium ferrioxalate as a chemical actinometer, and T was estimated to transform slowly in natural sunlight, with estimated half-lives ranging from 6.4 days (summer) to 19.3 days (winter) at latitude 40°N. The same photoproducts, characteristic of the lowest excited triplet states of cyclic α,β-enones, were observed across all experimental conditions (Table 4-1).

When androgenic steroid hormones are present in surface waters, their endocrine disrupting potential can be assessed by measuring the androgenic activity, or androgenicity, of the impacted waters. For example, two companion studies in eastern Nebraska, USA, observed significant androgenic activity, and masculinization of female fish, at sites impacted by livestock feedlot effluent [10, 63].

To the best of the authors' knowledge, no study has examined the effect of photodegradation on the androgenicity of AD and T solutions. In a competitive binding assay involving the androgen receptor (AR) and 202 natural, synthetic and environmental chemicals, one study determined that the hydrophobic 5α-steroidal backbone, and the H-bonding ability of the 3-keto and 17β-OH functional groups, made substantial contributions to AR binding affinity [4]. Because AD and T are expected to undergo photochemical reactions initiated by the conjugated 3-keto group in their A-ring, these features could change, forming photoproducts that have lower AR binding affinities and androgenic activities.

This study sought to compare the photodegradation rates, reaction quantum yields, and photoproducts of environmentally relevant concentrations of AD and T in natural sunlight, and to evaluate, for the first time, the effect of solar photodegradation on the *in vitro* androgenicity of AD and T solutions. This study also sought to determine the effect of DOM on AD photodegradation. Results from this study will be useful for predicting the androgenic activity of sunlit surface waters impacted by AD and T, and for developing management strategies to minimize their endocrine disrupting potential.

Experimental

Chemicals and Solutions

Ultrapure deionized water (18.2MΩ·cm) was obtained from a Milli-Q reagent water purification system (Millipore, Bedford, MA, USA) fed by reverse osmosis (RO) treated water. Androstenedione and T were purchased from Sigma Aldrich (St. Louis, MO, USA). 4-nitroacetophenone (PNAP) was purchased from Alfa Aesar (Ward Hill, MA, USA). Suwannee River fulvic acid (SRFA), Suwannee River humic acid (SRHA), and Nordic Reservoir NOM (Nordic) standards were purchased from the International Humic Substances Society (IHSS) (St. Paul, MN, USA). Monobasic potassium phosphate (KH_2PO_4), dibasic potassium phosphate (K_2HPO_4), and 5N sodium hydroxide (NaOH) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Pyridine, methanol (MeOH), acetonitrile (MeCN), and methyl tertbutyl ether (MtBE) were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Formic acid was purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA), and ammonium acetate (NH_4OAc) and 2N hydrochloric acid (HCl) were purchased from

Mallinckrodt Baker (Phillipsburg, NJ, USA). All standards and reagents were of the highest purity commercially available.

Stock solutions of AD (9.31 mg L^{-1}) and T (8.44 mg L^{-1}) were prepared directly in deionized water, stirred overnight, filtered through a 0.2 μm surfactant-free cellulose acetate filter unit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and stored at 4 °C in 500 mL amber glass bottles until use. Stock solutions of SRFA (25 mg L^{-1}), SRHA (25 mg L^{-1}) and Nordic (25 mg L^{-1}) DOM were prepared in deionized water and stored at 4 °C in 500 mL amber glass bottles until use. A stock solution of PNAP (1,565 mg L^{-1}) was prepared in MeCN and stored at −10 °C in amber glass vials until use. YAS media were prepared as described in the literature [64, 65].

Phosphate buffer solutions (1 mM PO_4, ~3 mM ionic strength) were prepared by dissolving KH_2PO_4 or K_2HPO_4 into deionized water, and adjusting to pH 7.9-8.1 with 2N HCl or 5N NaOH.

Kinetic and Reaction Quantum Yield Experiments

Dilute aqueous solutions of AD (9.3 μg L^{-1}) and T (8.4 μg L^{-1}) were prepared in a phosphate buffer solution (pH 8) to emulate the slightly basic conditions common to surface waters in the western USA. In addition, aqueous solutions (pH 8) of Suwannee River fulvic acid (SRFA), Suwannee River humic acid (SRHA), and Nordic Reservoir NOM (Nordic) were prepared in a phosphate buffer solution and amended with AD (9.3 μg L^{-1}). The DOM solutions were diluted as necessary to match their absorbance at 320 nm (α_{320} = 0.14 cm^{-1}), a wavelength where AD and T absorb sunlight at approximately the maximum rate (Figures 4-1 and 4-2). Then, the dissolved organic carbon (DOC) concentrations of the optically-matched DOM

solutions were determined with a Shimadzu TOC-V total organic carbon analyzer using a six-point calibration curve. Figure 4-2 contains UV absorption spectra of the final DOM solutions.

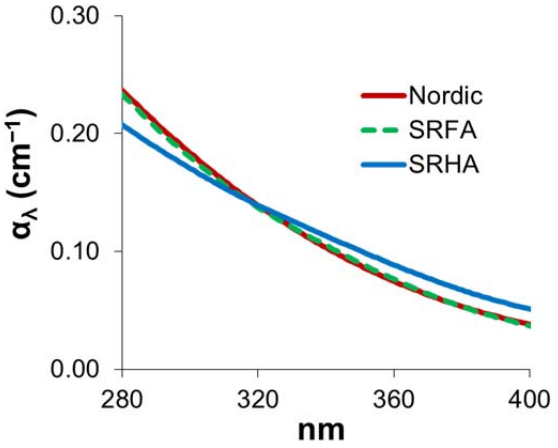


FIGURE 4-2. Absorption by dissolved organic matter solutions. Absorption coefficients of optically-matched (α_{320} = 0.14 cm^{-1}) Nordic NOM (15 mg L^{-1} DOC), Suwannee River fulvic acid (15 mg L^{-1} DOC), and Suwannee River humic acid (11 mg L^{-1} DOC) solutions.

In accordance with published methods for sunlight actinometry, aqueous solutions of PNAP (1.57 mg L^{-1}) were prepared in deionized water with pyridine concentrations of 0.25% and 0.75% to provide chemical actinometer solutions with different reaction quantum yields and half-lives [41]. The quantum yield of the PNAP actinometer solution is linear as a function of pyridine concentration (Φ_{PNAP} = 0.0169 [pyridine] up to 0.2 M pyridine in 10 μM PNAP), and generally stable from 25 to 43°C [41, 66]. However, the reaction between PNAP and pyridine has been reported to slow slightly with increasing temperature [66].

At various times over the four-month period from April to July, 2012, triplicate 7 mL AD or T samples (or duplicate 7 mL samples of AD in the presence of SRHA) and one or more of the chemical actinometer solutions were collected in quartz glass culture tubes (12 mm o.d. × 100 mm), sealed with Versa Vial™ PTFE/silicone closures (Supelco, Bellefonte, PA, USA), and exposed to natural sunlight in Henderson, NV, USA (latitude 36.04 °N) using a self-made rack angled 30° from the ground over a black wooden platform. For dark controls, 7 mL samples of each solution were collected in borosilicate glass culture tubes (13 mm o.d. × 100 mm), sealed

with PTFE-lined screw caps, covered with aluminum foil, and placed outdoors within 10 m of the self-made rack. 250 µL samples were collected from each culture tube over a total sunlight exposure period of 12 hours, spanning two days, and stored at 4 °C in 2 mL amber glass vials until analysis. Temperature was not controlled, and varied from a low of 19 °C to a high of 42 °C over the four-month experimental period.

Reaction Quantum Yield Calculations

The reaction quantum yields of AD and T were calculated using Equation I:

$$\phi_C = \phi_A \times \frac{k_{dC}}{k_{dA}} \times \frac{\sum I_{0\lambda}\varepsilon_{\lambda A}}{\sum I_{0\lambda}\varepsilon_{\lambda C}\, S_\lambda} \quad \text{(I)}$$

$$S_\lambda = \frac{(1 - 10^{-\alpha_\lambda l})}{2.303\ \alpha_\lambda l} \quad \text{(II)}$$

where: Φ_x is the contaminant's (x = C) or the actinometer's (x = A) reaction quantum yield (mol einstein^{-1}); k_{dx} is the first order rate constant for direct photodegradation of the contaminant (x = C) or the actinometer (x = A) (t^{-1}); $I_{0\lambda}$ is the irradiance at each incident wavelength λ (einstein L^{-1} t^{-1}); $\varepsilon_{\lambda x}$ is the contaminant's (x = C) or the actinometer's (x = A) molar absorptivity at each incident wavelength λ (L mol^{-1} cm^{-1}); S_λ is the light screening factor determined under Equation II; α_λ is the measured attenuation coefficient for the relevant DOM solution at each incident wavelength λ (cm^{-1}), and ℓ is the pathlength of the incident light (cm).

The procedure to account for light screening using Equation II is described in the literature [66, 67]. In deionized water, when no light screening occurs, the value of S_λ is 1 [31]. The pathlength (l) of the quartz glass culture tubes (12 mm o.d. × 100 mm) was assumed to be 1.0 cm in accordance with published literature values [66]. A longer path length, due to reflection or refraction in the cylindrical quartz glass culture tube, would slightly decrease S_λ, and increase the calculated reaction quantum yields. For example, increasing the path length from 1.0 to 1.5 cm

would decrease $S_{295\text{-}350}$ in the SRHA solution by approximately 7%, and increase the quantum yield calculated for the SRHA solution by the same amount.

Reaction quantum yields are generally assumed to be wavelength-independent [30, 41, 42]. Due to fast radiationless decay from higher vibrational and electronic states, luminescence and photochemical reactions in solution generally occur from vibrationally relaxed chromophores in their lowest excited electronic states [42]. In the case of luminescence, this generalization has been formalized: (a) in solution, emission occurs from the lowest excited singlet or triplet state, independent of the initial excited state (Kasha's Rule); and (b) the fluorescence quantum yield is independent of the excitation wavelength (Vavilov's Rule). These rules have been corroborated experimentally for photochemical reactions in solution, including the photodegradation of 17β-estradiol and 17α-ethinylestradiol at 254 and > 285 nm [42, 68]. Nevertheless, several exceptions to wavelength-independent reaction quantum yields exist, including molecules with multiple chromophores or non-equilibrating conformers [42, 43]. In Equation I, the reaction quantum yields of the contaminant and the actinometer are assumed to be constant, and represent either wavelength-independent reaction quantum yields or average values over all incident wavelengths. In the case of PNAP, Φ_{PNAP} at 313 and 366 nm were reported to be equal [41].

Equation I assumes that the ratio of light absorbed by the chemical and the actinometer is constant over changes in seasons, latitudes and sky conditions [41]. This ratio will not change with intensity, but is likely to change with the spectral distribution of incident light. In this study, the SMARTS (v.2.9.5) spectral radiation model was used to produce the solar irradiance values used in Equation I. Under the SMARTS model, direct beam irradiance is calculated using spectral transmittance functions for the main extinction processes in a cloudless atmosphere: Rayleigh scattering, aerosol extinction, and absorption by ozone, uniformly mixed gases, water vapor, and

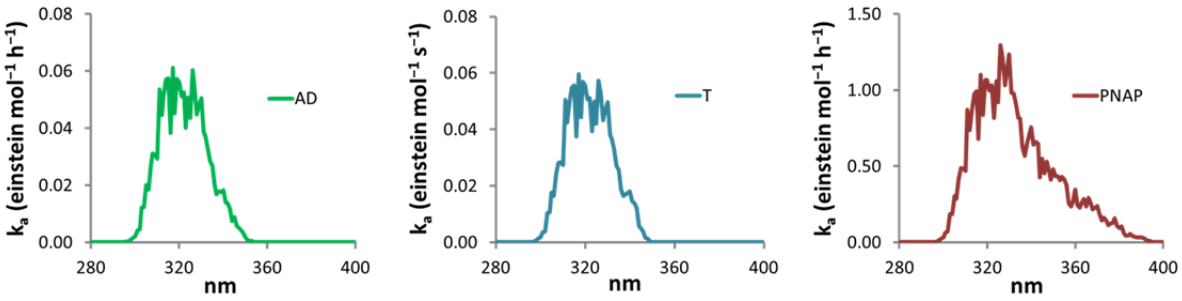


FIGURE 4-3. Comparison of specific absorption rates. Estimated specific absorption rates of androstenedione (AD, left panel), testosterone (T, center panel), and 4-nitroacetophenone (PNAP, right panel) in deionized water at 12:00 PM (PST) on May 16, 2012 at Henderson, NV, USA (SMARTS v.2.9.5).

nitrogen dioxide [69]. Therefore, the SMARTS model assumes fair weather. Many of the model's parameters are customizable. This study used an "urban" aerosol model (Shettle and Fenn), airport visibility to estimate turbidity, and the U.S. standard atmosphere (1976) for most other parameters.

Figure 4-3 contains an example of specific absorption rates calculated with Equation III using solar irradiance values from the SMARTS (v.2.9.5) spectral radiation model and molar absorptivities determined with absorption spectra.

$$k_{a\lambda x} = 2.303\, I_{0\lambda}\, \varepsilon_{\lambda x}\, l\, S_{\lambda} \qquad \text{(III)}$$

where: $k_{a\lambda x}$ is the contaminant's (x = C) or the actinometer's (x = A) specific absorption rate at each incident wavelength λ (einstein mol^{-1} t^{-1}); $I_{0\lambda}$ is the irradiance at each incident wavelength λ (einstein L^{-1} t^{-1}); $\varepsilon_{\lambda x}$ is the contaminant's (x = C) or the actinometer's (x = A) molar absorptivity at each incident wavelength λ (L mol^{-1} cm^{-1}); l is the pathlength (cm); and S_{λ} is the light screening factor under Equation II.

Androgenicity and Photoproduct Experiments

Multiple 7 mL samples of the unbuffered aqueous stock solutions of AD (9.31 mg L^{-1}) and T (8.44 mg L^{-1}) were collected in quartz glass culture tubes, sealed with PTFE/silicone

closures, and exposed to natural sunlight in Henderson, NV using the self-made rack described above. At 6 to 8h intervals, one culture tube was removed from the rack, and its contents were split into three portions: two 1 mL samples and one 5 mL sample.

The 1 mL samples were stored at 4 °C in 2 mL amber glass vials until analysis. The 5 mL sample was concentrated to 1 mL by solid phase extraction, and stored at 4 °C in 2 mL amber glass vials until analysis. The first 1 mL sample was analyzed by a yeast-based androgen receptor assay, and the remaining samples, which reflected initial AD and T concentrations 1,000 and 5,000 times higher than those used in the kinetic and reaction quantum yield experiments, were analyzed for photoproducts. To estimate photodegradation rates during the androgenicity and photoproduct experiments, a 100 μL aliquot was taken from the second 1 mL sample at each time interval and diluted 1,000 times with deionized water for quantification.

Yeast-Based Androgen Receptor Assay

Studies of photoproduct solution androgenicity were conducted using the yeast androgenicity screen (YAS) in an analogous fashion to the way in which the yeast estrogen screen (YES) was recently used to determine estrogenic activity of steroid estrogen photoproducts [35]. The YAS bioassay procedure followed that described in the literature [64, 65]. In brief, small aliquots (10 μL) of photolysis solutions taken at various irradiation times were mixed with 190 μL solutions of YAS media (OD_{620} = 0.1) in round-bottom 96-well plates. Following a 24 h incubation period at 30 °C, 50 μL of a cycloheximide (17 μM)/chlorophenol red galactopyranoside (CPRG, 10 ppm) solution was added to stop growth and to visualize β-galactosidase production. Following an additional 24 h incubation at 30 °C, the plates were centrifuged to pellet cells, and 150 μL aliquots of supernatant were transferred to flat-bottom 96-

well plates for analysis. A plate reader (BioTek Synergy HT) was used to measure absorbance values at 575 nm. Standards of AD and T were analyzed alongside the photolysis solutions. Photolysis solutions were diluted in such a way that at time = 0 h, the concentrations of AD and T were situated at the high end of the steep portion of the YAS activity curve. YAS standard curves can be viewed in Figure 4-4.

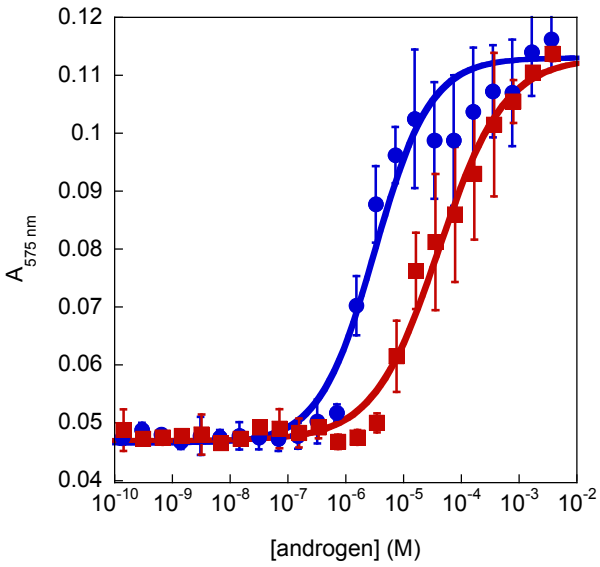


FIGURE 4-4. YAS standard curves. Results of the YAS bioassay using standard solutions of T (circles) and AD (squares).

Solid Phase Extraction

The 5 mL photoproduct sample was concentrated by solid phase extraction using a Supelco Visiprep 12-position vacuum manifold (Sigma Aldrich, St. Louis, MO, USA) and Oasis HLB SPE cartridges (6 cc, 200 mg, Waters Corporation, USA). The SPE cartridges were conditioned with 5 to 10 mL of 10% MeOH in MtBE, followed by 3 mL of MeOH. After the SPE cartridges were equilibrated with 3 mL of deionized water, the sample solutions were loaded onto the cartridges, and 6 mL of 10% MeOH in MtBE was drawn through the cartridges to elute the samples. The eluates were evaporated to 500 µL, diluted to 1 mL with deionized water, and stored at 4°C in 2 mL amber glass vials until analysis.

Analytical Methods

Solution pH was measured with an Accumet AP62 portable pH meter (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Absorption spectra were measured by a PerkinElmer Lambda 45 dual beam spectrophotometer using a 5 cm quartz cell, a 1 nm slit width, and a 120 nm min^{-1} scan rate (PerkinElmer Inc., Waltham, MA, USA). To eliminate solvent effects, all absorption spectra were measured in deionized water except PNAP (0.2% ACN in deionized water).

Androstenedione and T were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Agilent 1200 series HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) interfaced to an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an Applied Biosystems API 4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA). The mass spectrometer was operated in positive electrospray ionization (ESI^+) mode under the following parameters: 550°C temperature, 71V declustering potential, 10V entrance potential, 5500V ion spray voltage, 60 psig ion source gas 1, 50 psig ion source gas 2, 19 psig curtain gas, and 9 psig collision gas. The injection volume of each sample was 10 µL. The chromatographic method used a Kinetex C18 column (100 × 3.1 mm i.d., 2.6 µm particle size, Phenomenex, Torrance, CA), maintained at 50°C, and the following binary gradient: 90% 2.5 mM NH_4OAc in deionized water (A) and 10% methanol (B) for 0.5 minutes at 400 µL min^{-1}; stepped to 50% B at 0.51 minutes and increased to 55% B over 27 minutes at 400 µL min^{-1}; stepped to 100% B at 27.51 minutes and held for 7.5 minutes at 500 µL min^{-1} to flush the column; and equilibrated at 90% A for 5 minutes at 500 µL min^{-1}. Samples were quantified by multiple reaction monitoring (MRM) using a nine-point external calibration curve and the

following precursor/product ion transitions: AD (*m/z* 287 → *m/z* 97, 109 and 79) and T (*m/z* 289 → *m/z* 97, 109 and 79).

PNAP was quantified by liquid chromatography-UV diode array detection (HPLC-UV DAD) using a Varian 212 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) interfaced to an HTC-PAL autosampler and a Varian ProStar 335 UV-diode array detector. The chromatographic method used a Kinetex PFP column (100 × 3.1 mm i.d., 2.6 μm particle size, Phenomenex, Torrance, CA), maintained at room temperature, and the following binary gradient: 50% formic acid in deionized water (0.05%) (A) and 50% methanol (B) for 0.5 min at 300 μL min^{-1}; increased to 80% B over 6.5 minutes at 300 μL min^{-1}; stepped to 100% B and held for 3 minutes at 400 μL min^{-1} to flush the column; and equilibrated at 50% B for 4 minutes at 300 μL min^{-1}. The injection volume of each sample was 20 μL, and samples were quantified at 288 nm (PNAP) using an eight-point external calibration curve.

Photoproducts were analyzed by liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF MS) using an Agilent 1200 series HPLC system interfaced to an HTC-PAL autosampler and an Agilent 6520 quadrupole time-of-flight mass spectrometer. The mass spectrometer was operated in ESI^+ and ESI^- modes under the following parameters: 350°C temperature; 4500V (ESI^+) or 4000V (ESI^-) capillary voltage; 150V fragmentor voltage; 60V skimmer voltage; 750 Vpp Oct 1 RF; 25 psig nebulizer pressure; and 5 L min^{-1} drying gas (nitrogen) flow. The injection volume of each sample was 20 μL. The chromatographic method used the Kinetex C-18 column, maintained at room temperature, and the following binary gradient: 10% B for 0.5 minutes at 300 μL min^{-1}; increased to 100% B over 40 minutes at 300 μL min^{-1}; held at 100% B for 5 minutes at 300 μL min^{-1} to flush the column; and equilibrated at

10% B for 15 minutes at 300 μL min^{-1}. During MS/MS experiments, product ions were collected at 15 eV (low), 30 eV (medium), or 50 eV (high) collision energies.

Data Processing

All least squares linear regression analyses, sample means and confidence intervals were calculated using Microsoft Office Excel 2007 for Windows (Microsoft, Inc., Redmond, WA, USA). Agilent MassHunter Workstation Qualitative Analysis software (v. B.01.03) was used for all LC-QTOF MS data processing.

Results and Discussion

Photodegradation Rates and Reaction Quantum Yields

Photodegradation rates vary with the intensity of available sunlight, which changes with season, location, time of day, weather, and other environmental factors [30]. The following section describes experiments to compare reaction quantum yields and photodegradation rates for environmentally relevant concentrations of AD and T in Henderson, NV, USA (latitude 36.04 °N) during spring and summer, 2012. In the case of AD, these experiments were performed in the presence and absence of DOM.

Solar photodegradation rates were determined by exposing aqueous solutions of AD (9.3 μg L^{-1}) and T (8.4 μg L^{-1}), and optically matched SRFA (15 mg L^{-1} DOC), SRHA (11 mg L^{-1} DOC), and Nordic (15 mg L^{-1} DOC) solutions amended with AD (9.3 μg L^{-1}), to natural sunlight during the four-month period from April to July, 2012. AD and T underwent direct photodegradation, with half-lives ranging from 3.7 h (AD, June 29) to 10.8 h (T, April 6) (Table 4-2). Like T and 17β-trenbolone in previous studies, AD's photodegradation was not

TABLE 4-2. Photodegradation of androstenedione (AD) and testosterone (T). First order degradation rate constants, half-lives, and reaction quantum yields, with 95% confidence intervals (α = 0.5), of phosphate buffered aqueous, and optically-matched (α_{320} = 0.14 cm−1) Suwannee River fulvic acid (SRFA), Suwannee River humic acid (SRHA), and Nordic Reservoir NOM (Nordic), solutions (pH 8) of AD (9.3 µg L−1, n = 3) or T (8.4 µg L−1, n = 3) after exposure to sunlight in Henderson, NV, USA (latitude 36.04 °N).

Exposure Dates			**Daily Temp (max.)**	**k (h^{-1})**	**$t_{½}$ (h)**	**Φ_{solar} [a] PNAP 0.25%**	**PNAP 0.75%**
AD	5/16, 5/18	DI Water	38 °C, 32 °C	0.155 (±0.006)	4.5 (±0.2)	–	0.071 (±0.002)
	5/26, 5/27	DI Water	24 °C, 28 °C	0.137 (±0.004)	5.0 (±0.1)	0.070 (±0.004)	0.075 (±0.003)
	6/29, 6/30	DI Water	41 °C, 41 °C	0.190 (±0.008)	3.7 (±0.2)	–	0.071 (±0.002)
		SRFA		0.146 (±0.009)	4.8 (±0.3)	–	0.063 (±0.003)
		SRHA		0.139 (±0.008)	5.0 (±0.3)	–	0.061 (±0.002)
		Nordic		0.168 (±0.008)	4.1 (±0.2)	–	0.073 (±0.002)
T	4/6, 4/7	DI Water	19 °C, 24 °C	0.064 (±0.004)	10.8 (±0.6)	0.048 (±0.003)	0.049 (±0.003)
	5/4, 5/5	DI Water	30 °C, 29 °C	0.080 (±0.004)	8.7 (±0.4)	–	0.042 (±0.002)
	7/6, 7/7	DI Water	40 °C, 42 °C	0.091 (±0.002)	7.6 (±0.2)	–	0.0336 (±0.0009)

[a]4-nitroacetophenone (PNAP, 1.57 mg L^{-1}, 0.25 and 0.75% pyridine, n = 3) was used as a chemical actinometer, and AD's reaction quantum yield in the dissolved organic matter (DOM) solutions was calculated using screening factors to eliminate DOM's inner filter effect. Temperature data are from the U.S. National Weather Service (www.nws.noaa.gov).

enhanced in the presence of DOM [27, 28]. In fact, AD's half-life increased in each DOM solution (SRFA, 24%; SRHA, 35%; and Nordic, 11%) (Figure 4-5).

The reaction quantum yields of AD and T in deionized water were determined using published methods for sunlight actinometry: AD, 0.072 ± 0.005 (α = 0.05); and T, 0.043 ± 0.005 (α = 0.05) [31, 41, 66, 67]. AD degraded 66% more efficiently than T, presumably because its D-ring

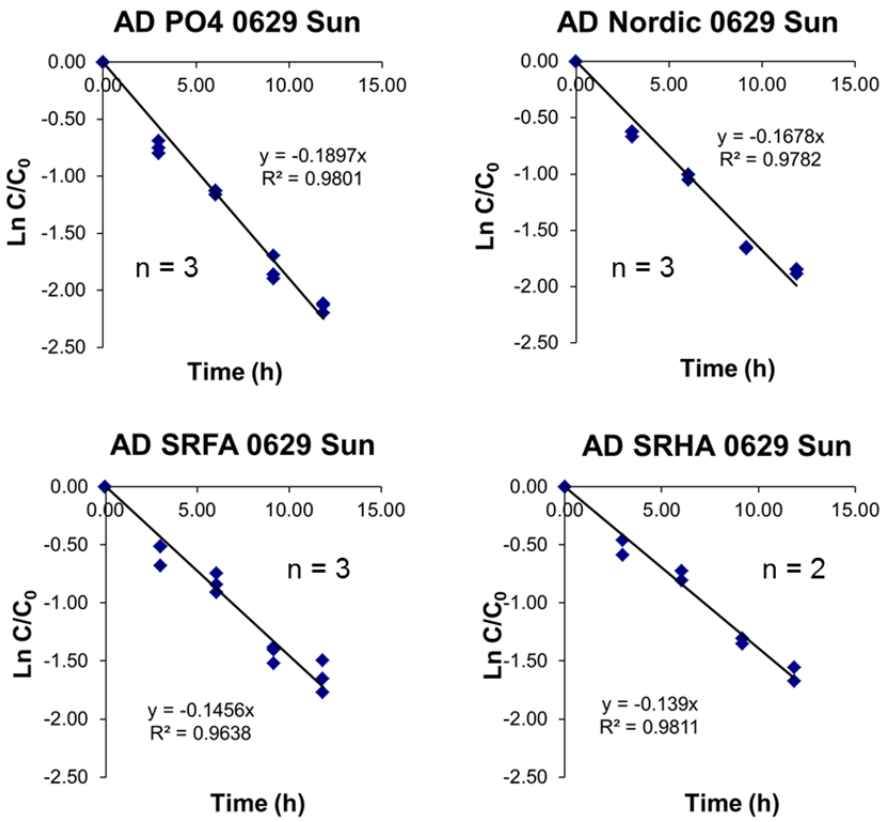


FIGURE 4-5. Photodegradation of androstenedione (AD) in the presence and absence of dissolved organic matter. Photochemical loss of AD (9.3 μg L^{-1}) in phosphate buffered (pH 8) solutions of deionized water (top left), Nordic Reservoir NOM (top right), Suwannee River fulvic acid (bottom left), and Suwannee River humic acid (bottom right) after 12h exposure to natural sunlight in Henderson, NV, USA (36.04°N) during June, 2012.

chromophore permitted simultaneous, independent photochemical reactions. Taken together, the reaction quantum yields were generally consistent across the range of observed temperatures (19 to 42 °C), but T's reaction quantum yield appeared to diminish with increasing temperature. This behavior is difficult to explain because AD and T are expected to undergo the same A-ring photochemistry.

T's reaction quantum yield in sunlight was approximately 18 times greater than the reaction quantum yield reported by Vulliet, et al. for 313 nm (Φ_{313nm} = 0.0024), even though sunlight and 313 nm are associated with the same electronic transition (n,π*) [27]. Different T concentrations were used (i.e., 5 mg L^{-1} vs. 8.4 μg L^{-1}), and self-screening and self-quenching

have been observed in comparable studies [39, 70]. Nevertheless, this behavior is unlikely to explain the magnitude of the difference between studies. Using the reaction quantum yield determined for T in sunlight (Φ_{solar} = 0.043 ± 0.005), T's molar absorptivity in Figure 4-1, the published method of Zepp and Cline, and midday, midspring values of Z_λ, the predicted half-life for T at latitude 40 °N was 13 ± 2 h [30]. This predicted half-life compared favorably with T's observed half-life (8.7 ± 0.4 h) on May 4 and 5 at latitude 36.04 °N (Table 4-2), and even more favorably after T's observed half-life was adjusted, using the ratio of observed to predicted actinometer photodegradation, to account for T's exposure to scattered light on all sides (12.4 ± 0.5 h) [41]. The similarity between the reaction quantum yields of AD and T in Table 4-2 also discounts the possibility that impurities could have enhanced T's photodegradation rate and reaction quantum yield in the sunlight experiments. These experiments demonstrate that T's reaction quantum yield in sunlight is greater than previously believed, meaning that T will degrade faster than expected in surface waters across all lighting conditions, subject to the influence of additional water constituents like DOM.

Light Screening and Physical Quenching by DOM

Photodegradation rates can be enhanced by reactions with DOM-derived intermediates (e.g., ^{3}DOM*, $^\bullet$OH and $^1O_2$), inhibited by DOM's inner filter effect, or inhibited by the physical or chemical quenching of photochemical intermediates [31-39]. These processes can be distinguished with light screening factors, which isolate the proportion of sunlight absorbed by DOM and eliminate DOM's inner filter effect from quantum yield calculations. An increase in AD's reaction quantum yield, relative to its reaction quantum yield in deionized water, would

signify the occurrence of indirect photodegradation with DOM-derived intermediates, and a decrease would signify the prevalence of quenching over indirect photodegradation.

After eliminating the influence of light screening, AD's reaction quantum yield in the Nordic solution (0.073 ± 0.002 mol einstein^{-1}) was not significantly different from that in deionized water (0.072 ± 0.005), and AD's reaction quantum yield in the SRFA (0.063 ± 0.003 mol einstein^{-1}) and SRHA (0.061 ± 0.002 mol einstein^{-1}) solutions was only slightly lower. These results suggest that light screening was primarily responsible for AD's increased half-life in the DOM solutions, and that any indirect photodegradation with DOM-derived intermediates was offset by quenching. Furthermore, AD's reduced reaction quantum yield in the SRFA and SRHA solutions suggests that physical quenching inhibited AD's direct photodegradation, apart from any quenching effect on indirect photodegradation.

DOM can physically quench direct photodegradation through energy and electron transfer processes, including nonreactive processes involving the formation of temporary charge-transfer complexes [34, 71]. Energy transfer is thermodynamically controlled, and occurs only when the donor's excited state energy equals or exceeds that of the acceptor [67, 71]. Because AD's direct photodegradation is believed to proceed from its lowest excited triplet state, a comparison of triplet state energies will determine if SRFA and SRHA can physically quench the direct photodegradation of AD. The energy of T's lowest excited triplet state has been estimated to exceed 290 kJ mol^{-1} [70], and AD's is expected to be similar because AD and T share the same A-ring chromophore. In comparison, DOM's predominant triplet state energy has been estimated to equal 250 kJ mol^{-1}, and the average triplet state energies of Armadale and Laurentian fulvic acids have been estimated to equal 170-180 kJ mol^{-1} [67, 72, 73]. These estimates suggest that SRFA and SRHA can quench AD's direct photodegradation through energy transfer. Alternatively,

nonreactive quenching has been observed to result from transitory interactions involving aromatic moieties and excited triplet states of ketones [74, 75]. Because AD contains two ketone chromophores, and SRFA and SRHA contain substantial proportions of aromatic carbon, nonreactive quenching is also plausible [76, 77].

The DOC concentrations (15 mg L^{-1}) and UV absorption spectra (280 to 400 nm) of the Nordic and SRFA solutions were almost identical, but SRFA inhibited AD photodegradation more (Figures 4-2 and 4-5). Similarly, after eliminating the influence of light screening, the effects of SRFA and SRHA on AD's reaction quantum yield were approximately the same, but their DOC concentrations were significantly different (15 vs. 11 mg L^{-1}). These results suggest that DOM's capacity for physical quenching, like its chemical and photochemical reactivity, can vary with DOM source [31, 78]. However, these observations are contingent on identical pH conditions (pH 8), and may not represent DOM's effect at other pH's. In particular, pH is known to influence DOM's optical and colloidal properties [79-81].

Photoproducts

When considering the ecological effects of AD and T in surface waters, the identities and androgenic activities of their transformation products must be considered [82]. The following section discusses the photoproducts of AD and T, and the photoinduced structural changes that are likely to affect their AR binding affinities.

A variety of photoproducts were detected when the AD and T sample solutions were analyzed by LC-QTOF MS, including rearrangement, hydration, and more highly oxidized photoproducts (Figure 4-6; Table 4-3). Because neutral water losses are common for androgenic steroids in positive ion electrospray mode, dimers and adducts were used to identify the most

likely molecular ion [83]. The absolute mass errors of the identified photoproducts, including their dimers and adducts, were generally less than 2 ppm.

Many of the T photoproducts have been previously identified (Table 4-1) [27, 55, 58, 61]. Because the common A-ring chromophore is expected to produce similar photoproducts, the AD and T sample solutions were examined for analogous photoproducts (i.e., T photoproduct = AD photoproduct + 2H). Using a revised chromatographic method with MeCN as the organic modifier, it was determined that Peak T-8 of the T sample actually comprised two photoproducts with identical molecular formulas and similar fragmentation patterns (i.e., the lumiketone and cyclopentenone products in Table 4-1) [61].

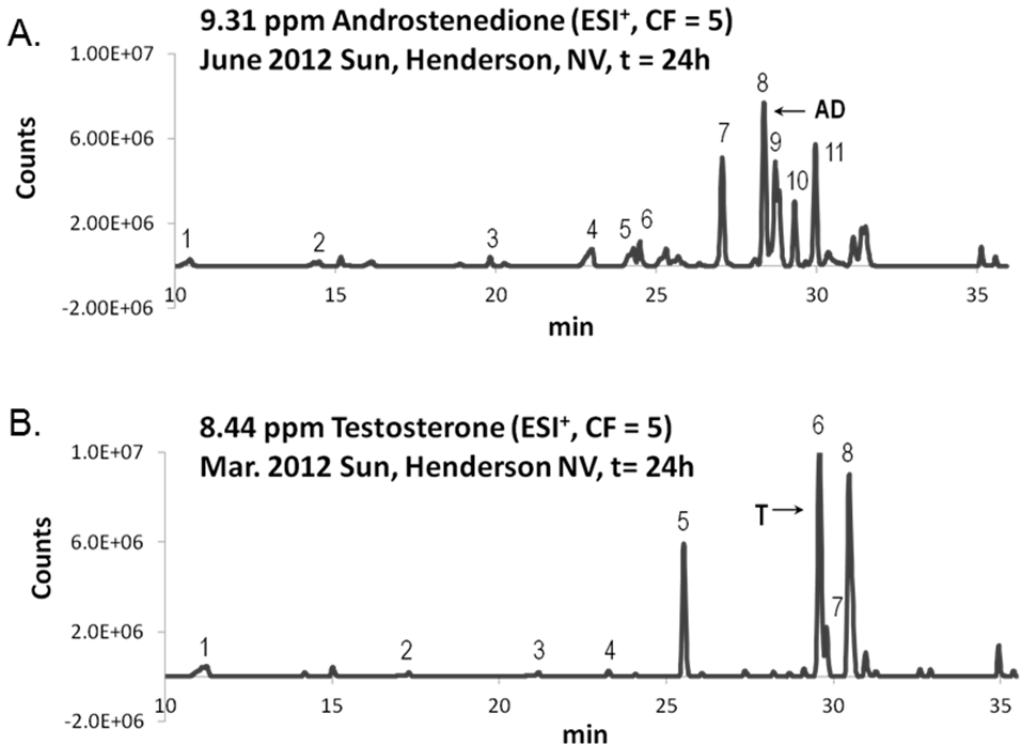


FIGURE 4-6. Ion chromatograms of androstenedione (AD), testosterone (T), and their photo-products. LC-ESI$^+$-QTOF MS total compound chromatograms for aqueous solutions of (A) AD (9.31 mg L^{-1}) and (B) T (8.44 mg L^{-1}) after 24h exposure to natural sunlight in Henderson, NV, USA (36.04°N). Each sample was concentrated 5 times by solid phase extraction (CF = concentration factor). Selected data for the numbered peaks are set forth in Table 4-3.

Several things stood out from the LC-QTOF analysis of the irradiated AD and T samples. First, AD produced more photoproducts than T, presumably because its D-ring chromophore generated independent photoproducts. Next, most of the T photoproducts had at least one, and sometimes two, analogues in the irradiated AD sample (e.g., T → one $C_{18}H_{28}O_5$ peak, and

TABLE 4-3. Proposed identities for androstenedione (AD) and testosterone (T) photo-products. Selected data for numbered peaks from LC-ESI+-TOF MS total compound chromatograms (Figure 4-6) for aqueous solutions of (A) AD (9.31 mg L−1) and (B) T (8.44 mg L−1) after 24h exposure to natural sunlight in Henderson, NV, USA (latitude 36.04 °N). The LK (lumiketone), CP (cyclopentenone) and SH (spiro-hydration) photo-products are illustrated in Table 4-1.

A. ID	**Peak**	**t_R (min)**	**Formula**	**Mass (amu)**	**Error (ppm)**	**Base Peak**
	A-1	10.4	$C_{18}H_{26}O_5$	322.1780	0.16	$(M+NH_4^+)$
	A-2	14.5	$C_{18}H_{24}O_4$	304.1674	0.05	$(M+H^+)$
	A-3	19.6	$C_{19}H_{26}O_4$	318.1832	0.19	$(M+H^+)$
	A-4	23.0	$C_{19}H_{30}O_4$	322.2147	0.62	$(M+NH_4^+)$
	A-5	24.1	$C_{19}H_{28}O_5$	336.1935	0.45	$(M+H^+-H_2O)$
	A-6	24.5	$C_{19}H_{28}O_3$	304.2040	0.64	$(M+NH_4^+)$
	A-7	27.1	$C_{19}H_{28}O_3$	304.2038	0.57	$(M+H^+)$
AD	A-8	28.4	$C_{19}H_{26}O_2$	286.1933	0.01	$(M+H^+)$
	A-9	28.7	$C_{19}H_{26}O_2$	286.1933	0.52	$(M+H^+)$
	A-10	29.3	$C_{19}H_{28}O_3$	304.2038	0.71	$(M+NH_4^+)$
	A-11	29.9	$C_{19}H_{26}O_2$	286.1933	0.72	$(M+H^+)$
B. ID	**Peak**	**t_R (min)**	**Formula**	**Mass (amu)**	**Error (ppm)**	**Base Peak**
	T-1	11.2	$C_{18}H_{28}O_5$	324.1937	0.13	$(M+NH_4^+)$
	T-2	17.3	$C_{18}H_{26}O_4$	306.1831	0.14	$(M+H^+)$
	T-3	21.2	$C_{19}H_{28}O_4$	320.1988	0.63	$(M+NH_4^+)$
	T-4	23.3	$C_{19}H_{30}O_5$	338.2090	0.91	$(M+H^+-H_2O)$
SH	T-5	25.5	$C_{19}H_{30}O_3$	306.2195	0.53	$(M+NH_4^+)$
T	T-6	29.6	$C_{19}H_{28}O_2$	288.2089	0.77	$(M+H^+)$
	T-7	29.8	$C_{19}H_{30}O_3$	306.2195	0.39	$(M+NH_4^+)$
LK, CP	T-8	30.5	$C_{19}H_{28}O_2$	288.2089	0.32	$(M+H^+)$

AD → two $C_{18}H_{26}O_5$ peaks, in Figure 4-7). Finally, while rearrangement and hydration photoproducts of polycyclic enones have been reported previously, the more highly oxidized photoproducts detected in this study (e.g., $C_{18}H_{28}O_5$ and $C_{18}H_{26}O_5$) have not, suggesting that more extensive photooxidation may occur under environmental conditions.

The A-rings of estrogens and androgens differ, but estrone shares the same D-ring chromophore as AD. Thus, estrone is a good model for highlighting the differences between AD and T photochemistry. In simulated sunlight in the absence of DOM, estrone's primary photoproduct was identified as lumiestrone, the 13α-methyl epimer of estrone [35, 37]. Cyclic ketones commonly form such photoproducts through ring cleavage adjacent to the ketone chromophore, stereochemical rearrangement, and subsequent ring closure (Table 4-1) [84]. Assuming that AD forms the analogous 13α-methyl epimer, then twin peaks in the irradiated AD sample (e.g., Figure 4-7) are likely to comprise 13α and 13β-methyl epimers of the corresponding T photoproduct, which does not undergo D-ring photochemistry or lose the original 13β-methyl configuration (Figure 4-1). Given the continued estrogenic activity of lumiestrone, and similar AR binding affinities between methyltestosterone and T, AD's

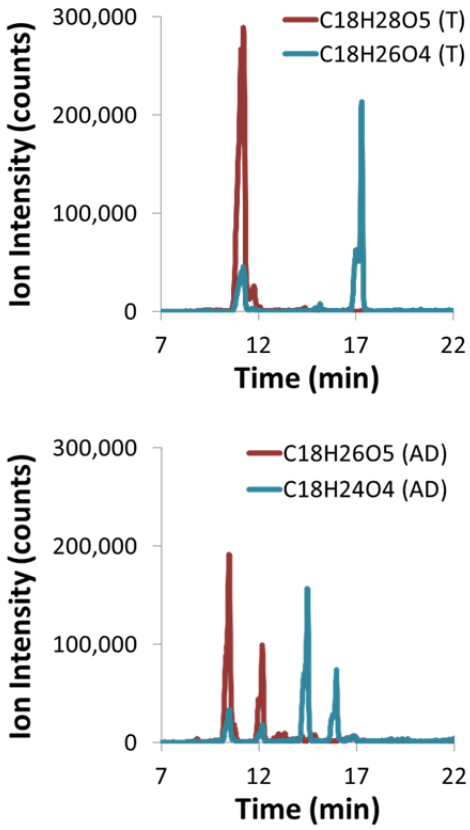


FIGURE 4-7. Comparison of ion chromatograms from selected photoproducts of androstenedione (AD) and testosterone (T). Extracted ion chromatograms (LC-ESI+-QTOF MS) of selected analogous photoproducts of T (top panel) and AD (bottom panel) after 24h exposure to natural sunlight in Henderson, NV, USA (latitude 36.04 °N).

13α-methyl epimer photoproducts are likely to exhibit androgenic activity similar to their 13β-methyl isomers [4, 35].

In negative ion electrospray mode, several of the more highly oxidized T photoproducts featured the loss of CO_2 during collision-induced fragmentation, implying the presence of a carboxyl group [85, 86] (Figure 4-8). Cyclic α,β-enones, like the A-ring chromophores of AD and T, rarely undergo ring cleavage, but their rearrangement photoproducts have been observed to undergo ring cleavage in *t*-butanol [58, 62, 87, 88] (Table 4-1). Therefore, the more highly oxidized photoproducts likely result from secondary reactions involving AD and T photoproducts. In fact, any photoproduct with a cyclic ketone or cyclic α,β-enone chromophore is a candidate for further photodegradation in natural sunlight. More work is needed to establish their molecular structures, but A-ring cleavage is likely to affect both the AR binding affinity and the androgenicity of these more highly oxidized photoproducts.

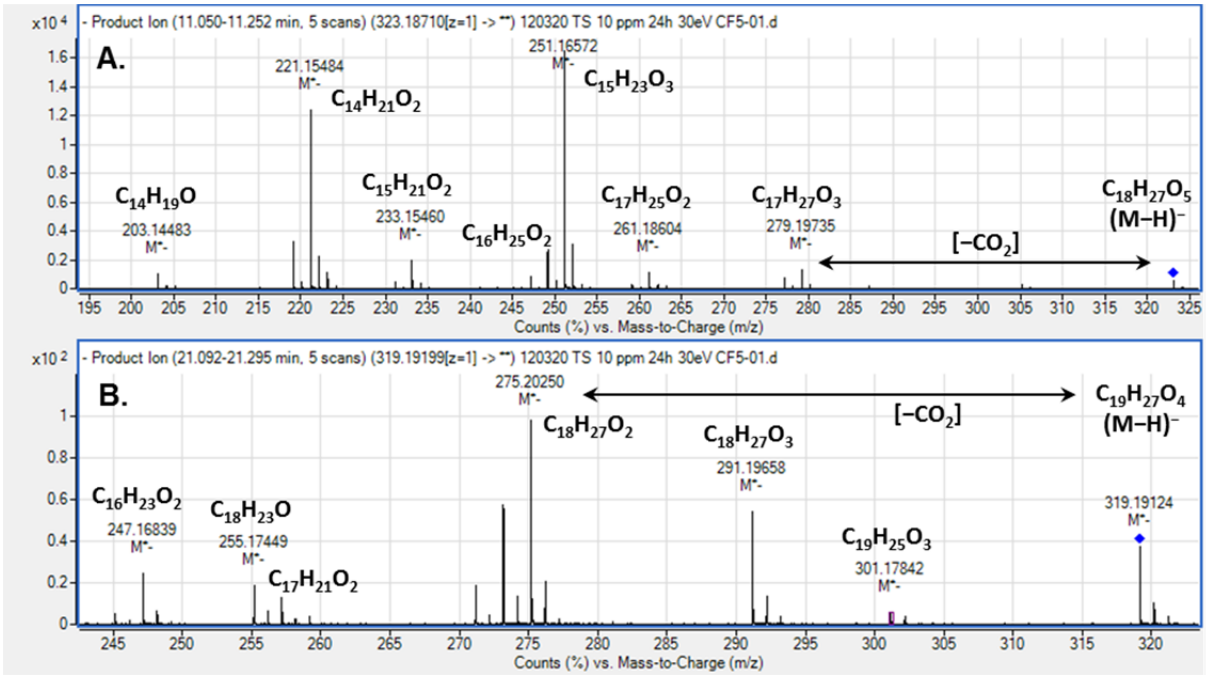


FIGURE 4-8. Product ion mass spectra of selected testosterone (T) photoproducts. LC-ESI⁻-QTOF MS product ion mass spectra at a collision energy of 30eV for (A) Peak T-1 ($C_{18}H_{28}O_5$) and (B) Peak T-3 ($C_{19}H_{28}O_4$).

In Vitro Androgenic Activity

Because degradation of a parent compound (or its initially formed products) does not necessarily lead to a concomitant reduction in biological activity, it is desirable to independently assess the androgenic activity of reaction product solutions. To that end, the following section describes the results of bioassay experiments conducted to determine whether photoproduct solutions retain the biological activity of AD and T.

In Figure 4-9, the YAS activities of photolysis solutions taken at various times during the direct solar photodegradation of AD (9.31 mg L^{-1}) and T (8.44 mg L^{-1}) were compared to their initial YAS responses (i.e. at t = 0 h), and to predicted YAS activities based on observed direct solar photodegradation rates (AD, 0.155 h^{-1}; T, 0.094 h^{-1}), initial androgen concentrations, and standard activity curves for AD and T (Figure 4-4). The rate of AD loss was 66% faster than T, exactly equal to the estimated difference between the reaction quantum yields of AD and T in sunlight. AD's observed YAS activities were generally at or slightly above AD's predicted YAS activities, and T's observed YAS activities were consistently below T's predicted YAS activities. Together, these results establish

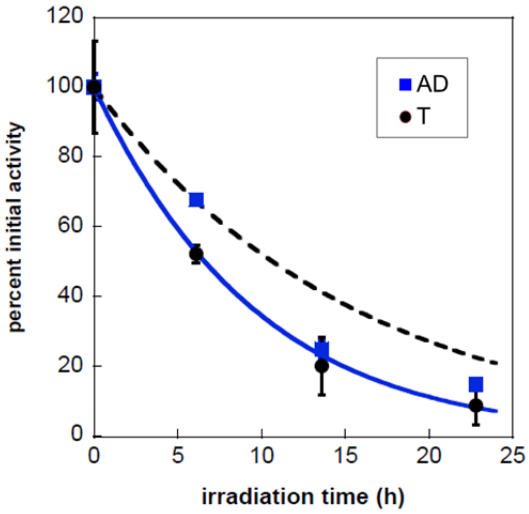


FIGURE 4-9. Reduction of initial YAS activity in androstenedione (AD) and testosterone (T) solutions. Percent of initial YAS activity of aqueous solutions of androstenedione (AD, 9.3 mg L^{-1}) and testosterone (T, 8.4 mg L^{-1}) after 23h exposure to natural sunlight in Henderson, NV, USA (latitude 36.04 °N) during July, 2011, and predicted YAS activities of AD (blue solid line) and T (black dashed line) based on observed direct solar photodegradation rates (AD, 0.155 h^{-1}; T, 0.094 h^{-1}), initial androgen concentrations, and standard activity curves for AD and T (Figure 4-4). Vertical error bars for standard errors of the means (n = 3) in the bottom panel may be obscured by the symbols used.

that the i*n vitro* androgenic activity of the AD and T photolysis solutions decreased approximately as fast as AD and T were removed.

As described in the discussion on photoproducts, three types of changes appear to have been initiated by the A-ring chromophores of AD and T: increased polarity, rearrangement of the steroid skeleton, and A-ring cleavage. Increased polarity would reduce the hydrophobic character of the 5α-steroidal backbone, which has the optimal octanol-water partition coefficient (log P) value for AR binding affinity [4]. In addition, skeletal rearrangement or A-ring cleavage would disrupt the structure of the steroidal backbone, which provides the ideal positional and spatial orientation to form 3-keto and 17β-OH anchors for AR binding [4]. Together, these changes could explain the reductions in YAS activity reflected in Figure 4-9.

Also as described in the discussion on photoproducts, the D-ring chromophore of AD appears to be producing a stereochemical rearrangement of the 13β-methyl group, analogous to the photochemical reaction of estrone to give lumiestrone [35]. Because the 5α-steroidal backbone is engaged in hydrophobic bonding, and 17α-methyl substitution (to form methyltestosterone) has almost no effect on T's relative binding affinity or androgenic activity, this change is not expected to affect the YAS activity of AD's photoproducts as much as the changes initiated by its A-ring chromophore [4]. Nevertheless, contrary to the case of lumiestrone, Figure 4-9 contains no clear evidence of continued YAS activity [35]. This may reflect the fact that most previously identified photoproducts of AD and T contain a cyclic ketone or cyclic α,β-enone chromophore, and are candidates for further solar photodegradation.

In summary, despite the formation of several photoproducts, the *in vitro* androgenic activity of the AD and T solutions decreased approximately as fast as AD and T were removed,

demonstrating that the major photoproducts of AD and T are inactive or rapidly degraded into inactive secondary products.

Environmental Relevance

In surface waters exposed to sunlight, AD and T have been shown to degrade by direct photolysis. DOM inhibited the direct photodegradation of AD, primarily by acting as an inner filter and, to a lesser extent, by physical quenching. Because photodegradation rates are a function of the intensity of available light, AD and T photodegradation will be most important when sunlight intensity is high (e.g., in clear weather during summer) and DOC concentrations are low.

The environmental importance of AD and T photodegradation will also be influenced by competing transformation and removal processes, including biodegradation and sorption to colloids or sediments, which are governed by different environmental parameters [19, 29]. For example, T's A-ring was mineralized at initial rates up to approximately 30% d^{-1} under aerobic batch conditions in stream sediments (30 ng T g^{-1} dry weight) located near wastewater treatment plant outfalls [24]. Even if the half-lives observed in this study ($t_{½}$ = 7.6 to 10.8 h) are adjusted to account for T's exposure to scattered light on all sides (e.g., increased by a factor of 2.2 in accordance with prior literature), it remains clear that direct photodegradation and biodegradation are competitive processes [41].

Relatively rapid photodegradation rates suggest that photodegradation can be incorporated into management practices to minimize the endocrine disrupting potential of AD and T (e.g., lagoons or treatment wetlands). The ideal configuration would feature large, shallow

(20-50 cm), open-water areas with good mixing and transient storage, and the required area would likely depend primarily on DOC concentration [89, 90].

Taken together, the results of this study show unambiguously that, subject to continuing inputs, direct solar photodegradation plays an important role in reducing the endocrine disrupting potential of surface waters impacted by AD and T. Given the complex nature of environmental photochemical processes, the extent of these solar reactions that lead to reduced biological activity will vary with environmental conditions.

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CHAPTER 5- CONCLUSIONS AND FUTURE WORK

The overarching goal of this dissertation was to examine the photodegradation of selected endocrine and pharmaceutically active chemicals under environmental relevant conditions. The selected micropollutants (androstenedione, testosterone, and lamotrigine) are potent, biologically active chemicals, and have a demonstrated presence in surface waters [1-9]. Each of the selected micropollutants also absorbs light from the photochemical spectrum in natural waters (290 to 700 nm), creating the potential for direct photodegradation.

Chapter 3 of the dissertation examined the direct photodegradation of lamotrigine (LTG) in simulated sunlight from pH 3.3, where LTG is 99.6% protonated, to pH 7.7 where LTG is 1% protonated. Lamotrigine is known to produce a phototoxic response in some patients, and its photochemical properties have been studied previously on that basis [10]. However, to the best of the author's knowledge, the research in this dissertation represents the first time that LTG's photochemistry has been studied in an environmental context.

Lamotrigine's half-life varied little (100 ± 3 to 112 ± 2 h) with solution pH, but its specific light absorption rate was 12 times higher, and its reaction quantum yield was 13 times lower, at pH 7.7 versus pH 3.3. Using these reaction quantum yields and a spectral radiation model (SMARTS v 2.9.5), LTG's estimated photodegradation rate in the estimated midday, midsummer sunlight of Denver, CO, USA (latitude 39.8617 °N) was more than twice as fast at pH 7.7 ($t_{½} = 230 \pm 6$ h) than pH 3.3 ($t_{½} = 500 \pm 20$ h), and substantially slower than the degradation rates observed in the solar simulator. Because the intensity of sunlight diminishes during other seasons and other times of day, LTG's average direct photodegradation rate in natural sunlight presumably would be even slower. Indirect photodegradation is common in natural waters [11], and could enhance LTG's photodegradation rate relative to the direct

photodegradation rates observed in this study. Accordingly, future work is planned to examine LTG's indirect photodegradation in the presence of dissolved organic matter (DOM) from various sources. Nevertheless, LTG's slow rate of direct photodegradation, and its low reaction quantum yield in comparison to other pharmaceutical micropollutants (e.g., e.g., naproxen, $\Phi = 0.012$) [12], might explain why LTG appears to be relatively persistent, and is detected in a high percentage of surface water samples (47 to 97%) [7-9]. In fact, LTG shares structural features with polychlorinated biphenyls (PCBs) and atrazine, which are well-known persistent organic pollutants. In addition, LTG has a reaction quantum yield comparable to carbamazepine, an antiepileptic drug that has been described as "the most frequently studied and detected compound in both North America and Europe and third in Asia" [13]. The results of this dissertation indicate that further research on the environmental fate of LTG is warranted, especially because LTG already has been identified as a high priority ecotoxicological risk [14].

The experiments in chapter 3 showed that solution pH affects the identities and relative abundances of LTG's photoproducts. Some photoproducts appeared only in solutions containing protonated LTG, and others appeared only in solutions containing neutral LTG. Different reaction mechanisms were proposed, and experiments to validate the proposed mechanisms are ongoing. Among other things, LTG's pH-dependent photochemical reactions suggested a potential for accelerated degradation in natural waters through photoinduced substitution reactions with π electron donors present in DOM, and through photoinduced electron transfer reactions with redox active chromophores in DOM.

Additional experiments are also planned to examine the photodegradation of lamotrigine-2-N-glucuronide (LTG-2NG), a metabolite of LTG that also has been detected in surface waters [7]. Because LTG-2NG is positively charged at the same location where LTG is

protonated [15, 16], its photodegradation is expected to follow the photodegradation of protonated LTG.

Chapter 4 of the dissertation examined the direct photodegradation of androstenedione (AD) and testosterone (T), two of the most commonly detected male sex hormones in surface waters, upon exposure to natural sunlight in Henderson, NV, USA (latitude 36.04 °N). In addition, chapter 4 sought to determine the effect of DOM on AD photodegradation. To the best of the author's knowledge, the research in this dissertation represents the first examination of AD's photochemistry under environmentally relevant conditions, and the first examination of the effect of photodegradation on male sex hormone endocrine disrupting potential.

Androstenedione and T underwent direct photodegradation, with half-lives ranging from 3.7 to 10.8 h. Androstenedione and T share many structural features, including a conjugated ketone chromophore that is likely to produce similar photochemical reactions in AD and T. Nevertheless, AD (ϕ_M = 0.072 ± 0.005 mol einstein^{-1}) degraded 66% more efficiently than T (ϕ_M = 0.043 ± 0.005 mol einstein^{-1}), presumably because AD contains a second ketone chromophore that permits independent photochemical reactions. Testosterone's reaction quantum yield was approximately 18 times greater than the reaction quantum yield previously reported for T during laboratory experiments with 313 nm monochromatic light (ϕ_M = 0.0024 mol einstein^{-1}) [17]. This implies that T will photodegrade in natural waters much faster than previously believed. The photodegradation rates of AD and T appear to be competitive with biodegradation rates in natural waters, given evidence that T undergoes ring cleavage from aerobic biodegradation at initial rates up to 30% d^{-1} in stream sediments (30 ng T g^{-1} dry weight) located near wastewater treatment plant outfalls [18].

In three model DOM solutions (Suwannee River fulvic acid, Suwannee River humic acid, and Nordic Lake natural organic matter), AD's half-life increased by 11 to 35%. Using screening factors to eliminate DOM's inner filter effect, reaction quantum yield calculations revealed that light screening was primarily responsible for AD's increased half-life, and suggested that physical quenching further inhibited AD's photodegradation in two out of the three DOM solutions (Suwannee River fulvic acid and Suwannee River humic acid). If any indirect photodegradation occurred in the AD solutions containing DOM, it was more than offset by the influence of screening and physical quenching.

Chapter 4's determination regarding physical quenching by DOM was not unprecedented (e.g., in the context of redox photochemistry) [19], but it was unusual considering that other steroid hormones have been reported to undergo enhanced photodegradation in the presence of DOM [20-22], and that reduced steroid photodegradation in the presence of DOM has been attributed to light screening [21]. However, physical quenching by DOM was plausible based on previously published reports of excited triplet state energies for T [23] and DOM [24-26], and reports of non-reactive quenching between aromatic moieties (e.g., in DOM) and excited triplet states of ketones (e.g., in AD) [27, 28]. A subsequent study did report the quenching of excited triplet states by DOM using four model triplet photosensitizers and a suite of DOM fractions from various sources [29]. However, that study also indicated that significant quenching (>10%) would not be expected to occur in aerated water at DOM concentrations below 22 to 72 mg C L^{-1}, unless ground-state complexes with DOM are present, because molecular oxygen competitively quenches excited triplet states. In chapter 4, physical quenching varied with DOM type (15 mg DOC L^{-1} Nordic NOM: 0%, 15 mg DOC L^{-1} Suwannee River fulvic acid: 11%; 11 mg DOC L^{-1} Suwannee River humic acid: 14%), but twice exceeded 10% at DOM

concentrations below 22 mg C L^{-1}. Because hydrophobic partitioning into organic carbon domains plays a dominant role in the sorption of T to soils and sediments [30], it is possible that DOM complexes contributed to the increased rates of physical quenching in this study, and future work is planned to explore the influence of DOM complexation on LTG photodegradation.

When the effect of photodegradation on the endocrine disrupting potential of AD and T was studied, the *in vitro* androgenic activity of the irradiated solutions decreased approximately as fast as AD and T were removed. Examination of the resulting photoproducts suggested that the reduced androgenic activity was related to steroid ring cleavage, and the formation of previously unreported, highly oxidized photoproducts.

The research in chapter 4 presented a simple technique for determining the relative influence of light screening and other photochemical effects of DOM, and suggested a rarely reported role for DOM during environmental photodegradation. The relatively rapid photodegradation rates for AD and T in natural sunlight, contrary to previous predictions, also suggested that photodegradation can be incorporated into management practices to minimize their endocrine disrupting potential (e.g., lagoons or treatment wetlands).

Overall, the results of this dissertation demonstrate that the effects of photodegradation can vary substantially between micropollutants, producing variable degradation rates and a wide variety of photoproducts. The results of this dissertation also confirm that different environmental conditions can have a significant effect on photodegradation processes. Because many pharmaceuticals and other micropollutants can occur in surface waters in more than one protonation or dissociation state, the results in chapter 3 demonstrate the importance of examining the influence of pH on photodegradation rates and the resulting photoproducts. The

results in chapter 4 add to the existing knowledge of DOM's influence on photodegradation in natural waters, and suggest that other DOM influences remain to be explored, including the effect of micropollutant complexation by DOM. The results of this study should assist in prioritizing micropollutant research, by indicating structural features that are relatively photostable, and by providing new insights into their photoproducts. Many of these photoproducts may already exist, unidentified and undetected, in surface waters.

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APPENDIX A

Degradation Kinetics of Testosterone by Manure-Borne Bacteria: Influence of Temperature, pH, Glucose Amendments, and Dissolved Oxygen

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Land application of manure may contribute endocrine disrupting compounds (EDCs) such as steroid hormones to the environment. Little attention has been paid to the potential for degradation of steroid hormones by manure-borne bacteria and their degradation kinetics and pathways. In a laboratory study, the potential for biodegradation of testosterone, 17β-estradiol (E2) and progesterone by swine (*Sus scrofa*) manure-borne bacteria was examined. In addition, the impact of temperature, pH (6, 7, and 7.5), glucose amendments (0, 3, and 22 mmol L^{-1}), and presence of oxygen on testosterone degradation kinetics was determined. Testosterone, 17β-estradiol and progesterone were biodegraded within 25 h of reaction initiation under aerobic conditions. The degradation of testosterone followed pseudo first-order and zero-order reaction kinetics under aerobic and anaerobic conditions, respectively, in tryptic soy broth (TSB) pre-enriched systems. The half-life ($t_{1/2}$) for the degradation of testosterone under anaerobic conditions was six times longer than aerobic conditions. Testosterone degradation was found to significantly increase (~ 17%) when incubated at 37°C vs. 22°C. The impact of pH ($t_{1/2}$ ranged from 4.4–4.9 h) and glucose amendments ($t_{1/2}$ ranged from 4.6–5.1 h) on the testosterone degradation rate were found to be small. Testosterone was transformed to dehydrotestosterone (DHT) (major degradation product), androstenedione (AD), and androstadienedione (ADD) under aerobic conditions as revealed by liquid chromatography-time-of-flight mass spectrometry (LC/TOF-MS). These results indicate that testosterone is rapidly degraded by manure-borne bacteria under a wide range of environmentally relevant conditions. However, the formed degradation products are still of potential concern due to their endocrine disrupting potential.

Environmental steroid hormones are endocrine-disrupting compounds (EDCs), which have the potential to adversely affect wildlife development and reproduction (Das et al., 2004; Vajda et al., 2008). The retention and removal of steroid hormones in the environment is expected to be largely the result of a combination of sorption and biotic and abiotic degradation. Biodegradation of steroid hormones has previously been studied in agricultural soils (Das et al., 2004), sewage sludge (Layton et al., 2000; Li et al., 2005), river water and sediments (Jürgens et al., 2002), and pure culture media (Shi et al., 2004; Yoshimoto et al., 2004).

Jürgens et al. (2002) showed that microorganisms in a river water sample were capable of transforming 17β-estradiol (E2) to estrone (E1) with a half-life ($t_{1/2}$) of 0.2 to 9 d, and degrading E1 with a half-life of 0.1 to 11 d. A Gram-positive bacterium (i.e., *Bacillus* sp.) isolated from soil was found to be capable of transforming progesterone to 6β- and 14α-hydroxyprogesterones, but no kinetic data were obtained (Das et al., 2002). Lee et al. (2003) measured testosterone first-order half-lives ($t_{1/2}$) in aerobic soil-water slurries that ranged from 0.3 to 7.3 d, and Casey et al. (2004) observed first-order testosterone degradation rate constants (k) of 0.4 to 0.6 h^{-1} in agricultural soils.

Several studies have examined the degradation of estrogens in soil and soil that has been amended with manure, and the impact of pH, carbon source, and temperature on the degradation kinetics (Li et al., 2008; Lucas and Jones, 2006; Raman et al., 2001). However, little work has been published on the degradation of androgenic steroid hormones by manure-borne microorganisms and the impact of environmental factors, such as temperature, moisture, pH, organic carbon and redox conditions, on the degradation kinetics. Jacobsen et al. (2005) investigated the impact of swine manure amendments to three different soil types on testosterone degradation at various temperatures. Under all conditions testosterone and its transformation products were dissipated within a few days. Addition of swine manure slurry to soil hastened the transformation of testosterone (4-androsten-17β-ol-3-one) to androstenedione (4-androsten-

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Abbreviations: AD, androstenedione; ADD, androstadienedione; CAFOs, concentrated animal feeding operations; DAD, diode array detector; DHT, dehydrotestosterone; E1, estrone; E2, 17β-estradiol; E3, estriol; EDCs, endocrine-disrupting compounds; EE2, 17α-ethynylestradiol; ESI^+, positive-ion electrospray ionization; HPLC, high performance liquid chromatography; NAS, nitrifying activated sludge; T, testosterone; TOF-MS, time-of-flight mass spectrometry; t_R, retention time; TSA, tryptic soy agar; TSB, tryptic soy broth; WWTPs, wastewater treatment plants.

3,17-dione). Two other testosterone transformation products, 5α-androstanedione (5α-androstan-3,17-dione) and androstadienedione (1,4-androstadien-3,17-dione), were also detected. Experiments with sterilized soil and sterilized swine manure slurry suggested that the transformation of ^{14}C-labeled hormonal parent compounds was mainly caused by microorganisms in the manure slurry, while mineralization of the hormones to $^{14}CO_2$ required viable soil microorganisms. In addition, Lorenzen et al. (2005) investigated the degradation of testosterone in three different soils, and found that 50% dissipated in 8.5 h (loam soil) to 21 h (silt loam soil) at 30°C, but that testosterone dissipated progressively more slowly at 12 and 4°C. They found only a minor impact of soil moisture (7–39%) on testosterone dissipation rates.

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

Materials and Methods

Chemicals

Testosterone, E2 (99.6%), and progesterone (98%) were purchased from Pfaltz & Bauer (Waterbury, CT), Calbiochem (La Jolla, CA), and Acros Organics (Morris Plains, NJ), respectively. Dehydrotestosterone (DHT), AD, ADD, and epitestosterone were purchased from Steraloids, Inc. (Newport, RI). Chemicals used to prepare the phosphate buffer solution (Na_2HPO_4, KH_2PO_4, NaCl, and NH_4Cl) and minimal growth media (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $MgSO_4$–$7H_2O$, $CaCl_2$–$2H_2O$, and $C_6H_{12}O_6$) were all of ACS grade and purchased from Fisher Scientific (Fair Lawn, NJ). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were purchased from MP Biomedicals (Solon, OH), and prepared according to the manufacturer's instructions. HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ) and Honeywell Burdick & Jackson (Muskegon, MI). Formic acid (88% A.C.S.) and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO) and Mallinckrodt Baker (Phillipsburg, NJ). LC-MS grade water was purchased from Honeywell Burdick & Jackson (Muskegon, MI). Deionized water was obtained using a Milli-Q reagent water purification system (Millipore, Bedford, MA).

Manure Collection

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

Biodegradation Experiments

To study the degradation of testosterone, E2, and progesterone by manure-borne bacteria, batch incubation experiments were conducted in minimal growth media with swine manure (system 1), and in a pre-enriched culture of swine manure-borne bacteria (system 2). All glassware was sterilized in an autoclave for 15 min at 121°C and 20 psi before use.

Biodegradation Experiments-System 1

In system 1, 0.5 g of sterilized (autoclaved for 15 min at 121°C and 20 psi) or unsterilized swine manure was mixed in 250-mL Erlenmeyer flasks with 100 mL of minimal growth media (pH 7) and an initial steroid hormone concentration of 3 mg L^{-1}. The minimal growth media (pH 7) was composed of 2 mmol L^{-1} $MgSO_4$–$7H_2O$, 3 mmol L^{-1} glucose, 0.1 mmol L^{-1} $CaCl_2$–$2\ H_2O$, 48 mmol L^{-1} Na_2HPO_4, 22 mmol L^{-1} KH_2PO_4, 9 mmol L^{-1} NaCl, and 19 mmol L^{-1} NH_4Cl. The sterilized swine manure was used as an abiotic control, and to estimate the extent of testosterone sorption during the batch incubation experiments. Blanks were prepared with testosterone in minimal media, but no manure, and all treatments were prepared in triplicate. Incubation was conducted in the dark at 22°C on a rotary shaker at 250 rpm. Samples were collected at regular intervals, and immediately filtered through 0.2-μm filters (0.2 μm, Spartan 13/A, regenerated cellulose, Schleicher & Schuell MicroScience, Inc., FL) into 2 mL amber glass vials for analysis. No more than 4% of any steroid hormone was retained on these filters.

Biodegradation Experiments-System 2

In system 2, the pre-enriched culture of swine manure-borne bacteria was prepared by mixing 1 g of swine manure with 100 mL of TSB in 250-mL Erlenmeyer flasks. The enrichment culture was incubated at 22°C on a rotary shaker at 250 rpm under oxic conditions. An Agilent 8453 UV-visible spectrophotometer was used to measure the optical density at 600 nm (OD_{600}) of samples collected from the enrichment culture, and the OD_{600} measurements were correlated with biomass concentration (colony-forming units [CFU] mL^{-1}). The TSB and TSA were used for preparation of serial dilutions and plate counts to determine the growth curve. When the culture reached the late log phase (14 h; OD_{600} = 3.8; ~10^8 CFU mL^{-1}), the cell suspension was centrifuged at 3000 g for 10 min, and resuspended in 100 mL of phosphate buffer solution (pH 7). Cells were centrifuged a second time, and resuspended in minimal growth media.

Next, a 1 mL portion of the cell suspension was inoculated into 250-mL Erlenmeyer flasks containing 99 mL of minimal growth media and either testosterone, E2, or progesterone, resulting in an initial cell density of approximately 10^6 CFU mL^{-1} and an initial steroid hormone concentration of 3 mg L^{-1}. To determine the impact of temperature, pH, glucose amendments, and the presence of molecular oxygen on testosterone degradation kinetics, triplicate incubations of the following treatments were also used: (A) 22 and 37°C; (B) pH 6, 7, and 7.5; (C) 0, 3, and 22 mmol L^{-1} glucose; and (D) aerobic vs. anaerobic conditions. For anaerobic conditions, the solutions used for the phosphate buffer and minimal growth media were boiled and purged with N_2 for 45 min

and sampled periodically in an anaerobic (O_2–free) glovebag. The flasks were incubated in the dark at 22°C on a rotary shaker operated at 250 rpm. Samples were collected at regular intervals, and immediately filtered through 0.2 μm regenerated cellulose filters into 2 mL amber glass vials for analysis.

Analytical Methods

To determine the degradation kinetics of testosterone, E2, and progesterone, samples were analyzed using an Agilent 1200 Series high performance liquid chromatography (HPLC) system with a diode array detector (DAD). The UV chromatograms were quantified at 220 nm for E2, 245 nm for progesterone, and 254 nm for testosterone. The analysis was performed using a Zorbax Eclipse XDB-C18 column (150 by 4.6 mm i.d., 5 μm particle size, Agilent, Santa Clara, CA), preceded by a guard column of the same packing material. For the androgens and E2, an isocratic analysis was performed with a mobile phase consisting of acetonitrile (45% for androgens; 40% for E2) and water (55% for androgens; 60% for E2), and a flow rate of 1 mL min^{-1}. The total run time was 20 min. For progesterone, a gradient method was used, with a binary mobile phase consisting of acetonitrile and water, and the mobile composition was adjusted as follows: held at 40% acetonitrile for 9 min, increased linearly to 100% acetonitrile over 5 min, held at 100% acetonitrile for 5 min, decreased linearly to 40% acetonitrile over 1 min, and held at 40% acetonitrile for 5 min. The flow rate was 1 mL min^{-1}, and the total run time, including conditioning, was 25 min. The injection volume of each sample was 20 μL. Testosterone, progesterone, and E2 samples were quantified by reference to a linear calibration, using least squares regression, of six external steroid hormone standards in methanol (0.15, 0.25, 0.5, 1, 2, and 3 mg L^{-1}). The reporting limit was based on the lowest calibration point.

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

Results

Steroid Hormone Degradation by Swine Manure-Borne Bacteria—Systems 1 and 2

The normalized concentration profiles of testosterone, E2, and progesterone spiked separately into systems 1 and 2 are illustrated in Fig. 1. No degradation of testosterone, E2, or progesterone was observed in minimal media in the absence of manure (data not shown). Sterilization of manure by autoclaving was performed to elucidate the role of sorption and the potential for abiotic degradation.

In system 1, some sorption of steroid hormones to swine manure was observed in sterilized controls (i.e., 7% of testosterone, 15% of E2, and 29% of progesterone) within the first hour of reaction. Steroid hormones in sterilized controls did not exhibit a significant loss after the first hour of incubation (Fig. 1A). Testosterone, E2, and progesterone were observed to degrade in system 1 within 4 to 12 h after a lag phase of approximately 5 to 9 h. The degradation of E2 appears to be faster than progesterone and testosterone. Specifically, no E2, progesterone, or testosterone was observed in system 1 after 9, 17, and 21 h of reaction initiation, respectively (Fig. 1A).

In system 2, no degradation or sorption of steroid hormones was observed in sterilized controls. Conversely, steroid hormones were observed to degrade in the TSB pre-enriched

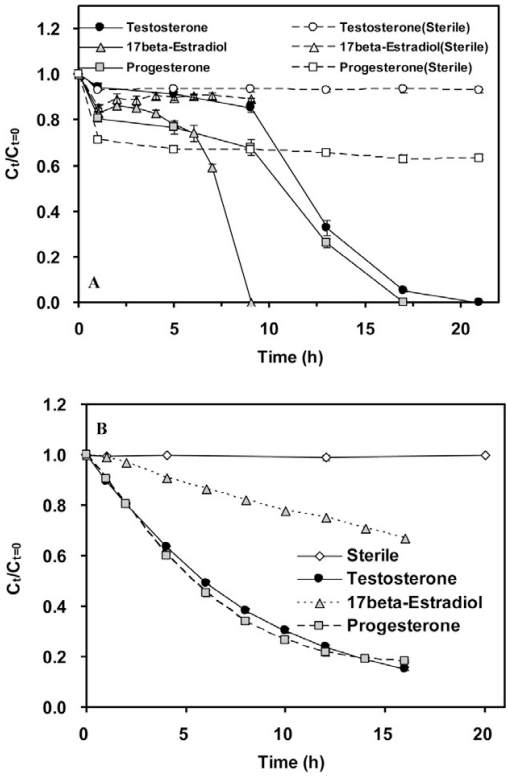

Fig. 1. Degradation of testosterone, 17β-estradiol, and progesterone under aerobic conditions in (A) system 1 (with swine manure) and (B) system 2 (TSB pre-enriched culture) at 22°C. Sterile represents sterilized system. Error bars represent the standard deviation of triplicate samples.

biologically active systems (Fig. 1B; system 2). Testosterone, E2, and progesterone degradation in system 2 were initiated without a lag phase. Testosterone and progesterone were transformed in a similar fashion, and followed pseudo first-order reaction kinetics. The degradation of E2 followed a zero-order reaction kinetics model during the observed time period. To compare the degradation rates for the three steroid hormones, their rate constants (k) and half-lives ($t_{1/2}$) were calculated based on an initial rate method for the first 8 h (Table 1), as described previously (Fendorf and Li, 1996; Loftin et al., 2008). Degradation rates and associated 95% confidence intervals were estimated with nonlinear regression analysis using the Statistical Analysis System's (SAS 9.2) exponential decay model. Multiple comparisons were conducted using ANOVA at $\alpha = 0.025$, and a p value < 0.05 was considered to indicate significance. The degradation rates followed the order progesterone > testosterone > > E2 at pH 7 and 22°C (Table 1; $R^2 > 0.99$).

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 (Fig. 2A). During the observed time period, the degradation of testosterone under anaerobic conditions followed a zero-order reaction kinetics model, in contrast to pseudo first-order reaction kinetics under aerobic conditions. The testosterone concentration decreased by 58% within 6 h of reaction time under aerobic conditions, in contrast to a decrease of only 15% under anaerobic conditions. The half-life of testosterone under anaerobic conditions was observed to be five to six times longer than under aerobic conditions (Table 1).

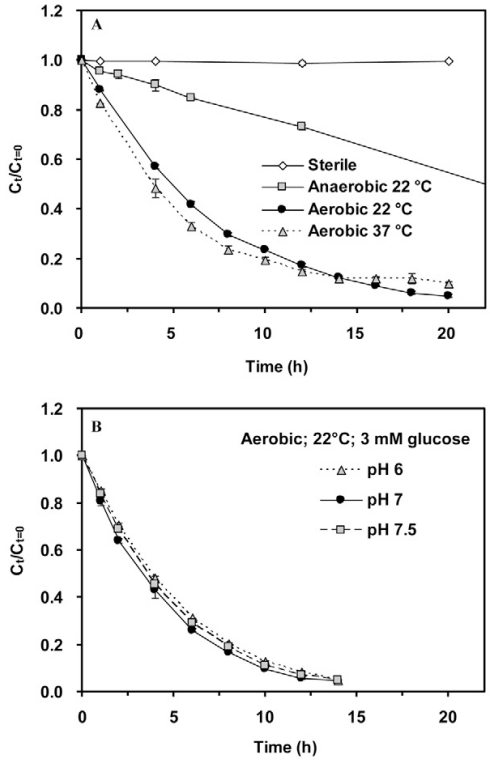

Fig. 2. (A) Influence of temperature and molecular oxygen at pH 7, and (B) pH on testosterone biodegradation in system 2. Error bars represent the standard deviation of triplicate samples.

Influence of Temperature on Testosterone Degradation—System 2

The degradation kinetics of testosterone (system 2) were investigated at 37 and 22°C to simulate conditions optimal for fecal bacteria and a temperature relevant for the conditions that swine feces is exposed to within the first 24 h of excretion. The degradation rate was significantly (p value < 0.025) slower (17%) at 22°C than 37°C based on the initial rate calculation (Table 1; Fig. 2A).

Influence of pH on Testosterone Degradation—System 2

Fresh swine feces (i.e., pH 6.8 for this study), fertile agricultural soils, and waters (e.g., rivers) often vary in pH from approximately 6 to 7.5. Therefore, this pH range was chosen to investigate the impact of pH on testosterone degradation by manure-borne bacteria. The normalized concentration profiles of testosterone obtained for experiments conducted at pH 6, 7, and 7.5 indicated that pH within the investigated range had only a minor impact on the degradation rate. The fastest degradation rate was observed at pH 7, and the degradation rate was approximately 11 and 6% slower in experiments conducted at pH 6 and 7.5, respectively (Table 1, Fig. 2B). 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).

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

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

Influence of Glucose Amendments on Testosterone Degradation—System 2

The impact of glucose amendments was investigated to provide insight into the microbial mechanism responsible for testosterone degradation. Glucose amendments (i.e., 0, 3, and 22 mmol L^{-1}) were found to have minor influence on testosterone degradation and metabolite formation within the 18-h time period investigated (Table 1, Fig. 3). Similar results were also observed in system 1 (data not shown). The difference between the observed rate constant at 0 and 22 mmol L^{-1} glucose amendment was only 4%, but this difference was not significant (p value > 0.05). A significant difference (p value < 0.025) was observed between 3 and 22 mmol L^{-1} glucose amendment.

Testosterone Degradation Products—System 2 (Aerobic Conditions)

In system 2 under aerobic conditions, HPLC-DAD and LC/TOF-MS analysis revealed three degradation products of testosterone (Fig. 4 and 5). The degradation products were identified through HPLC-DAD analysis by comparing their retention times (t_R) to the retention times of chemical standards, and confirmed by TOF-MS analysis (absolute mass error < 5 ppm). The degradation products were identified as DHT (absolute mass error 2.1 ppm; major degradation product), ADD (absolute mass error 1.68 ppm), and AD (absolute mass error 0.24 ppm; minor degradation product).

Discussion

Steroid Hormone Degradation by Manure-Borne Bacteria—Systems 1 and 2

Microorganisms such as bacteria common in feces (i.e., swine manure) can transform testosterone, E2, and progesterone to other potential endocrine active compounds under a range of physical (e.g., temperature) and chemical (e.g., pH and redox) conditions relevant for natural environments.

No sorption (i.e., <2%) in manure-free systems, and no degradation of steroid hormones in sterilized controls, were observed. In contrast, steroid hormones were rapidly degraded in biologically active systems, indicating that manure-borne bacteria were most likely responsible for the observed degradation. The observed lag phase in system 1 indicates that it takes approximately 5 to 9 h before the bacteria have adapted to the minimal media or produced a sufficient amount of enzymes for steroid hormone degradation. In addition, bacterial enumeration was performed as a part of one of the testosterone studies, and indicated an inverse relationship between the number of bacteria and testosterone concentration (data not shown). In contrast, the absence of a lag phase in system 2 (pre-enriched) indicates that steroid hormone degradation was not limited by induction or proliferation of steroid hormone-degrading microorganisms, which is in agreement with previous laboratory studies of testosterone degradation in agricultural soils (Lorenzen et al., 2005). E2 was degraded to below the detection limit within approximately 9 h in systems directly inoculated with swine manure (system 1),

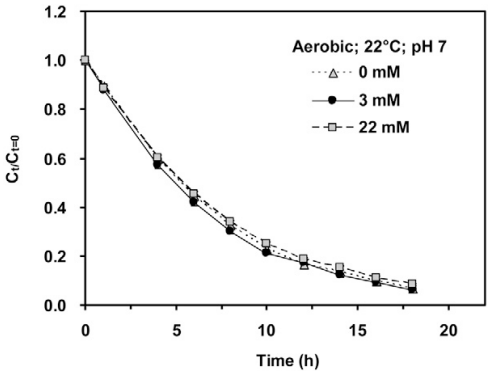

Fig. 3. Influence of glucose amendments on testosterone degradation in system 2. Error bars represent the standard deviation of triplicate samples.

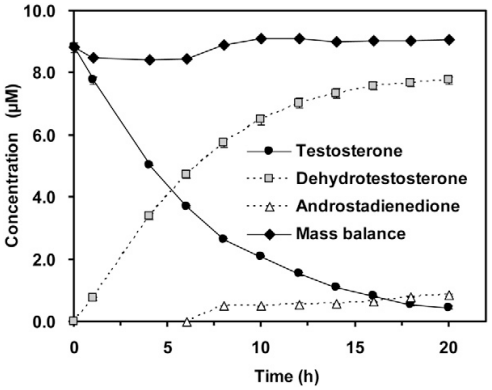

Fig. 4. Degradation of testosterone and formation of degradation products under aerobic conditions in system 2 at 22°C and pH 7. Error bars represent the standard deviation of triplicate samples.

but only 20% of the E2 was degraded within the same incubation period in systems inoculated with TSB pre-enriched microbial cultures (system 2) despite the higher cell density. The most likely reason for the slower and different (zero- vs. pseudo first-order kinetics) degradation pattern in system 2 is that the TSB pre-enrichment disfavored the bacteria that were responsible for the rapid degradation in system 1 (Vimont et al., 2007). The relative degradation rates of E2, testosterone, and progesterone observed in system 1 (E2 > progesterone > testosterone) are in contrast to results obtained from studies conducted with soils, biosolids, and swine manure-applied soils, broiler litter, and composted chicken (*Gallus gallus*) manure (Casey et al., 2004; Ermawati et al., 2007; Hakk et al., 2005; Hemmings and Hartel, 2006; Jacobsen et al., 2005; Lee et al., 2003). This may, in part, be due to stronger sorption of E2 (which contains an aromatic ring) than testosterone to soil and undiluted manure and compost, resulting in a lower bioavailability (Casey et al., 2004; Lee et al., 2003; Sangsupan et al., 2006). Alternatively, the bacteria in soil, composted chicken manure, and similar media might be unable to degrade E2 as efficiently, perhaps due to the difficulty of degrading the aromatic A-ring of estradiol (Hakk et al., 2005). Interestingly, the pseudo first-order rate constant

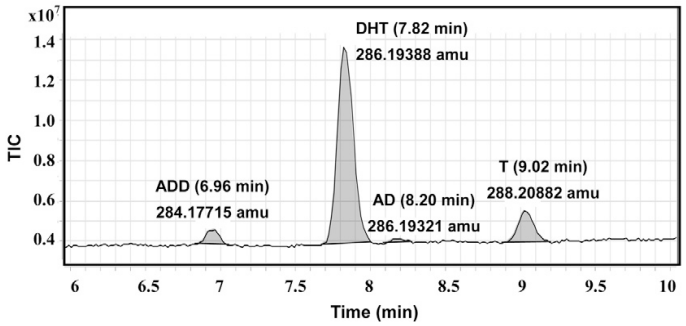

Fig. 5. Total ion current (TIC) chromatogram obtained by LC/TOF-MS analysis after 12 h of testosterone biodegradation (system 2) showing the presence of androstadienedione (ADD), dehydrotestosterone (DHT), androstenedione (AD), and testosterone (T). The molecular mass (amu) and retention time are shown for each compound.

obtained for E2 in system 2 (0.025 h^{-1} at 22°C) is similar to a previously reported pseudo first-order rate constant for E2 dissipation in a loam soil (0.02 h^{-1} at 19°C; (Colucci et al., 2001)). Overall, testosterone, E2, and progesterone were all observed to degrade in the presence of manure-borne bacteria, with half-lives from approximately 5 to 27 h.

Anaerobic versus Aerobic Degradation of Testosterone—System 2

Swine manure-borne bacteria were found to significantly degrade testosterone under both aerobic and anaerobic conditions (Fig. 2A). More than 80% of the testosterone was transformed under aerobic conditions within 12 h, in contrast to 27% under anaerobic conditions. The most plausible explanation for this observation is that manure-borne facultative anaerobic bacteria are more efficient at transforming testosterone under aerobic conditions than anaerobic conditions (obligate aerobic bacteria are not likely to be found in manure because the gastrointestinal tract is very anaerobic). In this study, the bacterial growth conditions excluded the presence of strict anaerobic bacteria, which might otherwise have contributed to the transformation of testosterone under anaerobic conditions. Despite less efficient degradation under anaerobic conditions, the findings support previous observations of estrogen and androgen removal in anaerobic swine manure lagoons, anaerobic digesters, and anoxic soils (Ermawati et al., 2007; Esperanza et al., 2007; Lorenzen et al., 2004; Shappell et al., 2007). The degradation of testosterone under anaerobic conditions followed a zero-order reaction kinetics model during the observed time period, suggesting that the degradation mechanisms under aerobic and anaerobic conditions may be different. A study by Harms and Bosma (1997) indicated co-metabolic degradation of 17α-ethynylestradiol (EE2) by nitrifying bacteria. On the other hand, both Gram-positive bacteria, including *Nocardia*, *Arthrobacter*, *Mycobacterium*, *Rhodococcus*, and Gram-negative bacteria, such as *Comamonas* and *Pseudomonas*, have been described as being capable of using testosterone and other steroids as sole carbon and energy sources (Horinouchi et al., 2003). It was beyond the scope of this study to characterize the microbial communities and the mechanisms responsible for testosterone degradation.

Influence of Temperature and pH on Testosterone Degradation—System 2

Testosterone was degraded approximately 17% faster at 37°C than at room temperature (i.e., 22°C), which is likely due to the fact that many fecal-derived enzymes have optimal activity at physiological temperature. Lorenzen et al. (2005) observed indistinguishable testosterone dissipation rates (i.e., testosterone was below detection within 25 h) at 23 and 30°C, but progressively more slow rates at 12 and 4°C. Previous studies of temperature on dissipation of E2 and EE2 in soil (Colucci and Topp, 2001; Xuan et al., 2008) showed a pattern similar to the study by Lorenzen et al. (2005). The negligible impact of temperatures above approximately 20°C on steroid hormone degradation in soils might be due to the fact that microbial communities in soils produce enzymes with similar activities at higher temperatures.

The impact of pH values relevant for a majority of agricultural soils and rivers (i.e., pH 6.0–7.5) indicated that the microbial activity was only slightly different within this pH range. However, testosterone was observed to degrade significantly faster at pH 7 (i.e., 6–11%) than at both pH 6 and 7.5 (Table 1). To the best of our knowledge this is the first study of pH impact on testosterone degradation by manure-borne bacteria. The results of this study suggest that steroid hormones are likely to biodegrade under a wide range of temperature and pH conditions in the environment.

Influence of Labile Carbon on Testosterone Degradation—System 2

Several studies have documented that the presence of a labile organic carbon source can influence the degradation of estrogens, indicating a co-metabolic process (Stumpe and Marschner, 2009). Li et al. (2008) performed semi-continuous aerobic batch experiments to investigate the impact of a coexisting organic carbon source (glucose) on the biodegradation of E2 and E1. When the initial glucose concentration was varied from 0 to 100 mg L^{-1}, the apparent disappearance rates of E2 and E1 ranged from 0.84 to 4.31 h^{-1} and 0.15 to 0.84 h^{-1}, respectively, assuming first-order kinetics (Li et al., 2008). Another study examined the effects of glucose concentration on E2 and EE2 mineralization in different soils and found that glucose induced faster E2 and EE2 degradation (Stumpe and

Marschner, 2009). The present study indicated no significant impact of glucose on the testosterone degradation. Glucose most likely did not have a substantial impact on testosterone degradation due to a large initial concentration of exogenous enzymes in the investigated system thus eliminating the need for co-metabolic processes involving glucose in short-term (< 24 h) incubations (Shackle et al., 2006). Alternatively, steroid hormones have been observed to mineralize (Haiyan et al., 2007; Lorenzen et al., 2005), which indicates that some bacteria have the potential to use steroid hormones as their sole carbon source.

Testosterone Degradation Products—System 2

Three degradation products (i.e., DHT, AD, and ADD) of testosterone were observed under aerobic conditions (Fig. 4 and 5). The main degradation product in this study was observed to be DHT. As far as the authors are aware, this is the first time that DHT has been reported as a degradation product of testosterone by manure-borne bacteria. However, DHT has previously been reported in sewage effluent (Thomas et al., 2002). The formation of DHT (major degradation product) is likely a result of 1(2)-dehydrogenase catalyzed testosterone transformation, while AD (minor degradation product) is likely formed by enzyme catalyzed 17β-dehydrogenation of testosterone (Donova, 2007). Actinobacteria such as *Mycobacterium and Nocardia* have been described as being capable of introducing 1(2)-dehydrogenation to 3-keto steroids, such as testosterone (e.g., conversion of T to DHT, or AD to ADD) and *Mycobacterium* have also been observed to oxidize steroids at position 17 (e.g., conversion of T to AD, or DHT to ADD) (Donova, 2007). Actinobacteria are not common in swine manure but similar transformation processes appear to be occurring in system 2. Previous studies have primarily reported that degradation of testosterone results in the initial formation of androstenedione (Das et al., 2004; Jacobsen et al., 2005; Lee et al., 2003; Lorenzen et al., 2005). Jacobsen et al. (2005) reported that microorganisms in a swine manure slurry were able to convert testosterone to 4-AD, 5α-AD, and ADD. The same three degradation products were observed in unmanured agricultural soils (Lorenzen et al., 2005). Interestingly, an unidentified testosterone metabolite was also observed in previous soil column studies (Das et al., 2004), but it was unclear if the compound was produced directly from testosterone. Lee et al. (2003) also observed a testosterone degradation product that they were unable to characterize, and hypothesized it to be androst-4-ene-3-one-16,17-diol (no confirmation was made). It was beyond the scope of this study to determine the degradation products of E2 and progesterone, although current studies in our laboratory are trying to elucidate the degradation pathways.

Conclusions

Testosterone, E2, and progesterone were rapidly (i.e., within 27 h) degraded by swine manure-borne bacteria under aerobic conditions. Testosterone was degraded significantly faster under aerobic ($t_{1/2} \approx 4$ h) than anaerobic ($t_{1/2} \approx 27$ h) conditions in tryptic soy broth pre-enriched systems. The biodegradation rate of testosterone was influenced to a smaller extent ($t_{1/2}$ ranged from 3.8–5.1 h) by different temperatures (22 and 37°C), pH (6, 7, and 7.5), and glucose (0, 3, and 22 mmol L^{-1}) amendments, indicating that testosterone has the potential for degradation by manure-borne bacteria under a wide range of environmentally relevant conditions. However, the formed degradation products (e.g., DHT, AD, and ADD) are still of potential concern due to their endocrine disrupting potential. Thus, future work needs to carefully elucidate the complete degradation pathways and mechanisms of testosterone, E2, and progesterone, to help advance our current understanding of the extent to which these hormones and their degradation products contribute to endocrine disruption in terrestrial and aquatic environments.

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APPENDIX B

Environmental Science & Technology

ARTICLE

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Testosterone-Mineralizing Culture Enriched from Swine Manure: Characterization of Degradation Pathways and Microbial Community Composition

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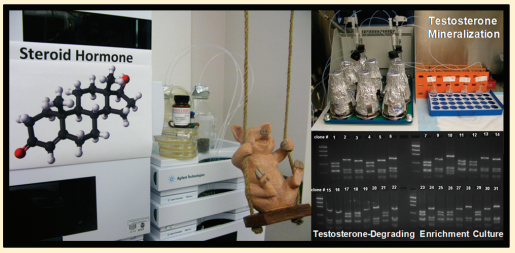
Supporting Information

ABSTRACT: Environmental releases and fate of steroid sex hormones from livestock and wastewater treatment plants are of increasing regulatory concern. Despite the detection of these hormones in manures, biosolids, and the environment, little attention has been paid to characterization of fecal bacteria capable of hormone degradation. The enrichments of (swine) manure-borne bacteria capable of aerobic testosterone degradation were prepared and the testosterone mineralization pathway was elucidated. Six DNA sequences of bacteria from the Proteobacteria phylum distributed among the genera *Acinetobacter, Brevundimonas, Comamonas, Sphingomonas, Stenotrophomonas,* and *Rhodobacter* were identified in a testosterone-degrading enriched culture with testosterone as the sole carbon source. Three degradation products of testosterone were identified as androstenedione, androstadienedione, and dehydrotestosterone using commercially available reference standards, liquid chromatography-UV diode array detection, and liquid chromatography-time-of-flight mass spectrometry (LC-TOF/MS). Three additional degradation products of testosterone were tentatively identified as 9α-hydroxytestosterone, 9α-hydroxyandrostadienedione or 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, and 9α-hydroxydehydrotestosterone or 9α-hydroxyandrostenedione using LC-TOF/MS. When ^{14}C-testosterone was introduced to the enriched culture, 49−68% of the added ^{14}C-testosterone was mineralized to $^{14}CO_2$ within 8 days of incubation. The mineralization of ^{14}C-testosterone followed pseudo-first-order reaction kinetics in the enriched culture with half-lives ($t_{1/2}$) of 10−143 h. This work suggests that Proteobacteria play an important environmental role in degradation of steroid sex hormones and that androgens have the potential to be mineralized during aerobic manure treatment or after land application to agricultural fields by manure-borne bacteria.



INTRODUCTION

Steroid hormones are an increasing public concern because of their ability to act as endocrine disruptors and thus adversely affect wildlife reproduction.[1] One major source of steroid hormones to the environment is the land application of animal manures as fertilizer or amendment. Livestock manure often contains a large amount of natural and synthetic chemicals, including hormones.[2,3] Previous studies have reported that steroid hormones could potentially contaminate the aquatic environment via surface runoff or leaching from agricultural fields amended with manure.[4−6] Androgenic hormones, including testosterone and its metabolites, have been detected in water bodies receiving feedlot effluent.[5] Demasculinization of male fish (lower testicular testosterone synthesis, altered head morphometrics, and smaller testis size) and defeminization of female fish (decreased estrogen/androgen ratio during in vitro steroid hormone synthesis) were observed when wild fathead minnows were exposed to cattle feedlot effluent.[7] The authors of that study did not observe overt characteristics in male or female fish suggesting environmental exposure to estrogens, and hypothesized that androgenic substances were at least partly responsible, because cells transfected with the human androgen receptor showed potent androgenic responses when exposed to the feedlot effluent.

Biodegradation has been suggested as an important steroid hormone removal mechanism in the environment.[8] Microbial transformation of testosterone (4-androsten-17β-ol-3-one; T) has been observed in several environmental matrices such as soils,[9] soil amended with manure or biosolids,[10,11] and stream sediments.[12] Hastened conversion of testosterone to androstenedione (4-androstene-3,17-dione; AD) and other metabolites, and mineralization of ^{14}C-testosterone were observed within a swine manured soil.[11] Differential sterilization experiments suggested that swine manure carried microorganisms that converted testosterone to steroidal transformation products, whereas mineralization of ^{14}C-testosterone required viable soil microorganisms.

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Incubation experiments were also conducted to examine the fate of 17β-estradiol (βE2) and testosterone in agricultural soils under aerobic and anaerobic conditions.[13] The results indicated that 6% of βE2 and 63% of testosterone could be mineralized to $^{14}CO_2$ in native soils under aerobic conditions.

Several bacterial species have been observed to have the ability to degrade testosterone and a variety of other steroids. For example, many actinobacteria, including species of *Arthrobacter, Mycobacterium, Nocardia,* and *Rhodococcus,* have the ability to utilize steroids as their sole source of carbon.[14−16] Steroids with the 3-oxo-4-ene structure, such as T and AD, are commonly subjected to B ring cleavage in a mechanism that involves 9α-hydroxylation and 1(2)-dehydrogenation.[14,16] However, actinobacteria can introduce hydroxyl groups in 11 other positions within the steroid molecule.[14] A gram-negative bacterium, *Comamonas testosteroni*, is also known for its ability to metabolize testosterone as a sole carbon and energy source.[17] Degradation of testosterone by *C. testosteroni* is thought to be initiated by dehydrogenation of the 17β-hydroxyl group to AD, which is then converted to androstadienedione (1,4-androstadiene-3, 17-dione; ADD), and proceeds via aromatization of the A-ring to complete mineralization.[17−19] A recent study found that a βE2-utilizing bacterium, *Sphingomonas* strain KC8 isolated from a wastewater treatment plant (WWTP), was capable of degrading and further utilizing testosterone as a growth substrate.[20] *Rhodococcus erythropolis* and *R. equi* were shown to degrade ethinylestradiol (EE2; synthetic estrogen) in the presence of a cosubstrate (adipic acid or glucose), removing up to 47 and 39% of the initial 1.4 mg L^{-1} EE2 in 13 and 65 h, respectively,[21] which suggests that the presence of an easily degradable carbon source is important for the removal of EE2 by some microbial species. In addition, the γ-proteobacterium *Steroidobacter denitrificans* strain FS^T, isolated and enriched from anoxic digested biosolids, was found capable of transforming testosterone under anoxic conditions.[22] Ten transformation products produced by *Steroidobacter denitrificans* strain FS^T were identified, including compounds such as 3β-hydroxy-5α-androstane-1,4-diene-3-one, 5α-androstan-3,17-dione, dehydrotestosterone (17β-hydroxy-androstane-1,4-diene-3-one; DHT), AD, and ADD.

Previous research demonstrated that testosterone is rapidly degraded by manure-borne bacteria under a wide range of environmentally relevant conditions with three degradation products (i.e., DHT, AD, and ADD) produced under aerobic conditions within 24 h of incubation.[23] However, little is known about the manure-borne bacteria responsible for testosterone mineralization and their degradation pathways under aerobic conditions without a readily available carbon source. The specific objectives of this study were (i) to enrich manure-borne bacteria capable of using testosterone as their sole carbon source under aerobic conditions, (ii) to sequence the 16S rRNA genes of an enriched microbial culture, and (iii) to elucidate the testosterone degradation/mineralization pathway by the enriched bacteria. It is expected that the knowledge gained from this study will help the development of best management practices for optimal hormone removal by fecal bacteria in, for instance, lagoons, wastewater treatment plants, and manure/biosolids amended soils.

EXPERIMENTAL SECTION

Enrichment Culture of Testosterone-Degrading Bacteria. Reagents and details on the collection of manure are described in the Supporting Information (SI) and elsewhere.[23] To study degradation of testosterone by manure-borne bacteria, batch incubation experiments were conducted. A half gram of sterilized (autoclaved for 15 min at 121 °C and 20 psi) or unsterilized swine manure was mixed in 250-mL Erlenmeyer flasks with 100 mL of minimal growth medium (pH 7) and an initial testosterone concentration of 3 mg L^{-1}. The minimal growth medium was composed of 2 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$, 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, and 19 mM NH_4Cl. Sterilized swine manure was used as an abiotic control. Incubation was conducted in the dark at 22 °C on a rotary shaker at 250 rpm, and all treatments were prepared in triplicate. Samples were obtained using sterile 1-mL syringes (Becton Dickinson, Franklin Lakes, NJ) at regular intervals (1−4 samples per day) and immediately filtered through 0.2-μm filters (0.2-μm, Spartan 31/A, Schleicher & Schuell MicroScience, Inc., FL) into 2-mL amber glass vials for analysis. To further enrich the testosterone-degrading bacteria present in swine manure, five serial transfers were conducted. The serial transfers were performed at the same time in both sterile and unsterile systems. When less than 40% of the initial testosterone concentration was observed in the unsterile system, a 1-mL aliquot of the cell suspension from the sterilized or unsterilized manure systems was transferred into 250-mL Erlenmeyer flasks containing 99 mL of fresh minimal growth medium and an initial testosterone concentration of 3 mg L^{-1}. Incubation was again conducted in the dark at 22 °C on a rotary shaker at 250 rpm. This process was repeated five times over a 26-day period. Tryptic soy agar (TSA) was used for preparation of plate counts to determine the microbial growth curve during the degradation process. The culture suspension from the fifth transfer was transferred into a 50-mL sterilized plastic centrifuge tube (Thermo Fisher Scientific Inc.) and stored at −80 °C prior to DNA extraction. Subsequent experiments were conducted with enriched cultures from the fifth transfer.

^{14}C Mineralization Laboratory Assays. A 0.5-mL portion of cell suspension from the enrichment culture was added into 49.5 mL of fresh minimal growth medium containing 3 mg L^{-1} testosterone. To determine the amount of CO_2 produced during mineralization of testosterone, approximately 25 million dpm of [4-^{14}C]-testosterone was added. $^{14}CO_2$ was trapped by purging air through a Teflon tube into the microbial medium via a 2-hole rubber stopper. The air from the outlet of the test flasks was passed through a second Teflon tube that was connected to a scintillation vial containing a scintillation cocktail (C-14 trapping cocktail OX-161, R.J. Harvey Instrument Co., Tappan, NY 10983) that trapped the produced $^{14}CO_2$. An abiotic control containing a sterilized cell suspension was also set up to elucidate whether the conversion of testosterone to $^{14}CO_2$ was biologically facilitated. Six additional samples, including sterilized controls and enriched cultures, were set up with air sparging to allow for measurements of ^{14}C in the aqueous phase. The flasks were incubated in the dark on a rotary shaker at 250 rpm, and all treatments were prepared in triplicate. Trapped $^{14}CO_2$ and aqueous ^{14}C were subsequently counted on a scintillation counter (Packard 2500R, PerkinElmer, USA), and the scintillation cocktail used in the $^{14}CO_2$-trap was replaced after each sample collection to prevent saturation/evaporation.

DNA Extraction. DNA was extracted from cell pellets obtained from 35 mL of the testosterone-degrading culture from the enrichment culture using the UltraClean microbial DNA isolation kit (Mo Bio, Carlsbad, CA). DNA from lysed cells was bound to a silica spin filter, washed, and eluted with 50 μL

Table 1. Testosterone-Degrading Culture Enriched from Swine Manure (Testosterone Was the Sole Carbon and Energy Source) and DNA Sequencing Results of Clones of PCR Products Obtained with the 16Sr RNA Gene Primers U341F and 1492R

genbank accession number	% matching sequences	BLAST identity	order	class	% match
AB219359.1	46	*Sphingomonas* sp. JEM-1	Sphingomonales	α-proteobacteria	95.50−98.86
GQ246710.1	25	*Rhodobacter* sp. M2T8B7	Rhodobacterales	α-proteobacteria	97.58−98.56
GQ259481.1	15	*Comamonas testosteroni* strain TDKW	Burkholderiales	β-proteobacteria	97.98−98.97
GQ246681.1	7	*Acinetobacter* sp. M1T8B5	Pseudomonadales	γ-proteobacteria	97.75−98.59
AB294556.1	5	*Stenotrophomonas maltophilia*	Xanthomonadales	γ-proteobacteria	98.37
FJ197848.1	2	*Brevundimonas* sp. 39 (2008)	Caulobacterales	α-proteobacteria	99.36

of 10 mM Tris buffer at pH 8. DNA quality was verified by agarose gel electrophoresis. The extracted DNA was stored at −80 °C for subsequent studies.

16S rRNA Gene Polymerase Chain Reaction (PCR) Assays. The DNA extract was diluted 1:10 with sterile deionized water and used as template for 16S rRNA gene PCR assays. The PCR application was performed in a Mastercycler prothermal cycler (Eppendorf, Ontario, Canada). Primers U341F[24] and 1492R[25] were used to amplify ∼1150 bp of the 16S rRNA gene. Reagents and details on the PCR thermal cycle are described in the SI.

Cloning and Sequence Analysis. The U341F-1492R PCR products were cloned and transformed into competent *E. coli* cells with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). PCR products were ligated at room temperature for 30 min and transformed cells were plated in LB agar plates with 50 μg mL^{-1} kanamycin and incubated at 37 °C for 18 h. The inserts were PCR amplified directly from the colonies using the vector-specific M13F/M13R PCR primers. The reaction mixture included 10 mM dNTPs, 20 μM of each primer, 1X TaqMaster PCR enhancer (5 Prime), 1X GenScript buffer (GenScript, Piscataway, NJ), 1.75 U GenScript *Taq* DNA polymerase, 0.25 μL formamide, and 15.9 μL deionized water to bring the volume to 25 μL. The amplification conditions were 3 min at 94 °C, followed by 35 cycles of 20 s at 94 °C, 30 s at 55 °C, 70 s at 72 °C, and final extension step for 7 min at 70 °C.

Amplified rDNA restriction analysis (ARDRA) was performed on the M13 PCR products with the restriction enzyme *Msp1*. M13 PCR products from the different *Msp1* restriction digest patterns were sequenced at the CSU Proteomics and Metabolomics facility. Rarefaction curves were calculated for the different ARDRA patterns. The DNA sequences obtained from cloning were aligned to the sequences of the closest identified microorganisms by the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

Quantitative and Qualitative Analysis. Filtrate samples were analyzed using an Agilent 1200 Series high-performance liquid chromatography (HPLC) system with a diode array detector (DAD) monitoring UV absorbance at 254 nm, as described in detail in the SI. Testosterone and three expected degradation products, AD, ADD, and DHT, were identified in the filtrate samples by matching retention times to commercially available reference standards, and then quantified by external calibration. The identities of testosterone, AD, ADD, and DHT were confirmed with high-resolution, accurate mass data obtained when the filtrate samples were analyzed by LC/TOF-MS using an Agilent 1200 series HPLC system interfaced to a HTC-PAL autosampler (CTC analytics, Zwingen, Switzerland) and an Agilent 6510 quadrupole time-of-flight mass spectrometer, as described previously.[23] More details can be found in the SI. The elemental compositions of three additional degradation products were determined using high-resolution, accurate mass data from LC/TOF-MS analysis of the filtrate samples, and tentative identifications were proposed after considering known patterns of testosterone degradation.

RESULTS

Characterization of the Microbial Community in a Testosterone-Degrading Culture Enriched from Swine Manure. Amplified rDNA restriction analysis (ARDRA) of 60 M13 PCR products with restriction enzyme *Msp1* produced 10 different restriction patterns. The rarefaction curve based on ARDRA patterns approached saturation indicating that nearly all the diversity of the sample had been covered (Figure S1). The DNA sequences of 30 M13 PCR products, representing all 10 restriction patterns, were determined to identify the bacterial species. The DNA sequencing results revealed that the microorganisms in the sample were distributed among six different genera—*Acinetobacter, Brevundimonas, Comamonas, Sphingomonas, Stenotrophomonas,* and *Rhodobacter*—of three classes: α-proteobacteria, β-proteobacteria, and γ-proteobacteria (Table 1). With over 46% of the total bacterial sequences, *Sphingomonas* sp. JEM-1 represented the dominant DNA sequence in the microbial enrichment. The second most abundant DNA sequence had identity to *Rhodobacter* sp. M2T8B7 (∼25%). Approximately 15% of the sequences were similar to *C. testosteroni* strain TDKW.

Testosterone Degradation by Swine Manure-Borne Bacteria. Within enriched cultures under aerobic conditions, no sorption was observed and testosterone degradation occurred within 29 h after a lag phase of approximately 22 h (Figure S2). Sampling continued for 96 h, but no testosterone was observed after 51 h of incubation. In contrast, no degradation or sorption of testosterone was observed in sterile controls. The extent of microbial growth was determined at the beginning of the cultivation period and after 24, 48, and 96 h incubation. The number of colony-forming units (CFU) mL^{-1} was ∼2×10^4 for 0 h, 6×10^4 for 24 h, 1.2×10^6 for 48 h, and 5×10^6 for 96 h, respectively, as determined by TSA plating.

After 48 h incubation, HPLC-DAD and LC/TOF-MS analysis revealed six degradation products of testosterone within the enriched culture (Table 2; Figure S3). Three of the products were identified as ADD, DHT, and AD using the retention times of commercially available reference standards and high resolution, accurate mass data (mass error <2 ppm). Using elemental compositions from high-resolution, accurate mass data and considering known patterns of testosterone degradation,[19] the other three degradation products were tentatively identified as 9α-hydroxytestosterone (9α-OH-T), 9α-hydroxyandrostadienedione (9α-OH-ADD) or 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA), and 9α-hydroxydehydrotestosterone

Table 2. Proposed Identities (ID) of Products of Testosterone Degradation by a Microbial Enrichment from Swine Manure after 48 h of Incubation, Determined by LC/TOF-MS Analysis, Showing Proposed Formulas, Accurate Masses, and Associated Mass Errors (Peak Number Refers to Peaks in Figure S3)

peak	t_R (min)	proposed formula	ID	mass	mass error (ppm)
1	17.46	$C_{19}H_{28}O_3$	9α-hydroxytestosterone (9α-OH-T)[a]	304.20384	0.48 (MH^+)
					0.65 (MNa^+)
2	20.41	$C_{19}H_{24}O_2$	androstadienedione (ADD)	284.17763	0.15 (MH^+)
					1.07 (MNa^+)
3	20.77	$C_{19}H_{24}O_3$	9α-hydroxyandrostadienedione (9α-OH-ADD)[a]/3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA)[a]	300.17254	1.59 (MH^+)
					0.09 (MNH_4^+)
					0.35 (MNa^+)
4	22.15	$C_{19}H_{26}O_3$	9α-hydroxydehydrotestosterone (9α-OH-DHT)[a]/ 9α-hydroxyandrostenedione (9α-OH-AD)[a]	302.18819	0.6 (MH^+)
					0.91 (MNH_4^+)
					0.65 (MNa^+)
5	23.62	$C_{19}H_{26}O_2$	dehydrotestosterone (DHT)	286.19328	0.57 (MH^+)
					1.54 (MNa^+)
6	24.45	$C_{19}H_{26}O_2$	androstenedione (AD)	286.19328	0.05 (MH^+)
					1.21 (MNa^+)
7	27.26	$C_{19}H_{28}O_2$	testosterone (T)	288.20893	1.26 (MH^+)
					0.49 (MNa^+)

[a] Tentative ID.

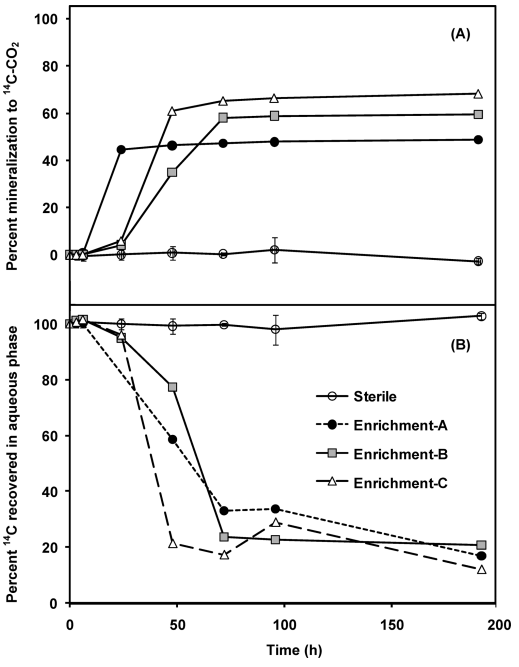

Figure 1. Percent of (A) ^{14}C-testosterone mineralization to $^{14}CO_2$ by a microbial culture enriched from swine manure and (B) the ^{14}C recovered in the aqueous phase. Experiments were conducted with enriched cultures from the fifth serial transfer at 22 °C. Enrichments A, B, and C represent triplicate enrichment cultures.

(9α-OH-DHT) or 9α-hydroxyandrostenedione (9α-OH-AD). Among the six degradation products, DHT was the major testosterone degradation product within the first 48 h. For this reason, the degradation product tentatively identified as 9α-OH-DHT is more likely than 9α-OH-AD.

The mass balance determined using HPLC-DAD data decreased substantially (~96% at 31 h to 12% at 48 h) after 27 h of incubation (Figure S2). This could be due to nondetectable degradation products or the conversion of testosterone to CO_2. To verify whether testosterone was completely mineralized to CO_2, a mineralization experiment was performed. Within 2 d, 35−60% of the added ^{14}C-testosterone had been mineralized to $^{14}CO_2$ (Figure 1A). At the end of an 8-d incubation period, using the microbial enrichment, testosterone mineralization reached a maximum of 49−68%. In contrast, no mineralization was observed in sterilized controls. The mineralization data were well described ($R^2 > 0.986$) using first-order kinetics, based on the first 24-h time period, as described previously.[10]

A first-order mineralization rate was determined for the removal of testosterone by mineralization to $^{14}CO_2$ with rate constants k and half-lives ($t_{1/2}$) ranging from 0.005 to 0.072 h^{-1} and 10 to 143 h, respectively. The recovery of ^{14}C at the end of the experiment, as determined by measuring the radioactivity remaining in the aqueous phase (Figure 1B) and adding these values to the amount of $^{14}CO_2$ trapped, were >96% and 79−83% for sterilized controls and enriched cultures, respectively. Based on the degradation products observed by LC/TOF-MS (Table 2; Figure S3) and the evidence of mineralization (Figure 1), the proposed conversion pathways of testosterone by enriched swine manure-borne bacteria are shown in Figure 2. Bacteria are capable of introducing hydroxyl groups across the steroid nucleus, but *C. testosteroni*, in particular, has been shown to produce many of the observed degradation products via 9α-hydroxylation en route to cleavage of the A-ring and mineralization.[14,18] As a result, 9α-hydroxylation is assumed in Figure 2.

DISCUSSION

Characterization of the Microbial Community in a Testosterone-Degrading Culture Enriched from Swine Manure.

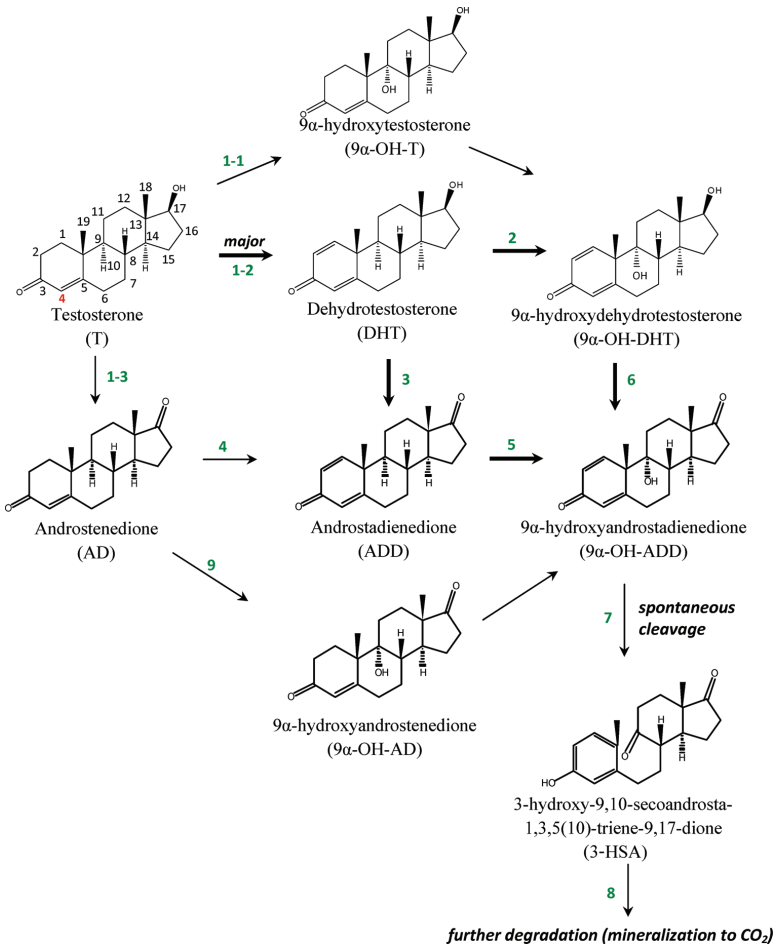

Figure 2. Proposed testosterone transformation pathways by the microbial culture enriched from swine manure, assuming 9α-hydroxylation in accordance with refs 16 and 19 (see Table 2 and Figure S3).

In this study, partial 16S rRNA gene sequences corresponding to *Acinetobacter* sp. M1T8B5, *Brevundimonas* sp. 39 (2008), *C. testosteroni* strain TDKW, *Rhodobacter* sp. M2T8B7, *Sphingomonas* sp. JEM-1, and *Stenotrophomonas maltophilia* were found in a testosterone-degrading culture enriched under aerobic conditions from swine manure (Table 1). At the phylum level, all of the 16S rRNA gene sequences derived from the swine manure were assigned to Proteobacteria, as was the case in previous studies.[26,27] To the best of our knowledge, this is the first report of a microbial culture enriched from animal manure capable of using testosterone as their sole carbon source.

In one study involving aerated pig slurry, 16S rRNA gene sequences ($n = 48$ clones) belonging to the phylum Proteobacteria (i.e., ~17% of α-, 10% of β-, and 8% of γ-proteobacteria) were among the most abundant. In addition, among Proteobacteria, some of the predominant bacterial sequences found were associated with the genera *Acinetobacter, Comamonas, Sphingomonas,* and *Stenotrophomonas.*[26] Another study of the microbial community in piggery wastewater sampled from an anaerobic digester reported that the following bacterial lineages were dominant: α-proteobacteria, β-proteobacteria, γ-proteobacteria, and Firmicutes with >93% identity with sequences in the NCBI database.[27] Similar results were reported in studies that investigated a bacterial population in dairy waste where the majority of the operational taxonomic units (OTUs) from the circulated dairy wastewater[28] and aerobic reactor effluent[29] were associated with the phylum Proteobacteria. A wide array of diversity in isolates, including *Brevibacterium, Acinetobacter,* and *Comamonas* spp., was also found in a study that investigated the diversity of tetracycline resistance gene among bacteria isolated from a swine lagoon.[30]

The microorganisms of the genera *Acinetobacter, Novosphingobium, Nitrosomonas,* and *Sphingomonas* have been shown elsewhere to degrade several organic compounds including estrogens.[31−36]

A βE2-degrading bacterium isolated from a WWTP in Japan was suggested to be *Novosphingobium* spp.[31] The ammonia-oxidizing bacterium *Nitrosomonas europaea* was found to significantly degrade E1, βE2, E3, and EE2.[32] Additionally, sequences corresponding to the microorganism *Sphingomonas* sp. JEM-1 found in the present study have also been isolated from soil and activated sludge and described as being capable of utilizing 7-ketocholesterol as a sole carbon and energy source, resulting in its mineralization.[33]

Some of the microorganisms identified through DNA sequencing in the testosterone-degrading enrichment culture from swine manure have also been found in soil and WWTPs, and are known degraders of estrogens and androgens. For example, members of the genera *Comamonas* and *Sphingomonas* are well-known for their broad catabolic potential and ability to degrade sterols and steroids such as cholesterol, estrogens, and androgens.[17,18,20,37] Fourteen phylogenetically diverse βE2-degrading bacteria, distributed among eight different genera (*Aminobacter, Brevundimonas, Escherichia, Flavobacterium, Microbacterium, Nocardioides, Rhodococcus,* and *Sphingomonas*) and three phyla (Proteobacteria, Actinobacteria, and Bacteroidetes), were also isolated from activated sludge.[37] In addition, βE2-utilizing bacterium, *Sphingomonas* strain KC8, can degrade and further utilize testosterone as a growth substrate.[20] The maximum specific substrate utilization rates reported were 0.50, 0.37, and 0.17 mg-substrate/mg-protein/d for E1, βE2, and testosterone, respectively. Some bacteria of the genera *Comamonas* (e.g., *C. testosteroni*) do utilize the steroid 9α-hydroxylase system, which requires molecular oxygen for action. [17] It is also reasonable to expect that some species of the genera *Sphingomonas* may possess an enzyme system that degrades testosterone and structurally similar compounds. To the best of our knowledge, *Rhodobacter* has never been described as capable of steroid hormone degradation.

Testosterone Degradation by Swine Manure-Borne Bacteria. The results of this testosterone degradation study indicate that the enriched microbial culture can degrade testosterone without addition of another readily available organic carbon source. When inoculated into fresh medium, a lag phase of 22 h was observed (no observed microbial growth) before the onset of testosterone degradation (Figure S2). No detectable testosterone was observed after 51 h in the enriched culture. However, growth did not cease after the depletion of testosterone, suggesting that the enriched microbial culture can grow not only on testosterone but also on its degradation products.

The six testosterone degradation products (i.e., 9α-OH-T, ADD, 9α-OH-ADD/3-HSA, 9α-OH-DHT/9α-OH-AD, DHT, and AD; Table 2, Figure 2, and S3) identified in this study have previously been reported as intermediates in testosterone degradation pathways. Microorganisms in swine manure were found capable of converting testosterone to AD, 5α-androstan-3,17-dione (5α-AD), and ADD; [11] however, the authors did not attempt to enrich the testosterone-degrading culture. The same three degradation products were also detected in unmanured agricultural soil.[9]

The conversion of testosterone to DHT (major degradation product; step 1-2, Figure 2), AD (step 1-3, Figure 2), and ADD (step 4, Figure 2) by the enriched culture was also observed in a previous study in which swine-manure borne bacteria had been pre-enriched or grown in tryptic soy broth (TSB), indicating a similar biodegradation pathway for both microbial culture systems.[23] Importantly, the enrichment culture in the present study was obtained using minimal medium with testosterone as the sole carbon and energy source. A pathway of testosterone degradation by *C. testosteroni* TA441 has previously been proposed and *C. testosteroni* was found to grow on testosterone, progesterone, dehydroepiandrosterone, and epiandrosterone.[18,38] The major proposed metabolites were AD, ADD, and 9α-OH-ADD with the latter undergoing spontaneous cleavage; however, in contrast to our study DHT and 9α-OH-DHT were not suggested as degradation products.[38] Kim et al.[16] reported degradation of testosterone to AD and 9α-OH-T (steps 1-1 and 1-3, Figure 2), followed by degradation to 9α-OH-AD and ADD (steps 4 and 9, Figure 2) by *Rhodococcus equi* ATCC 14887.

Testosterone Mineralization. The oxidation of ^{14}C-testosterone to $^{14}CO_2$ requires ring cleavage and thus complete inactivation of testosterone. In enriched cultures, the mineralization observed was >48% within 8 d. The high percentage of ^{14}C-testosterone converted to $^{14}CO_2$ suggests that testosterone served as an energy source. Although the trend in testosterone mineralization was similar in all replicates, mineralization rates varied among them, probably due to biological variability through the serial transfer process. In contrast, no testosterone was converted to $^{14}CO_2$ in the sterilized controls (Figure 1), clearly indicating that the microbial enrichment was responsible for testosterone mineralization. These findings were similar to another study,[11] in which 47% and 36% of the testosterone was mineralized to $^{14}CO_2$ in (swine) manured and unmanured soils, respectively, following 6-d incubation under aerobic conditions. Interestingly, based on a series of sterilization experiments, in which sterilized soil was added to nonsterilized manure and vice versa, 4-d incubations suggested that the swine manure slurry carried microorganisms that converted testosterone to steroidal transformation products, whereas mineralization of ^{14}C-testosterone required viable soil microorganisms.[11] Because our study shows that mineralization of ^{14}C-testosterone by manure-borne bacteria is possible, future studies should include a more detailed investigation of the role of manure-borne bacteria after land application to soils including a study of competitive interactions with native soil microbial communities. Another study also observed that approximately 50% of the applied ^{14}C-testosterone to an agricultural soil was mineralized to $^{14}CO_2$ after 5-d incubation.[9] The mineralization of ^{14}C-testosterone, E1, and βE2 in breeder and broiler litter under different conditions was determined by Hemmings and Hartel,[39] and they reported that after 23 wk, an average of 27% of the ^{14}C-testosterone applied to breeder litter was mineralized to $^{14}CO_2$ at 25 °C.

The mineralization of ^{14}C-testosterone in this study followed pseudo-first-order reaction kinetics. Mineralization of testosterone, βE2, and EE2 was also observed in biosolids from a WWTP resulting in 55−65% conversion of ^{14}C-testosterone to $^{14}CO_2$ under aerobic conditions within 1 d.[10] First-order mineralization reaction kinetics with k values of approximately 0.912 h^{-1} and 0.252 h^{-1} were reported for ^{14}C-testosterone and ^{14}C-βE2, respectively. The k values determined in this study (0.005−0.072 h^{-1}) are similar to the observations from Fan et al.,[13] who found the first-order mineralization rate constant for testosterone in native soil to be 0.012 h^{-1}.

The percent of ^{14}C-testosterone mineralized to $^{14}CO_2$ ceased after 72 h of incubation. The total recovery of ^{14}C at the end of the experiment was between 79 and 83%. Inadequate trapping of $^{14}CO_2$ during the experiments is the most likely reason for the lack of a full recovery of ^{14}C. Additionally, uptake of ^{14}C by the cells may also contribute to the lack of full recovery. Taken

together, these results suggest that testosterone can be degraded by swine manure-borne bacteria, in the absence of a readily available carbon source, and be further mineralized to $^{14}CO_2$.

Environmental Significance. In this study, the microorganisms in a testosterone-degrading culture enriched from swine manure were identified using 16S rRNA gene-based methods, and the degradation pathway was examined. Six DNA sequences of bacteria from the Proteobacteria phylum were identified. The bacteria identified here were to a large extent similar to hormone-degrading bacteria previously characterized in urban wastewater treatment plants indicating that these fecal bacteria are likely to play an important role in mineralizing hormones in both animal manures and sewage sludge or biosolids.

Another important finding of this study is that manure-borne bacteria are capable of using testosterone as the sole carbon and energy source resulting in mineralization to CO_2 under aerobic conditions. This suggests that biodegradation can be an important mechanism for removing steroid hormones from aerated manure treatment systems. Relatively little information is available on biodegradation of hormones and veterinarian pharmaceuticals in aerated lagoons, aerobic digesters, compost, and other manure treatment systems. A recent study investigated the removal of estrogens (i.e., E1, 17α-estradiol (αE2), and βE2) and estrogenic activity in dairy shed effluent within two passive secondary biological treatment systems that both included anaerobic and aerobic stages.[40] In both systems, the greatest reduction in estrogenic activity occurred in aerobic ponds, suggesting that active aeration is likely to provide the most cost-effective and acceptable solution to dairy farms. This finding is supported by previous research where testosterone was degraded significantly faster under aerobic ($t_{1/2} \approx 4$ h) than anaerobic ($t_{1/2} \approx 27$ h) conditions.[23] The use of aerated lagoons or aerated caps to provide an oxygenated zone on the surface layer of anaerobic lagoons would be another potentially viable option for more effectively treating hormones in lagoons. The findings in this study should also be considered when optimizing methods of manure and biosolids land application for optimal hormone removal as the management approach (e.g., surface application vs injection) can result in different redox conditions that will ultimately influence the biodegradation kinetics and pathways.

■ ASSOCIATED CONTENT

Supporting Information. Detailed description of the chemicals, manure collection, 16S rRNA PCR assays, analytical methods, the rarefaction curve, testosterone degradation plot, and a total ion current chromatogram showing the degradation products. This material is available free of charge via the Internet at http://pubs.acs.org.

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APPENDIX C

Environmental Science & Technology

ARTICLE

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Artificial Sweetener Sucralose in U.S. Drinking Water Systems

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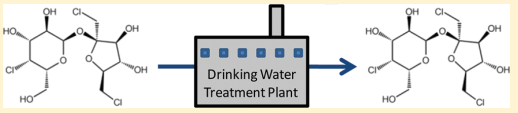
Supporting Information

ABSTRACT:



The artificial sweetener sucralose has recently been shown to be a widespread of contaminant of wastewater, surface water, and groundwater. In order to understand its occurrence in drinking water systems, water samples from 19 United States (U.S.) drinking water treatment plants (DWTPs) serving more than 28 million people were analyzed for sucralose using liquid chromatography tandem mass spectrometry (LC-MS/MS). Sucralose was found to be present in source water of 15 out of 19 DWTPs (47−2900 ng/L), finished water of 13 out of 17 DWTPs (49−2400 ng/L) and distribution system water of 8 out of the 12 DWTPs (48−2400 ng/L) tested. Sucralose was only found to be present in source waters with known wastewater influence and/or recreational usage, and displayed low removal (12% average) in the DWTPs where finished water was sampled. Further, in the subset of DWTPs with distribution system water sampled, the compound was found to persist regardless of the presence of residual chlorine or chloramines. In order to understand intra-DWTP consistency, sucralose was monitored at one drinking water treatment plant over an 11 month period from March 2010 through January 2011, and averaged 440 ng/L in the source water and 350 ng/L in the finished water. The results of this study confirm that sucralose will function well as an indicator compound for anthropogenic influence on source, finished drinking and distribution system (i.e., tap) water, as well as an indicator compound for the presence of other recalcitrant compounds in finished drinking water in the U.S.

INTRODUCTION

Artificial sweeteners are a class of organic contaminants recently discovered in wastewater, groundwater, surface water, bank filtrate and drinking water.[1−7] These compounds are widely added to foods, personal care products and pharmaceutical formulations, and enter the environment in part due to incomplete removal during wastewater treatment. While artificial sweeteners undergo comprehensive toxicological testing prior to their use in consumer products, their unintended presence in water is still cause for concern. This is because the long-term health effects resulting from chronic exposure to low levels of these compounds and other trace organic contaminants in water, such as pharmaceuticals and personal care products,[8,9] are largely unknown.[10,11] However, the true importance of the artificial sweeteners will likely be as indicator compounds for wastewater influence on other water types.[1,3,6] In any case, the quantification of these compounds in various water types, including finished drinking water, is the first step in understanding the significance of this issue.

A great deal of analytical work for artificial sweeteners has concerned measuring their concentration in food products.[12−17] However, there are a growing number of studies of artificial sweetener occurrence in wastewater and surface water. A large volume of this work has been completed in Europe, where acesulfame and sucralose were found to be the most persistent through wastewater treatment and in the environment.[1,3,4] Acesulfame was found to occur at higher levels than sucralose in the waste-, surface, ground, and tap water of Switzerland in Buerge et al.[1] and wastewaters and surface waters of Germany in Scheurer et al.[3] Both studies proposed acesulfame as an indicator compound for domestic wastewater influence on other water types. The occurrence of sucralose in surface waters has been measured in 27 European countries in Loos et al.,[4] as well as smaller studies in Switzerland[1] and Germany[3] that included groundwater. This compound has also been proposed as an indicator compound of domestic wastewater influence on surface water,[3] although acesulfame was found to be the predominant artificial sweetener in these studies. Both compounds were the subject of a recent study of bench-scale and full-scale drinking

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Table 1. Summary of Treatment Processes Employed at the DWTPs Included in This Study (Adapted From Ref [9]) and the Concentration of Sucralose Determined in the Source, Finished and Distribution System Water for Each Site[a]

DWTP No.	treatment scheme	influence	source (ng/L)	finished (ng/L)	% removal	distribution (ng/L)
1	C/F → Sed. → Cl_2 → Filt. → NH_2Cl	ww	2400	2400	0	2400
2	ClO_2 → C/F → Sed. → Filt. → UV → Cl_2	ww	2900	1500	48	n/a
3	ClO_2 → C/F → Sed. → Filt. → UV → Cl_2	ww	790	1100[b] (680)	14	1100[b] (680)
4	Cl_2 → C/F → Sed. → Cl_2 → Filt. → Cl_2	ww	220	210	5	200
5	Cl_2 → O_3 → C/F → Filt. → Cl_2	ww	240	n/a	n/a	n/a
6	Cl_2 → O_3 → C/F → Filt. → Cl_2	ww	240	190	21	180
7	Cl_2 → C/F → Sed. → Cl_2 → Filt. → NH_2Cl	ww	2200	2000	9	n/a
8	Cl_2 → C/F → Sed. → Cl_2 → Filt. → NH_2Cl	ww	1500	1500	0	n/a
9	Cl_2 → C/F → Sed. → Cl_2 → Filt. → NH_2Cl	ww	400	370	8	n/a
10	Cl_2 → C/F → Sed. → Filt. → NH_2Cl	n	<MRL	<MRL	n/a	<MRL
11	Cl_2 → C/F → Sed. → Filt. → NH_2Cl	n	<MRL	<MRL	n/a	<MRL
12	Cl_2 → NH_2Cl	n	<MRL	<MRL	n/a	<MRL
13	Cl_2 → O_3 → C/F → Sed. → NH_2Cl	ww	150	130	13	120
14	Filt. → O_3 → NH_2Cl	n	<MRL	<MRL	n/a	<MRL
15	Cl_2 → C/F → Sed. → Cl_2 →Filt. → NH_2Cl	ww	100	n/a	n/a	93
16	C/F → Sed. → O_3 → dm-Filt. → NH_2Cl	r	81	55	32	53
17	Cl_2 → C/F → Sed. → Cl_2 → Filt. → NH_2Cl	r	47	49	(+4%)	48
18	O_3 → C/F → Sed. → GAC/sand bio-Filt. → NH_2Cl	r	72	108	(+50%)	n/a
19	O_3 → C/F → Sed. → GAC/sand bio-Filt. → NH_2Cl	r	62	98	(+58%)	n/a

[a] C/F: coagulation/flocculation; Sed.: sedimentation; Cl_2: free-chlorine (hypochlorite); Filt.; filtration; NH_2Cl: chloramine; ClO_2: chlorine dioxide: pre-ox.: O_3; ozonation; dm-Filt.: dual-media filtration; GAC/sand bio-Filt.; granular activated carbon/sand biofiltration; ww: wastewater; n: none; r: recreational; <MRL: below method reporting limit; n/a: not available for analysis. [b] The finished water for DWTP-3 is a blend, and the value in parentheses has a blending factor applied.

water treatment for their removal, where acesulfame was found to somewhat persistent, while sucralose was effectively removed by granular activated carbon in Scheurer et al.[7]

There is a growing body of work related to the artificial sweetener sucralose in the United States and Canada. Sucralose was measured in the marine and coastal waters of the U.S., as well as wastewater treatment plant effluent and river water subject to its discharge, where concentrations reached 1.9 μg/L in Mead et al.[2] Later, sucralose was detected in five out of five wastewater samples from three locations, at a median concentration of 1.5 μg/L in Ferrer and Thurman.[5] It was also detected in eight out of 22 surface water samples, in eight out of eight alluvial groundwater samples from two locations, and suggested as a conservative tracer of sewage effluent in surface and groundwater. Most recently, sucralose was demonstrated to be a viable indicator compound for wastewater loading in surface waters, where it was consistently found in wastewater, wastewater-influenced surface water and septic samples in Oppenheimer et al.[6] Sucralose occurrence has also recently been measured in urban groundwater in Canada, although acesulfame was found more consistently in Van Stempoort et al.[18] Sucralose has been found to be persistent through wastewater treatment processes in municipal plants in Torres et al.,[19] and some bench scale studies in Soh et al.[20]

Sucralose, a chlorinated form of sucrose, is an artificial sweetener that is approximately 600 times sweeter than sucrose and is widely used as a food additive.[21] It is not extensively adsorbed and metabolized in humans, resulting in the majority being excreted without transformation.[22] Sucralose is very soluble in water (282 g/L at 20 °C, Log $P = -0.51$),[23] so it is not expected to be extensively adsorbed to organic solids in the environment. Further, sucralose has been shown to be resistant to treatment processes used in the production of drinking water at the bench scale, such as oxidation by free chlorine and ozone.[20] Because of these properties, it is likely that sucralose may be widespread in drinking waters in the U.S., persisting through traditional drinking water treatment processes.

The objective of this work was to quantify the amount of sucralose present in select U.S. drinking water systems. To this end, an archived set of extracts from the study reported in Benotti et al.,[9] of the source, finished, and distribution system water from 19 U.S. DWTPs from 2006 to 2007, was analyzed for this compound. The relative ineffectiveness of commonly employed drinking water treatment strategies to remove sucralose is presented. The utility of this compound as an indicator for anthropogenic influence on drinking water systems, and as an indicator for other recalcitrant compounds in finished drinking water, is also discussed. To date, this manuscript represents the first study to quantify the artificial sweetener sucralose in U.S. drinking water systems.

EXPERIMENTAL SECTION

Sample Collection. The sampling sites consisted of the source, finished and distribution systems of 19 U.S. DWTPs. This is the same sample set analyzed by Benotti et al.[9] Only 10 of the 19 DWTPs had sites in the distribution system sampled, and one finished water sample extract from one DWTP was lost during storage and handling. The treatment process employed at each plant is summarized in Table 1. The hydraulic retention time was taken into account when collecting samples so that the data should represent, as closely as possible, the same plug of water moving through the treatment process.

Samples were collected in 1 L silanized, baked amber glass bottles containing the preservative sodium azide and ascorbic acid to quench any residual oxidant. All samples were cooled on ice, shipped overnight and stored at 4 °C in the laboratory for a maximum of 14 days until extraction. Travel blanks were included with each sampling event, and consisted of laboratory-grade water that was shipped with the bottles, and transferred to a sampling bottle during the sampling procedure. All samples were extracted using the SPE method described in Vanderford et al.,[24] and resulted in a 1000-fold concentration. Following extraction, the sample extracts in methanol were capped in airtight sample vials and stored at −80 °C until the time of analysis, which totaled 36−48 months depending on sampling and extraction dates. Prior to analysis, extracts were allowed to warm to room temperature and a positive displacement pipet was used to transfer 40 μL to an autosampler vial containing 60 μL of a solution of the isotope-labeled internal standard, sucralose-d_6 (Toronto Research Chemicals, Toronto, ON). After thorough mixing using a glass pipet, the sample was diluted 2.5 times to a final composition of 46% MeOH, 54% DI water, and an internal standard concentration of 200 ng/mL. The original extract was capped and returned to the −80 °C freezer, and the prepared extract was stored at 4 °C until analysis.

The source waters of the DWTPs included in this study display a range of anthropogenic influence. Only one of the DWTPs utilizes a groundwater source (DWTP-12), while the remaining employ surface water. The source water from DWTPs 1 to 9, 13, and 15 are impacted by wastewater inputs. The source waters for DWTPs 16 to 19 are reservoirs with recreational usage, but no known wastewater inputs. The remaining DWTPs have no known wastewater inputs or recreational usage.

Analysis Method. All extracts were analyzed by isotope dilution liquid chromatography−tandem mass spectrometry (LC-MS/MS). The chromatography was performed on an Agilent (Santa Clara, CA) 1200 system equipped with a Restek (Bellefonte, PA) Allure Organic Acids column, 150 × 3.2 mm with 5 μm particles. Mass spectrometric analysis was performed using an AB SCIEX (Foster City, CA) API-4000 QTrap operating with negative electrospray ionization in MRM mode. Sucralose was quantified using the $(M\text{-}H)^-$ precursor ion at m/z 395.0 and the Cl^- product ion at m/z 35.0 and confirmed using the $(M\text{-}H)^-$ precursor ion at m/z 397.0 and the Cl^- product ion at m/z 35.0. The internal standard was measured using the $(M\text{-}H)^-$ precursor ion at m/z 403.0 and the Cl^- product ion at m/z 35.0 in order to avoid significant contribution from the naturally occurring isotopes of sucralose. A product ion spectrum of the precursor ion at m/z 395.0 collected at a collision energy of 22 eV is shown in Figure 1. Further details on the analytical method can be found in the Supporting Information. Representative LC-MS/MS chromatograms for source water, finished water and travel blank extracts from a sampling event for DWTP-8 can also be found in the Supporting Information (Figure SI-1).

RESULTS AND DISCUSSION

Analytical Parameters and Data Quality. The percent recovery was determined by analyzing 10 samples of laboratory reagent water spiked with 250 ng/L of sucralose and with 200 ng/L of isotopically labeled internal standard, taken through the entire sample extraction procedure. The absolute recovery of sucralose was determined by external calibration (i.e., − no internal standard correction) to be 85 ± 7%. This represents the recovery of the sucralose in the samples through the extraction procedure. Relative recovery was determined by ratioing the sucralose to the internal standard in order to account for any analyte ionization suppression and extraction efficiency, which was calculated to be 99%. Since the internal standard was added postextraction in the procedure used for the archived extracts, this only corrects for ion suppression, but not for the extraction recovery. The results reported in this manuscript are reported as such, with no correction for extraction efficiency. It should be noted that the artificial sweeteners saccharin and acesulfame were also considered for analysis; however, the extraction procedure used in the original study[9] resulted in very low recoveries. Therefore, they could not be quantified in these extracts.

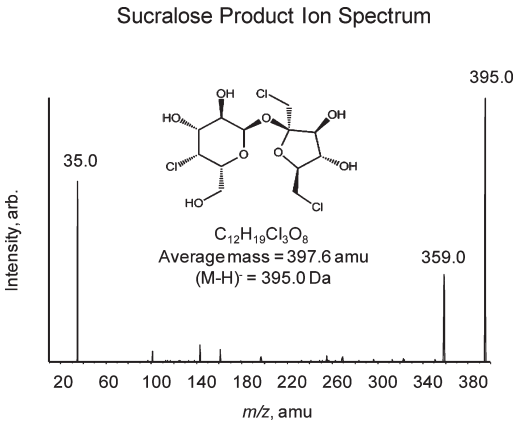

Figure 1. Sucralose product ion spectrum in negative ion mode with a collision energy of −22 V. The molecular structure, empirical formula, and average mass are included. The precursor ion is the $(M\text{-}H)^-$ species at m/z = 395.0 Da.

The minimum detection limit (MDL) was determined from the analysis of eight samples of laboratory reagent water spiked with 10 ng/L of sucralose and 200 ng/L of the internal standard. The samples were subjected to the entire sample preparation procedure and quantified by the LC-MS/MS method using internal calibration. The MDL was calculated from the resulting data by multiplying the standard deviation of these eight replicate measurements by the appropriate Student's T-value for $n - 1$ degrees of freedom, and determined to be 1.6 ng/L. The low calibrator and reporting limit was set to a factor 6 times higher, or 10 ng/L. A similar procedure has been used previously to determine MDL values for pharmaceutical compounds in water matrices in Vanderford et al.[24]

Because the analyses were performed on a set of sample extracts archived for 36−48 months at −80 °C, there are concerns with sample integrity during long-term storage. Sucralose has been shown to be a relatively inert and stable molecule, and is known to be very soluble in the storage solvent, methanol.[25] There were several duplicate samples collected throughout the study for source, finished drinking and distribution water. The majority differed by less than 10% of the average of the two values, although one distribution duplicate sample had a 15% difference. This error is well within that expected in typical water analysis methods, and indicates acceptable data quality. It also shows that the long-term storage conditions did not cause

significant differences between samples. Additionally, sucralose was not detected in any of the travel blanks, demonstrating the integrity of sample collection and handling procedures.

Sucralose Occurrence in Source and Finished Water. Sucralose was detected in the majority of source water samples, and displayed a strong dependence on the relative amount of anthropogenic influence. Those source waters that were impacted by wastewater all tested positive and displayed the highest concentrations, ranging from 100 ng/L (DWTP-15) to 2900 ng/L (DWTP-2), as shown in Table 1. Source waters that had no known wastewater inputs but were subject to recreational usage, such as boating and swimming, also tested positive but displayed lower concentrations than those impacted by wastewater. This sample group also displayed very similar levels to one another, ranging from 47 (DWTP-17) to 81 ng/L (DWTP-16), with sucralose likely being present from improper disposal of foodstuffs and human waste.[22] Those source waters that had no known wastewater inputs or recreational usage did not contain quantifiable levels of sucralose (DWTP-10 to 12, 14).

The concentrations reported in this work are comparable with previously published levels of artificial sweeteners [2−6] in surface and groundwater. Sucralose concentrations range higher than those reported in European surface waters,[3,4] but are comparable to those measured in American surface and ground waters,[2,5,6] likely due to the time period for its approved usage (beginning in 1998 in the U.S. versus 2004 in the E.U.), as well as differences in artificial sweetener usage patterns. For results obtained in U.S. waters, the concentrations reported here range up to 2900 ng/L, compared with 1900 ng/L by Mead et al., 2400 ng/L by Ferrer and Thurman, and 10,000 ng/L by Oppenheimer et al. Interestingly, these results are from surface water samples, with the exception of Ferrer et al., which is from a groundwater sample, demonstrating that this compound can be of concern even for those DWTPs with groundwater sources.

All of the DWTPs that used source water that tested positive for sucralose also had quantifiable amounts in the finished water, with the highest level at 2400 ng/L (DWTP-1). In fact, most DWTPs showed very little removal, including those that employed ozone as an oxidant. Counting any increases in concentration between finished and source water as 0% removal, the average removal across the plants with representative samples was 12%. DWTP-2 displayed the highest removal (48%), and employed ClO_2 preoxidation and UV treatment prior to chlorination. Although DWTP-3 has significantly less removal (14%), this still suggests that advanced oxidation processes (AOP) should be explored further for the removal of sucralose from drinking water.

Persistence of Sucralose in Distribution Systems. The distribution system water of the DWTPs whose finished water tested positive for sucralose also tested positive (Figure 2). Further, within expected experimental error, every distribution system sample tested at the same levels as the finished water. These results indicate that residual free chlorine or chloramines are not effective at removing sucralose during transit from the DWTP to the distribution sampling point. As mentioned in the Experimental Section, these sampling sites were chosen to be relatively far removed from the DWTP, so it can be expected that sucralose persists completely through the distribution system.

Every distribution water (i.e., tap water) that was produced from source water with measurable amounts of sucralose, also had sucralose present (Table 1, Figure 2). Therefore, it is

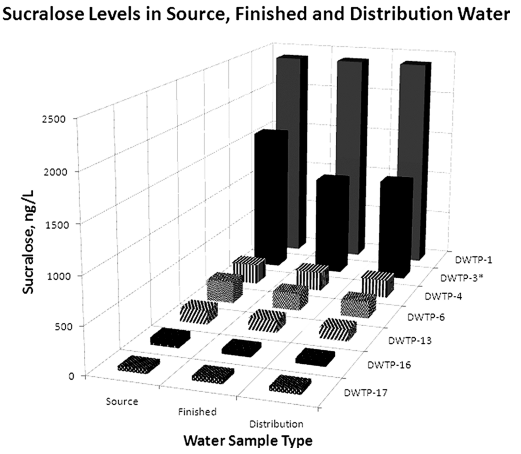

Figure 2. Comparison of sucralose concentration the source, finished and distribution water samples from seven utilities. These seven DWTPs represent those that had quantifiable amounts of sucralose in the source water, and had at least one finished and one distribution sample available for testing. *DWTP-3 was corrected for relative input to blend.

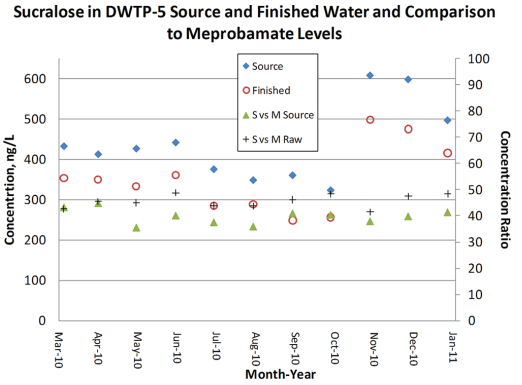

Figure 3. Sucralose concentrations in the source and finished water of DWTP-5 measured monthly by isotope dilution LC-MS/MS, and it is ratio to the meprobamate concentrations in the same sample sets. The average daily output of the DWTP during this time was 130 million gallons/day.

reasonable to expect that human exposure to sucralose via tap water is widespread in the U.S. Since the exact amount of tap water consumed daily on an individual basis varies, an average consumption amount of 2 L is estimated by the U.S. Environmental Protection Agency (US EPA).[26] Using this volume of tap water, the largest exposure to sucralose was from DWTP-1, where an individual would have been exposed to 4.8 μg of sucralose per day. The toxicological relevance of chronic exposure to this amount warrants further studies, but is beyond the scope of this paper.

11-Month Monitoring Program at DWTP-5. In order to test whether the presence of sucralose is consistent over time, the

Table 2. Comparison of the Average Concentrations, Median Concentrations and Concentration Range of Sucralose with Compounds That Were Consistently Present for Each Water Type with Either Wastewater (ww) or Recreational Usage (r) Influence from Supporting Information in Benotti et al.[9]

water type	influence	analyte	average concentration, ng/L	median concentration (ng/L)	concentration range (ng/L)	MRL (ng/L)
source						
	ww	sucralose	1000	400	150−2900	10
	ww	carbamazepine	17	4.8	1.9−51	0.50
	ww	phenytoin	13	8.7	2.5−29	1.0
	ww	meprobamate	20	10	3.2−73	0.25
	ww	sulfamethoxazole	35	15	1.6−110	0.25
	ww	atrazine	180	32	1.4−870	0.25
	r	sucralose	66	67	47−81	10
	r	carbamazepine	1.5	1.7	0.60−1.8	0.50
	r	meprobamate	3.0	3.1	1.6−4.3	0.25
	r	sulfamethoxazole	1.7	1.6	0.62−3.2	0.25
	r	atrazine	310	230	0.50−780	0.25
finished						
	ww	sucralose	1000	1100	130−2400	10
	ww	phenytoin	8.7	6.2	1.1−19	1.0
	ww	meprobamate	17	13	3.3−42	0.25
	ww	atrazine	150	59	0.88−870	0.25
	r	sucralose	78	77	49−108	10
	r	meprobamate	1.4	1.5	1.0−1.8	0.25
	r	atrazine	120	110	0.38−240	0.25
distribution						
	ww	sucralose	680	190	93−2400	10
	ww	phenytoin	5.3	3.6	1.1−15	1.0
	ww	meprobamate	14	6.1	2.7−38	0.25
	ww	atrazine	186	113	0.94−915	0.25
	r	sucralose	51	51	48−53	10
	r	meprobamate	1.5	1.5	1.3−1.6	0.25
	r	atrazine	0.47	0.47	0.36−0.58	0.25

concentration in the raw and finished drinking water at DWTP-5 was monitored over the 11-month period from March, 2010 until January, 2011 (Figure 3). This DWTP has an ozone contact time of 24 min and a chlorine contact time of ~1.5 h. The amount of sucralose was quantified by isotope dilution LC-MS/MS using the method outlined in the Experimental Section, with the exception that the isotope was spiked into the sample prior to extraction, and the methanol extract was analyzed without dilution. This procedure corrects both for losses due to extraction and handling, as well as any ion suppression during analysis. The concentration of sucralose was relatively stable in both sample sets over that time, indicating that the flux of sucralose to that body of water was relatively consistent. There was an increase in concentration between October and November, which is due to seasonal fluctuations in the mixing dynamics of the treated wastewater stream and the reservoir, as determined previously through water quality parameters.[27,28] The removal efficiency is consistently low through the treatment process (20% average removal), demonstrating the resistance of the compound to oxidation by both ozone and chlorine.

Sucralose as an Indicator Compound. Indicator compounds for drinking water have been defined as anthropogenic compounds that can be used to represent certain classes of compounds based on their similarities in occurrence and behavior under various environmental and treatment conditions.[29] They have also been defined as chemical compounds that denote the presence of different types of water influence, usually wastewater.[6] The data presented here confirm that sucralose is a reliable indicator compound for anthropogenic influence on source water across the United States. It is important to note that it is not only present in water that is directly impacted by wastewater, but also water where recreational use is permitted (with no known wastewater inputs). Given the poor removal of sucralose during the various treatment schemes, it is also a viable indicator compound in finished and distribution water for other resistant compounds, such as meprobamate and atrazine.[9,30] In fact, when the concentration of sucralose is ratioed to that of meprobamate for data collected over a 11-month period at DWTP-5, relatively consistent values are obtained for both the source (average ratio = 41, %RSD = 8.4) and finished (average ratio = 46, %RSD = 5.7) water (Figure 3).

A comparison of the sucralose data with those from the much broader study by Benotti et al.[9] reveals important trends. Table 2 shows the concentrations of the compounds that were

consistently present based on type (source, finished or distribution) and influence (wastewater or recreational usage). Sucralose, atrazine and meprobamate were the only analytes from this compound set that were present in source, finished and distribution water, regardless of the influence type. The source of sucralose and meprobamate is likely from direct human activities, whereas the majority of atrazine present is likely from agricultural application and runoff. Therefore, sucralose and meprobamate should represent better indicators of wastewater and/or direct human input to source water, and subsequently finished drinking and distribution (i.e., tap) waters. While the concentration of sucralose was much higher than the concentration of meprobamate for all of the waters tested, from an analytical standpoint, the concentrations are comparable in relation to the respective method reporting limits (MRLs). A comparison with the meprobamate data presented by Benotti et al.[9] is included in the Supporting Information (Table SI-1). Given expected decreases in the input of meprobamate from human usage,[31−33] sucralose will likely be the more important indicator compound in the future.

■ ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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APPENDIX D

Inorganic Chemistry

COMMUNICATION

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Two New Methods of Synthesis for the Perbromate Ion: Chemistry and Determination by LC-MS/MS

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Supporting Information

ABSTRACT: Historically, the synthesis of perbromate ion through conventional oxidation routes has proven elusive. Herein, we report perbromate ion formation through the reaction of hypobromite and bromate ions in an alkaline sodium hypobromite solution. Formation was established via LC-MS/MS analysis of the bromate and perbromate ions in the reaction solutions over a 13-day period. Furthermore, it was discovered that the perbromate ion was also formed as a result of the electrospray ionization process. Selective reduction of the bromate ion prior to analysis was used to confirm the two formation pathways.

Perbromate has captivated inorganic chemists attempting its synthesis since the 19th century.[1] It was not until 1968 that substantial amounts of perbromate ions were synthesized using fluorine gas and xenon difluoride to oxidize bromate ions.[2,3] The development of these methods of synthesis allowed investigation of the properties of perbromate, including reactions, stability, and thermodynamic properties.[3−7] In brief, it was found that once perbromate is formed, it is stable in aqueous solutions, which allowed the development of various characterization methods.[2,8−12] Unfortunately, the difficulties associated with these methods of synthesis have prevented the widespread study of perbromate chemistry.

It has been hypothesized that the main difficulties in synthesizing perbromate (oxidation potential, E^o = 1.763 V) are due to unfavorable reaction rates and a relatively high energy barrier between Br(V) and Br(VII), which limit the choice of a suitable oxidation agent.[4,6,13] While other studies reported the unsuccessful oxidation of a bromate ion to perbromate using ozone and other strong oxidants, oxidation was recently reported by researchers employing boron-doped conductive diamond electrodes.[14] Hydroxyl radicals generated during electrolysis of water converted small quantities of bromate to perbromate, which was characterized using cyclic voltammetry and thermogravimetric analysis.

Until the early 2000s, there were no known reports of perchlorate ions (ClO_4^-) in sodium hypochlorite solutions. Since then, several studies have established the presence of perchlorate in sodium hypochlorite solutions, as well as increases in perchlorate concentration in these solutions over time.[15−17] The potential regulation of perchlorate in drinking water and the widespread use of sodium hypochlorite in drinking water treatment prompted a specific study to evaluate the factors impacting its rate of formation in sodium hypochlorite solutions.[18] Sensitive analytical methodologies suited for quantitation of perchlorate in sodium hypochlorite[19] were developed and employed to determine the thermodynamic properties for perchlorate formation. It was found that hypochlorite (OCl^-) and chlorate (ClO_3^-) concentrations, as well as ionic strength, are all important factors in perchlorate formation.[18,20] Comparatively, hypobromite is a weaker oxidant (E^o = 0.760 V) than OCl^- (E^o = 0.890 V), although it is more reactive than hypochlorite.[21] However, hypobromite has also been shown to decompose to bromate in a similar fashion to hypochlorite.[22] Given the similarities in chemistries, it was hypothesized that perbromate (BrO_4^-) could form through a similar pathway involving reactions of hypobromite and bromate, as shown by Scheme 1.

Scheme 1. Decomposition of Hypobromite and Formation of Perbromate

$$OBr^- + OBr^- \rightarrow BrO_2^- + Br^-$$
$$BrO_2^- + OBr^- \rightarrow BrO_3^- + Br^-$$
$$BrO_3^- + OBr^- \rightarrow BrO_4^- + Br^-$$

The primary objective was to monitor the formation of bromate and perbromate via LC-MS/MS. Changes in bromate were also monitored using a modified ion chromatography (IC) method for cross-validation.[23] A previously described liquid chromatography tandem mass spectrometric (LC-MS/MS) method for bromate[19] was modified to include perbromate-specific multiple reaction monitoring (MRM) transitions of $^{81}BrO_4^-$ (m/z 145) to $^{81}BrO_3^-$ (m/z 129) and $^{79}BrO_4^-$ (m/z 143) to $^{79}BrO_3^-$ (m/z 127).

An alkaline solution of sodium hypobromite was prepared over the course of ∼2.0 h by slowly adding 25 mL of liquid bromine (Br_2) to a chilled 500 mL aqueous solution containing 8.25% sodium hydroxide (NaOH) by weight. The reaction vessel was maintained at 10−15 °C, while the oxidation reduction potential (ORP, mV) was continuously monitored to ensure that a rapid depletion of hydroxide ions did not occur. The prepared solution was standardized by iodometric titration, resulting in $[OBr^-]$ = 0.953 M. This solution was also found to contain $[BrO_3^-]$ = 2.27 mM, determined by the described LC-MS/MS

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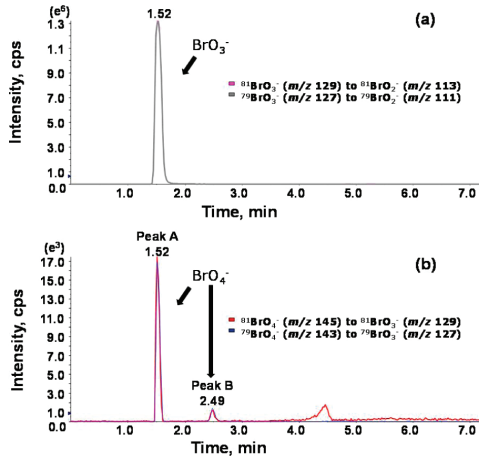

Figure 1. Extracted ion chromatograms of (a) bromate and (b) perbromate in the sodium hypobromite sample (diluted by a factor of 1000) after 13 days of incubation at 40 °C (note, cps = counts per second).

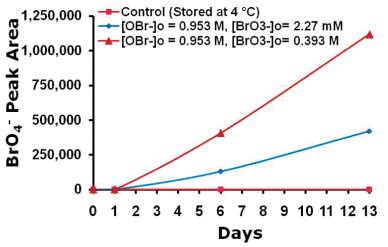

Figure 2. Formation of perbromate in sodium hypobromite solutions stored at 40 °C (control: $[OBr^-]_o$ = 0.953M, $[BrO_3^-]_o$ = 2.27 mM).

method. The stock solution of sodium hypobromite (pH 12.5) was split into three aliquots. The first was incubated at 40 °C in a water bath to increase the rate of decomposition of hypobromite. $NaBrO_3$ was added to the second aliquot to achieve a BrO_3^- concentration of 0.393 M (in duplicate) and incubated at 40 °C. The third aliquot was kept at 4 °C for the duration of the study, as a control sample. Changes in BrO_3^- and BrO_4^- were monitored over an incubation period of 13 days.

Figure 1 shows overlaid extracted ion chromatograms for bromate and perbromate in the sodium hypobromite sample (diluted by a factor of 1000) after 13 days at 40 °C. Two perbromate peaks, one coeluting with bromate at 1.5 min (peak A) and a second peak at 2.5 (peak B) min, were observed. The two peaks displayed both MRM transitions in the correct ratio for the naturally occurring isotopic abundance of Br. Peak B was determined to be due to perbromate formed during the reaction and was used to monitor the evolution of perbromate in incubated sodium hypobromite samples.

Figure 2 shows the formation of perbromate over incubation time at 40 °C in sodium hypobromite solutions at various initial bromate concentrations. As shown by Figure 2, perbromate

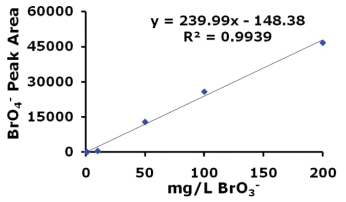

Figure 3. Change in perbromate peak A as a function of bromate concentration in aqeuous bromate solutions at pH 11.2.

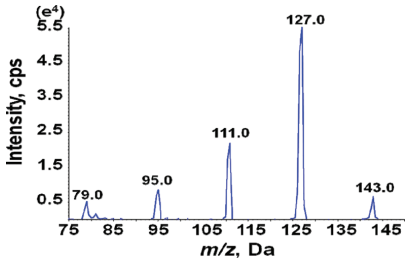

Figure 4. Product ion mass spectrum of $^{79}BrO^{4-}$ (m/z 143).

formation is strongly dependent on the initial concentration of bromate. As hypobromite decomposes, the bromate concentration increases (shown in the Supporting Information, Figure SI-1), allowing the formation of perbromate. In the sample spiked with bromate at the start of incubation, perbromate formed at a faster rate. These findings support the hypothesis that perbromate formation is dependent on reaction of the hypobromite and bromate.

The perbromate that coeluted with the bromate peak (peak A, 1.5 min, Figure 1) was found to be directly dependent on the presence of bromate. To investigate this phenomenon, various bromate standard solutions were prepared at a pH matching that of the diluted hypobromite samples. Bromate standard solutions of 1, 10, 50, 100, and 200 mg/L were adjusted to pH 11.2 using sodium hydroxide and analyzed by the described LC-MS/MS method. Figure 3 shows the increase in perbromate peak A area as a function of bromate concentration. Note that perbromate peak B was not detected in these solutions. These results confirm that perbromate peak A is due to perbromate formed solely from reactions involving bromate in the electrospray ionization process and represent a second viable synthesis route. Figure 4 shows the product ion spectrum for $^{79}BrO_4^-$ (m/z 143), from infusion of a 1000 mg/L BrO_3^- standard solution, and reveals BrO_3^-, BrO_2^-, BrO^-, and Br^- product ions. The product ion spectrum for $^{81}BrO_4^-$ (m/z 145) (Figure SI-2) and analytical details (Table SI-1) are provided in the Supporting Information.

Attempts were made to collect product ion mass spectra of perbromate peak B in the samples with LC-MS/MS. However, perbromate peak B lacked sufficient intensity to generate such spectra of acceptable quality.

To further confirm the two formation pathways for perbromate, the selective reduction of bromate was conducted in hypobromite samples. Earlier studies have shown that both bromate and perbromate are reduced by sulfite. However, at acidic pH, the reaction of perbromate is slow,[5] while the reaction

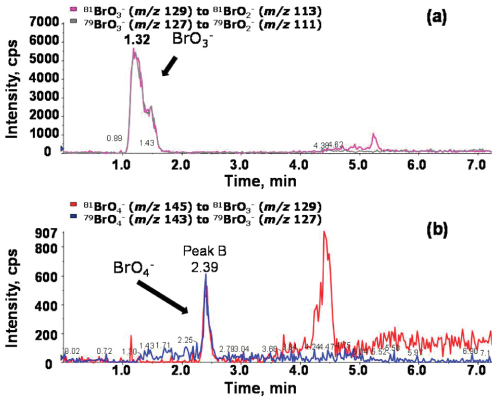

Figure 5. Extracted ion chromatograms of (a) bromate and (b) perbromate in sodium hypobromite sample spiked with 0.1 M HCl and 0.001 M SO_3^{2-}.

of bromate proceeds readily.[24,25] Given that sample solutions needed to be diluted by a factor of 1000 for LC-MS/MS analysis, it was possible to spike other chemicals in the process of dilution. Thus, hypobromite solutions were prepared, in duplicate, containing 0.1 M HCl and 0.001 M SO_3^{2-} (Figure 5).

Figure 5 illustrates that, when bromate is mostly reduced by sulfite, perbromate peak A is not present due to the decreased concentration of bromate. However, the later eluting perbromate peak B is still present and not significantly reduced by the sulfite. Therefore, the presence of perbromate peak B must be attributed to the oxidation of bromate by the hypobromite, further supporting the conclusions drawn from Figure 2.

This work shows that perbromate can form in concentrated sodium hypobromite solutions, likely via a pathway similar to perchlorate formation, although more work is needed to determine the rate law. The results also demonstrate that small amounts of perbromate are produced during electrospray ionization, and bromate can be selectively reduced without significantly affecting perbromate concentrations. The two methods of synthesis discussed in this study merit investigation beyond the scope of this work; however, the synthesis and analytical methods employed will likely be of interest to those involved in inorganic, analytical, and environmental chemistry.

■ ASSOCIATED CONTENT

Supporting Information. Formation of bromate over time during decomposition of hypobromite and product ion mass spectrum of $^{81}BrO_4^-$ (m/z 145). This material is available free of charge via the Internet at http://pubs.acs.org.

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