2013

Fate of 17α-Estradiol, 17β-Estradiol, and Estrone in Agricultural Soils and Sediments

Michael L. Mashtare Jr

Purdue University

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By Michael L. Mashtare Jr

Entitled
Fate of 17α-Estradiol, 17β-Estradiol, and Estrone in Agricultural Soils and Sediments

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

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Head of the Graduate Program

11/22/2013
Date
FATE OF 17α-ESTRADIOL, 17β-ESTRADIOL, AND ESTRONE IN AGRICULTURAL SOILS AND SEDIMENTS

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Michael L. Mashtare Jr.

In Partial Fulfillment of the
Requirements for the Degree

of

Doctor of Philosophy

December 2013

Purdue University

West Lafayette, Indiana
This dissertation is dedicated to my husband, Steven J. Landry, and my parents, Michael and Susan Mashtare, who have stood by me through it all.

*Valete, et gratias pro piscibus agimus!*
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ABSTRACT

Mashtare, Michael L., Jr. Ph.D., Purdue University, December 2013. Fate of 17α-Estradiol, 17β-Estradiol, and Estrone in Agricultural Soils and Sediments. Major Professor: Linda S. Lee.

The shift to concentrated animal production facilities and increasing rural-urban migration has increased the localized land application of nearly 1 billion tons of manure and biosolids annually. Although these applications provide nutrients and contribute to soil tilth, they also serve as a source for an estimated 49 tons of the natural manure-borne estrogens, 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), and estrone (E1). While these estrogens are critical to endocrine systems, the low concentrations observed in the environment can disrupt endocrine function in non-target organisms, e.g., altering secondary sex characteristics which can lead to changes in wildlife communities.

Research presented here focuses on understanding natural endocrine fate, specifically: (1) the sorptive behavior of 17α-E2 and 17β-E2 on agricultural soils using batch sorption experiments on seven autoclave-sterilized soils, with a range of properties, where both solution and soil phase concentrations were measured; (2) the aerobic biodegradation of 17α-E2 and 17β-E2 in soils using aerobic soil microcosms on two soils with different taxonomic properties, sacrificed over a 3-week period; and (3) the biotransformation of 17α-E2, 17β-E2, and E1 in sediments using anaerobic microcosms under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions. For all degradation studies, sterile controls were used to discern between biotic and abiotic transformations. Water, soil, and sediment extracts were analyzed for hormones using negative electrospray ionization tandem mass spectrometry. Electron acceptor concentrations and gas production were also quantified.
17α-E2 and 17β-E2 exhibited stereospecific sorption with the highest β/α sorption ratio being 1.9. Sorption was best correlated to soil organic carbon (OC) with average log OC-normalized distribution coefficients (L kg_oc⁻¹) of 2.97 ± 0.13 for 17α-E2 and 3.14 ± 0.16 for 17β-E2. No statistical difference, however, was observed between the aerobic degradation rates of the isomers, which were relatively fast (t½ < 0.5 d), with residuals persisting with time. Under all anaerobic conditions, stereospecific degradation was observed with the magnitude of t½ following 17β-E2 < 17α-E2 < E1. The observed t½ of 17β-E2 was rapid under all conditions (< 1.5 d), while 17α-E2 exhibited higher persistence with an observed t½ of 4.3 d to 69.3 d depending on the redox condition. Interconversion between 17α-E2 and 17β-E2 was observed, as was the reversible transformation from E1 back to its E2 precursors with a preferential formation of the more potent 17β-E2 (e.g., up to 33 mol % in iron-reducing conditions within 1 d). Under both aerobic and anaerobic conditions, E1 was the primary metabolite of both isomers and sterile controls support that the observed transformations were primarily biotically mediated. The anaerobic studies mirror the trends observed in a reconnaissance field study that monitored hormone concentrations in sediments taken quarterly over a 2-year period in an agro-impacted ditch and stream network using quarterly grab samples.

These findings are significant and suggest that careful attention is needed when evaluating resource and risk management strategies for these compounds. For example, given that 17α-E2 is more likely to be leached from agricultural soils than 17β-E2, assuming the isomers exhibit the same sorption behavior, as has been previously assumed, would underestimate the transport behavior for the α-isomer. Likewise, although the bulk of E2 appears to degrade within a day under aerobic conditions, using a first-order degradation model for E2 would fail to predict the residual concentrations remaining in the soil profile. In stream networks receiving hormone-containing discharge, hormones are likely to persist in anaerobic sediments. Given evidence of interconversion between 17α-E2 and 17β-E2 and the reversible transformation to E2 from E1 under reducing conditions, sediments may serve as both a source and a sink of hormones to the water column. Therefore, quantifying just the inputs into the water column from discharge and run off may not be sufficient for understanding the persistence of these compounds. This
suggests that further research is needed in water and nutrient management strategies, including controlled tile drains and bioreactors, where an anaerobic environment conducive to these transformations may be an unintended consequence.
CHAPTER 1. INTRODUCTION

1.1 Introduction

This introduction briefly sets the framework for the research needs on the sorption and transformation of the natural estrogens, 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), and estrone (E1), in agricultural soils and sediments. Detailed research objectives and an outline describing the organization of this dissertation follows.

Concerns over the environmental fate and impact of the natural manure-borne estrogens, 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), and estrone (E1), have increased with the shift of animal production facilities to more concentrated units and increasing rural-urban migration. Currently ~50% of the world’s population lives in urban areas with this number expected to increase to over 60% by 2030 (Cohen, 2006). The concentration of our waste streams has resulted in more localized land application of nearly 1 billion tons of manure and biosolids annually. Subsequently, an estimated 49 tons of natural estrogens are introduced into the soil environment where they may eventually reach surface and groundwater (as reviewed by Aga, 2008). These estrogens have been detected in soils, sediments, and surface waters in and near agricultural land. While estrogens are critical to our endocrine systems, their presence in the environment, even at low concentrations, have the potential to disrupt the endocrine function in non-target organisms which can alter secondary sex characteristics and lead to changes in wildlife communities (as reviewed by Hanselman et al., 2003, and Borch and Young, 2009). These low concentration effects and their widespread detection in the environment have raised concerns in recent years and have led to considerable research on the fate of 17β-E2. However, it is the stereoisomer, 17α-E2, and E1, the primary metabolite of E2, which often dominate in mammalian wastes and are frequently detected in surface water and sediments around agricultural land and wastewater treatment facilities (as reviewed
by Khanal et al., 2006; Cai, et al., 2012; Liu et al, 2012). Because of their perceived weak estrogenicity relative to 17β-E2 based on mammalian assays, few studies have looked at the fate of 17α-E2 and E1 (as reviewed by Aga et al., 2009). Recent studies, however, suggest that aquatic species may be significantly more sensitive to low exposures of 17α-E2 and E1 than previously believed (Thrope, et al., 2003; Huang et al., 2010; Shappel et al, 2010; Dammann, 2011).

While stereo-chemical assessments are standard in the pharmaceutical industry, they are less prevalent in environmental fate and effects studies (Stanley and Brooks, 2009). The primary goal of my research was to characterize the environmental fate of the stereoisomers, 17α-E2 and 17β-E2, and their primary metabolite, E1. **The specific hypotheses driving my research were that (1) the stereochemistry of these compounds (17α-E2, 17β-E2) would affect their behavior in the environment, and (2) the metabolite E1 could be converted back to the parent hormones, 17α-E2 and 17β-E2, under the right conditions, specifically reducing conditions.** The first hypothesis was based on stereospecific responses observed for other agro-chemicals, organic compounds, and hormones in the environment. Stereoselectivity in sorption (Oudou and Hansen, 2002; Khan et al., 2009; Heeb et al., 2010), aerobic degradation (Marucchini and Zadra, 2002; Li et al., 2012), and anaerobic degradation (Gerecke et al., 2006; Chen and Liu, 2009) have been reported for various organic compounds including pesticides, flame retardants, and hormones. The second hypothesis was based on the observed reversible transformation of E1 to E2 in isolated enzymatic studies (Renwick and Engel, 1967), in laboratory studies which reported the re-accumulation of E2 following its oxidation to E1 in lake sediments and sewage sludge (Czajka and Londry, 2006; Dytczak et al. (2008), and from our field observation study at the

![Figure 1.1. Estrogen concentrations (dry wt. basis) detected in sediment from ASREC in March 2009.](image-url)
During a 2-year reconnaissance field study from 2009-2010, quarterly grab samples were collected from the ditch and stream network to monitor hormone concentrations in the agricultural sediments. All 3 estrogens were routinely detected in ditch and stream sediments, with 17α-E2 and 17β-E2 concentrations significantly greater than E1. The highest concentrations of 17α-E2, 17β-E2, and E1 were 2.34 µg/kg, 3.98 µg/kg, and 0.50 µg/kg of dry sediments, respectively. This was surprising given that a 3-year EPA-funded study showed the tile drain discharge and stream surface water to contain significantly more E1 than E2 (Gall et al., 2011). We hypothesized that this disparity was due to the reversible transformation of E1 to 17α-E2 and 17β-E2 in the stream bed sediments which led to the development of Aim 3, discussed below.

The specific aims of my research were designed to fill data gaps by examining stereospecific differences in E2 sorption, aerobic degradation, and anaerobic degradation in soils and sediments, and quantifying the interconversion and reversible transformation of 17α-E2, 17β-E2, and E1 in sediments under controlled redox conditions. While the importance of these parameters may vary by scale, this information is needed to improve our ability to predict the fate of these hormones in the environment.

1.2 Specific Aims

- **Aim 1. To quantify the sorption coefficients of 17α-E2 and 17β-E2 in soil.**
  
  Batch sorption experiments (Figure 1.2) were used to test the hypothesis that E2 would exhibit stereoselective sorption in soils with 17α-E2 sorbing less strongly (having a smaller sorption coefficient, $K_d$) than 17β-E2.

  ![Figure 1.2. Batch sorption study.](image-url)
- **Aim 2. To quantify the aerobic degradation kinetics of 17α-E2 and 17β-E2 in soil.**

Aerobic soil microcosm studies (Figure 1.3) were used to test the hypotheses that (1) under aerobic conditions, E2 would exhibit rapid stereospecific degradation in soils (half-lives, $t_{1/2} < 3$ d) with 17α-E2 having a longer $t_{1/2}$ than 17β-E2, and (2) that E1 would be the primary metabolite of both isomers.

- **Aim 3. To quantify the anaerobic transformation kinetics of 17α-E2, 17β-E2, and E1 in agro-impacted stream sediment.**

Anaerobic sediment microcosm studies (Figure 1.4) under controlled redox conditions were used to test the hypotheses that (1) under anaerobic conditions, E2 would exhibit stereospecific degradation in stream sediments with the $t_{1/2}$ of 17α-E2 longer than 17β-E2; (2) degradation rates would vary under different anaerobic conditions as follows: iron-reducing > sulfate-reducing > methanogenic > nitrate-reducing conditions; and (3) interconversion would be observed between the isomers with E1 as the intermediate, reversibly transforming to its precursors with a preferential formation of 17β-E2 under all reducing conditions.
1.3 **Organization**

- **Chapter 2.** A literature review on the natural estrogens, 17α-E2, 17β-E2, and E1, including the physiochemical properties, biosynthesis pathways, excretion rates, environmental pathways, degradation pathways and probable metabolites, toxicology, and environmental fate, is presented. Several pieces of this unpublished work have been incorporated into published works (Chapters 3-5) as well as this introduction and Chapter 6.

- **Chapter 3:** This chapter focuses on the sorption of 17α-E2 and 17β-E2 in agricultural soils. Sorption isotherms were measured on seven surface soils with a wide range of taxonomic properties to assess whether the stereoisomers exhibited the same sorption affinities. [Published: Mashtare, M. L.; Khan, B.; and Lee, L. S. 2011. Evaluating stereoselective sorption by soils of 17α-estradiol and 17β-estradiol. *Chemosphere.* 82: 847-852. Reproduced with permission from Elsevier Ltd, Copyright 2010.]

- **Chapter 4:** This chapter focuses on the transformation of 17α-E2 and 17β-E2 in aerobic agricultural soils. Aerobic biotransformation rates were quantified in batch microcosms over a 3-week period to assess whether the E2 stereoisomers exhibited the same degradation patterns. The appropriateness of a first order exponential decay model in modeling the fate of these hormones is discussed. [Published: Mashtare, M. L.; Green, D. A.; and Lee, L. S. 2013. Biotransformation of 17α- and 17β-estradiol in aerobic soils. *Chemosphere.* 90: 647-652. Reproduced with permission from Elsevier Ltd, Copyright 2012.]

- **Chapter 5:** This chapter focuses on the transformation of 17α-E2, 17β-E2, and E1 in agricultural sediments under nitrate-reducing and sulfate-reducing conditions. Anaerobic microcosms under controlled redox conditions were used to assess whether the stereoisomers exhibited the same degradation patterns and whether transformation occurred between isomers including reversible transformation of E1 to E2. [Published: Mashtare, M. L.; Lee, L. S.; Nies, L. F.; and Turco, R. F. 2013. Transformation of 17α-estradiol, 17β-estradiol, and estrone in sediments under nitrate- and sulfate-reducing conditions. *Environ. Sci.*]
Chapter 6: This chapter focuses on the transformation of 17α-E2, 17β-E2, and E1 in agricultural sediments using a similar approach for similar purposes as defined in Chapter 5, but under iron-reducing and methanogenic conditions. In addition, more extensive efforts were made with abiotic controls, with and without sediment, to discern between biotic and abiotic contributions for all reducing conditions. [Manuscript in preparation: Mashtare, M. L.; Jenkinson, B.; Lee, L. S.; Nies, L. F.; and Turco, R. F. 2013. Anaerobic biotransformation in sediments of 17α-estradiol, 17β-estradiol, and estrone under iron-reducing and methanogenic conditions.]

Chapter 7: The major findings from these studies are summarized, followed by a discussion on the environmental implications and future research needs.
2.1 Physiochemical Properties of 17α-Estradiol (17α-E2), 17β-Estradiol (17β-E2) and Estrone (E1)

As shown in Figure 2.1, all three hormones consist of a four (tetracyclic) carbon ring backbone with an aromatic A-ring, and OH groups located at the C3 position (Fang et al., 2008). At the C-17 position, E1 has a ketone, while both E2 isomers have an OH group. The structural difference between 17α-E2 and 17β-E2 is the orientation of the hydroxyl group on C17 of the D-ring with the OH group being oriented out of the general tetracyclic plane (Table 1). Structures and physiochemical properties are shown in Table 2.1.

2.2 Biosynthesis and Metabolic Pathways for 17α-E2, 17β-E2, and E1

The biosynthesis of 17α-E2 and 17β-E2, E1 begins with cholesterol which is transformed to androstenedione via 2 multi-step pathways:

i. Cholesterol $\Rightarrow$ pregnenoione via cholesterol side-chain cleavage (CYP11A) $\Rightarrow$ progesterone via 3β-hydroxysteroid dehydrogenase (3β-HSD) $\Rightarrow$ 17α-hydroxyprogesterone via 17α-hydroxylase and 17,20 lyase activity (CYP17) $\Rightarrow$ androstenedione via 3β-HSD

ii. Cholesterol $\Rightarrow$ pregnenoione via CYP11A $\Rightarrow$ 17α-hydroxypregnenoione via CYP17 (also transformable to hydroxyprogesterone and subsequently androstenedion via 3β-HSD) $\Rightarrow$ dehydroepiandrosterone via CYP17 $\Rightarrow$ androstenedione via 3β-HSD
An abbreviated schematic of the relevant pathways following androstenedione formation are shown in Figure 2.2.

Table 2.1. Physical Properties of 17αE2, 17β-E2, E1.

<table>
<thead>
<tr>
<th>Structure</th>
<th>17α-E2</th>
<th>17β-E2</th>
<th>E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>C₁₈H₂₄O₂</td>
<td>C₁₈H₂₄O₂</td>
<td>C₁₈H₂₂O₂</td>
</tr>
<tr>
<td>MW (g mol⁻¹)</td>
<td>272.39</td>
<td>272.39</td>
<td>270.37</td>
</tr>
<tr>
<td>Log Kᵦw</td>
<td>3.57ʰ, 3.73ʰ</td>
<td>3.94ᵃᵇ, 4.01ᶜ, 3.76ʰ</td>
<td>3.43ᵃᵇ, 3.13ᶜ, 3.53ʰ</td>
</tr>
<tr>
<td>Log Kᵦo</td>
<td>2.97ⁱ</td>
<td>3.14ⁱ</td>
<td>3.47ᶜ</td>
</tr>
<tr>
<td>Log Kᵦwⁱ</td>
<td>1.87</td>
<td>1.95</td>
<td>NA</td>
</tr>
<tr>
<td>Log Kᵦwⁱ</td>
<td>1.13</td>
<td>0.82</td>
<td>3.20</td>
</tr>
<tr>
<td>VP (Pa)ᵃᵇ</td>
<td>3E-8</td>
<td>3E-8</td>
<td></td>
</tr>
<tr>
<td>Kᵦ (atm³ molecule⁻¹)ᶠ</td>
<td>3.64E-11</td>
<td>3.8E-10</td>
<td></td>
</tr>
<tr>
<td>Sₜ (20°C, mg L⁻¹)</td>
<td>3.9ʲ</td>
<td>13ᵃ</td>
<td>13ᵃ</td>
</tr>
<tr>
<td>Sₜ (23°C, mg L⁻¹)ᵇ</td>
<td>3.1±0.02</td>
<td>2.1±0.03</td>
<td></td>
</tr>
<tr>
<td>Sₜ (25°C, mg L⁻¹)ᵈ</td>
<td>3.85</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>pKₐ</td>
<td>10.23ᵇᶜᵈ</td>
<td>10.40ᵇ, 10.34ᶜᵈ</td>
<td></td>
</tr>
<tr>
<td>Size (nm)ᶠ</td>
<td>0.398</td>
<td>0.398</td>
<td>0.396</td>
</tr>
<tr>
<td>MP (°C)ᵍ</td>
<td>173-179</td>
<td>254.5-256</td>
<td></td>
</tr>
</tbody>
</table>


Androstenedione is the immediate precursor to estrone (E1) via aromatase (CYP19). 17α-E2 is formed from E1 via 17α-HSD. Testosterone is formed from androstenedione via 17β-HSD and serves as the immediate precursor for 17β-E2 via CYP19. 17β-E2 is transformed to E1 via 17β-HSD. Estriol (E3) is formed directly from either 17β-E2 via 16α-OHase, or through E1 via 16α-OHase and 17β-HSD through the intermediate, 16α-hydroxyestrone (16α-OH-E1, Figure 2.3). Reversible transformations
have been observed between testosterone and androstenedione, 17α-E2 and E1, and 17β-E2 and E1, via their respective hydroxysteroid dehydrogenase (HSD).

**Figure 2.2.** Biosynthesis pathways of 17α-E2 and 17β-E2. Adapted from Sanderson and van den Berg (2003) and Renwick and Engel (1967).

The enzymes required for reversible transformations (e.g., HSD) of E2 and E1 have been isolated in lab studies from a range of mammals including humans, rats, mice, swine, chickens, and guinea pigs, (Peltoketo et al., 1999); bacteria, fungi, yeasts, algae, and protozoa (Donova et al., 2005). This reversibility is important for steroid hormone level regulation, physiology, and disease. Specifically, 17-HSD controls the formation of active estrogens, while also having the ability to deactivate them by transforming them to compounds with lower biological activity when needed, to optimize development, growth, and reproductive function (Labrie et al., 1997). The rate of hormone formation from its precursors, however, is dependent on the level of expression of the enzyme present in the tissue. While oxidation appears to be metabolically favored, the properties and structures of enzymes likely impact the behavior of the enzymes, in some cases driving the synthesis almost exclusively in 1 direction or the other. There is also some evidence in fungi, that reversible reduction is dependent on the NADPH/NADP+ and NADH/NAD+ ratio (Donova et al., 2005). As noted earlier, biologically, reversible transformations are
possible between testosterone and androstenedione, 17α-E2 and E1, and 17β-E2 and E1, via their respective HSD, demonstrating the versatility and importance of these enzymes. It is unclear, however, how many of these pathways are relevant in environmental systems.

While 17β-reduction is relatively common (although oxidation is preferred), 17α-reduction is more rarely observed (Donova et al., 2005). In a pure enzyme study with isolates from chicken livers, Renwick and Engel (1967) found that 17β-HSD activity was faster, had a higher saturation limit, and was more stable than 17α-HSD. After identifying unique microbial fingerprints of nitrified activated sludge repeatedly amended with 17α-E2, 17β-E2, or E1, results from a study by Yu et al. (2005) suggests that while non-specific HSD do exist (overlap), some microbes may be better equipped than others to degrade each respective hormone, expressing selectivity for specific substrates.

2.3 Excretion of 17α-E2, 17β-E2, and E1

All humans and animals naturally produce and excrete estrogenic hormones. Excretion rates and type vary by species, sex, age, and whether undergoing veterinary or pharmaceutical supplementation (Hanselman et al., 2003). Veterinary use includes the administration of estrogen as a growth promoter for cattle, swine, and poultry as implants using E2, or the synthetic analogues benzoate or palmitate esters of estradiol which are then readily hydrolyzed to the biologically active E2 form (Ivie et al., 1986; Casey et al., 2003). Pharmaceutical applications include hormone replacement in the form of E1 and E2 (de Mes et al., 2005). Steroids, however, are not typically biosynthesized by prokaryotes, although Methlococcus capsulatus and Nannocystis exedens are some noted exceptions (Ismail and Chiang, 2011).

While the primary focus in most environmental fate studies are the free hormones because of their estrogenic potency, most estrogens and their metabolites are enzymatically conjugated with sulphate and glucuronide esters in the C-3 and C-17 positions after biosynthesis. Excretion data for humans and select animals is provided in Table 2.2.
Table 2.2. Excretion rates for humans and select animals.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Reproductive stage</th>
<th>Excretion rates (ug/d)</th>
<th>Estrogens measured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fecal Excretion</td>
<td>Urinary Excretion</td>
</tr>
<tr>
<td>Dairy cattle</td>
<td>Not-pregnant</td>
<td>400-600</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Pregnant</td>
<td>300-11400</td>
<td>700-163000</td>
</tr>
<tr>
<td>Sows</td>
<td>Not-pregnant</td>
<td>600-900</td>
<td>400-600</td>
</tr>
<tr>
<td></td>
<td>Pregnant</td>
<td>1000-1600</td>
<td>4400-108000</td>
</tr>
<tr>
<td>Poultry</td>
<td>Non-laying</td>
<td>400-1400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Laying</td>
<td>1400-2700</td>
<td></td>
</tr>
<tr>
<td>Human (f)</td>
<td>Non-pregnant</td>
<td>11.5 (26-54)c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnant</td>
<td>859 (7693)c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Menopause</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Human (m)</td>
<td></td>
<td>5.5</td>
<td></td>
</tr>
</tbody>
</table>

a Adapted from Hanselman et al. (2003).
b Adapted from Ying et al., (2002), menopausal rates do not include hormone replacement therapy.
c Excretion rates when pregnant increase with gestation period, thus higher values represent near-term pregnancy. For animals, data is normalized to 1000 kg LAM. For humans, they are on a per (average) person basis.
d Measured hormones do not necessary indicate whether hormones were detected (i.e., some hormones may not be accounted for in some studies, while in other studies they may have analyzed for a hormone that was not detected, or in negligible concentrations).

2.4 Environmental Pathways

The primary environmental pathway of human-borne estrogens is through waste water treatment plant (WWTP) discharge and the land application of biosolids (Belfroid et al, 1999; Fine et al., 2003). As previously noted, estrogens are typically conjugated prior to excretion. Although this conjugation increases the solubility and potential
mobility in the environment, until recently there has been little concern because the conjugated forms were thought to be biologically inactive (de Mes et al., 2005; Kvanli et al., 2008). Measurements of WWTP inflow and effluent estrogen concentrations, however, often show a marked increase of free biologically active estrogens in the effluent compared to the inflow levels. Bacteria, such as E. Coli, found in sludge and feces has been found through enzymatic activity to readily hydrolyze many of the conjugated metabolites back to free unconjugated forms (de Mes et al., 2005; Fujii et al, 2002; Kvanli et al, 2008). The glucuronide conjugated forms are typically rapidly deconjugated via glucuronidase (Duong et al., 2011), however, the sulfonated forms have been found to be more resistant to biodegradation and thus may be more persistent (D’Ascenzo et al., 2003).

While some studies have suggested that sludge was able to fully degrade these free compounds, Fujii et al. (2002) inferred that in many cases these estrogens were merely sorbed to the activated sludge as evidenced by their frequent detection in sludge and biosolids (Higgins, et al., 2010). E1 and E2 have also been detected in effluents in relevant concentrations released from WWTPs (de Mes et al., 2005; Vader et al., 2000). However, the disposal and land application of manure onto agricultural land is the most significant contributor to the release of estrogens into the environment. While excretion type varies by animal (with both conjugated and free form excretion of E1 and E2), the contribution to global estrogen exposure from cattle is an order of magnitude greater than that of humans (reviewed by Hanselman et al., 2003; Raman et al., 2004; Sarmah et al., 2006).

Because of moderate to strong sorption of hormones by soils and their rapid degradation based on laboratory studies, transport to surface waters from land applied biosolids and manure was not expected. Residues in ground and surface waters, however, have been routinely detected with higher concentrations near agricultural land and WWTPs (reviewed by Hanselman, et al, 2003). Despite growing concern about the potential ecological impacts, however, routine monitoring of waterways for estrogenic compounds is not currently mandated. While attempts (H. R. 1311 and H. R. 1712) to amend the Federal Water Pollution Act to include estrogenic compounds in the water
monitoring guidelines have failed, 17α-E2, 17β-E2, and E1 were added to the EPA’s Drinking Water Contaminant Candidate list in 2009.

2.5 Degradation Pathways and Probable Metabolites

The degradation of 17α-E2 and 17β-E2 in environmental samples has been shown to primarily form E1 which is theorized to further metabolize via cleavage of rings A, B, or D, resulting in humus-like macromolecules. Enzymes needed to metabolize these transformations are frequently found in soils and are produced by bacteria, fungi, and plants (reviewed by Hanselman et al., 2003).

Figure 2.3. Synthesis of E1 and E2 transformations. Adapted from Xuan et al. (2008); Combalbert and Hernandez-Raquet (2010), Zheng et al. (2012), Mashtare and Lee (2013).

Figure 2.3 summarizes the generally accepted E1 and E2 transformations in environmental media from the literature. With the exception of estratetraenol (proposed by Nakai et al., 2011) and E3 formation from 17α-E2 (proposed by Xuan et al., 2008), the transformation pathways are identical to the biotransformation pathways discussed earlier. Three dominant ring cleavage pathways are discussed briefly below.
Coombe et al. (1966) and Shi et al. (2004) suggested that 2 common bacteria found in soils, *Nocardia sp.* (nitrifying bacteria) and *Nitrosomonas spp.* (ammonia oxidizing bacteria) rapidly degrade E1 with the eventual cleavage of ring A (followed later by cleavage of ring B). Degradation products I, II, and III were observed in isolated pure culture studies (Figure 2.4).

**Figure 2.4.** Cleavage of Ring A and B. Adapted from Coombe et al. (1966).

Results by Jurgens et al. (2002) and Bradley et al. (2009) both support the ring A cleavage pathway after noting mineralization and CO$_2$ being the end product using 17β-E2 and E1. Cleavage was observed in most, but not all sediments tested, however, suggesting that the microbial communities needed for E1 degradation were not present...
(or in sufficient numbers) in the all sediments. Although Shi et al. (2004) reported 3 unidentified polar metabolites which were quickly removed, no intermediates were detected prior to cleavage in the Jurgens et al. (2002) or Bradley et al. (2009) studies. A formal pathway for Ring A and B cleavage as proposed by Coombe et al. (1966) is shown in Figure 2.4.

To determine whether ring A and B cleavage was viable, a meta-cleavage inhibitor (3-chlorocatechol) was used by Kurisu et al. (2010) in their soil isolates study (Figure 2.5a) in an attempt to identify the intermediate products prior to cleavage. They found that the cleavage of ring A was supported via formation of 4-OH-E2. Detection of other metabolites also supported an alternative pathway suggesting possible ring B cleavage (which was also proposed by Haiyan et al., 2007, Figure 2.5b). Interestingly, none of the metabolites of E1 were detectable when 3-chlorocatechol was not added, suggesting that transformation was essentially instantaneous and thus detection of these hormones in environmental samples may pose a unique challenge.

Lee and Liu (2002) proposed a pathway in which the initial cleavage of E1 occurs on Ring D (Figure 2.6). They hypothesized that ring D cleavage was the most likely initial step in degradation after detecting a new intermediate “X1,” later identified as a lactone. Ternes et al. (1999) reported ring D cleavage in their study with activated sludge.

Given the prevalence of E1 and E2 in the environment, adaptation is generally not an issue in soils or areas near wastewater treatment facilities or agricultural settings because of long-term exposure to estrogenic compounds. When exposing the estrogenic compounds to a microbial community from industrial sludge, however, the microbial population was not able to degrade the estrogenic compounds. This suggests that in most areas, no adaptation is required for the degradation of E1 and E2 because of prolonged exposure to the compounds in nature; however, it also suggests that exposure of estrogenic compounds to soils or sludge that are unfamiliar with the compounds may
Figure 2.5. Meta-cleavage inhibition and Ring B cleavage. Meta-cleavage pathway is adapted from Kurisu et al. (2010) and Ring B cleavage study is adapted from Haiyan et al. (2007).

Figure 2.6. Ring D cleavage. Adapted from Lee and Liu (2002).
require an adaptation period (Colucci et al, 2001; Layton et al, 2000). What is also unclear, is the degradation efficacy in oxygen-limited environments. Select reported half-lives from the literature, excluding the half-lives reported in this dissertation and subsequent publications, are shown in Table 2.3.

Table 2.3. Select half-lives of 17αE2, 17β-E2, and E1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>17α-E2</th>
<th>17β-E2</th>
<th>E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Not determined</td>
<td>0.2-8.7</td>
<td>0.1-10.9</td>
</tr>
<tr>
<td>Soil</td>
<td>Not determined</td>
<td>0.2-0.5^b</td>
<td>0.6-1.7^b</td>
</tr>
<tr>
<td>Sediments (aerobic)^a</td>
<td>Not determined</td>
<td>0.11</td>
<td>0.42</td>
</tr>
<tr>
<td>Sediments (anaerobic)</td>
<td>Not determined</td>
<td>0.66^a-27^c</td>
<td>11.5-14.3^a</td>
</tr>
<tr>
<td>Photolysis</td>
<td>Not determined</td>
<td>3.5^d-10^a</td>
<td>0.2^h</td>
</tr>
<tr>
<td>Mixed lagoon water (anaerobic)^f</td>
<td>2.26</td>
<td>0.78</td>
<td>27.24</td>
</tr>
<tr>
<td>Soil (80:20 sterile to unsterile mixture)^g</td>
<td>1.9</td>
<td>0.92</td>
<td>2.7</td>
</tr>
</tbody>
</table>


2.6 Toxicology

Exposure to low (ng L⁻¹) concentrations of these natural estrogens has been shown to disrupt endocrine function, activate hormone responses, and alter secondary sex characteristics in non-target organisms at low concentrations routinely detected in the environment (less than 1 ng/L to 5 ng/L) (Aga, 2008; Hanselman, 2003; Young and Borsch, 2009). Observed ecological effects include endocrine disruption of frogs, turtles and other aquatic species, the feminization of fish including the formation of ova in the testes of Japanese medaka, increase in female phenotypes in fish populations, and vitellogenin production in male zebra fish (Hansen, 1998; Fujii et al, 2002; Hanselman, 2003; Sarmah et al., 2006). While much of the focus has been on 17β-E2, the most potent natural estrogen in mammalian species, recent research shows that aquatic species may
be significantly more sensitive to low exposures of 17α-E2 and E1 than previously believed (Huang et al., 2010; Shappel et al., 2010; Dammann, 2011). While the impact on human health at environmentally relevant concentrations is unclear, estrogens have been linked to the increased risk of cancers of the reproductive organs in both males and females and adverse effects on male fertility (de Mes, 2005).

2.7 Environmental Fate Summary

The environmental fate of 17β-E2 has been extensively studied. The sorptive behavior of 17β-E2 has been previously characterized in soils (Lee et al., 2003; Casey et al., 2005; Ying and Kookana, 2005; Hildebrand et al., 2006; Bonin and Simpson, 2007; Caron et al., 2010), sediments (Lai et al., 2000, Yu et al., 2004), soil minerals (Van Emmerik et al., 2003), and in the presence of dissolved organic matter (Lee et al., 2012). Degradation studies on 17β-E2 have been conducted in aerobic soils (Colucci et al., 2001; Das et al., 2004; Jacobsen, 2005; Ying et al., 2005; Lucas and Jones, 2006; Xuan et al., 2008); anaerobic/saturated soils (Ying and Kookana, 2005; Fan et al., 2007; Carr et al., 2011); aquifer materials (Ying and Kookana, 2008); marine sediments (Ying and Kookana, 2003b), riverwater and sediments (Jurgens et al., 2002; Sarmah et al., 2006; Bradley et al., 2009); activated sludge (Ternes et al., 1999; Joss et al., 2004; Dytczak et al., 2008), anaerobic lake sediments (Czajka and Londry, 2006); and a dairy lagoon water mixture (Zheng et al., 2012). In general, 17β-E2 was found to exhibit moderate to moderately-high sorption, degrade rapidly in the aerobic environment, and persist longer under anaerobic conditions. As previously discussed, the biological removal efficacy of estrogens within WWTP varied depending on microbial communities present. Select physiochemical properties and degradation rates have been summarized in Tables 2.1 and 2.3.

Less has been reported about the environmental fate of 17α-E2 and E1. Prior to our work, laboratory studies on 17α-E2 had been limited to a single sorption study exploring the partitioning behavior of 17α-E2 in activated sludge (Gomes et al., 2011), and degradation studies in soil bacteria cultures (Turffitt, 1947a, 1947b), a dairy lagoon water mixture (Zheng et al., 2012), and an 80/20 % by wt. sterile/unsterile soil mixture
Laboratory degradation studies on E1 focused on aerobic soils (Colucci et al., 2001; Ying et al., 2005); aerobic stream sediments (Bradley et al., 2009); anaerobic soils (Ying et al., 2005); anaerobic river water and sediments (Jurgens et al., 2002); activated sludge (Joss et al., 2004); and a dairy lagoon water mixture (Zheng et al., 2012). Select physiochemical properties and degradation rates have been summarized in Tables 2.1 and 2.3.

Evidence of interconversion between the E2 isomers and reversible transformations from E1 to E2 under anaerobic conditions have been increasingly reported in the literature. Recent field studies have reported the apparent conversion of 17α-E2 to 17β-E2 in a simulated feedlot under saturated conditions (Mansell et al., 2011) and in dairy manure and waste lagoons (Zheng et al., 2008). Hutchins et al. (2007) also reported unexpected elevated concentrations of 17α-E2 in swine and poultry lagoons suggesting interconversion between 17β-E2 and 17α-E2. Reversible transformation from E1 to 17β-E2 has also been observed in algae ponds (Shi et al., 2010). These observations, however, have only been inferred based on changes in the relative hormone concentrations at the field sites. Laboratory studies supporting the potential for interconversion had been limited to activated sludge (Dytczak et al., 2008) and anaerobic lake sediments (Czajka and Londry, 2006), which reported 17β-E2 to 17α-E2 conversions, presumably through E1. Neither of these studies, however, looked at the transformations of 17α-E2 or E1. More recently, Zheng et al. (2012) reported the interconversion and reversible transformation of these hormones in a dairy lagoon water mixture supporting the results reported in Chapter 5 and 6 of this dissertation. It is clear, however, that significant gaps remain in our understanding of 17α-E2 and E1.
CHAPTER 3. EVALUATING STEREOSELECTIVE SORPTION BY SOILS OF 17α-ESTRADIOL AND 17β-ESTRADIOL

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3.1 Abstract

The application of manure and biosolids onto agricultural land has increased the risk of estrogenic exposure to aquatic systems. Both 17α-estradiol (17α-E2) and 17β-estradiol (17β-E2) have been routinely detected in surface and ground waters with higher concentrations reported near concentrated animal feeding operations and agricultural fields. Although movement through the soil to a water body is highly dependent on hormone-soil interactions, to date, only the interaction of 17β-E2 with soils has been characterized despite 17α-E2 often being the more common form excreted by livestock such as beef cattle and dairy. In predicting the transport of estradiol, sorption characteristics for the stereoisomers have been assumed to be the same. To evaluate this assumption, sorption of 17α-E2 and 17β-E2 was measured on seven surface soils representing a range in soil properties. Soils were autoclave-sterilized to minimize loss due to biotransformation, and both solution and soil phase concentrations were measured. Overall, E2 sorption is best correlated to soil organic carbon (OC) with an average log OC-normalized distribution coefficient (log $K_{OC}$, L kg$_{OC}^{-1}$) of $2.97 \pm 0.13$ for $\alpha$E2 and $3.14 \pm 0.16$ for $17\beta$-E2 with $17\beta$-E2 consistently exhibiting higher sorption than 17α-E2 with the highest $\beta/\alpha$ sorption ratio of 1.9. Assuming that the two isomers sorb the same is not a
conservative decision making approach. The lower sorption affinity of 17α-E2 increases the likelihood that it will be leached from agricultural fields.

3.2 Introduction

In recent years, natural steroidal estrogen hormones, including 17α-estradiol (17α-E2) and 17β-estradiol (17β-E2), have been routinely detected in surface and ground waters (Khanal et al., 2006). Although naturally produced by humans and animals, pharmaceutical and veterinary supplementation has been linked to an increase in the production and excretion of estrogens, particularly by livestock (Lange et al., 2002). The type and quantity of estrogen production varies by species, sex, physiological and developmental state, and age. For example, beef cattle and dairy primarily excrete 17α-E2, while swine and poultry primarily excrete 17β-E2 (Hanselman et al., 2003). The primary pathways of steroid estrogens into the environment are through the discharge of sewage wastewater; the overflow, leakage and runoff from storage facilities and feedlots; and the land application of manure, effluent, and biosolids (Ying et al., 2002; Khanal et al., 2006; Zhao et al., 2010). Manure is a good fertilizer source and optimally should be applied based on plant nutrient needs; however, manure as well as lagoon effluent are often applied at higher rates as a convenient means of disposal, which may enhance transport to surface waters and groundwater (Khanal et al., 2006; Zhao et al., 2010).

While the impact of these hormones on human health at environmentally relevant concentrations is unknown, effects of 17β-E2 on non-target organisms including endocrine disruption affecting sexual development, behavior, and reproduction, have been reported (Ying et al., 2002; Hanselman et al., 2003; Khanal et al., 2006). For example, the formation of vitellogenin was observed in male rainbow trout when exposed to concentrations as low as 1 ng L\(^{-1}\) (Hansen et al., 1998). Also the exposure of male Japanese medaka to 10 ng L\(^{-1}\) induced production of female-specific proteins resulting in the formation of intersex (ova in testes) and altered sex species (Metcalf et al., 2001).

While considerable research has focused on the environmental fate of 17β-E2 because of its known high estrogenic potency, 17α-E2 has largely been ignored assuming it to be a weak, biologically inactive estrogen. Hajek et al. (1997), however, found that
weak environmental estrogens such as 17α-E2 may have profound effects including teratogenic and morphological changes in mammalian species depending on the stage of development when exposed. They also noted the potential for partial metabolism of 17α-E2 to the more potent 17β-E2. Still, most toxicological studies have reported 17α-E2 to be up to 500 times less estrogenic than 17β-E2 in mammalian species and the same potency has been assumed for aquatic species (reviewed in Huang et al., 2010). However, recent studies found aquatic species to be more sensitive than humans, which underscores the need to better understand the fate and transport behavior of 17α-E2 in the environment. Huang et al. (2010) found 17α-E2 to be only 11–30 times less potent to medaka fish compared to 17β-E2. Likewise, Shappel et al. (2010) reported 17α-E2 was only 8–9 times less potent than 17β-E2 on flathead minnows.

Despite the dependence of hormone-soil interactions in predicting the transport from field application or runoff to surface and groundwater, there are no published studies on the sorptive behavior of 17α-E2. Environmental models have either ignored 17α-E2 or assumed that the sorptive behavior was the same as 17β-E2. This may not be a conservative assumption. For example, in their study on the synthetic androgens, 17α-trenbolone and 17β-trenbolone, Khan et al. (2009) found that 17α-trenbolone exhibited half the sorption of 17β-trenbolone despite the previously held assumption that the sorptive behavior of the isomers would be the same. While stereo-chemical assessments are standard in the pharmaceutical industry, use in environmental fate and effects studies is less prevalent despite mounting evidence that stereoisomers may have profoundly different impacts on microbial and ecological communities (Stanley and Brooks, 2009). For example, Xuan et al. (2008) reported a 17α-E2 aerobic soil half-life to be twice that of 17β-E2 under favorable conditions with half-lives typically less than 1 week (Colucci et al., 2001; Lee et al., 2003; Xuan et al., 2008). Likewise, while 17α-trenbolone was assumed to be a weak androgen based on mammalian studies, Ankley et al. (2003) and Jensen et al. (2006) found 17α-trenbolone to invoke similar effects on fathead minnows as the potent β-isomer. These examples further highlight the need for stereo-chemical assessments.
The objective of this study was to assess if 17α-E2 and 17β-E2 exhibited the same sorption affinities. Both 17α-E2 and 17β-E2 consist of the standard four (tetracyclic) carbon rings with an aromatic A-ring, and OH groups located on the C3 and C17 positions. The structural difference between stereoisomers is the orientation of the hydroxyl group on C17 of the D-ring with the OH group being oriented out of the general tetracyclic carbon plane (Figure 3.1). Sorption of each stereoisomer was estimated by independently quantifying solution and sorbed phase concentrations on seven autoclaved soils with a wide range of taxonomic properties.

![17α-estradiol and 17β-estradiol structures](image)

**Figure 3.1. 17α- and 17β-estradiol structures.**

### 3.3 Materials and Methods

#### 3.3.1 Soils

Seven soils previously used in other studies and representing a range of pH, organic carbon content, soil texture, cation exchange capacity, and dominant clay types were selected (Table 3.1). Raub12, Toronto-4, Coloma-32, and Drummer-36 are agricultural soils from the Purdue Agronomy Research Farm (West Lafayette, IN, USA). Raub-12 and Toronto-4 are silt-loams previously characterized by Huang and Lee (2001). Coloma-32 is a sandy soil and Drummer-36 is a silty clay loam previously characterized by Khan et al. (2009). EPA-14 is a clayey soil from an eroded hillside in southeastern Ohio (Means et al., 1980). 7CB is a high organic matter sandy loam from Northern Costa Rica (Lee et al., 2004). Oakville-24 is a sandy soil from Northern Indiana (Liu and Lee,
Prior to use, the soils were air-dried, gently crushed to pass through a 2-mm sieve, thoroughly mixed, and stored at room temperature in closed containers.

**Table 3.1. Soil Properties.**

<table>
<thead>
<tr>
<th>Soil (ID)</th>
<th>pH(^d)</th>
<th>pH(^e)</th>
<th>OC(^f) (%)</th>
<th>Clay (%)</th>
<th>Sand (%)</th>
<th>Silt (%)</th>
<th>CEC(^h) (cmolc/kg)</th>
<th>Dominant Clay Type(^h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7CB(^a)</td>
<td>6.3</td>
<td>5.4</td>
<td>7.5</td>
<td>6.8</td>
<td>68.8</td>
<td>24.4</td>
<td>41.0</td>
<td>K</td>
</tr>
<tr>
<td>Coloma-32</td>
<td>5.9</td>
<td>5.8</td>
<td>0.6</td>
<td>5</td>
<td>88</td>
<td>7</td>
<td>4.3</td>
<td>I</td>
</tr>
<tr>
<td>(C32)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drummer-36</td>
<td>7.4</td>
<td>6.1</td>
<td>2.3</td>
<td>36</td>
<td>17</td>
<td>47</td>
<td>15.5</td>
<td>S</td>
</tr>
<tr>
<td>(D36)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPA-14(^c)</td>
<td>4.5</td>
<td>3.9</td>
<td>0.5</td>
<td>63.6</td>
<td>2.1</td>
<td>34.4</td>
<td>18.9</td>
<td>K &gt;&gt; S, I</td>
</tr>
<tr>
<td>Oakville-24</td>
<td>5.7</td>
<td>4.9</td>
<td>0.5</td>
<td>4</td>
<td>92</td>
<td>4</td>
<td>2.7</td>
<td>K</td>
</tr>
<tr>
<td>(O24)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raub-12</td>
<td>6.7</td>
<td>6.1</td>
<td>1.4</td>
<td>23.8</td>
<td>10.5</td>
<td>65.7</td>
<td>23.0</td>
<td>S</td>
</tr>
<tr>
<td>(R12)(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toronto-4</td>
<td>4.4</td>
<td>4.4</td>
<td>1.3</td>
<td>20</td>
<td>12</td>
<td>68</td>
<td>11.2</td>
<td>S</td>
</tr>
<tr>
<td>(T4)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Properties determined by MDS Harris Laboratories, Lincoln, NE

\(^b\) Properties determined by A&L Great Lakes Laboratories, Fort Wayne, IN

\(^c\) Determined by the Institute for Environmental Studies, University of Illinois at Urbana-Champaign.

\(^d\) pH of a 1:1 soil (g):water (mL) slurry.

\(^e\) pH of water at experimental soil mass (g):0.005 M CaCl\(_2\) solution volume (mL) ratio.

\(^f\) Percent organic carbon determined by loss-on-ignition method.

\(^g\) Determined by hydrometer method.

\(^h\) Cation exchange capacity determined by a modified ammonium acetate method.

\(^i\) I: illite, K: kaolinite, S: smectite.

### 3.3.2 Chemicals

All estrogens (17α-E2, 17β-E2, estrone) and the internal standard 17β-estradiol-D3 (17β-E2-D3) were obtained from Sigma Aldrich, St. Louis MO, USA. Acetonitrile, methanol, and calcium chloride (CaCl\(_2\)) were purchased from Mallinckrodt, Phillipsburg, NJ, USA. Dichloromethane (DCM) was purchased from Fisher Scientific, Pittsburgh, PA,
USA. All chemicals were analytical-reagent grade or higher (>99% purity, except for 17α-E2, 17β-E2, 17β-E2-D3 which were >98% purity) and used as received. Ultrapure water was prepared using a Mega-Pure System, MP-3A from Barnstead, Dubuque, IA, USA. Hormone stock solutions were prepared in pure methanol and stored at 4°C in the dark. Aqueous hormone solutions were prepared just prior to application by diluting the stock solutions in a sterile 0.005 M CaCl$_2$ solution, mixing with a vortex mixer, and sonicating for 10 min. The volume fraction of methanol in aqueous estrogen solutions applied to soils was less than 0.1%.

3.3.3 Batch Sorption Experiments

Sorption isotherms for 17α-E2 and 17β-E2 were measured from aqueous 0.005 M CaCl$_2$ solutions using four to five solution concentrations in duplicate or triplicate ranging from 0.004 mg L$^{-1}$ to 0.22 mg L$^{-1}$ (plus a blank). Soil mass (g) to solution volume (mL) ratios ranged from 1:10 to 1:70 for the different soil-solute combinations to ensure that the concentrations in both the sorbed and solution phase extracts were well above the method limit of quantification (MLOQ). Soils were wet-autoclaved to minimize biotransformation using a method described by Wolf et al. (1989). Hildebrand et al. (2006) found that autoclaving had minimal impacts on estrogen sorption to soils and was more effective than chemical sterilization methods at reducing degradation. Wet-autoclaving has also been shown to be more effective in inhibiting enzyme activity compared to other methods including irradiation (Peterson, 1962; Parham and Deng, 2000; McNamara et al., 2003). Air-dried soils were weighed into 10 or 35 mL glass tubes, adjusted to field capacity, and incubated for 72 h at room temperature (22 ± 2°C). The soils were autoclaved at 103.4 kPa and 121°C for 1 h. The soils were readjusted to field capacity, incubated for 24 h, and autoclaved again for 1 h. All glassware and the CaCl$_2$ solution used to make the estrogen solutions were sterilized by autoclaving. Single-estrogen solutions were added to tubes containing the autoclave-sterilized soils and capped with Teflon-lined screw caps. Tube sizes were selected to minimize headspace and the potential for volatilization. Each tube was wrapped in aluminum foil to minimize photolysis. Samples were equilibrated end-over-end at 35 rpm for 24 h at
room temperature (22 ± 2°C) and centrifuged at 1600 rpm for 20 min. Casey et al. (2005), Khan et al. (2009), and Ying et al. (2003) found that hormones appeared equilibrated within 24 h based on 2-5 d studies.

Both aqueous and soil phases were analyzed for estrogen concentrations. A 5 mL aliquot of the aqueous supernatant was extracted using 3 mL of DCM. A solvent exchange and concentration step was performed by taking 1 mL of DCM from each sample, evaporating the DCM, and redissolving the residual precipitate in 0.5 mL methanol containing an internal standard (17β-E2-D3). The remaining soil plug was extracted using 30 mL of methanol. A 0.5 mL aliquot of MeOH was taken from each sample, and a 0.5 mL aliquot of an internal standard (17β-E2-D3) dissolved in methanol was added. The resulting concentration of the internal standard in all samples was the same. Completely replicated isotherms for both isomers were measured for Toronto-4 and Drummer-36 soils at a time different than the original isotherm for additional quality assurance.

3.3.4 Analysis

A Shimadzu high pressure liquid chromatography (HPLC) system with a Sciex API3000 mass spectrometer in multiple reaction monitoring mode was used for estrogen analysis. Separation was performed using 20 µL injections on a Phenomenex Synergi RP MAX column (150 x 2.0 mm, dp-4 µm) with a gradient elution using water:methanol (90:10) containing 2 mM ethanolamine [solvent A] and acetonitrile [solvent B] at 0.3 mL min⁻¹. Initial mobile phase composition was 40% solvent B followed by a linear gradient to 60% solvent B from 0 to 5.5 min after which solvent B was ramped to 100% for 2 min to wash the column and then re-equilibrated at 40% solvent B for 2.4 min prior to the next injection. Solutions were also analyzed for estrone, a primary estradiol metabolite, as an additional check for any significant degradation during equilibration. The chromatographic retention times were 5.3 min, 5.7 min, and 6.4 min for 17β-E2, 17α-E2, and estrone, respectively. 17α-E2 and 17β-E2 (precursor ion 271, product ion 145) and estrone (precursor ion 269, product ion 145) were quantified using independent external calibration curves with check standards run
approximately every 10 samples. An internal standard, 17β-E2-D3 (precursor ion 274, product ion 145), was used for confirmation but not for quantification given that deuterated 17α-E2 was not available. For all estrogens, the limit of detection was 0.015 µL L⁻¹ and the MLOQ was 0.03 µL L⁻¹.

3.3.5 Statistical Analysis

Statistical analyses were carried out using Minitab v15. Linear regressions were performed between linear sorption coefficients and individual soil properties for both 17α-E2 and 17β-E2, as well as between % OC and CEC, % OC and % clay, and % clay and CEC. In addition, a paired t-test was performed to test the statistical significance of the difference between log Kₑₒ values for 17α-E2 and 17β-E2. All statistical tests used α = 0.05 as the level of significance.

3.4 Results and Discussion

Little to no degradation of 17α-E2 or 17β-E2 to estrone was observed during the 24 h equilibration. The total mass recoveries from solution and sorbed phases were 101 ± 8% for 17α-E2 and 99 ± 10% for 17β-E2 (Table 3.2 and Table A.1 in Appendix A). Sorption isotherms constructed from measured solution (Cₑ, mg L⁻¹) and sorbed phase (Cₛ, mg kg⁻¹) concentrations were well fit with the linear isotherm model: Kₑ = CₛCₑ⁻¹ where Kₑ (L kg⁻¹) is the linear distribution coefficient, with coefficients of determination (R²) of 0.81-1.00 (Table 3.2). Isotherms for all seven soils including replicate experiments are presented in Figure 3.2. For all but one soil, the Kₑ values for 17β-E2 are significantly greater than 17α-E2 (t-test, p < 0.05) with the β/α sorption ratio across soils between 1.4 and 1.9 (Table 3.2). For the Oakville-24 soil, 17β-E2 sorption was greater than 17α-E2; however, the difference was not considered significant at the 95% confidence level (p = 0.056).

Coefficients of determination for the regressions between Kₑ and various soil properties are presented in Table 3.3 and Figure A.1 in Appendix A. The best regression fit by far for both stereoisomers is between Kₑ and soil organic carbon (OC) (R² = 0.98-0.99). This result is in agreement with earlier studies on 17β-E2 (Lee et al., 2003; Casey
et al., 2005; Bonin and Simpson, 2007; Caron et al., 2010). OC-normalized sorption coefficients ($K_{oc}$, L kg$_{oc}^{-1}$ = $K_d f_{oc}^{-1}$ where $f_{oc}$ is the OC fraction) yields average log $K_{oc}$ values of 2.97 ± 0.13 for 17α-E2 and 3.14 ± 0.16 for 17β-E2 (Table 3.2). A paired t-test confirmed that the difference between the log $K_{oc}$ values for 17α-E2 and 17β-E2 across all soils is significant (p < 0.05). Qiao et al. (2010) observed similar trends in the association of these estradiol isomers with dissolved Leonardite and Aldrich humic acids. While log $K_{oc}$ values for 17α-E2 have not been previously reported, the log $K_{oc}$ values for 17β-E2 (2.96-3.47) fall within the range reported by Lee et al. (2003) of 3.21-3.46 (n = 2), Casey et al. (2005) of 2.75-4.13 (n = 5 after 24 h), and Caron et al. (2010) of 2.60-3.49 (n = 121).

Table 3.2. Summary of the sorption coefficients estimated from linear model fits to multiple-concentration sorption isotherms for 17α-E2 and 17β-E2 from seven autoclaved sterilized soils.a

<table>
<thead>
<tr>
<th>Soil ID</th>
<th>17α-Estradiol</th>
<th>17β-Estradiol</th>
<th>17α-Estradiol</th>
<th>17β-Estradiol</th>
<th>17α-Estradiol</th>
<th>17β-Estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MR±SDb</td>
<td>$K_d$ (L kg$^{-1}$)</td>
<td>$R^2$ c</td>
<td>Log $K_{oc}$d</td>
<td>MR±SDb</td>
<td>$K_d$ (L kg$^{-1}$)</td>
</tr>
<tr>
<td>7CB</td>
<td>117±17</td>
<td>62.4</td>
<td>0.99</td>
<td>2.92</td>
<td>113±6</td>
<td>101.9</td>
</tr>
<tr>
<td>C32</td>
<td>95±12</td>
<td>9.9</td>
<td>0.98</td>
<td>3.19</td>
<td>107±3</td>
<td>18.8</td>
</tr>
<tr>
<td>D36</td>
<td>101±8</td>
<td>22.8</td>
<td>0.98</td>
<td>3.00</td>
<td>96±12</td>
<td>32.6</td>
</tr>
<tr>
<td>EPA-14</td>
<td>93±12</td>
<td>2.8</td>
<td>0.81</td>
<td>2.77</td>
<td>86±10</td>
<td>4.4</td>
</tr>
<tr>
<td>O24</td>
<td>97±13</td>
<td>6.2</td>
<td>0.94</td>
<td>3.08</td>
<td>94±7</td>
<td>6.6</td>
</tr>
<tr>
<td>R12</td>
<td>101±8</td>
<td>11.4</td>
<td>0.96</td>
<td>2.93</td>
<td>96±9</td>
<td>17.9</td>
</tr>
<tr>
<td>T4</td>
<td>101±10</td>
<td>10.7</td>
<td>0.99</td>
<td>2.92</td>
<td>105±8</td>
<td>14.8</td>
</tr>
<tr>
<td>Average log $K_{oc}$ ± SDe</td>
<td>2.97 ± 0.13</td>
<td>3.14 ± 0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Each isotherm is represented by at least four concentrations plus zero in duplicate or triplicates.

b Average percent mass recovery ± standard deviation.

c Coefficient of determination from linear regression.

d Sorption coefficient normalized to organic carbon.

e Standard deviation.

f Ratio of sorption coefficients between 17α-estradiol and 17β-estradiol for each soil.
**Figure 3.2.** 17α-E2 and 17β-E2 sorption isotherms with seven soils. Replicated isotherms conducted at a different time are included for T4 and D36. Error bars represent standard deviations (small errors are hidden by the symbols). Lines represent linear isotherm model fits.

The regression fit between $K_d$ and CEC reflects a moderate correlation ($R^2 = 0.67$); however, this apparent relationship is an artifact of the strong relationship between % OC and % CEC ($R^2 = 0.74$) and between $K_d$ and % OC ($R^2 = 0.98-0.99$) (Table 3.3). Estradiol is a weak acid, thus will not exist as a cation, therefore, there is no mechanistic reason that CEC would be directly associated with estradiol sorption as suggested by Casey et al. (2003). Anion exchange to iron oxides is also not expected given that estradiol has a $pK_a$ of 10.5-10.7 (Hanselman et al., 2003), thus will not exist as an anion at environmentally relevant pH values (4-8). Not surprisingly, the regression between $K_d$ and pH was not
significant \((p > 0.05; R^2 = 0.09)\), in agreement with a review by Hanselman et al. (2003) where no consistent correlation between pH and sorption of estrogens in the bulk soil matrix was noted. More recently, pH-dependent hormone sorption to three pure humic materials was reported (Neale et al., 2009); however, effects on sorption were due to pH-induced changes in the speciation and conformation of the humic acids and subsequent change in the hydrophobicity of the humic acid domain, and not necessarily compound specific. We did not find evidence that differential sorption between the stereoisomers was pH dependent. For example, the Toronto-4 and Raub-12 soils, which have similar soil properties except for soil pH, with values of 4.4 and 6.1 respectively, had \(\beta/\alpha\) sorption ratios of 1.4 and 1.6 and are essentially the same. While we cannot draw a definitive conclusion from this limited soil set, this does suggest that pH has little impact on stereoselective sorption.

The strong correlation between \(K_d\) and soil OC (Tables 3.2 and 3.3; Figure A.1 in Appendix A) for both isomers (Table 3.2) suggests hydrophobic partitioning is the primary driver of estradiol sorption in which case, similar differences would be expected in their aqueous activities (i.e., escaping tendency from water). While accurate solubilities and melting points are not available for both isomers to reliably estimate aqueous activities directly, relative aqueous activities can be extrapolated from hexane–water partition coefficients \((K_{hw})\) reported by Qiao et al. (2010). Hexane is apolar and will not have specific interactions with estradiol; therefore, differences in \(K_{hw}\) are a direct reflection of their escaping tendencies from water (aqueous activity coefficient based on their pure organic liquid reference state). However, the opposite is true with \(17\beta\)-E2 having a lower \(K_{hw}\) (0.82) than \(17\alpha\)-E2 (1.13). Likewise, the retention time of \(17\beta\)-E2 is smaller (5.3 min) than that of \(17\alpha\)-E2 (5.7 min) in our own HPLC analysis with a reverse-phase column Phenomenex Synergi RP-MAX column and a polar mobile phase. If hydrophobic forces dominate in sorption, the retention order in reverse-phase liquid chromatography is typically positively correlated with \(K_{oc}\) (Woodburn et al., 1989) unlike the estradiol isomer sorption trend reported here. This suggests that the greater sorption of \(17\beta\)-E2 cannot be explained by simple hydrophobic-partitioning processes alone.
Table 3.3. Summary of the coefficients of determination ($R^2$) for linear regressions between linear sorption coefficients ($K_d$, L kg$^{-1}$) for 17α- and 17β-estradiol and individual soil properties or between two soil properties.

<table>
<thead>
<tr>
<th>Correlation</th>
<th>17α-estradiol</th>
<th>17β-estradiol</th>
<th>Between soil properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ vs. % OC</td>
<td>0.990$^c$</td>
<td>0.982$^c$</td>
<td></td>
</tr>
<tr>
<td>$K_d$ vs. pH$^a$</td>
<td>0.085</td>
<td>0.089</td>
<td></td>
</tr>
<tr>
<td>$K_d$ vs. % clay</td>
<td>0.107</td>
<td>0.117</td>
<td></td>
</tr>
<tr>
<td>$K_d$ vs. % sand</td>
<td>0.049</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>$K_d$ vs. % silt</td>
<td>0.005</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>$K_d$ vs. CEC$^b$</td>
<td>0.671</td>
<td>0.670</td>
<td></td>
</tr>
<tr>
<td>% OC vs. CEC</td>
<td></td>
<td>0.741$^c$</td>
<td></td>
</tr>
<tr>
<td>% OC vs % clay</td>
<td></td>
<td></td>
<td>0.079</td>
</tr>
<tr>
<td>% Clay vs. CEC</td>
<td></td>
<td></td>
<td>0.013</td>
</tr>
</tbody>
</table>

$^a$ pH of water at experimental mass (g): 0.005 M CaCl$_2$ solution volume (mL) ratio.

$^b$ High $R^2$ is due to the correlation between % OC and % CEC and the correlation between $K_d$ and % OC.

$^c$ p < 0.05.

In sorption studies with 17β-E2, Yamamoto and Liljestrand (2003) suggested that hydrogen-bonding and electron donor–acceptor (EDA) interactions with the phenolic groups in soil organic matter may also contribute to sorption by soils. Further support for such interactions is reflected in the higher toluene-water partition coefficients ($K_{tw}$) for 17β-E2 compared to 17α-E2 (Qiao et al., 2010). Qiao et al. (2010) also measured octanol–water partition coefficients ($K_{ow}$) for both 17α-E2 and 17β-E2, which were orders of magnitude higher than their corresponding $K_{hw}$ or $K_{tw}$ values. In addition, log $K_{ow}$ values were similar for the two isomers (3.73 ± 0.03 for 17α-E2 and 3.76 ± 0.03 for 17β-E2). The latter infers that H-bonding interactions may be somewhat greater with the β-isomer otherwise the $K_{ow}$ for 17β-E2 should have been lower reflecting the different escaping tendencies from water reflected in the $K_{hw}$ values. H-bonding, EDA, and aromatic type interactions have the potential to be stereospecific (Gu et al., 1995; Meyer et al., 2003). In the case of 17α-E2 and 17β-E2, a more planar orientation with the sorbent, the aromatic-ring containing steroid backbone, is likely to be thermodynamically favorable, thus the C-17 OH group of the β-isomer that lies in the plane may be better.
positioned to optimize sorption whereas the C-17 OH group in the α-isomer would be oriented outside the sorbing plane.

3.5 Summary and Environmental Implications
Both 17α-E2 and 17β-E2 have been routinely detected in surface and ground waters with higher concentrations of both stereoisomers reported near concentrated animal feeding operations and agricultural fields (Khanal et al., 2006; Zhao et al., 2010). In beef cattle and dairy, 17α-E2 is excreted in larger quantities than 17β-E2 (Hanselman et al., 2003). A recent study by Gadd et al. (2010) found 17α-E2 concentrations in dairy shed effluents are also higher (30 times) than 17β-E2. This study found that 17α-E2 typically sorbs on average 50% less than 17β-E2. Sorption of both stereoisomers appears to be driven primarily by hydrophobic forces; however, stereoselective sorption is likely governed by aromatic interactions and H-bonding. Assuming that the two isomers sorb the same, as is currently done, is not a conservative decision making approach. The lower sorption affinity of 17α-E2 increases the likelihood that it will be leached from agricultural fields.

3.6 Acknowledgements
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CHAPTER 4. BIOTRANSFORMATION OF 17α- AND 17β-ESTRADIOL IN AEROBIC SOILS

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4.1 Abstract

Considerable research has focused on the fate of 17β-estradiol (17β-E2) given its high estrogenic potency and frequent detection in the environment; however, little is known about the fate behavior of 17α-estradiol (17α-E2) although it often dominates in some animal feces, and recently has been shown to have similar impacts as the β-isomer. In this study, the aerobic biotransformation rates of 17α-E2 and 17β-E2 applied at 50 µg kg⁻¹ soil and metabolite trends were quantified in batch microcosms at ~21 °C and 70-85% field capacity using two soils with different taxonomic properties. Soils were extracted at designated times over a 3-week period and analyzed over time using negative electrospray ionization tandem mass spectrometry. For a given soil type, the two isomers degraded at the same rate with half lives across soils ranging between 4 and 12 h. Estrone (E1) was the only metabolite detected and in all cases subsequent dissipation patterns of E1 are statistically different between isomers. Autoclaved-sterilized controls support that E2 dissipation is dominated by microbial processes. A first order exponential decay model that assumed sorption did not limit bioavailability was not able to accurately predict hormone residuals at later times, which indicates caution is required when trying to model fate and transport of hormones in the environment.
4.2 Introduction

The detection of estrogenic compounds in the environment has raised concern in recent years because of their potential to disrupt endocrine function, activate hormone responses, and alter secondary sex characteristics in non-target organisms at environmentally relevant concentrations (low ng/L range) (as reviewed by Hanselman et al., 2003, and Young and Borch, 2009). While natural estrogens are produced by humans and vertebrates, the excretion rate and type (e.g., 17α-estradiol and 17β-estradiol) varies by species, sex, age, reproductive status, and administration of veterinary pharmaceuticals. For example, dairy, beef cattle, and sheep typically excrete larger concentrations of 17α-estradiol (17α-E2), while swine and poultry excrete primarily 17β-estradiol (17β-E2) (Hanselman et al., 2003). The consolidation of concentrated animal feeding operations and high transportation costs result in the land disposal of manure, effluent, and biosolids onto nearby agricultural land, increasing the density of application of these manure-borne hormones (Khanal et al., 2006; Zhao et al., 2010).

Considerable research has focused on the fate of 17β-E2 given its high estrogenic potency and frequency of detection in the environment. Numerous laboratory studies have examined the biotransformation of 17β-E2 in aerobic soils (Colucci et al., 2001; Das et al., 2004; Ying et al., 2005; Xuan et al., 2008; Carr et al., 2011); soils and sediments amended with biosolids, manure (Jacobson et al., 2005) and nutrients (Stumpe and Marschner, 2009); activated sludge (Ternes et al., 1999); anaerobic lake sediments (Czajka and Londry, 2006); aquifer materials (Ying and Kookana, 2003a, 2008) and marine sediments (Ying and Kookana, 2003b) under aerobic and anaerobic conditions; dairy lagoon water (Zheng et al., 2012); and river water and sediments (Jürgens et al., 2002). Few studies have looked at the environmental fate of the 17α-E2 isomer, because it has largely been considered weakly estrogenic based on mammalian assays. However, recent studies on medaka and flathead minnows suggest that 17α-E2 may be significantly more potent to aquatic species than previously believed (Huang et al., 2010; Shappel et al., 2010). To our knowledge, laboratory degradation studies on 17α-E2 have been limited to soil bacteria cultures (Turfitt, 1947a, 1947b), a 80/20 % by wt. sterile/unsterile soil mixture (Xuan et al., 2008), and dairy lagoon water (Zheng et al., 2012).
Environmental fate and transport models have either ignored 17α-E2 or assumed that the behavior is the same as 17β-E2. Recent studies, however, show differences between the stereoisomers with regards to their affinity for soil (Mashtare et al., 2011) and anaerobic degradation rates in dairy lagoon water (Zheng et al., 2012). Stereoselective degradation has been observed for other organic agrochemicals in soils (Marucchini and Zadra, 2002; Li et al., 2012) as well. The objectives of this study were to assess if the biotransformation rates and primary metabolite formation of 17α-E2 and 17β-E2 were the same in aerobic soils using two soils with distinctly different taxonomic properties. Sterile controls were used to differentiate between microbial and abiotic processes.

4.3 Materials and Methods

4.3.1 Soils

A silty clay loam (Drummer, D-46) and a sandy loam (Coloma, C-45) were selected for our study. D-46 was obtained from the Purdue Animal Science Research and Education Center (West Lafayette, IN) from an agricultural field where effluent and manure is periodically applied. C-45 was obtained near State Road 26 just north of the Purdue University airport (West Lafayette, IN). Multiple sub samples at each site were collected after removal of vegetation to ensure a representative mix of the top 8 cm of soil. After collection, the soils were gently passed through a 2 mm sieve and stored in closed containers at 4 °C in the dark. Soil characterization including pH, % organic matter (OM), particle size analysis, dominant clay minerals, field capacity, and cation exchange capacity (CEC) is summarized in Table 4.1. Soil moisture content at the time of sampling was 10.1 % for C-45 and 20.3% for D-46, which is 67.2% and 84.6% of their field capacities, respectively.
4.3.2 Chemicals

17α-E2, 17β-E2, estrone (E1) and estriol (E3) (all of > 98% purity), and the internal standard 17β-estradiol-D3 (17β-E2-D3) (> 99% purity) were obtained from Sigma Aldrich, St. Louis MO, USA. Analytical reagent grade acetonitrile, methanol, and talc were purchased from Mallinckrodt, Phillipsburg, NJ, USA. Ultrapure water was prepared using a Mega-Pure System, MP-3A from Barnstead, Dubuque, IA, USA. Hormone stock solutions were prepared in pure methanol and stored at 4 °C in the dark.

Table 4.1. Selected soil properties

<table>
<thead>
<tr>
<th>Soil</th>
<th>OM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CEC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pH&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Clay&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Sand&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Silt&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Clays&lt;sup&gt;e&lt;/sup&gt;</th>
<th>% Moisture at Field Capacity&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-45</td>
<td>1.2%</td>
<td>12 cmol·kg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7.8</td>
<td>19%</td>
<td>50%</td>
<td>31%</td>
<td>I, RIS, C, K</td>
<td>15%</td>
</tr>
<tr>
<td>D-46</td>
<td>2.9%</td>
<td>13 cmol·kg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>6.9</td>
<td>28%</td>
<td>17%</td>
<td>55%</td>
<td>S, I, K</td>
<td>24%</td>
</tr>
</tbody>
</table>

<sup>a</sup> % organic matter determined by loss on ignition at 360 °C (Brown, 1998); <sup>b</sup> cation exchange capacity determined using the Mehlich III Extraction method (1 M NH₄OAc buffered at pH 7.0) (Brown, 1998); <sup>c</sup> pH of a 1:1 soil (g): water (mL) slurry (Brown, 1998); <sup>d</sup> determined by hydrometer method (Gee and Bauder, 1986); <sup>e</sup> I=illite, RIS=randomly interstratified illite-smectite, C=chlorite, K=kaolinite, and S=smectite as identified by XRD. <sup>f</sup> Moisture content at field capacity determined by small soil core method.

4.3.3 Aerobic and Soil Microcosms

Soil (10 g ± 0.1 g dry wt. basis) was transferred to sterilized 120 mL amber glass bottles. Soil moisture of the C-45 soil was adjusted to 75% of field capacity using sterilized ultrapure water. D-46 was maintained at 84.6% of field capacity. Soil microcosms were then capped with rubber stoppers and allowed to re-acclimate in the dark at room temperature (21°C ± 2°C) for 7 d prior to hormone amendment. Soil moisture contents were maintained at their respective pre-incubation levels for the duration of the experiment. To discern abiotic transformations, sterile controls were prepared by autoclaving as described by Wolf et al. (1989). Briefly, prepared soil microcosms were autoclaved for 1 h for each of 3 consecutive days, prior to hormone
amendment. After each autoclaving cycle, soil moisture was re-adjusted to pre-incubation levels. For each soil-isomer combination, sterile and non-sterile soil microcosms were prepared. Triplicate microcosms were extracted at each sampling time. Soil blanks, in triplicate, were used to quantify background hormone concentrations.

**4.3.4  Hormone Addition, Extraction, and Concentration.**

Soil microcosms were re-acclimated for 7 d followed by the addition of hormones using a talc carrier as described by Khan et al. (2010). Briefly, 7 mL of a 10 mg L\(^{-1}\) single hormone-containing solution in methanol was added to 14 g of talc in a petri dish, mixed well and periodically as methanol evaporated. The solvent-free talc was then transferred to a glass bottle, capped, and vortexed to homogenize the amended talc. 100 mg of either 17α-E2 or 17β-E2 amended talc was added to each biotic and abiotic soil microcosm, lightly shaken to mix the talc and soil, recapped, and stored in the dark until sampling. Total amended talc did not exceed 1% of the soil weight and yielded an estimated applied hormone concentration of 0.184 μmol hormone kg\(^{-1}\) soil.

Biotic and abiotic microcosms were sacrificed in triplicate at nine designated times over ~19 d. Microcosms were extracted by adding methanol (70 mL), sealing with an aluminum-lined crimp cap, rotating end-over-end for 24 h, and centrifuging for 25 min at 500 g. An aliquot of the methanol extract (~1.7 mL) from each microcosm was transferred to an HPLC vial, evaporated to dryness under a gentle stream of nitrogen, and re-dissolved in 0.5 mL of methanol spiked with an internal standard (17β-E2-D3). Preliminary studies indicated an extraction efficiency > 97% from the first extraction; however, soils were extracted a second time in case extraction efficiency changed with incubation time. Second extractions were done with new methanol (20 mL) after removing excess methanol from the first extraction, and gravimetrically determining the residual methanol.

**4.3.5  Hormone Analysis**

Hormone analysis was done by high performance reverse-phase liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS)
in negative mode using a Shimadzu high pressure liquid chromatography system coupled to a Sciex API-3000 mass spectrometer operated in multiple reaction monitoring mode. Separation was performed using 25 μL injections on a Phenomenex Gemini C-18 column (150 mm x 2 mm, dp= 5 μm) with gradient elution using methanol:water (10:90) containing 2 mM ethanolamine [solvent A] and acetonitrile containing 2 mM ethanolamine [solvent B] at 0.35 mL min\(^{-1}\). Initial mobile phase composition was 30% solvent B followed by a linear gradient to 50% solvent B from 0 to 8.5 min after which solvent B was ramped to 90% for 2 min to elute highly retained sample components and then re-equilibrated at 30% solvent B for 2 min prior to the next injection. The chromatographic retention times for E3, 17β-E2, 17β-E2-D3, 17α-E2, and E1 were 3.5 min, 8.2 min, 7.6 min, 7.6 min, and 8.8 min, respectively. E3 (\(m/z\) 287 \(\rightarrow\) 145), 17α-E2 and 17β-E2 (\(m/z\) 271 \(\rightarrow\) 145), and E1 (\(m/z\) 269 \(\rightarrow\) 145) were quantified using independent external calibration curves with check standards run approximately every 12 samples. The internal standard, 17β-E2-D3 (\(m/z\) 274 \(\rightarrow\) 145), was used to assess matrix effects in the MS, which were found to be negligible. A deuterated 17α-E2 internal standard was not available. For all estrogens, the limit of detection was 0.015 μg L\(^{-1}\) and the method limit of quantitation (MLOQ) was 0.03 μg L\(^{-1}\).

4.3.6 Gas Analysis

\(O_2\) and \(CO_2\) levels in the headspace were determined by manual injection on an Agilent 7890A gas chromatograph (GC) equipped with a thermal conductivity detector (TCD). Concentrations are reported as a percent normalized to ambient air concentrations.

4.3.7 Degradation Rates

A pseudo first-order exponential decay model was used to estimate biotransformation rates (\(k_a\) and \(k_b\), d\(^{-1}\)) of the hormones (Eqs. 1 and 2).

\[
C_t = C_0e^{-k_at} \quad (1)
\]

\[
M_t = [(k_aC_0)(e^{-k_at} - e^{-k_bt})](k_b-k_a)^{-1} + M_0e^{-k_bt} \quad (2)
\]
where \( C_0 \) and \( C_t \) represent the extracted hormone concentrations on a mole basis at time = 0 and time \( t \) (d), respectively, and \( M_0 \) and \( M_t \) represent the extracted metabolite mole concentrations. This model assumes that transformation is irreversible, sorption has negligible impact on bioavailability, and steady-state biomass.

4.3.8 Statistical Analysis

Statistical analysis used Minitab v16 (State College, PA: Minitab, Inc.). An ANOVA was conducted to determine the significance of differences between the observed temporal changes in biotransformation of \( 17\alpha\)-E2 and \( 17\beta\)-E2 as well as between the rate of E1 metabolite production and subsequent loss from \( 17\alpha\)-E2 and \( 17\beta\)-E2 within and between soil types. Nonlinear regressions were used to fit the data to Eq. 1 and 2, which generated rate coefficients \((k_a \text{ and } k_b)\) and associated confidence intervals for \( 17\alpha\)-E2, \( 17\beta\)-E2, and E1 in each soil. The confidence intervals on the rate coefficients were used to assess whether the rates of decay were significantly different within and between soil types. Linear regressions were used to determine whether the rate of loss in abiotic microcosms was significantly different than zero. Statistical tests used \( \alpha = 0.05 \) as the level of significance.

4.4 Results and Discussion

Changes in hormone concentrations over time in unsterile and sterile microcosms and metabolite formation and loss are plotted (Figure 4.1) and discussed in terms of mol % relative to the E2 isomer applied. Observed half-lives \((t_{1/2})\) for the aerobic degradation of \( 17\alpha\)-E2, \( 17\beta\)-E2, and the metabolite E1 in unsterile soils are summarized in Table 4.2 along with the degradation rates \((k_a \text{ and } k_b)\), \( t_{1/2} \) values, and coefficients of determination \((R^2)\) from fitting Eqs. 1 and 2 to the data. Both isomers exhibited rapid degradation in unsterile soils with similar degradation rates for a given soil. Changes in concentrations in sterile soil controls supported microbial processes as the primary dissipation pathway for both E2 isomers.
Table 4.2. Summary of modeled aerobic degradation rates ($k_a$ and $k_b$, d$^{-1}$) assuming pseudo first order decay, half-lives ($t_{1/2}$, d) of 17α-E2, 17β-E2, and subsequent metabolite E1 in C-45 (monitored for 18.1 d) and D-46 soils, which were monitored for 18.1 and 19.1 d, respectively), and coefficients of determination ($R^2$) of model fits. $k_a$ and $k_b$ were fitted simultaneously with Eq. 1 and 2. Bracketed values represent estimates of $k_a$ and $k_b$ and associated $t_{1/2}$ values calculated in sequence.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Applied hormone</th>
<th>$t_{1/2}$ (d) observed</th>
<th>$k_a$ (d$^{-1}$)</th>
<th>$R^2$</th>
<th>$t_{1/2}$ (d) from $k_a$</th>
<th>$k_b$ (d$^{-1}$)</th>
<th>$R^2$</th>
<th>$t_{1/2}$ (d) from $k_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C45</td>
<td>17α-E2</td>
<td>0.44</td>
<td>1.15 [1.32]</td>
<td>0.94</td>
<td>0.60 [0.44-0.62]</td>
<td>0.89 [0.91]</td>
<td>0.97</td>
<td>0.78 [0.62-0.92]</td>
</tr>
<tr>
<td></td>
<td>17β-E2</td>
<td>0.45</td>
<td>1.56 [1.44]</td>
<td>0.96</td>
<td>0.44 [0.41-0.57]</td>
<td>0.39 [0.44]</td>
<td>0.97</td>
<td>1.77 [1.39-1.81]</td>
</tr>
<tr>
<td>D46</td>
<td>17α-E2</td>
<td>0.16</td>
<td>3.22 [3.71]</td>
<td>0.88</td>
<td>0.22 [0.15-0.24]</td>
<td>4.98 [6.92]</td>
<td>0.88</td>
<td>0.14 [0.07-0.14]</td>
</tr>
<tr>
<td></td>
<td>17β-E2</td>
<td>0.23</td>
<td>2.98 [2.98]</td>
<td>0.89</td>
<td>0.23 [0.18-0.29]</td>
<td>2.64 [2.68]</td>
<td>0.85</td>
<td>0.26 [0.18-0.36]</td>
</tr>
</tbody>
</table>

*a Bracketed values represent a range of half-lives determined using the confidence intervals calculated for the estimates of $k_a$ and $k_b$ using Eq. 1 and 2 in sequence.
4.4.1 Aerobic Biotransformation of 17α-E2 and 17β-E2

The dissipation of 17α-E2 and 17β-E2 in the unsterile microcosms was rapid with no lag time and $t_\frac{1}{2} < 0.5$ d in both soils (Table 4.2). The changes in E2 concentrations over time are statistically the same between isomers for a given soil, similar to what was observed for the synthetic androgens 17α- and 17β-trenbolone (Khan et al., 2009, 2010). E2 exhibited a factor of ~2 shorter $t_\frac{1}{2}$ in D-46 compared to C-45. The higher moisture content, OM and silt-clay fraction of the D-46 soil, likely enhanced the pool of biomass and enzymatic activity (Kanazawa and Filip, 1986; Sessitsch, et al., 2001; Skujiņš, and Burns. 1976; Drążkiewicz, 1995). The activity of the enzyme dehydrogenase has been positively correlated to 17β-E2 oxidation (Chun et al., 2005). Additionally, CO$_2$ production in the D-46 soil controls was ~4 times higher than the C-45 soils indicating a higher biomass activity in the D-46 microcosms.

The pseudo first-order exponential decay model fits to the data for the E2 isomers in both soils with degradation rates of 1.15 - 3.22 d$^{-1}$ ($R^2$ values of 0.88-0.96, Table 4.2), which result in $t\frac{1}{2}$ estimates similar to those observed (Table 4.2). Model fits in all systems are poor after 2 to 3 half-lives and do not predict the persistent residuals observed at later times, which averaged 6.7 ± 1.5 mol% across all soil-isomer combinations (Figure 4.1). Sorption-limited bioavailability, residence time, and oxygen and nutrient depletion have been found to hinder the degradation of compounds in soil (Harms and Bosma, 1997). In soil controls, O$_2$ levels were > 70% of O$_2$ in ambient air after 19 d, thus oxygen was not considered limiting. Nutrients were also unlikely to be limiting given the organic matter present, the relatively high nutrient holding capacity, and CO$_2$ production indicating an active microbial community. Sorption-limited bioavailability can occur with increasing contact time with soil as contaminants penetrate further into microscopic pores (physical entrapment) or are irreversibly bound (Steinsberg et al., 1987; Harms and Bosma, 1997; Boivin et al., 2005). Hormone recovery in the first extractions at $t=0$ were > 97%, but by the end of the incubation period, ~10% of the hormone recovered was in the second extraction. Fan et al., (2007) observed decreasing extractability over time for 17β-E2 and testosterone in soils, with accumulation of 17β-E2 in the humic acids. Xuan et al. (2008) suggested that the slow
desorption of 17β-E2 from their nonsterilized soil may have limited its availability in solution and thus deviated from first order degradation with time.

**Figure 4.1.** Loss of 17α- and 17β-E2 (top) and production/loss of the metabolite E1 (bottom) over time in live and sterile aerobic Coloma (left) and Drummer (right) soils represented as mole % based on moles of parent hormone added at t=0. Lines represent fits of a pseudo first order exponential decay model (Eq. 1 and 2) for both the parent compounds (top) and the metabolite E1 (bottom), respectively. Error bars represent the mean ± standard deviation (n=3).
Sorption-limited degradation was not apparent for hormones in aqueous soil slurries over a 3-d period (Lee et al., 2003) or in hormone mineralization studies in soil microcosms over a 21-d period (Stumpe and Marschner, 2009).

The observed t\(\frac{1}{2}\) values of 17β-E2 are similar to those reported by Coluci et al. (2001) of < 0.5 d in 3 soils (a loam, sandy loam, and silty loam), Ying et al. (2005) of < 1 d in a loam soil (estimated from their Figure 4.1) and Xuan et al. (2008) of <0.2 d in a silt loam. Carr et al. (2011) reported longer half-lives 17β-E2 of ~2.2 d with Friona loam soils, but their aerobic soil microcosms were maintained at drier conditions (30% of field capacity). Khan et al. (2010) and Xuan et al. (2008) observed increasing degradation rates of hormones with increasing soil moisture content (sub-saturation). For the only 17α-E2 aerobic soil study available in the literature (Xuan et al., 2008), a t\(\frac{1}{2}\) value of 1.9 d was reported, but this was done in a sterile-unsterile soil mix with only 20% wt% being unsterilized soil, thus the initial biomass was likely much lower. The same group measured the effect of unsterile to sterile soil ratios on degradation rates with 17β-E2, and not surprisingly, observed decreasing t\(\frac{1}{2}\) values with increasing amounts of unsterile soil. Applying a similar relationship to 17α-E2 results in a t\(\frac{1}{2}\) for their unsterile soil of < 0.4 d.

### 4.4.2 Metabolite Formation and Degradation in Unsterile Microcosms

E1 and E3 were monitored in all samples; however, only E1 was detected. E3 was not detected in any samples nor was 17α-E2 detected in soils amended with 17β-E2 and vice versa. As E2 concentrations decreased, E1 concentrations increased, peaking between 0.33 – 1 d before declining, presumably from subsequent microbial degradation. E1 peaks at lower levels in D-46 (32 mol%, t = 0.33 d) as compared to C-45 (60 mol%, t = 1 d) and, likewise, subsequent loss of E1 was faster in D-46 (Figure 4.1), consistent with a higher biomass activity in D-46. The observed temporal formation of E1 from 17α-E2 versus 17β-E2 and subsequent E1 loss are statistically different in both soils and between soils. As was observed for E2, degradation of E1 in D-46 is much faster than in C-45, which results in E1 peak concentrations being higher in C-45.
The first-order exponential decay fits to the E1 data ($k_b$ in Eq. 2) are also statistically different between isomers for a given soil or between soils (Table 4.3; model fits are plotted in Figure 4.1), although the model fits do not predict peaks and tails well in all systems. The $t_{1/2}$ values for the subsequent loss of E2 formed from the E2 isomers are $<1.8$ d, in general agreement with reported E1 half-lives by Coluci et al. (2001) of $<1.7$ d and Carr et al. (2011) of $<1.1$ d. However, model rates do not do a good job of predicting the persistent residuals observed at later times, especially for D-46, with E1 remaining long after the predicted loss from the soils.

The difference in E1 formation/loss behavior between $17\alpha$-E2 and $17\beta$-E2 was not expected given that degradation rates of both isomers are essentially the same. Xuan et al. (2008) observed significant E3 formation in their 20/80 unsterile/sterile soil mix from $17\alpha$-E2 (the only sorbent tested for the $\alpha$-isomer), but not for $17\beta$-E2 in any of the sterile/unsterile soil mixes or in the 100% unsterile soil. Rapid degradation of a daughter metabolite such as E3 can limit the ability to monitor their production (Ying and Kookana, 2005) as may have been the case in our soil microcosms. Regardless, it is clear that E1 is the first primary metabolite for both hormones, consistent with studies by Turfitt (1947b) in soil bacteria cultures and Renwick and Engel (1967) in isolated pure enzyme studies.

### 4.4.3 Abiotic Transformation

Average recoveries over time in autoclaved-sterilized soils of $17\alpha$-E2 and $17\beta$-E2 were $97.9 \pm 8.9\%$ and $97.4 \pm 11.8\%$, respectively, in the D-46 soil, and $96.4 \pm 12.0\%$ and $88.7 \pm 8.9\%$, respectively, in the C-45 soil. No metabolites were observed in the abiotic microcosms except in a single sample ($17\alpha$-E2, D-46, $t = 19$ d), where E1 was detected corresponding to $3.8$ mol% conversion and which may be due to incomplete sterilization (e.g., not all spores were destroyed) or introduction of microbes during hormone amendment. Slow loss from abiotic microcosms over time for all E2 isomers in both soils is statistically significant. The lack of any known metabolites in the abiotic microcosms and the slow loss of E2 over time suggest that loss is likely due to
irreversible sorption rather than abiotic degradation. The latter reasoning is consistent with the apparent persistence of hormone residuals in unsterile soils (Figure 4.1).

Previous studies have reported abiotic conversion of $17\beta$-E2 in soils. Colucci et al. (2001) observed a 75% conversion of $17\beta$-E2 to E1 within 3 d in autoclaved soil microcosms (autoclaved for only 2 consecutive days). Fan et al. (2007) observed a 12 % transformation of applied $17\beta$-E2 to an unknown polar compound in their soil which had been autoclaved once and amended with HgCl$_2$. Sheng et al. (2009) assessed the potential for manganese oxides in soil to oxidize $17\beta$-E2 to E1 in ground autoclaved soils before and after selective removal of manganese oxides. E1 was produced with the concurrent release of Mn(II) in soils prior to manganese oxide removal exemplifying one potential abiotic transformation process in soils.

4.5 Summary and Environmental Implications

This study suggests that the degradation rates of $17\alpha$-E2 and $17\beta$-E2 in aerobic soils can be assumed to be the same and that E1 is the primary metabolite of both isomers with biotransformation being the primary mechanism of E2 oxidation in aerobic soils. The first order exponential decay model with the assumption that sorption does not significantly impact degradation did not accurately predict hormone residuals at later times, which indicates that caution should be used when trying to model fate and transport of hormones in the environment, given that most models default to such simplifying assumptions. With continual land application of biosolids, manure, and effluent, these natural estrogens may continue to accumulate, providing additional opportunity for these compounds to enter aquatic habitats. Once hormones are discharged or run off into the surface water, sediments may serve as a sink or source, depending on water turbulence and biogeochemical processes (Duong et al., 2009), for which little is known.
4.6 Acknowledgements

This work was funded in part by a U.S.D.A. AFRI Water and Watersheds Award No. 104117 and by a Purdue University College of Agriculture Undergraduate Research Grant. We also wish to acknowledge Stephen Sassman for his analytical chemistry support, Marianne Bischoff for her help with the GC, and Dr. Steven J. Landry for his statistical support.
CHAPTER 5. TRANSFORMATION OF 17α-ESTRADIOL, 17β-ESTRADIOL, AND ESTRONE IN SEDIMENTS UNDER NITRATE- AND SULFATE-REDUCING CONDITIONS

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5.1 Abstract

The natural manure-borne hormones, 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), and estrone (E1), are routinely detected in surface water near agricultural land and wastewater treatment facilities. Once in the stream network, hormones may enter the sediment bed where they are subject to anaerobic conditions. This study focuses on the difference in anaerobic transformation rates and formation of metabolites from 17α-E2, 17β-E2, and E1 (applied at ~3.66 μmol kg\(^{-1}\) of sediment on a dry weight basis) under nitrate- and sulfate-reducing conditions. Sediment extracts were analyzed using negative electrospray ionization tandem mass spectrometry. Under both redox conditions, degradation was stereospecific and followed similar trends in half-lives: 17β-E2 < 17α-E2 < E1, with degradation considerably slower under sulfate-reducing conditions. Both E2 isomers were predominantly converted to E1; however, isomeric conversion also occurred with peak concentrations of ~1.7 mol% of 17β-E2 formed in 17α-E2 amended sediments and peak concentrations of ~2.4 mol% of 17α-E2 formed from 17β-E2. In E1-amended systems, E1 transformed to E2 with preferential formation of the more potent 17β isomer up to ~30 mol% suggesting that isomer interconversion is through E1.
Sediments, therefore, may serve as both a sink and a source of the more estrogenic compound E2. Transformation of amended hormones in autoclaved sediments was markedly slower than in non-autoclaved sediments. Results support the inclusion of isomer-specific behavior and the potential for reversible transformation and interconversion in anaerobic sediments in modeling fate in stream networks and developing risk management strategies.

5.2 Introduction

The persistence and subsequent transport of estrogenic compounds from the agronomic land application of manure, effluent, and biosolids have contributed to increased estrogen detection in drainage ditches and surface water (Aga, 2008; Khanal et al., 2006). Once in the water, these compounds including 17α-estradiol (17α-E2), 17β-estradiol (17β-E2) and their primary metabolite, estrone (E1) (Figure B.1 in Appendix B) have the potential to alter secondary sex characteristics, disrupt endocrine function, and activate hormone responses in sensitive aquatic species at low ng L\(^{-1}\) concentrations, which are common in environmental samples (reviewed by Hanselman et al., 2003; Aga, 2008; and Young and Borch, 2009).

Most of the studies addressing the degradation of the natural estrogens have focused on 17β-E2 including aerobic stream sediments (Bradley et al., 2009); aerobic soils (Colucci et al., 2001; Das et al, 2004; Ying and Kookana, 2005; Xuan et al., 2008; Bradley et al., 2009; and Mashtare et al., 2013); anaerobic or saturated soils (Ying and Kookana et al., 2003b; Fan et al., 2007; Carr et al., 2011); sediments impacted by wastewater treatment plant discharge (Bradley et al., 2009); marine sediments (Ying and Kookana, 2003b), river water and sediments (Jurgens et al., 2002); activated sludge in membrane reactors (Joss et al., 2004); alternating anoxic and aerobic conditions (Dytczak et al., 2008), and anaerobic lake sediments (Czajka and Londry, 2006). The latter two studies (Czajka and Londry, 2006; Dytczak et al., 2008) observed the conversion of 17β-E2 to 17α-E2 under reducing conditions, which Hutchins et al. (Hutchins et al., 2007) hypothesized as a reasonable explanation for the unexpected elevated concentrations of 17α-E2 in swine and poultry lagoons.
Much less is known on the environmental fate of 17α-E2 and E1, although these compounds are more frequently detected in beef cattle and dairy lagoons, stream and ditch water, and stream sediments than 17β-E2 (Kolpin et al., 2002; Hanselman et al., 2003; Snow et al., 2009; Gall et al., 2011). To date, laboratory degradation studies of 17α-E2 have been limited to soil bacteria cultures (Turfitt, 1947a, 1947b), dairy lagoon water (Zheng et al., 2012), a sterile/unsterile soil mixture (Xuan et al., 2008), and aerobic soils (Mashtare et al., 2013). Laboratory degradation studies of E1 have focused on aerobic soils (Colucci et al., 2001; Ying and Kookana, 2005); aerobic stream sediments (Bradley et al., 2009); anaerobic soils (Ying and Kookana et al., 2005); anaerobic river water and sediments (Jurgens et al., 2002); activated sludge (Joss et al., 2004); and dairy lagoon water (Zheng et al., 2012). Limited field studies have reported the apparent conversion 17α-E2 to 17β-E2 and E1 in a simulated feedlot under saturated conditions (Mansell et al., 2011) and in dairy manure and waste lagoons (Zheng et al., 2008) based on changes in relative concentrations in environmental samples. However, a direct assessment of this conversion potential has not been well explored and little is known about how 17α-E2 and E1 will behave in anaerobic sediment systems.

The objectives of this study were to unequivocally assess: (1) differences in transformation rates of 17α-E2, 17β-E2, and E2 in sediments under nitrate-reducing and sulfate-reducing conditions; (2) if interconversion between the E2 isomers occurs; (3) if E1, the primary metabolite from E2 degradation, is transformed back to E2; and (4) if a particular isomer is preferentially formed from E1. To achieve these objectives, separate sediment microcosm treatments were prepared for 17α-E2, 17β-E2, and E1.

5.3 Materials and Methods

5.3.1 Sediment

Sediment samples were collected from the ditch and stream network at the Purdue University Animal Science Research and Education Center (ASREC) and Little Pine Creek (West Lafayette, IN) in July and October 2010, and February and April 2011. Sample collection times and locations were selected to achieve a representative
composite of sediments in the network. After each collection, stream water-saturated sediments were stored in closed containers at 4 °C in the dark. Immediately prior to initiating the anaerobic microcosms (May 2011), all collected sediments were passed through a 2 mm sieve and thoroughly mixed to create a homogenous sediment sample. Homogenized sediment properties are summarized in Table 5.1.

**Table 5.1. Selected sediment properties**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM (%)</td>
<td>4.0</td>
</tr>
<tr>
<td>CEC (cmol_c kg^{-1})</td>
<td>25</td>
</tr>
<tr>
<td>pH</td>
<td>7.07</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>20</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>42</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>38</td>
</tr>
<tr>
<td>Clays</td>
<td>RIS &gt;&gt; I &gt; K</td>
</tr>
</tbody>
</table>

a % organic matter determined by loss on ignition at 360 °C (Brown, 1998); b cation exchange capacity determined using the Mehlich III Extraction method (1 M NH_{4}OAc buffered at pH 7.0) (Brown, 1998); c pH of a 1:1 soil (g): water (mL) slurry (Brown, 1998); d determined by hydrometer method (Gee and Bauder, 1986); e RIS=randomly interstratified illite-smectite, I=illite, K=kaolinite, as identified by XRD.

### 5.3.2 Chemicals

All estrogens (17α-E2, 17β-E2, E1) and the internal standard 17β-estradiol-D3 (17β-E2-D3) were obtained from Sigma Aldrich, St. Louis MO, USA. Acetonitrile (ACN), ethyl ether (Et2O), and methanol (MeOH) were purchased from Mallinckrodt, Phillipsburg, NJ, USA. Food grade protein gelatin used as an electron donor was purchased from Kroger Co., Cincinnati, Ohio, USA. All chemicals were analytical-reagent grade or higher purity (>99%) except for 17α-E2, 17β-E2, 17β-E2-D3, which were >98% purity, and used as received. Ultrapure water was prepared using a Mega-Pure System, MP-3A from Barnstead, Dubuque, IA, USA. Hormone stock solutions were prepared in pure methanol and stored at 4 °C in the dark.

A synthetic freshwater medium (pH adjusted to 7 with HCL) was prepared as described by Homklin et al (2011) by dissolving 1.0 g of NaCl, 0.4 g of MgCl_{2}×6H_{2}O, 0.1 g of CaCl_{2}×2H_{2}O, 0.25 g NH_{4}Cl, 0.2 g of KH_{2}PO_{4}, 0.5 g of KCl, 1 mL of trace element mixture, 2.52 g of NaHCO_{3}, 0.36 g Na_{2}S nonhydrate into a total volume of 1 liter of Milli-Q water. The trace element mixture was prepared by mixing 12.5 mL HCl (7.7
M), 2.1 g FeSO₄·7H₂O, 30 mg H₃BO₃, 100 mg MnCl₂·4H₂O, 190 mg CoCl₂·6H₂O, 29.36 mg Ni(NO₃)₂, 2.9 mg CuSO₄·5H₂O, 144 mg ZnSO₄·7H₂O and 36 mg Na₂MoO₄·2H₂O into 1 liter of Milli-Q water. The freshwater medium was autoclaved and cooled under nitrogen. Sodium nitrate or sodium sulfate was added to each bottle to achieve an initial 20 mM electron acceptor solution. The headspace was then purged with nitrogen, bottles capped, sealed with parafilm, and transferred into a large 4-glove vinyl anaerobic chamber (Figure B.2 in Appendix B). The chamber is equipped with an automated airlock pass-through chamber, oxygen and hydrogen analyzer, 3 fan boxes equipped with palladium catalysts, and a dark storage incubator. At the start of the experiment, 0.5 mL of a 500 mg/L methanol hormone stock solution was transferred into a glass flask, the methanol evaporated, and hormones re-suspended in 500 mL of a 20 mM electron acceptor-solution and mixed on a magnetic stir plate to achieve ~0.5 mg hormone L⁻¹. Each hormone solution (17α-E₂, 17β-E₂, and E₁) was prepared separately as well as a no-hormone blank. A sample from each solution was saved and extracted using diethyl ether (Et₂O) at a 4:4.4 water:Et₂O liquid-liquid exchange for analysis on the LC/MS/MS.

5.3.3 Batch Sediment Pre-incubation

Sediments were pre-incubated to ensure the onset of nitrate-nitrite-reducing or sulfate-reducing conditions prior to hormone addition (defined as time t=0). Homogenized wet sediment was transferred to a plastic container and covered with 500 mL of 20 mM nitrate or 20 mM sulfate solution. The protein gelatin electron donor (3 g) was dissolved in the freshwater medium prior to amendment to provide a complex food source and promote diverse anaerobic microbial community development as described by Kourtev et al (2006, 2009). The container was tightly sealed by wrapping electrical tape and parafilm around the lid edges followed by purging the headspace with nitrogen for 5 minutes using two 16-gauge syringe needles (one for N₂ input and one for exhaust) after which the syringe holes were sealed with electrical tape. The sediment was mixed by gently rocking the container and then placed into a small foil-covered plexi-glass anaerobic chamber to pre-incubate (Figure B.2 in Appendix B). The chamber was
maintained under positive N\textsubscript{2} pressure to minimize the risk of O\textsubscript{2} contamination and a methylene blue indicator solution was used to confirm anaerobic conditions. After a week in the small anaerobic chamber, the sediment-water mixtures were covered in foil and transferred to a larger vinyl chamber as previously described (Figure B.2 in Appendix B) for which an atmosphere of N\textsubscript{2} with ~3-5\% H\textsubscript{2} was maintained.

5.3.4 Individual Anaerobic Microcosm Preparation

Glass centrifuge tubes (35 mL) and Teflon-lined screw caps were rinsed with acetone and wet autoclaved for 30 minutes, transferred to a 105 °C oven until dry and cooled to room temperature. Water and solvent-resistant labels were applied and the microcosm tubes were transferred into the large anaerobic chamber for at least 24 hours prior to the start of the experiment to allow sufficient time to degas.

The water above the sediment surface in the pre-incubation batch container was siphoned off followed by mixing the sediment with a metal spatula. Approximately 8.5 g of wet sediment (~5 g dry wt basis) was transferred into each centrifuge tube and lightly capped until time of amendment. Sediment slurry was sampled in triplicate for gravimetric moisture content determination. Pore water was extracted by centrifuging wet sediments and saved along with the siphoned off water for nitrate-nitrite or sulfate analysis to monitor nitrate-nitrate and sulfate reduction activity. A hormone solution (10 mL) or blank solution was added to each microcosm, tightly capped, gently shaken to suspend the sediments in the solution, and the time of amendment for each microcosm recorded. For hormone amended microcosms, the initial hormone concentration was ~3.66 μmol kg\textsuperscript{-1} dry wt. basis. Microcosms were placed in the dark anaerobic storage incubation chamber until time of sacrifice.

In addition, a separate set of sediment microcosms were prepared using autoclaved sediment. Wet sediment (~8.5 g of sediment) was transferred into 35 mL centrifuge tubes and autoclaved for 1 h on each of 3 consecutive days as described by Wolf et al (1989). These microcosms were transferred to the anaerobic chamber 24 hours prior to the start of the experiment and amended with hormones as described above.
For each hormone and reducing condition, five hormone-amended microcosms were analyzed at each sampling time: four for hormone analysis (including 1 autoclaved microcosm), and 1 for electron acceptor analysis. Triplicate soil blanks (no hormones) were also analyzed at each sampling time. Microcosm sets were sacrificed after 0, 1, 3, 7, 14, and ~21 days and at two later times between 45 – 80 days.

5.3.5 Electron Acceptor Analysis

Microcosms for electron acceptor analysis were gently shaken and centrifuged at 1600 rpm for at least 20 min. Supernatant (1 mL) was transferred into a micro-centrifuge tube and centrifuged at 13000 rpm for 30 min to remove fine particulates followed by analysis on a Seal AQ2. AQ2 Methods No: EPA-114-A Rev. 6 and EPA-123-A Rev. 4 were used for nitrate-nitrite and sulfate analyses, respectively. If electron acceptor concentrations were determined to be less than 10% of the amended concentration, the remaining microcosms in the anaerobic chamber were re-amended with a sterilized and degassed concentrated electron acceptor solution until concentrations approached ~20 mM, which was only required for the nitrate-reducing systems.

5.3.6 Hormone Extraction and Analysis

At each sampling time, microcosms for hormone analysis were transferred into the small anaerobic chamber for liquid extraction. Diethyl ether (Et₂O) was added to each microcosm to minimal headspace and capped tightly. Each bottle-cap was wrapped with parafilm, tubes covered with foil to minimize photodegradation potential, equilibrated end-over-end at 35 rpm for ~24 h at room temperature (22 ± 2 °C) and centrifuged at 1600 rpm for 20 min. Approximately 1.2 mL (by weight) of Et₂O was transferred into an HPLC vial, evaporated, and residues re-dissolved in 0.5 mL of MeOH containing an internal standard (17β-E2-D3). For the sulfate-reduction experiment, a second extraction was carried out by removing the excess Et2O from the first extraction and repeating the extraction steps above.

Estrogen analysis was performed on a Shimadzu high performance reverse-phase liquid chromatography coupled to a Sciex API3000 mass spectrometer operated in
positive electrospray ionization (ESI) mode with multiple reaction monitoring. Separation was performed using 20-25 μL injections on a Phenomenex Gemini C-18 column (150 mm x 2 mm, dp= 5 μm) with a gradient elution using water:methanol (90:10) containing 2 mM ethanolamine [solvent A] and acetonitrile containing 2 mM ethanolamine [solvent B] at 0.35 mL min\(^{-1}\). Initial mobile phase composition was 30% solvent B followed by a linear gradient to 50% solvent B from 0 to 8.5 min after which solvent B was ramped to 100% for 2 min to wash the column and then re-equilibrated at 30% solvent B for 2 min prior to the next injection. The chromatographic retention times for E3, 17β-E2, 17β-E2-D3, 17α-E2, and E1 were 3.8 min, 7.9 min, 7.9 min, 8.5 min, and 9.2 min, respectively. E3 (m/z 287 → 145), 17α-E2 and 17β-E2 (m/z 271 → 145), and E1 (m/z 269 → 145) were quantified using independent external calibration curves with check standards run approximately every 12 samples. The internal standard, 17β-E2-D3 (m/z 274 → 145), was used to assess matrix effects in the MS, which were found to be negligible. A deuterated 17α-E2 internal standard was not available. For a 25 uL injection, the limit of detection for all estrogens was 0.015 μg L\(^{-1}\) and the method limit of quantitation (MLOQ) was 0.03 μg L\(^{-1}\).

### 5.3.7 Estimating Degradation Rates

A first-order exponential decay model was fit to the data to estimate net (apparent) degradation rates (\(k_a\) and \(k_b\), d\(^{-1}\)) of the hormones following the assumption of a simple set of consecutive reactions (Eq. 1) and using solutions defined in Eqs. 2 and 3 (derivation detailed in the supplemental information).

\[
\begin{align*}
A \xrightarrow{k_a} B \xrightarrow{k_b} C \\
[A]_t &= [A]_0 e^{-k_at} \\
[B]_t &= \{(k_a[A]_0(e^{-k_at} - e^{-k_bt}))/(k_b-k_a)\}^{-1}
\end{align*}
\]

where \([A]_0\) and \([A]_t\) represent the extracted applied hormone concentrations on a mole basis at time t=0 and time t (d), respectively, and \([B]_t\) represents the mole concentration
of the extracted primary daughter metabolite at time $t$ (d). At time=0, all compounds except the applied hormone is assume to be zero (i.e., $[B]_0 = [C]_0 = 0$). This model assumes a steady-state biomass, and does not account for irreversible transformations or effects of sorption on bioavailability.

5.3.8 Statistical Analysis

Minitab v16 (State College, PA: Minitab, Inc.) was used for statistical analysis. An ANOVA was used to determine the significance of differences between the observed temporal changes in transformation of 17α-E2 and 17β-E2 as well as between the rate of E1 metabolite production and subsequent loss from 17α-E2 and 17β-E2 within and between redox conditions. Statistical tests used $\alpha = 0.05$ as the level of significance.

5.4 Results and Discussion

All hormone concentrations are presented and discussed on a mol % basis relative to the parent hormone applied. Electron acceptor concentrations are presented in mM. Applied hormone concentrations over time for nitrate-reducing and sulfate-reducing conditions are shown in Figures 5.1A and 5.1B, respectively, with the corresponding electron acceptor trends shown in Figures 5.1C and 5.1D. Metabolite formation and isomeric conversion for each E2-isomer are summarized in Figure 5.2 with reversible transformation of E1 to 17α-E2 and 17β-E2 under both reducing conditions shown in Figure 5.3. The pseudo first-order exponential decay model fits to the hormone data are summarized in Table 5.2 along with select apparent degradation rates ($k_a$ and $k_b$), modeled $t_{1/2}$ values, and coefficients of determination ($R^2$) from fitting Eqs. 2 and 3.
5.4.1 17α-E2 and 17β-E2 Transformation under Nitrate- and Sulfate-
Reducing Conditions

The loss 17β-E2 was rapid and significantly faster than 17α-E2 under both nitrate-
and sulfate-reducing conditions with faster dissipation of both isomers under nitrate-
reducing conditions (Table 5.2). The continuous reduction over time in electron acceptor
concentrations indicates that the targeted redox activity was occurring and that the
microbial communities in the live microcosms remained active during incubation
(Figures 5.1C and 5.1D). However, 17α-E2 and 17β-E2 t½ values were ~16 and ~6 times
longer, respectively, under sulfate-reducing conditions than under nitrate-reducing
conditions. Sulfate utilization (Figure 5.1D) was slower relative to nitrate (Figure 5.1C)
with nitrate concentrations falling to below 20% of amended concentrations by day 7
while it took 80 d to achieve a similar loss in the sulfate-reducing systems suggesting
lower microbial activity in the latter. E3 was not detected in either system. The mass
balance of total estrogens (Figure B.3 in Appendix B) decreased with time under both
redox conditions suggesting that either E1 or the E2 isomers were mineralized, degraded
to unknown metabolites, or irreversibly sorbed.

For 17β-E2 under nitrate-reducing conditions, the t½ of < 0.3 d is similar to values
of < 0.66 d (n=2) measured in river sediments by Jürgens et al. (2002) whereas Czajka
and Londry (2006) observed t½ of 21 d in a sandy lake sediment. Likewise, under sulfate-
reducing conditions we observed a t½ ≤ 1.5 d for 17β-E2 whereas others have reported
much longer half-lives including 9 d and 70 d in sandy (Czajka and Londry, 2006) and
marine (Ying and Kookana, 2003b) sediments, respectively. The shorter half-lives
observed in our study are likely due, in part, to differences in sediment composition (OM
and clay content, thus smaller particle size) (Marshall, 1976) and the addition of a
complex protein, both which have been shown to promote microbial diversity (Kourtev et
al., 2006, 2009). In addition, the agro-impacted sediments used in our study have regular
exposure to hormones from land-application of manure and lagoon effluent as well as
plant sterols/phytoestrogens and other xenoestrogens (e.g., atrazine), which may have
fostered a microbial community better adapted for xenobiotic degradation. Jacobsen et al
(Jacobsen et al., 2005). observed enhanced degradation of 17β-E2 in soils that been
exposed to manure and biosolids suggesting an adaptation of the microbial community has occurred.

For 17α-E2, t½ values were ~16 and ~46 times longer than 17β-E2 under nitrate-reducing and sulfate-reducing conditions, respectively. We hypothesize that the isomer differences may be due to disparities in the stereospecificity of the available enzymes, which would alter the loss kinetics. In an isolated enzyme study, Renwick and Engel (1967) observed that 17β-E2 dehydrogenase (which can reversibly transform 17β-E2 to E1) has an activity ~3.5 times faster, possesses a higher saturation limit, and exhibits higher stability than 17α-E2 dehydrogenase. The greater difference between isomer t½ values under sulfate-reducing conditions suggests a pronounced decrease in available enzymes specific to 17α-E2 degradation. Longer t½ values of 17α-E2 relative to 17β-E2 were also observed in an anaerobic blended dairy lagoon water study by Zheng et al. (2012).

For nitrate-reducing conditions, a significant decrease in nitrate utilization was observed between 14 d and 46 d (Figure 5.1B). The loss of nitrate was ~12% d⁻¹ from 0-14 d, 2% from 15-46 d, and increased to 6.2% d⁻¹ after 48 d when microcosms were re-amended with protein suggesting that from day 15 to 26 the system demonstrates a shift to less available carbon sources such as to SOM. Therefore, a comparison was made of decay rate estimates for the entire incubation period (59 d) and for the first 14 d of data to determine whether the decrease in nitrate-reduction significantly impacted the observed transformation rates (Table 5.1, Figure 5.1A). The model fits to the dissipation patterns of the amended E2 isomers were good (R² of 0.98-0.99) with no significant differences observed suggesting that the decrease in nitrate-use had no discernible impact on the transformation rates of the amended hormones. The model fits were also generally able to predict the tails of the amended hormones suggesting that any impact of sorption-limited bioavailability on degradation was likely limited given that sorption is not accounted for in the first order fits (Eqs. 2 and 3).
5.4.2 E1 Formation from 17α-E2 and 17β-E2 and Interconversion

Under nitrate-reducing conditions, the first-order exponential decay model fits to the formation of E1 from each isomer and E1’s subsequent loss were good (R² of 0.66-0.98) (Table 5.2, Figures. 5.2A and 5.2C). The estimated $t_{1/2}$ of E1 that formed from 17α-E2 is approximately a factor of two slower than the subsequent degradation of E1 formed from 17β-E2. Under aerobic conditions, Mashtare et al. (2013) also observed differences in E1 formation and loss between the E2 isomers. Half-lives estimated for fits to the first 14 d under nitrate-reducing conditions compared to the entire incubation period are 25 to 50 % shorter, suggesting the decrease in nitrate-reduction may have slowed down the apparent loss of E1 formed from E2 in the microcosms.

Table 5.2. Summary of observed half-lives ($t_{1/2}$, d) under nitrate-reducing and sulfate-reducing conditions and estimated anaerobic degradation rates ($k_a$ and $k_b$, d⁻¹), half-lives ($t_{1/2}$, d), and associated coefficients of determination ($R^2$) for 17α-E2, 17β-E2, and E1 assuming pseudo first-order decay (Eqs. 2 and 3) under nitrate-reducing conditions (monitored for ~59 d). Bracketed modeled values are fit to the first 14 d of data when nitrate reduction was fastest.

<table>
<thead>
<tr>
<th>Reducing Condition</th>
<th>Applied Hormone</th>
<th>t_{1/2} (d) observed</th>
<th>$k_a$ (d⁻¹)</th>
<th>$R^2$</th>
<th>t_{1/2} (d) from $k_a$</th>
<th>$k_b$ (d⁻¹)</th>
<th>$R^2$</th>
<th>t_{1/2} (d) from $k_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>17α-E2</td>
<td>4.34</td>
<td>0.17 [0.17]</td>
<td>0.99 [0.98]</td>
<td>4.18 [4.16]</td>
<td>0.031 [0.060]</td>
<td>0.66 [0.93]</td>
<td>21.9 [11.6]</td>
</tr>
<tr>
<td></td>
<td>17β-E2</td>
<td>0.27</td>
<td>5.67 [5.67]</td>
<td>0.98 [0.99]</td>
<td>0.12 [0.12]</td>
<td>0.016 [0.022]</td>
<td>0.96 [0.98]</td>
<td>42.7 [32.0]</td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td>35.9</td>
<td>0.021 [0.024]</td>
<td>0.73 [0.25]</td>
<td>32.4 [28.6]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfate</td>
<td>17α-E2</td>
<td>69.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17β-E2</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td>~3 dᵇ, &gt;80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Bracketed modeled values are fit to the first 14 d of data when nitrate reduction was fastest. ᵇ E1 concentrations dropped to ~42 mol % but then rose above 60 mol % for the rest of the 80-d incubation period.
In addition to E1 formation, apparent interconversion between E2 isomers occurred by day 1 sampling under both redox conditions. 17α- and 17β-E2 concentrations formed from isomeric conversion peaked and then decreased over time under nitrate-reducing conditions, but generally continued to accumulate over time under sulfate-reducing conditions (Figure 5.2). In 17α-E2 amended nitrate-reducing microcosms, 17β-E2 peaked at 0.62 mol % on day 21 (Figure 5.2C) while under sulfate-reducing conditions, 17β-E2 peaked earlier (14 d) and at 1.72 mol % (Figure 5.2D). Likewise, in the 17β-E2

![Figure 5.1](image-url)

**Figure 5.1.** Composite of single hormone-amended experiments showing the loss of 17α-E2 (▲), 17β-E2 (■), and E1 (●) in mol % over time under (A) nitrate-reducing and (B) sulfate-reducing conditions; and associated changes in electron acceptor concentrations (mM) over time shown for (C) nitrate and (D) sulfate. Re-amendment of nitrate (+) is shown for 8 and 15 d and re-amendment with both nitrate and protein (*) at 48 d. Solid lines (—) represent first-order decay model fits (Eq. 2) over the first 14 d with extrapolation after 14 d represented by dotted lines (⋯). Dashed lines (---) represent the model fits to the entire incubation period. Error bars represent the standard deviation (n=3).
amended microcosms, greater isomeric conversion occurred with 17α-E2 concentrations reaching 2.4 mol % (Figure 5.2A), whereas in nitrate-reducing conditions, only 0.24 mol % of the isomer was formed (Figure 5.2D). Formation of 17α-E2 from 17β-E2 in laboratory studies has previously been reported for anaerobic lake sediments under methanogenic, sulfate-reducing, and iron-reducing conditions (not observed in nitrate-reducing) (Czajka and Londry, 2006) and in anoxic activated sludge (Dytczak et al., 2008). Isomeric conversion from 17α-E2 to 17β-E2 in sediments has not been previously reported; however, it was observed in blended dairy lagoon water (Zheng et al., 2012) and simulated feedlot runoff (Mansell et al., 2011). The greater accumulation of E2 isomers
in sediments under sulfate-reducing versus nitrate reducing conditions is likely due to both the slower degradation rates of E2 and reversible transformation of E1 back to E2 as discussed below.

5.4.3 E1 Fate in E1-Amended Sediments

In E1-amended nitrate-reducing microcosms, E1 half-life was 35.9 d (Table 5.2, Figure 5.3A), which is similar to the subsequent loss of E1 (\( t_{1/2} \) ranged between 22 and 43 d) after forming from either E2 isomer. In the E1-amended sulfate-reducing microcosms, E1 dropped to ~42 mol % within 3 days. However, E1 concentrations subsequently rose to >60 mol % by 14 d presumably due to the reversible transformation between E1 and E2 (Figure 5.3B) and remained above ≥ 60% through the 80-d incubation period. In anaerobic sludge (without nitrate) membrane reactors spiked with E1, a constant E1-E2 ratio was observed within hours, supporting that the reversible transformation of E1 and E2 results in an apparent increase in persistence (Joss et al., 2004). Czajka and Londry (2006) also reported that the E1 formed from 17β-E2 in their sandy lake sediments under nitrate-, iron-, and sulfate-reducing conditions as well as methanogenesis did not appear to dissipate substantially within their 383 d incubation period. The slow or apparent non-loss of E1 in these studies relative to E2 suggests that the processes and microbial populations responsible for degrading 17β-E2 are different than those capable of E1 degradation. While denitrifying bacterium has been isolated from activated sludge that can degrade E2 as a sole source of carbon (Fahrbach et al., 2006), only a limited number of bacteria are known to be able to degrade both E1 and E2 (Li et al., 2012). The current study is the first to investigate E1-amended anaerobic sediments; however, E1 fate as the starting compound was assessed in anaerobic sludge (Joss et al., 2004) and in lagoon water (Zheng et al., 2012) for which \( t_{1/2} \) of E1 was reported to be >52 d.

E1 is presumed to be the intermediate in the isomeric conversion of the parent hormones observed in the E2-amended microcosms. Reversible transformation from E1 was observed under each redox condition with both precursors formed in the E1-amended microcosms (Figures 5.3A and 5.3B). Under nitrate-reducing conditions, formation of 17α-E2 peaked at 0.19 mol % while 17β-E2 peaked at 2.14 mol %. This reversible
transformation was more pronounced under sulfate-reducing conditions with E1 to 17α-E2 conversion steadily increasing to 2.6 mol % by 81 d, while 17β-E2 peaked at 28.9 mol % within 3 d, before declining to a pseudo steady state concentration of ~7 mol %.

The formation and loss of 17β-E2 under sulfate-reducing conditions occurred with a near stoichiometric loss and gain of E1 (Figure 5.3B). While a preference for 17β-E2 formation from E1 was observed under both redox conditions, the temporal accumulation of these compounds appears to be influenced by the decay rate of the isomer. This reversible transformation may also help explain the sizeable remaining mass of E1 and the E2 isomers observed under sulfate-reducing conditions. Zheng et al. (2012) also observed reversible transformations from E1 to both E2 isomers in blended dairy lagoon water with preferential formation of 17β-E2. This preference for 17β-E2 formation is also consistent with the relative activities and stability of 17α-E2 and 17β-E2 dehydrogenases (Renwick and Engel, 1967).

5.4.4 Autoclaved Sediments

In an attempt to discern differences between biotic and abiotic transformations, we employed autoclaved sediments in a single microcosm set. Autoclaving as a sterilization procedure is commonly used in our lab and traditionally used for aerobic soils (Wolf et al., 1989) but may have been inadequate for effective sterilization of anaerobic systems. Bradley and Chapelle (2012) in regards to biodegradation of chlorinated solvents comment that while heat-sterilization under high pressure (e.g., autoclaving), greatly suppresses biological activity, it may not completely inhibit biological activity in sediments. Both Slepova et al. (2007) and Hyun et al. (1983) found thermophilic bacteria and their spores to be extremely heat resistant. In addition, Carter et al. (2007) found that although autoclaving appears to kill aerobic soil microbes, microbial enzymes remained active.

Sterility was not confirmed in our experiments and only a single microcosm for each amended hormone was sacrificed at each sampling point, thus providing no measure of variability. Nevertheless, some interesting trends were observed in the autoclaved sediments. In general, transformation of the applied hormones was slower than the non-
autoclaved (live) sediments under both nitrate-reducing (Figure B.5 in Appendix B) and sulfate-reducing (Figure B.6 in Appendix B) conditions. One exception is that the apparent loss of amended E1 under sulfate-reducing conditions was similar to the live sediments, but no formation of 17β-E2 was observed until day 14 resulting in an apparent pseudo steady state of 17β-E2 (~16 mol %) and E1 (~30 mol %). Metabolite formation was generally slower across all autoclaved microcosms. With the exception of 17α-E2 formed from E1 under nitrate-reducing conditions (~1.2 mol %), peak E2 isomeric conversion and E1 to E2 conversion was generally smaller across the incubation period under both redox conditions. Mass balances in all autoclaved microcosms (Figure B.4 in Appendix B) are higher than in the live microcosms under nitrate-reducing conditions. In autoclaved sulfate-reducing conditions, E2 mass balance in E2 amended microcosms was similar to the live soils, but lower for E1. The latter could be due to transformation of E1 to an unknown metabolite or loss to irreversible sorption, which can increase with increasing residence time. Van Emmerik et al. (2003) reported irreversible sorption of 17β-E2 in a smectite clay, which is the dominant clay type in the sediment used in our study. Although abiotic transformation of 17β-E2 to E1 in aerobic soils was reported by Colucci et al. (2001), we are unable to definitively differentiate between biotic and abiotic transformation in our study. However, if the slower transformation patterns in the autoclaved sediments are due to abiotic transformation, which is not unexpected, much of the observed transformations in the live systems appear microbially-mediated. Nevertheless, further work is needed to elucidate the difference between biotic and abiotic transformation of these compounds.

5.4.5 Environmental Implications

In soils under aerobic conditions, the rapid dissipation (t½ generally < 3 d) of 17α-E2, 17β-E2, and E1 has been observed (Colucci et al., 2001; Das et al., 2004; Ying and Kookana, 2005; Xuan et al., 2008; Mashtare et al., 2013). For hormones entering the surface water via discharge or run off, sediments may serve as a sink or source for hormones (Duong et al., 2009). Once in the sediment bed, this study suggests that the anaerobic degradation rates of 17α-E2 and 17β-E2 cannot be assumed to be the same. For
Figure 5.3. Mol % of 17α-E2 (▲) and 17β-E2 (■) (right axes) in E1-amended systems under (A) nitrate-reducing and (B) sulfate-reducing conditions. Lines represent E1 decay patterns (left axis). Error bars represent the standard deviation (n=3).

In our study, the half-life of 17α-E2 in stream sediment is 16–46 times longer than 17β-E2 depending on the extant redox condition, with higher persistence under sulfate-reducing conditions. Interconversion was observed between 17α-E2 and 17β-E2, presumably with E1 as the intermediate, which was observed to reversibly transform back to its precursors. Of particular concern is the apparent preferential formation of 17β-E2, the more potent of the estradiol isomers, although the loss rate of 17β-E2 is more rapid. The slower degradation rate of 17α-E2, to which some aquatic species have been shown to be more sensitive than their mammalian counterparts, suggests that both isomers, and their primary metabolite, E1, which exhibited even slower degradation than E2, have the potential for prolonged environmental persistence under highly reduced conditions.

The potential for isomeric conversion and reversible transformations from E1, suggest the risk to aquatic species may not be adequately predicted by looking at inputs/discharge into the water column alone. For example, E1, often the dominant hormone detected in impacted surface water (Kolpin et al., 2002; Gall et al., 2011), may transform back to the more potent 17β-E2 and 17α-E2 once partitioned into the sediment bed. The lower partition coefficients of E2 (Mashtare et al., 2011) relative to E1 suggest that sediments may then serve as a long-term source of the E2 isomers re-entering the water column via diffusion and under turbulent conditions where they may come into
contact with sensitive species. Thus, understanding the transformation potential of these compounds once in the sediment bed is paramount in developing an effective risk management strategy.

5.4.6 Acknowledgements

This work was funded in part by USDA AFRI Water and Watersheds Award 104117. We also wish to acknowledge Stephen Sassman for his analytical chemistry support, Chandeepa T. Cooray Bulathsinhala for his help in sediment collection and preparation, Leila Nyberg for her assistance with the anaerobic chamber, Dr. Steven J. Landry for his statistical support, and Nicole De Armond for her help with the Seal AQ2.
CHAPTER 6. ANAEROBIC BIOTRANSFORMATION IN SEDIMENTS OF 17α-ESTRADIOL, 17β-ESTRADIOL, AND ESTRONE UNDER IRON-REDUCING AND METHANOGENIC CONDITIONS


6.1 Abstract

Although 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), and estrone (E1) have been routinely detected in surface water and sediments near wastewater treatment plants, concentrated animal feeding operations, and agricultural fields, little is known about the transformation processes of these natural estrogens in sediments under anaerobic conditions which may dominate, especially under low base flow and stagnant water conditions. The purpose of this study was to characterize the relative biotransformation rates and formation of metabolites for 17α-E2, 17β-E2, and E1 (applied at ~3.66 µmol kg⁻¹ of sediment on a dry weight basis) under iron-reducing and methanogenic conditions for up to 95 days. Sediment extracts were analyzed using negative electrospray ionization tandem mass spectrometry. Under both redox conditions, the E2 isomers exhibited differences in degradation rates, with the half-lives of 17β-E2 < 17α-E2 < E1, with no clear correlation between the loss of the amended hormone and the dominant redox condition. Although E1 was the primary metabolite of both E2 isomers, interconversion was observed between 17α-E2 and 17β-E2 under both redox conditions with E1 hypothesized as the intermediate which reached up to ~33 mol %. Under methanogenic conditions, pseudo-steady state concentrations of ~5 mol % 17α-E2 resulted in 17β-E2 amended sediments while ~10 mol% 17β-E2 formed in 17α-E2 amended sediments. Abiotic controls support that these transformations are primarily biologically mediated. There was a negligible loss of total estrogens in these microcosm studies suggesting that
these estrogens will persist in the sediment bed under highly reducing conditions. The potential for E2 interconversion and reversible transformation from E1 to the more potent precursors, 17α-E2 and 17β-E2 supports the need to account for these transformation patterns when developing resource management strategies and risk assessment models.

6.2 Introduction

The release of the natural estrogens, 17α-estradiol (17α-E2), 17β-estradiol (17β-E2) and estrone (E1), through discharge or runoff from municipal treatment plants, concentrated animal feeding operations and agricultural fields (reviewed by Hanselman et al., 2003; Khanal et al., 2006; Aga, 2008; Liu, 2012; and Snow, 2013) continue to be a concern due to their ability to adversely affect the health and fecundity of aquatic species at low environmentally detected concentrations (reviewed by Aga, 2009; Young and Borsch, 2009). Once discharged into a water body, they may enter sediment beds, which can in turn serve as a sink and source for these hormones in the water column with subsequent hydrologic events.

Previous studies on the transformation processes of these natural estrogens in freshwater sediments under anaerobic conditions has been limited primarily to 17β-E2 in lake sediments (Czajka and Londry, 2006) and 17β-E2 and E1 (formed from 17β-E2) in river sediments (Jürgens et al., 2002). Information on the fate of 17α-E2 and E1 in sediments under anaerobic conditions is sparse despite being the most frequently detected forms in beef cattle and dairy lagoons and in agricultural stream and ditch water and sediments (Kolpin et al., 2002; Hanselman, 2003; Snow et al., 2009 and 2013; Gall et al., 2011). Mashtare et al. (2013 and Chapter 5 in this dissertation) recently evaluated the transformation potential of 17α-E2, 17β-E2, and E1 under nitrate-reducing and sulfate-reducing conditions in agricultural sediments; however, questions still remain about the fate of these hormones under iron-reducing conditions and methanogenic conditions, 2 dominant processes in freshwater sediments (reviewed by van Bodegom et al., 2004). Understanding the fate of these hormones under these reducing conditions is especially important given that seasonal cycling between iron-reduction and methanogenesis has
been observed in freshwater anaerobic sediments (reviewed by Bullock et al., 2013), and may dominate under low base flow and standing water conditions. Questions also remain about the biotic and abiotic contributions to hormone attenuation in anaerobic sediments. This study also explores the role that abiotic processes may have in the transformation of these estrogens in the presence and absence of sulfate, nitrate, and ferric iron.

The objectives of this study were to assess the transformation potential 17α-E2, 17β-E2, and E1 in sediments under iron-reducing and methanogenic conditions. Under these conditions, we specifically sought to determine if the transformation rates of 17α-E2, 17β-E2, and E1 are different and if interconversion between the E2 isomers occurs, which isomer is preferentially formed and if E1 is the primary metabolite. Additional work was done in autoclaved and chemically sterile systems under all reducing conditions to confirm if the transformations of E1 and E2 are primarily biotically or abiotically initiated.

6.3 Materials and Methods

6.3.1 Sediment

Sediments were collected, stored, and prepared as described previously by Mashtare et al. (2013). Briefly, batch sediments were collected from the ditch and stream network at the Purdue University Animal Science Research and Education Center (ASREC) and Little Pine Creek (West Lafayette, IN) in October 2012 and June 2013, and stored in closed containers in the dark at 4°C. Sample collection times and locations were selected to achieve a representative composite of sediments in the network. Prior to pre-incubation of the sediments (June 2013), all collected sediments were passed through a 2-mm sieve and thoroughly mixed to create a homogenous sediment sample.

6.3.2 Chemicals

All estrogens (17α-E2, 17β-E2, E1) and the internal standard 17β-estradiol-D3 (17β-E2-D3) were obtained from Sigma Aldrich, St. Louis MO, USA. Acetonitrile (ACN), ethyl ether (Et2O), and methanol (MeOH) were purchased from Mallinckrodt, Phillipsburg, NJ, USA. Food grade protein gelatin used as an electron donor was
purchased from Kroger Co., Cincinnati, Ohio, USA. Ethyl alcohol was purchased from AAPER Alcohol and Chemical Co, Shelbyville, KY, USA. Glucose was purchased from Sigma Aldrich, St. Louis MO, USA. All chemicals were analytical-reagent grade or higher purity (>99%) except for 17α-E2, 17β-E2, 17β-E2-D3, which were >98% purity, and used as received. Ultrapure water was prepared using a Mega-Pure System, MP-3A from Barnstead, Dubuque, IA, USA. Hormone stock solutions were prepared in pure methanol and stored at 4 °C in the dark.

A synthetic freshwater medium (pH adjusted to 7 with HCl) was prepared as described by Homklin et al (2011) by dissolving 1.0 g of NaCl, 0.4 g of MgCl$_2$×6H$_2$O, 0.1 g of CaCl$_2$×2H$_2$O, 0.25 g NH$_4$Cl, 0.2 g of KH$_2$PO$_4$, 0.5 g of KCl, 1 mL of trace element mixture, 2.52 g of NaHCO$_3$, 0.36 g Na$_2$S nonhydrate into a total volume of 1 liter of Milli-Q water. The trace element mixture was prepared by mixing 12.5 mL HCl (7.7 M), 2.1 g FeSO$_4$×7H$_2$O, 30 mg H$_3$BO$_3$, 100 mg MnCl$_2$×4H$_2$O, 190 mg CoCl$_2$×6H$_2$O, 29.36 mg Ni(NO$_3$)$_2$, 2.9 mg CuSO$_4$×5H$_2$O, 144 mg ZnSO$_4$×7H$_2$O and 36 mg Na$_2$MoO$_4$×2H$_2$O into 1 liter of Milli-Q water. The freshwater medium was autoclaved and cooled under nitrogen. For the iron-reducing study, a 50 mM ferric iron citrate solution was prepared in freshwater medium. For the methanogenesis study, a solution of glucose, ethanol, and methanol at an initial concentration of 1, 27, and 39 mM, respectively, in freshwater medium was prepared. Nitrate and sulfate solutions were prepared for the abiotic study using sodium nitrate and sodium sulfate, respectively, as previously described by Mashtare et al. (2013). Gelatin was added to each solution as an electron. The headspace was then purged with nitrogen, bottles capped, sealed with parafilm, and transferred into a large 4-glove vinyl anaerobic chamber. The chamber was equipped with an automated airlock pass-through chamber, oxygen and hydrogen analyzer, 3 fan boxes equipped with palladium catalysts, desiccation unit, and a dark storage incubator. At the start of the experiment, 0.5 mL of a 500 mg/L methanol hormone stock solution was transferred into a glass flask, the methanol evaporated, and hormones re-dissolved in 500 mL of the appropriate aqueous solution, as noted above, and mixed on a magnetic stir plate to achieve ~0.5 mg hormone/L. Each hormone solution (17α-E2, 17β-E2, and E1) was prepared separately as well as a no-hormone
blank. A sample from each solution was saved and extracted using diethyl ether (Et$_2$O) at a 4:4.4 water:Et$_2$O liquid-liquid exchange for analysis on the LC/MS/MS.

6.3.3 Batch Sediment Pre-incubation

Sediments were pre-incubated as described previously by Mashtare et al. (2013) to ensure the onset of iron-reducing or methanogenic conditions prior to hormone amendment (defined as time $t = 0$). Briefly, homogenized wet sediment was transferred to a plastic container and covered with 500 mL of a 50mM ferrous citrate solution or a methanolic solution (1 mM glucose, 27 mM ethanol, and 39 mM methanol). A protein gelatin electron donor (3 g) was dissolved in the freshwater medium prior to amendment. The pre-incubation container was tightly sealed and the headspace purged with nitrogen for 5 minutes using two 17-gauge syringe needles (one for N$_2$ input and one for exhaust) after which the syringe holes were sealed with electrical tape. The sediment was mixed by gently rocking the container, covered in foil, and transferred to a large vinyl chamber as previously described for which an atmosphere of N$_2$ with ~3-5% H$_2$ was maintained.

6.3.4 Individual Biotic Anaerobic Microcosm Preparation

Anaerobic microcosms were prepared as described previously by Mashtare et al. (2013). Briefly, acetone-rinsed glass centrifuge tubes (40 mL) and Teflon-lined screw caps were wet autoclave-sterilized, oven-dried at 105 °C, cooled, and degassed in the anaerobic chamber for at least 24 h prior to the start of the experiment. The headspace of the anaerobic chamber and sediment pre-incubation container were sampled immediately prior to the start of the experiment for CH$_4$ and CO$_2$ determination. The container was then opened, excess water removed, the sediment thoroughly mixed, and approximately 8.5 g of wet sediment (~5 g dry wt basis) was transferred into each centrifuge tube and tightly capped until time of amendment. Background hormone concentrations were determined in the pore water and pre-incubated sediments. Gravimetric moisture content of the sediment slurry was determined in triplicate. Either 10 mL of hormone (initial hormone concentration ~3.66 µmol kg$^{-1}$ dry wt. basis) or blank solution was added to each microcosm, tightly capped with Teflon septa, gently shaken to suspend the
sediments in solution, and stored in a dark anaerobic incubation chamber until time of sacrifice. Separate microcosms were prepared for gas and metals analysis and sealed with rubber septa to minimize excess gas leakage. For each hormone and reducing condition, 4 hormone-amended microcosms were analyzed at each sampling time: 3 for hormone analysis and 1 for iron and manganese analysis. CO$_2$ and CH$_4$ measurements were sampled from the anaerobic chamber (ambient) and the headspace of at least 1 microcosm for each amended hormone at each sampling point. Triplicate soil blanks (no hormones) were also analyzed at each sampling point. Microcosm sets were sacrificed after 0, 1, 3, 7, 14, 28, and 3 later times between 42 and 95 d. Results through 95 d are presented for the methanogenic study while results from the first 42 d are presented for the ongoing iron-reducing study.

6.3.5 Individual Abiotic Anaerobic Microcosm Preparation

Microcosms were prepared, as described above, after pre-incubating sediment in the anaerobic chamber for 3 weeks without the addition of an electron acceptor. After transferring ~8.5 g of wet sediment to sterile glass centrifuge tubes, however, the abiotic microcosms were wet autoclaved twice for 1 h with a 5 d incubation period between autoclaving cycles. Immediately following each cycle, microcosms were transferred back into the anaerobic chamber to minimize oxygen exposure to the sediments while cooling. No increase in ambient O$_2$ was noted in the chamber during the transfer or degasing of the microcosms. For each hormone, 4 solutions were prepared for the abiotic study: 3 electron acceptor solutions (20 mM nitrate, 20 mM sulfate, and 50 mM ferric iron citrate) and synthetic stream water without the addition of an electron acceptor to serve as a surrogate for methanogenic conditions. All solutions were wet autoclave sterilized and degassed prior to amendment. Sodium azide (50 mM) was added as a chemical sterilizer. The pH of the iron solution was adjusted to pH ~7 and the remaining solutions to ~8 to approximate the final pH observed in the biotic systems. For each hormone and each of the 4 solutions, abiotic microcosms were prepared in duplicate (12 prepared solutions, total). Additionally, to assess hormone stability in each of the aqueous solutions, 10 mL of each hormone solution was transferred into separate
autoclave sterilized glass scintillation vials to be sacrificed at each sampling period. At each sampling point, CO$_2$ and CH$_4$ measurements were sampled from the anaerobic chamber (ambient) and the headspace of a subsample of the hormone-amended sterile sediment microcosms to assess whether biotic respiration was occurring. Abiotic sediment microcosm and liquid microcosm sets were sacrificed after 0, 1, 3, 7, and 14 d.

6.3.6 Gas Analysis

CO$_2$ and CH$_4$ concentrations in the headspace were determined by manual injection on an Agilent 7890A gas chromatograph (GC) equipped a flame ionization detector (FID) and thermal conductivity detector (TCD).

6.3.7 Metal Analysis

After centrifugation, aqueous samples were transferred to plastic centrifuge tubes and acidified with trace metal grade HNO$_3$ for total iron and manganese analysis on an Elan DRC-e inductively coupled plasma-mass spectrometer (ICP-MS). Iron (Fe$^{2+}$) concentrations were also confirmed using the ferrozine method as described by Lovely and Phillips (1987).

6.3.8 Hormone Extraction and Analysis

Hormones were extracted from the microcosms as described previously by Mashare et al. (2013). Briefly, a single step extraction was carried out by adding diethyl ether (Et$_2$O) to minimal headspace, capping tightly, covering tubes with foil, equilibrating end-over-end at 35 rpm for ~24 h at room temperature (22 ± 2 °C) and centrifuging at 1600 rpm for 20 min. Approximately 1.2 mL (by weight) of Et$_2$O was transferred into an HPLC vial, evaporated, and residues re-dissolved in 0.5 mL of MeOH containing an internal standard (17β-E2-D3). A second extraction was carried out for all microcosms by removing the excess Et$_2$O from the first extraction and repeating the extraction steps above.

Estrogen analysis was performed on a Shimadzu high performance reverse-phase liquid chromatography coupled to a Sciex API3000 mass spectrometer operated in
positive electrospray ionization (ESI) mode with multiple reaction monitoring. Separation was performed using 20-25 μL injections on a Phenomenex Gemini C-18 column (150 mm x 2 mm, dp= 5 μm) with a gradient elution using water:MeOH (90:10) containing 2 mM ethanolamine [solvent A] and acetonitrile containing 2 mM ethanolamine [solvent B] at 0.35 mL min\(^{-1}\). Initial mobile phase composition was 30% solvent B followed by a linear gradient to 50% solvent B from 0 to 8.5 min after which solvent B was ramped to 100% for 2 min to wash the column and then re-equilibrated at 30% solvent B for 2 min prior to the next injection. The chromatographic retention times for E3, 17β-E2, 17β-E2-D3, 17α-E2, and E1 were 3.8 min, 7.1 min, 7.1 min, 7.7 min, and 8.2 min, respectively. E3 (m/z 287 →145), 17α-E2 and 17β-E2 (m/z 271 →145), and E1 (m/z 269 →145) were quantified using independent external calibration curves with check standards run approximately every 12 samples. The internal standard, 17β-E2-D3 (m/z 274 →145), was used to assess matrix effects in the MS, which were found to be negligible. A deuterated 17α-E2 internal standard was not available. For a 25 μL injection, the limit of detection for all estrogens was 0.015 μg L\(^{-1}\) and the method limit of quantitation (MLOQ) was 0.03 μg L\(^{-1}\).

6.3.9 Statistical Analysis

Minitab v16 (State College, PA: Minitab, Inc.) was used for statistical analysis. An ANOVA was used to determine the significance of differences between the observed temporal changes in transformation of 17α-E2 and 17β-E2 within and between redox conditions. A linear regression was used to determine whether there was a decrease in the mass balance of hormones in the microcosms over time. Statistical tests used α = 0.05 as the level of significance.

6.4 Results and Discussion

All hormone concentrations are presented and discussed on a mol % basis relative to the hormones at t=0. CO\(_2\) and CH\(_4\) concentrations are presented as a %. For live (non-autoclaved) microcosms, hormone concentrations over time are shown in Figures 6.1A
and 6.1B for iron-reducing and methanogenic conditions, respectively, with the corresponding gas production trends shown in Figures 6.1C and 6.1D. Metabolite formation and isomeric conversion for each E2-isomer are summarized in Figure 6.2. The reversible transformation of E1 to 17α-E2 and 17β-E2 under both reducing conditions are shown in Figure 6.3. Mass balances for both conditions are shown in Figure 6.4. For abiotic (autoclaved and chemically sterilized) microcosms, hormone concentrations over time in nitrate, ferric iron, sulfate, and synthetic stream water (without the addition of an electron acceptor) are shown in Figures 6.5A-C. Stability of no sediment controls for the different solutions are shown in Figures 6.5D-F. Corresponding gas production trends are shown in Figures 6.5G and 6.5H.

### 6.4.1 17α-E2 and 17β-E2 Transformation Under Iron-Reducing and Methanogenic Conditions

Under both iron-reducing and methanogenic conditions, the loss of 17β-E2 was significantly faster than 17α-E2 (Figures 6.1A and 1B, Table 6.1). There was no clear correlation between the loss of E2 and the reducing condition present, with the loss of 17α-E2 ~1.4 times faster and 17β-E2 ~1.4 times slower under methanogenic conditions than iron-reducing conditions. A similar lack of correlation between the loss of 17β-E2 and electron acceptor use in lake sediments was observed by Czajka and Londry (2006). Borsch and Young (2009) suggested that a lack of correlation indicates the hormones are not being utilized directly as a food (energy) source but for co-factor regeneration. Under iron-reducing conditions, CO₂ production outpaced CH₄ production until 7 d where CH₄ began to dominate (Figure 6.1C). This suggests our targeted redox activity of iron-reduction was likely mixed with methanogenesis. Although unintended, this mixed system may more closely mimic natural systems where methanogenesis likely dominates in the streambed under an iron-reducing layer, or where pockets of both conditions may be occurring simultaneously in the sediment bed (reviewed by Bethke et al., 2008). Re-amendment with iron-citrate at 29 d resulted in a similar gas production pattern as observed with the initial amendment suggesting that iron-reduction was again briefly dominant. Interestingly, re-amendment with ferric iron had no discernible effect on
hormone transformation in the systems. Under methanogenic conditions, CH₄ production dominated CO₂ production over the duration of the study suggesting our targeted redox activity of methanogenesis was occurring over the course of the study. Re-amending the microcosms with the methanolic solution at 42 d had minimal to no effect on hormone transformations in the E2-amended systems. E3 was not detected in either system. The average mass balance of total estrogens for 17α-E2 and 17β-E2 was 98.4 ± 3.5 mol % and 100.2 ± 7.8 mol % under iron-reducing conditions and 99.8 ± 5.3 mol % and 104.6 ± 5.9 mol % under methanogenic conditions, respectively (Figures 6.4A-D). Changes in the mass balances were not statistically significant suggesting that the potential for long-term persistence of total estrogens is high under these conditions.

Table 6.1. Summary of observed half-lives (t₁/₂, d) under anaerobic conditions.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Reducing Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrate-reducingᵃ</td>
</tr>
<tr>
<td>17α-E2</td>
<td>4.3 d</td>
</tr>
<tr>
<td>17β-E2</td>
<td>0.3 d</td>
</tr>
<tr>
<td>E1</td>
<td>35.9 d</td>
</tr>
</tbody>
</table>

ᵃMashtare et al. (2013). ᵇIndicates that > 50 mol % remained at end of study period (time shown).

For 17α-E2, the observed t₁/₂ were 39 d and 28 d under iron-reducing and methanogenic conditions, respectively, while the observed t₁/₂ of 17β-E2 was 0.8 d under iron-reducing conditions and 1.1 d under methanogenesis (Figures 6.1A and 6.1B). Under iron-reducing conditions, 17α-E2 concentrations are at 49% by 42 d and presumably still decreasing while 17β-E2 reached a pseudo-steady state of ~9 mol % by 14 d. Under methanogenic conditions, concentrations of 17α-E2 and 17β-E2 continue to decrease until reaching a pseudo-steady state of ~14 mol % by 56 d and ~10 mol % by 28 d, respectively. A similar trend for 17β-E2 was noted by Mashtare et al. (2013) in sediments under sulfate-reducing conditions where a pseudo-steady state of ~9 mol % was reached by 21 d. This suggests that under these reducing conditions, residuals of E2
are likely to be persistent in the sediment bed. The observed $t_{1/2}$ of 17β-E2 is shorter than those reported by Czajka and Londry (1986) of 6.3 d and 15 d under iron-reducing and methanogenic conditions, respectively. However, as we previously discussed (Mashtare et al., 2013), the shorter half-lives of 17β-E2 observed in our study are likely due to differences in sediment composition, adaptation of the microbial communities in our agro-impacted sediments (Jacobsen et al., 2005) and the addition of a complex protein. The latter has been shown to promote microbial diversity and serve as a readily available carbon source for iron-reducers and methanogens (Jain and Zeikus, 1989; Kourtev et al., 2006). Degradation of 17β-E2 was much faster than 17α-E2 under both methanogenic (~25 times faster) and iron-reducing (~49 times faster) conditions, similar to differences observed between nitrate and sulfate reducing conditions (Mashtare et al., 2013) (Table 6.1). As we previously hypothesized (Mashtare et al., 2013), differences in the rate of isomer transformation is likely due to disparities in the stereospecificity of the available enzymes as observed by Renwick and Engel (1965) and the relative abundance of the available enzymes under each redox condition. Regardless of mechanisms, clearly under all anaerobic conditions evaluated, 17α-E2 is more resistant to degradation than its isomeric counterpart, 17β-E2.

6.4.2 E1 Formation from 17α-E2 and 17β-E2 and Interconversion

The near stoichiometric formation of E1 was observed with the loss 17α-E2 and 17β-E2 under both redox conditions confirming that E1 is the primary metabolite of both isomers. Interconversion between the E2 isomers and continued accumulation over time was observed under both redox conditions (Figures 6.2A-D). In 17α-E2 amended iron-reducing microcosms, 17β-E2 peaked at ~1.6 mol % through 42 d (Figure 6.2A) while under methanogenic conditions, 17β-E2 peaked at 7 mol % by 56 d after the re-amendment of methanolic stock and then decreased to ~6 mol % by 95 d (Figure 6.2B). In the 17β-E2 amended microcosms, 17α-E2 reached ~2.7 mol % through 42 d in the iron-reducing microcosms (Figure 6.2C) and ~5 mol % under methanogenesis by 28 d (Figure 6.2D). The interconversion of the E2 isomers mirrors our previous results in agricultural sediments under nitrate-reducing and sulfate-reducing conditions (Mashtare
et al., 2013) and has been observed in blended dairy lagoon water (Zheng et al., 2012) as well as simulated feedlot runoff (Mansell et al., 2011).

Figure 6.1. Composite of single hormone-amended experiments showing the loss of 17α-E2 (▲), 17β-E2 (■), and E1 (●) in mol % over time under (A) iron-reducing and (B) methanogenic conditions; and associated CO₂ (○) and CH₄ (●) production under (C) iron-reducing and (D) methanogenic conditions. Re-amendment (+) of ferric citrate (C) or methanolic solution (D) is shown for 28 d and 42 d, respectively. Error bars represent the standard deviation (n=3).
Figure 6.2. Mol % of 17α-E2 (▲) (left axis), 17β-E2 (■) (left axis), and E1 (●) (right axis), in sediment amended with either 17α-E2 (upper graphs A and B) or 17β-E2 (lower graphs C and D) under iron-reducing conditions (left graphs A and C) and methanogenic conditions (right graphs B and D). Re-amendment (+) of ferric citrate (C) or methanolic solution (D) is shown for 28 d and 42 d, respectively. Error bars represent the standard deviation (n=3).

6.4.3 E1 Fate in E1-Amended Sediments

Under both iron-reducing and methanogenic conditions, E1 dropped to ~59 mol % and 64 mol %, respectively, within 1 d (Figures 6.3A and 6.3B). This loss of E1 was accompanied by the concomitant formation of 17β-E2 which peaked at ~33 mol % under iron-reducing conditions and ~28 mol % under methanogenic conditions. Under both reducing conditions, a gradual loss of 17β-E2 was observed, reaching a pseudo-steady
state of ~9 mol % within 14-28 d. A near stoichiometric re-formation of E1 was observed with no discernible subsequent loss of E1 observed for the duration of either study. Czajka and Londry (2006) also reported negligible loss of E1 formed from 17β-E2 in their iron-reducing and methanogenic studies with anaerobic lake sediments over their 383-d incubation period. The formation of 17α-E2 reached ~2.7 mol % through 42 d under iron-reducing conditions and peaked at ~5 mol % under methanogenic conditions by 14 d. Estriol (E3) was not detected in either system. This preferential formation of 17β-E2 from E1 was also observed in sediments under nitrate-conditions and sulfate-reducing conditions (Mashtare et al., 2013), in blended dairy lagoon water by Zheng et al. (2012), and is consistent with the relative activities and stability of 17α-E2 and 17β-E2 dehydrogenases (Renwich and Engel, 1967). The decay rate of each E2 isomer, however, will likely affect their long-term accumulation within the sediment bed. Changes in the mass balances of the E1-amended microcosms were not statistically significant,

![Figure 6.3. Mol % of 17α-E2 (▲) and 17β-E2 (■) (left axes), in E1-amended systems under (A) iron-reducing and (B) methanogenic conditions. Lines represent E1 decay patterns (right axis). Error bars represent the standard deviation (n=3).](image-url)
suggesting little to no mineralization or loss to irreversible sorption in the amended systems. The average mass balance of total estrogens was $99.9 \pm 5$ mol % and $97.4 \pm 5$ mol % under iron-reducing and methanogenic conditions, respectively, (Figures 6.4E and 6.4F).

While the addition of ferric iron had little effect on hormone transformation in the E1 microcosms, a 4 mol % increase in $17\beta$-E2 by 56 d was observed under methanogenic conditions after the addition of the methanolic stock at 42 d. A smaller increase (~1 mol %) was noted in the $17\beta$-E2 amended microcosms, presumably because of the high E1 concentrations. Sampling closer to the re-amendment period (within 1-3 d) would help clarify whether the transformation patterns closely mirror those at t=0 or indicate only a modest increase in reversible transformation. Nevertheless, this suggests that an influx of nutrient and carbon-rich water into E1-rich sediments may promote the heightened transformation of E1 to $17\beta$-E2 under highly reduced conditions.

6.4.4 Abiotic Conditions

Our previous attempt (Mashtare et al., 2013) to discern between biotic and abiotic transformations under anaerobic conditions was inconclusive because of uncertainty about whether the microcosms remained sterile (abiotic) over time. In this study, we employed a longer incubation period (5 d) between autoclaving cycles and used sodium azide as a chemical sterilizer to help retard microbial activity. CO$_2$ and CH$_4$ concentrations in the headspace of the abiotic microcosms were used to assess microbial activity.

In contrast with our previous results (Mashtare et al., 2013), we found no discernible evidence of transformation of $17\alpha$-E2, $17\beta$-E2, or E1 in our abiotic anaerobic microcosms over the course of 14 d (Figures 6.5A-F). Recovery of the applied hormones averaged $99 \pm 0.8$ mol %, $94 \pm 1.3$ mol %, and $94 \pm 0.8$ mol % across the sampling period for $17\alpha$-E2, $17\beta$-E2, and E1, respectively, in the sediment microcosms. Headspace CH$_4$ concentrations (Figure 6.5G) remained unchanged relative to the ambient chamber suggesting no, or limited, methanogenic activity. After an initial increase of ~1%
Figure 6.4. Mass balance of live (non-autoclaved) microcosms amended with 17α-E2 (upper graphs A and B), 17β-E2 (middle graphs C and D), or E1 (lower graphs E and F). Lines represent mol % of total estrogens over time under iron-reducing (left graphs A, C, and E) and methanogenic conditions (right graphs B, D, and F). Error bars represent the standard deviation (n=3).
Figure 6.5. Composite of abiotic sediment microcosms (top row A, B, and C) and sterile liquid stability microcosms (middle row D, E, and F) showing the loss of 17α-E2 (left columns A and D), 17β-E2 (middle columns B and E), and E1 (right columns C and F) as a mol % over time in nitrate-amended (▲), sulfate-amended (■), iron-amended (●), and water-amended (♦) microcosms. Headspace gas measurements (bottom row G and H) are shown for CO₂ (○) and CH₄ (●). Error bars represent the standard deviation (n=2).
following hormone amendment, likely because of an abiotic release of CO$_2$ from the carbonate-rich sediments, CO$_2$ concentrations (Figure 6.5H) remained unchanged for the duration of the study suggesting microbial activity, if any, was minimal. No E1 or E3 formation was observed in any of the abiotic samples, nor was any interconversion or reversible transformation from E1 to E2. Temporal changes in mass balances were not statistically significant. Czajka and Londry (2006) observed similar trends in the sterile controls of their 17β-E2 anaerobic lake sediment study. The hormones also remained stable in their respective aqueous solutions (no sediment). Applied hormone recoveries in the aqueous no-sediment stability study (Figures 6.5D-F) averaged 98 ± 6 mol %, 101 ± 1 mol %, and 101 ± 6 mol % across the sampling period for 17α-E2, 17β-E2, and E1, respectively, with no evidence of metabolite formation. Changes in mass balances over time were not statistically significant. Similar results were observed by Zheng et al. (2012) in the sterile controls of their lagoon water study. The lack of metabolite formation, interconversion, reversible transformation, or significant changes in mass balance with time in the abiotic systems support that the transformations observed in our live microcosms were primarily biologically initiated.

6.4.5 Environmental Implications

In environmental risk assessment, the focus is often on 17β-E2, despite E1 being the dominant hormone detected in agro-impacted surface water (Kolpin et al., 2002; Gall et al., 2011). The assumption, however, that non-reversible transformations will dominate in a hormone impacted streambed, and that the estrogenic output will be less than the input may not be a conservative approach, especially under iron-reducing and methanogenic conditions. This study shows that once the hormones are partitioned into the sediment bed, E1 can reversibly transform to the more potent E2 precursors, with preferential formation of 17β-E2, under highly reduced conditions. It also shows that the potential for interconversion between the E2 isomers, presumably through E1, may result in a mixture of the stereoisomers that would otherwise not be expected based on estrogens quantified in receiving waters soon after discharge. For example, although 17α-E2 is the primary isomer excreted from dairy and 17β-E2 from swine (Hanselman et
al., 2003), unexpected elevated concentrations of 17β-E2 have been detected in dairy manure and waste lagoons (Zheng et al., 2008) and 17α-E2 in swine waste lagoons (Yost et al., 2013). The slow loss of total estrogens with time also suggests the potential for long-term persistence under iron-reducing and methanogenic conditions, with the $t_{1/2}$ of E1 significantly greater than 17α-E2 which is significantly greater than 17β-E2. These results emphasize the importance of accounting for these biologically mediated transformations of E1 and E2 when developing resource management and risk assessment models. For example, while flow controlled tile-drains provide an opportunity to manage water resources, the anaerobic conditions created in the soil profile may provide the conditions necessary to slow E2 and E1 degradation and instead allow the anaerobic microbial community present in the saturated subsurface to form the more potent isomers that would otherwise not be expected. Likewise, changes in redox conditions due to seasonal shifts, and changes in base flow conditions during drier periods which may reduce oxygen recharge in agricultural streams and ditch networks, may provide conditions that facilitate the transformation of the estrogens present in the sediment profile to their more biologically potent forms, presenting a potential danger to the nearby aquatic species.

6.4.6 Acknowledgements

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CHAPTER 7. CONCLUSIONS AND FUTURE WORK

7.1 Major Findings

This dissertation focused on the fate of the natural estrogens 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), and estrone (E1) in agricultural soils and sediments. Sorption isotherms were measured on surface soils to assess whether the stereoisomers exhibited the same sorption affinities. The transformation of 17α-E2 and 17β-E2 was quantified in aerobic agricultural soils to assess whether the stereoisomers exhibited differences in degradation patterns. The transformation of 17α-E2, 17β-E2, and E1 in agricultural sediments under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions were quantified to determine whether the stereoisomers exhibited the same degradation patterns under anaerobic conditions and whether transformation occurred between isomers and reversibly from E1 to E2. The major findings are as follows:

1. Overall, 17α-E2 and 17β-E2 were found to have different sorption coefficients with sorption best correlated to soil organic carbon. The average log OC-normalized distribution coefficients (log $K_{oc}$, L kg$^{-1}$) are $2.97 \pm 0.13$ for 17α-E2 and $3.14 \pm 0.16$ for 17β-E2 with 1.9 being the highest β/α sorption ratio.

2. No statistical difference was observed between aerobic degradation rates of 17α-E2 and 17β-E2 with observed $t/2 < 0.5$ d. E1 was determined to be the primary metabolite of both E2 isomers with autoclaved-sterilized controls supporting that E2 dissipation under aerobic conditions in soils is dominated by microbial processes. While the first order exponential decay model was able to predict the $t/2$ of both parent compounds, failure to
predict residual concentrations at later times suggests degradation may have been retarded by sorption-limited bioavailability.

3. Stereospecific degradation was observed for the isomers under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions with magnitude of $t_{1/2}$ being $17\beta$-E2 $< 17\alpha$-E2 $< E1$ under all reducing conditions. The observed $t_{1/2}$ of $17\beta$-E2 was rapid under all conditions (<1.5 d), while $17\alpha$-E2 exhibited higher persistence with an observed $t_{1/2}$ of 4.3 d to 69.3 d depending on the reducing conditions present. E1 was the primary metabolite of both isomers.

4. Under all anaerobic conditions, interconversion between $17\alpha$-E2 and $17\beta$-E2 was observed. E1 demonstrated reversible transformation back to its E2 precursors with preferential formation of the more potent $17\beta$-E2. The reversible transformation from E1 was especially pronounced under more highly reducing conditions (i.e., iron-reducing, sulfate-reducing, and methanogenesis), with $17\beta$-E2 peaking between ~28-33 mol % within 1-3 d of amendment of E1. Sterile controls support that these transformations are biologically mediated.

7.2 Implications

The implications of these findings are:

1. $17\alpha$-E2 is more likely to be leached from agricultural soils than $17\beta$-E2; therefore, assuming the isomers exhibit the same sorption behavior, as has been previously assumed, may not be a conservative approach. The relative importance of these differences, however, may vary based on scale, whether macropore flow and facilitated transport are dominant, and on the physiochemical properties of the soil (i.e., organic carbon content and clay mineral type).

2. The relatively rapid aerobic degradation of the E2 isomers can be assumed to be the same under aerobic conditions; however, persistent
residuals were apparent and suggest caution is warranted when trying to model the environmental fate of these hormones with regards to common modeling assumptions such as the negligible impact of sorption on bioavailability.

3. Under anaerobic conditions, assuming non-reversible E2 to E1 degradation would likely be an erroneous assumption given the potential for interconversion between 17α-E2 and 17β-E2 and the reversible transformation to E2 from E1 under reducing conditions. These hormones are also likely to be persistent in sediments, which then can serve as both a source and a sink. Transformation potential, however, is dependent on the condition(s) present.

Taken together, these findings suggest that careful attention needs to be taken when evaluating resource and risk management strategies for these compounds. For example, Frey et al. (2013) reported higher concentrations of veterinary antibiotics (e.g., tylosin, chlortetracycline, tetracycline, and oxytetracycline) from controlled tile drains relative to free flowing tiles, presumably because the anaerobic conditions in the controlled tiles hindered the degradation of these compounds. Likewise, while Ilhan et al. (2011) reported that wood chip bioreactors reduced concentrations of agrochemicals (atrazine, enrofloxacin, and sulfamethazine) discharged from tiles, they noted removal was primarily through sorption with limited to no biodegradation observed under the nitrate-reducing conditions present. These bioreactors could then serve not only as a sink, but as a potential source of these compounds. This suggests that while controlled tiles and bioreactors may provide a valuable resource management strategy, the reduced conditions present may have the unintended consequence of slowing the degradation of these compounds, promoting isomeric interconversion and reversible transformations from E1 to the more potent, and potentially mobile, E2 isomers.

This work also suggests that focusing on the inputs/discharge into the water column may not be sufficient in understanding the persistence of the estrogens and potential risk to aquatic species. For example, once E1, the dominant hormone detected in impacted surface water (Gall et al., 2011; Kolpin et al., 2002) or E2 (which is likely to be
rapidly converted to E1 through aerobic degradation or potentially photolysis, see Chapter 2) is discharged into the water column, it will likely partition to the sediment bed. Under reducing conditions, E1 may transform back to the potentially more potent E2 isomers. Because of their lower partition coefficients (see Chapter 2), the E2 isomers may partition back into the water column where they may pose a potential risk to the aquatic species. Likewise, under turbulent conditions or when the sediment bed is disturbed, the re-suspension of the sediments would facilitate desorption of the hormones back into the water column. Once in the water column, aerobic degradation processes could transform E2 to E1, which has shown longer persistence than 17β-E2 in aerobic soils (Colucci et al., 2001), and oxic water and sediments (Bradley et al., 2009). These hormones can be further transported downstream where they would likely partition back into the sediment bed and where E1 could potentially convert back to E2 or persist long-term as E1. Under this cycle, it is likely that estrogens will continue to persist longer than would otherwise be predicted and are subject to transformation back to their more potent forms.

7.3 Future Work

1. Quantifying what impact newer management strategies, including controlled tile drains, may have on the fate of these hormones in the saturated soil column.

2. Future work should also include integrating the results of these studies into a model (i.e., HERD) to characterize and predict the fate of these hormones in the source zone (soil), water column, and sediment bed of an impacted agricultural system (e.g., Purdue’s Animal Sciences and Research Education Center).

3. Quantifying the role that photolysis and other biological activities (e.g., algae) that may play in the fate of these compounds would improve our understanding of transformation properties in the water column. For example, Qu et al. (2013) recently reported in Science that metabolites
formed through photolysis of the synthetic androgen trenbolone acetate reverted back to the parent hormone at night in the absence of light.

4. The use of flow-through microcosms would reduce the risk of the buildup of potential toxins (e.g., hydrogen sulfide) that may have impacted the microbial communities present in our closed anaerobic systems. It would also allow the continued inflow of electron donors and acceptors more closely mimicking a natural system. This would allow a better understanding of how other factors including water turbulence may affect the fate of these compounds in the water column and sediment bed.

5. While mineralization and irreversible sorption were assumed to contribute to the loss of mass balance with time in our studies, the use of radiolabeled hormones, which are now available for both isomers, could be useful in determining isomeric differences in mineralization rates and whether hormones are being incorporated into biomass. In addition, loss to irreversible sorption and formation of bound residues could be confirmed, as well as any yet unidentified metabolites.

6. While there was a strong correlation between organic carbon and the stereospecific sorption of E2, unpublished results of a single experiment with Ca\(^{2+}\)-montmorillonite (a 2:1 clay) showed a $\beta/\alpha$ sorption ratio $>2$ (isotherm shown in Figure 7.1). This suggests that the clay fraction may also play an important role in the sorptive behavior of the stereoisomers of hormones, particularly in low OC soils. Bonin and Simpson (2007) observed that smectites can contribute significantly to sorption of estrogens suggesting that including only

![Figure 7.1 Isotherm of E2 and a smectitic clay](image-url)
organic carbon content in estimating sorption may under predict sorption by soils.

7. E1 aerobic degradation rates were estimated from the loss of E1 formed from E2 and not independently by monitoring E1 loss from a soil amended with E1 at t=0. Direct measurement of E1 degradation would be helpful given the differences in the estimated decay rates of E1 formed from 17α-E2 versus 17β-E2 in this study. E1 degradation appeared to be dependent on its precursor (17α-E2 versus 17β-E2) although the degradation rates of both E2 isomers were not statistically different.

8. Sorption from single-solute systems, like those employed in our sorption studies, may not be representative of the behavior in mixed hormone systems as is typically present in impacted surface and pore waters. This could lead to potentially over-predicting sorption for the estrogens. Competitive effects between natural and synthetic estrogens in soils and sediments have been reported in bi-solute systems (Yu et al., 2004) and tri-solute systems (Bonin and Simpson, 2007), with strong competition observed with pure minerals. Understanding the sorption behavior of these compounds, including E1, would be helpful in assessing their behavior in soils and sediments.
LITERATURE CITED
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APPENDICES
Appendix A  **Supporting Information: Evaluating Stereoselective Sorption by Soils of 17α-Estradiol and 17β-Estradiol**

- Table A.1. Isotherm data for 17α- and 17β-estradiol with seven soils and two completely replicated isotherms (Rep) for D36 and T4 soils for both isomers.
- Figure A.1. Linear regressions between linear sorption coefficients ($K_d$, L kg$^{-1}$) for estradiol isomers and individual soil properties.
- Figure A.2. Linear regressions between different soil properties.
Table A.1. Isotherm data for 17α- and 17β-estradiol with seven soils and two completely replicated isotherms (Rep) for D36 and T4 soils for both isomers. C_i is the applied estradiol concentration (mg L^{-1}), C_s is the sorbed estradiol concentration (mg kg^{-1}) after a 24-h equilibration, C_w is the solution estradiol concentration (mg L^{-1}) after a 24-h equilibration, and MR is the mass recovery. Zero applied concentration data (0,0) not shown.

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Fig. A.1. Linear regressions between linear sorption coefficients ($K_d$, L kg$^{-1}$) for $17\beta$-estradiol (open squares) and $17\alpha$-estradiol (open triangles) and individual soil properties.
Fig. A.2. Linear regressions between different soil properties.
Appendix B  Supporting Information: Transformation of 17α-Estradiol, 17β-Estradiol, and Estrone in Sediments under Nitrate- and Sulfate-Reducing Conditions

- Figure B.1. Structures of hormones.
- Figure B.2. Photos of experiment.
- Extraction efficiencies
- Simple first-order modeling of two consecutive reactions
- Figure B.3. Mass balance in microcosms.
- Figure B.4. Mass balance of autoclaved microcosms.
- Figure B.5. Autoclaved sediments under nitrate-reducing conditions.
- Figure B.6. Autoclaved sediments under sulfate-reducing conditions.
**Figure B.1.** Structures of 17α-estradiol, 17β-estradiol, estrone, and estriol
Figure B-2. Photos of experiment. The small chamber is a 2-glove rigid plexiglass anaerobic chamber with a manual pass-through chamber. Anaerobic conditions were achieved using vacuum/nitrogen flush cycles until anaerobic conditions are confirmed with a methylene blue indicator. Positive pressure with nitrogen was maintained to minimize the risk of oxygen contamination. The chamber was covered with foil to decrease the risk of photolysis during pre-incubation. The large chamber is 4-glove vinyl anaerobic chamber equipped with an automated airlock pass-through chamber, oxygen and hydrogen analyzer, 3 fan boxes equipped with palladium catalysts, and a storage incubator. The storage incubator has smokey brown glass doors designed to minimize external light contamination but was covered with foil to create a completely dark environment. Hydrogen levels were maintained at ~5% and oxygen at 0ppm.
**Extraction efficiencies**

Approximately 8 g of sediment slurry (~5 g dry wt basis) was added to 40 mL glass centrifuge tubes and autoclave-sterilized for 1 h across 2 consecutive days. 10 mL of a hormone solution (~0.5 mg L\(^{-1}\) of 17\(\alpha\)-E2, 17\(\beta\)-E2, or E1) prepared in ultra pure water was added to each tube, capped with a Teflon-lined cap, covered in foil, and rotated end-over-end for 24 h. After equilibration, a 1-step extraction with ethyl ether (Et\(_2\)O) was tested for each in duplicate. Solvent (20 mL) was added to each tube, the samples were shaken, vortexed, and then rotated for 24 h. A second extraction (20 mL) was repeated after removing excess solvent from extraction 1. Solvent extract was transferred to an HPLC vial after each extraction and evaporated under the hood, residues re-dissolved with 0.5 mL of MeOH containing an internal standard (17\(\beta\)-E2-D3), and analyzed on the LC/MS/MS. Single-extraction extraction efficiencies were between 98-103% with combined extraction efficiencies > 100%.
Simple First-Order Modeling of Two Consecutive Reactions

The simplest complex reaction consists of two consecutive steps that are assumed to be irreversible: \[ A \xrightarrow{k_1} B \xrightarrow{k_2} C \]

The first-order rate equations for the concentrations of A, B, and C are:

- \[ \frac{d[A]}{dt} = -k_a[A] \] \hspace{1cm} \text{Eq. S-1}
- \[ \frac{d[B]}{dt} = k_a[A] - k_b[B] \] \hspace{1cm} \text{Eq. S-2}
- \[ \frac{d[C]}{dt} = -k_b[B] \] \hspace{1cm} \text{Eq. S-3}

At time \( t = 0, [A] = [A]_0, [B] = 0, [C] = 0 \). Integrating of Eq. (S-1) gives:

\[ [A] = [A]_0 \exp(-k_at) \] \hspace{1cm} \text{Eq. S-4}

which is the same as Eq. 2 in the manuscript. Substituting Eq. (S-4) into Eq. (S-2) gives

\[ \frac{d[B]}{dt} + k_b[B] = k_a[A]_0 \exp(-k_at) \] \hspace{1cm} \text{Eq. S-5}

The solution to Eq. (S5) is as follows

\[ [B]_t = \left( \frac{k_a}{k_b - k_a} \right) \left( \exp(-k_at) - \exp(-k_bt) \right) [A] + [B]_0 \exp(-k.bt) \] \hspace{1cm} \text{Eq. S-6}

With the assumption that \([B]_0=0\), which is true for our studies in that metabolites were below LOQ at time 0, thus reducing the solution to Eq. S-2 as follows and the same Eq. 3 in the manuscript:

\[ [B]_t = \left( \frac{k_a}{k_b - k_a} \right) \left( \exp(-k_at) - \exp(-k_bt) \right) [A] \] \hspace{1cm} \text{Eq. S7}

We are not modeling \([C]\) since although we assume it is estriol in aerobic systems, we rarely detected estriol and have not identify additional metabolites, thus no solution is needed for the current work.
Figure B.3. Mass balance in microcosms. Lines represent total mass balance (E1 and E2) under nitrate-reducing and sulfate-reducing conditions. Error bars represent the standard deviation (n=3).
Fig. B.4. Mass balance in autoclaved sediment microcosms. Lines represent total mass balance (E1 and E2) under nitrate-reducing and sulfate-reducing conditions. Black lines represent the mass balance of single points (n=1) in autoclaved sediments. Grey lines represent mass balance in non-autoclaved (live) sediments. Error bars represent the standard deviation (n=3) in non-autoclaved sediments.
Figure B.5. Mol % of 17α-E2 (▲), 17β-E2 (■), and E1 (●) in single-hormone amended autoclaved microcosms under nitrate-reducing conditions. Lines represent mol % in live sediments. Error bars represent the standard deviation (n=3).
Figure B.6. Mol % of 17α-E2 (▲), 17β-E2 (■), and E1 (●) in single-hormone amended autoclaved microcosms under sulfate-reducing conditions. Lines represent mol % in live sediments. Error bars represent the standard deviation (n=3).
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Michael L. Mashtare Jr.
Ecological Sciences and Engineering Interdisciplinary Graduate Program (ESE-IGP)
Department of Agronomy
Graduate School, Purdue University

Education
2013 Purdue University, West Lafayette, IN
Ph.D., ESE-IGP, Department of Agronomy

2009 Purdue University, West Lafayette, IN
Bachelor of Science with Distinction, NRES

Teaching Experience
Fall 2013 Co-Instructor & Facilitator
Ecological Science & Engineering IGP Colloquium: Sustainability,
Resilience and Human Impacts (GRAD 590)

Spring 2013 Teaching Assistant / Lab Instructor
Soil Fertility Laboratory (AGRY 365) and Intro to Soil Science (AGRY 255)

Fall 2012 Teaching Assistant / Lab Instructor
Environmental Soil Chemistry (AGRY 385)

Fall 2011 Teaching Assistant / Lab Instructor
Environmental Soil Chemistry (AGRY 385)

Spring 2010 Teaching Assistant (Volunteer)
Intro to Soil Science (AGRY 255) / Soil Resource Center and Field Trips

Fall 2009 Teaching Assistant (Volunteer)
Intro to Soil Science (AGRY 255) / Soil Resource Center and Field Trips

Other Teaching Involvement
- Guest Lecturer in Environmental Organic Chemistry (AGRY 544: Spring 2012); Soil Fertility (AGRY 365: Fall 2010); Soil Chemistry (AGRY 385: Fall 2010, Fall 2011, Fall 2012, and Fall 2013)
- ESE-IGP Colloquium mentor (2010-2012)
- Tutor in organic chemistry (CHM 255, 256, 257) for the Purdue Span Plan Adult Student Services program for non-traditional students
### Teaching and Professional Development

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### Academic Awards, Honors, and Grants

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Mentorship Experience
2010-2013  Graduate advisor for the Purdue Undergraduate Environmental Science Club
2011-2012  Undergraduate NRES senior capstone project mentor (Dara A. Green)
2010-2012  ESE-IGP Colloquium mentor
2009     Mentored an NSF Fellow from Ohio State University (Dr. Marcella Card) in batch sorption techniques
2008-2010 Community Connections Program mentor working with youth to clean up and restore a local community park in Lafayette and plant a community garden

Leadership, Service, and Outreach
2012-2013 Reviewer for Journal of Environmental Quality; Environmental Toxicology and Chemistry
2012-2013 ESE-IGP GSO Constitutional founding committee member and interim president
2012, 2013 ESE Keystone Planning Committee
2011 ESE Symposium Webmaster
2010 ESE Symposium Planning Committee (Marketing Chair and Webmaster)
2009-2013 Purdue Invitational Soil Judging Contest volunteer (scoring)
2008-2011 Webmaster for ESE-IGP

Membership in Academic, Professional, and Scholarly Societies
American Society of Agronomy/Crop Science Society of America/Soil Science Society of America; American Association for the Advancement of Science; American Chemical Society, Division of Environmental Chemistry; Gamma Sigma Delta Honor Society; Phi Kappa Phi Honor Society; Sigma Xi Scientific Research Society; Society of Environmental Toxicology and Chemistry

Analytical Equipment Experience
Atomic absorption spectrophotometer; GC/MS (Agilent 7890A); HPLC coupled with a Sciex API-3000 triple quadrupole MS/MS (Shimadzu); HPLC/UV/FL (Shimadzu); IC (Dionex); NMR spectrometer (Varian Inova-300); Seal AQ-2 Discrete Analyzer; X-ray diffraction (PANalytical X’Pert PRO)
Publications


Conference/Symposium/Meeting Podium (Oral) Presentations


Conference/Symposium/Meeting Poster Presentations


Mashtare, M. L. and L. Bowling. 2009. Self-sustainability of three cities and the impact on their hydrological systems. Purdue University Ecological Sciences and Engineering Poster Symposium, West Lafayette, IN.