Aerobic- and Redox-Specific Biodegradation of Trenbolone Acetate Metabolites

A Thesis submitted in partial fulfillment of the Requirements for the degree of Master of Science in Civil and Environmental Engineering

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Abstract

Synthetic growth promoters and their metabolites (SGPMs) can be released into the aquatic environment from agricultural crop, rangeland, and concentrated animal feeding operation (CAFO) runoff at concentrations higher than 1,000 ng/L, potentially impacting ecosystem health at concentrations as low as 10 ng/L. The available research on the environmental fate of these compounds and their transformation mechanisms is currently incomplete. To investigate aquatic degradation rates of the trenbolone acetate (TBA) metabolites 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), and trendione (TBO), GC/MS/MS analysis was used to measure steroid loss as a function of time in biologically active microcosms. The effect of temperature and inocula was assessed at incubation temperatures of 5, 20, and 35°C using inocula collected across multiple seasons and water sources. High resolution LC/MS/MS was used to characterize transformation products and degradation pathways. Results of thorough kinetic analyses indicate that aerobic biodegradation yields half-lives of approximately 1.8, 0.68, and 1.3 days for 17α-TBOH, 17β-TBOH, and TBO, respectively at 20°C. Results also indicate that seasonal changes in microbial activity can change these observed half-lives by as much as a factor of two, while changes in inocula source can drastically effect transformation observations. Incubation of 17α-TBOH at 5°C yielded a longer half-life (7.3 days) than samples incubated at 20°C, indicating that temperatures of 20°C and lower may have a detrimental effect on the enzyme-mediated transformations of TBA metabolites. Metabolite interconversion analysis indicates that up to 53% of TBO mass may transform into 17β-TBOH, whereas only up to 7% of TBO mass may transform into 17α-TBOH. This stereo-specific pathway mimics patterns observed for
analogous compounds in the estradiol and testosterone families of environmental steroids. Preference for 17β-TBOH and TBO biotransformation over 17α-TBOH biotransformation could lead to preferential 17α-TBOH accumulation in affected water bodies. Although the results are expected to be system-specific, outcomes of this study will improve environmental risk assessment concerning the impact of synthetic growth promoters and their metabolites on aquatic organisms by improved characterization of the range of environmental transformations.
Acknowledgments

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Introduction

Emerging contaminants represent a class of compounds, nearly always synthetic, that in some cases have been shown to cause cancer, birth defects, and endocrine disruption, among other health complications. Harmful emerging contaminants include plastics and additives such as the endocrine disruptor bisphenol-A – known for being banned from use in water bottles – flame retardants, and pharmaceutical and personal care products (PPCPs). Many of these compounds are pervasive in modern life – an example would be flame retardants – and subsequently make their way into the environment. Those contaminants that are capable of negatively impacting reproductive function in wildlife are referred to as endocrine disrupting compounds (EDCs) and represent a wide range of chemicals such as certain organochlorine, pesticides, alkylphenolic compounds, and natural and synthetic steroid hormones.

Since the 1970s, the occurrence of endocrine disrupting compounds in the environment has been a topic of scientific concern (1-8). One of the first studies on ecological effects of EDCs reported feminization of both wild and caged fish; later studies reported masculinization of mosquito fish in the presence of paper mill effluent (9), which eventually lead to the discovery of fathead minnow masculinization in the presence of 17β-trenbolone (10). Investigation into estrogenic hormones present in sewage treatment effluent also led to observations of vitellogenin – a characteristic of female reproduction – in male fish exposed to these contaminants (2).

Major sources of EDC input to surface waters include municipal wastewater effluent and agricultural runoff (2-8, 11-15), and as most wastewater treatment facilities do not optimize treatment for EDC removal, the resulting wastewater effluent can often
be estrogically or androgenically active (11-22). In other areas, concentrated animal feeding operations (CAFOs), rangelands, and manure-disposal operations are primary sources of EDCs associated with animal agriculture (3, 23-25). Indeed, studies report that synthetic EDCs originating from animal manure can exhibit increased environmental persistence in aquatic systems, thereby increasing risk to aquatic life. Agricultural runoff can thus lead to biological uptake of EDCs, which have undesirable effects on aquatic reproductive health (4, 7, 10, 26-30). This poses problems for ecological systems as well as for both indirect reuse and drinking water systems.

Agricultural growth promoters are often administered as implants, most commonly given to beef cattle in CAFOs. Table 1 lists commercially available single-compound and combination growth implants. Estradiol is one of the most commonly used natural growth promoters; trenbolone acetate (TBA) is the most widely-used synthetic growth promoter (10, 31). When administered as a constant-release ear implant, TBA is 10-50 times more potent than testosterone and is primarily metabolized and excreted as 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), and trendione (TBO) (Figure 1). The metabolic pathway for known TBA metabolites is as indicated by the arrows in Figure 1: TBA is metabolized to 17β-TBOH, which is subsequently metabolized to TBO. TBO is then metabolized to the presumed major metabolite, 17α-TBOH, which represents approximately 95% of the known metabolite mass in manure from implanted cattle (31, 32). A direct pathway between 17α-TBOH and 17β-TBOH (such as one characterized by an isomerase enzyme) is not likely to be part of the TBA metabolite pathway because no such pathway has been observed for similar compounds. A single implanted animal may excrete up to 80 µg/day of 17α-
TBOH, which could potentially lead to runoff concentrations as high as several thousand ng/L \((31, 33)\).

**Table 1:** Commercially available implant formulations used by the beef cattle industry \((34)\)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Implant Dose [mg]</th>
<th>Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single compound Implants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>24</td>
<td>Mild estrogen</td>
</tr>
<tr>
<td>Trenbolone Acetate</td>
<td>40-200</td>
<td>Strong androgen</td>
</tr>
<tr>
<td>Zeranol</td>
<td>36-72</td>
<td>Moderate to strong estrogen based on dose</td>
</tr>
<tr>
<td><strong>Combination Implants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol benzoate / progesterone</td>
<td>10-20 / 100-200</td>
<td>Mild to strong estrogen/progestin, based on dose</td>
</tr>
<tr>
<td>Estradiol benzoate / Testosterone propionate</td>
<td>20 / 200</td>
<td>Mild estrogen/androgen</td>
</tr>
<tr>
<td>Estradiol / Trenbolone Acetate</td>
<td>8-28 / 40-200</td>
<td>Strong estrogen/androgen</td>
</tr>
</tbody>
</table>

High concentrations of TBA metabolites in receiving waters would be expected to impact aquatic organisms. Jensen et al. \((2006)\) and Ankley et al. \((2003)\) report reductions in fathead minnow fecundity at concentrations as low as 11 ng/L for 17α-TBOH and 27 ng/L for 17β-TBOH \((10, 27)\). Physiological abnormalities and genotoxicity have also been reported in fish populations after exposure to TBA metabolites \((35, 36)\). Seki et al. \((2006)\) reports reduction of plasma vitellogenin in female medka, fathead minnow, and zebra fish at 17β-TBOH concentrations as low as 40 ng/L \((35, 36)\). They also reported complete female masculinization at 1 – 10 μg/L 17β-TBOH \((35, 36)\).
Figure 1: Beef cattle metabolize TBA into 17β-TBOH, which is then metabolized into TBO and 17α-TBOH, respectively. 17α-TBOH is the major metabolite excreted by cattle.

Abiotic and biotic transformation and interconversion of TBA metabolites into degradates and uncharacterized compounds have been reported in recent studies (29, 37). In addition to abiotic transformation, sorption, and biodegradation studies have been used to assess the environmental fate of TBA metabolites (38-40). Khan et al. (2008) reported a variety of soil-based 17α-TBOH and 17β-TBOH biodegradation half-lives at varying soil moisture contents (Table 2), but this study did not address seasonal and temperature effects of data (38). Much of the experimental TBA metabolite concentrations were higher than what is observed in CAFO runoff, suggesting questionable environmental significance (31, 33, 38, 39). Khan et al. (2009) investigated stereoselective sorption of TBA metabolites to agricultural soils and found that 17α-TBOH was most likely to desorb from soil and leach into waterways (39). Regardless, the relative magnitude of $K_{OW}$ for each metabolite indicate that all three metabolites substantially desorb into
waterways. This implies that measuring aqueous biodegradation data for these metabolites would be critical to advancing research probing their environmental fate.

**Table 2:** Published partitioning and soil- and photo-based biodegradation rate constants

<table>
<thead>
<tr>
<th>TBA Metabolites</th>
<th>17α-TBOH</th>
<th>17β-TBOH</th>
<th>TBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log $K_{OC}^a$</td>
<td>2.77 ± 0.12a</td>
<td>3.08 ± 0.10a</td>
<td>3.38 ± 0.19a</td>
</tr>
<tr>
<td>Log $K_{OW}^a$</td>
<td>2.72 ± 0.02a</td>
<td>3.08 ± 0.03a</td>
<td>2.63 ± 0.05a</td>
</tr>
<tr>
<td>$k_{soil}[h^{-1}]^b$</td>
<td>0.166 ± 0.033c</td>
<td>0.094 ± 0.012c</td>
<td>0.014 ± 0.002c</td>
</tr>
<tr>
<td>$t_{1/2}[h]^b$</td>
<td>0.086 ± 0.010d</td>
<td>0.157 ± 0.008d</td>
<td>0.053 ± 0.12d,e</td>
</tr>
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</table>

- Experimental values from Khan et al. (2009);
- Soil degradation constants for soils with varying moisture contents, each dosed with 0.1 mg 17α-TBOH or 17β-TBOH per kg soil;
- Soil moisture content 6%;
- Soil moisture content 29%;
- Experimental values for TBO degradation as a result of interconversion from 17α-TBOH and 17β-TBOH;
- Experimental values from Qu et al. (2012);
- Experimental values from Xuan et al. (2008); Experimental values from (37-39, 41, 42)
Testosterone and estradiol are among the environmental steroids most commonly studied using lab-scale biodegradation systems. Pure cultures of *Comamonas testosteroni* (previously *Pseudomonas testosteroni*) are often used to investigate the enzymatic pathways active in testosterone microbial biodegradation. Enzymes such as 17β-hydroxysteroid dehydrogenase (17β-hsd), Δ1-dehydrogenase (Δ1-dh), 9α-hydrogenase, and C4-hydroxylase are directly involved in testosterone biodegradation and have been shown to exhibit stereospecificity to the 17β position (43, 44). A 17β-hsd enzyme isolated from certain mutant *Mycobacterium* strains has exhibited both oxidative and reductive capabilities at the 17β position. However, pathways are not always entirely predictable; bi-directional C17 oxygen conversion – conversion of a single site to a variety of different products – has been reported in systems containing *C. testosteroni*, *C. radicicola*, *Cochliobolus lunatus*, and *Pleurotus ostreatus*. Other sites, such as the C3 position, are also subject to transformation by *C. testosteroni*, *Streptomyces hydrogenans*, and *Mycobacterium* in similar bi-directional patterns (43). It is believed that many of these microorganisms contain similar steroid-degrading genes. Horinouechi et al. (2012) report a steroid-degrading “hot spot” in *C. testosteroni* comprised of an A-ring aromatizing and cleaving gene cluster and a B-, C-, and D-ring β-oxidizing gene cluster (45); differences in the enzymes responsible for A, B, and C ring cleavage are likely responsible for the observed variation in TBA and estrone transformation discussed above. For this reason, it is expected that TBA metabolite degradation rate constants will vary from observed estradiol and testosterone metabolite degradation rate constants.

Published data indicate that specific environmental conditions play a critical role in steroid biodegradation rates. Soil-based, aerobic biodegradation rates reported for the
estradiol system were significantly different from aqueous aerobic biodegradation rates and anaerobic aqueous biodegradation rates for the same compounds. In agricultural waste-receiving lagoons, steroid biodegradation may take place at a wide variety of redox states, ranging from completely aerobic to completely anaerobic. Lee (2011) attempted to quantify TBA metabolite biodegradation at fixed redox states, but found that unsterile controls and highly variable data led to unclear results (46).

In order to appropriately supplement existing TBA soil biodegradation data, aqueous TBA biodegradation studies must be done at aerobic and anaerobic redox states, and sterile controls must be used to isolate biodegradation systems from other degradation pathways. The limitations of published data, coupled with the implications of sorption data, necessitate a critical and comprehensive mechanistic study of TBA metabolite biodegradation in aquatic systems at environmentally relevant concentrations and redox conditions (38, 39, 41, 47).

The objectives of this study were to experimentally determine the aerobic biodegradation rate constants and half-lives of TBA metabolites at environmentally relevant conditions (e.g., 1,400 ng/L) to provide a more complete understanding of the fate of TBA under agricultural conditions. Transformation pathways and TBA metabolite interconversion were assessed to compare results with published TBA, estradiol, and testosterone biodegradation data (38, 41, 47). Source water to provide microbial inocula was gathered from a wetland area in Reno, NV for the majority of the data presented; additional inocula was gathered from different sites in the Reno, NV area to assess degradation as a function of source water. Temperature was manipulated as a control variable, and experiments were repeated with source water gathered during
different seasons to understand seasonal effects on metabolite biodegradation. A nutrient and carbon analysis was used to characterize the source water, and DAPI staining and fluorescein diacetate hydrolysis analyses were used to assess microbial activity. Finally, transformation of the three metabolites was investigated using a metabolite mass balance, and a select number of samples were analyzed using high resolution LC/MS/MS analysis to detect additional, previously unreported, transformation products.
Experimental

Chemicals

TBA metabolites (17α-TBOH, 17β-TBOH, and TBO) were purchased from Steraloids (Newport, RI, USA). 17β-TBOH-16,16,17-d3 was used as an internal standard and was purchased from BDG Synthesis (Wellington, NZ). HPLC grade methanol, acetone, and dichloromethane were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were purchased from Sigma Aldrich (Milwaukee, WI). N-methyl-N-(trimethylsilyl)-trifluoro-acetamine (MSTFA) and iodine (99.999% pure) were used to derivitize samples prior to GC analysis (33). Fluorescein diacetate (3’6’-diacetyl-fluorescein), fluorescein disodium salt, DAPI (4’,6-diamidino-2-phenylindole), and sodium pyrophosphate were used in microbial activity measurements. Dichlorodimethylsiloxane and toluene were used to silanize all steroid-related glassware.

Sample Collection and Microcosm Conditions

Biodegradation Studies

Surface water samples were gathered from Steamboat Creek in Reno, NV (39º25’08.25”N, 119º44’25.91”W) by disturbing the sediment at the bottom of the sampling site and collecting two gallons of turbid sample water in plastic sampling containers. Samples were immediately transported to the laboratory for use in biodegradation studies; these water samples served as the microbial inocula for each microcosm. Water samples were not centrifuged to remove particulates, as particulates represented a negligible contribution to the overall composition of the inocula. Prior to experimentation, inocula underwent a 1:1 dilution – and were acclimated to for up to 12 hours – with a mineral media of: 0.85 g/L KH2PO4, 2.175 g/L K2HPO4, 3.34 g/L
Na₂HPO₄·H₂O, 0.05 g/L NH₄Cl, 0.275 g/L CaCl₂, 0.225 g/L MgSO₄·7H₂O, 0.002 g/L FeCl₃·4H₂O (47, 48). All mineral media chemicals were purchased from Sigma Aldrich. Microcosms (70 mL) were assembled as follows: 70 mL of 1:1 inocula:media mixture was spiked with 100 µL of a 1 g/L metabolite standard (either 17α-TBOH, 17β-TBOH, or TBO in methanol) to obtain a nominal TBA metabolite concentration of 1,400 ng/L per microcosm. Control microcosms were composed of a 1:1 inocula:media mixture, autoclaved at 120ºC for two hours, and cooled to room temperature. All samples were loosely covered with aluminum foil to allow for air circulation but to minimize air-borne contamination.

All microcosm experiments were conducted in triplicate and incubated in a dark room on a 100 rpm shake table at 20ºC unless otherwise noted. All glassware was baked and silanized. Experiments were conducted over 15 days, and both aerobic and control microcosm samples were collected 0, 1, 3, 6, 10, and 15 days after incubation. On sample-collection days, collected samples were spiked with 17β-TBOH-d₃ internal standard (final concentration 1,400 ng/L), immediately followed by 10 minutes of mixing to ensure full incorporation of the internal standard into each sample and subsequent pH measurement. After pH measurement (lag time less than two hours), samples were extracted onto C-18 solid phase extraction (SPE) cartridges; cartridges were stored at -4ºC prior to GC/MS/MS analysis.

**Multiple-Inocula Studies**

To facilitate identification of transformation products, multiple-inocula experiments were conducted using inocula taken from the secondary clarifier effluent at the Truckee Meadows Water Reclamation Facility in Sparks, NV, from the Sparks
Marina swimming area in Sparks, NV, from the Truckee River access point at Mayberry Park in Reno, NV (39°30’09.30”N, 119°53’49.95”W), and from a duck pond at Idlewild Park in Reno, NV (39°31’17.69”N, 119°49’55.90”W). Samples were spiked to an initial 2.61 µM (700 µg/L) TBO concentration, and samples were collected 0, 2, 6, and 13 days after incubation. All additional sample treatment was identical to sample treatment for biodegradation studies with the exception of GC/MS/MS prep. Multiple-inocula samples were prepared for LC/MS/MS analysis, which is why a higher metabolite concentration was used for these samples (700µg/L for LC/MS/MS samples vs. 1,400 ng/L for GC/MS/MS samples).

**Amendments for Anaerobic Systems**

Anaerobic samples were prepared as described above, with an addition of L-cysteine, titanium (III) citrate, or dithiothreitol designed to fix the microcosm redox state at 0 to -200mV, -200 to -400mV, and less than -400 mV, respectively (48). Samples were sealed with sterilized caps and wrapped in parafilm to ensure an anaerobic environment. Immediately prior to pH measurement, redox potential was taken for each sample with an ORP probe (Beckman Coulter, model number PSI 570).

**Microbial Activity Analysis**

Fluorescein diacetate (FDA) hydrolysis – a cell activity measurement – and DAPI cell staining – a population count – were used to assess microbial activity. Both methods were performed concurrently with GC/MS/MS analysis. Final pH of each sample was taken immediately prior to elution to monitor overall microcosm conditions (49). All microbial activity figures can be found in Supporting Information.
Microbial activity was assessed by FDA hydrolysis by measuring FDA concentration as a function of experimental incubation time. A 5 g/L stock FDA solution in acetone was made and stored at -4°C. 200 µL of this stock solution was added to a 3 mL sample aliquot and allowed to react for 60 minutes, after which samples were immediately filtered through a 0.2 µm polyamide syringe filter (Chromafil, Germany). Sample absorbance was measured at 490 nm with a UV Spectrophotometer (Varian Cary-300 Bio). A calibration curve was prepared from a 20 mg/L fluorescein disodium salt stock solution using either the control or the aerobic 1:1 inocula:media mixture as dilution water (50).

Active and microbial population was also assessed by direct count using DAPI stain by taking three images per sample, using ImageJ software to quantify image luminescence, and plotting luminescence as a function of time. A 1 g/L DAPI solution and a 5% sodium pyrophosphate solution were each made in water and kept at 4°C. 3 mL DAPI stock and 1.5 mL sodium pyrophosphate stock were added to 3 mL sample aliquots and shaken for 60 minutes. Stained samples were immediately filtered through black polycarbonate filters using a vacuum manifold before UV light microscopy (EX330-380, BA420) with an oil immersion objective lens (UPlan-FI 100× oil) on a Nikon Eclipse microscope. A SPOT RT Camera was used to capture images (51, 52).

Nutrient Analysis

Total organic carbon (TOC) was measured with a Shimadzu Total Organic Carbon Analyzer (TOC-VCSH, Shimadzu Scientific Instruments, Kyoto, Japan). Procedures outlined in the Shimadzu TOC manual were used to prepare samples for TOC measurement.
GC/MS/MS Analysis

GC/MS/MS analysis is described elsewhere (33). Briefly, at 0, 1, 3, 6, and 15 days after incubation, three aerobic and three control microcosms per metabolite were extracted onto SPE cartridges. All cartridges were pre-conditioned by rinsing four times with 5 mL aliquots of methanol, and once with a 5 mL aliquot of distilled water. Cartridge conditioning and sample extraction was performed in an extraction manifold at a flow of 1-5 mL/min and all cartridges were stored in a freezer at -8 °C until elution(33).

SPE cartridge elution was performed using 9 mL of a 95:5 (v/v) methanol:water mixture in 3 mL aliquots. The eluent was dried in a vacuum oven at 20 °C. After drying, each sample was resuspended in 1 mL of a 95:5 (v/v) dichloromethane:methanol solution, transferred to glass GC vials and dried-down under N₂. Derivitization was performed by adding 50 µL MSTFA-I₂ (1.44 mg I₂ / mL MSTFA) to each sample, vortexing, and then drying-down under N₂ again. Each sample then received 100 µL MSTFA and was capped before 40 minutes of incubation at 60 °C, after which samples were placed on the GC/MS/MS for analysis (33). With these sample volumes, the method limit of quantification was 14 ng/L. Samples that failed to meet either this limit of quantification or meet or exceed a signal to noise ratio of 10 were excluded from quantitative analysis.

High-Resolution LC/MS/MS Analysis

Samples were extracted onto pre-conditioned SPE Cartridges as described above and stored until analysis commenced. SPE cartridge elution and vacuum oven drying was done as described above; samples were then resuspended in 1 mL of a 90:10 (v/v) methanol:water solution and transferred to LC vials for analysis. High-resolution
LC/MS/MS analysis was used to screen for additional TBA metabolites and samples were analyzed using an LTQ-Orbitrap XL (ThermoElectron, Bremen, Germany) (37).
Results and Discussion

Aerobic Degradation Study

Using inocula collected in mid-April, control samples of 17α-TBOH, 17β-TBOH, and TBO exhibited 85 – 100%, 77 – 95%, and 69 – 110% recovery ranges over the 15 day experiments, respectively. A rapid initial mass loss of 34% and 30% of 17α-TBOH and 17β-TBOH mass occurred within two hours of metabolite addition to aerobic samples, which is an observation that cannot be explained by sorption equilibrium to particles because 17α-TBOH and 17β-TBOH control samples did not exhibit similar loss. Despite some limited loss in control samples, aerobic TBO samples still exhibited consistent, reproducible transformation relative to controls.

In samples spiked with 17α-TBOH, the concentration decreased from 1,400 ng/L to 171 ng/L over the first six days of incubation, which is an 88% loss of initial metabolite mass (Figure 2). Between 6 and 15 days of incubation, the 17α-TBOH concentration exhibited a statistically insignificant increase to 196 ng/L. In these same 17α-TBOH microcosms, TBO increased from 31 ng/L to 173 ng/L over the first three days, corresponding to 12% of the original metabolite mass; TBO levels then decreased to 33 ng/L at six days of incubation and rose slightly to 73 ng/L over the next eight days. This suggests a 12% maximum TBO mass yield from 17α-TBOH. 17β-TBOH was observed after three days of incubation, at which time 36 ng/L of 17β-TBOH was detected, and an additional increase to 89 ng/L was observed after 15 days of incubation. This suggests a 7% maximum 17β-TBOH mass yield from 17α-TBOH, which is lower than the maximum TBO mass yield because TBO is an intermediate in the pathway from
17α-TBOH to 17β-TBOH, and neither interconversion is exclusive. No significant metabolite interconversion was observed in 17α-TBOH control samples.

![Graph](image)

**Figure 2:** Aerobic biodegradation of 17α-TBOH at 20°C. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1,400 ng/L. Error bars represent standard deviations of triplicate samples.

In samples spiked with 17β-TBOH, initial metabolite concentration decreased from 1,400 ng/L to 123 ng/L over three days of incubation, which is a 93% loss of initial metabolite mass (**Figure 3**). Between 3 and 15 days of incubation, the 17β-TBOH concentration decreased to 14 ng/L, representing a 99% loss of the original metabolite mass over the experiment. Within the first 24 hours of incubation, TBO increased to 218 ng/L, corresponding to 16% of the original metabolite mass. TBO levels then decreased to 34 ng/L, suggesting a 16% maximum TBO mass yield from 17β-TBOH. 17α-TBOH was not quantifiable in any sample, indicating a steric or enzymatic limiting for the TBO to 17α-TBOH pathway. No significant inter-metabolite transformation was observed in 17β-TBOH control samples.
Figure 3: Aerobic biodegradation of 17β-TBOH at 20°C. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1,400 ng/L. Error bars represent standard deviations of triplicate samples.

In samples spiked with TBO, initial metabolite concentration decreased from 1,400 ng/L to 14 ng/L over 15 days of incubation, which is a 99% loss of initial metabolite mass (Figure 4). In the first day, 17β-TBOH increased to 90 ng/L, corresponding to 6% of the original metabolite mass. 17β-TBOH levels then decreased slightly for the remainder of the experiment, reaching a no-detect concentration at day 15. 17α-TBOH was detected after ten days of incubation at an average 17α-TBOH concentration of 26 ng/L, although 17α-TBOH was not observed in any other sample on any other day. This observation supports the concept of a steric or enzymatic hindrance in TBO/17α-TBOH pathways that is not present in the more efficient TBO/17β-TBOH pathways. Sterile controls exhibited a 32% loss in TBO, which cannot be attributed to sorption because samples contained negligible particulate mass and because no similar loss was observed in 17α-TBOH or 17β-TBOH control samples. 17β-TBOH was
observed up to a 2% mass yield in sterile controls (28 ng/L) during TBO loss, but this observation does not account for all of the lost TBO mass. It is possible that the C3 and C17 sites exhibited aqueous reactivity, but ultimately there was no significant inter-metabolite conversion was observed in TBO control samples.

Figure 4: Aerobic biodegradation of TBO at 20°C. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1,400 ng/L. Error bars represent standard deviations of triplicate samples.

TBA metabolite interconversion – specifically conversion of 17α-TBOH and 17β-TBOH into TBO – as a result of biodegradation has been reported in a previous study, but at much higher yields (38). Khan et al. (2008) reported TBO yields of approximately 40% and 60% from 17α-TBOH and 17β-TBOH biodegradation respectively in agricultural soils spiked with 0.1 mg steroid per kg soil (38). Upon reaching peak TBO yields (approximately one day after incubation), Khan et al. (2008) observed subsequent TBO degradation to unknown products (38). Summing TBA metabolites in Khan’s soil study implies 90% TBA metabolite mass conservation at the point of peak TBO
conversion (1 day) (38). For comparison, this study reports maximum TBO yields of 16% – observed around three days after incubation – from the same metabolites. These data imply additional pathways that led to transformation of TBA metabolites to uncharacterized degradates in aqueous systems. For example, Khan et al. (2008) reported higher TBO yield for soil samples than what was observed in this study for aqueous samples (38). This is likely due to a difference in the microbial composition of the microcosms (i.e., soils are often more biologically active than aqueous samples), likely providing more enzymatic pathways for biotransformation, or higher experimental steroid concentration. As the soil transformation studies contained much higher particle concentrations, soil samples studied by Khan et al. (2008) likely contained a higher density of 17β-reducing microorganisms than this study’s aqueous samples, leading to greater conversion of 17α-TBOH and 17β-TBOH to TBO (39).

FDA, DAPI, pH, and total carbon data shown in Supporting Information suggest that microbial activity increased throughout the course of the study. Despite the phosphate buffer, the pH of all aerobic control samples rose approximately 0.5 pH units to a pH of 8.0 after six days of incubation, which may by attributable to loss of the carbonate buffer system in conjunction with ammonia volatilization (53), while the pH of all aerobic microcosms remained stable at a pH of approximately 7.5. A slight dip in pH was observed ten days after incubation for all aerobic samples and is believed to be evidence of microorganisms decomposing organics to acetic acid, which then further decomposed, causing the pH to rise once more (49, 54). The overall increasing pH for both aerobic and control samples may be attributable to nitrogen cycling (53). Total organic carbon (TOC), inorganic carbon (IC) and total carbon (TC) levels were negligible
for raw water samples, but steroid/methanol addition in fully assembled microcosms led to initial TOC and IC levels around 400 mg/L. **Figures SI-9 – SI-11** show carbon content changes over the course of an aerobic experiment.

**Seasonal Variability in Degradation**

To compare biodegradation across different seasons and inocula characteristics for the most potent of the known TBA metabolites, the 17α-TBOH experiments were repeated using an inocula sample gathered in January, 2013. Inocula was gathered from the same site at an ambient temperature of approximately -2°C and was transported to the laboratory where it acclimated for eight hours at 20°C prior to initiating the experiment.

![Graph showing aerobic biodegradation of 17α-TBOH in April and January.](image)

**Figure 5**: Aerobic biodegradation of 17α-TBOH in April- or January-gathered inocula at 20°C. Hollow symbols represent the average (January and April) of sterile control samples (autoclaved). Error bars represent standard deviations of triplicate samples.

Samples with January inocula exhibited a 17α-TBOH decrease from 1,400 ng/L to 138 ng/L over 15 days of incubation, which is a 90% loss of initial metabolite mass (**Figure 5**). The TBO concentration increased to 213 ng/L after three days of incubation,
accounting for 15% of the original metabolite mass, and then subsequently decreased (Figure SI-4). This suggests a 12% maximum $17\alpha$-TBOH-to-TBO mass yield in January inocula. $17\beta$-TBOH was only observed after six days of incubation when 65 ng/L of $17\beta$-TBOH was detected (5% of the initial metabolite mass (Figure SI-4). $17\beta$-TBOH was not observed in any other sample. This suggests a 5% maximum $17\alpha$-TBOH-to-$17\beta$-TBOH mass yield. No significant metabolite interconversion was observed in $17\alpha$-TBOH control samples.

These samples indicated that $17\alpha$-TBOH degraded more slowly in inocula gathered in January than in inocula gathered in April. In samples with April inocula, $17\alpha$-TBOH persisted for only six days before near complete disappearance (90% final loss ± 1%). For the January inocula, 15 days elapsed before $17\alpha$-TBOH reached 90% disappearance. However, both the April and January experiments exhibited similar metabolite interconversion. Both sets reached a maximum TBO yield of 12% from $17\alpha$-TBOH interconversion after three days of incubation; both sets also exhibited similar $17\beta$-TBOH yields (6% and 5% for April and January inocula, respectively). Persistence of the TBA metabolites after fifteen days of incubation may be due to either slow abiotic processes or limited biotransformation at low concentrations. Trace levels of TBA metabolites (less than 14 ng/L) were occasionally observed in control samples between ten and fifteen days of incubation, and likely due to abiotic mechanisms or microbial contamination. The similarities imply that TBO and $17\beta$-TBOH interconversion pathways from $17\alpha$-TBOH might be less dependent on seasonal changes to microbial communities, although the rates do show some seasonal dependence. These data should
be validated by additional biodegradation studies monitoring metabolite conversion at a high concentration.

Slower 17α-TBOH degradation in January is likely a result of a less microbially-active system than that observed in April. FDA data suggest that the microbial activity of the samples were lower in January than in April. Furthermore, some of the metabolite-degrading microorganisms likely remained dormant in the January sample; this is possibly a result of the optimum temperatures for the microorganisms being above 20°C, such as the optimum temperatures exhibited for psychrotrophs and mesotrophs.

**Temperature Dependence of Degradation**

To compare 17α-TBOH biodegradation rates across different incubation temperatures, additional microcosms containing January-gathered inocula were also maintained at either 5°C or at 35°C. An inverse relationship between 17α-TBOH transformation rates and temperature, consistent with traditional microbial degradation patterns, was expected for this data set (Figure 6).

In samples incubated at 5°C, 17α-TBOH concentration decreased from 1,400 ng/L to 600 ng/L over 6 days of incubation, which is a 57% loss of initial metabolite mass. Between 6 and 15 days of incubation, 17α-TBOH concentration exhibited a statistically insignificant increase from 600 ng/L to 650 ng/L, representing a gain of 5% of the original metabolite mass. After six days of incubation, TBO was detected at 90 ng/L and steadily increased to 368 ng/L by 15 days of incubation, corresponding to a 26% maximum TBO mass yield from 17α-TBOH (Figure SI-5). 17β-TBOH was not quantifiable in any samples, suggesting an unfavorable 17α-TBOH to 17β-TBOH pathway at low temperatures, potentially arising from a temperature-limited TBO/17β-
TBOH interconversion pathway. The increased TBO yield in the absence of observable 17α-TBOH biodegradation indicates that there is a potential transformation pathway from an uncharacterized metabolite to TBO that is active at low temperatures. Moreover, cessation of 17α-TBOH biodegradation accompanied by simultaneous increase in TBO concentration indicates that the samples may have gone anaerobic.

**Figure 6**: Aerobic biodegradation of 17α-TBOH in January-gathered inocula at 5°C, 20°C, and 35°C. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1,400 ng/L. Error bars represent standard deviations. Data points at 6, 10, and 15 days of incubation for 5°C samples are statistically similar by ANOVA, and data points at 1, 3, 6, 10, and 15 days of incubation for 35°C samples are statistically similar by ANOVA. All data points are statistically unique for 20°C samples.

In samples incubated at 35°C, 17α-TBOH concentration decreased from 1,400 ng/L to 740 ng/L over one day, which is a rapid 47% loss of initial metabolite mass. Between one and 15 days of incubation, however, no additional significant decreases were observed. TBO was not observed in any samples, and 17β-TBOH was observed only in one sample after six days of incubation, suggesting unfavorable conversion
pathways at these conditions (Figure SI-6). Cessation of 17α-TBOH biodegradation accompanied by nearly nonexistent metabolite interconversion indicates that the inocula, which was sampled in January, may not have been well-equipped for biodegradation at higher temperatures.

**Degradation in Various Inocula Sources**

In samples using Truckee River inocula, TBO concentration decreased from 700 µg/L to 340 µg/L over 13 days which is a 51% loss of initial metabolite mass (Figure 7). During TBO degradation, 17β-TBOH concentration steadily rose to 330 µg/L (Figure SI-12); 17α-TBOH was detected only after 13 days of incubation and at a concentration of 36 µg/L (Figure SI-13). The TBA metabolite mass balance closes (100% closure in the experiment), indicating that the established metabolite interconversions adequately describe biodegradation done by enzymes in this inocula (Figure 8). No controls were used for this study.

In samples using Idlewild Park inocula, TBO concentration decreased to 240 µg/L over 13 days, a 65% overall loss. However, degradation was unsteady, dropping to 176 µg/L and 93 µg/L TBO after two and five days of incubation, respectively. During TBO degradation, 17β-TBOH concentration steadily rose to 330 µg/L (Figure SI-12); 17α-TBOH was detected after five days of incubation at a concentration of 5 µg/L and after 13 days of incubation at a concentration of 46 µg/L (Figure SI-13). Because of the unusual pattern in TBO recovery, it is difficult to draw conclusions from the TBA metabolite mass balance for inocula from Idlewild Park.

In samples using Sparks Marina inocula, TBO concentration decreased from 700 µg/L to 160 µg/L over 13 days of incubation, which is a 77% overall loss of initial
metabolite mass. Degradation was rapid over the first two days, and slowed between two and 13 days of incubation. During TBO degradation, 17β-TBOH concentration steadily rose to and peaked at 370 µg/L after five days in incubation, then subsequently decreased to 190 µg/L (Figure SI-12). 17α-TBOH was detected only after 13 days of incubation and at a concentration of 27 µg/L (Figure SI-13). The TBA metabolite mass balance indicates an overall 54% loss of metabolite mass (Figure 8).

**Figure 7**: Aerobic biodegradation of TBO in five unique inocula sources.

In samples using TMWRF inocula, TBO concentration decreased from 700 µg/L to 8 µg/L over two days of incubation, which is a 99% loss of initial metabolite mass; concentrations steadily decreased for the remainder of the study. A 17β-TBOH concentration of 71 µg/L was observed in Day 0 samples, indicating rapid TBO to 17β-TBOH interconversion, and then decreased to 3 µg/L over 13 days (Figure SI-12). 17α-TBOH was detected only at trace levels (less than 2%) (Figure SI-13). The TBA
metabolite mass balance indicates a substantial 99% loss of TBA metabolites with this diverse, biologically active inocula (Figure 8).

In samples using Steamboat Creek inocula, TBO concentration decreased from 700 µg/L to 27 µg/L over 13 days of incubation, which is a 96% loss of initial metabolite mass. 17β-TBOH concentration increased to 120 µg/L after five days of incubation and subsequently decreased to 43 µg/L at 13 days (Figure SI-12). 17α-TBOH was detected at trace levels at two days, and increased to 15 µg/L after 13 days (Figure SI-13). The TBA metabolite mass balance indicates a substantial 88% loss of TBA metabolites (Figure 8).

![Figure 8](image-url)

**Figure 8:** TBA metabolite mass balance for samples spiked with TBO for six unique April-gathered inocula sources.

Mass balance values at two days of incubation likely indicate experimental error in sample processing, as all values are lower than expected and complete metabolite recovery was observed in Truckee River samples at the next time point (Figure 8).
Variation in biodegradation rates across multiple inocula supports the idea that TBA metabolite biodegradation is system-specific and subject to wide uncertainty. The Truckee River is fed by snowmelt in the Sierra Mountains and is known for its relatively high quality. The Sparks Marina is a public recreation area used for boating, fishing, and swimming. TMWRF water has been optimized for microbial activity, so it is reasonable that TMWRF water exhibited the most rapid and complete TBA metabolite removal. Steamboat creek is the same source used in the rest of the experiments in this study, and represents an active wetland, most like what might be used to remove TBA metabolites from agricultural runoff.

**Anaerobic Biodegradation Studies**

All anaerobic and control samples poised with l-cysteine exhibited rapid decay across the fifteen day time-series for all metabolites. A first order decay was observed for all control samples, starting with an average apparent metabolite concentration of 1,600, 920, and 1,720 ng/L and finishing with an average metabolite concentration of 250, 140, and 100 ng/L after 15 days of incubation for 17α-TBOH, 17β-TBOH, and TBO respectively. This implies a reaction between the l-cysteine poising agent and TBA metabolites (Figure SI-14 – Figure SI-22).

The large error bars on 17α-TBOH and TBO data points, indicate that a reaction also took place between l-cysteine and the 17β-TBOH-d3 internal standard. Because the error bars on the 17β-TBOH data points were so small – representing repeatable data-gathering – l-cysteine may have reacted with 17β-TBOH and 17β-TBOH-d3 at a similar rate and at similar sites. Overall, this means that l-cysteine cannot be used as a poising agent for these compounds.
Titanium (III) citrate data exhibited a rapid, approximately 60% loss in all Day 0 control samples. Moderate loss of another 30% of metabolite mass was observed over the remainder of experimentation. However, unlike the l-cysteine data set, TBA metabolite decay in the presence of titanium (III) citrate was not accompanied by persistent metabolite interconversion. Also, many titanium (III) citrate samples had no metabolite or internal standard detects in GC/MS/MS analysis, which implies that titanium (III) citrate is also a poor ORP poising agent to use in the presence of TBA metabolites (Figure SI-23 – Figure SI-31).

No metabolite or internal standard response was observed in GC/MS/MS analysis for anaerobic sample poised with dithiothreitol. However, all anaerobic samples exhibited a stable control pH and a drop in anaerobic pH from 7.5 to 6.5 between three and 10 days of incubation, indicating biological activity (49). All samples poised with l-cysteine and dithiothreitol exhibited a stable and repeatable ORP at approximately -200 mV throughout the course of the experiment. Samples spiked with titanium (III) citrate exhibited a stable ORP around -200 mV until day 10, when average sample ORP dropped below -250 mV and subsequently rose to -50 mV on day 15. ORP measurements for day 10 and day 15 samples were highly variable, leading to large standard deviations (Figure SI-32 – Figure SI-34).

**Determination of Biotransformation Rate Constants and Half Lives**

Degradation of organic contaminants is frequently approached with a pseudo-first order decay model (38, 41, 47, 55-57). This model is most applicable when contaminant concentrations trend towards zero in an exponential decay. The differential form of the
pseudo-first order model and its solution are represented by Equations 1 and 2, respectively.

\[ \frac{dc}{dt} = -kC \]  
\[ C = C_1 e^{-k_1 t} \]  

In Equation 2, \( C \) represents the observed metabolite concentration at time \( t \), \( C_1 \) represents the initial metabolite concentration at time \( t_0 \), and \( k_1 \) represents the corresponding biodegradation rate constant. The pseudo-first order model assumes that degradation proceeds until the metabolite concentration is zero.

When data does not exhibit a clear trend towards zero – in the case of degradation plateaus or for low-level contaminant persistence – modified exponential decay models are more applicable (58-60). These models are presented as Equation 3 (non-zero asymptote model) and Equation 4 (double-exponent model), and were used extensively in organic contaminant degradation analyses of the Great Lakes (58, 59).

\[ C = C_1 e^{-k_1 t} + C_2 \]  
\[ C = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} \]

In Equation 3, \( C_2 \) represents a stable, non-zero asymptotic concentration, and in Equation 4, \( C_1 + C_2 \) represents the initial metabolite concentration at time \( t_0 \); \( k_1 \) and \( k_2 \) represent the corresponding biodegradation rate constants. Again, \( C \) represents the observed metabolite concentration at time \( t \) for both equations.

It should be pointed out that the presence of \( C_2 \) – a constant, non-degrading term – in Equation 3 forces the non-zero asymptote model to assume the presence of two contaminant pools: one rapidly decaying and one persistent. The double-exponential
model also assumes two contaminant pools, but the second contaminant pool in this model may also decay (indicated by the exponential portion of the second term).

Because some biotransformation data for this study seemed to exhibit pseudo-first order traits (such as a trend towards zero, seen in Figure 4), while other data exhibited low-level persistence (seen in Figure 2) or plateaus (seen in Figure 6), all three models (Equations 2-4) were used to assess biotransformation kinetics. Metabolite biotransformation rate constants \((k_\alpha, k_\beta, \text{and} \ k_o,\ \text{for} \ 17\alpha\text{-TBOH,} \ 17\beta\text{-TBOH,} \ \text{and} \ \text{TBO biotransformation, respectively})\), were calculated from linear regressions of Equations 2-4. Half-lives were calculated by solving Equations 2-4 iteratively for a 50% loss in TBA metabolite concentration. Numerical values for all biodegradation constants and half-lives are shown in Table 3 and were compared to relevant published biodegradation data. Blank fields in Table 3 indicate that the parameter was not used in that specific model.

Best-fit was determined by comparison of \(R^2\) values for each model for a given data set. The non-zero asymptote model showed the best fit for all data with two exceptions: the 35°C data set was better estimated by the double-exponent model, and the TBO data set was equally well-estimated by all models. A pseudo-first order degradation constant was also calculated for the observed minor loss in TBO controls and was found to be 0.03 days\(^{-1}\). This value is less than 10% of the calculated degradation constant for aerobic TBO biodegradation and thus was considered to have had minimal to no impact on the calculated biodegradation rate constants for each kinetic model. From this point forward, only the best-fit biotransformation model will be discussed for each data set.
Table 3: Biodegradation kinetic parameters as calculated by Equations (2) – (4)\textsuperscript{a}

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<th>Temp. (°C)</th>
<th>Eqn. #</th>
<th>( C_1 ) (mg/L)</th>
<th>( k_1 ) (day(^{-1}))</th>
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<th>( k_2 ) (day(^{-1}))</th>
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\[\textsuperscript{a}Nonlinear regression calculated in Microsoft Excel using the Solver add-in\]

The two-fold difference in biotransformation constants for April 17α-TBOH and 17β-TBOH data surprisingly indicates that stereochemistry plays a significant role in the degradation process with increased persistence expected for 17α-TBOH metabolites in particular. Because TBO was such an important intermediate for both 17α-TBOH and 17β-TBOH degradation (Figures 2 and 3), and because it also exhibited an estimated half-life intermediate to those which were observed for 17α-TBOH and 17β-TBOH, the \( C_{17} \) position in TBA metabolites probably plays a critical role in governing microbial
transformation pathways (43). Because TBO showed reduced transformation compared to $17\alpha$-TBOH and $17\beta$-TBOH (Figures 2-4), the C$_{17}$ ketone seems to inhibit reactivity through select biotransformation.

A nearly twofold difference in $k_1$ was observed between January (0.15 d$^{-1}$) and April (0.27 d$^{-1}$) data for $17\alpha$-TBOH. Predicted half-lives for these data are 4.1 and 1.8 days, respectively. Assuming that the remaining metabolites would likely follow a similar seasonal shift in biotransformation rate, we might expect, for $17\beta$-TBOH and TBO, an aerobic half-life of approximately 3 days in winter and approximately 1 day in moderate summer climates.

The predicted half-lives for January 5°C and 20°C data sets also differed by nearly a factor of two (7.3 and 4.1 days, respectively), implying that temperature may play a significant role in $17\alpha$-TBOH biotransformation (Figure 5). The predicted half-life for the 35°C data set (6.0 days) were significantly different from the corresponding $k$ and $t_{1/2}$ for the 5°C and 20°C data sets, and more closely resembled the degradation parameters observed in $17\beta$-TBOH and TBO data sets, which were gathered in April.

Because the non-zero asymptote model exhibited the best kinetic fit for nearly every data set, it is possible that complete degradation of TBA metabolites is hindered by a pseudo-equilibrium. A pseudo-equilibrium suggests the presence of multiple contaminants, each undergoing interconversions between structurally similar contaminants at similar rates. Such a phenomenon would be consistent with a persistent contaminant pool characteristic of the non-zero asymptote model. The presence of a persistent contaminant pool would also support the spontaneous TBO growth observed in
the 5°C data set (Figure SI-5) and support the suggestion that TBA metabolites experience structural conservation instead of mineralization.

Because a low-concentration plateau was observed for all samples in the April data set (<10% TBA metabolite recovery, persisting for up to eight days, shown in Figures 2-4), it is likely that the non-zero asymptote model is truly the best fit for this data, indicating that even rapid biodegradation of TBA metabolites might result in development of structurally similar biodegradation products. Further confirmation of this theory would be best executed by gathering biodegradation data at various incubation temperatures for much higher concentrations and for much longer time-scales. Such a study would be best done if additional TBA metabolites are already identified.

Degradation rate constants and half-lives were compared against values calculated from earlier data sets to assess procedural consistency. Inocula collected from an aeration basin indicates that TBA-metabolizing enzymes may be common, but susceptible to variable effectiveness as a function of microbial activity. Aeration basin data led to predicted half-lives nearly half the magnitude of half-lives reported in Table 3. This difference implies that 17α-TBOH degradation rate is highly reliant on the type of microbial community and activity, and suggests that 17α-TBOH-specific enzymes may be active in only select types of microorganisms. Environmentally, this could lead to relative accumulation of 17α-TBOH in aquatic systems and adversely impact ecosystem health.

All observed biodegradation rate constants (0.31, 0.71, and 0.53 d⁻¹ for 17α-TBOH, 17β-TBOH, and TBO, respectively) were significantly lower than those reported by Khan et al. (2008) for soil-based biodegradation (1.6 to 4.0, 2.3 to 3.8, and 0.3 to 1.3
d for 17α-TBOH, 17β-TBOH, and TBO respectively at 20°C (38). Under similar experimental conditions, this study reports significantly longer half-lives for each compound. This is likely a result of a soil-based microbial community being larger or more active with more opportunity for direct biodegradation and biologically facilitated degradation than what is possible in a surface water sample. This difference is critical because at least 20% of all 17α-TBOH mass remains dissolved in a typical soil-water subsurface system (39).

Metabolite Interconversion Kinetic Estimations

Specific metabolite interconversion rate constants (e.g.: \(k_A\), \(k_B\), \(k_C\), and \(k_D\)) (Figure 1) were estimated from observed conversions using an adaptation of similar analyses done for the estradiol system (47). 17α and 17β are abbreviations for 17α-TBOH and 17β-TBOH, respectively (47). All points representing metabolite growth were used to quantify \(k_A - k_D\). For \(k_A\), TBO metabolite growth from 17α-TBOH decay was represented by three data points (Figure 2). For \(k_B\), 17β-TBOH metabolite growth from TBO decay was represented by two data points (Figure 4), and for \(k_D\), TBO metabolite growth from 17β-TBOH decay was also represented by two data points (Figure 3). Insufficient data existed to calculate \(k_C\) (Figure 4), so \(k_C\) was deemed negligibly small compared to \(k_A\), \(k_B\), and \(k_D\).

\[
\begin{align*}
17\beta & \rightleftharpoons TBO \\ 17\alpha & \rightleftharpoons TBO
\end{align*}
\]

\[
\frac{d[17\beta]}{dt} = -k_A[17\beta] + k_B[TBO] \tag{6}
\]

\[
\frac{d[TBO]}{dt} = k_A[17\beta] - (k_B + k_C)[TBO] + k_D[17\alpha] \tag{7}
\]

\[
\frac{d[17\alpha]}{dt} = -k_C[17\alpha] + k_D[TBO] \tag{8}
\]
Equations 7-9 are incomplete because mass balance analysis of TBA biodegradation indicates that additional biotransformation products exist. For example, TBO biodegradation results in more products than just $17\alpha$-TBOH and $17\beta$-TBOH, and there should be additional transformation products and constants, $k_E$, $k_F$, etc., added to this system of equations once these uncharacterized products are identified. Zheng et al. (2012) used transformation and interconversion data to estimate metabolite interconversion rates analogous to this study’s $k_A$, $k_B$, $k_C$, and $k_D$ because they were able to account for all significant biotransformation metabolites in their estrogen system (47).

For this study, metabolite interconversion constants, $k_A$, $k_B$, and $k_D$, were estimated by fitting transformation data to a pseudo-first order growth model and were estimated to be 0.51, 0.60, and 0.63 day$^{-1}$, respectively (Figure SI-37). No conversion of TBO to $17\alpha$-TBOH was observed above our quantification limits, so $k_C$ was assumed negligible relative to other constants. Zheng et al. (2012) calculated three sets of $k_A - k_D$, each associated with samples spiked with a single estradiol metabolite, at aqueous steroid concentrations 2,000 times higher than TBA metabolite concentrations used in this study. The lower concentrations and lower yields reported in this study did not allow for calculation of $k_A - k_D$ for each metabolite; instead, $k_A$ was calculated from samples spiked with $17\alpha$-TBOH, $k_3$ was calculated from samples spiked with TBO, and $k_D$ was calculated from samples spiked with $17\beta$-TBOH. In order to calculate $k_A - k_D$ for systems starting with each metabolite, it is suggested that these aquatic, aerobic degradation studies be repeated at higher concentrations – ideally at the same concentrations used by Zheng et al. (2012) – sampling frequently up to five days.
an investigation should also attempt to quantify additional biodegradation products – perhaps by using radioactive tracers – in an effort to close the TBA metabolite mass balance and complete the descriptive system of differential equations.

**Effects of Environmental Variability**

Ultimately, it is not surprising that this study’s data differs from results reported by Khan et al. (2008) (38). Studies have already established that pH, redox state, dissolved oxygen levels, organic carbon content, temperature, and overall variability in microbial community can significantly affect biodegradation of organic contaminants (41, 47, 55, 61-63). These same studies also emphasize that laboratory investigations often only provide a rough estimate of kinetic parameters; controlled, laboratory systems often cannot completely account for the constant biological flux of the environment. Due to these constraints, it is often the goal of researchers to simulate environmental conditions as best as possible.

In a fantastic example of how differences in inocula can affect biodegradation, Lartiges et al. (1995) investigated how using either laboratory water, river water, or sea water (collected near Bordeaux, France) as inocula led to highly variable pesticide biodegradation constants and half-lives (62). Their study showed that pesticide recovery could vary by as much as 20% of the original mass load, solely as a function of inocula source (62). They also concluded that half-lives for the investigated compounds could vary as much as three-fold, depending upon the inocula source (62). Further temperature-manipulation of their data indicated that dropping the incubation temperature of their samples from 22°C to 6°C also resulted in highly variable data (62). For example, the observed half-life for methyl parathion (an organophosphorous pesticide) in
river water was 95 and 23 days for incubation temperatures of 6°C and 22°C, respectively (62). Incubation of the same compound at the same temperature, but in sea water, led to half-lives of 233 and 30 days, respectively (62). Lartiges et al. (1995) concluded that water quality characteristics such as pH, temperature, chemical composition, particulate composition, etc. each play a substantial role in environmental degradation kinetics of organic contaminants, leading to high variability of contaminant degradation as a function of inocula source (62).

Bartholomew et al. (1983) investigated how biodegradation rates of organic pollutants varied for inocula gathered from various sampling sites in the Newport River Estuary in North Carolina, USA, highlighting how simple spatial and temporal differences can affect biodegradation observed in a single water system (61). Using Monod kinetics, they determined that microorganism metabolic rate was highly dependent upon sampling location and season of sample-gathering (61). For example, microorganism metabolic rates during chlorobenzene degradation for inocula gathered in September was 13, 10, and <1 ng/(L·h) for samples taken upstream of the estuary, in the estuary, and in the Atlantic Ocean at the mouth of the estuary, respectively (61). Metabolic rates were below the level of detection for chlorobenzene biodegradation using inocula gathered in February, but metabolic rates for nitrilotriacetic acid biodegradation using the same February-inocula were 56, 175, and 85 ng/(L·h) (61). The variability in metabolic rate as a function of season further supports our observations of seasonal variability in TBA metabolite biodegradation. Moreover, the estuarine environment led to the fastest decay of nitrilotriacetic acid (compared with the upstream environment and the ocean environment), but the upstream environment consistently led to the fastest
decay of chlorobenzene (61). Thus, both structural organic contaminant characteristics and fundamental environmental community characteristics strongly influenced organic contaminant biodegradation. Ultimately, this implies that accurate determination of TBA metabolite biodegradation kinetics may need to be assessed on a case-by-case basis. Furthermore, this puts our data well within observed environmental variability ranges for organic contaminant biodegradation.

Comparison with the Estradiol System

Results from this TBA metabolite biotransformation study are consistent with the stereospecific transformation pattern exhibited by the estrogens (47). Preference for conversion between 17β-estradiol and estrone over conversion between 17α-estradiol and estrone were similar to results observed for TBA metabolites. These data indicate that 17α-TBOH is expected to be the most persistent TBA metabolite in aqueous systems, which is consistent with increased observation of 17α-TBOH in agricultural receiving waters relative to other TBA metabolites (31, 32). Given that the observed fecundity threshold for fathead minnows is 10 ng/L for 17α-TBOH, the higher persistence of this most-potent compound implies additional risk for ecosystem health (10).

Similarities between analogous steroid systems diverge when examining mass conservation through biotransformation. According to Zheng et al., at least 80% of the total estradiol system mass has been conserved over 50 days under anaerobic conditions (47), but higher TBA metabolite transformation under aerobic conditions and reduced TBA metabolite interconversion was observed in this study. Loss of estrogen mass observed by Zheng et al. was attributed to mineralization in the tricarboxylic acid cycle, but no such data are available for any TBA study (29). It should be noted that A, B, and
C ring transformation is often accomplished by specific enzymes (45), and published literature would lead us to expect TBA metabolite degradation constants uniformly different from those observed in estradiol systems for analogous compounds. It would also seem that the C17 site on steroidal contaminants is more available to the relevant enzymes than the other common degradation sites (C3, C5, and C12) (45, 47). Conversely, because the present study did not observe analogous metabolite interconversion, the C3, C5, or C12 positions may represent additional active transformation sites. Furthermore, the 17α-TBOH 5°C data show potential formation of TBO from an uncharacterized compound, consistent with observations in photolytically active systems (29, 37). It is unlikely that this was the only case of such a transformation, and subsequent studies may investigate the biological formation of similar products.

Considerable research has been done to investigate the environmental fate of estrogenic conjugates (estriol, estrogen, and estradiol families) (64, 65). Estrogens, and other hormones, are known to be transformed into less potent, conjugated forms (commonly as glucuronides and sulfates) in order to increase their water-solubility and enhance their removal in animal waste (64-69). Their introduction into the environment and subsequent de-conjugation in rivers and wastewater treatment facilities lead to increased estrogenic activity and increased risk to wildlife reproductive health (64, 65). Because the microcosms in this study contained sulfate salts, it is possible – in theory – that microorganisms used the sulfate salts to form sulfate conjugates of TBA metabolites. However, the majority of known TBA mass is excreted through cattle feces, and TBA metabolite levels in cattle urine are considered negligible (31, 32). Additionally, hormone conjugation to sulfates and glucuronates is a trait largely reserved for mammals,
birds, reptiles, and some amphibians; such conjugation has not been observed in insects or in environmental microorganisms (69). Plants have been known to conjugate hormones, but there seems to be no evidence of conjugation pathways comparable to those observed in the intestines of animals (70, 71). It is thus unlikely that TBA metabolite conjugation occurred in the systems discussed in this study. Should one wish to investigate the environmental effects of possible TBA metabolite conjugates, experimentation should start with identification of TBA conjugates in cattle urine.
Environmental Implications

These reported degradation constants and half-lives further characterize environmental fate parameters for TBA metabolites. Because 17α-TBOH accounts for 95% of the total excreted TBA metabolite mass, 17α-TBOH is the most environmentally relevant of the known TBA metabolites and was a primary focus of these biodegradation studies (31, 32). Results indicate that a two-fold difference in half-life occurred between samples with January-gathered and April-gathered inocula ($t_{1/2, April} < t_{1/2, Jan}$). All observed half-lives were greater than those reported for TBA metabolites in soil systems, which is a discrepancy that likely reflects the different microbial conditions in soil and water samples (8). This suggests that both microbial activity and population composition can significantly influence biodegradation rates and product formation. Metabolite interconversion patterns observed in estrogen systems were consistent with results reported in this TBA metabolite study, suggesting the conservation of some metabolite interconversion pathways for steroid systems (47). These interconversion pathways likely arise from the presence of promiscuous, stereo-specific enzymes in receiving waters and soils that preferentially favor interconversion between the 17β alcohol group and the associated ketone in lieu of the 17α alcohol (43). As this interconversion pattern seems to be conserved through multiple steroid systems, it is likely that the functional enzyme responsible for observed TBA metabolite interconversion is widespread and perhaps already identified (45).

As expected, TBA metabolite transformation was generally faster in warmer conditions, implying an increase in TBA metabolite environmental persistence in colder months, consistent with results reported for estrogens (47). The most persistent
metabolite was 17α-TBOH because 17β-TBOH and TBO had substantially shorter half-lives than 17α-TBOH at 20°C (conservation of the relative magnitude of $k$ between each metabolite for temperatures above 20°C is assumed).

Ecological risk assessments can now include aqueous biodegradation rate constants for TBA metabolites. If a source water is known to degrade similar suites of steroids, then it is likely that the same water will degrade TBA metabolites, but at a slower rate. However, it is generally poor practice to rely on natural attenuation to remove problematic contaminants. These data should be combined with a full fate and transport model to provide a complete assessment of abiotic and biological soil and water effects on TBA removal. Ultimately, treatment methods may be developed that concurrently optimize conditions for TBA-degrading enzymes and abiotic photodegradation, which could drastically enhance TBA removal and facilitate the development of similar removal systems for other environmentally-persistent EDCs.
Works Cited


Supporting Information

Figure SI-1: pH variability for microcosms aerobically degrading $17\alpha$-TBOH, $17\alpha$-TBOH, and TBO at 20°C. Inocula was gathered in April 2013.

Figure SI-2: FDA variability for microcosms aerobically degrading $17\alpha$-TBOH, $17\alpha$-TBOH, and TBO at 20°C. Inocula was gathered in April 2013.
**Figure SI-3:** DAPI variability for microcosms aerobically degrading 17α-TBOH at 20°C. Inocula was gathered in April 2013. CTCF refers to Corrected Total Cell Fluorescence, as measured by ImageJ using the protocols developed by Sherr, et al. (51, 52)

**Figure SI-4:** Aerobic biodegradation of 17α-TBOH at 20°C in January-harvested inocula. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1,400 ng/L. Error bars represent standard deviations.
Figure SI-5: Aerobic biodegradation of 17α-TBOH at 5°C in January-harvested inocula. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1,400 ng/L. Error bars represent standard deviations. TBO growth between 6 and 10 days of incubation is statistically significant by ANOVA, but TBO growth between 10 and 15 days of incubation is not statistically significant by ANOVA.
Figure SI-6: Aerobic biodegradation of 17α-TBOH at 35°C in January-harvested inocula. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1,400 ng/L. Error bars represent standard deviations.

Figure SI-7: pH variability for microcosms aerobically degrading 17α-TBOH at 5, 20, and 35°C. Inocula was gathered in January 2013.
**Figure SI-8:** FDA variability for microcosms aerobically degrading 17α-TBOH at 5, 20, and 35°C. Inocula was gathered in January 2013.

**Figure SI-9:** TOC levels for Steamboat Creek Microcosms. Inocula gathered in July, 2013.
Figure SI-10: TC levels for steamboat creek microcosms. Inocula gathered in July, 2013.

Figure SI-11: IC levels for steamboat creek microcosms. Inocula gathered in July, 2013.
Figure SI-12: 17β-TBOH interconversion from TBO biodegradation for five unique April-gathered inocula samples.

Figure SI-13: 17α-TBOH interconversion from TBO biodegradation for five unique April-gathered inocula samples.
Figure SI-14: $17\alpha$-TBOH degradation in sterile controls in the presence of l-cysteine

Figure SI-15: $17\alpha$-TBOH degradation in biological samples in the presence of l-cysteine
**Figure SI-16:** pH and ORP variability for 17α-TBOH microcosms anaerobically degrading 17α-TBOH in the presence of l-cysteine

**Figure SI-17:** 17β-TBOH degradation in sterile controls in the presence of l-cysteine
Figure SI-18: $17\beta$-TBOH degradation in biological samples in the presence of l-cysteine

Figure 19: pH and ORP variability for $17\beta$-TBOH microcosms anaerobically degrading $17\beta$-TBOH in the presence of l-cysteine
**Figure SI-20**: TBO degradation in sterile controls in the presence of l-cysteine

**Figure SI-21**: TBO degradation in biological samples in the presence of l-cysteine
Figure SI-22: pH and ORP variability for TBO microcosms anaerobically degrading TBO in the presence of l-cysteine

Figure 23: 17α-TBOH degradation in sterile controls in the presence of Ti(III) citrate
Figure 24: 17α-TBOH degradation in biological samples in the presence of Ti(III) citrate

Figure SI-25: pH and ORP variability for 17α-TBOH microcosms anaerobically degrading TBO in the presence of Ti(III) citrate
Figure SI-26: $17\beta$-TBOH degradation in sterile controls in the presence of Ti(III) citrate

Figure SI-27: $17\beta$-TBOH degradation in biological samples in the presence of Ti(III) citrate
Figure SI-28: pH and ORP variability for 17β-TBOH microcosms anaerobically degrading 17β-TBOH in the presence of Ti(III) citrate

Figure SI-29: TBO degradation in sterile controls in the presence of Ti(III) citrate
Figure SI-30: TBO degradation in biological samples in the presence of Ti(III) citrate

Figure SI-31: pH and ORP variability for TBO microcosms anaerobically degrading 17β-TBOH in the presence of Ti(III) citrate
Figure SI-32: pH and ORP variability for 17α-TBOH microcosms anaerobically degrading TBO in the presence of DTT

Figure SI-33: pH and ORP variability for 17β-TBOH microcosms anaerobically degrading TBO in the presence of DTT
**Figure SI-34**: pH and ORP variability for TBO microcosms anaerobically degrading TBO in the presence of DTT

**Figure SI-35**: Maximum metabolite mass yield for all sample sets at specified conditions
**Figure SI-36:** To estimate $k_A$, $k_B$, and $k_D$, the slope of regression lines were forced through zero to match Equation (2).

**Table SI-1:** Metabolite Mass Balance

<table>
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<th>Days of Incubation</th>
<th>17α-TBOH</th>
<th>17α-TBOH</th>
<th>17α-TBOH</th>
<th>17α-TBOH</th>
<th>17β-TBOH</th>
<th>TBO</th>
</tr>
</thead>
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<td></td>
<td>Jan. 5°C</td>
<td>Jan. 20°C</td>
<td>Jan. 35°C</td>
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<td>91%</td>
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