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Degradation and metabolite formation of estrogen conjugates in an agricultural soil

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ABSTRACT

Estrogen conjugates are precursors of free estrogens such as 17ß-estradiol (E2) and estrone (E1), which cause potent endocrine disrupting effects on aquatic organisms. In this study, microcosm laboratory experiments were conducted at 25 °C in an agricultural soil to investigate the aerobic degradation and metabolite formation kinetics of 17ß-estradiol-3-glucuronide (E2-3G) and 17ß-estradiol-3-sulfate (E2-3S). The aerobic degradation of E2-3G and E2-3S followed first-order kinetics and the degradation rates were inversely related to their initial concentrations. The degradation of E2-3G and E2-3S was extraordinarily rapid with half of mass lost within hours. Considerable quantities of E2-3G (7.68 ng/g) and E2-3S (4.84 ng/g) were detected at the end of the 20-d experiment, particularly for high initial concentrations. The major degradation pathway of E2-3G and E2-3S was oxidation, yielding the primary metabolites 17ß-estrone-3-glucuronide and 17ß-estrone-3-sulfate, respectively. Common metabolites were E2, the second primary metabolite, and E1, the secondary metabolite. Additionally, ring B unsaturated estrogens and their sulfate conjugates were tentatively proposed as minor metabolites. The persistence of E2-3G and E2-3S (up to 20 d) suggests that the high rate of application of conjugated estrogen-containing substances could be responsible for the frequent detection of free estrogens in surface and subsurface water.

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1. Introduction

Concern has been raised over free estrogens (estrone [E1], 17ß-estradiol [E2] and 17α-ethynylestradiol [EE2] particularly) in the past decade due to their endocrine disrupting effects on environmental biota [1,2]. Estrogens are excreted by vertebrates primarily as sulfate or glucuronide conjugates, which are biologically inactive and more water soluble than the active free estrogens [3,4]. Nevertheless, microbially-mediated deconjugation of the conjugates to liberate potent free forms makes it imperative to understand the fate, persistence and dissipation of conjugates in the environment. Studies in this regard, however, are rare and incomplete.

Human waste and livestock manure are important sources of estrogen conjugates. Up to 97% of conjugated estrogens from human waste were removed from wastewater treatment plants [5]. Estrogen conjugates tend to persist longer in concentrated animal feeding operations where they accounted for a considerable part to the total estrogen load [6]. Application of livestock manure or lagoon water to agriculture fields could be an important source of estrogen contamination [7,8]. Laboratory batch studies revealed the effective removal of estrogens biologically and physically in sediment and soils [9,10], still measurable estrogens were frequently reported in aquatic systems [11,12]. Investigators ascribed these conflicting outcomes, at least partially, to the persistence of the estrogen precursors in soil-water systems [13].

So far, a handful of studies have dealt with the sorption and degradation of conjugated estrogens in soils. For example, Scherr, Sarmah, Di and Cameron [14] discussed the degradation kinetics of 17ß-estradiol-3-sulfate (E2-3S) in response to soil type and temperature. Shrestha, Casey, Hakk, Smith and Padmanabhan [13] investigated the fate and transformation of 17ß-estradiol-3-glucuronide (E2-3G) in the aqueous phase and solid phase of soil-water slurries. However, the previous work neither investigated the two conjugates under the same condition nor had new metabolites identified. The present study thus investigated the degradation of prototype estrogen conjugates E2-3G and E2-3S in laboratory-based soil microcosms at two initial concentrations with the high concentration for the sake of metabolite identification. To accomplish this, high performance liquid chromatography—tandem mass (HPLC–MS/MS) analysis with mul-

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tiple reaction monitoring (MRM) mode was performed to quantify the target metabolites, while collision-induced dissociation (CID) mass spectrometry coupled with gas chromatography—mass spectrometry (GC–MS) were applied for the identification of unknown intermediates.

2. Materials and methods

2.1. Chemicals and materials

Analytical standards of E1, E2, estrone-3-sulfate (E1-3S) were purchased from Sigma Aldrich (St. Louis, MO, USA), while E2-3G sodium salt, estrone-3-glucuronide (E1-3G), and E2-3S sodium salt were obtained from Steraloids Inc. (Newport, RI, USA). The purity of all compounds was ≥ 98%. The standards E2-3G sodium salt and E2-3S were prepared in ultrapure water at a concentration of 200 μg/mL, which are within their aqueous solubility range [15]. The stock solutions of other compounds were prepared in methanol (MeOH) at the concentration of 1000 μg/mL. The standards were dissolved without visible powder left. Organic solvents MeOH and acetone, used for extraction, were of HPLC grade; MeOH, acetonitrile (ACN) and ultrapure water, used for mobile phase, were of LC/MS grade. An ACS grade ammonium hydroxide (NH_4OH) solution containing NH_4OH-water (28.8:71.2, v/v) and ethyl acetate were obtained from Mallinckrodt (St. Louis, MO, USA). Unless otherwise specified, the organic solvents and ultrapure water were supplied by Fisher Scientific (Fair Lawn, NJ, USA). The derivatization reagent N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Pyridine and sodium azide (NaN_3) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All glassware was baked in a muffle furnace (Fisher Scientific, Pittsburgh, PA, USA) at 400 °C for 4 h. Teflon centrifuge tubes used for extraction were autoclaved before use.

Arlington sandy loam soil, a typical agricultural soil in Riverside County, was used in the study, which was collected from fallow field at the University of California-Riverside. The moisture content of the soil was 4% based on oven drying method and the water holding capacity was 21% [16]. Field-moist soil was gently passed through a 2-mm sieve and stored at 5 °C in the dark.

2.2. Biodegradation experiment

Field soil (2.5 ± 0.1 g dry weight basis) was weighed into 10-ml pre-sterilized glass vials. The moisture of the soil was adjusted to 60% of water holding capacity by adding sterilized ultrapure water and maintained at this level throughout the experiment. The vials were covered with aluminum foil with holes in it to allow air circulation. The moisturized soil was re-acclimated in the dark at 25 °C in an incubator for 5 days prior to hormone amendment to revive microbial activity. An aliquot of 50 μL of 200 μg/mL or 2 μg/mL of E2-3G and E2-3S standard solution was spiked individually into the incubation media yielding an initial spiking concentration of 4 μg/g or 40 ng/g for each compound, respectively. Since these standards were prepared in water, microbial activities should not be influenced by organic solvent. The experimental setup was detailed in Fig. S1. Soils were sampled at different time intervals (0, 4, 8, 12, 24, 36, 72, 144, 240, and 480 h) for instrument analysis. Soils fortified with low initial concentrations (40 ng/g) were merely subject to HPLC–MS/MS analysis in MRM mode while those at the high fortification level (4 μg/g) were analyzed by HPLC–MS/MS in both MRM and scan mode. Additionally, soils sampled at the incubation time of 24, 72 and 240 h were also analyzed by GC–MS for metabolite confirmation. For the sterile controls of each treatment, soils were autoclaved thrice at 120 °C for 51 min over three successive days, followed by the addition of 10 mM NaN_3 to maintain sterility. Nonsterile soil without hormone addition was simultaneously prepared and used as matrix control. The vials were weighed each day to check for water loss, and sterilized ultrapure water was added to obtain the original weight. Each treatment was conducted in triplicate.

2.3. Extraction

At each sampling event, the incubated soil in a vial was transferred to a 50 mL Teflon centrifuge tube and extracted by shaking on a reciprocal shaker (250 rpm for 15 min) thrice with 5 mL of water-MeOH (60:40, v/v), 5 mL of water-MeOH (25:75, v/v) and 5 mL of acetone-MeOH (50:50, v/v), sequentially. The slurry was centrifuged (8000 rpm) at room temperature for 5 min. The supernatant of each extraction was decanted into 15 mL glass centrifuge tubes, dried on a Labconco RapidVap Vacuum Evaporator (Kansas, MO, USA) and re-dissolved in 1 mL of 20% MeOH in water for sole HPLC–MS/MS analysis. For samples that are directed for both HPLC–MS/MS and GC–MS analysis (Fig. S1), the extracted residue was reconstituted in 1 mL of pure MeOH and filtered through a 0.22 μm Teflon syringe filter (Fisher Scientific, Rockwood, TN, USA). An aliquot of 100 μL of the filtrate was dried, reconstituted in 20 μL ethyl acetate and derivatized by the addition of 50 μL of pyridine followed by 30 μL of BSTFA. The vial was then tightly capped and incubated in a 70 °C oven (Fisher Scientific, Pittsburgh, PA, USA) for 60 min. The reaction solution was subsequently dried under nitrogen stream and re-constituted in 100 μL of ethyl acetate for GC–MS analysis. Another aliquot of 500 μL of the remaining filtrate was dried under nitrogen gas and re-dissolved in 500 μL of 20% MeOH for HPLC–MS/MS analysis.

2.4. Instrumentation

An Agilent 1100 HPLC coupled with an Agilent 6410 triple quadrupole MS/MS system equipped with an electrospray ionization (ESI) interface was used for the determination of estrogen conjugates and their potential polar metabolites. Chromatographic separation was performed on an Agilent ZORBAX Extend-C18 (3.0 × 150 mm, 3.5 μm) column (Agilent Technologies, USA) at a flow rate of 0.35 mL/min. The column temperature was set at 40 °C. The injection volume was 20 μL. The mobile phase consisted of (A) NH_4OH-water (0.15:99.85, v/v) (pH* 9) and (B) ACN-MeOH (80:20, v/v). The gradient and instrument parameters are included in Table S1. Nitrogen gas from the nitrogen generator was used as a drying and nebulizing gas. High purity nitrogen gas (99.999% purity) was used as collision gas. Three HPLC–MS/MS modes were carried out for qualification and quantification: full scan to confirm the parent ions (pseudomolecular ions), product ion scan to monitor the CID tandem mass spectra of parent ions and MRM to confirm and quantify the potential metabolites with available authentic standards. All the spectra were acquired in negative ionization mode (ESI^-). Collection and treatment of data were performed using Mass Hunter software (Version B.01.04). Mass parameters and the method validation data of target products were presented in Table S2 [11,17]. For quality control, a procedural blank of distilled water fortified with known concentrations of target compounds was processed the same time with soil samples to check for recovery. A solvent blank was run on instrument before and after real samples to assure no carryovers of previous injection. The adjusted recovery ranged from 71.6% to 93.6%; while the method detection limit was 2.2–89.5 ng/kg soil for the six target compounds.

The GC–MS experiment was performed on an Agilent 6890 GC connected with an Agilent 5975 MSD. Separation was fulfilled on an Agilent DB-5 MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The injector temperature was set at 280 °C and the

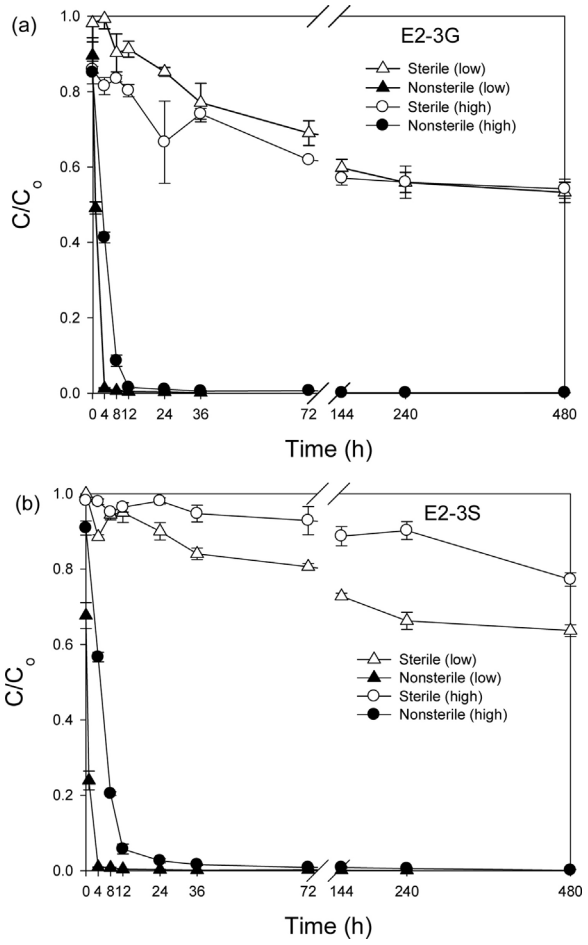

Fig. 1. Degradation curves for (a) E2-3G and (b) E2-3S at low (40 ng/g soil) and high (4 μg/g) fortification levels. *Error* bars indicate standard deviation ($n = 3$).

injection volume was 2 μL in splitless mode. The oven temperature was maintained at 80 °C for 2 min, increased to 200 °C at a rate of 30 °C/min, and then increased to 300 °C at a rate of 15 °C/min, holding for 7 min. High purity helium gas was used as the carrier gas and maintained at a constant flow of 1 mL/min. The mass spectrometer was operated in electron ionization mode with a mass scan range of 50–800 amu. The temperature of ion source, quadrupole and interface was kept at 230, 150 and 285 °C, respectively. Polar organic metabolites were converted to their trimethylsilyl (TMS) derivatives which were identified based on mass spectrum interpretation and NIST mass spectrum library search program (database version 05 provided by National Institute of Standards and Technology).

3. Results and discussion

3.1. Parent compound degradation

Degradation of parent conjugates was far faster in nonsterile soils than in sterile controls (Fig. 1), indicative of microbial degradation as the primary dissipation pathway for both conjugates. The degradation data obeys the simple first-order model and values of model-fitting parameters including half-lives ($t_{1/2}$), degradation rate constant (k), and coefficients of determination (R^2) of fitting are presented in Table 1. For soils with high initial substrate concentration (4 μg/g), concentrations of E2-3G and E2-3S diminished by more than 95% within the initial 24 h and reached a steady state after 36–72 h. Both of them persisted in soils with notable levels remaining after 20 days (Table S3). Degradation of E2-3G and E2-3S was faster for low initial concentrations with steady state reached sooner (between 4 and 8 h). The concentrations declined to less than 0.1 ng/g after 36 h (Table S3). This concentration-dependent degradation in soils was also observed for E2-3G [13] and E2-17S [18], which was attributed to enzymatic saturability. On the contrary, Chen and Hu [19] reported a trend of increasing degradation rates with the initial concentrations of E2-3G and E2-3S, arising from the stimulatory effect of higher initial substrate concentration on the microorganisms in activated sludge. These discrepancies could be ascribed to differences of microbial population densities, types and activeness in incubation media. In the current study, the observed half-lives of E2-3G were 3.05 h and 1.04 h for high and low initial concentrations, respectively, which is similar to those reported by Shrestha, Casey, Hakk, Smith and Padmanabhan [13] of 1.5–3.3 h in a topsoil, and Chen and Hu [19] of ~1 h in activated sludge (collected from a sewage treatment plant). For E2-3S, the calculated half-lives were 4.12 h and 0.67 h, respectively, for high and low initial concentrations. Scherr, Sarmah, Di and Cameron [14] reported a $t_{1/2}$ value of 0.424–1.84 h for E2-3S at 25 °C in pasture soils (Hamilton clay loam, Matawhero silt loam and Gibsons fine sandy loam), consistent with the values obtained in our study. However, a half-live of 2.5 d was also reported for E2-3S in a laboratory-based wastewater microcosm [5], in contrast to the value of ~ 1 h observed by Chen and Hu [19]. The contrasting observations could be caused by different experimental conditions controlled in their studies. The half-lives of E2-3S and E2-3G were 4.12 and 0.67 h, and 3.05 and 1.04 h, respectively, for high and low initial concentrations, suggesting that E2-3S was degraded more slowly than E2-3G for high initial concentrations, while an opposite trend was observed at low initial concentration. Higher rates of degradation for E2-3G versus E2-3S have been reported in previous studies [5,19], where 10–400 μg/L of the parent compounds were applied in aqueous samples. Our results suggested the relative persistence of sulfate to glucuronide conjugates could be concentration dependent.

Throughout the experiment, degradation of E2-3S and E2-3G occurred gradually in sterile controls with mass loss reaching ~45% and 35%, respectively, after 20 d incubation. Sorption and abiotic degradation are likely two dissipation pathways. Prior studies suggested that sorption of E2-3G to soil matrix was negligible [13] and sulfate conjugates were not readily sorbed compared to their free counterparts [20]. Therefore, the major dissipation pathway in sterile controls could be abiotic degradation, including but is not limited to metal oxide and mineral surface catalyzed degradation. The overall absence of discernable biodegradation products (E1-3G, E1-3S, E2, and E1) in the sterile control suggests that the abiotic degradation process could produce unknown metabolites which we cannot identify based on current conditions. Existing Mn or Fe oxides/hydroxides and clay minerals in soils acted as natural oxidizing agents, which catalyzed the degradation of steroid hormones primarily via hydroxylation and self-polymerization. Manganese dioxide (MnO_2), one of the strongest oxidizing agents, was reported in numerous studies to oxidize steroids via dehydrogenation and hydroxylation [20,21]. Additionally, in a previous study, E2 was effectively removed from aqueous phase via surface catalyzed oxidative oligomerization by Fe^{3+} –saturated montmorillonite [22]. It is likely the abiotic degradation produces hydroxylated and polymerized metabolites in our research which were not identified. One aspect of our future work will be focused on transformation pathways of free estrogens via mineral surface catalyzed degradation.

3.2. Hydrolysis and oxidation

Free estrogens E1 and E2 and estrogen conjugate E1-3G/E1-3S were identified to be major degradation products of the conjugates

Table 1
Degradation rate constants (k, h^{-1}) and corresponding half-lives ($t_{½}$, h) of E2-3G and E2-3S and their major primary metabolites in soils at two fortification levels.

Name	Spiking level (ng/g)	k (h^{-1})	$t_{½}$ (h)	R^2	E1-3G			E1-3S			E2		
					k' (h^{-1})	$t_{½}$ (h)	R^2	k' (h^{-1})	$t_{½}$ (h)	R^2	k' (h^{-1})	$t_{½}$ (h)	R^2
E2-3G	4000	0.226 (0.009)	3.07	0.985	0.081 (0.005)	8.56	0.529				0.051 (0.0005)	13.59	0.58
E2-3G	40	0.67 (0.055)	1.03	0.995	0.858 (0.048)	0.81	0.999						
E2-3S	4000	0.168 (0.003)	4.12	0.982				0.008 (0.0002)	86.64	0.549	0.125 (0.013)	5.54	0.89
E2-3S	40	1.044 (0.140)	0.67	0.999				0.259 (0.007)	2.68	0.965			

Note: Degradation rates of parent compounds were estimated using the first-order exponential kinetic model: $C_t/C_o = e^{-kt}$, where C_t and C_o are concentrations of parent compounds at time t (h) and time = 0, respectively, and k (h^{-1}), expressed in terms of mean (standard error), is the degradation rate constant. Degradation rates of major primary metabolites were estimated using the equation: $C_t'/C_0 = (1 - C_t/C_0)\, e^{-k't}$, where C_t' is the concentration of metabolites at time t and K' (h) is the degradation rate constant of metabolites. The value of half-life was determined by the equation: $t_{1/2} = \mathrm{Ln}2/k$. First-order kinetic model was performed based on nonlinear modeling platform of JMP Pro 11; At 0 h, the yields of parent compounds were not fully recovered because of partial degradation during the course of extraction.

by HPLC–MS/MS based on their MRM transition and retention time, which is consistent with their authentic standards. The formation of the two free estrogens was further verified by GC–MS according to the analysis of their mass spectra of the derivatized extracts and authentic standards (Fig. S3).

Fig. 2 depicts the concomitant formation of the major metabolites as a ratio to initial application of hormone conjugates in all the experiment treatments. The initial degradation of E2-3G was accompanied by formation of the primary metabolites, E1-3G and E2, the maximum percentage of which peaked at 1 h for low initial concentration (Fig. 2a). After arriving at its maximum ratio (0.32) at 1 h, E1-3G degraded rapidly with a ratio <0.01 remaining after 4 h and was below detection limit after 72 h. The second primary metabolite E2, after reaching its maximum ratio at 1 h (0.0023), disappeared completely after 4 h. However, there was some broadening and late arrival of the metabolite peaks (~4–8 h) for high initial concentration (Fig. 2b), with detectable concentration of E1-3G and E2 remaining, albeit at trace level (~1.9 and 2.7 ng/g, respectively; Table S4), until 20 d of incubation. The comparison suggested longer persistence of E2-3G would be expected (>20 d) when the applied concentrations of the parent compounds are high. The results coincide with the observations by Shrestha, Casey, Hakk, Smith and Padmanabhan [13] who showed E2-3G (initial concentrations are 3.7, 9.1 and 22.5 μg/mL) persisted in the aqueous phase for up to 28 d. The trend in the formation and dissipation of E1 for low initial concentration resembled that for high initial concentration. The formation of E1 was right accompanied by the dissipation of E1-3G and E2, suggestive of the conversion of E1-3G and E2 into E1. Previous studies have demonstrated that E2 degraded into E1 in soils [23] and that estrogen conjugates such as E1-3G and E1-3S deconjugated to liberate their free counterparts in wastewater treatment plants [5]. Likewise, E2-3S primarily degraded to E1-3S and further to E1 and the dynamic of metabolite formation was in analogy with E2-3G dynamic for low initial concentration (Fig. 2c). For high initial concentration (Fig. 2d), the peak of E1-3S and E1 occurred later (around 24 h and 36 h, respectively) and broader than that of corresponding metabolites of E2-3G, which is in accordance with the faster decay of E2-3G as indicated by the half-lives. E2 concentrations were either below detection limits or extraordinarily low if detected (ratio <0.003), probably because of its fast oxidation to E1. Generally, the primary metabolites persisted longer than their parent compounds with the exception of the case in which E2-3G was fortified at low concentration (Table 1). The predominant detection of E1-3S or E1-3G over E2 suggested that oxidation was the primary degradation pathway of E2-3G and, in particular, E2-3S, while hydrolysis was secondary, in line with the previous studies on E2-3S degradation [14]. However, Shrestha, Casey, Hakk, Smith and Padmanabhan [13] regarded hydrolysis as the primary dissipation process of E2-3G, and oxidation secondary. One possible explanation for the disparities was our soil microcosms were more aerated than the slurries involved in that study.

3.3. Proposed degradation pathways for E2-3G and E2-3S

In addition to E1, E2, and E1-3G (Fig. S2), GC–MS total ion chromatograms (TIC) suggested there is a fourth degradation product of E2-3G (Peak B in Fig. S3) which was tentatively identified to be Δ-^{9,11}-dehydroestrone (DHES) using NIST 05 database searching program. The metabolite Δ-^{9,11}-DHES should have a parent ion at m/z 267 ($[M\text{-}H]^-$) based on HPLC-ESI–MS/MS analysis. However, three metabolites with a parent ion at m/z 267 were present on the TIC and extracted ion chromatograms (EIC) at 9.81, 13.016 and 17.65 min, respectively (Fig. S2a & S4a). In order to interpret the structures of these metabolites, a collision energy (CE) of 20 eV was applied in the collision cell (performed in tandem mass product ion scan mode) to obtain the CID tandem mass spectra of the parent ion at m/z 267 (Fig. S4). The great similarity of CID spectra among the three metabolites suggested that the other two metabolites could have similar chemical structure with that of Δ-^{9,11}-DHES. Since the CID fragmentation pathways are consistent with that of Δ-^{8,9}-DHES and different from that of equilin [24], the three metabolites were tentatively proposed to be the B ring unsaturated estrogens, Δ-^{8,9}-DHES, Δ-^{9,11}-DHES and 6,7-DHES, and their CID fragmentation pathways are proposed in Fig. S5. The proposed metabolites (Δ-^{8,9}-DHES, Δ-^{9,11}-DHES and 6,7-DHES) share one similar characteristics, i.e, having a double bond conjugated with the aromatic ring, which possibly accounts for the similar fragmentation pathways of them. Without standard, the yields of the three metabolites were estimated based on the peak abundance of their parent ions assuming the instrument condition was constant throughout the experiment (Fig. S6). The yields of the three metabolites were suggested to peak after 24-h of incubation via the investigations of soils fortified with 4 μg/g of E2-3G. Since no glucuronide conjugates of the three metabolites were found at each sampling event as confirmed for the absence of corresponding pseudomolecular ions at m/z 443 (addition of glucuronide moiety, i.e. $[M-H+C_6O_6H_8]^-$) by the HPLC–MS/MS with scan mode, the three metabolites could be converted from E2 or E1 rather than from their corresponding conjugates via hydrolysis. Mizuguchi, Sadaka, Ogasawara and Shimada [25] and Havlikova, Novakova, Matysova, Sicha and Solich [26] have suggested the existence of the intermediate, Δ-^{9,11}-DHES, during the degradation of E1 or E2.

For E2-3S, three degradation products were found and two (E1-3S and E1) (Fig. S7) were identified by GC–MS and HPLC–MS/MS scan methods. The HPLC–MS/MS scan analysis revealed the presence of Product A with a parent ion at m/z 347 (pseudomolecular ion, $[M\text{-}H]^-$). The formation of the fragment ion at m/z 267 (Fig. S7), due to loss of the sulfate moiety ($[M\text{-}HSO_3]^-$), suggests Product A could be converted from E2-3S or E1-3S via dehydrogenation. The CID mass spectrum at the collision energy of 50 eV matches that of the three metabolites of E2-3G (Fig. S4 and S7), suggesting that Product A could be the sulfate conjugates of Δ-^{8,9}-DHES, Δ-^{9,11}-DHES or 6,7-DHES and the highest yield was observed around 8–24 h incubation (Fig. S8), paralleled by E1-3S. The yields of Δ-

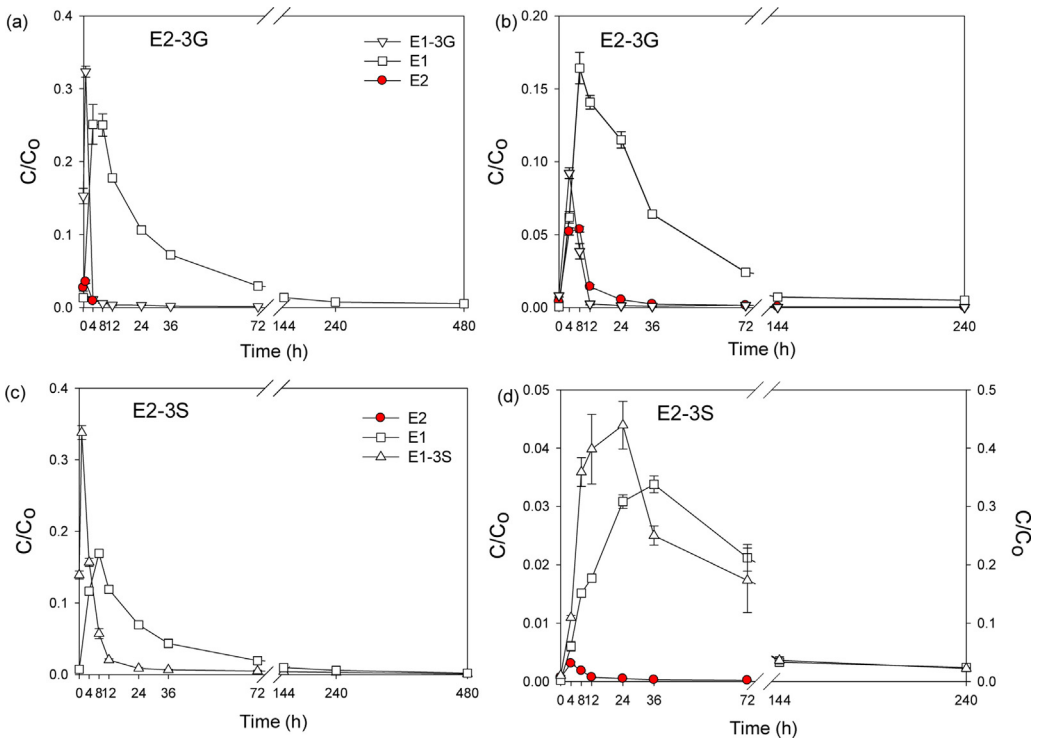

Fig. 2. Metabolite formation of E2-3G at (a) low and (b) high fortification levels and E2-3S at (c) low and (d) high fortification levels. *Error* bars indicate standard deviation ($n = 3$). Scale on the right axis applies to E1-3S for subfigure (d).

^{8,9}-DHES, Δ-^{9,11}-DHES or 6,7-DHES, however, were much lower in soils incubated with E2-3S relative to E2-3G, suggesting formation of them via deconjugation or dehydrogenation was minor.

On the basis of the degradation products identified, the degradation pathways of E2-3G and E2-3S are tentatively proposed in Fig. 3. Apparently, deconjugation (hydrolysis) and oxidation (majorly dehydrogenation) are the two major biodegradation mechanisms for the degradation of E2-3G and E2-3S in soils. The oxidation products E1-3G or E1-3S were identified as the major and E2 as the second primary metabolites based on their maximum occurrence. This suggests that the oxidation at position 17 of the D ring occurred to a greater extent than the hydrolysis of the thio-ester at position 3. Hydrolysis of E1-3G or E1-3S at position 3, as well as oxidation of E2 at position 17, both lead to the formation of E1 which was further degraded via ring cleavage and entering tricarboxylic acid cycle [27].

3.4. Environmental implications

Based on the results from the present study, E2-3G or E2-3S at higher initial concentrations (4 μg/g) are expected to be more recalcitrant to biological degradation with detectable amount persisted in soils after 20-d of incubation. The direct application of manures or wastewater from concentrated animal feeding operations to soils results in large loadings of estrogen conjugates, which, though, do not possess endocrine disrupting effect per se, they could contribute significant amount of free estrogens into the environment, posing a potential risk to aquatic life. The results from the current study suggested that the long persistence of E2-3G and E2-3S (up to 20 d) increase its potential mobility and could be partially responsible for the frequent detection of free estrogens in the surface and subsurface water after manure slurry is applied. Moreover, newly proposed metabolites, the ring B unsaturated estrogens and their sulfate esters, have potent estrogenic effects [28], with Δ-8,9-DHES, for instance, exhibiting high binding affinities in comparison to E1 to estrogen receptors [29]. These newly proposed metabolites could express biologic effects in soil environment by mechanisms similar to those described for the classic estrogens. In addition, soils in the current study were controlled well under an aerated condition which favors the biodegradation of the applied parent compounds. Under field conditions, however, soils may not be fully aerated and the moisture may not arrive at its optimum level, thus the residence time of parent compounds and the metabolites could be further prolonged in relative to that in lab conditions. Further research is required to understand the persistence of those conjugates and their degradation products in field and aquatic environments.

4. Conclusions

This work described the microbial degradation mechanisms of the prototype estrogen conjugates E2-3G and E2-3S in laboratory-based soil microcosms. The degradation process obeys the first-order kinetics. The degradation rates of the conjugates were extraordinarily rapid with half of mass lost within hours. The conjugate exhibited a higher degradation rate at low initial concentrations than at high initial concentrations. Oxidation and hydrolysis are two major degradation mechanisms for E2-3G and E2-3S in soil microcosms. In addition to the widely accepted degradation products, several new metabolites, namely ring B unsaturated estrogens and their sulfate conjugates, were tentatively proposed as minor metabolites which exhibited estrogenic potencies even as high as E1 does, leading to an increasing biological potency of soil environment as degradation occurs. One thing worth noting is that each extracted compound was a sum in the aqueous and reversibly-bound phase, while irreversibly sorbed fraction was not included, so a mass balance result in the three phases cannot be obtained due to the absence of isotope labelled compounds and the

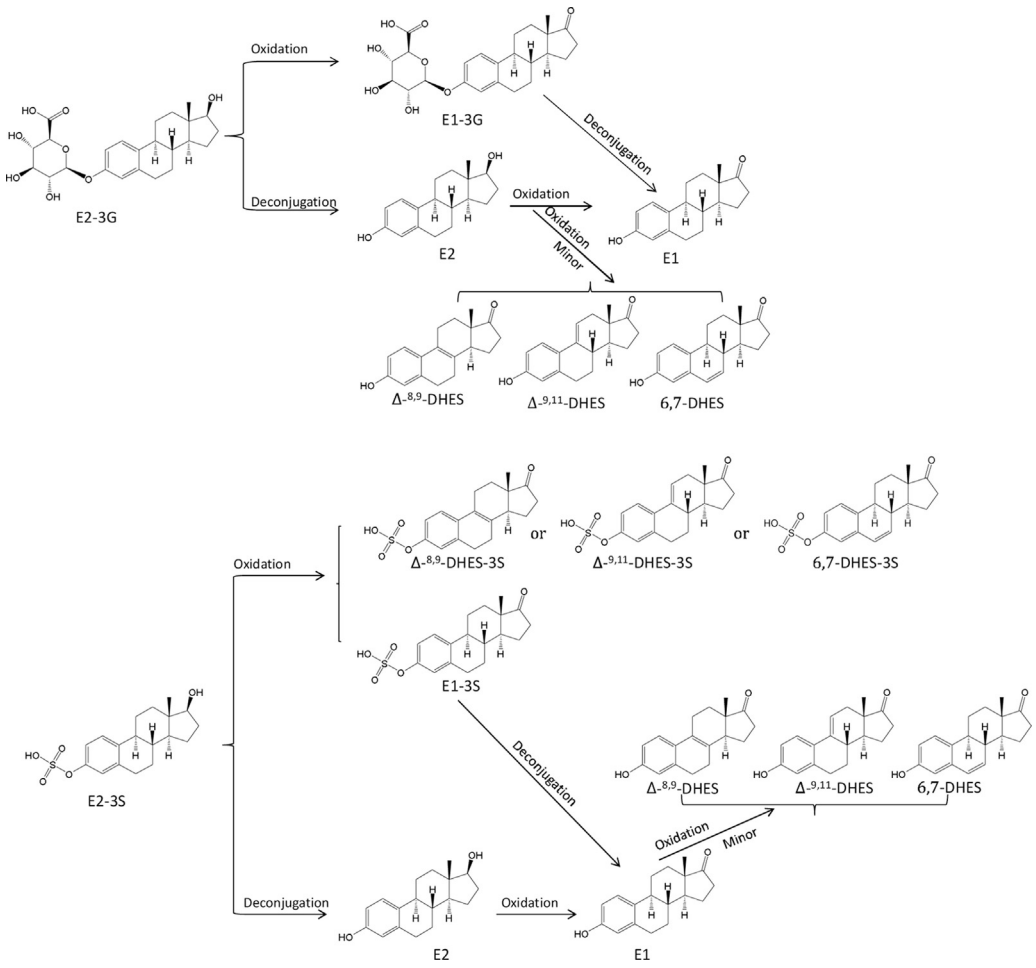

Fig. 3. Tentatively proposed degradation pathways of E2-3G and E2-3S.

corresponding detection equipment in our lab. Future work needs to address the fate and transport of estrogen conjugates under field conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2017.07.058.

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