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Degradation and metabolite formation of 17ß-estradiol-3-glucuronide and 17ß-estradiol-3-sulphate in river water and sediment

Li Ma [a, b], Scott R. Yates [b, *]

[a] Department of Environmental Sciences, University of California, Riverside, CA 92521, United States
[b] Contaminant Fate and Transport Unit, Salinity Laboratory, Agricultural Research Service, United States Department of Agriculture, Riverside, CA 92507, United States

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ABSTRACT

Laboratory degradation tests with two model estrogen conjugates, 17ß-estradiol-3-glucuronide (E2-3G) and 17ß-estradiol-3-sulphate (E2-3S), using river water and sediment as inoculum under aerobic conditions were investigated. Throughout the 14-day incubation, degradation of E2-3G in river water, at environmentally-relevant level (25 ng/L), obeyed first-order kinetics with the formation of 17-ß estradiol and estrone; in contrast, E2-3S was slowly converted to estrone-3-sulphate stoichiometrically. Degradation of the two conjugates across the spiking concentrations (0.01−1 μg/g) was much faster in sediment than in river water where 25 ng/L of conjugate standards were spiked, possibly due to relatively high population densities of microorganisms in sediment. De-conjugation of the thio-ester bond at C-3 position and oxidation at C-17 position were the predominant degradation mechanisms for E2-3G and E2-3S, respectively, with negligible presence of metabolites estrone-3-glucuronide for E2-3G and 17ß-estradiol for E2-3S. In addition, delta-9(11)-dehydroestrone and 6-ketoestrone were determined as new metabolites of the two conjugates. Also, a lactone compound, hydroxylated estrone and a few sulfate conjugates were tentatively identified. With the observation of new metabolites, biodegradation pathways of E2-3G and E2-3S were proposed. The formation of new metabolites may pose unknown risks to aquatic biota.

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1. Introduction

Estrogens are potent endocrine disrupting compounds which could exert adverse effects on aquatic wildlife at environmentally-relevant ng/L level, including intersexuality, feminization, and reproductive and behavioral problems (Jobling et al. 1998, 2006; Kidd et al., 2007; Pelissero et al., 1993). Estrogens exhibit up to thousands times higher estrogenic potency than other major pharmaceuticals and personal care products as reflected by their predicted no-effect concentration values (Li et al., 2013) or the yeast estrogen screen results (Beek et al., 2006; Yost et al., 2013). Estrogens were frequently detected at low concentrations in the range of ng/L to μg/L in surface waters (Kolpin et al., 2002; Belfroid et al., 1999; Khanal et al., 2006; Ma et al., 2016; Gorga et al., 2015). Consequently, investigation of estrogen contamination has drawn worldwide attention in the past few decades. The routine administration of sex hormones to human and livestock as medicine leads to large estrogen loadings to environment. It has been revealed that estrogen hormones were excreted in significant amounts from vertebrates in biologically inactive conjugates (D'Ascenzo et al., 2003; Hanselman et al., 2003). Estrogen sulfate or glucuronide conjugation at C-3 and C-17 position of the basic parent estrogen structure commonly exist in the environment. The sulfate or glucuronide moieties make the estrogen conjugates more water soluble and mobile, posing an environmental risk once they are hydrolyzed to biologically active free estrogens (Khanal et al., 2006; Pinto et al., 2014; Nazari and Suja, 2016).

Estrogen conjugates enter the aqueous environment through sewage discharge and animal waste disposal. A substantial portion can be converted back to their parent forms somewhere before and/or after entering sewage treatment plants (STPs) (Kumar et al., 2012; Liu et al., 2015). Arylsulfatase and ß-glucuronidase enzymes synthesized by *Escherichia coli* were suggested to be responsible for the de-conjugation of estrogen sulfate and glucuronide, respectively (Ternes et al., 1999; Duong et al., 2011). Estrogen conjugates normally occurred in wastewater treatment

* Corresponding author.
E-mail address: scott.yates@ars.usda.gov (S.R. Yates).

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effluents at up to several ng/L with estrogen sulfate outweighing glucuronide (Ma et al., 2016; Ben et al., 2017; Naldi et al., 2016). Animal waste disposal could cause considerable conjugate loadings to surface water considering one third of total estrogens in waste holding ponds of concentrated animal feeding operations were detected as conjugates (Hutchins et al., 2007). For example, up to 26.8 ng/L of 17ß-estradiol-3-sulphate (E2-3S) was observed in an agricultural watershed receiving poultry litter (Dutta et al., 2012). Estrogen conjugates that survive decomposition or bypass STP treatment enter river water and could liberate free estrogens and various intermediates with blurry biological activity, leading to potential risks following water reuse. However, the majority of studies on estrogen conjugates to date have focused on their fate and degradation behavior based on laboratory batch studies using soil or activated sludge as incubation media (Chen and Hu, 2009; Scherr et al. 2008, 2009; Shrestha et al., 2012; Bai et al., 2013) or in aqueous media on their occurrence and removal efficiency (Ma et al., 2016; D'Ascenzo et al., 2003; Reddy et al., 2005; Griffith et al., 2014; Kumar et al., 2011). In contrast, relatively little is known about their degradation behavior and transformative pathways in river water environment.

The main goal of the study was to investigate the degradation of two model conjugates,17ß-estradiol-3-glucuronide (E2-3G) and E2-3S, and their transformation products in river water and sediment. The degradation behavior of the two conjugates was investigated in river water at environmentally-relevant (ng/L) level and in sediment at three amendment levels. Finally, degradation mechanisms were proposed. Electrospray collision-induced dissociation (CID) tandem mass spectrometry together with gas chromatography—mass spectrometry (GC-MS) were used for structure elucidation of metabolites at a specific degradation time. Knowledge on degradation kinetics and the formation of microbial metabolites is important for assessing the environmental behavior of estrogen conjugates.

2. Materials and methods

2.1. Chemicals

Analytical standards E2-3G sodium salt, E1, 17ß-estradiol (E2), and E1-3S were bought from Sigma-Aldrich (USA), while E2-3S sodium salt, estrone-3-glucuronide (E1-3G), delta-9(11)-dehydroestrone (9,11-dehydro-E1), 8,9-dehydro-E1 and 6-ketoestrone (6-keto-E1) were purchased from Steraloids Inc. (Newport, RI). The purity of all standards was $\geq$ 98%. The individual stock standard solution was prepared at the concentration of 1000 μg/mL by dissolving 10 mg of each standard in 10 mL of methanol (MeOH). High performance liquid chromatography-mass spectrometry (HPLC-MS) grade MeOH, acetonitrile (ACN) and ultrapure water were obtained from Fisher Scientific (Fair Lawn, NJ). ACS grade ammonium hydroxide solution (NH_4OH, 28.8%, v/v) and ethyl acetate were obtained from Mallinckrodt (St. Louis, MO). The derivatization reagent N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was supplied by Sigma-Aldrich and pyridine by Fisher Scientific.

2.2. Experimental setup

Bulk water samples (dissolved organic carbon around 5.0 mg/L and pH 6.5) from Santa Ana River were collected on a sunny day of February, 2017. Collections were made at a site (33°58′12.4″ N, 117°29′18.2″ W) ~ 2.3 km downstream of a major STP. Sediment from the same site (with majority of coarse sand dotted with visible black organic substances) was collected on two occasions (April 2017 and June 2017). Water samples at surface (0–15 cm) were collected in 4-L amber solvent bottles and transported to the lab and used within 1 day of collection, while the top 5 cm of sediments were sampled in amber jars, passed through a 2-mm sieve, and used within 48 h.

The aerobic degradation potential of E2-3G and E2-3S in river water was measured at the concentration of 25 ng/L. To minimize the effect of solvent, 1 mL of 1 μg/mL conjugate standard solution in MeOH was air dried and reconstituted in 1 mL ultrapure water. Then 100 μL of the 1 μg/mL MeOH free standard solution of test substance was amended separately to 4-L fresh river water filled in the solvent bottle. Then the fortified river water was mixed thoroughly. The headspace in the bottle was aerated at regular sampling event. Autoclaved river water (90 min, 120°C) fortified with the same concentration of test substances together was used as sterile control. Fresh river water samples without fortification were taken as matrix control for background subtraction. The aerobic degradation of estrogen conjugates was evaluated via the incubation of samples in darkness in a temperature controlled room (20 ± 3°C) for consecutive 14 days. Duplicate samples were sacrificed at 0, 1, 2, 4, 7, 9, 11, and 14 d after fortification while the sterile bottles were only sampled at 0, 1, 7, and 14 d. Fig. S1 depicted the setup of the aerobic degradation study in river water. At each sampling event, 0.5-L water was taken from each bottle and filtered with a glass-fiber filter (GF/F, 0.7 μm, Whatman) to remove suspended solids. The target compounds were concentrated immediately by passing the filtrate through Waters Oasis HLB solid-phase extraction cartridges (6 cc, 200 mg, Waters) which were preconditioned with 6-mL MeOH and 6-mL ultrapure water. After the sample loading, the cartridges were rinsed and eluted following the procedure described elsewhere (Ma et al., 2016). The eluents were evaporated on a Labconco RapidVap Vacuum Evaporator (Kansas, MO) and the residues were re-dissolved in 0.5 mL of 20% MeOH in water and filtered through 0.22 μm Teflon syringe filter (Fisher Scientific) for HPLC-MS/MS analysis in multiple reaction monitoring (MRM) mode. The achieved method detection limit and recovery of target compounds performed on sterile samples ranged 0.01–0.37 ng/L and 71.6–98.5%, respectively (Table S1).

Biodegradation of estrogen conjugates in sediment was evaluated at three concentration levels under aerobic condition. Specifically, 5 g of fresh sediment and 2 mL of river water from the same site (sampled in April 2017) were added to a 20-mL EPA vial to make slurry, where 50 μL of 1, 10 and 100 μg/mL of MeOH free E2-3G or E2-3S was added separately to give a nominal concentration of 0.01, 0.1 and 1 μg/g (wet weight basis) for each compound in fresh sediment. After the fortification, the lid was tightened and the vial was vortex mixed and put in a 20 ± 1°C incubator for 14 days. The large headspace above the slurry ensured aerobic condition. Autoclaved sediments (120 °C for 51 min) at the highest fortification level served as sterile control while unfortified fresh sediments were used as matrix control. Samples were taken at 0, 1, 2, 4, 6, 9 and 14 d (Fig. S2). In addition, samples at the incubation time of 2, 4 or 8 h were appended to the existing sampling event dependent on the fortification level. Sterile controls were only sampled at 0, 48, 216 and 336 h. At each sampling event, two vials were sacrificed from each treatment. The slurry was microwave (CEM MARS, Matthews, NC, USA) extracted twice with 5 mL of 75% MeOH (60°C for 10 min; 600 W). The extracts were combined and dried on a Labconco RapidVap vacuum evaporator and re-dissolved in 0.5 mL of 20% MeOH for HPLC-MSMS analysis in MRM mode. The achieved method detection limit and recovery of target compounds performed on sterile samples ranged 0.01–0.46 ng/g and 72.4–93.3%, respectively (Table S1).

To monitor the degradation metabolites, conjugates were fortified separately in the sediment slurry (sampled in June 2017) at an initial concentration of 10 μg/g (wet weight basis). Sterile control

and matrix control were also included. Duplicate samples were taken at 2, 8, 24, 48, 72, and 144 h of incubation (20 ± 1°C) and extracted the same way as described in the previous paragraph. The dried extract residue was reconstituted in 1 mL of MeOH and filtered through 0.22 μm Teflon syringe filter. One aliquot of 200 μL of the filtrate was dried under nitrogen gas and re-dissolved in 200 μL of 20% MeOH for HPLC-MS/MS analysis (in full scan and product ion scan mode). Another aliquot of 200 μL of the filtrate was dried, reconstituted in 20 μL ethyl acetate and derivatized by the addition of 50 μL of pyridine followed by 50 μL of BSTFA. The vials were then tightly capped and incubated in a 70°C oven for 60 min. The reaction solution was subsequently dried under nitrogen stream and reconstituted in 100 μL of ethyl acetate for GC-MS analysis.

2.3. Instrument analysis

An Agilent 1100/6410 triple quadrupole LC-MS/MS system equipped with an electrospray ionization (ESI) interface was used for the qualitative and quantitative analysis. Separation was performed on an Agilent ZORBAX Extend-C18 column (3.0 × 150 mm, 3.5 μm). The column was thermostated to 40°C. The flow rate and injection volume was 0.35 mL/min and 20 μL, respectively. The mobile phase consists of (A) 0.15% (v/v, pH ~ 9) NH_4OH in water and (B) ACN/MeOH (80/20, v/v). The programed gradient and instrument parameters were displayed in Table S2. Nitrogen gas from the nitrogen generator was used as drying and nebulizer gas while high purity nitrogen gas (purity ≥ 99.999) was applied as collision gas. Quantification of target compounds was performed on LC-MS/MS in MRM mode. To help identify the potential degradation products, full scan chromatograms were acquired to confirm the precursor ions (pseudomolecular ions), while the ensuing product ion scan spectra of the precursor ion were obtained via an application of different collision energy in the collision cell. The data was collected in negative ionization mode. To assist auxiliary identification, UV chromatograms were simultaneously recorded at 242 nm.

An Agilent 6890 GC equipped with a 5975 MS detector was used to identify potential metabolites. The instrument was equipped with an Agilent DB-5 MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). Two μL of sample was injected in splitless mode at 280°C. Helium gas was used as the carrier gas and the flow rate was maintained constant at 1 mL/min. The oven temperature was kept at 80 °C for 2 min, programmed from 80 to 200°C at 30°C/min, and then increased to 300°C at 15°C/min, holding for 7 min. The total run time was 20 min. The mass spectra were scanned from m/z 50 to 800 at a rate of 1.5 scans per second. The temperature of ion source, quadrupole and interface was set to 230, 150 and 285°C, respectively. Polar organic metabolites were converted to their trimethylsilyl (TMS, m/z 73.0) derivatives which were identified based on National Institute of Standards and Technology (NIST) 14 mass spectrum library searching program and mass spectrum interpretation.

3. Results and discussion

3.1. Aerobic degradation in river water

The time course for E2-3G and E2-3S degradation in river water is illustrated in Fig. 1. Comparison of their degradation trend against sterile controls suggests that biodegradation is mainly responsible for the loss of estrogen conjugates. Degradation of E2-3G obeys simple first-order exponential kinetic model. The calculated degradation rate constant (k) and half-life ($t_{1/2}$) were 0.0355 h^{-1} and 18 h, respectively. Kumar et al. (2012) also recorded a similar

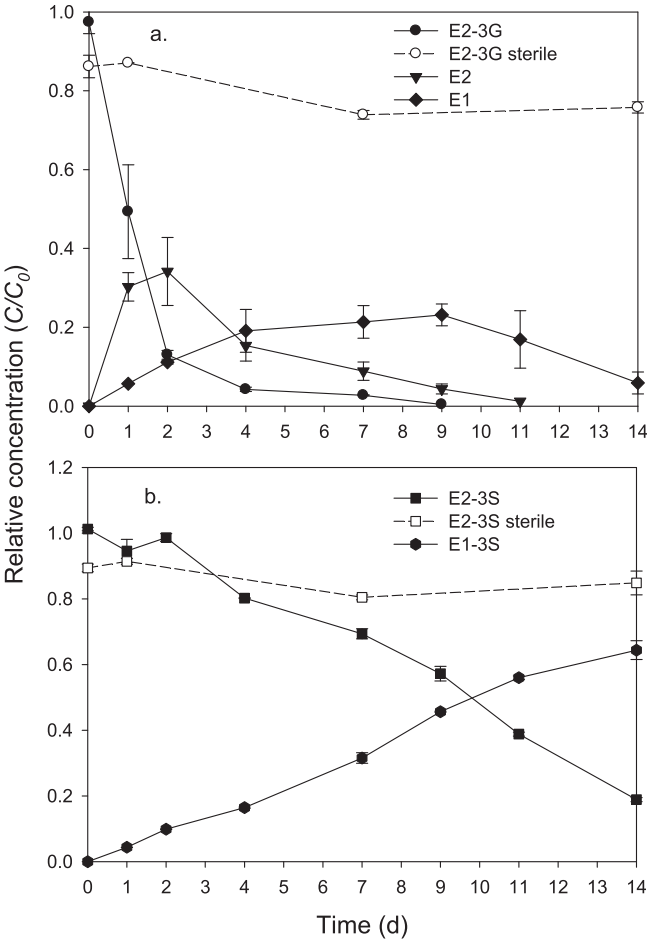

Fig. 1. Biodegradation and metabolite formation of E2-3G (a) and E2-3S (b) in river water at the initial concentration of 25 ng/L. Data points and error bars represent means and standard deviations of duplicate. The relative concentration expressed as C/C_0 was the concentration (ng/L) ratio of target compounds at different sampling event time to that at the initial time.

half-life of 15.4 h for E2-3G (370 ng/L) in incubated river water. About half of fortified E2-3G was removed within the first day and was non-detectable after 9 days of incubation, suggesting that, even at the environmentally relevant ng/L level, E2-3G can still be transformed. The loss of E2-3G was accompanied by the concomitant formation of the primary degradation product E2, which was further oxidized to E1. The maximum percentage of E2-3G converted to E2 was 34% (equal to 56% in molar percent). Estrone approached a plateau accounting for ~20% over the period of 4–9 d. The absence of E1-3G suggests that the dominant degradation pathway for E2-3G under the present conditions was hydrolysis of the thio-ester bond at the C-3 position rather than oxidation at C-17 position.

Degradation of E2-3S was much slower than E2-3G and the removal of E2-3S was concurrent with the sole detection of E1-3S (Fig. 1b), with ~19% of E2-3S remaining after 14 days. The molar percentages (similar to weight percentages) of E2-3S and E1-3S summed to around unity, and remained unchanged for 9 days, implying a stoichiometric conversion of E2-3S to E1-3S initially; the two represented 83% of the original conjugates after 14 days. To our knowledge, this is likely the first study of degradation of E2-3S in river water, at such low levels, therefore hardly any other finding with this regard can be used for comparison. However, the stoichiometric oxidation at the C-17 site for E2-3S was also observed

for E2 as investigated by Yu et al. (2007) who ascribed the pattern to the inability of the E2 degraders to catalyze the metabolite E1. While based on the previous results in a E2-3S degradation study that as arylsulphatase (i.e., arylsulfatase) was inhibited, the formation and persistence of the major metabolite E1-3S were more pronounced (Scherr et al., 2009), the slow degradation and accumulation of E1-3S in our study were expected since arylsulfatase in river water would be at insufficient levels. In contrast to E2-3G, E2-3S underwent a 2-day lag phase, indicative that acclimation was necessary for the micro-organisms to adapt before degradation began. Similar result was reported by D'Ascenzo et al. (2003), who investigated the degradation behavior of E2-3S at an initial concentration of 25 μg/L in domestic wastewater, and also observed a 10 h of lag phase prior to progressive biodegradation. After the lag phase, degradation of E2-3S generally followed a linear trend. Other sulfate conjugate such as 17α-estradiol-3-sulfate (Zheng et al., 2013) in diluted dairy wastewater also displayed similar degradation profile aerobically. These results are in line with the incomplete transformation of sulfate conjugate in STPs (Kumar et al., 2012) and its frequent detection in aqueous environments (Ma et al., 2016; Isobe et al., 2003).

3.2. Aerobic degradation in sediment

As illustrated in Fig. 2, the rapid depletion in the nonsterile treatments and the lack of abiotic loss in the sterile controls suggest that sorption to sediment is minimum while microorganisms play a key role in the dissipation of E2-3G and E2-3S in sediment. The data all fit well to a simple first-order kinetic model for three amendment levels and corresponding model parameters are presented in Table 1.

The half-lives (Table 1) ranged, respectively, 0.81–3.94 h for E2-3G and 3.11–22.36 h for E2-3S (Table 1), which are within the range reported in raw sewage (Kumar et al., 2012) and in soils (Scherr et al., 2009; Shrestha et al., 2012). One conspicuous finding is as the initial concentration of E2-3G or E2-3S increased, k value decreased (Table 1). Such degradation trend was also reported before for E2-3G and E2-3S in agricultural soils (soil slurries or moist soil) (Shrestha et al., 2012; Ma and Yates, 2017) and activated sludge (ng/mL to μg/mL range) (Ternes et al., 1999). Elsewhere, the inversed trend was proposed by Marcus and Talalay (1955) to be explained by the formation of nonactivated biomolecular complexes (enzyme-substrate complex involving two substrate molecules) when degradation of testosterone and related androgens by a purified bacteria enzyme was investigated. Conversely, Chen and Hu (2009) observed an increasing k value with increasing initial concentrations of E2-3G and E2-3S (10–400 μg/L) in sludge slurries. Apart from the stimulatory effect of high initial substrate concentration on the microorganisms, the researchers provided a possible reason that the degradation intermediates of the conjugates did not compete with amended compound itself for binding with degraders; because the activated sludge with relatively high concentration of mixed liquor suspended solids in the study would carry large bacterial population and result in effective degradation of the conjugates and their metabolites even at high initial concentration. The explanation can be supported by the findings in another study that the first-order rate constant (k) of E2-3S was significantly correlated to soil organic carbon content ($r \geq 0.978$, $p < 0.1$) and to soil microbial biomass carbon ($r \geq 0.995$, $p < 0.05$) (Scherr et al., 2009). In our case, the conjugates and their metabolites serving as carbon source could be saturated for microbiota in the sandy sediment.

Major degradation products of the two conjugates were displayed in subfigures of Fig. 2. As initial concentration increased, there was an overall increase, some broadening and late arrival of the metabolite peaks, a further hint that the metabolites together with their amended compounds as substrate could be saturated for the microbiota. Additionally, degradation rate of the conjugates and their major metabolites at the medium initial level (0.1 μg/g) was close to that at the lowest level (0.01 μg/g), while the difference gap widened as concentration scale become larger (1 μg/g). The finding is comparable to the previous observation that between 0.03 and 10 μg/L estrogen was degraded at similar rates in sewage while the rate was obviously slowed when estrogen concentration reached 100 μg/L (Xu et al., 2009). This was ascribed by the investigator to the possible availability of a key cometabolite at 100 μg/L concentration limiting estrogen removal. The figure also indicates that the metabolites, at the highest initial concentration in particular, persisted longer with certain amount detected at the end of the incubation. The conversion of E2-3G to E2 was not complete as E2 accounted for 6–17% (approximate 8–22% molar percent) of original fortified conjugate at the highest point. Earlier, ~6% formation of E2 from E2-3G was reported in incubated surface soils (Shrestha et al., 2012; Ma and Yates, 2017). Similar to the case in river water, no discernable proportion of E1-3G was detected (Fig. S3). Nevertheless, the absence of E1-3G seems counter to intuition given a wide range of bacteria was responsible for oxidation of E2 to E1 at C-17 position (Yu et al., 2007; Colucci et al., 2001; Jiang et al., 2010; Tanaka et al., 2009). Moreover, prior batch studies of E2-3G degradation in soils observed considerable quantities of E1-3G (Shrestha et al., 2012). This observation suggests that transformation mechanisms of estrogen conjugates in river water could be different from other compartments possibly due to differences of microbial population densities, types and activeness as well as the dissolved oxygen content in the incubation media. The proportions of E1-3S converted from E2-3S accounted for 33–100% of the applied E2-3S, similar to the range of 55–100% reported by Scherr et al. (2009) in a soil microcosm study. Since the formation of E2 was trivial (<0.3%, Fig. S4), the secondary metabolite E1 (<8%) was thus converted from E1-3S. The low proportions of E1 and E2 in the present study as metabolites of E2-3S are congruent with a prior batch study with raw sewage and river water (Kumar et al., 2012), indicative oxidation as the predominant transformation pathway of E2-3S in river sediment. Another noticeable finding is the maximum proportion of E1 decreased as the initial concentration of fortified E2-3S rose, suggested that other transformation pathways become more important than de-conjugation when E1-3S as substrate is saturated. The formation of new metabolites of E1-3S with high level (10 μg/g) of E2-3S amended discussed in the next section provides certain evidence for this. If this were the case, degradation of E1-3S may not simply form E1, in particular when the initial concentration is high, while other metabolites might need to be calculated for modelling and risk assessment.

3.3. New metabolite formation

In addition to the abovementioned general metabolites, microbial assisted degradation of E2-3G in the sediment leads to four new products as indicated on the GC-MS chromatograms (Fig. 3a). These degradation metabolites are labeled herein asI,II, III and IV. The mass spectra of the new products were subject to NIST library searching. By comparison, TMS derivatives of products IIand III exhibited highly similar fragment patterns to that of 9,11-dehydro-E1 and 6-keto-E1, respectively (Fig. S5). And the TMS derivatives of the authentic standards also showed identical retention times and spectra (figure not shown). ProductsIIand III were, thus, determined to be 9,11-dehydro-E1 and 6-keto-E1, respectively. With respect to the TMS derivatives of productI(molecular ion at m/z 430) and IV (molecular ion at m/z 358), no corresponding TMS derivatives with similar mass spectra in the library were found. However, their

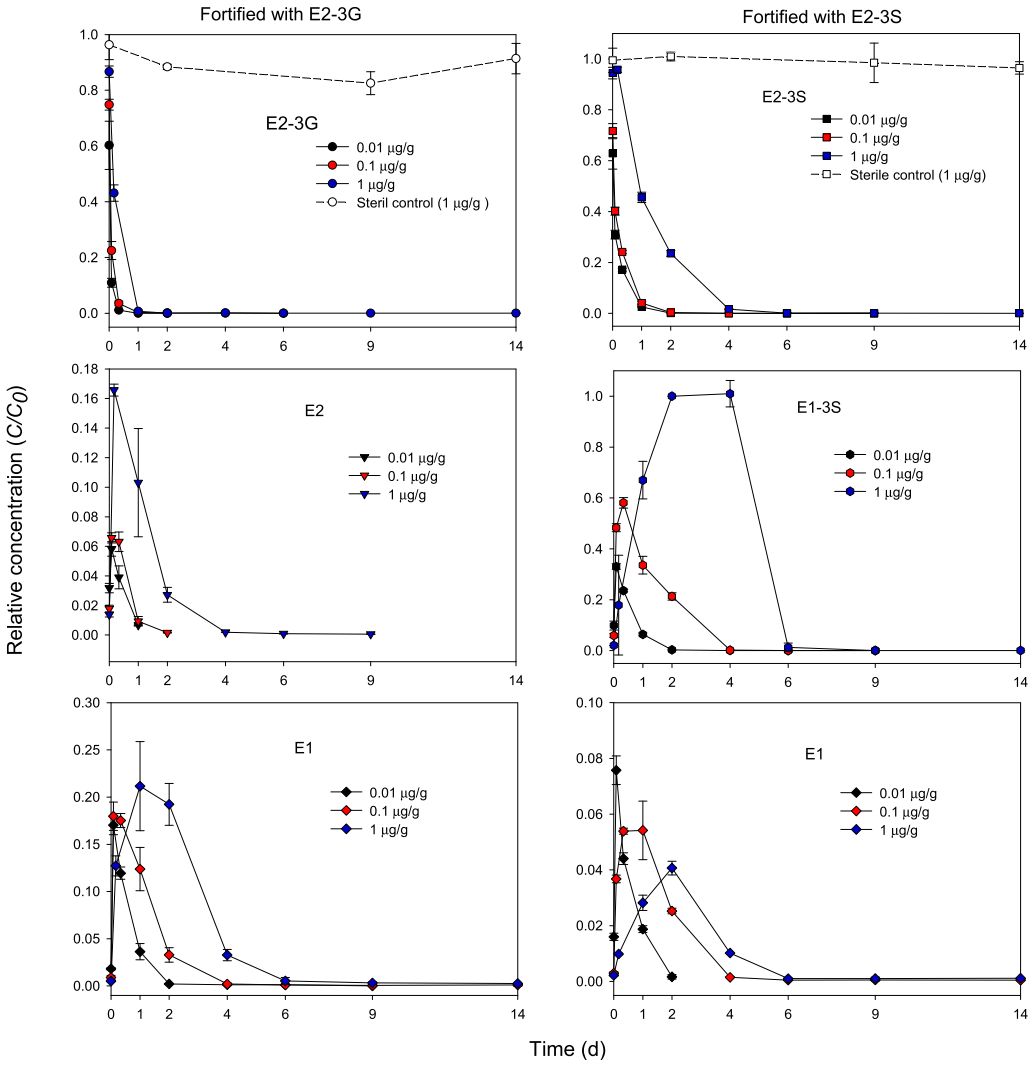

Fig. 2. Extracted concentrations of fortified compounds and their major metabolites in sediment through time under different initial concentrations. Data points and error bars represent means and standard deviations of duplicate. At 0 d, the yields of fortified compounds were not fully recovered because of partial degradation and physical mass loss during the course of extraction. The relative concentration expressed as C/C_O was the concentration (ng/g) ratio of target compounds at different sampling event time to that at the initial time.

Table 1
Parameter estimates for E2-3G and E2-3S degradation in sediment using first-order kinetic model under multiple concentrations.

Initial concentration (μg/g)	k (h^{-1})	r^2	$t_{1/2}$ (h)
E2-3G			
0.01	0.852 (0.023)	0.999	0.81
0.1	0.597 (0.085)	0.998	1.16
1	0.176 (0.022)	0.999	3.94
E2-3S			
0.01	0.223 (0.053)	0.967	3.11
0.1	0.156 (0.012)	0.972	4.44
1	0.031 (0.0003)	0.992	22.36

Note: Degradation rates of fortified compounds were estimated using the first-order exponential kinetic model: $C/C_o = e^{-kt}$, where C and C_o are concentrations of fortified 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. 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 13; At 0 h, the yields of fortified compounds were not fully recovered because of partial degradation during the course of extraction.

spectra suggested that they could have two and one OH moieties, respectively, to react with BSTFA. Herein, a molecular ion at m/z 286 (430+2H-2TMS forland 358+H-TMS for IV) was tentatively assigned to productland IV. To assist identification, the underivatized counterpart of the sediment extract was reanalyzed, and one new peak in the extract, with a retention time of 15.33 min and a molecular ion at m/z 286, showed its mass spectra identical to that of estrolactone with one OH group attached (Fig. S6). Product IV was therefore tentatively identified to be estrolactone. The structure of productIwas unclear, nevertheless, the molecular ion (m/z 430) represents +14 with respect to that of derivatized E2 (m/z, 272-2H+2TMS), possibly corresponding to keto-E2 or OH substituted E1. Based on the peak areas, yields of product IIand III peaked around 72–144 h of incubation. According to our knowledge, estrolactone as a biodegradation product of either E2-3G or E2 has never been reported before. A similar metabolite with a lactone structure at the D ring was proposed as a biodegradation product of E2 (Lee and Liu, 2002). Keto-E1 has been tentatively identified as degradation intermediate of E2 by soil isolates of *Rhodococcus* sp. and *Sphingomonas* sp (Kurisu et al., 2010). 9,11-dehydro-E1 was not reported as estrogen metabolites by others.

Although data was simultaneously collected on HPLC using mass and UV detector, the UV spectra (Fig. 3b) for E2-3G were included

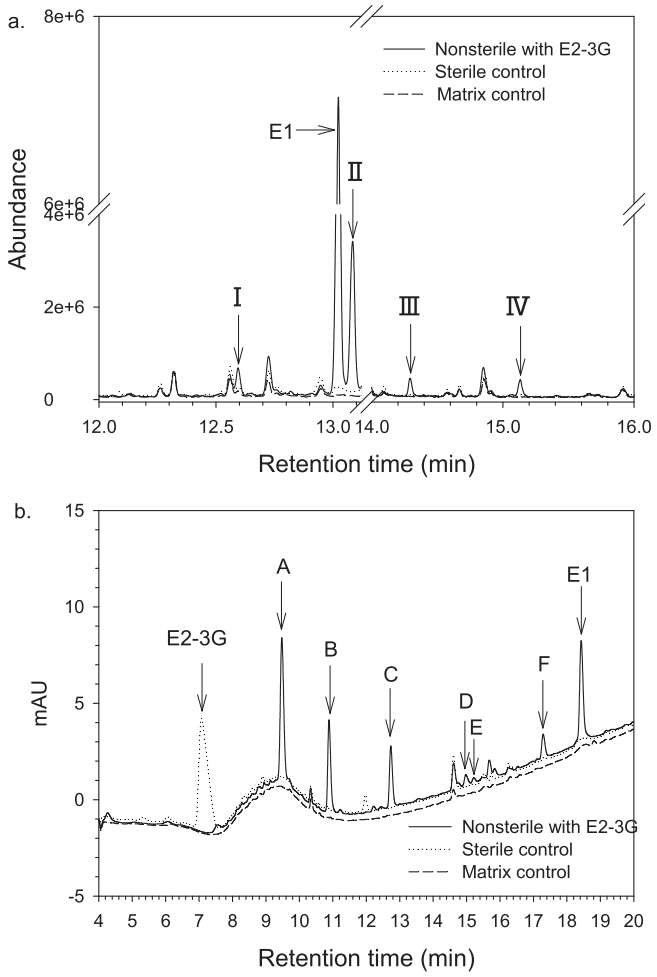

Fig. 3. Total ion chromatograms (TIC) of gas chromatography-mass spectrometry (GC-MS) (a) and UV spectra at 242 nm (b) of E2-3G at the initial concentration of 10 μg/g and its degradation products after 72 h of incubation.

here since the spectra were able to show the new metabolites (product A to F) clearly. These degradation metabolites are labelled herein as A-F to differentiate them from GC-MS peaks. The ambiguous identification of different metabolites was achieved via the application of CID fragmentation mechanisms. A precursor ion of each new metabolite (selected based on the full scan spectra) was subject to CID (in a product ion scan mode), and the subclass precursor, normally a product ion with the greatest signal intensity, was selected for further fragmentation. Corresponding spectra of all new products listed in alphabetical order were shown in Fig. S7. Products B and F, with pseudomolecular ions ($[M-H]^-$) at m/z 283 and 267, were identified to be 6-keto-E1 and 9,11-dehydro-E1 (in accordance to product III and IIby GC-MS), respectively, considering the identical CID spectra (Fig. S8) and retention times with that of the standards. Without an authentic reference compound, structure of other products can only be tentatively deducted from their molecular ions and fragmentation patterns. Product A and C, with a pseudomolecular ion ($[M-H]^-$) at m/z 285, upon CID, exhibited a characteristic ion at m/z 267, which can be explained by a neutral loss of H_2O to form a double bond, implying there is an second OH group in the structure in addition to the existing one at C-3 position. Considering water loss from the aromatic ring is not favored and the subsequent fragmentation pattern of the ion at m/z 267 is similar to that of 8,9-dehydro-E1 (Fig. S9) and 9,11-dehydro-E1 (Fig. S8) authentic standards, the second OH is likely located at B or C ring. Formation of major fragments (m/z 251, 237, 223, 209, 195 and 171) of the ion at m/z 267 was proposed as shown in Fig. S7. We determined product A and C to be hydroxy-E1 although the position of the OH group is not located. B-ring or C-ring OH-substituted E1 hasn't been reported to be potential microbial degradation product of E2 or E1 in soil environment, however, hydroxylation of E2 and/or E1 at various positions was reviewed by Zhu and Conney (1998) to be catalyzed by enzymes present in animal or human tissues, as exemplified by human cytochrome P450 enzymes. In the current study, hydroxylation could be performed by soil bacteria as soil bacterial isolates were reported to oxidize E2 or E1 via hydroxylation at A- or D-ring, which is then followed by ring-cleavage (Kurisu et al., 2010; Casas-Campillo and Bautista, 1965). Products D and E were not identified in the present study due to limited data available.

Like E2-3G, E2-3S showed the presence of 6-keto-E1, 9,11-dehydro-E1 and estrolactone as potential degradation products on GC-MS chromatograms (Fig. S10). Nevertheless, the HPLC-MS/MS spectra suggested of additional distinctive products (Fig. 4). Products G-M have pseudomolecular ions ($[M-H]^-$) at m/z 365 or 363 which were selected as initial precursor ions for fragmentation (Fig. S11). The formation of characteristic ions at m/z 267($[M-HSO_3-H_2O]^-$), 285($[M-HSO_3]^-$) or 283 ($[M-HSO_3]^-$) and 80 (SO_3^-) suggested products G-M should be all sulfate conjugated metabolites. To elucidate the structure of the products, those characteristic ions (m/z 267, 285 and 283) were fragmented further and the following spectra were compared with those acquired in Figs. S7—S9. Product G and K were tentatively identified to be the sulfate conjugated hydroxy-E1. Product L was determined to be sulfate conjugated keto-E1. Products H, I, J, and M were uncertain. To the best of our knowledge, this is the first report of sulfate conjugated hydroxy-E1 and sulfate conjugated keto-E1 as important biodegradation products of E2-3S. The formation of multiple sulfate conjugated metabolites explained, to some degree, why percentages of E1 declined as the initial concentration of fortified E2-3S rose as exhibited in Fig. 2. Based on the tentatively identified and confirmed metabolites, tentative pathways describing conjugate degradation were proposed in Fig. 5. The degradation of E2-3G occurred mostly through oxidation of metabolites E2 and/or E1 via hydroxylation, desaturation, and lactonization to form hydroxy-E1, 9,11-dehydro-E1, and estrolactone. Keto-E1 could be converted from hdyroxy-E1 or directly from E2 (Li et al., 2012). Further degradation could be promoted by ring cleavage. Unlike E2-3G, the

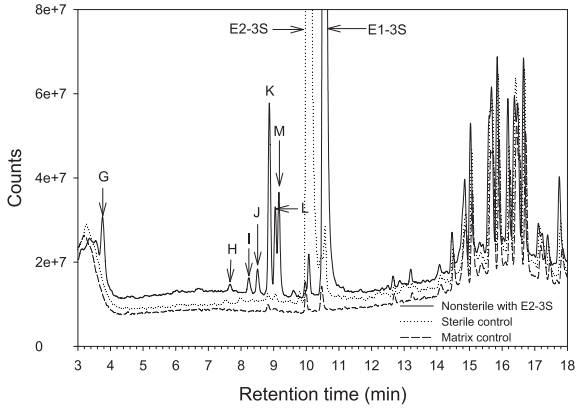

Fig. 4. Total ion chromatograms (TIC) of liquid chromatography-tandem mass spectrometry (LC-MS/MS) of E2-3S at the initial concentration of 10 μg/g and its degradation products after 144 h of incubation.

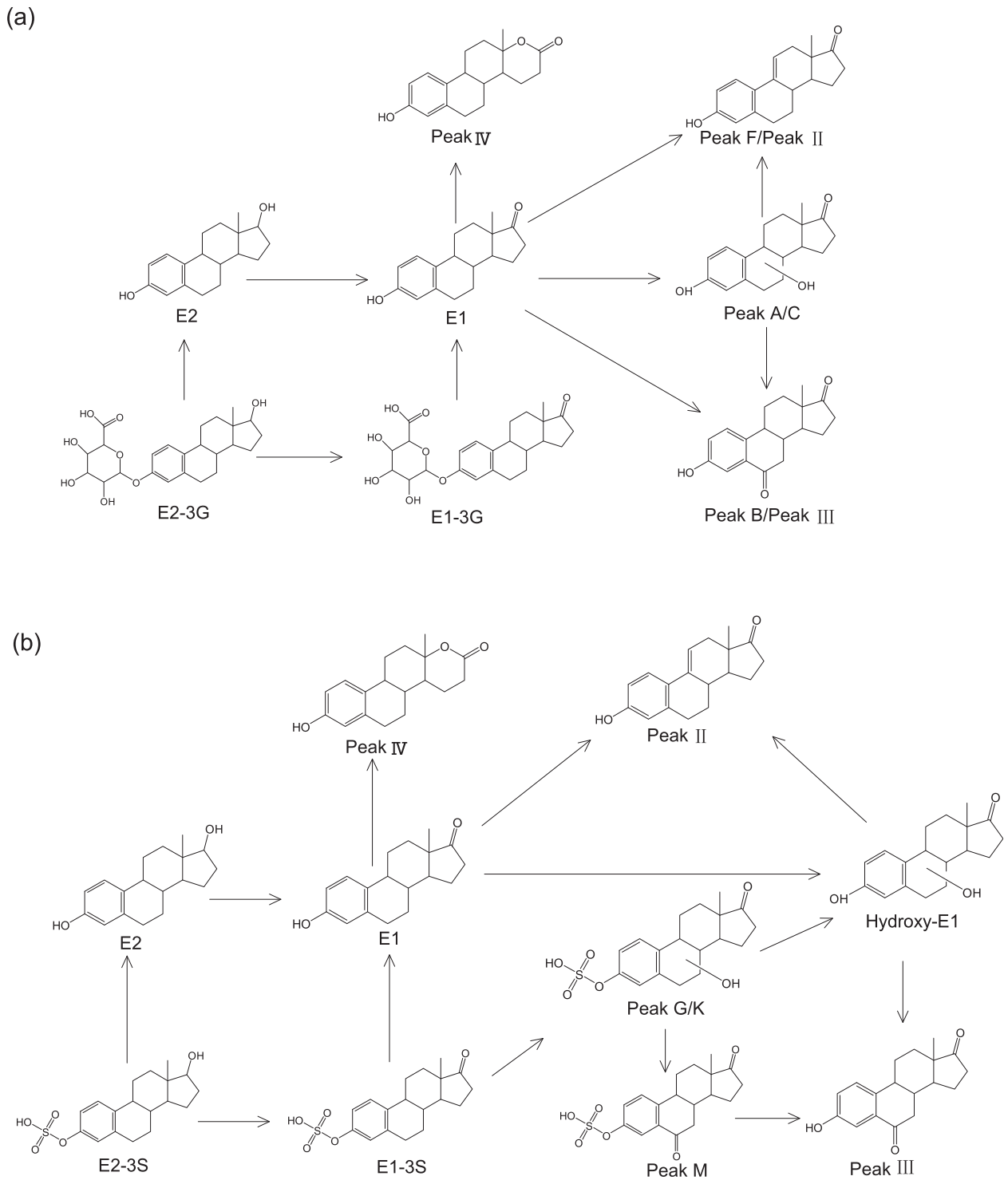

Fig. 5. Proposed pathways for the biotransformation of E2-3G (a) and E2-3S (b) under aerobic condition.

degradation of E2-3S features the formation of different sulfate conjugate intermediates accompanied by the dissipation of E1-3S. E2-3S was initially oxidized to E1-3S, which was partially deconjugated to form E1; while considerable part of it was hydroxylated and ketonized, understandable since sulfated estrogens are relatively recalcitrant due to limited arylsulfatase activity in the environment (D'Ascenzo et al., 2003; Kumar et al., 2012; Scherr et al., 2008).

4. Conclusions

This study reports for the first time the degradation and new metabolite formation of E2-3G and E2-3S in river water environment. Results showed faster degradation rates in sediment than in river water, for glucuronide conjugate than for sulfate conjugate and at low fortification level than at high level, which were likely attributable to biomass densities. It is clearly that low biomass

availability and higher estrogen conjugate loadings prolonged their persistence. Also, environmentally occurred estrogen conjugates could persist in river water for long (9 days for E2-3G and >14 days for E2-3S). Therefore, existing conjugates may serve as sources of free estrogens via gradual liberation of free estrogens during the time course of their persistence.

New metabolites formed via hydroxylation, dehydrogenation, lactonization and ketonization in addition to de-conjugation, and the biologically active part of the estrogen structure is still preserved. Some of the metabolites appeared to be labile since they existed in a transitional manner, but their role in the biodegradation of estrogen conjugates remains to be elucidated. The negligible presence of E1-3G and E2 as primary metabolites, respectively, of E2-3G and E2-3S seems to be in a worse situation as E1-3G itself does not contribute to estrogenicity, substituting E1-3G with E2 enhances the estrogenicity; while the exclusive formation of E1-3S tends to prolong the estrogenicity because E1-3S is more persistent than E2. However, since we didn't monitor the time course of estrogenicity, this assumption remains untested. In addition, we only utilized sandy sediment in the study, whether other sediment exhibits similar degradation behavior is unknown. The study involved the degradation behavior in aerobic condition (air exchange), while real field condition could be a bit different since anaerobic condition may exist. De-conjugation could play a role for E2-3S under anaerobic condition. Therefore, a parallel field study needs to be conducted in the future to obtain a better comparison.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.03.071.

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