FATE AND TRANSFORMATION OF A CONJUGATED NATURAL HORMONE 17β-ESTRADIOL-3-GLUCURONIDE IN SOIL-WATER SYSTEMS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Shrestha, Suman Lal, Ph.D., Department of Civil Engineering, College of Engineering and Architecture, North Dakota State University, September 2011. Fate and Transformation of a Conjugated Natural Hormone 17β-Estradiol-3-Glucuronide in Soil-Water Systems. Major Professors: Dr. G. Padmanabhan and Dr. Francis X.M. Casey.

The objectives of the study were to investigate the sorption and degradation behavior of a glucuronide conjugated natural hormone, 17β-estradiol-3-glucuronide (E2-3G), and the conjugate-derived estrogens in soil-water systems. The effects of soil organic matter content and microbial activity on the fate and sorption of E2-3G were investigated using soil-water batch experiments with natural and sterilized topsoil (0-6 cm) and subsoil (18-24 cm) from an agricultural farm.

A radiolabeled version of E2-3G was synthesized using an immobilized-enzyme approach for conducting the fate and transformation experiment. The aqueous dissipation of ^{14}C in the batch experiments followed a biphasic pattern, where there was an initial rapid dissipation phase followed by a second slower phase where apparent sorption equilibrium was achieved. Significant differences in total aqueous ^{14}C dissipation were observed for the different initial concentrations for both soils. The persistence of intact E2-3G was exacerbated at higher initial concentrations, which may indicate the saturability of the enzymatic hydrolysis.

Speciation analysis indicated that E2-3G metabolized into estrone glucuronide (E1-3G), E2, and estrone (E1). Other unidentified polar metabolites were detected and estriol (E3) was not detected. The sorbed phase fraction contained E1 and E2. Concentration of E1 was at least 1.8 times greater than E2 in the sorbed phase of the natural topsoil, while E2 concentration was greater in the sorbed phase of the subsoil.

Compared to the subsoil, more rapid E2-3G deconjugation in the topsoil was observed, which resulted in rapid aqueous phase dissipation dominated by aglycone (E2 and E1) hydrophobic sorption dynamics. In case of the subsoil, transformation of E2-3G to free estrogens was slower, and E2-3G transformation and sorption of aglycones were equivalent processes. For the topsoil and subsoils, the first 24 h and up to 14 d, respectively, were critical periods for the potential estrogenic contribution to the environment from intact glucuronide conjugates. The estrogenicity, expressed as E2 equivalent (EEQ) concentration, was calculated for the natural topsoil showing a maximum EEQ at 24 h (33 to 972 μg eq-E2 L^{-1}), and significant EEQ was observed even up to 28 days.

A comprehensive kinetic biogeochemical model was developed to describe various non-equilibrium degradation and sorption processes of E2-3G and its metabolites that included the simultaneous chemical and biological transformation of the compounds and their mass exchanges between the aqueous phase and the reversible and irreversible sorption sites. The batch soil-water process parameters for E2-3G and its metabolites were uniquely identified by optimizing all the experiments simultaneously, using a global optimization strategy. The E2-3G hydrolysis in the natural topsoil was two orders of magnitude greater than the subsoil. The sorption capacity for E2-3G was one and three orders of magnitude lower than the E2 in the topsoil and subsoils, respectively.

This study shows that estrogen conjugates may be significant contributors in the environmental detection of potent free estrogens. Agricultural application of manure containing conjugates, especially at the subsurface, may result in greater mobility of intact conjugates in the environment.

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DEDICATION

To my late father Mr. Punya Lal Shrestha, who had a dream to see me engaged in high academic endeavors.

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GENERAL INTRODUCTION

Steroidal estrogens occupy a prominent spot in the list of the emerging contaminants because they have the ability to disrupt sensitive organisms at very low concentrations. Estrogens have been demonstrated to disrupt the endocrine systems of sensitive organisms at concentrations as low as 10 parts per trillion. Synthetic steroid hormones used in the livestock industry were first recognized as a possible environmental threat in 1980 (Knight, 1980). Several studies conducted in 1990s (Shemesh and Shore, 1993; Shore et al., 1993; Shore et al., 1995) were then the first to connect animal operations with natural hormone detections in the environment and indicated the possible toxicological implications. Other early reports included the casual observations of hermaphrodite roach fish (*Rutilus rutilus*) in UK rivers in mid-1980s by anglers. Several ensuing scientific studies in early 1990s linked these observations to estrogens emitted from the effluent of sewage treatment works (Purdom et al., 1994). In the US, a national reconnaissance of 139 rivers across 38 states found reproductive hormones in approximately 40% of the samples that were analyzed (Kolpin et al., 2002). The samples from this reconnaissance study were taken downstream of waste treatment facilities and animal feeding operations.

As a result of the aforementioned field observations, multiple laboratory studies ensued to identify the fate and transport of estrogenic compounds. These studies overwhelmingly suggest that estrogens are highly hydrophobic and should be strongly bound to the organic rich topsoil, and should completely degrade within a few hours to days (Casey et al., 2003; Fan et al., 2007; Shore et al., 1993). Nonetheless, the continued detection of these compounds in the water bodies around the world (Belfroid et al., 1999; Kolpin et al., 2002; Lei et al., 2009) indicates that there are other unknown significant processes contributing to

estrogen fate and transport in the environment. This dissertation presents the results of investigations into the fate and transformation of an estrogen conjugate in agricultural soil-water systems, which may provide an explanation to the frequent and widespread detections of steroid estrogens in the environment.

Organization of the Dissertation

This dissertation is organized into six parts that consists of a general introduction, one published and three manuscripts to be submitted to peer-reviewed journals, and a general conclusion. The general introduction includes a literature review on natural steroidal hormones especially estrogens and their conjugates. It also includes discussion on the sources and occurrences of estrogens in the environment, their toxicological effects, their environmental fate and transport, and the potential for estrogen conjugates to contribute estrogens in the environment. The first manuscript presents a method on laboratory syntheses and characterization of radiolabeled 17β-estradiol conjugates. The second manuscript provides methods developed to investigate fate and transformation of a labile estrogen conjugate, 17β-estradiol-3-glucuronide. The third manuscript focuses on the potential estrogenic contributions to environmental systems from an applied estrogen conjugate source. The fourth manuscript presents the possibility of transport of intact conjugates as a potentially significant contributor of steroidal estrogens in the environment. A detailed qualitative and quantitative analysis of the metabolites in the aqueous and reversibly sorbed phase is reported. Furthermore, a model was also developed to capture the degradation and sorptive potential of the estrogen conjugate and its metabolites in soil-water systems. This fourth manuscript addresses the puzzle of consistent estrogen detection

in the water bodies using the batch soil-water experiments and modeling. The dissertation is finished with the general conclusion, which summarizes the entire research. Paper references are listed at the end of each paper in which they are cited; however, references for the General Introduction and General Conclusions are listed together at the end of the dissertation.

Literature Review

Estrogens: Natural steroidal hormones

Estrogens refer to a group of female sex hormones; estradiol, estrone, and estriol; that all have steroidal structures with four rings made of seventeen carbon atoms. The most important estrogens in terms of their hormonal binding strengths and their ability to bring about physiological changes are 17β-estradiol (E2) and estrone (E1). Estrogens stimulate the female secondary sex characteristics, impact the growth and maturation of long bones, and are responsible for health of reproductive tissues, breast, skin, and brain (Ying et al., 2002). Although male and female mammals produce estrogens, they are found in higher amounts in females. In humans, estrogens are secreted by the adrenal cortex, the ovary, placenta, and the testis (Nelson and Bulun, 2001). In livestock, the granulosa cells of the ovarian follicles and the placenta in the female, and the testes in males are the major sites of estrogen secretion (Lange et al., 2002).

Conjugation of estrogens

After hormones perform their intended action, the body disposes them by way of Phase I and Phase II metabolisms (Dutton, 1980). In Phase I, hormones undergo a wide range of reactions such as hydroxylation, oxidation, and reduction in order to 'functionalize' the

compound. Functionalization produces or uncovers a chemically reactive functional group on the molecule on which a Phase II reaction can occur. The Phase II reaction then attaches a polar moiety, increasing its water solubility (Gibson, 2001). The Phase II reaction, also known as a conjugation reaction, is a major detoxification pathway in the metabolism of endogenous and exogenous steroid hormones, drugs, toxicants, and non-nutritive small organic molecules (Dutton, 1980). A typical conjugation reaction involves the covalent attachment of a charged, polar moiety to a hydrophobic compound, such as an estrogen, which increases its water solubility, allowing it to be easily excreted in urine or bile. Estrogens are typically conjugated with glucuronic acid or sulfate moiety, or both (Khanal et al., 2006). Glucuronidation reactions are catalyzed by uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes, which are localized within the endoplasmic reticulum, and are divided into two subfamilies, UGT1 and UGT2 (Kiang et al., 2005; King et al., 2000). Glucuronidation consists of the transfer of a glucuronosyl group from uridine diphosphate (UDP) glucuronic acid to the substrate molecule (e.g. an estrogen) bearing a reactive oxygen, nitrogen, sulfur, or carboxyl functional group (Bock and Kohle, 2005; King et al., 2000). Glucuronidation is a major detoxicatory pathway in all vertebrates (Dutton, 1980), the reasons for its being the most widespread conjugation reaction are the relative abundance of the co-factor for the reaction (UDP glucuronic acid) and ubiquitous nature of the enzyme, UDP-glucuronic acid (Gibson and Skett, 2001). Sulfation reactions occur with cytosolic sulfotransferases, which serve to inactivate hydrophobic substrates such as estrogens (Nishiyama et al., 2002). Glucuronidation or sulfation of estradiol can occur at either the C-3 phenol or C-17 hydroxyl, or simultaneously at both sites (Khanal et al., 2006).

Conversion of conjugated estrogens to 'free' estrogens

Conjugated estrogens can be hydrolyzed to form 'free' estrogens either by acid (Carignan and Lodge, 1980) or enzymatic hydrolysis (Khanal et al., 2006). Acid and enzymatic hydrolysis are common laboratory methods used to indirectly quantify aqueous conjugated estrogens. However, hydrolysis reactions in the environment are primarily governed by the bacterial enzymes β-glucuronidase or sulfatase for glucuronide and sulfate conjugation, respectively (Khanal et al., 2006).

Studies from several wastewater treatment plants (WWTPs) indicated that estrogen glucuronides can easily transform back into their free estrogen forms. However, sulfate conjugates of estrogens are found to be more recalcitrant, persisting longer in septic tanks and manure storage ponds (Liu et al., 2009). There is a large variation in the reported deconjugation efficiencies for estrogen glucuronides in WWTPs. For example, researchers in separate studies found estrone-3-glucuronide removal by deconjugation at 84% (D'Ascenzo et al., 2003), 100% (Reddy et al., 2005), and 0-51% (Kobayashi et al., 2006). Reported removal efficiencies for estradiol-3-glucuronide were highest at 100% (D'Ascenzo et al., 2003; Reddy et al., 2005) and lowest at 0-19% (Kobayashi et al., 2006). Deconjugation efficiencies for estrone-3-sulfate in WWTPs ranged from 27.6 % (N. Nakada et al., 2006) to 99.1% (Reddy et al., 2005), and from 48.4% (N. Nakada et al., 2006) to 100% (D'Ascenzo et al., 2003; Reddy et al., 2005) for E2-3S. Compared to WWTPs, there is not much data on conjugated estrogens in the waste of animal feeding operations (AFO), where animal excreta are stored in pits or lagoons prior to their land application. Waste storage ponds and lagoons at AFOs are typically used as holding reservoirs or anaerobic reactors for animal excreta, and unlike sewage treatment plants,

there is generally no treatment before the waste is applied to the land as fertilizers (Hutchins et al., 2007). Hutchins et al. (2007) found estrogen conjugates contributed to 27 – 35% of the total estrogens in swine (*Sus scrofa domesticus*) nursery, beef (*Bos taurus*) feedlot, and poultry (*Gallus gallus*) primary lagoons; 57% for a dairy lagoon; and 95% for a tertiary poultry lagoon. The predominant estrogen conjugates were E1 and E2 sulfates or glucuronides, which were determined indirectly from enzyme hydrolysis. Furthermore, direct analysis using LC/MS/MS indicated the sulfate conjugates were still intact, indicating their persistent nature (Hutchins et al., 2007).

Sources of steroidal estrogens and their conjugates in the environment

Humans

The amount of natural estrogens in human excretion varies with sex and age. The highest estrogen excretion rates occur in pregnant females with 259 μg d^{-1} of E2, 600 μg d^{-1} of E1, and 6,000 μg d^{-1} of E3 (Ying et al., 2002). In menstruating females, excretion rates are reported to be 3.5, 8.0, and 4.3 μg d^{-1} for E2, E1, and E3, respectively (Ying et al., 2002). For menopausal females, E2, E1, and E3 excretion rates are 2.3, 4.0, and 1.0 μg d^{-1}, respectively. Male excretion rates are 1.6, 3.9, and 1.5 μg d^{-1} for E2, E1, and E3, respectively (Ying et al., 2002). Even though the excretion of estrogens are generally reported in terms of free estrogens, the majority of the steroid estrogens are excreted in their conjugated forms from humans (Gomes et al., 2009). An Italian study found E3 (21 μg d^{-1}) was the only free estrogens detected in the urine of 73 women ranging in age from 18 to 74 years (D'Ascenzo et al., 2003). In this Italian study, all estrogens were conjugated, predominantly as glucuronides. Sulfates estrogen conjugates were 23, 20, and 22% of the

total estrogen derivatives excreted from menstruating, menopausal, and pregnant women, respectively. In a study of gender based distribution of estrogen conjugates, glucuronides conjugates, not sulfates, were found to be more dominant in males (85%) than in females (65%) in human urine (de Mes et al., 2005).

Synthetic hormonal formulations, such as 17α-ethynylestradiol (EE2) and mestranol (MeEE2), used as female oral contraceptives are excreted into urine predominantly as glucuronide conjugates (Ranney, 1977). A daily excretion rate of 35 μg d^{-1} for EE2 has been reported for females using EE2 contraception (Ying et al., 2002). Considering all types of oral contraceptive usage, the environmental load of synthetic estrogens has been estimated to be 720 Kg $year^{-1}$, assuming a mean rate of excretion of 60% (Combalbert and Hernandez-Raquet, 2010). Though the figures are reported in the free forms of synthetic estrogens, actual release from the human body occur in the conjugated forms, as glucuronides (Ranney, 1977).

Wildlife

In comparison to humans and livestock, not much is known about the environmental loading of estrogens from wildlife. In one study, ^{14}C labeled E2 was injected into female cotton-top tamarins (*Saguinus oedipus oedipus*), and estrogen excretion was measured. Estradiol was excreted predominantly through the urinary route (87%), and estrone was excreted in the urine (57%) and feces (43%). Furthermore, 59% of ^{14}C E2 was excreted as an E2 conjugate and 41% as an E1 conjugate (Ziegler et al., 1989).

Livestock

In livestock, the estrogen excretion amount, route, and type including conjugation pattern varies with sex, age, and species. Cattle excrete estrogens mostly in feces (56%) (Ivie et al., 1986), whereas swine (96%) and poultry (69%) excrete estrogens predominantly through the urinary route (Hanselman et al., 2003). Estrogen excretion for non-pregnant cattle is evenly distributed between urinary and fecal routes, at about 500 μg d^{-1} per 1000 Kg live animal mass. During late stages of pregnancy in cattle, estrogen excretion through urinary and fecal routes increase by a factor of 326 and 10, respectively, compared to non-pregnant cattle (Hanselman et al., 2003). In a survey of hormone activities in animal manures, Lorenzen et al. (2004) used recombinant yeast assays to measure E2 estrogenic activity in the manure and detected the highest levels from finishing pigs (5965 ng g^{-1} dry weight) and the lowest levels from steers (0.43 ng g^{-1} dry weight). Hormonal implant use and diet are also factors contributing to the estrogenic activities in the manure (Lorenzen et al., 2004).

The potential contribution of estrogens to the environment by farm animals dwarfs that of humans. In the U.S. and the European Union alone, an estimated 82 tons of estrogens were excreted by livestock in 2000 (Lange et al., 2002). Even though considerable land area in the United States is still devoted to rangeland grazing of cattle, commercialization of animal agriculture in US has resulted in the increase of confined animal feeding operations (CAFOs). A confined animal feeding operation is defined by the US EPA as an operation where animals are confined at least 45 days in a 12-month period and there is no grass or vegetation in the confined area during the normal growing season. Concentrated animal feeding operations meet the requirement to be an AFO and a size threshold (number

of animals) distinguishes a CAFO as large, medium, or small. A large CAFO, for example, would house 1000 or more cattle; 10,000 or more sheep or lambs; 10,000 or more swine (weighing 55 pounds or less); or 30,000 or more laying hens or broilers (USEPA, 2004). In the United States, CAFOs generate more than 40 times the amount of waste than human bio-solids generated from WWTPs (Graham and Nachman, 2010). In the United Kingdom, the combined farm animal population was estimated to generate about four times more estrogens than the human population (Johnson et al., 2006). In China, an estimated 3.2 billion tons of animal waste was generated in 2003, which was 3 times the industrial solid waste generated in the country (Li et al., 2009). A study of manure born estrogens from dairy and beef cattle operations in northeast China found that 24 CAFOs generated 16 times more estrogens than the human population in the same study area (Wei et al., 2011).

Most endocrine disruption studies have focused on the aquatic environment downstream point sources such as sewage treatment plants (STPs) (Desbrow et al., 1998; Jin et al., 2008; Wang et al., 2010), industrial wastewater discharges (Pothitou and Voutsa, 2008; Snyder et al., 2001), and runoff receiving waters from urban areas (Cargouet et al., 2004; Vulliet et al., 2011). However, data on CAFOs are limited and so are the studies on the associated endocrine disruption studies of aquatic organisms downstream CAFOs. Table 1 presents some examples of studies that measured estrogens related to CAFO waste. These data highlight potential of environmental loading of potent hormones originating from CAFOs.

Table 1. Estrogen concentrations in different concentrated animal feeding operations.

Facility	Estrogen Concentrations (ng L^{-1})				Study
	E1	E2	E3	Total	
Creek near a CAFO (mixed[†])	7.4 -1267	0 - 313.6	0 - 210		(a)
Primary lagoon, swine CAFO				1000 - 21,000	(b)
Secondary dairy cattle effluent				370 - 550	
Secondary beef cattle effluent				22 - 24	
Dairy farm effluent	0 - 3123	0 - 331			(c)
Swine lagoon	24,900*	3000*	10,420*		(d)

[†] Broiler chickens, laying hens, pigs, and cattle; *max values; (a) = Chen et al. (2010), (b) = Hutchins et al. (2007), (c) = Sarmah et al. (2006), (d) = Fine et al. (2003)

Ecotoxicology

Relative potencies

In terms of the capacity to disrupt the endocrine systems of sensitive organism, E2 is the most potent among natural estrogens. Conjugated estrogens have very little potency compared to free estrogens. Table 2 presents the relative potencies of some estrogen glucuronides and the free estrogens, based on E-screen assays (Gadd et al., 2010).

Table 2. Relative potencies of glucuronide conjugated and free estrogens.

Compound	Relative Potency*
E2-3G	1.30×10^{-3}
E1-3G	2.90×10^{-5}
E2	1.00
E1	2.40×10^{-2}

*Gadd et al. (2010)

Impact on different organisms

Fish. Vitellogenin production is a reproductive disruption in the male fish resulting from exposure to estrogens. Vitellogenin is a female-specific protein that is a precursor to an egg yolk protein, and is synthesized in the liver of oviparous vertebrates (Jobling et al., 1998). In female fish the expression of genes to produce vitellogenin is regulated by endogenous estrogens such as E2 (Desforges et al., 2010). Even though estrogen levels in male organisms are insufficient to produce vitellogenin under normal circumstances, production of the vitellogenin can be induced by exogenous estrogens (Bjorkblom et al., 2008). Concentrations of E2 as low as 10 ng L^{-1} can induce vitellogenin production in the male fish in the laboratory when continuously exposed for 21 d (Routledge et al., 1998). Kitamura et al (2009) conducted a study on the induction of vitellogenin in Japanese Medaka (*Oryzias latipes*) and observed the lowest effect concentrations (LOECs) of E1 and E2 to be 31.6 and 5.0 ng L^{-1}, respectively; and the E2 equivalent (EEQ) LOEC value of E1 to be 5.2 ng-E2 L^{-1}. In the field, 100% intersex male roach fish (*Rutilus rutilus*) was observed in the sewage-contaminated rivers, Nene and Aire, in the UK, compared with 4% intersex males from laboratory controls and field reference locations (Jobling et al., 1998). The reproductive capability of male fish with morphological defects caused by endocrine disruption was investigated by Jobling et al. (2002). Compared to an unaffected male fish, they found gamete quality was reduced by as much as 50% in terms of sperm motility and 75% in fertilization success. In a 7-year, whole-lake experiment at the Experimental Lakes Area in northwestern Ontario, Kidd et al. (2007) observed a near extinction of fathead minnow (*Pimephales promelas*) as a result of

chronic exposure to low concentrations of EE2 (5-6 ng L^{-1}), which impacted gonadal development in males and altered oogenesis in females.

Birds. Eggshell thinning, leading to reproductive failure, is one of the most serious ecotoxicological effects observed in avian wildlife (Berg et al., 2004). Exposure to exogenous estrogens can disrupt the expression of carbonic anhydrase, a key enzyme in the process of shell formation that controls the supply of the carbonate ions, in the adult shell gland (Holm et al., 2001). Berg et al. (2004) showed that eggshell thinnning in domestic hen (Gallus domesticus) is induced when embroy is exposed to EE2. Hens exposed to EE2 in ovo (20 ng g^{-1} per egg) produced eggs with thinner shells and reduced strength. Exposure to exogenous estrogens can also result in the disruption in sexual development. Biau et al. (2007) exposed chick embryos to 600 ng g^{-1} E1 and 600 ng g^{-1} E3, which are relatively higher concentrations compared to environmental values, and found morphological defects of the urogenital system. The impacts of E3 were greater than E1, where E3 caused the persistence of Müllerian ducts in 48% of male embryos and hypertrophic oviduct in 71% of females. The E1 caused 18% persistence of the Müllerian ducts in male embryos and 49% of hypertrophic oviduct in female embryos. Numerous studies on quail (Coturnix coturnix) have shown that the male reproductive behavior can be permanently demasculinized by in ovo exposure to E2 (Abdelnabi and Ottinger, 2003; Panzica et al., 1998). Estrogens are also shown to alter the behavioral response of female songbirds. A dose-response study on the development of the female zebra finch (Poephila guttata) song system, administered 50, 15, 5 and 0-μg of E2 via subcutaneous implants, and found that 15 μg E2 was sufficient to masculinize several aspects of the song system (Grisham et al., 2008). Svec et al. (2009) explored the behavioral response to E2 exposure

on song pattern of female zebra finch and found decreased long-distance calls and visual scanning behavior.

Reptiles. In many reptiles, incubation temperature of the egg impacts the gonadal sex determination (Bull, 1987). Estrogen can also serve as the physiologic equivalent of incubation temperature, where exposure to estrogens has been shown to have similar effects as temperature-dependent sex determination (TSD) (Crews et al., 1995). In a study on leopard geckos (Eublepharis macularius), a TSD reptile, Janes et al. (2007) exposed eggs to 5 μL of E2 (6.1 μM) and hatched them at a male-producing incubation temperature of 32.5°C. The estrogen-treated groups produced more females than the negative control group. Similarly, significantly more males were produced when eggs were treated with E2 and were incubated at cooler, female-producing, temperature (Janes et al., 2007). Birth defects in wildlife and the associated role of environmental contaminants have been studied extensively (Hamlin and Guillette, 2010) . A study in Lake Apopka, one of the most polluted lakes in Florida, found juvenile alligators to be feminized, and exposure to estrogenic compounds was implicated as the cause (Guillette et al., 1994). Also, ovarian abnormalities were observed in female alligators, with elevated in vivo E2 concentrations compared to normal female alligators (Guillette et al., 1994).

Amphibians. Since amphibians spend their embryonic life stage in aquatic medium, their embryos are exposed to environmental contaminants. Population declines in amphibians (Stuart et al., 2004) have resulted in increased concern over environmental exposures to exogenous estrogens. Courtship behaviors in frogs are found to be related to the sex hormones concentrations, where there are relations between hormonal concentration variation and "inactive" and "approaching" frogs. In a study on the courtship

behavior of crested newts (Triturus carnifex), "inactive" males showed higher levels of testosterone compared to those involved in various degree of courtship, while E2 was low in such males (Zerani et al., 1992). In a study on basal water absorption (BWA) on Japanese tree frogs (Hyla japonica), injected E2 reduced the BWA in males, whereas injected testosterone increased the BWA in females (Kohno et al., 2004). According to these authors, since BWA regulates the concentration of body fluids under normal conditions, these results have important implications in understanding normal osmoregulation in frogs.

Humans. Impacts on human health from the exposure to environmental concentrations of natural hormones are not clear; however, there is an urgency to quantify exposures, which has instigated several U.S. congressional bills (Congress, 2005; Congress, 2008). Humans are not sensitive to low doses of estrogens typically found in the environment. Aherne and Briggs (1989) reported that the synthetic hormone EE2 concentrations found in sewage effluent, reservoirs, rivers and potable water were unlikely to present significant risks to human health. Christensen (1998) estimated a daily worst case environmental intake of EE2 of 85 ng day^{-1} for a 70-Kg person, and concluded that it was unlikely to contribute to any significant risk. Andersson and Skakkebaek (1999) contended that there could be possible biological significance from estrogen intake from meat consumption, and raised concern for exposure to exogenous estrogens and in particular for prepubescent males. The benefits of hormone replacement therapy (HRT) (estrogen, progesterone, or both) to relieve menopausal symptoms in women are outweighed by the risk of breast cancer (Rossouw et al., 2002). Typical HRT consists of a combined estrogen and progesterone, such as conjugated equine estrogens (CEE) (0.625

mg d^{-1}) plus medroxyprogesterone acetate (2.5 d^{-1}) in one tablet (Rossouw et al., 2002). To date, the prescription and marketing of HRT in the USA and other countries have dramatically decreased as a result of its risks (Krieger et al., 2005). Medroxyprogesterone acetate is a synthetic variant of the human hormone progesterone (Cordeaux et al., 2010), while CEE is a complex formulation containing the conjugates of at least 10 estrogens such as E1, 17α-E2, E2, and equilin, to name a few (Zhao and Brinton, 2006). An indication of the relative hormone concentration in HRT can be made by comparing the HRT dose of estrogens (0.625 mg d^{-1}) to the conjugated estrogens (E2, E1, and E3) excreted daily by a cycling woman (about 54 μg d^{-1} (D'Ascenzo et al., 2003)), which is about 12 times greater. Compared to the environmental concentrations of E2 that have been reported for soil, at 150 ng Kg^{-1} on control fields and 650 ng Kg^{-1} on manured plots (Finlay-Moore et al., 2000), or surface water, at 9 ng L^{-1} median concentration (Kolpin et al., 2002), the concentration of estrogens in HRT is very high.

Outside HRT, studies have shown that increased prenatal exposures to endogenous estrogens may also increase the risk of breast cancer (Park et al., 2008). In one study, young women who were considered at high risk for breast cancer because of family history, also exhibited a significantly different urinary estrogen profile than the control group (Fishman et al., 1979). The authors linked the low urinary estrogen glucuronide with women at risk for familial breast cancer, and suggested that a change in conjugation could be the endocrine feature distinguishing women at risk of familial cancer risk. One of the expected outcome of the US congressional Breast Cancer and Environmental Research Act of 2008 of the USA is to eliminate knowledge gaps in research to improve the research portfolio in breast cancer (Congress, 2008).

Fate and transport of estrogen conjugates

Studies on fate and transport of estrogen conjugates are few, and focus on municipal waste treatment by STPs and/or WWTPs (Chen and Hu, 2010; D'Ascenzo et al., 2003; Gomes et al., 2009; Schlusener and Bester, 2008). D'Ascenzo et al. (2003) qualified and quantified the conjugated forms of estrogen in six activated sludge STPs in Italy and observed complete deconjugation of glucuronide conjugates of estrogen; however, the removal efficiency of estrone-3S (E1-3S) was only 64%, indicating its relatively recalcitrant character. D'Ascenzo et al. (2003) also found E1 was the main degradation product in the STP effluent and attributed it to the oxidation of E2 and partial deconjugation of E1-3G and E1-3S. Schlusener and Bester (2008) studied removal efficiencies of E2-3S, E1-3S, and other free estrogens, in three different WWTPs in Germany. They found elimination efficiencies differed greatly, where activated sludge WWTP was better able to eliminate steroid hormones compared to the trickling filter WWTP. Gomes et al. (2009) investigated the fate of sulfate and glucuronide conjugated estrogens in batch studies using activated sludge grown from sewage, and found glucuronide conjugates of estrogen were deconjugated after 8 h, while sulfate conjugates persisted after 8 h in significant concentrations. The authors concluded that deconjugation was a biotic process based on the results from biotic and abiotic experiments. Also, Gomes et al. (2009) observed sulfate conjugated ethinylestradiol 3-sulfate (EE2-3S) 8 h after crude sewage samples were spiked with EE2-3G alone, and attributed the presence of EE2-3S to the initial cleavage of the glucuronide moiety followed by the sulfate conjugation of the free estrogen. These results were not obtained for the crude sewage spiked with E1-3G, but only occurred when it was spiked with EE2-3G. Deconjugation first order reaction rates of

0.32, 0.24, and 0.35 h^{-1} for 17α-EE2-3G, E3-16α-G, and E1-3G, respectively were determined for these batch studies. Chen and Hu (2010) investigated the adsorption of natural estrogens and their conjugates by activated sludge, and found that while the adsorption capacities of E1 and E2 was influenced by the pH (highest at neutral pH, lower at pH 2 and 11.5), the adsorption of the estrogen conjugates, E1-3S and E2-3S, were similar at pH 5, 7, and 9. Freundlich sorption isotherm accurately described both estrogen and estrogen conjugate sorption in the batch studies. Soil sorption coefficient normalized to organic carbon content of the soil, or log K_{oc} values, for E1, E2, E1-3S, and E2-3S were reported at 3.31, 3.12, 2.21, and 2.46 L Kg OC^{-1}, respectively.

To date, studies on the fate and transformation of estrogen conjugates in agricultural soil has remained mostly an untouched research area. So far, two studies exist that investigate fate and transport of estrogen sulfates in pasture soils (Scherr et al., 2009a; Scherr et al., 2009b). Incubation laboratory experiments were conducted on E2-3S using three pasture soils at three temperatures (7.5, 15, and 25°C) and showed first-order kinetics of E2-3S degradation, as well as temperature dependence of the rate constants (Scherr et al., 2009b). The result also showed that the rate constants across the soils were significantly correlated to the arylsulphatase activity at 7.5 and 15°C. The authors concluded that the arylsulphatase activity in the soil microbial biomass was responsible for the degradation of E2-3S. Scherr et al. (2009a) also conducted a study on sorption of E1-3S in a $CaCl_2$ solution and an artificial urine (pH 7.2, EC 1.4 ds m^{-1}; $KHCO_3$, KCl, K_2SO_4 , $(NH_2)_2CO$, and $C_2H_5NO_2$) using pastoral soils of New Zealand. All the three soils used in the study were of high OC content (4 – 8.2%), and no sterilization agents were used. Given the apparent non-linearity observed in the sorption, the authors reported a concentration-

dependent effective distribution coefficient ($K_d^{eff} = K_f C_w^{N-1}$) for E1-3S were an order of magnitude lower than that for free E1.

Research Gap

Estrogens are hydrophobic compounds. Extensive laboratory studies on these compounds over the past decade have shown E2 and E1 to be highly sorptive and immobile in soil (Casey et al., 2003; Casey et al., 2005; Fan et al., 2007; Hildebrand et al., 2006; Karnjanapiboonwong et al., 2010), and E2 to be highly labile (Colucci et al., 2001; Fan et al., 2007). These results are, however, disparate with the relatively high detection frequencies of E2 and other estrogens in surface water (Kolpin et al., 2002; Lei et al., 2009). Detection of E2, above the LOAEL in over 40% of rivers (139) sampled across 38 U.S. states (median concentration 9 ppt; maximum concentration 93 ppt) (Kolpin et al., 2002) and similar trend elsewhere (Lei et al., 2009), indicate that there are other overlooked processes contributing to estrogen fate and transport in the environment. Estrogen conjugates are more polar than their free counterparts, which may make them more mobile and increase their potential for downstream transport to the receiving water bodies. Furthermore, urinary excretion of estrogens is predominantly as conjugates. Animals in the U.S. in 2000 contributed about 21 tons of estrogen conjugates to the environment (Lange et al., 2002). The research question, therefore, is whether conjugates could contribute to environmental detections of free estrogens.

Dissertation Objective

The hypothesis of the study was that if conjugated estrogens eliminated by animals are more persistent and mobile in soil compared to their unconjugated free forms, then estrogen conjugates could contribute significantly to the environmental estrogen loads. To test this hypothesis, the sorption, fate and transformation of a manure-borne estrogen conjugate in soil-water system, and the effect of soil sterility and OC were investigated. The specific objectives were (1) to synthesize a radiolabeled estrogen glucuronide conjugate and conduct in-depth characterization of the synthesized material; (2) to develop a laboratory analysis method capable of detecting both the estrogen conjugate and its possible metabolites by using high performance liquid chromatography (HPLC) and liquid scintillation counting (LSC); (3) to identify/verify the study compound and its metabolites, by means of liquid chromatography- mass spectrometry/quadrupole time of flight (LC-MS/QTOF); (4) to investigate the sorption behavior of conjugate and conjugate-derived estrogens; (5) to investigate the degradation of the conjugate with respect to the soil organic carbon; (6) to investigate the degradation/transformation pathways of E2-3G in the biotic and abiotic conditions, and (7) to develop a mathematical model to determine the process parameters involved in the fate and transformation of the model estrogen conjugate.

PAPER 1. SYNTHESIS AND CHARACTERIZATION OF RADIOLABELED 17β-ESTRADIOL CONJUGATES[1]

Abstract

The use of radioactive tracers for environmental fate and transport studies of emerging contaminants, especially for those that are labile, offers convenience in tracking study compounds and their metabolites, and in calculating mass balances. The aim of this study was to synthesize radiolabeled glucuronide and sulfate conjugates of 17β-estradiol (E2). The conjugates 17β-[4-^{14}C]estradiol-3-glucuronide ([^{14}C]E2-3-G) and 17β-[4-^{14}C]estradiol-17-sulfate ([^{14}C]E2-17-S) were synthesized utilizing immobilized enzyme and chemical syntheses, respectively. Microsomal proteins from the liver of a phenobarbital induced pig (*Sus scrofa domestica*) were harvested and used to glucuronidate E2. Synthesis of [^{14}C] E2-17-S consisted of a three-step chemical process – introducing a blocking group at the C-3 position of [^{14}C] E2, sulfation at C-17 position, and subsequent deblocking to yield the desired synthetic product. Successful syntheses of [^{14}C] E2-3-G and [^{14}C] E2-17-S were achieved as verified by liquid chromatography, radiochemical analyses, quadrupole-time-of-flight (QTOF) mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Radiochemical yields of 84 and 44 percent were achieved for E2-3-G and

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[1] This paper has been reproduced from my published paper Shrestha et al. (2011) as per the permitted use from the publisher. Other co-authors are Ms. Xuelian Bai, Dr. David Smith, Dr. Heldur Hakk, Dr. Francis Casey, Dr. Gerald Larsen, and Dr. G. Padmanabhan. The bulk of the work has been done by me as part of my Ph.D. research to synthesize the study compound required for my experiment. The style of the manuscript has been modified for keeping uniformity with the rest of the dissertation.

E2-17-S, respectively. Synthetic products were purified using high performance liquid chromatography (HPLC) and radiochemical purities of 98% or greater were obtained.

Introduction

Medical research has used radiolabeled estrogenic compounds to study breast and uterine cancers (Mull et al., 2002), estrogenic receptors (Cummins, 1993), and as imaging agents in breast tumors (Dence et al., 1996). More recently though, the radiolabeled hormonal compounds (e.g. [^{14}C]estradiol, [^{14}C]estrone, and [^{14}C] testosterone (Casey et al., 2003; Casey et al., 2004; Fan et al., 2006; Fan et al., 2007; Sangsupan et al., 2006) and 6,7-^{3}H-estradiol (Sangsupan et al., 2006)) have been used to study the fate and transport of steroids in the environment. Exposures to exogenous reproductive hormones have been associated with adverse effects in certain aquatic (Desbrow et al., 1998; Larsson et al., 1999; Teles et al., 2005) and terrestrial (Lintelmann et al., 2003; Park et al., 2009; Preziosi, 1998) species. Human waste treatment and animal feeding operations (AFOs) are sources of estradiol (E2), estrone (E1), and estriol (E3) to the environment. Estradiol is the most potent of these natural estrogens (Legler et al., 2002; Matsui et al., 2000; Palme et al., 1996; Payne and Talalay, 1985).

Laboratory studies suggest estrogens should have little to no mobility and should not persist in the environment because they bind rapidly and strongly to soil and degrade within hours (Casey et al., 2003; Fan et al., 2007; Holthaus et al., 2002). Field studies, however, have indicated that estrogens are present in the environment at frequencies and concentrations that imply they are moderately mobile and persistent (Kolpin et al., 2002; Schuh, 2008). Estrogen conjugates, which have different water solubilities, sorption

coefficients, and degradation rates relative to their "free" estrogen counterparts, may offer insights into why steroidal estrogens are frequently detected in the environment (Kolpin et al., 2002). Swine (*Sus scrofa domesticus*), poultry (*Gallus domesticus*), and cattle (*Bos taurus*) excrete 96%, 69%, and 42%, respectively, of the urinary estrogens as conjugates (Hanselman et al., 2003). In fact, appreciable amounts of 17β-estradiol (17β-E2) in conjugated forms have been measured in swine manure slurry (liquid urine and feces) from AFO manure storage lagoons (Hutchins et al., 2007). Conjugates form a major portion of total environmental estrogen loading from AFOs and might play a significant function in the detections of "free" steroidal estrogens in the environment. Although, estrogen conjugates are biologically inactive (Khanal et al., 2006), they can potentially be cleaved by microbial enzymes to form the more potent parent compound (Khanal et al., 2006).

Conjugation reactions are a common vertebrate mechanism in which hormones, drugs, toxicants, and non-nutritive organic molecules are eliminated (Amdur et al., 2001). During conjugation a charged, polar moiety is attached to a hydrophobic compound (e.g. estrogen), which increases its water solubility and excretion in urine or bile (Amdur et al., 2001). Estrogens are typically conjugated with glucuronic or sulfuric acid at the C-3 and/or the C-17 positions (Khanal et al., 2006) (Figs. 1 & 2). Glucuronidation of estrogen is catalyzed by uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes in the endoplasmic reticulum and sulfation is catalyzed by cytosolic sulfotransferases (SULTs) (Nishiyama et al., 2002).

The environmental fate of estradiol conjugates has not been extensively studied, possibly because radiolabeled conjugates are not commercially available. The availability of radiolabeled conjugated hormones would enable studies to be conducted that would

improve the understanding of the fate and transport of these labile compounds in the environment. The objective of this paper is to provide a method to synthesize carbon-14 labeled 17β-E2-3-G and 17β-E2-17-S.

Experimental

Materials

[^{14}C] labeled E2 (55 mCi/mmole) was purchased from American Radiolabeled Chemicals (St Louis, MO). Unlabeled E2, UDP glucuronic acid, magnesium chloride, ethanol, potassium phosphate monobasic, potassium phosphate dibasic, potassium hydroxide, hydrochloric acid, ethyl acetate, pyridine, sodium hydroxide, chlorosulfonic acid and acetic acid were obtained from Sigma-Aldrich. Triethylamine (Fluka); benzoyl chloride (Bayer); trisodium phosphate (Mallinkrodt, Paris, KY) were obtained from other sources. Acetonitrile (ACN) was obtained from EMD Chemicals (Gibbstown, NJ). Scintillation fluid EcoLite™ was obtained from MP Biomedicals (Santa Ana, CA). SPE cartridges Bond Elut™ C18 (6 g, 20 mL) and Sep-Pak® Vac C18 were obtained from Varian (Harbor City, CA) and Waters (Milford, MA), respectively.

Uridine 5'-diphospho-glucuronosyltransferase (UGT)

A castrated, cross-bred hog weighing 24.4 Kg was used as the source of the UGT enzymes, following USDA Animal Care and Use Committee guidelines. The hog was intramuscularly (2 d) then intraperitoneally dosed (2 d) with approximately 20 mg Kg^{-1} phenobarbital for four consecutive days, after which, the hog was euthanized. The liver was homogenized and microsomes were isolated via differential centrifugation. Proteins

were solubilized and immobilized onto Sepharose beads (Pallante et al., 1986) and were stored in a 1:1 suspension with 0.1 M Tris buffer (pH 7.4) at 4°C until use.

Liquid scintillation counting

Radioactivity was quantitated with a Packard 1900 CA scintillation analyzer (Downers Grove, IL), and samples were dissolved in EcoLite™ scintillation cocktail.

High-performance liquid chromatography

Analytical HPLC for E2-3-G was performed using a Waters 600E System Controller and pump (Milford, MA), equipped with a Jasco FP 920 fluorescence detector (Jasco, Easton, MD) with the following conditions: Phenomenex-C18, 4.6 × 250 mm, 5 µm; A: 10% ACN in 50 mM ammonium acetate (pH 4.5), B: 90% ACN in 50 mM ammonium acetate (pH 4.5); gradient: 20 to 100% B, 29 min, 100% B, 3 min hold, 1.0 ml/min, excitation and emission wavelengths of 280 and 312 nm, respectively. Prep-HPLC was performed on Jones Chromatography-C18, 10 × 250 mm, 5 µm; A: 5% ACN in 50 mM ammonium acetate (pH 4.5), B: 90% ACN in water; isocratic 85% solvent A, 15% solvent B; 4.7 mL/min.

For E2-17-S, analytical HPLC was performed on a Gilson System 45NC Gradient Analytical instrument (Gilson Medical Electronics, Middleton, WI) equipped with a variable wavelength UV detector with the following conditions: Radial-Pak-C18, 8 × 100 mm (Waters Associates, Milford, MA); A: 10:90 methanol/water, B: 90:10 methanol/water; gradient: 20% B to 100% B, 28 min., 4 min hold; 1.0 ml/min 220 nm. HPLC for E2-3-benzoate was conducted using following conditions: Radial-Pak-C18, 8 ×

100 mm; A: 10:90 methanol/water, B: 90:10 methanol/water; gradient: 20% B to 100% B, 30 min, 15 min hold; 1.0 ml/min, UV 220 nm.

Mass spectral analysis

Negative ion LC/MS was performed with a Waters Alliance 2695 HPLC (Symmetry-C18, 2.1 × 100 mm; A: 40% ACN in water, B: 60% ACN in water; gradient: 40 to 100% B, 10 min, 5 min hold, 0.2 mL/min), and a Waters Micromass QTOF (API-US in a ES- mode, MassLynx software, FWHM: 6500, source temperature 120°C, desolvation temperature 350°C, cone voltage 35 V, capillary voltage 2500 V, collision energy 5 eV for sulfate and 20 eV for glucuronide conjugates.

NMR spectra

A Bruker AM-400 spectrometer (Billerica, MA) operating at either 400.13 MHz or 100.61 MHz was used to record the ^{1}H- and ^{13}C-NMR spectra respectively. ^{1}H-NMR spectra were run in fully coupled mode with 128 scans and an acquisition time of 3.9713 s. ^{13}C-NMR spectra were run in CPD mode, with 64K scans obtained with an acquisition time of 1.307 s. The chemical shifts for the NMR spectra for E2 were ^{13}C NMR (MeOH-d_4) δ: 155.84, 138.8, 132.32, 127.22, 116.05, 113.72, 82.49, 51.26, 45.34, 44.35, 40.5, 38.00, 30.72, 30.68, 28.83, 27.53, 24.03, 11.71. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.06 (d), 6.53 (d), 6.47 (s) (Table 7 in Appendix I).

Synthesis of 17β-[4-^{14}C]estradiol-3-glucuronide

Five mL of 0.1 M phosphate buffer (pH 7.4) was added to 20 mL of pre-rinsed microsomal proteins immobilized on Sepharose beads. Forty microliter of 2.63 M magnesium chloride, 63 mg of UDP glucuronic acid (5 mM final concentration), and 164.7

μg of [^{14}C] labeled E2 (0.60 μmole; 33 μCi; dissolved in 567 μL ethanol) and 6477 μg of unlabeled E2 (23.78 μmole, dissolved in 540 μL ethanol) were added to the reaction flask. The reaction flask was slowly stirred on a Roto-Vap (Büchi, Flawil, Switzerland) without vacuum at 37°C for 24 h determined *a priori*. The enzymatic glucuronidation reaction is shown in Fig. 1. The aqueous fraction was collected by filtration. 17*β*-[4-^{14}C]estradiol-3-glucuronide was partially purified on a Bond Elut™ C18 SPE cartridge preconditioned with ACN and nanopure water by eluting with 20:80 ACN-water. The final radiochemical purity was 99% obtained after preparative HPLC. ^{13}C NMR (MeOH-d_4) δ: 176.52, 156.99, 135.66, 127.20, 117.96, 115.41, 102.65, 82.47, 77.71, 76.68, 74.74, 73.59, 51.26, 45.41, 44.32, 40.34, 38.97, 37.97, 30.69, 30.04, 28.40, 27.51, 24.00, 11.67. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.18 (d), 6.87 (d), 6.81 (s) (Table 7 in Appendix I). LC/MS-QTOF: M-H = 447.21, m/z 271.17, 175.03, 113.02 (Fig. 19 in Appendix I).

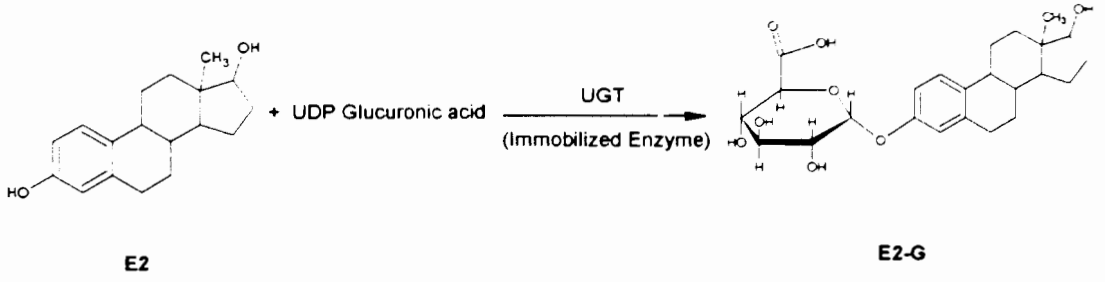

Figure 1. Glucuronidation of the hydroxyl group at C-3 of 17*β*-estradiol by uridine 5'-diphospho-glucuronosyltransferase (UGT).

Synthesis of 17β-[4-^{14}C]estradiol-17-sulfate

Synthesis of [^{14}C] E2-17-S consisted of a three-step chemical process that involved introducing a blocking group at the C-3 position of [^{14}C] E2, sulfation at C-17 position, and subsequent deblocking to yield the desired synthetic product (Fig.2).

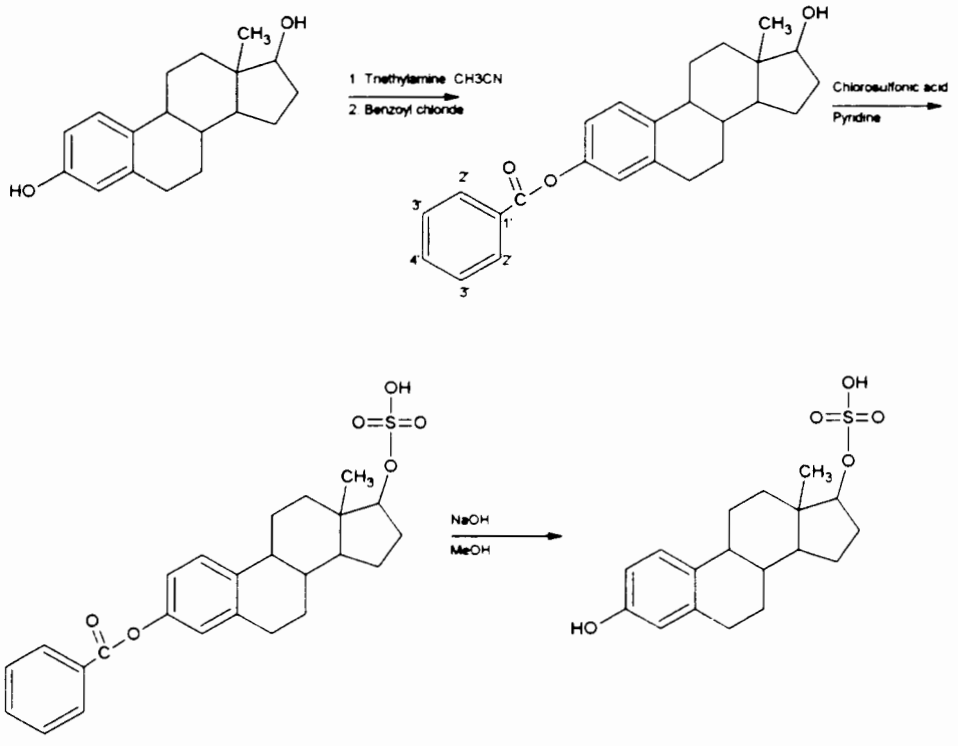

Figure 2. Chemical synthesis of [^{14}C]17β-estradiol-17-sulfate conjugate from [^{14}C]17β-estradiol.

[^{14}C]17β-estradiol-3-benzoate

Radiolabeled E2 (259.5 µg, 0.95 µmole, 47.7 µCi) was mixed with unlabeled E2 (11.43 mg, 42 µmole) in ethanol and the solvent was evaporated (Hooijerink et al., 2005). The residue was re-dissolved in 2 mL of acetonitrile, and 13 µL triethylamine and 11 µL benzoyl chloride (13.3 mg, 94.7 µmole) were added; the reaction mixture was stirred at

room temperature for 2 h and subsequently dried under a stream of nitrogen. To the resulting residue, 4 mL of 0.1 M trisodium phosphate solution was added and the mixture was sonicated for 30 min resulting in a light yellow suspension. The suspension was extracted with ethyl acetate (3 mL × 3), and the organic solvent was evaporated under a stream of nitrogen. The residue (E2-3-benzoate) was dissolved in ethyl acetate (3 mL) and water (1 mL) for further purification using HPLC. The yield of E2-3-benzoate was 59.3% and radiochemical purity was 98%. ^{13}C NMR (MeOH-d_4) δ: 165.94, 150.13, 139.49, 139.38, 134.86, 130.99, 130.99, 129.83, 129.83, 127.47, 122.63, 119.79, 82.45, 51.33, 45.55, 44.34, 40.14, 37.99, 30.69, 30.56, 28.27, 27.48, 24.04, 11.68. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.44 (d), 6.94 (d), 6.89 (d); δ (benzoate protons): 8.14 (d), 7.66 (dd), 7.54 (dd). LC/MS-QTOF: M-H = 375.21, m/z 361.21, 356.85, 334.82, 332.82.

[^{14}C]17β-estradiol-3-benzoate-17-sulfate

Sulfur trioxide-pyridine complex was synthesized in-house (Itoh et al., 1999) by adding chlorosulfonic acid (138 µL, 2.07 µmoles) with stirring to dry pyridine (1.66 mL) at 0°C. The solution was allowed to warm to room temperature, followed by dilution with dry pyridine (623 µL). 17β-estradiol-3-benzoate was dissolved into 1.1 mL of pyridine, and the solution was heated to 50°C, to which sulfur trioxide-pyridine complex, also heated to 50°C, was added. The mixture was stirred for 30 min at 50°C followed by solvent evaporation under nitrogen, addition of water (4 mL), and adjusting to pH 8 (1 M NaOH). The mixture was partially purified with a Sep-Pak® Vac C18 cartridge and E2-3-benzoate-17-sulfate eluted with methanol. ^{13}C NMR (MeOH-d_4) δ: 166.95, 150.11, 139.42, 139.27, 134.95, 130.85, 130.85, 129.89, 129.89, 127.52, 122.64, 119.84, 88.22, 50.78, 45.41,

44.23, 40.29, 37.98, 30.53, 29.22, 28.18, 27.47, 24.11, 12.24. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.30 (d), 6.90 (d), 6.86 (s); δ (benzoate protons): 8.13 (d), 7.66 (dd), 7.52 (dd). LC/MS-QTOF: M-H = 455.10, m/z 351.12.

17*β*-[4-^{14}C]estradiol-17-sulfate

Hydrolysis (Kirdani, 1965) of E2-3-benzoate-17-sulfate was accomplished by adding 5% NaOH in methanol (5 mL), stirring for 1 h at room temperature, then neutralization with 10% acetic acid, and evaporation under nitrogen. After purification by HPLC, 21 μCi (18.9 μmoles; 7.1 mg; 98% pure) of E2-17-S was obtained (overall yield: 44%). ^{13}C NMR (MeOH-d_4) δ: 155.89, 138.76, 132.54, 127.26, 116.04, 113.76, 88.19, 50.78, 45.3, 44.24, 40.34, 38.00, 30.71, 29.22, 28.48, 27.48, 24.10, 12.19. ^{1}H NMR (MeOH-d_4) δ (aromatic A-ring protons): 7.06 (d), 6.53 (d), 6.47 (s) (Table 7 in Appendix I). LC/MS-QTOF: M-H = 351.07, m/z 96.96.

Results and Discussion

Synthesis of 17*β*-[4-^{14}C]estradiol-3-glucuronide

A one-step enzymatic synthesis of E2-3G is described that permitted regioselective attachment of a glucuronide acid moiety to E2. Since the reaction occurred in a buffered solution, reaction progress (Scheme 1, as shown in Fig. 1) could be readily followed by reversed-phase HPLC. The radiolabeled parent peak (E2) at 27.57 min dropped steadily in intensity while the increase in peak intensity at 5.55 min occurred for the desired product (E2-3G) (Fig. 3). The reaction was essentially complete by 24 h. C-18 SPE purification yielded a radiochemical purity of 95%; semipreparative HPLC improved radiochemical purity to 99%. LC/MS-QTOF analysis of the peak at 5.55 min showed ions at m/z 447.21,

271.17, 175.03, and 113.02, representing the molecular ion of E2-3G and ions of E2, glucuronic acid, and a glucuronide fragment, respectively (Fig. 19 in Appendix I).

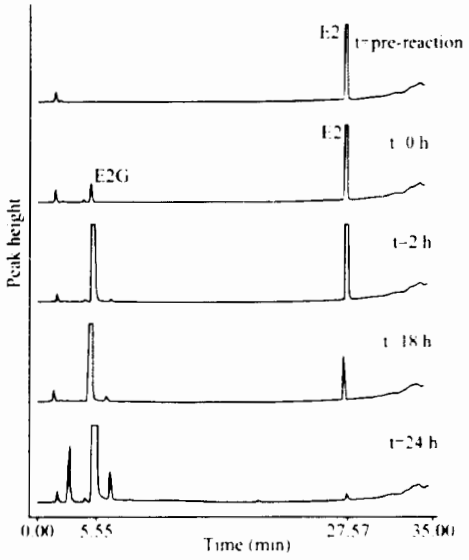

Figure 3. Progress of enzymatic synthesis of 17β-estradiol-3-glucuronide (E2-3G) with time and the concurrent consumption of 17β-estradiol (E2).

To determine the site of conjugation, ^{13}C-NMR spectra of E2 and E2-G were compared to each other and with literature values of E2 (Dionne et al., 1997; Kashima et al., 2010) and bisphenol A glucuronide (Kurebayashi et al., 2003). Glucuronidation was indicated by the presence of an additional 6 carbons in the ^{13}C-NMR spectrum of E2-G; and the site of conjugation was indicated by the downfield shift of C-3 from 132.32 to 135.66 ppm in the spectrum of E2-G (Table 7 in Appendix I). Chemical shifts in the ^{1}H-NMR spectrum of E2-G were also consistent with glucuronidation at C-3. For example, protons ortho and meta to C-3 were shifted downfield 6.53 to 6.87, 7.06 to 7.18, and 6.47 to 6.81 ppm) for E2 and E2-G, respectively (Table 7 in Appendix I). In addition, as one of the most diagnostic

components of a sugar conjugated spectrum, an anomeric singlet at 4.30 ppm also confirms the formation of E2-3-G. Chemical shift assignments for the C-17 remained invariant for E2 and E2-3-G.

Diglucuronide conjugation was theoretically possible due to two hydroxyl groups in E2, one a phenolic in the A-ring, and the other an aliphatic on the D-ring. However, only one site of conjugation was expected because enzyme-catalyzed reactions are usually regio- and stereospecific (Alonen et al., 2009). UGT enzymes are divided into two distinct subfamilies, UGT1 and UGT2 (Kiang et al., 2005; King et al., 2000). Phenobarbital treatment of hepatoma cell lines is known to induce hepatic bilirubin UGTs (Brierley et al., 1996), which show a strong selectivity for phenolics (Lepine et al., 2004).

Product yield of E2-3-G was 84%, and was attributed to the induction of UGT's by Phenobarbital (Watanabe and Yoshizawa, 1982). The same microsomal proteins also were active at glucuronidating hydroxylated polybrominated diphenyl ether metabolites, triclosan, and ractopamine hydrochloride.

Synthesis of 17β-[4-^{14}C]estradiol-17-sulfate

The synthesis of E2-17-S, presented in Scheme 2 (as shown in Fig. 2), was initiated by blocking the more reactive C-3 hydroxyl in E2, which was accomplished with a 59.3% product yield to form the intermediate E2-3-benzoate. The negative ion LC/MS analysis of E2-3-benzoate resulted in a molecular ion at 375.21, a methyl loss fragment at 361.21, and a water loss at 356.85. Losses of propanyl and propenyl groups were consistent with fragments at m/z 332.82 and 334.82, respectively. ^{1}H-NMR analysis of E2 and E2-3-benzoate indicated shifts in protons ortho- and meta- to C-3 occurred in E2-3-benzoate relative to E2 (6.53 to 6.94; 7.06 to 7.44; 6.47 to 6.89 ppm) (Table 7 in Appendix I).

Benzoate protons were present at 8.14, 7.54, and 7.66 ppm of E2-3-benzoate. The ^{13}C-NMR spectrum confirmed that the blocking had occurred at C-3 because carbons ortho- and meta- to C-3 of E2-3-benzoate were shifted downfield relative to their chemical shift position in E2 (113.72 to 119.79; 132.32 to 134.86; 116.05 to 122.63 ppm) (Table 7 in Appendix I).

The formation of E2-3-benzoate-17-S was confirmed by a molecular ion at 455.09 in the LC/MS spectrum, and was accompanied by a prominent fragment at m/z 351.12, which was consistent with a benzoate fragment loss. Sulfation at C-17 was suggested by significant downfield chemical shifts for the C-17 proton (3.67 to 4.31 ppm) and carbon (82.45 to 88.22 ppm) in the ^{1}H and ^{13}C NMR spectra of E2-3-benzoate andE2-3-benzoate-17-S, respectively. A radiochemical purity of 95% was achieved and was considered satisfactory for the next step.

The purification of the final product (E2-17-S) yielded 21 μCi (18.9 μmoles; 7.1 mg) of 98% radiochemical purity. The formation of E2-17-S was confirmed by a molecular ion at 351.07 and the sulfate moiety ion at m/z 96.96 in the LC/MS spectrum of E2-17-S. ^{13}C NMR analyses of E2-17-S indicated a significant upfield chemical shift of C-3 relative to E2-3-benzoate-17-S (132.54 from 134.95 ppm), as well as for carbons ortho to C-3 (116.04 from 122.64; 113.76 from 119.84 ppm). Upfield shifts of the aromatic protons between E2-17-S and E2-3-benzoate-17-S were observed (6.53 from 6.90; 7.06 from 7.30; 6.47 from 6.86 ppm), but no chemical shift difference was observed for the C-17 proton (Table 7 in Appendix I). Collectively, the physical data provide convincing evidence that sulfation of E2 had occurred at C-17. The overall yield of E2-17-S was 44%, which possibly could

have increased if the reaction conditions had been optimized; however optimization was not an immediate objective.

Conclusions

[^{14}C]Radiolabeled 17β-E2-3-G and 17β-E2-17-S were successfully synthesized using enzymatic and chemical approaches, respectively, which permitted their use for laboratory scale fate and transport experiments in soil-water systems. Though the current study objective was measuring and modeling the movement of endocrine disrupting compounds in the environment, these studies are but a small portion of the potential studies in which radiolabeled conjugates could be used. Since glucuronidation and sulfation are the major conjugation pathways in vertebrates for not only steroid hormones, but other xenobiotics (Dutton, 1980), it is hypothesized that radiolabeled glucuronides and sulfates of other emerging contaminants can also be synthesized following the approaches provided in this paper, or with appropriate modifications of them.

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PAPER 2. A HIGH MASS RECOVERY ANALYTICAL METHOD FOR FATE AND TRANSPORT STUDIES OF LABILE ESTROGENS

Abstract

The aim of this research was to present a suit of analytical methods that provide simultaneous analysis of a glucuronide conjugate of estrogen, 17β-estradiol-3-glucuronide (E2-3G), and its metabolites under the context of an environmental fate and transport study in a complex matrix. The challenge for these types of studies is achieving adequate quantification and qualifications of labile compounds in complex matrices while maintaining excellent mass balances. A radioassay technique was used to track the estrogen conjugate and its metabolites in the aqueous, sorbed, and gaseous phases in a matrix of soil and water. An analytical method was developed to separate E2-3G and its metabolites using high performance liquid chromatography (HPLC). Liquid scintillation counting (LSC) was used to quantify the distribution of E2-3G and its metabolites in the aqueous and reversibly sorbed phases. Reversibly and irreversibly sorbed fractions were accounted for by solvent extraction and a sample oxidizer, respectively, followed by LSC, while gas chromatography followed by LSC was used to measure radioactivity in the gas phase. Mass spectral analysis using liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to confirm the E2-3G metabolite peaks eluted from the HPLC. The combined HPLC-LSC method was capable of simultaneously quantifying the study compound, E2-3G, and its metabolites, estrone-3-glucuronide (E1-3G), 17β-estradiol (E2) and estrone (E1). Estriol (E3) was not detected, but could be used with this method. The total mass recovery achieved from the aqueous and bound phases ranged from 99.0 to

105.5 % for all eight time points throughout 336 h. The limits of quantitation (LOQ) were 0.24±0.03 μg L^{-1} for E2-3G and E1-3G and 0.15±0.02 μg L^{-1} for E2 and E1 in the aqueous phase. The sorbed phase LOQs were 1.22±0.17, 1.21±0.17, 0.74 ±0.10, and 0.73 ±0.10 μg Kg^{-1} for E2-3G, E1-3G, E2, and E1, respectively.

Introduction

Natural and synthetic steroidal estrogens have been frequently detected in the environment (Kolpin et al., 2002) and pose a great concern because of their high potential to disrupt the endocrine system of aquatic organisms (Routledge et al., 1998) at concentrations as low as 1 ng L^{-1} (Hansen et al., 1998). There is a need for methods to study these labile compounds and their metabolites to understand their fate and transport in the environment (Gorog, 2011). However, the labile nature of estrogens, and their conjugates, poses many analytical challenges that make studying them difficult.

Gas chromatography (GC) has been a popular analytical technique for organic pollutants, but its use is mainly suited for non-polar and moderately polar organic compounds. Although polar compounds such as alkylphenols and steroid sex hormones have been analyzed using GC methods (Pacakova et al., 2009), derivatization of steroid compounds is usually required to improve the stability of the analytes (Gabet et al., 2007). Also, direct analysis of conjugated steroids is not possible using the GC technique without hydrolyzing the conjugates to their free forms. The difference of GC derived concentration values before and after the hydrolysis step is presumed to be contributed from the conjugate. High performance liquid chromatography (HPLC) in combination with photo spectrometric (PS) detectors has been used as a cheaper alternative to the more sensitive

mass spectrometers (MS) (Ingerslev and Halling-Sørensen, 2003). Use of HPLC will eliminate the need of derivatization as well as the hydrolysis of the conjugates, although some researchers have preferred to hydrolyze the conjugates (Mao et al., 2004) perhaps because there was insufficient separation between the conjugates and free estrogens. However, matrix effects can affect the resolution of the analytes, especially when extracting from a complex matrix, such as soil (Tso et al., 2011). Analytical advances using LC mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) have also been utilized in the environmental analysis of estrogens, which offer detection limits in the low ng L^{-1} range. However, the higher costs of these instruments pose constraints. Additionally, even these high-end analytical tools cannot be used to quantify what is irreversibly bound on soil or what has been converted to gas (Fan et al., 2007).

Use of alternative techniques, such as radioassay, may provide solutions to the analytical problems and allow detailed experiments in the laboratory that can discern the complicated fate and transport processes. Liquid scintillation counting, a method to quantitate radioactivity, is not subject to matrix issues, unlike LC MS techniques. No other analytical methods can directly quantify what is irreversibly sorbed and what is converted to gas via methanogenesis or mineralization (Fan et al., 2007). Since all major fate dispositions (i.e., dissolved, bound, and gaseous) are directly quantifiable with radiological methods, an excellent mass balance closure can be achieved. Aqueous phase radioactivity can be measured directly using LSC. Reversibly bound fractions, extracted using organic solvent, can also be quantified using LSC. The irreversibly bound fractions can be measured by combusting soil samples in a oxidizer, trapping the radioactive CO_2, and conducting LSC on the trapped CO_2 (Zitnick et al., 2011). Gas phase (mineralized) fraction

of the labile estrogen can be quantitated by sampling head-space gas and either by trapping the radioactive CO_2 gas followed by LSC (Fan et al., 2007), or by analyzing air samples with GC followed by LSC.

There is, however, a major drawback of the radioassay method in regard to speciation analysis. Even though thin layer chromatography (TLC) can be used for speciation information (Fan et al., 2007), it requires high concentrations (Zitnick et al., 2011) compared to other method (e.g. HPLC), and it is often difficult to obtain good quantification of all metabolites. Combination of HPLC separation capability with the quantitation capability of LSC can provide a very convenient laboratory method to speciate and effectively track metabolites. Furthermore, HPLC and LSC are relatively inexpensive and common equipment, where GC- or LC-MS/MS are more expensive and less available.

There is a need for a simple laboratory method that can provide a complete mass balance as well as detailed speciation of labile emerging contaminants, so that critical fate and transport experiments can be conducted with ease. Even though the combinations of radioassay with HPLC have been used for some environmental contaminants such as pesticides (McDonald et al., 2006), such a method has not been developed in case of highly labile sex hormones, and especially for estrogen conjugates. In this study, a suit of analytical methods with an excellent mass recovery and complete speciation capabilities is presented to investigate the fate of a glucuronide conjugated estrogen, E2-3G, in an agricultural soil-water system.

Material and Methods

Reagent and chemicals

The study compound 17β-[4-^{14}C]-estradiol-3-glucuronide (E2-3G) (99% radiochemical purity; specific activity 103.13 Bq μg^{-1}) was synthesized using radiolabeled and unlabeled E2 and other chemicals as described by Shrestha et al. (2011). Acetonitrile, ammonium acetate, hydrochloric acid, calcium chloride dihydrate and formaldehyde (all analytical grades) were purchased from Sigma-Aldrich and were used as received. Carbo-Sorb E and Permafluor were purchased from PerkinElmer (Waltham, MA) and scintillation cocktail, Ecolite, was obtained from MP Biomedicals (Santa Ana, CA). All aqueous solutions were prepared using analytical reagent grade nanopure water.

Individual as well as composite standards of E2-3G, E2, and E1, were prepared in methanol ensuring enough mass to produce satisfactory detection. The standard had mass concentrations of 0.73, 0.50, and 2.5 ng μL^{-1} and 5, 182, and 1045 dpm μL^{-1} radioactivity for E2-3G, E2, and E1 respectively. A 50 µL injection volume was used as the standard volume. The standards were stored at -20° C when not used.

Batch soil experiments

The laboratory methods presented in this study are in the context of a soil batch studies, but can be extrapolated to other media, such as sediment, manures, or biosolids. The soil used for the batch studies came from the surface horizon (0 – 6 cm) of a Hamar series soil (sandy, mixed, frigid typic Endoaquolls). The sand:silt:clay distribution was 83:10:7, organic carbon content was 1.35%, pH was 7.0, and CEC was 9.3 meq $100g^{-1}$. The soil was air-dried and sieved through a 2 mm sieve and stored in a jar until used.

The mass balance of the total radioactivity in the soil-water batch experiments took into consideration the radioactive fractions in gaseous and aqueous phases, as well as the reversibly and irreversibly sorbed phase fractions. Figure 4 presents the mass balance schematic along with the analytical techniques used to quantify each compartment.

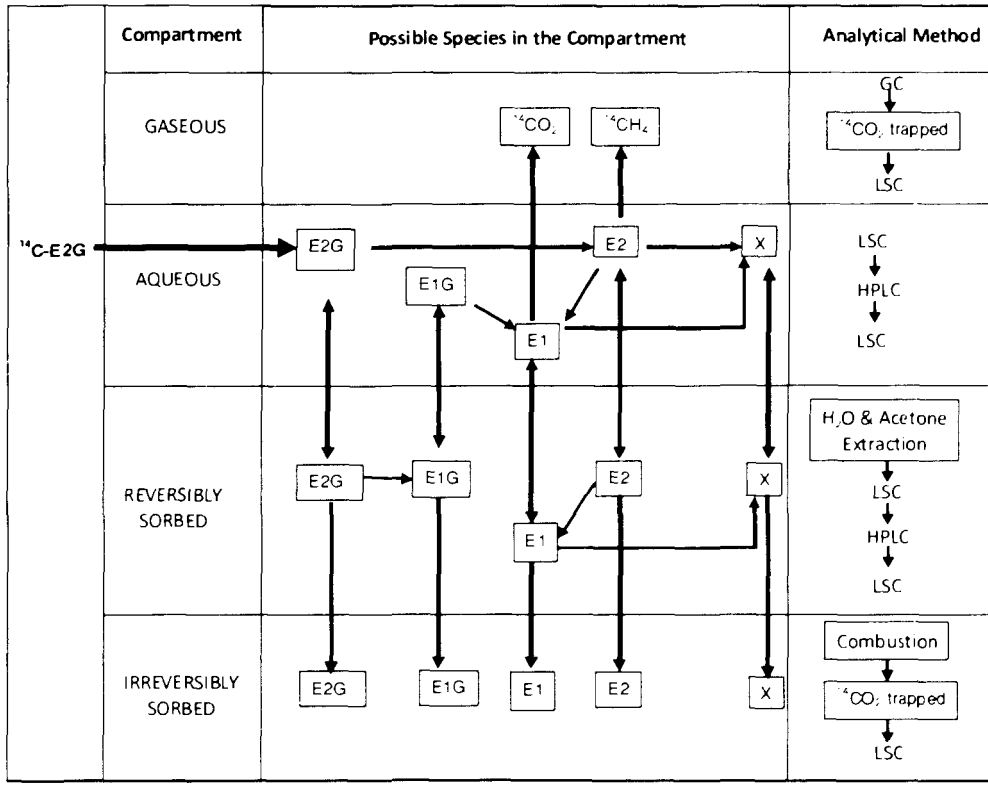

Figure 4. Mass balance and analytical methods used in each compartment. Possible metabolites of E2-3G (abbreviated as E2G) are shown in each compartment; X is unidentified metabolites registering radioactivity. Radiolabeled E2-3G is administered to the aqueous phase of the soil-water slurry to initiate the batch study.

Radiolabeled E2-3G was spiked into vials containing 1.6 g of soil and 8 mL of 0.01 M $CaCl_2$ in each vial, to attain a final concentration of 3.7 $\mu g\ mL^{-1}$. Three vials were designated for aqueous phase sampling through time, while additional 7 vials (hereafter referred to as 'stop' vials) were devoted for the analysis of sorbed phase radioactivity by

destructive sampling of one vial at each sampling time. Method blanks contained no analyte (E2-3G), while control blanks contained no substrate (soil) and were dosed at 0.5 mg L^{-1} to check the stability of the analyte during the experimental period. Triplicate dosed vials, method blanks, and control blanks, along with seven 'stop' vials were agitated (360° every 5 seconds) on a rotor and were stopped briefly to take samples at 4, 8, 24, 48, 72, 168, and 336 h.

At each sampling time, the vials were removed from the rotor and centrifuged at 1700 rpm (380×g) for 20 min. One of the 'stop' vials was also removed and retired at each sampling time. From the triplicate vials, a 100-µL aliquot and 120 µL duplicate aliquots were taken from each vial using sterile syringes, for the analysis of the bulk radioactivity and speciation (metabolite formation), respectively, in the aqueous phase. The 120 µL duplicate aliquots were filtered through a 0.45 µm PTFE glass filter, to which 37 µL of 37% (13.3 M) formaldehyde (2.7% final volume) was added to inhibit any microbial activity, and were stored at -20°C until analysis. Liquid scintillation counting was used to analyze the bulk radioactivity, while HPLC and LSC were used to investigate the speciation in the aqueous phase (Fig. 4).

The aqueous phase from the retired 'stop' vial was separated from the soil to analyze the sorbed phase radioactivity. To preserve the samples, formaldehyde was applied to the aqueous and soil separates (2.7% final volume) and the samples were then stored at -20°C until analysis. Water and acetone extraction, followed by analysis with LSC and HPLC were used to analyze reversibly sorbed phase radioactivity and speciation (Fig. 4). Irreversibly sorbed radioactivity was measured by combusting sample soil from the 'stop' vials that had already been extracted with water and acetone.

To analyze the gas phase radioactivity, the headspace of the retired 'stop' vials and the triplicate dosed vials were sampled (500 µL). 'Stop' vial headspace was sampled immediately after the vial was retired, while the triplicate dosed vials were sampled at the end of the batch study. The air samples were analyzed with GC and LSC (Fig. 4).

Analytical techniques

Liquid scintillation counting (LSC)

A 1900 CA scintillation counter (Packard, Downers Grove, IL) was used for LSC with EcoLite scintillation cocktail. The LSC was calibrated with ^{14}C standards before use. For each sample, 100 µL in the case of bulk radioactivity analysis and 1 mL for fraction collected HPLC eluent, 4 mL of the scintillation fluid were added in a 5-mL HDPE scintillation vial. The vials were capped, shaken (~10 seconds in a vortex shaker), and stored overnight in the dark to stabilize the effects of induced chemiluminescence and photoluminescence. The vials were wiped with an antistatic sheet before loading into the LSC to minimize static charge build up on the plastic vials due to dry weather or by handling with latex gloves. Sample vial activity was counted for a period of 10 min. Measured activity was corrected for background activity in blank vials. The background level of radioactivity was measured every day with 5 scintillation vials containing 4 mL of the scintillation cocktail.

High performance liquid chromatography (HPLC)

The HPLC comprised of a Waters 600E System Controller and pump (Milford, MA), a C18 reverse phase analytical column (Phenomenex, 4.6 × 250 mm, 5 mm), a Jasco FP 920 fluorescence detector (Jasco, Easton, MD), a Waters 717 Plus auto-sampler , and a Waters

746 Data Module integrator. The HPLC was coupled with a fraction collector (Gilson FC 204, Middleton, WI). Both ultraviolet (UV) and fluorescence (FLD) detectors were investigated for suitability, with an on-column injection of 40 ng of the study compound. Initial settings for the UV and the FLD wavelengths were selected from the published literature but were later modified to suit our experimental conditions. The column temperature was maintained at 25°C during analysis.

A gradient elution was adopted based on the hydrophobicity of E2-3G and its expected metabolites, E2 and E1. Various gradients were tested to allow the analysis of both the conjugate and the expected free metabolites in a single run. Mobile phases used for gradient elution comprised of solvent A (90% 50 mM ammonium acetate at pH 4.5 and 10% acetonitrile), and solvent B (90% acetonitrile with 10% 50 mM ammonium acetate at pH 4.5). Both the solvents A and B were degassed daily by helium sparging at a gas flow rate of 30 mL min^{-1} for 30 min prior to use with the HPLC. A flow rate of 1.0 mL min^{-1} with an operating pump pressure of approximately 3.98 MPa was used. Run times were systematically adjusted based on mobile phase responses. At least one injection of mobile phase was made to remove co-extractives from the injector and the column before a standard was injected.

Peaks in the chromatogram were synchronized with fraction collection by correcting the lag time between the column and fraction collector. Using LSC of the fractions collected from a radioactive injection bypassing the column, a lag time of 0.6 min was determined for the conduit system. Interference in the target peak of E2-3G from 0.01 M $CaCl_2$ solution, soil solution matrix, and the bactericide formaldehyde were also tested, but were determined to be negligable.

Analysis of reversibly sorbed fraction

Soil bound radioactivity (i.e. radiolabeled E2-3G or its metabolites) on the reversible sorption locations was extracted from the soil with nanopure water (3×4 mL) and acetone (3×4 mL) using a sonication bath for 30 min followed by centrifuging at 380 × *g* for 30 min. The nanopure water and acetone from the extractions were decanted to separate vials and 500 µL aliquots were analyzed in triplicate for bulk radioactivity in the LSC.

Calculated volumes of extracted supernatants (bulk radioactivity > 200 dpm) were evaporated in gentle stream of nitrogen, reconstituted in 50:50 acetonitrile and nanopure water, and filtered through a 0.45 µm filter to prepare for HPLC analysis.

Quantification of irreversibly sorbed fraction

To quantify the irreversibly sorbed phase fraction (i.e. unextractable radioactivity bound to soil), activity in the water-and-acetone-extracted soil residues were determined by total sample oxidation using a Packard 307 sample oxidizer (Packard Chemicals, Meridan, CT). The soil residues were completely air-dried under a hood for a week. Five replicates of 0.1 g of soil from each sample were weighed in paper combustion cones, to which 100 µL of Combustaid was added to enhance combustion, and then the samples were capped with combustion pads for analysis in the sample oxidizer. Trapping efficiency of ^{14}C from the combustion process was established before sample combustion. The recovery of duplicate aliquots of ^{14}C-Spec-chec solution combusted in the oxidizer was established to be greater than 98%. Blanks were also run before and after the sample combustion. Resulting $^{14}CO_2$ gas from the sample combustion was eluted from the sample oxidizer with 8 mL of Carbosorb E, which was then combined with 12 mL of Permafluor scintillation

cocktail in 20 mL glass scintillation vials. The vials were capped, shaken, and stored overnight before counting for the activity in the LSC. Samples were counted with automatic background deduction.

Gas chromatography (GC)

Gas phase radioactivity due to possible methanogenesis or mineralization of E2-3G (or its metabolites) was investigated by GC analysis of the head-space samples. The GC was an HP 5790A gas chromatograph (Avondale, PA) and the column was a 10-foot glass column (1.5 mm id and 7.0 mm od) packed with 3% OV-17. Temperature gradient was 30°C at the initial condition, hold for 2 min, then raised to 180°C with a ramping rate of 30 deg min^{-1}, and held at 180°C for 5 min. Five hundred microliter of the head-space air sample was injected into the GC. The effluent of the column was split so that 42% would be directed to the flame ionization detector for the determination of chemical mass, and the remaining 58% was directed into a 740°C oven containing Cu(II)O, which converted the [^{14}C]methane into [^{14}C]CO_2. The radiolabeled carbon dioxide was trapped by Carbo-Sorb E (8 mL), then diluted with of Permafluor (12 mL) and counted for radioactivity by LSC. The integrating recorder was an HP 3390A.

Liquid chromatography-tandem mass spectrometry verification

To identify metabolite peaks resulting from the transformation of E2-3G, LC-MS/MS analysis was employed. A Waters Alliance 2695 HPLC system coupled to a Waters Micromass quadrupole time-of-flight (Q-TOF) tandem mass spectrometer was used to identify the metabolites of E2-3G in this study. A Waters 2996 photodiode array detector was used in the HPLC. The LC separation was carried out using a reverse phase Symmetry

C18 column (3.5µm, 2.1× 100 mm) equipped with a 2.1×10 mm guard column. Five to 20 µL of each sample was injected onto the column. The mobile phase, operating at a flow rate of 0.2 mL min^{-1}, consisted of nanopure water and acetonitrile at 60:40 v/v as solvent A and 40:60 v/v as solvent B. A linear gradient from 40-100% solvent B in 10 min, a 5 min hold at 100% B, and return to 40% solvent B in 0.1 min was used to resolve the compounds. A 9.9 minute equilibration time was provided before a new injection was made. The column effluent flow was split 3:1 to waste and to the MS/MS system.

The Q-TOF tandem mass spectrometer (QTOF API-US) was equipped with electrospray ionization (ESI). Negative ionization (NI) mode was used for this study since NI is generally a method of choice for estrogen detection by LC/ESI MS-MS (Díaz-Cruz et al., 2003). The Q-TOF operating conditions were as follows: a source temperature of 120°C, a desolvation temperature of 350°C, a capillary voltage of 2500 V, and a cone voltage of 35 V. The TOF provided accurate mass measurement (within 2mDa) of collision induced fragments of quadrupole-selected parent ions, at a moderately high resolving power at full width at half maximum of 6500. Data were acquired in the continuum mode with a mass range of *m/z* 100–500 Da and a scan time of 0.1 s, and processed using MassLynx v.4.2 software. The MS/MS acquisitions were performed with a collision energy of 20 eV, and then at 50 eV to allow both identification of small neutral losses and the observation of diagnostic fragment ions.

Results and Discussion

Simultaneous analysis of conjugate and free estrogens

Both UV and fluorescence detectors were investigated for better detection of E2-3G and free estrogens E2 and E1. Although UV detection is almost exclusively used to analyze steroid hormone formulations in drugs, fluorescence detection is reported to offer high sensitivities for estrogens possessing native fluorescence, such as ethinylestradiol (Gorog, 2011). Most of the estrogens are reported to have a weak native fluorescence (Mao et al., 2004; Wang et al., 2011), requiring fluorescent derivatization prior to analysis, such as pre-column derivatization with p-nitrobenzoyl chloride (Mao et al., 2004). In our study, 40 ng of E2-3G and E2 produced satisfactory peaks in the chromatogram; however, the FLD response of estrone was low due to its lower efficiency of native fluorescence (Bramhall and Britten, 1976). Since quantitation was based on the scintillation counting of trapped radioactive peaks, sufficient FLD response for the E1 peak in the HPLC chromatograph was not an absolute requirement. Satisfactory peak detection was obtained with an injection of 36.5, 25, and 125 ng for E2-3G, E2, and E1, respectively, without fluorescent derivatization (Fig. 5-A). Bravo et al. (2005) also successfully analyzed E2 with intrinsic fluorescence using HPLC flow injection.

Fluorescence detection was chosen because it demonstrated less matrix interference compared to the UV detection method. The FLD excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 280 and 312 nm, respectively, were sufficient for the detection of E2-3G and free estrogens. These values were close to Mao et al. (2004) (λ_{ex} and λ_{em} at 282 and

315 nm, respectively), who analyzed free estrogens including E2 and E1; however, they enhanced the native fluorescence by derivatization with p-nitrobenzoyl chloride.

A gradient elution scheme was adopted due to the wide variation in the hydrophobicity of the compounds. Some researchers have opted for two different isocratic elution schemes to be used separately for either analyzing the conjugate or the free estrogens. Gatti et al. (1998), for example, used triethanolamine (TEA) phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (70:30, v/v) at a flow rate of 1.0 mL min^{-1} for conjugated estrogens, while for free estrogens the mobile phase composition was TEA phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (66:34, v/v) at a flow rate of 1.3 mL min^{-1}. The mobile phase gradient of this study was optimized in a systematic manner for each of the compounds using standards of E2-3G, E2, and E1, and then with a composite standard of all three estrogens. Solvent elution was also tested with different gradient shape (linear, concave) in order to resolve the expected hydrophobic metabolites (E2 and E1) with reasonable retention times, and at the same time capture the early eluting E2-3G at a retention time in excess of the solvent front. At the selected excitation and emission wavelengths, the optimum separation among E2-3G, E2 and E1 were achieved with the gradient: 20% to 100% B in 29 min in concave gradient (shallow at first, steep towards the end; curve number 7), 100% B for 3 min, and a linear return to 20% B over 3 min, all at 1.0 mL min^{-1}. For the HPLC run of 35 min, the elution times for E2-3G, E2, and E1 are presented in Figure 5-A.

The HPLC results showed an unknown peak at 8.40 min in samples collected at 4 h (Fig. 22 in Appendix II) and 8 h of the batch study. Figure5-B presents a typical aqueous phase speciation in samples collected at 8 h. The FLD response was poor, even though the radiochromatogram showed considerable mass. Since this was consistent with the low FLD

response observed with E1, we suspected this peak to be the estrone glucuronide. Further, the elution time of this peak near E2-3G indicated it may be a conjugate as well. Accurate mass of the M-H ion for this peak was obtained using the Q-TOF. A pooled sample of the unknown from multiple replicate samples was evaporated to dryness, and reconstituted in 50:50 acetonitrile and water to yield a concentration of 0.5 ng L^{-1}. Other HPLC peaks that corresponded to the E2-3G, E2, and E1 standards were also collected by HPLC methods and prepared for MS/MS verification.

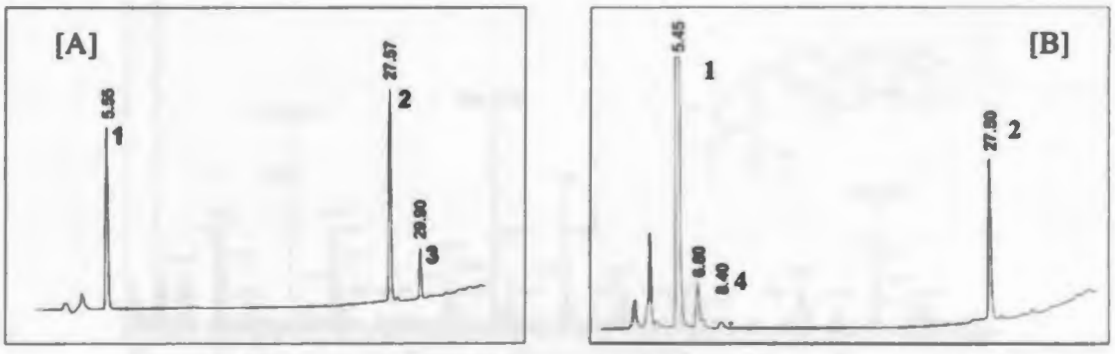

Figure 5. Chromatographic separation of the standard, and a typical chromatogram of speciation of an aqueous sample taken at 8 h from the batch study. The left panel shows standard elution of 17β-estradiol-3-glucuronide (peak 1) and its expected metabolites 17β-estradiol (peak 2), and estrone (peak 3) with on-column mass of 36.5, 25, and 125 ng, respectively. The panel on the right shows speciation of an 8 h aqueous sample in the batch study with an unknown radioactive peak (peak 4) at 8.40 min.

Compound identification/verification

The E2-3G metabolites that eluted between 27-29 and 29-31 min coincided with elution times for E2 and E1, respectively. They were characterized using LC-QTOF mass spectral analysis. The 27-29 min fraction showed ions at m/z (271, 183, 145) representing the molecular ion of E2, and two prominent fragments respectively. The 29-31 min fraction

was identified as E1, with ions at m/z (269.17, 183, 145), corresponding to the molecular ion of E1, and the same two prominent fragment ions.

The radioactive peak at 8.40 min in the HPLC chromatogram was identified as E1-3G, with a mass spectrum consisting of ions at 445.21 (molecular ion of E1-3G), 269.17 (molecular ion of E1), 175.03 (glucuronic acid), and 113.02 (a glucuronide fragment), respectively (Fig. 6).

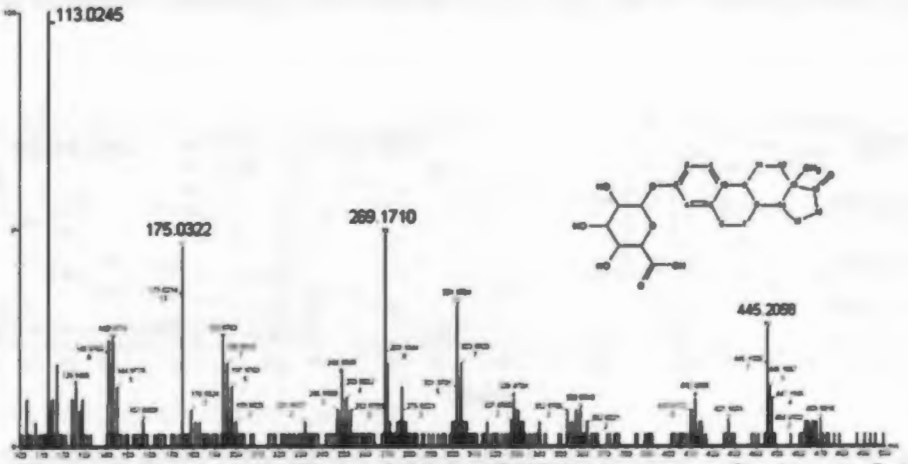

Figure 6. Quadrupole Time of Flight (QTOF) spectrum of unknown 8.40 min peak in the HPLC of 8 h aqueous sample in the batch study.

Mass balance

Combining the HPLC separation of the analyte and liquid scintillation counting of the HPLC fractions allowed for the tracking of the E2-3G and its metabolites with ease. The limit of quantitation (LOQ) was defined as 3 times the standard deviation of the background noise using repetitive counting of the 4 mL of scintillation cocktail. The aqueous phase LOQ were 0.24±0.03 $\mu g\ L^{-1}$ for E2-3G and E1-3G, and 0.15±0.02 $\mu g\ L^{-1}$ for E2 and E1. The sorbed phase LOQ were 1.22±0.17, 1.21±0.17, 0.74 ±0.10, and 0.73 ±0.10 $\mu g\ Kg^{-1}$ for E2-3G, E1-3G, E2, and E1, respectively.

The time course results of E2-3G and its metabolites for aqueous and sorbed phases based on the HPLC-LSC analyses are presented in Figure 7. The matrix effects from soil did not affect our results using the HPLC-LSC combined analysis. The HPLC chromatographic peaks were used only to associate the radioactivity measured in each fraction-collected vial. Since no quantitative information was derived from the chromatograms, no calibration or validation studies were performed. However, as a quality control, the standards containing radiolabeled E2-3G, E2, and E1 were run every day.

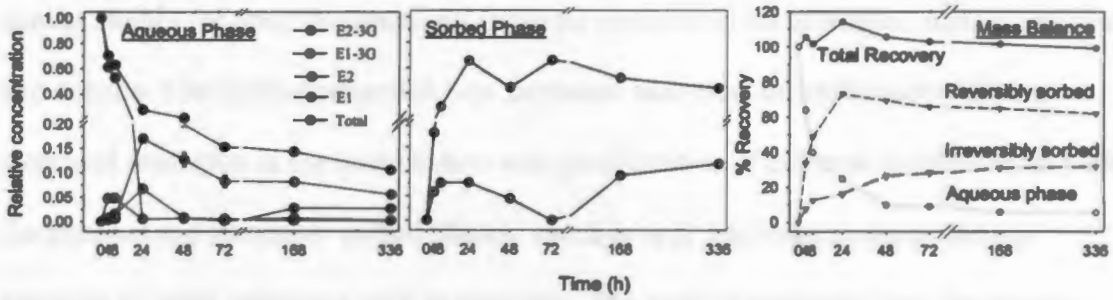

Figure 7. Speciation in the aqueous and sorbed phase in the batch study, along with mass balance recovery through time. Aqueous phase values are average of triplicate vials (± std. error), others are single observations. Mass balance of ^{14}C is shown as concentrations in total aqueous, reversibly sorbed, irreversibly sorbed, and total recovery. Scale in X axes has been adjusted to highlight the changes in early time points.

Mass balance of the radioactivity in the aqueous, reversibly sorbed, and irreversibly sorbed fractions produced an excellent total recovery, ranging from 99.0 to 105.5 percent (Fig. 7). Total aqueous radioactivity was calculated as the sum of the bulk radioactivity in the aqueous phase sampling and the additional radioactivity recovered from the soil by aqueous extraction. Gas phase radioactivity was negligible and did not contribute to the total mass balance recovery. Fan et al. (2007), in their incubation experiment of E2, found 6% and 0.9% of applied dose of E2 mineralized to $^{14}CO_2$ under the aerobic and anaerobic

conditions, respectively using soil from the same location. For the current study, the conditions would have had a limited supply of oxygen, hence mineralization was probably not a significant process.

Conclusions

A simple, robust, and reliable method to study fate and transformation of E2-3G was presented in this study. Although this method was developed primarily for the study of the fate of E2-3G in a laboratory soil-water batch experiment, it can be easily expanded for similar studies for other estrogens and different environmental substrates, such as sediment and manure. The method presented here increased analytical capabilities by offering increased resolution in the qualification and quantification of different metabolites in both the aqueous and reversibly sorbed phases, which is very important to study fate and transport of labile pollutants such as estrogens. The method presented here can separate both polar and nonpolar estrogens and their conjugates in a single run, offering the versatility of simultaneous monitoring all these estrogenic compounds. Furthermore, by not requiring a hydrolysis step prior to chromatographic separation through HPLC, this method saves the experimental time and adds to the accuracy of the analysis. The simplicity of the method is reflected in not requiring specific solvents to use for conjugate and free estrogens separately, and not needing fluorescent derivatization of the study compound. In addition, the combination of HPLC and LSC offers relatively easier tracking of the metabolites as well as the direct measurement of the mass in all compartments with an excellent mass balance.

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PAPER 3. ESTROGENIC CONTRIBUTIONS IN AGRICULTURAL SOIL-WATER SYSTEMS BY AN ESTROGEN CONJUGATE

Abstract

Animal agriculture produces large amounts of estrogen conjugates, which can be transformed in the environment into highly potent endocrine disrupting free estrogens. The objective of this study was to obtain a better understanding of the conjugate derived estrogenicity in agricultural soil-water systems using a prototype conjugate of 17β-estradoil (E2). Batch experiments were conducted using natural topsoil and subsoil with radiolabeled [^{14}C] 17β-estradiol-3-glucuronide (E2-3G). Biphasic dissipation of total aqueous phase ^{14}C was observed in the soil-water batch results. The composition of the total ^{14}C included glycones (E2-3G and its metabolite estrone glucuronide (E1-3G)) and aglycones (E2 and estrone (E1)). Hydrolysis of the glycones dominated the initial phase of the biphasic dissipation (~24 h for the topsoil, ~168 h for the subsoil), and sorption equilibria of the aglycones dominated the second portion of the biphasic dissipation. Calculated bioavailable estrogenicity values (E2 equivalent; EEQ) for the topsoil were maximum value (52.3 to 972.0 μg eq-E2 L^{-1}) when glycones deconjugation was completed, and were minimum when apparent sorption equilibrium occurred (72 h). The EEQ was persistent in the topsoil for the entire duration of the experiment, with 29.5 to 516.5 μg eq-E2 L^{-1} in the aqueous phase at 672 h. In the subsoil, with lower organic matter (OM) and microbial activities, the E2 concentrations were two or more times greater than E1 concentrations, which caused significantly high aqueous EEQ in the subsoil even though molar concentrations of aglycones were generally less compared to the topsoil. Further, intact

glycones still ranged from 1 to 21% of the applied dose at 336 h in the subsoil, which would cause a greater potential for estrogen mobility and EEQ in the environment.

Introduction

In the environment, exogenous estrogens are highly potent endocrine disrupters, with lowest observed adverse effect levels (LOAEL) of less than 10 ng L^{-1} for some aquatic organisms (Routledge et al., 1998). Detections of estrogen compounds at concentrations greater than the LOAELs in river systems (Kolpin et al., 2002; Lei et al., 2009), effluent streams (Zhao et al., 2010), shallow and deep groundwater (Bartelt-Hunt et al., 2011; Fine et al., 2003), and throughout the deep (>30 m) vadose zone below an animal feeding operation (AFO) lagoon (Arnon et al., 2008) have raised concerns over 'ecosystem health' (Huschek and Hansen, 2006). Endocrine disruptions in aquatic organisms may take several forms such as intersex in fish (Jobling et al., 2002), poor osmoregulation and altered courtship behaviors in frogs (Kohno et al., 2004; Zerani et al., 1992), and reproductive abnormalities in alligators (Guillette and Iguchi, 2003).

The ecotoxicology of endocrine disrupting compounds (EDCs) that interfere with the female sex-steroid (estrogen) signaling is typically investigated using biomarkers such as vitellogenin expression in male fish (Hansen et al., 1998). Estrogenicity, or the estrogenic potential of an EDC, is quantitatively expressed as 17β-estradiol (E2) equivalents (EEQ) (Hutchins et al., 2007). Vitellogenin is normally synthesized by females and is a precursor protein in the production of eggs. Based on E-screen assays, the estrogenicity or relative potency of the natural estrogens E2, estrone (E1), and estriol (E3) are 1.0, 0.024 and 0.054, respectively (Gadd et al., 2010). 17α-ethynyl estradiol (EE2), the synthetic estrogen used in

contraceptive pills, has an EEQ value of 1.2 (Legler et al., 2002). These EEQ values from exogenous steroidal hormones are much greater than other estrogenic environmental pollutants such as 4-nonylphenol and 4-tertbutyl-phenol (detergent components in sewage effluents) that have EEQs of 9.00×10^{-6} and 1.60×10^{-4}, respectively (Jobling and Sumpter, 1993). Bisphenol A (BPA), a chemical widely used in many industrial applications, has an EEQ of 0.004 (Sumpter and Johnson, 2005) .

A number of studies have been conducted on the fate and transport of estrogens generated from municipal waste, with a focus on sewage treatment plants (STPs) and waste water treatment plants (WWTPs) (Chen and Hu, 2010; D'Ascenzo et al., 2003; Gomes et al., 2009). Even though potential estrogen contribution to the environment by animal agriculture far exceed that from human sources (Combalbert and Hernandez-Raquet, 2010), very few studies (e.g. Chen et al., 2010; Zheng et al., 2008) have been conducted on the fate and transport of steroid hormones with respect to animal agriculture. Estrogens are relatively immobile in soil resulting from their low aqueous solubilities (e.g. 1.51 mg L^{-1} for E2 and 1.30 mg L^{-1} for E1 (Shareef et al., 2006)) and high sorption potentials, (e.g. the organic carbon (OC) normalized partition coefficients ($\log K_{oc}$) for E2 and E1 are of 2.94 and 2.99, respectively (Casey et al., 2005)). On the other hand, conjugates of estrogens are quite water soluble (e.g., theoretical value of 0.35 g L^{-1} for E2-3G calculated from ALOGPS 2.1 (Tetko et al., 2005)), and therefore have much higher potentials to be mobile in the soil-water systems compared to their free deconjugated forms.

Estrogen conjugates are produced by the body by attaching polar moieties (a glucuronide, a sulfate, or both) to the estrogen molecule (Gibson and Skett, 2001). Estrogen conjugates are largely innocuous, with relative potency values of 1.30×10^{-3},

2.90×10^{-5}, 2.60×10^{-3}, and 1.20×10^{-5} for 17β estradiol-3-glucuronide (E2-3G), estrone-3-glucuronide (E1-3G), 17β estradiol-3-sulfate (E2-3S), and estrone-3-sulphate (E1-3S), respectively (Gadd et al., 2010). The polar moieties are readily cleaved from the steroidal estrogen molecules by the enzymes, β-glucuronidase and aryl-sulfatase, transforming the compounds into a highly potent EDC. Furthermore, the enzymes that cause this deconjugation are ubiquitous in soil (Khanal et al., 2006). D'Ascenzo et al. (2003) found very little or no persistence of glucuronide estrogen conjugates and estimated their removal efficiencies to be 84 to 100% in six STPs. Sulfate conjugates, however, are found to be more recalcitrant with 28 to 48% removal efficiencies at STPs (Nakada et al., 2006). To date, only two studies have conducted fate and transport studies on estrogen conjugates in the context of agricultural soil-water systems, and both investigated estrogen sulfates in pasture soils (Scherr et al., 2009a; Scherr et al., 2009b). Incubation experiments on E2-3S showed first-order kinetics of E2-3S degradation, as well as temperature dependence of the rate constants (Scherr et al., 2009a). Sorption studies on E1-3S indicated a concentration-dependent effective distribution coefficient ($K_d^{eff} = K_f C_w^{N-1}$) for E1-3S that was an order of magnitude lower than that for free E1 (Scherr et al., 2009b).

Estrogens are excreted by humans mostly as conjugates (Gomes et al., 2009), where as swine (*Sus scrofa domesticus*), poultry (*Gallus gallus*), and cattle (*Bos taurus*) will excrete 96, 69, and 46% of their estrogens as conjugates, respectively (Hanselman et al., 2003). Gadd et al. (2010) measured conjugated estrogens concentrations between 12 ng L^{-1} and 320 ng L^{-1} in most samples from the effluent of 18 dairy farm sheds. Hutchins et al. (2007) found estrogen conjugates to contribute at least a third of the total estrogen load in the lagoons of AFOs. The authors found estrogen conjugates contribute 27% to 35% of total

estrogen loads for swine nursery, beef feedlot, and poultry primary lagoons; 57% for a dairy lagoon; and 95% for a tertiary poultry lagoon (Hutchins et al., 2007). Detection of E1 in rivers that received discharges from concentrated AFOs have been partially attributed to the cleavage of glucuronide conjugates (Chen et al., 2010; Ternes et al., 1999). Also, Chen et al. (2010) attributed increases in spring detections of E2 and E3 downstream a concentrated AFOs compared to winter to higher microbial activities that caused more conversion of conjugates to free estrogens.

The objective of this study was to investigate the dissipation of a glucuronidated estrogen, using17β-estradiol-3 glucuronide (E2-3G) as a model compound, and the resulting estrogenicity in agricultural soil-water systems with varying organic carbon contents. Such investigations may give valuable information on the biological availability of estrogenicity so that best management practices can be developed for proper handling and application of animal manure on agricultural lands.

Material and Methods

Soil

Soil from the Hamar series (sandy, mixed, frigid typic Endoaquolls) was collected near Milner, North Dakota, which was representative of soils upon which manures from a local hog farm were regularly applied as a soil nutrient amendment. Samples were collected from the top 6 cm (topsoil) and from 18–24 cm (subsoil) of the field and stored immediately at 4°C upon arrival to the laboratory. The soils were collected from a location that had not received an application of animal manure in over five years, and were the same used in the previous laboratory (Fan et al., 2007; Schuh et al., 2011; Zitnick et al., 2011)

and field studies (Schuh et al., 2011; Thompson et al., 2009) on the topic of free estrogen fate and transport. Soil physical and chemical properties of this Hamar soil are presented in Table 3. Prior to conducting the batch experiments, the soil was air dried for 48 h, large clods were gently broken, and then the soil was passed through a 2-mm sieve.

Table 3. Selected physical and chemical properties of soil samples.

	Topsoil (0 – 6 cm)	Subsoil (18 – 24 cm)
OM content (%)	2.10	0.40
Total Carbon (TC), %	1.35	0.32
Inorganic carbon (IO), %	0.00	0.00
Organic carbon (OC), %	1.35	0.32
pH	7.0	7.4
Cation-exchange capacity, CEC (meq $100g^{-1}$)	9.3	9.8
Particle size distribution (sand:silt:clay)	83:10:7	90:4:6
Bulk density (g cm^{-3})	1.4	1.8
Mn (mg Kg^{-1})	254.6	154.4
Soil surface area (m^2)	49.81	49.41

Chemicals

The study compound, 17β-[4-^{14}C]-estradiol-3-glucuronide (E2-3G) was synthesized with a 99% radiochemical purity and specific activity of 103.13 Bq μg^{-1} (Shrestha et al., 2011). Ammonium acetate, hydrochloric acid, acetonitrile, calcium chloride dihydrate and formaldehyde (all analytical or better grades) were purchased from Sigma-Aldrich (St. Lois, MO) and were used as received. CarboSorb E (2-methoxy ethylamine) and the scintillation cocktail used after soil combustion were obtained from PerkinElmer

(Permafluor; Waltham, MA), and the general purpose scintillation fluid EcoLite was purchased from MP Biomedicals (Ecolite; Santa Ana, CA).

Synthesized E2-3G was dissolved in ethanol and stock solutions of 18 to 889 mg L^{-1} were prepared for the batch studies. Radiolabel standards were prepared in ethanol for E2-3G, E2 and E1 with an on-column mass of 40, 25, and 125 ng, respectively, for the HPLC. While the stock solution and standards were not in use, they were stored at -20°C to minimize evaporation and degradation.

Batch sorption experiment

Batch sorption experiments were conducted in triplicate at 25°C. In 10-mL clear glass vials, 1.6 g of soil and 8 mL of 0.01 M $CaCl_2$ solution were added, fitted with Teflon caps, and then E2-3G doses were spiked to obtain concentrations of 0.0, 0.5, 3.7, 9.1, and 22.5 mg L^{-1}. These concentrations were selected to ensure HPLC resolution of E2-3G and its possible metabolites in the aqueous and sorbed phase in the batch samples throughout the study period. A weak salt solution (i.e., 0.01 M $CaCl_2$) was used to ensure that soil aggregates would not be dispersed. The amount of ethanol introduced by way of spiking the doses was 0.125%. Ethanol concentrations of less than 0.5% are not shown to affect the sorption of an organic pollutant to soil (Wauchope and Koskinen, 1983). Control blank vials were dosed at 0.5 mg L^{-1} with 8 mL of 0.01 M $CaCl_2$ and contained no soil. To analyze the sorbed-phase speciation, a series of 'stop' vials were prepared identical to other batch vials at initial E2-3G concentration of 3.7 mg L^{-1} in order to retire and destructively sample one vial for each treatment (i.e. topsoil and subsoil) at each sampling time. Batch soil-water slurries were agitated by mechanical rotation (360° every 5 s), stopping only to take samples at 4, 8, 24, 48, 72, 168, 336, 504, and 672 hours. At each sampling, vials were

centrifuged at 380 × *g* for 20 minutes, and triplicate 100 µL aliquots were withdrawn through septa using sterile syringes and were assayed for radioactivity. Additional 120 µL aliquots were also withdrawn for HPLC analysis, filtered through a 0.45 µm glass filter into HPLC vials, to which formaldehyde (2.7% final volume) was added to inhibit any microbial activity and were stored at –20°C until analyzed. To each retired stop-vial, the aqueous layer was transferred into a separate vial and formaldehyde (2.7% final volume) was added to both the aqueous and sorbed phases.

To complete the mass balance, gas phase was also sampled from the stop-vials at each sampling time, and at the end of the batch experiments for other experimental vials. Head space samples of 500 µL were collected through the septa of the vials using sterile syringes for subsequent analysis.

Analytical methods

Analytical techniques used in this study are described in detail in Paper 2 in this dissertation. Bulk radioactivity (i.e. total ^{14}C) in the aqueous phase was measured by liquid scintillation counting (LSC; 1900 CA scintillation counter, Packard, Downers Grove, IL). To assay the radioactivity, each sample was mixed with 4 mL of scintillation fluid (EcoLite) and the disintegration per minute (dpm; 1 Bq = 60 dpm) was counted for 10 minutes with zero background deduction. Background dpm was determined by averaging the dpm counts of 5 blank vials. The limit of detection (LOD)/limit of quantitation (LOQ), determined by measuring replicate blank samples, were 0.24±0.03 µg L^{-1} for E2-3G and estrone glucuronide (E1-3G)), and 0.15±0.02 µg L^{-1} for E2 and E1 in the aqueous phase; and 1.22±0.17, 1.21±0.17, 0.74 ±0.10, and 0.73 ±0.10 µg Kg^{-1} for E2-3G, E1-3G, E2, and E1, respectively, in the sorbed phase.

High performance liquid chromatography (Waters 600 E System Controller, Waters 717 Plus auto-sampler, Waters 746 Data Module integrator, Jasco FP 920 fluorescence detector, C18 Phenomenex analytical column (250×4.6 mm) with 5 μm particle size) was used with a gradient elution to separate estradiol glucuronide from its metabolites. Mobile phases A and B consisted of 10% acetonitrile (ACN) in 100 mM ammonium acetate buffer (pH 4.5), and 90% ACN in 100 mM ammonium acetate buffer (pH 4.5), respectively. The gradient conditions were 20% B increased to 100% B over 29 minutes with a slightly concave curve, then 29 to 32 min isocratic, and linear return to the starting condition over 3 min (flow rate 1 mL min^{-1}). The excitation and emission wavelengths of 280 and 312 nm, respectively, were used on the fluorescence detector. The standards E2-3G, E2, and E1 eluted from the HPLC at 5.7, 27.6, and 29.9 min, respectively. A Gilson FC 204 Fraction Collector was used to collect the eluent in 1 min interval for subsequent radioassay. Overall, over 11,000 fractionated samples were collected for LSC analysis.

Identification of unknown metabolites and compound verification in the aqueous and reversibly sorbed phases were done using liquid chromatography tandem mass spectrometry (LC-MS/MS) on a Waters/Micromass API US Q-TOF mass spectrometer, interfaced to Waters Alliance 2695 HPLC (Symmetry-C18, 2.1× 100 mm, Waters 2996 photodiode array detector). Solvent A was 40% ACN in nanopure water, solvent B was 60% ACN in nanopure water, and the gradient was 40 to100% B over 10 min, 5 min hold at 100% B with 0.2 mL min^{-1} solvent flow. Analytes were characterized in negative ionization (ES-) mode. The glucuronide conjugate and metabolites were analyzed at collision energy of 20 and 50 eV, respectively. The MassLynx software was used for acquiring and analyzing the mass spectrometry information.

Reversibly sorbed fraction in the soil was analyzed by acetone extractions from the soil followed by LSC for total ^{14}C, and HPLC and LSC for speciation. The soil was extracted first with nanopure water (4 mL×3) by ultrasonication for 30 minutes and centrifugation at 380 × *g* for 30 minutes, decanting the aqueous fraction in a separate glass vial; and then extracted with acetone (4 mL×3), following similar procedure. The extracted soils were completely dried under a hood for a week and then the irreversibly sorbed fraction was determined by combusting 6 replicates of 0.1 g of soil in an oxidizer (Model 307 Oxidizer; Packard, Meridan, CT). The radiolabelled carbon dioxide produced in the oxidizer was trapped by Carbo-Sorb (8 mL), and assayed in the LSC with 12 mL of scintillation cocktail Permafluor.

To account for the gas phase radioactivity, headspace air samples from the batch vials were analyzed in a gas chromatograph (GC) (HP 5790A, Avondale, PA). The GC had a 10-foot glass column (1.5 mm id and 7.0 mm od) packed with 3% OV-17. Temperature gradient was 30°C at the initial condition, hold for 2 min, then linearly raised to 180°C (30 deg min^{-1}), and held at 180 °C for 5 min. The radiolabeled carbon dioxide was trapped and assayed following the same method as for irreversibly sorbed fraction. The integrating recorder was an HP 3390A.

Data analysis

Aqueous phase speciation data were obtained as dpm values for each minute fraction from the 50 µL injections to the HPLC, followed by the LSC. Only the values greater than LOQ (average background dpm + 3 times standard deviation) were considered for further analysis. Average background radioactivity on each measurement day was deducted to obtain the net dpm. Radioactivity fraction collected from the HPLC effluent at time

increments of 5-7, 7-9, 27-29, and 29-31 min were determined to be E2-3G, estrone glucuronide (E1-3G), E2, and E1, respectively, using HPLC and LC-MS/MS analyses. The dpm values for all the HPLC fractions were adjusted to 100% recovery, based on the bulk LSC analysis of 100 μL of the sample replicates. The adjusted dpm values were then converted to the molar concentrations, using the specific activity of E2-3G and molar weights of respective metabolites. Estrogenicity, defined as the concentration of equivalent E2 to cause the same estrogenic effect (ng eq-E2/L), was calculated using the relative potency of each metabolite.

Two-factor analysis of variance (ANOVA) with replication was performed to analyze the concentration effect on the total ^{14}C dissipation through time. Initial applied concentration and time of sampling were the two factors considered in each soil. An alpha level of 0.05 was used and $p \leq 0.01$ were considered significant.

Results and Discussion

Aqueous phase dissipation

In both the topsoil and subsoils, the total ^{14}C in the aqueous phase declined through time (Fig. 8). The aqueous phase dissipation of ^{14}C consisted of two phases: (i) a rapid first-order dissipation phase (Table 4), and (ii) a flat segment, where apparent sorption equilibrium was achieved (Fig. 8). The initial first-order decline in the total aqueous ^{14}C was greater in the topsoil compared to the subsoil (Table 4). Additionally, the onset of the second phase occurred at 72 h for the topsoil, which was much earlier than observed ($\geq$ 504 h) in the subsoil (Fig. 8). Additionally, significant differences in total aqueous ^{14}C

dissipation were seen for the different initial concentrations for topsoil ($p \leq 0.001$) and subsoil ($p \leq 0.001$).

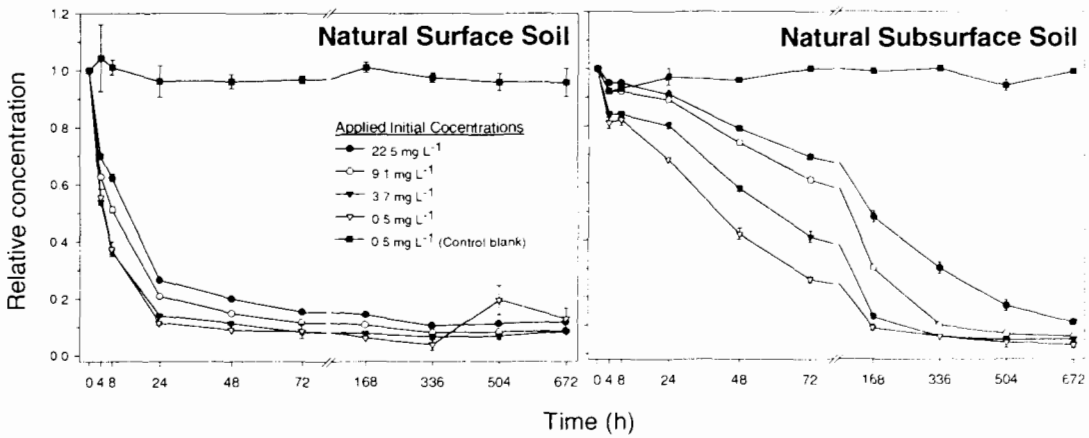

Figure 8. Aqueous phase concentration (normalized) of ^{14}C through time in natural topsoil and subsoil with different initial concentrations of 17β estradiol-3-glucuronide. Data represent average of at least two independent observations of three replicates, with error bars representing standard errors. Scale in X axis has been adjusted to highlight the changes in early time points.

Table 4. Dissipation of ^{14}C aqueous concentration in rapid dissipation phase.

Topsoil				Subsoil			
Time of best fit	Concentration ($mg\ L^{-1}$)	Rate Constant (h^{-1})	r^2	Time of best fit	Concentration ($mg\ L^{-1}$)	Rate Constant (h^{-1})	r^2
0-24	3.7	-0.0872	0.9083	0-168	3.7	-0.0122	0.9909
	9.1	-0.0679	0.9546		9.1	-0.0071	0.9929
	22.5	-0.0561	0.9799		22.5	-0.0045	0.9903

The distinctions observed in total aqueous ^{14}C between the topsoil and subsoils (Fig. 8 and Table 4) were caused by differences in E2-3G transformation processes (Fig. 9) and hydrophobic sorption interactions (Lee et al., 2003). The higher OC topsoil would have higher microbial activities compared to the lower OC subsoil (Watts et al., 2010). Natural

soils with higher OC would have greater abundance of enzymes, such as β-glucuronidase, that would hydrolyze the glucuronide from the estrogen conjugate to form free deconjugated estrogens (Khanal et al., 2006). Furthermore, the difference in the aqueous phase dissipation of the different concentrations (Fig. 8) was attributed to the rate-limited capacity of the enzymatic hydrolysis process, where the capacity of the enzymes to hydrolyze the glucuronic acid is saturated. Additionally, once the polar, glucuronide conjugate of estrogen is transformed to a non-polar, lower-solubility, free estrogen; greater distinctions between the hydrophobic sorption interactions (Lee et al., 2003) will be observed. The higher OC of the topsoil would have higher hydrophobic sorption of the free estrogens compared to the lower OC subsoil.

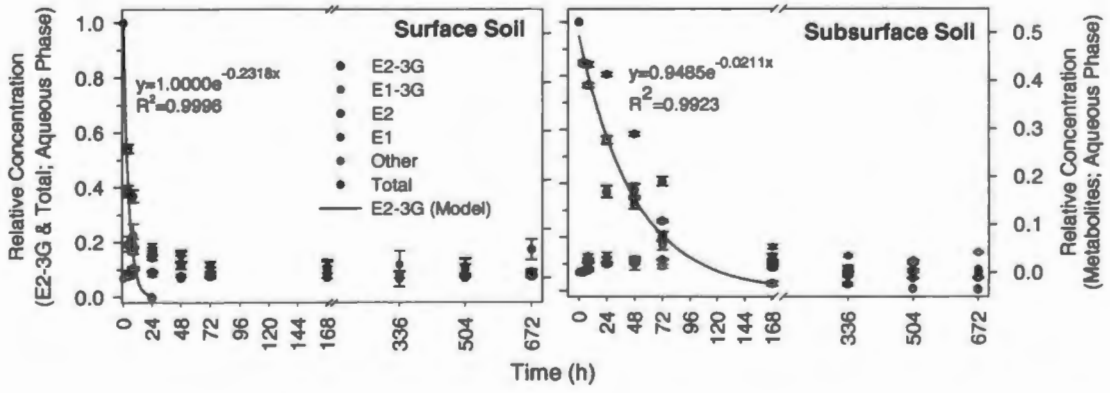

Figure 9. Aqueous phase speciation pattern in natural topsoil and subsoil. 17β estradiol-3-glucuronide (E2-3G) in aqueous phase dissipated following a 1st order decay with best fit at 24 h for topsoil and 168 h for subsoil. Primary y-axis represents relative aqueous phase concentration of E2-3G and total concentrations, while the metabolites of E2-3G are shown in secondary y-axis. Data represent average of at least two independent observations of three replicates, at E2-3G applied concentration of 3.7 mg L^{-1}. Error bars represent standard errors. Scale in X axis has been adjusted to highlight the changes in early time points.

Applied E2-3G metabolized to estrone glucuronide (E1-3G), E2, and E1 (Fig. 9) and some unknown metabolites in the aqueous phase that comprised of up to 8.8% in the topsoil and 6.6% in the subsoil of the applied ^{14}C doses. Oxidation on manganese-oxides reaction sites (Sheng et al., 2009) caused the transformations of E2-3G to E1-3G and of E2 to E1, which has been observed under sterile conditions in these same soils (Zitnick et al., 2011). The deconjugation of the glucuronic acid from E2-3G to form E2, and from E1-3G to form E1 was a result of enzymatic hydrolysis. Based on their similar hydrophobicity, E2-3G and E1-3G were broadly categorized as glycones, and E2 and E1 as aglycones. For the topsoil and for all the initial concentrations, the glycones essentially vanished from the aqueous phase within the first 24 h (Fig. 10). For the subsoil, the dissipation of the glycones varied for the different initial concentrations, taking between 168 h to 672 h to completely dissipate in the aqueous phase. The earlier peaking of the aglycones

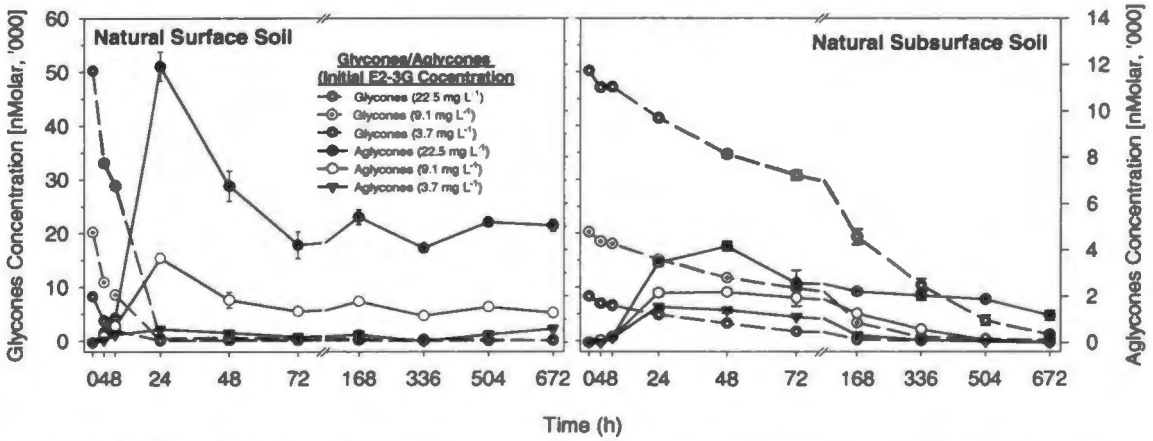

Figure 10. Trends of metabolite concentration in aqueous phase through time in natural topsoil and subsoil with different initial concentrations of 17β estradiol-3-glucuronide. Dashed lines represent glycone and solid lines represent aglycone concentrations. Glycones are shown in primary axis and the secondary axis for aglycones, both in nmole L^{-1} (in thousands). Data points are average of at least two independent observations of three replicates. Error bars represent standard errors. Scale in X axis has been adjusted to highlight the changes in early time points.

(before complete dissipation of the glycones) in the aqueous phase of the subsoil was attributed to the smaller hydrophobic sorption potential of this low OM soil. The lower hydrophobic sorption potential was also indicated by a more gradual decrease in the aqueous phase concentration of the aglycones through time in the subsoil compared to the topsoil (Fig. 10). The composition of the total ^{14}C in the aqueous phase was almost entirely aglycones after 24 h for the topsoil; however, glycones persisted in the aqueous phase in the subsoil up to 336 h to 672 h (Fig. 10).

Estrogenicity

Distinctions between the glycone and aglycone aqueous concentrations in topsoil and subsoils are important, as the mobility in the environment and estrogenic activities are tied to these observations. Furthermore, the aqueous concentrations were considered because they are the most important bearing in mind exposures to organisms and toxicological implications. The sum of the estrogenic activities of glycone and aglycones were calculated through time (Fig. 11) using the relative potencies, or EEQ values of 0.0013, 2.9×10^{-5}, 1.00, and 0.0240 for E2-3G, E1-3G, E2, and E1, respectively, reported by Gadd et al. (2010). The calculated initial estrogenicity introduced into the aqueous phase by the E2-3G dosing was 2.9 µg eq-E2 L^{-1}, 7.2 µg eq-E2 L^{-1}, and 17.8 µg eq-E2 L^{-1} for the initial concentrations of 3.7 mg L^{-1}, 9.1 mg L^{-1}, and 22.5 mg L^{-1}, respectively. The EEQ for the control blank (0.5 mg L^{-1}) was at 0.4 µg eq-E2 L^{-1}.

The aqueous estrogenicity increased earlier in the topsoil compared to the subsoil, which suggested the higher enzymatic activities of the topsoil could cleave the polar moieties from the glycones to form more potent aglycones at a faster rate. By 8 h, the estrogenicity in the aqueous phase in the topsoil had increased by 12-fold and was more

than double that of the subsoil. For the topsoil, the maximum EEQ value coincided with the completion of the deconjugation of E2-3G at 24 h (Figs. 10 and 11). Thereafter, EEQ in the aqueous phase declined steeply as the more hydrophobic aglycones (E2 and E1) were rapidly sorbed (Fig. 10). When apparent equilibrium was achieved at 72 h the EEQ reached its lowest value but then increased (Fig. 8 and Fig. 11). The increase was greater than indicated by the change in molar concentration of total aglycones. For example, for initial concentration of 22.5 mg L^{-1}, aglycones in the aqueous phase of the topsoil comprised of 62 nmole L^{-1} of E2 and 4,146 nmole L^{-1} of E1 at 72 h; however, at 168 h, E2 desorbed and its concentration increased to 1,360 nmole L^{-1} in the aqueous phase, while E1 sorbed and its aqueous concentration became 4,048 nmole L^{-1}. Such a change in E2 concentration, with its much higher relative estrogenicity than E1, could increase the EEQ value substantially. A major finding of this study was the persistence of estrogenicity in the aqueous phase even at 28 days after the start of the experiment.

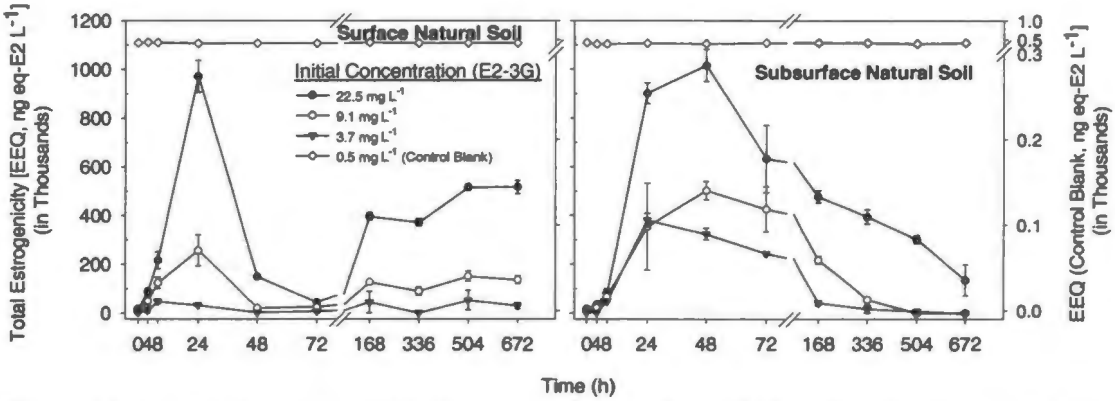

Figure 11. Trends in estrogenicity in aqueous phase through time in natural topsoil and subsoil with different initial concentrations of 17β estradiol-3-glucuronide. Estrogenicity values for all treatments are shown in ng eq-E2/L on the primary axis, and the secondary axis for the control blank. Data points are averages of at least two independent observations of three replicates, with error bars representing standard errors. Scale in X axis has been adjusted to highlight the changes in early time points.

The trend of estrogenicity in the subsoil was markedly different from the topsoil, which resulted from lower OM content and reduced microbial activities of the subsoil (Fig. 11). In the topsoil with greater OM content, E2 and E1 concentrations from 0 to 48 h were almost similar, whereas in the subsoil, E2 concentrations were almost two or more times greater than E1 concentrations (Fig. 9). Oxidation of E2 would occur slower in the subsoil because of lower microbial activity (Colucci et al., 2001). More importantly, lower OM values in the subsoil would mean lesser hydrophobic interaction with soil OC (Das et al., 2004), which would reduce abiotic oxidative surface reactions that convert E2 to E1 (Sheng et al., 2009).

Even though EEQ started to decline after 48 h in the subsoil (Fig. 11), intact E2-3G still composed the major portion of the total ^{14}C in the aqueous phase (Figs. 9 and 10), which, if hydrolyzed, would raise the EEQ by a factor of 769. Since glycones are more polar than aglycones, they have much greater potential to be transported downstream as a precursor to the potent aglycones. Thus, the greater mobility and EEQ conversion potential may add to the risk of potential EEQ contributions to environmental systems from an applied estrogen conjugate source in the subsoil.

Conclusions

The major contributing factors in the fate of the conjugate-derived estrogenicity in soil appeared to be the soil OM and, with the positive association of microbial activities with soil OM (Watts et al., 2010), microbial activities. The initial concentration of the E2-3G also influenced the dissipation of estrogenicity. Results from this study show that an estrogen conjugate can result in the significant increase in estrogenicity to environments

compared to free estrogens, which dissipate on the order of a few hours (Casey et al., 2003; Colucci et al., 2001). Compared to the subsoil, more rapid E2-3G deconjugation in the topsoil was likely caused by the higher microbial activities, which resulted in rapid aqueous phase dissipation dominated by aglycone hydrophobic sorption dynamics. In case of the subsoil, transformation of E2-3G to free estrogens was slower, and E2-3G transformation and sorption of aglycones were equivalent processes. Respectively, for the topsoil and subsoil, the first 24 h and up to 14 d were critical periods for the potential estrogenic contribution to the environment from intact glucuronide conjugates. For the topsoil, the persistence of estrogenicity in the aqueous phase up to 28 days indicated that even with high sorption capacity of the high OC topsoil, conjugate-derived estrogenicity may be a significant source of bioavailable estrogenicity. The risk of estrogenicity to the environment from surface application of manures containing conjugates could be more from surface runoff. Furthermore, the results of this study may have practical management implications of manure especially for the practice of subsurface injection of manures. When manure containing conjugates are injected into lower OC subsoils, the speciation of E2-3G in the lesser oxidative environment may favor more E2 than E1, which would result in the increased estrogenicity. Also, longer persistence of intact E2-3G in the subsoil may increase the potential mobility of E2-3G through the soil and to downstream locations where it can be hydrolyzed to potent E2 or E1. With the majority of estrogen excretion as conjugates from animals, results of this study highlight the need to consider the overlooked risk of glycone derived EEQ to the environment.

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PAPER 4. THE POTENTIAL ROLE OF CONJUGATES IN THE FATE AND TRANSPORT OF ESTROGENS IN THE ENVIRONMENT

Abstract

Natural and synthetic estrogens are the most potent endocrine disrupters in the environment. Although these hydrophobic compounds are very labile and immobile in soils and sediments, they are frequently detected in the environment at relatively high concentrations, which suggests they are mobile and persistent. Using soil batch experiments and numerical modeling we demonstrate how a prototype estrogen conjugate, 17β-estradiol-3-glucuronide (E2-3G), can persist intact to greatly increase the potential mobility of the highly potent estrogens, 17β-estradiol (E2) and estrone (E1). Intact conjugates are much more soluble than free estrogens, and therefore may be readily transported to receiving waters where they can undergo deconjugation to form endocrine-disrupting free estrogens. Given that mammals excrete estrogen conjugates in large quantities, results of this study provide a mechanism to explain the concentrations and detection frequencies of estrogens found in environmental samples contrary to the expectation.

Introduction

From the feminization of male fish downstream waste water treatment plants (WWTPs) (Routledge et al., 1998) to the collapse of a fish population in an experimental lake, (Kidd et al., 2007), steroidal estrogens have drawn much concern and considerable debate about their potential impact on the environment and human health (Stone, 1994). Lowest

observed adverse effect levels (LOAEL) for natural and synthetic estrogens are reported to be below 10 parts per trillion (ppt) for aquatic organisms (Routledge et al., 1998). A survey of 139 rivers across the U.S. frequently detected reproductive hormones (>40% detection rate) and found E2, the most potent natural estrogen, well above the LOAEL (median = 9 ppt; maximum = 93 ppt) (Kolpin et al., 2002). There is an urgency to understand the fate and transport of these compounds because of their potency, and the frequency and concentrations they are found in the environment. Legislation in the U.S. (Congress, 2005; Congress, 2008) illustrates the increased awareness of this issue and the need to minimize adverse human and environmental impacts.

The global human population of 6.9 billion (Bureau, 2011) is estimated to release 4.4 Kg yr^{-1} estrogen per million inhabitants (Combalbert and Hernandez-Raquet, 2010), or 30,500 Kg yr^{-1} total; and an additional 700 Kg yr^{-1} of synthetic estrogens from contraceptive usage (Combalbert and Hernandez-Raquet, 2010). Much focus has been given to human waste management with a number of studies conducted on estrogen removal efficiencies of WWTPs. However, potential estrogen contributions to the environment by farm animals dwarf that of humans. An estimated 81,000 Kg yr^{-1} of estrogens are released by farm animals in the U.S. and European Union alone (Lange et al., 2002). Indeed, animal feeding operations (AFOs) were recognized as potential sources of water contamination by synthetic steroidal hormones over thirty years ago (Knight, 1980). Subsequent studies have found associations between AFOs and natural steroidal estrogens in surface (Finlay-Moore et al., 2000) and subsurface (Arnon et al., 2008) water.

Laboratory studies find estrogens to be short-lived (Colucci et al., 2001; Fan et al., 2007) and immobile (Das et al., 2004; Fan et al., 2008) in soil, which contradicts the

relatively high detection frequency and concentrations in surface waters (Kolpin et al., 2002; Lei et al., 2009). These divergent results between laboratory and field studies may be related to estrogen conjugates, the environmental fate of which is not clearly understood. Mammals enzymatically conjugate glucuronic acid or sulfate moieties to the hydrophobic estrogen molecule, increasing its solubility and facilitating urinary or biliary excretion (Khanal et al., 2006). Mammals excrete estrogens primarily as conjugates. For example, humans excrete estrogens predominantly as conjugates (D'Ascenzo et al., 2003), while swine (*Sus scrofa domesticus*), poultry (*Gallus gallus*), and cattle (*Bos taurus*) excrete 96, 69, and 42% of their estrogens as conjugates, respectively (Hanselman et al., 2003). Although estrogen conjugates are considered innocuous (Zhu and Conney, 1998), they may hydrolyze to form free, potent estrogens (Khanal et al., 2006). Further, being more water soluble, the transportability of the intact estrogen conjugates in the environmental waters would be greater than that of the more hydrophobic free estrogens. Conjugated estrogens from human wastes are effectively hydrolyzed and metabolized by the rich biota in WWTP sludge (D'Ascenzo et al., 2003). However, animal-excreted conjugates may have an entirely different fate and transport processes. In waste holding ponds of concentrated AFOs, estrogen conjugates can account for a third of the total estrogens (Hutchins et al., 2007). Under the context of AFO waste management, where large amounts of conjugates are produced and untreated manures are applied directly to soils, the potential environmental threat of conjugated estrogens is largely unknown.

The research hypothesis was that if conjugated estrogens eliminated by animals are more persistent and mobile in soil compared to their free forms (e.g. E2, E1, estriol (E3)), then estrogen conjugates could contribute significantly to environmental estrogen loads.

Using soil-water batch experiments and numerical modeling, the sorption and fate of a prototype estrogen conjugate, a ^{14}C radiolabeled E2-3G with a specific activity of 103.13 Bq μg^{-1} (Shrestha et al., 2011), was investigated. Experiments were conducted using natural and sterilized topsoil (0-6 cm) and subsoil (18-24 cm) to identify the effects of soil microbial activity and soil organic carbon (OC) content on the fate of E2-3G and its metabolites. The soil (Hamar series; *sandy, mixed, frigid typic Endoaquolls*) was collected near a swine farm in southeastern North Dakota and was used in previous laboratory (Fan et al., 2007; Zitnick et al., 2011) and field studies (Schuh et al., 2010; Thompson et al., 2009) that identified the fate and transport of free steroidal hormones (e.g., E2, E1, testosterone).

Results and Discussion

Biphasic aqueous phase dissipation

Aqueous-phase dissipation is the reduction of aqueous concentrations through time from individual or multiple processes (e.g. sorption, degradation). Aqueous dissipation of E2-3G and its metabolites were found to result from interactions between biotic hydrolysis, abiotic oxidation, and physicochemical sorption processes. Experimental factors that influence the dissipation were the soil OC, soil sterility, and initial E2-3G concentration. Furthermore, aqueous dissipation of E2-3G and its metabolites generally followed a biphasic kinetic pattern, where there was an initial rapid decline in aqueous concentrations followed by a second slower phase (Fig. 12). The initial dissipation phase, which lasted about 24 h for the topsoil and 168 h for the subsoil, was dominated by the hydrolysis of the glucuronide moiety from the estrogen steroid molecule. The second slower phase was

dominated by sorption equilibrium of the deconjugated hydrophobic compounds, E1 and E2 (Fig. 12-B, 12-E, 12-H, and 12-K).

Modeling fate and transformation processes

A model was developed to capture the kinetic biogeochemical sorption and transformation processes of E2-3G in the batch studies (Fig. 13 and Supplemental Information). Using this model, fate and transformation processes for E2-3G and its metabolites in the aqueous and bound phases were discerned and quantified by employing an inverse global-optimization method (Runarsson and Xin, 2000). Several model restrictions were used to maximize uniqueness of process parameters (see Supplemental Information). The model provided parameter estimates that compared well with independently determined values (Yu et al., 2004) and had narrow 95% confidence intervals (Table 5). Additionally, the model's fit to the data was considered highly satisfactory, with a modified index of agreement (d_1) (Willmott et al., 1985) value of 0.86 for all data.

Hydrolysis of the conjugates

Hydrolysis was observed through time as a rapid decline in the aqueous conjugates, E2-3G and estrone-glucuronide (E1-3G), along with the concomitant formation of E2 and E1 (Fig. 12-A, 12-D, 12-G, and 12-J). Aqueous phase hydrolysis is primarily a biological process governed by bacterial β-glucuronidases (Fan et al., 2008; Harms and Bosma, 1997). Hydrolysis rates of the conjugates were greater in the topsoil (e.g. $\omega_{w,E2\text{-}3G} = 3.55\ h^{-1}$ for natural topsoil) compared to the subsoil ($\omega_{w,E2\text{-}3G} = 0.012\ h^{-1}$), with higher OM content in the topsoil associated with higher biological activity (Aon and Colaneri, 2001).

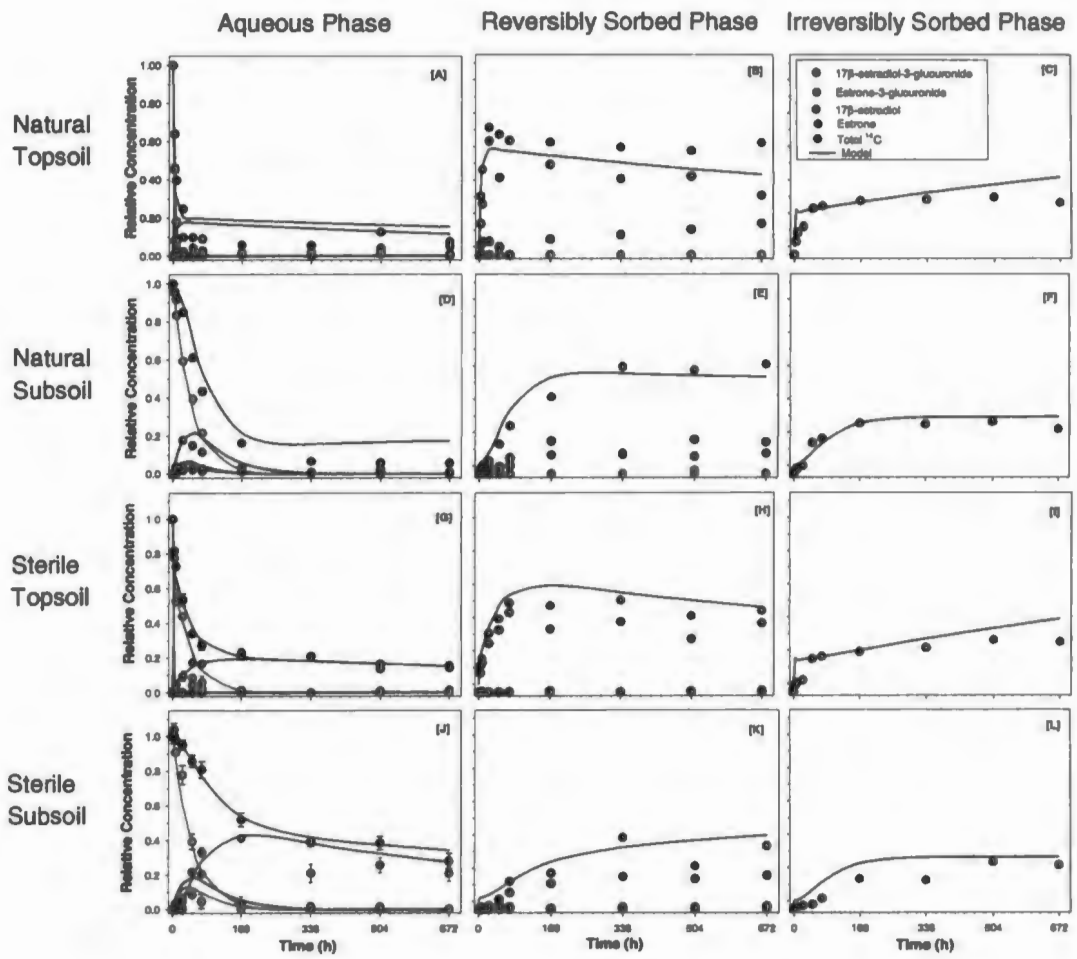

Figure 12. Batch concentration of aqueous and reversibly and irreversibly sorbed estrogens in natural and sterile topsoil (0–6 cm) and subsoil (18–24 cm). Symbols in the aqueous phase represent means of triplicates (±standard errors). The plots represent the data with initial concentration of 17β estradiol-3-glucuronide of 3.7 mg L^{-1}.

Although we did not find degradation rates of E2-3G in the soil-water batch studies to compare our value with, Gomes et al. (2009) found deconjugation first order reaction rate of 0.35 h^{-1} for E1-3G in batch study conducted with activated sludge grown from sewage, which is about two times the value obtained in this study for E1-3G using natural topsoil

(Table 5). The difference can be explained in light of possibly higher microbial activity in their study matrices grown from sewage than the soil used in this study, which was an agricultural soil without prior application of animal manure for past five years before the sample collection.

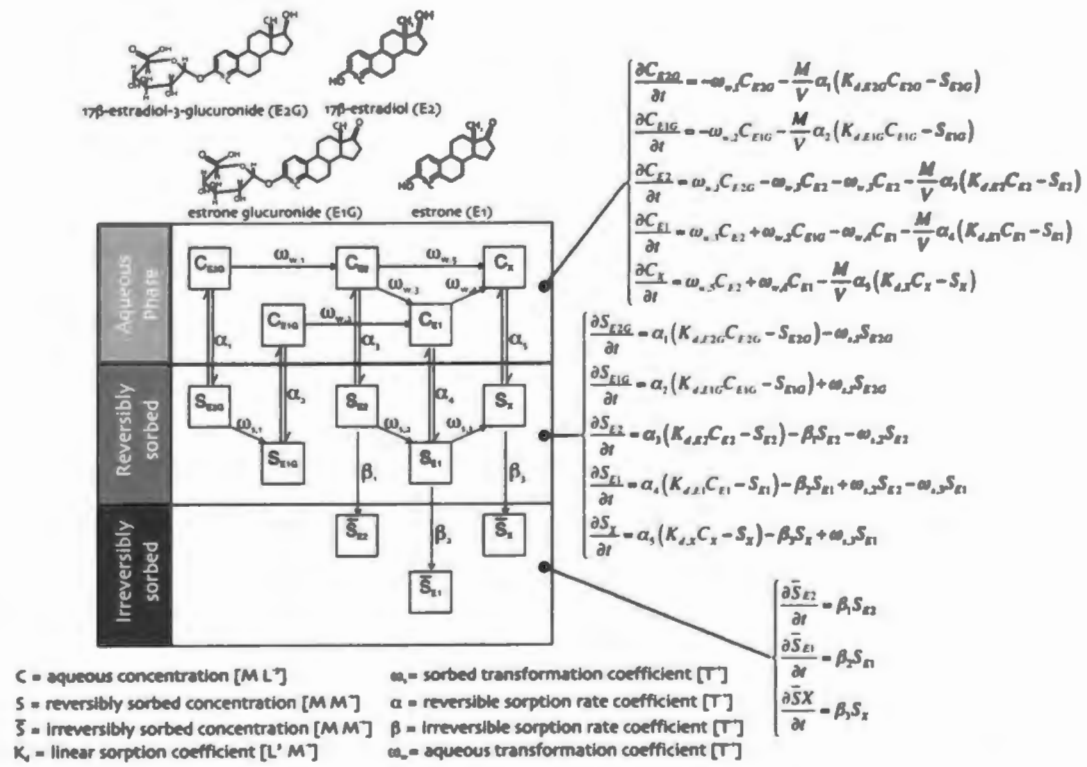

Figure 13. Conceptual model and governing equations for the fate and transformation of 17β estradiol-3-glucuronide and its metabolites in soil and water in the batch study.

Hydrolysis was also observed in the sterile soils (Fig. 12-G & 12-J), albeit at a distinctly slower rate. Irradiation sterilization (7.6 kGy) would have been sufficient to kill all soil biota but it may not have denatured enzymes released from lysed cells that could have hydrolyzed the glucuronide conjugates.

Oxidation of the conjugate and free estrogen

Under conditions of lower microbial activity (e.g. sterile, subsoil), abiotic oxidation was observed, similar to that reported by Colucci et al. (2001). Such a conversion has been reported to occur on Mn-oxide reaction sites in the soil (Xu et al., 2008). Manganese-oxide concentrations ranged from 154 to 255 $\mu g\ g^{-1}$ in the soil, following the method of Chao (1972). Oxidation was observed as the conversion of E2-3G to E1-3G and E2 to E1 in both natural and sterile soils (Fig 12-A, 12-D, 12-G, & 12-J). The oxidation rates in the topsoil (e.g. $\omega_{S,E2\text{-}3G}$=0.815 h^{-1}) with higher OC was lower compared to the subsoil ($\omega_{S,E2\text{-}3G}$=1.734 h^{-1}), because higher OC in the topsoil reduces the oxidative potential of the Mn-oxide reaction sites (Xu et al., 2008).

Sorption

Compared to the free estrogens (E2 and E1), estrogen conjugates had low potential to bind to the soil, and thus have higher mobility potentials in the environment. Sorption of free estrogens is primarily a hydrophobic process (Das et al., 2004). Compared to E2 the sorption partitioning coefficients (K_d) of E2-3G were one and three orders of magnitude lower for the topsoil and subsoil, respectively (Table 5). Moreover, estrogen conjugates extraction from the sorbed phases were nearly non-existent, indicating their low sorption potentials (Fig. 12-B, 12-E, 12-H, and 12-K). There is not much data in the literature on the fate and transformation of E2-3G to compare our value with. Nevertheless, the K_d value of E2-3G for the natural topsoil obtained in this study through inverse modeling (435 L Kg^{-1}) compares well with the experimental value of 114 L Kg^{-1} for sorption capacity of E2-3G obtained by Kobayashi et al. (2006) (reviewed in (Liu et al., 2009).

Factors that had the greatest influence on estrogen conjugate fate and transport were soil OC, soil microbial activities, and initial E2-3G concentrations. Values of sorption partitioning (K_d), sorption kinetics (α), and biotic transformation rates (ω_w) were one to two orders of magnitude greater in the high OC topsoil compared to the subsoil (Table 5). Additionally, E2-3G persisted in the aqueous phase seven times longer in the natural subsoil (persistence=168h, half-life=31 h) with lower OC and lower biological activity compared to the natural topsoil (persistence=24 h, half-life=4 h) (Fig. 12-A and 12-D). The persistence of intact E2-3G was exacerbated at higher initial concentrations (Fig. 14), which may indicate the saturability of the enzymatic hydrolysis. Half-lives for aqueous E2-3G in the natural subsoil increased from 31 to 133 h as initial concentrations increased from 3.7 to 22.5 $\mu g\ mL^{-1}$, taking 21-28 d to completely dissipate E2-3G at the highest initial concentration (Fig. 14).

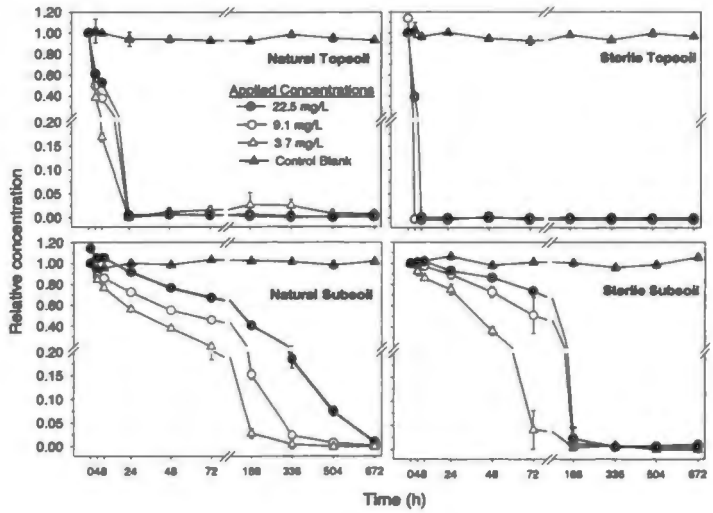

Figure 14. Aqueous phase concentration of 17β estradiol-3-glucuronide in natural and sterile topsoils and subsoils. Symbols represent means of triplicates (±standard errors). Scales in X and Y axes have been adjusted to highlight the changes

Table 5. The model parameter estimates for 17β estradiol-3-glucuronide and its metabolites in natural and sterile soils using stochastic ranking evolution strategies.

Natural Soil										
		K_d	α	ω_w	ω_s	K_d	α	β	ω_w	ω_s
		$L g^{-1}$	h^{-1}			$L g^{-1}$	h^{-1}			
		17β estradiol-3- glucuronide				17β estradiol				
Topsoil	Optimized value	0.435	0.059	3.545	0.815	3.040	0.080	1.048	9.79×10^{-4}	0.788
	95% CI¶	0.380-0.491	0.050-0.068	3.368-3.722	0.640-0.989	3.017-3.062	0.079-0.080	0.827-1.270	8.23×10^{-4}- 1.13×10^{-3}	0.620-0.956
		17β estrone-3- glucuronide				Estrone				
	Optimized value	0.043	0.181	0.178		3.024	0.681	3.56×10^{-6}	5.20×10^{-4}	0
	95% CI	0.039-0.046	0.136-0.227	0.175-0.181		3.005-3.044	0.551-0.811	$(3.51-3.61) \times 10^{-6}$	$(4.70-5.69) \times 10^{-4}$	
		17β estradiol-3- glucuronide				17β estradiol				
Subsoil	Optimized value	0.007	0.008	0.012	1.734	2.990	3.33×10^{-3}	4.149	4.15×10^{-7}	3.553
	95% CI	0.005-0.008	0.006-0.010	0.012-0.013	1.537-1.931	2.969-3.011	$(3.31-3.36) \times 10^{-3}$	3.857-4.441	$(3.37-4.93) \times 10^{-7}$	3.289-3.816
		17β estrone-3- glucuronide				Estrone				
	Optimized value	0.034	0.002	0.099		2.946	2.29×10^{-6}	3.5×10^{-6}	0.083	8.89×10^{-6}
	95% CI	0.029-0.039	0.002-0.003	0.089-0.109		2.910-2.982	$(2.23-2.34) \times 10^{-6}$	$(2.27-4.73) \times 10^{-6}$	0.079-0.087	$(8.82-8.97) \times 10^{-6}$
Sterile Soil										
		K_d	α	ω_w	ω_s	K_d	α	β	ω_w	ω_s
		$L g^{-1}$	h^{-1}			$L g^{-1}$	h^{-1}			
		17β estradiol-3- glucuronide				17β estradiol				
Topsoil	Optimized value	0.435	0.059	1.571	0.815	3.040	0.080	1.048		0.788
	95% CI	0.380-0.491	0.050-0.068	1.489-1.653	0.640-0.989	3.017-3.062	0.079-0.080	0.827-1.270		0.620-0.956
		17β estrone-3- glucuronide				Estrone				
	Optimized value	0.043	0.181	0.025		3.024	0.681	3.56×10^{-6}		0
	95% CI	0.039-0.046	0.136-0.227	0.025-0.025		3.005-3.044	0.551-0.811	$(3.51-3.61) \times 10^{-6}$		
		17β estradiol-3- glucuronide				17β estradiol				
Subsoil	Optimized value	0.007	0.008	0.010	1.734	2.990	3.33×10^{-3}	4.149		3.553
	95% CI	0.005-0.008	0.006-0.010	0.010-0.010	1.537-1.931	2.969-3.011	$(3.31-3.36) \times 10^{-3}$	3.857-4.441		3.289-3.816
		17β estrone-3- glucuronide				Estrone				
	Optimized value	0.034	0.002	0.039		2.946	2.29×10^{-6}	3.50×10^{-6}		8.89×10^{-6}
	95% CI	0.029-0.039	0.002-0.003	0.038-0.040		2.910-2.982	$(2.23-2.34) \times 10^{-6}$	$(2.27-4.73) \times 10^{-6}$		$(8.82-8.97) \times 10^{-6}$

†K_d is the linear sorption distribution coefficient; α and β are the sorption mass transfer coefficient between liquid and reversibly sorbed phases, and liquid and irreversibly sorbed phases, respectively; ω_w and ω_s are first-order transformation rates in the liquid and on the solid phase, respectively. ¶CI = confidence interval

Conclusions

Comparing results from the present study with the results from our previous field and laboratory studies, the apparent disparities between the two can be explained in light of the conjugate-aided transport of the estrogens. Although the soils used for all these experiments were the same, the field studies frequently detected E2 in soil drainage, groundwater, and widely distributed throughout the soil profile (Schuh et al., 2011a; Schuh et al., 2011b; Thompson et al., 2009), whereas the laboratory studies found unconjugated estrogens (E2 and E1) degraded within minutes and bound strongly and irreversibly to the

soil (Fan et al., 2007; Fan et al., 2008; Zitnick et al., 2011). Other researchers have made similar observances for field (Kolpin et al., 2002; Lei et al., 2009) and laboratory (Colucci et al., 2001; Das et al., 2004) studies. When conjugates are considered, the current study shows that they can persist intact in natural soil for long durations of time (21-28 d) and their mobility potential is much higher than free estrogens (E2 and E1). Implications of these results on AFO manure management are unclear; however, under typical manure management schemes they can possibly explain the observations of unexpectedly high detection frequencies and concentrations (Kolpin et al., 2002; Schuh et al., 2010; Thompson et al., 2009).

Based on the occurrence and fate and transformation of estrogen conjugates, major factors such as (i) the potential for large conjugated estrogens inputs from AFOs (~21,000 Kg y^{-1} in USA alone (Hanselman et al., 2003; Lange et al., 2002)), (ii) their persistence in soil-water (up to 28 d), and (iii) their low soil sorption potentials suggest their contribution to the overall fate and transport of estrogens in the environment has been greatly overlooked. The current study presents results for a glucuronide estrogen conjugate alone; however, these results may have even greater implications for the more recalcitrant sulfate conjugates that can persist longer in the environment (Hutchins et al., 2007).

Supplementary Information

Materials and methods

Soil

Soils, belonging to Hamar soil series (sandy, mixed, frigid typic Endoaquolls), were collected from a swine (*Sus scrofa domestica*) farm in southeastern North Dakota from the

surface (0 – 6 cm) and sub-surface (18 – 24 cm), and are referred to as topsoil and subsoil, respectively. Selected soil characteristics are given in Table 3 of Paper 3 of this manuscript. Prior to conducting sorption and speciation studies, soils were air-dried for 48 h. Also, soil aggregates were gently broken and then passed through a 2-mm sieve.

Chemicals

The study compound, 17β-[4-^{14}C]-estradiol-3-glucuronide (99% radiochemical purity and specific activity 103.13 Bq μg^{-1}), abbreviated as E2-3G, was synthesized using radiolabeled 17β-estradiol as described in detail by Shrestha et al. (2011). Ammonium acetate, hydrochloric acid, acetonitrile, calcium chloride dihydrate and formaldehyde (all analytical grades) were purchased from Sigma-Aldrich and were used as received for the synthesis and experimentation. Carbo-Sorb E and Permafluor were obtained from PerkinElmer (Waltham, MA) and scintillation fluid was purchased from MP Biomedicals (Ecolite; Santa Ana, CA).

Study design

The experimental variables for the soil batch studies were soil organic carbon (OC) content (n=2; 1.35 vs. 0.32%), soil biological activity (n=2; natural vs. sterile), initial concentration of E2-3G (n=5; 0.0, 0.5, 3.7, 9.1, and 22.5 $\mu g\ mL^{-1}$), and sample time (n=9; 4, 12, 24, 48, 72, 168, 336, 504, and 672 h). For each concentration level, triplicate vials were prepared with 1.6 g of soil and 8 mL of 0.01 M $CaCl_2$ solution in each vial. Blanks were identically prepared with no E2-3G added, while control blank vials contained no soil. Additionally, a separate series of experimental vials, referred to as 'stop vials', were prepared following the same protocol for all soil treatments (topsoil, subsoil, natural, and

sterile) with an initial E2-3G concentration of 3.7 μg mL^{-1}. The 'stop' vials were retired after each sampling time and extracted to obtain sorbed-phase concentrations of E2-3G and its metabolites.

Soil was sterilized by irradiation, which has minimal impacts on soil structure and organic matter compared to other methods (e.g. autoclaving) (Berns et al., 2008). Sterilization was achieved by irradiating the sealed vials containing soil and 0.01 M $CaCl_2$ for 14 h with 7.6 kGy. The E2-3G dose volumes of 57 μL each were prepared in 70% ethanol for sterile vials (200 μL in 20% ethanol for natural soil, both resulting in the total organic solvent of 0.5% of aqueous volume) and different initial concentrations of E2-3G were introduced into the sterilized vials by injection through the septa using sterile syringes.

Batch vials were mechanically rotated (360° every 5 s), stopping only to take samples. At each sample time, vials were removed from the mechanical rotator and centrifuged at 1700 rpm (380 × g) for 20 min. After centrifugation, 100-μL aliquots were withdrawn through the septa using sterile syringes to measure bulk radioactivity in the aqueous phase. Additional 120-μL aliquots were removed from each vial to identify E2-3G metabolites. The 120-μL aliquots were first passed through a 0.45 μm PTFE glass filter into HPLC vials, after which formaldehyde (2.7% final volume) was added, and then samples were stored at −20 ℃ until analysis. The aqueous phase of each 'stop' vial was decanted and formaldehyde (2.7% final volume/weight) was added to both the soil and aqueous phases before they were stored at −20 ℃. At the end of the experiment, headspace air was sampled and analyzed by gas chromatography (GC) combined with liquid scintillation counting (LSC).

Soils from the 'stop' vials were extracted with water (3×4 mL) and acetone (3×4 mL) using sonication for 30 min followed by centrifuging at 380 × g for 30 min. Extraction solvents were pooled, and triplicate 500 µL aliquots from each were assayed for bulk radioactivity. The acetone extract was evaporated under a stream of nitrogen, reconstituted with 1:1 acetone:nanopure water, and filtered through a 0.45 µm PTFE glass filter prior to HPLC analysis. The air-dried soil residues that remained after extraction were then analyzed by combustion analyses of five soil replicates (~0.1 g) for each sample using methods identical to (Casey et al., 2003).

Analytical techniques

Liquid scintillation counting. A Packard 1900 CA scintillation analyzer (Downers Grove, IL) was used to quantify radioactivity. Samples were dissolved in 4 mL of Ecolite scintillation cocktail and counted for 10 minutes. The limit of detection/limit of quantitation were determined by measuring replicate blank samples and were 0.24±0.03 µg L^{-1} (E2-3G and estrone glucuronide (E1-3G)) and 0.15±0.02 µg L^{-1} (E2 and E1) for the aqueous phase and 1.22±0.17, 1.21±0.17, 0.74 ±0.10, and 0.73 ±0.10 µg Kg^{-1} for E2-3G, E1-3G, E2, and E1, respectively in the sorbed phase.

High-performance liquid chromatography. Speciation analysis in the aqueous and reversibly sorbed phases were achieved by HPLC using a Waters 600E Systems Controller and pump (Milford, MA), a Jasco FP 920 fluorescence detector (Easton, MD), and a RP-HPLC column Phenomenex-C18, 4.6 ×250 µm. Solvent A was 10% acetonitrile (ACN) in 50 mM ammonium acetate (pH 4.5) and solvent B was 90% ACN in 50 mM ammonium acetate (pH 4.5). Gradient elution consisted of 20–100% B over 29 min, a 3

min hold at 100% B, and a return to 20% B in 3 min. The solvent flow was 1.0 mL min^{-1}; excitation and emission wavelengths were set at 280 and 312 nm, respectively. The injection volume of the analyte was 50 µL, and the eluent was fraction collected at 1 min interval. With this method, E2-3G and the free estrogens E2 and E1 eluted at 5.55, 27.57, and 29.90 min, respectively. The eluents were then analyzed by LSC to quantify the radioactivity in the known HPLC peaks of E2-3G, E2, and E1. Major Radioactive peaks were characterized by mass spectral analysis.

Mass spectral analysis. Negative ion LC/MS/MS was performed to characterize analytes using a Waters Alliance 2695 HPLC (Symmetry-C18, 2.1× 100 mm). Solvents were A: 40% ACN in nanopure water, B: 60% ACN in nanopure water and the gradient was 40-100% B over 10 min, 5 min hold at 100% B with 0.2 mL min^{-1} solvent flow. A Waters Micromass quadrupole time-of-flight (API-US) mass spectrometer was used in an ES- mode, with FWHM at 6500, source temperature 120°C, desolvation temperature 350°C, cone voltage 35 V, and capillary voltage 2500 V. The collision energy was found to be optimum at 20 and 50 eV to analyze glucuronide conjugate and metabolites. The MassLynx software was used for acquiring and analyzing the mass spectrometry information.

Gas chromatography. Radioactivity in headspace samples from batch vials was analyzed using an HP 5790A gas chromatograph (Avondale, PA) with a 10-foot glass column (1.5 mm id and 7.0 mm od) packed with 3% OV-17. Temperature gradient scheme was 30°C at the initial condition, hold for 2 min, raised at 30 deg min^{-1} to 180 deg, and 5 min hold. The effluent of the column was split so that 42% would be directed to the flame ionization detector for the determination of chemical mass, and the 58% was directed into a

740°C oven containing Cu(II)O, which would convert the [^{14}C]methane into [^{14}C]CO_2. The radiolabeled carbon dioxide was trapped by bubbling into Carbo-Sorb E (8 mL), then diluted with Permafluor (12 mL) and counted for radioactivity by LSC. The integrating recorder was an HP 3390A.

Soil combustion analysis. A tissue oxidizer (Packard Model 307 Oxidizer; Packard Chemicals, Meridan, CT) was used for soil combustion analysis. Each sample was combusted and the resulting CO_2 gas was trapped in the scrubber solution Carbo-Sorb E and mixed with Permafluor to measure the radioactivity by LSC. Blanks and standards were run before and after the samples were combusted.

Fate and transformation model

A conceptual model (Fig. 13) was developed to elucidate the fate and transformation processes of E2-3G (abbreviated as E2G in the model) and its metabolites E1-3G (abbreviated as E1G in the model), E2, and E1 in the soil batch experiments. The objective was to investigate the conversion of the relatively more hydrophilic conjugated estrogens (E2-3G and E1-3G) to its more hydrophobic free forms, or the deconjugated estrogens (E2 and E1) in different OC contents and soil microbial activities. This is crucially important in understanding the risk of conjugate derived estrogenicity to the environment. Further, the modeling would also provide an insight into the dominant processes involved in the conversion and the bioavailability of the free estrogens that were derived from their conjugated precursors under the different experimental conditions.

A one-site sorption model with simultaneous transformation and sorption of E2-3G and its metabolites was considered and was similar to the approach of Fan et al. (2008). The

one-site consideration assumed that all sorption sites are time-dependent or rate limited, and was backed by experimental observation in this study. Mass distribution was limited to aqueous, reversibly sorbed, and irreversibly sorbed phases. Gas phase was not considered because mineralization was not discernable from the headspace analysis. The following system of differential equations describes the model.

$$\begin{cases} \dfrac{dC_{E2G}}{dt} = -\omega_{w,1} C_{E2G} - \dfrac{M}{V}\alpha_1 \left(K_{d,E2G} C_{E2G} - S_{E2G}\right) \\ \dfrac{dC_{E1G}}{dt} = -\omega_{w,2} C_{E1G} - \dfrac{M}{V}\alpha_2 \left(K_{d,E1G} C_{E1G} - S_{E1G}\right) \\ \dfrac{dC_{E2}}{dt} = \omega_{w,1} C_{E2G} - \omega_{w,3} C_{E2} - \omega_{w,5} C_{E2} - \dfrac{M}{V}\alpha_3 \left(K_{d,E2} C_{E2} - S_{E2}\right) \\ \dfrac{dC_{E1}}{dt} = \omega_{w,3} C_{E2} + \omega_{w,2} C_{E1G} - \omega_{w,4} C_{E1} - \dfrac{M}{V}\alpha_4 \left(K_{d,E1} C_{E1} - S_{E1}\right) \\ \dfrac{dC_X}{dt} = \omega_{w,5} C_{E2} + \omega_{w,4} C_{E1} - \dfrac{M}{V}\alpha_5 \left(K_{d,X} C_X - S_X\right) \end{cases} \quad (1)$$

$$\begin{cases} \dfrac{dS_{E2G}}{dt} = \alpha_1 \left(K_{d,E2G} C_{E2G} - S_{E2G}\right) - \omega_{s,1} S_{E2G} \\ \dfrac{dS_{E1G}}{dt} = \alpha_2 \left(K_{d,E1G} C_{E1G} - S_{E1G}\right) + \omega_{s,1} S_{E2G} \\ \dfrac{dS_{E2}}{dt} = \alpha_3 \left(K_{d,E2} C_{E2} - S_{E2}\right) - \beta_1 S_{E2} - \omega_{s,2} S_{E2} \\ \dfrac{dS_{E1}}{dt} = \alpha_4 \left(K_{d,E1} C_{E1} - S_{E1}\right) - \beta_2 S_{E1} + \omega_{s,2} S_{E2} - \omega_{s,3} S_{E1} \\ \dfrac{dS_X}{dt} = \alpha_5 \left(K_{d,X} C_X - S_X\right) - \beta_3 S_X + \omega_{s,3} S_{E1} \end{cases} \quad (2)$$

$$\begin{cases} \dfrac{d\bar{S}_{E2}}{dt} = \beta_1 S_{E2} \\ \dfrac{d\bar{S}_{E1}}{dt} = \beta_2 S_{E1} \\ \dfrac{d\bar{S}X}{dt} = \beta_3 S_X \end{cases} \quad (3)$$

The mass balance of the batch experiment was expressed as

$$\begin{cases} C_{total} = C_{E2G} + C_{E1G} + C_{E2} + C_{E1} + C_X \\ S_{total} = S_{E2G} + S_{E1G} + S_{E2} + S_{E1} + S_X + \bar{S}_{E2} + \bar{S}_{E1} + \bar{S}_X \\ -M \dfrac{dS_{total}}{dt} = V \dfrac{dC_{total}}{dt} \end{cases} \quad (4)$$

Equations 1, 2, and 3 represent concentrations in aqueous (C; μM L^{-1}), reversibly sorbed (S; μM g^{-1}), and irreversibly sorbed ($\bar{S}$; μM g^{-1}) fractions, respectively. Individual species are represented by their subscripted symbols, while symbol X represents unidentified polar metabolites. The symbol K_d represents the linear distribution coefficient between the reversibly sorbed and aqueous phases (L g^{-1}); ω_w and ω_s represent the first-order transformation rate constants in the aqueous and sorbed phases (h^{-1}), respectively; α and β are the mass transfer rate constants (h^{-1}) between the aqueous and the reversibly-sorbed, and the reversibly-sorbed and the irreversibly-sorbed phases, respectively. In the equations, K_d is used with the respective subscripted names to represent individual species, while the rate constants (ω_w, ω_s, α, and β) are subscripted using numbers representing individual species as shown in Fig. 13 in the main document. Equations 1 through 4 were expanded to cover the soils in different horizons (topsoil and subsoil) in natural and sterile conditions in order to simultaneously optimize the parameters in all the experiments. The objective of the simultaneous optimization was to apply additional constraints on the inverse solution so as to improve the reliability of parameter estimates (Casey and Simunek, 2001).

Several model restrictions, based on known biogeochemical processes and scientific judgment, were applied in the conceptual model to improve the parameter uniqueness. The

model restrictions applied in this study are presented in Table 6 and also discussed in detail in the following paragraphs.

Biodegradation was assumed only to occur in the aqueous phase in that the compound needs to be available for the degrading bacteria (Harms and Bosma, 1997). Differences in the free estrogen concentrations through time in the aqueous phase for different soil treatments (Fig. 12-A, 12-D, 12-G, & 12-J) indicated that hydrolysis was different in the upper and lower horizon soils as well as in the natural and sterile soils. In sterile soils, enzymatic hydrolysis still occurred for E2-3G and E1-3G, which was possibly due to residual enzymes released from lysed bacterial cells. Speciation in the sterile soils indicated that for the topsoil, the aqueous and sorbed phases consisted only of E1 produced from the hydrolysis of E1-3G, and not the oxidation of E2 (Fig. 12-G & 12-H). In the subsoil, with OC an order of magnitude lower than the topsoil, the aqueous phase contained E2 and E1 (Fig. 12-J) that were also assumed to be the hydrolyzed products of E2-3G and E1-3G, respectively. Hence, biodegradation was considered only for the conjugated estrogens in the sterile soil.

Oxidation was considered to be an abiotic surface process attributed to MnO_2 (Sheng et al., 2009) and was assumed to be the same for natural and sterile soils, since sterilization would not impact the minerals. Oxidation was considered different for the upper and lower horizon soils because higher OC in the topsoil would reduce oxidation by interfering with Mn-oxide surface reactions sites and/or competing with the sequestration of the hydrophobic compounds (Barrett and McBride, 2005; Stepniewska et al., 2004; Zitnick et al., 2011). The α, K_d, and β were considered to be functions of soil OC content, not impacted by sterility, and were held constant between sterile and natural soils.

Table 6. Model restrictions applied in the simultaneous optimization of all soil treatments.

Model Restriction	Reference/Assumption
1. Biodegradation occurs in the aqueous phase.	(Harms and Bosma, 1997)
2. Oxidative conversion of estrogen occurs as a surface reaction.	(Sheng et al., 2009)
3. Biotic degradation (hydrolysis) in the natural topsoil is greater than that in the natural subsoil.	Greater microbial activities are associated with higher OM soil (Watts et al., 2010).
4. Abiotic degradation (oxidation) in the subsoil is greater than that in the topsoil.	(Barrett and McBride, 2005)
5. Sorption capacity of the topsoil (higher OM content) is greater than that of subsoil (lower OM content).	Sorption of the estrogens are governed by hydrophobic processes (Das et al., 2004)
6. Sorptive potential of soil (K_{oc}) follows the order of E1 ≥ E2 > X ≥ E1-3G ≥ E2-3G; where X is unidentified polar compounds.	Estrogen conjugates are more water soluble than free estrogens due to their polar moieties (Hanselman et al., 2003). The reverse-phase HPLC elution of estrogens, as obtained in this study, would result in more polar compounds to elute before less polar compounds.
7. For sterile soil, hydrolyses of estrogen conjugates were due to enzymatic hydrolysis.	Experimental evidence of hydrolysis of conjugate in sterile soil is assumed to be due to remnant hydrolyzing enzymes from lysed microbial cells during γ-sterilization.
8. Abiotic degradation (oxidation) is same in the natural and sterile soil.	Sterilization would minimally affect soil OM. The γ-sterilized soils and fractions result in fewer/smaller changes in the soil OM (Berns et al., 2008)
9. Mass transfer rate constants α and β, and sorption capacity K_d is same for the natural and sterile soils.	Sterilization by γ-radiation would not impact soil OC.

In total, there were 42 unknown process parameters in the model. The resulting equations were solved using a finite difference method, CVODE (Cohen and Hindmarsh, 1994) using a time step of 0.1 h. The batch model was applied inversely to match the model solutions to the experimental data by optimizing the model process parameters. The inverse problem was sought to minimize the objective function J that was defined as

$$J = \sum_{i=1}^{l}\sum_{j=1}^{n}\left\{\left[C\left(^{14}C\right)_i - \bar{C}\left(^{14}C\right)_i\right]_j\right\}^2 \qquad (5)$$

In equation (5), n is the number of experiments; l is the number of data points for each experiment; $C\left(^{14}C\right)_i$ is the experimental concentration of ^{14}C for each species in aqueous, reversibly sorbed, and irreversibly sorbed fractions; $\bar{C}\left(^{14}C\right)_i$ is the predicted concentration of ^{14}C for each species in the respective fractions. For the batch experiment, n was set to 4 to represent different soil treatments (topsoil, subsoil, natural, and sterile), and l was set to 9 representing the nine time points (4, 8, 24, 48, 72, 168, 336, 504, and 672 h).

Several parameters restrictions, based on known soil-physical relations related to sorption and on the experimental findings on relative chromatographic elution were used in the model to improve parameter uniqueness. Following constraints were applied to the parameter optimization (Table 6).

$$\begin{cases} (K_d)_{i,\,topsoil} > (K_d)_{i,\,subsoil} \\ (\omega_s)_{i,\,topsoil} < (\omega_s)_{i,\,subsoil} \\ (K_d)_{E1} \geq (K_d)_{E2} > (K_d)_X \geq (K_d)_{E1G} \geq (K_d)_{E2G} \end{cases} \qquad (6)$$

where i represents each species.

Topsoils were considered to have higher sorptive capacities for all estrogen species with greater hydrophobic partitioning by higher OC contents in the topsoils (Das et al., 2004). For the reason already described above, higher OC in the topsoil would reduce the oxidation of estrogens by interfering with the surface reaction with Mn-oxide sites (Barrett and McBride, 2005; Stepniewska et al., 2004; Zitnick et al., 2011). Further, sorption capacity of soil would be lesser for the conjugates which would be more water soluble than unconjugated estrogens due to their polar functional groups (Hanselman et al., 2003). Elution order in the RP-HPLC was used to indicate the order of hydrophobicity (hence K_d) of different species as shown in equation (6).

Owing to the large number of the process parameters to be optimized, a global optimization method was chosen over traditional inverse local optimization methods to avoid local optima. Stochastic ranking evolutionary strategy (SRES) (Runarsson and Xin, 2000), was used to solve this identification problem. SRES has been successfully used previously to estimate the process parameters to describe the fate and transport for unconjugated sex hormones (Fan and Casey, 2008; Fan et al., 2008) and in uniquely estimating 36 parameters of a nonlinear biochemical dynamic model to describe the metabolite concentrations with time (Moles et al., 2003). The program used in this paper was written in ANSI C using the libSRES library (Ji and Xu, 2006) and run on a PC/Pentium IV (2.99 GHz, 1GB of RAM) with a Windows XP operating system. To optimize the computational time, the program was initially run with a lower bound of 1.00×10^{-8} and an upper bound of 1.00 until the parameter values stabilized, and then the parameter values were refined by introducing new upper and lower bounds as 3 and 0.3

times the old values; with the refining continued till there was no more changes in the parameter estimate.

Table 5 presents the model parameter estimates for the study compound and its metabolites for the topsoil and subsoil with natural and sterile conditions. The final simulation was run 20 times to compute the 95% confidence interval. The goodness-of-fit of the model with observed data was evaluated using modified index of agreement, d_1 (Willmott et al., 1985), a parameter given by

$$d_1 = 1.0 - \frac{\sum_{i=1}^{n}|O_i - P_i|}{\sum_{i=1}^{n}(|P_i - \bar{O}| + |O_i - \bar{O}|)} \qquad (7)$$

where O and P are the observed and predicted data, $\bar{O}$ is the mean observed value, and n is the number of observation. The parameter d_1 takes a value from 0 to 1, with 1 indicating a perfect fit. It may be interpreted similarly to R^2 albeit considered superior, being less sensitive to outliers (Helmke et al., 2004), and has been applied to evaluate goodness of fit in soil studies (Helmke et al., 2004), and in hydrologic models (Legates and McCabe, 1999).

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GENERAL CONCLUSIONS

The conjugate 17β-[4-^{14}C]estradiol-3-glucuronide ([^{14}C]17β-E2-3-G) was synthesized using an enzymatic approach, with a high radiochemical yield of 84% for use in the fate and transport experiments. The synthesis techniques developed in this study may also be used to synthesize radiolabelled conjugates of other emerging contaminants for their radio-assay based environmental fate and transport studies.

A suit of analytical methods developed to study the labile conjugate allowed adequate quantification and qualifications of its fate in complex matrices (i.e., soil), while maintaining excellent mass balances. The methods included development of an HPLC analysis that allowed simultaneous detection and quantification of the polar estrogen conjugate and its hydrophobic metabolites, E2 and E1. Using this method with topsoil, total mass recoveries from the gaseous, aqueous, and bound fractions ranged from 99.0 to 105.5%. These experiments can be expanded to include other difficult environmental matrices of concern (e.g. manure, sediment), and used for other labile estrogens, with some appropriate modifications. Furthermore, this method greatly expands the ability to study these labile compounds.

The soil-water batch experiments using the natural topsoil and subsoil indicated a biphasic kinetic pattern of aqueous dissipation of E2-3G and its metabolites, E1-3G, E2, and E1. The initial dissipation phase (~24 h for the topsoil, ~168 h for the subsoil) was dominated by the hydrolysis of the glucuronides, while the second slower phase was dominated by sorption equilibria of the E1 and E2. Conjugate-derived estrogenicity in the aqueous phase (biologically available) was a function of soil organic matter as well as initial concentration of the applied E2-3G. The persistence of intact E2-3G was

exacerbated at higher initial concentrations, which may indicate the saturability of the enzymatic hydrolysis. These results from soil were in contrast to other studies that indicate rapid estrogen conjugate degradation in raw sewage from municipal treatment plants, and would have implications on the management of manure containing estrogen conjugates and the application of these manures to agricultural soil.

Hydrolysis and oxidation were the two main processes for the transformation of E2-3G and its metabolites. Depending upon whether the biotic or abiotic condition was dominant, the transformation pathways were distinctly different, and impacted on the metabolite formation, as shown in Fig. 15. In the natural topsoil, aqueous phase hydrolysis was primarily a biological process resulting in E2, which was subsequently oxidized to E1. In the sterile soil, oxidation, primarily a surface process, was the major pathway that resulted in an intermediate conjugate E1-3G, and was subsequently hydrolyzed to E1.

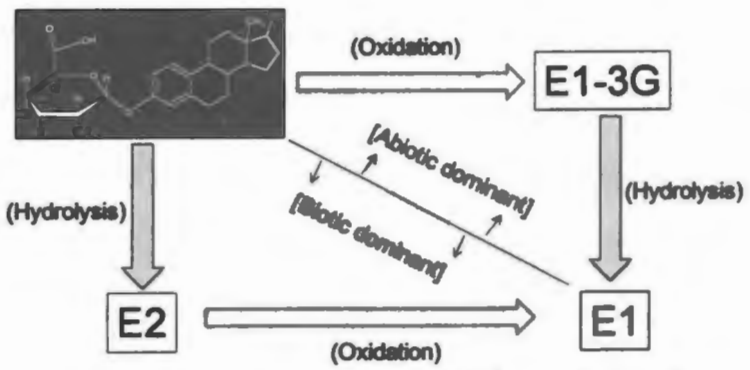

Figure 15.Transformation pathways of 17β-estradiol-3-glucuronide and its metabolites in the biotic and abiotic conditions.

Glycones (E2-3G and E1-3G) are innocuous, with their relative potential for estrogenicity at one to five orders of magnitude lower than their unconjugated forms; but they are more water soluble compared to aglycones (E2 and E1). Aqueous phase

dissipation of glycones indicated a critical period of 1 day for the topsoil and up to 14 days for the subsoil, where there is an increased potential of transport of intact glycones in the environment. Aqueous phase concentrations of the aglycones (E2 and E1) accounted for the bulk of the estrogenic activity (EEQ). For the topsoil, maximum values of EEQ ranged from 52.3 to 972.0 μg eq-E2 L^{-1} in the aqueous phase throughout the experiment (28 days) for the applied E2-3G concentration range, and 29.5 to 516.5 μg eq-E2 L^{-1} EEQ remained in the aqueous phase on day 28, indicating that conjugate-derived EEQ may be a significant source of bioavailable estrogenicity even with high OC topsoils (1.4%). For the natural subsoil, half-lives for aqueous E2-3G were longer than the topsoil, ranging from 31 h to 133 h for initial concentrations of 3.7 μg mL^{-1} to 22.5 μg mL^{-1}. The longer persistence of intact E2-3G in the subsoil may increase the potential mobility of E2-3G through the soil and to downstream locations where it can be hydrolyzed to potent E2 or E1.

A kinetic biogeochemical model was developed to account for the complex processes involving the simultaneous sorption and degradation of the highly labile E2-3G and its metabolites. Primarily, the modeling approach was sought to obtain a clear knowledge on the conversion of the relatively more hydrophilic conjugated estrogens (E2-3G and E1-3G) to its hydrophobic free forms, or the deconjugated estrogens (E2 and E1) in different experimental conditions of OC contents and soil microbial activities. Such information is essential in better understanding the risk of conjugate derived estrogenicity to the environment. The basic conceptual model is presented in Fig. 16.

Sink/Source	$\frac{dC}{dt} = -Sink + Source - \frac{M}{V}\alpha(K_dC - S)$ [Aqueous Phase]
ω_W (Biotic degradation)	$\frac{dS}{dt} = \alpha(K_dC - S) - Sink + Source$ [Reversibly Sorbed Phase]
ω_S (Abiotic degradation)	$\frac{d\bar{S}}{dt} = \beta S$ [Irreversibly Sorbed Phase]

Figure 16. Basic model formulation considering simultaneous sorption, and biotic and abiotic degradation of 17β-estradiol-3-glucuronide and its metabolites. Symbols $\boldsymbol{C}$, $\boldsymbol{S}$, and $\bar{\boldsymbol{S}}$ represent aqueous, reversibly sorbed and irreversibly sorbed concentrations; $\boldsymbol{M}$ and $\boldsymbol{V}$ are the mass of soil and volume of water used in the batch experiments; α and β are reversible and irreversible sorption rate coefficients, and $\boldsymbol{K_d}$ is the linear sorption coefficient.

The model results showed significant impacts of soil OM and microbial activities, with the dominant processes of hydrolysis, oxidation, and sorption. The hydrolysis rate of E2-3G in the sterile topsoil was less than half the value in the natural topsoil; however, the difference was not significant in the subsoil. Values of sorption partitioning, sorption kinetics, and biotic transformation rates for E2-3G were one to two orders of magnitude greater in the high OC topsoil compared to the subsoil. The sorption capacity of the glycone, E2-3G, was one and three orders of magnitude lower than the aglycone, E2, in the topsoil and subsoils, respectively, which indicated the highly polar nature of E2-3G. Although E2 can undergo both biotic and abiotic transformations into E1, the modeling indicated abiotic transformations (0.674 h^{-1}) were much more significant than biotic transformations (9.86×10^{-4} h^{-1}).

The contribution of estrogens to the environment from animals far exceeds that from humans. Further, majority of estrogens excreted by animals are in the form of conjugates. Results from the current studies suggest the potential contribution of estrogen glucuronide to the overall fate and transport of estrogens in the environment, under the context of agricultural soil-water systems. When conjugates are considered, estrogens in the conjugated forms may be introduced into the environment days after they are applied in soils and may hydrolyze to the free forms, in stark contrast with the transport potential of land applied free form estrogens to water bodies where, studies have shown the free form estrogens to be short-lived and immobile. Thus, the current laboratory study provides an important mechanistic link between dispensing of estrogen that occurs with manure application onto to agricultural lands or perhaps through grazing situation, and the consistent detections of estrogen in the water bodies that cannot be explained if only the transport of free form estrogens are considered. The risk of glycone derived estrogenicity to the environment, which is mostly overlooked in the fate and transport of estrogens, may be too great to ignore.

There were a few limitations in the study. The enzymatic activities in the soils were not measured but it was assumed that the natural topsoil had higher enzymatic activity than the natural subsoil. However, result from literature was used to back the assumption of higher microbial activity in the natural topsoil. The hydrolysis of the conjugate that occurred in the sterile soils was assumed to be caused by the antecedent enzymes in the soil or the enzymes that were lysed during the gamma-irradiation for soil sterilization. Additional experiment with autoclaved soil would have offered a definite answer to that assumption. Modeling results for total ^{14}C in the aqueous phase for natural soils were comparatively

inferior to that of the subsoils, especially in the later time points, indicating additional more complicated processes for natural soils. However, the objective of the modeling exercise was not to only numerically match the data points without understanding the processes involved. Overall, the current study has provided a very important ground work to understand the fate and transformation of conjugated estrogens in the environment.

Further Studies

The fate and transformation of E2-3G have been investigated in this research using soil-water batch studies. The degradation rate obtained in this study may represent a saturated field situation. Since enzymatic degradation of organic compound occurs in aqueous phase (Harms and Bosma, 1997), the saturated condition would have enhanced the degradation rate compared to a natural soil-water system in the field condition. Therefore, an incubation study with the soil water content at field capacity may give more representative degradation rates.

Soil irradiation was employed in this study to kill all biota in the soil prior to conducting sterile soil experiments. This method would help to preserve the soil structure as it does not impact soil water content compared to an autoclaving method. However, some deconjugation of E2-3G was observed in the irradiated soil. The possibility of chemical hydrolysis was ruled out since the soil pH was near neutral range whereas considerably lower pH (< 2) is required for an acid hydrolysis to occur. Perhaps some enzymes, lysed from the bacterial cell during the irradiation process, or some antecedent enzymes may have caused the deconjugation. Therefore, a similar experiment with autoclaved soil may help provide further insight into the mechanism of E2-3G hydrolysis.

Based on the results of this study, application of E2-3G in lower OC soils, such as in the subsurface, may enhance the intact conjugate transport due to the longer persistence (from lesser microbial activity in the soil) and higher water solubility (compared to free estrogens) of the conjugate. In this study, intact conjugate was available up to 14 days in the subsoil. A column study with the subsoil would provide further information on the transportability of the E2-3G in the vadose zone.

Radiolabelled conjugate synthesis methods were developed in this study for conjugates of E2. The method can be extrapolated to synthesize radiolabeled conjugates of other endocrine disrupting compounds such as anabolic steroid trenbolon acetate, and other compounds such as antibiotics that are used in animal agriculture, for example. This will enable the conduct of radio-assay based fate and transport studies on such compounds for which radiolabel version is commercially unavailable.

In this study, the process parameters involved in the fate and transformation of E2-3G and its metabolites in soil-water systems have been obtained by following a modeling approach that used linear sorption and one-site compartment. More compartment complexities can be introduced in the model by considering two-site or three-site assumptions.

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APPENDIX I. THE EXPERIMENTAL DATA ON SYNTHESIS OF 17β-ESTRADIOL-3-GLUCURONIDE

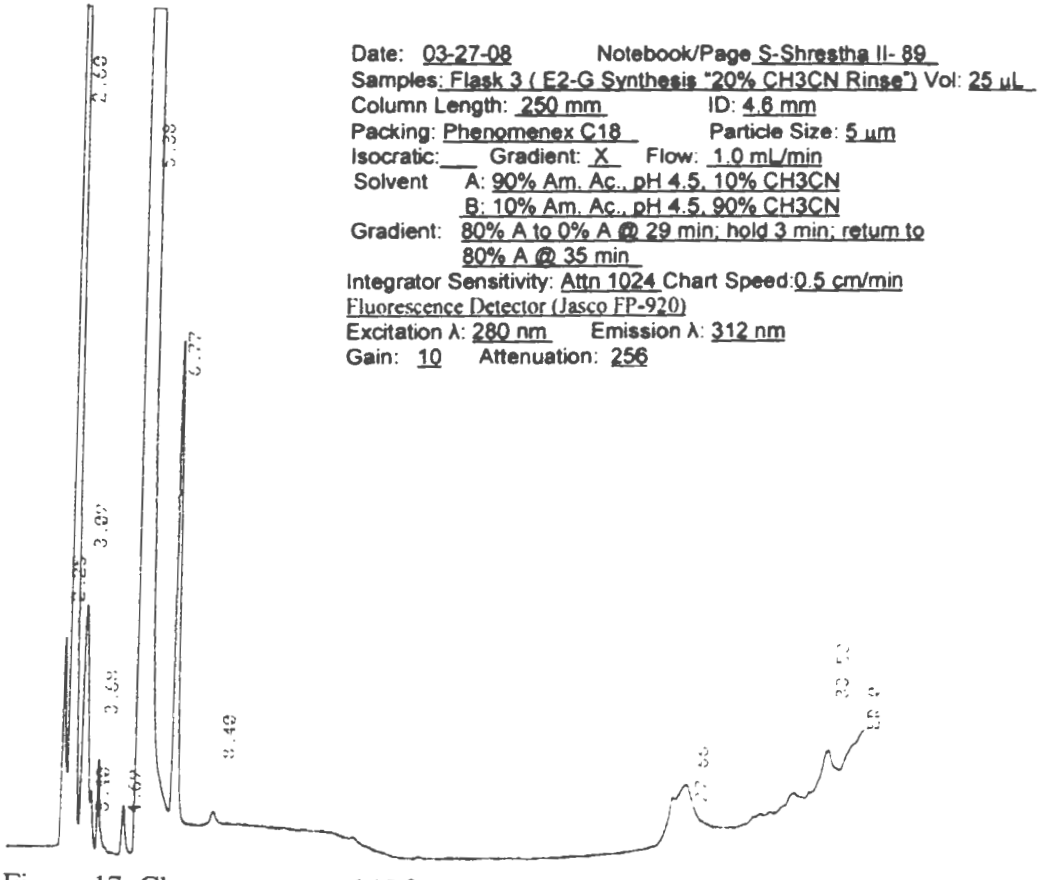


Figure 17. Chromatogram of 17β-estradiol glucuronide before purification.

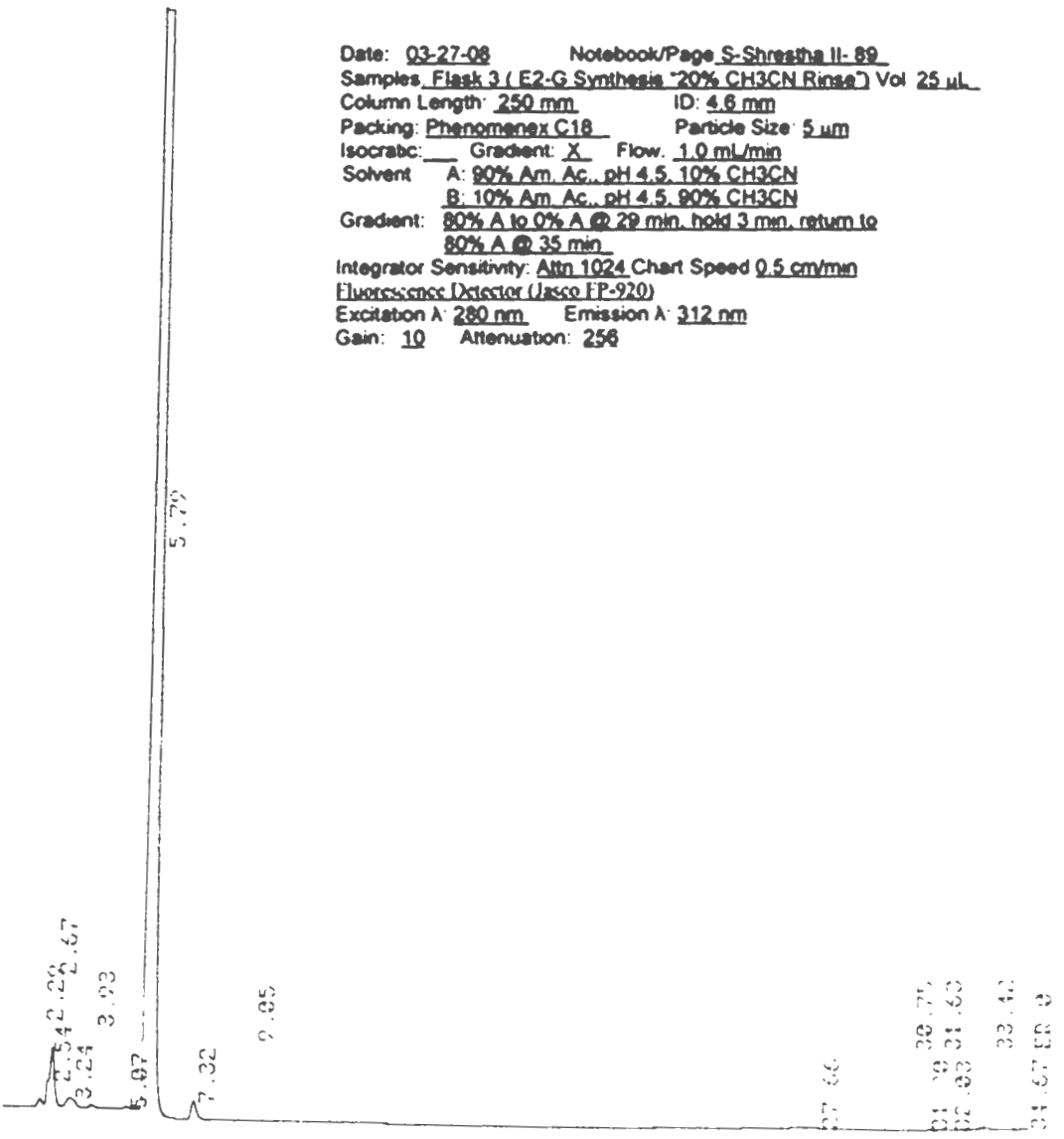


Figure 18. Chromatogram of 17β-estradiol glucuronide after purification.

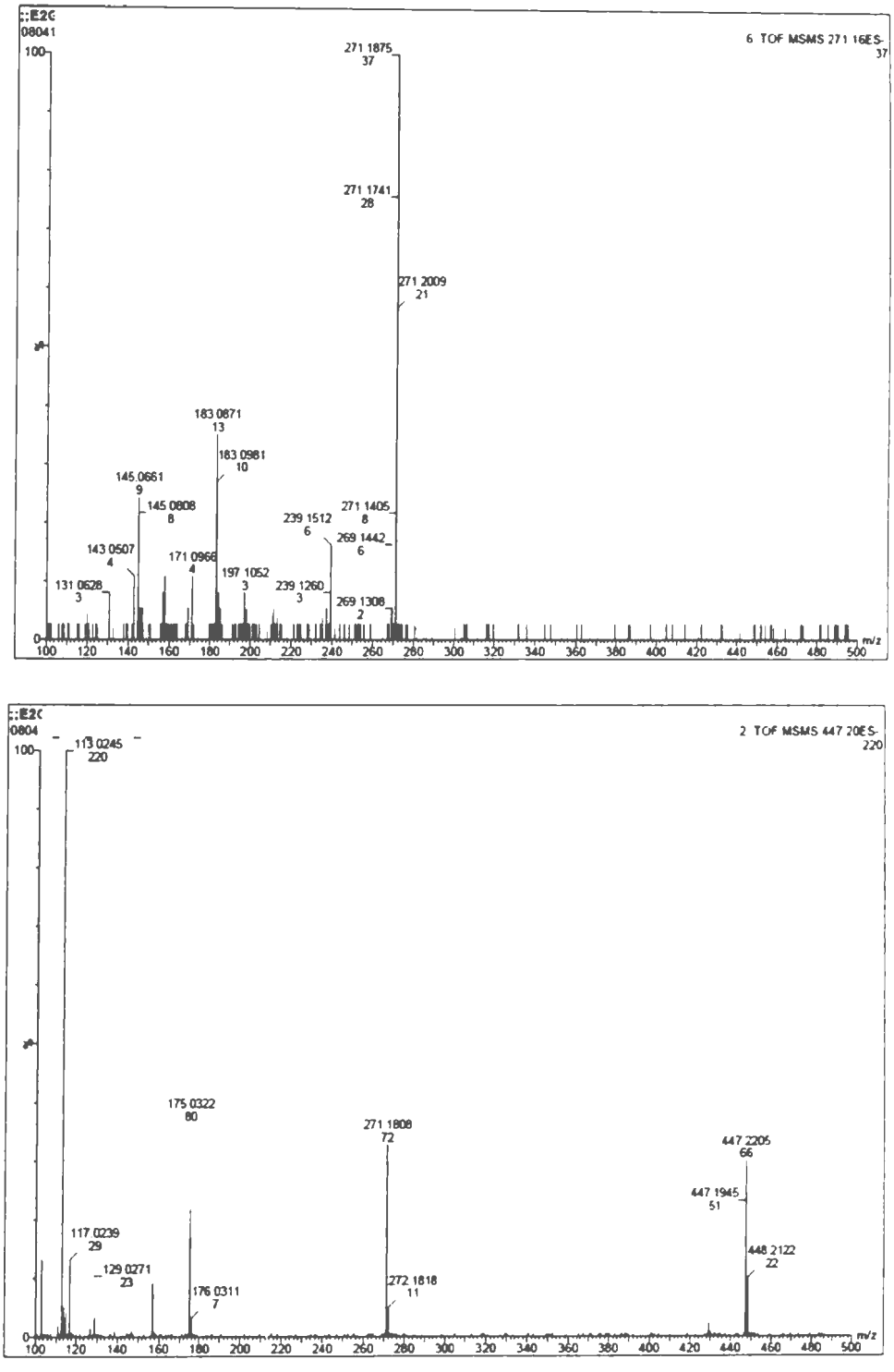
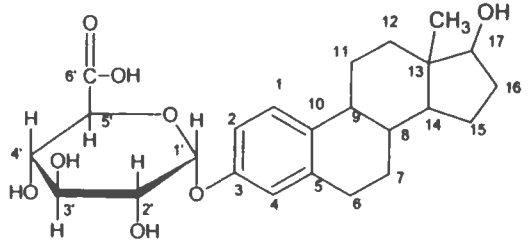


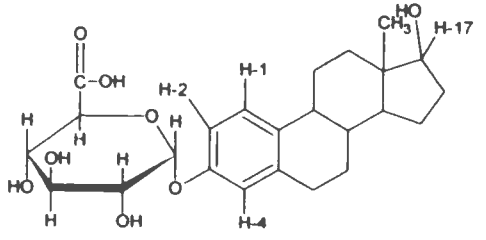
Figure 19. Mass spectra of 17β-estradiol glucuronide (bottom) and 17β-estradiol (top) obtained from the synthesized compound.

Table 7. Chemical shift assignments of NMR spectra of 17β-estradiol-3-glucuronide and 17β-estradiol-17-sulfate.

Carbon/Proton	E2[a]	E2-3G	E2-3-B	E2-3-B-17-S	E2-17-S	Remark
Carbon	127.22	127.20	127.472	127.52	127.26	E2-3-B = E2-3-Benzoate E2-3-B-17-S = E2-3-Benzoate-17-Sulfate E2-17-S = E2-17-Sulfate
1	113.72	115.41	119.791	119.839	113.762	
2	155.84	156.99	150.127	150.105	155.892	
3	116.05	117.96	122.626	122.638	116.053	
4	138.80	138.97	139.490	139.424	138.762	[a]Carbon assignments based on the values provided by Dionne et al. (1997) for 17β-estradiol
5	30.72[b]	30.69	134.857	134.947	132.535	
6	28.83	28.40	28.268	28.177	28.477	
7	40.50	40.34	40.144	40.292	40.335	[b]The signal for C6 is masked under solvent peaks (Acetone –d6) in values provided by Dionne et al. (1997). The assignment for C6 is based on the NMR spectrum obtained under MeOH solvent.
8	45.34	45.41	45.553	45.408	45.297	
9	132.32	135.66	30.694	30.533	30.71	
10	27.53	27.51	27.480	27.471	27.48	
11	38.00	37.97	37.992	37.981	38.00	s= singlet; d = duplet; t= triplet
12	44.35	44.32	44.343	44.225	44.236	
13	51.26	51.26	51.327	50.779	50.778	
14	24.03	24.00	24.043	24.106	24.1	
15	30.68	30.04	30.560	29.223	29.219	
16	82.49	82.47	82.455	88.216	88.186	
17	11.71	11.67	11.677	12.238	12.192	
18						
Sugar moiety carbons'						
1'	-	102.65				
2'	-	74.74				
3'	-	77.71				
4'	-	73.59				
5'	-	76.68				
6'	-	176.52				
Protons[a]						
H_1						
H_2	6.527(d,a)	6.870(d,a)	6.9375(d)	6.9035 (d)	6.527 (d)	
H_4	7.058(d,b)	7.177(d,b)	7.4405(d)	7.3015 (d)	7.0605 (d)	
H_{17}	6.467(s,c)	6.807(s,c)	6.8920(d)	6.855 (s)	6.4665 (s)	
			3.669 (t)	4.311 (t)	4.295 (t)	
Benzoate moiety carbons'						
B 1			165.936	166.953		
B 2			130.995	130.853		
B-3			130.995	130.853		
B-4			129.827	129.892		
B-5			129.827	129.892		
B 6			139.385	139.265		
B 1						
B 2						
B 3						



[Carbon (top figure) and Hydrogen (bottom) number.]



APPENDIX II. THE EXPERIMENTAL DATA OF FEW REPRESENTATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

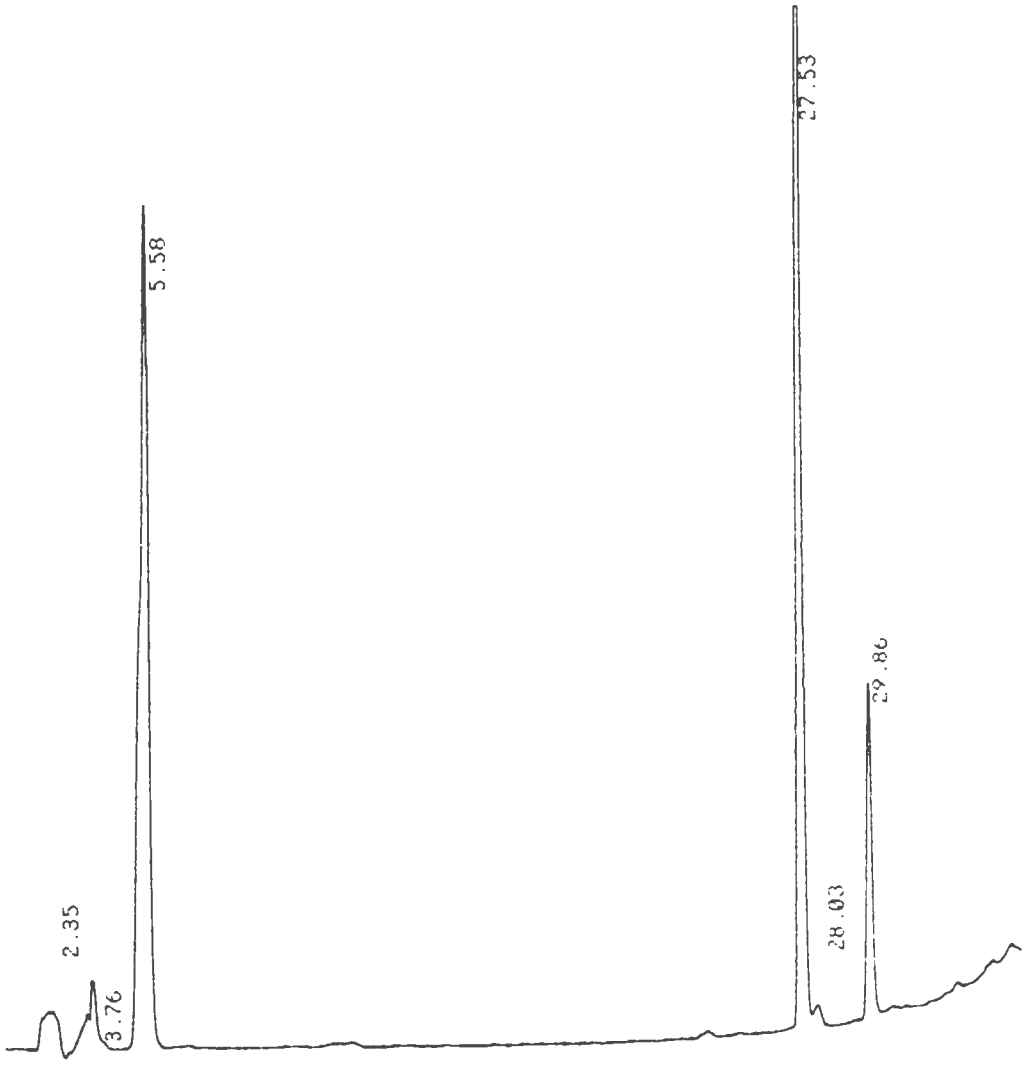


Figure 20. Chromatogram of the standard mix containing 17β-estradiol-3-glucuronide, 17β-estradiol, and estrone from method development.

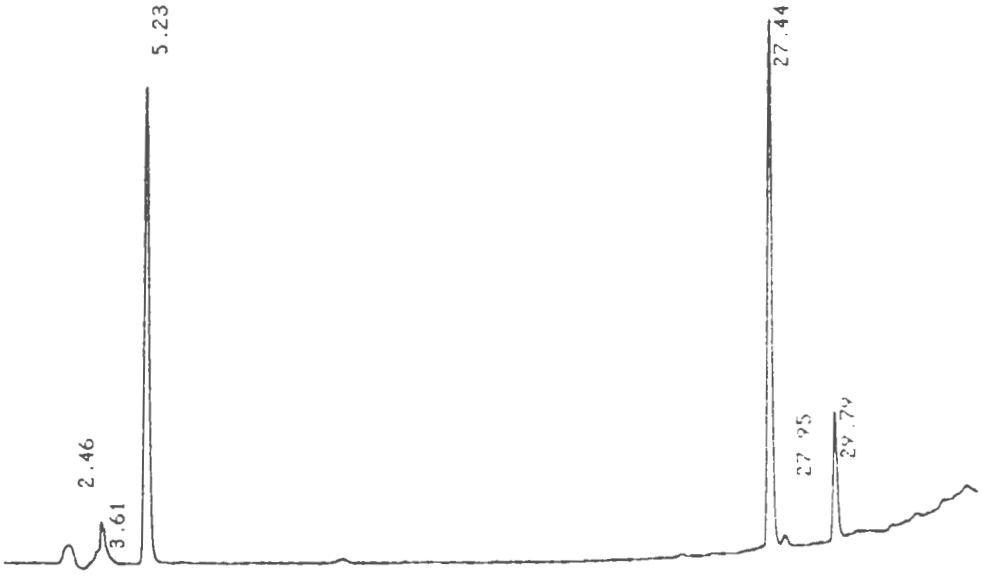


Figure 21. Chromatogram of the standard mix during batch study as a quality control.

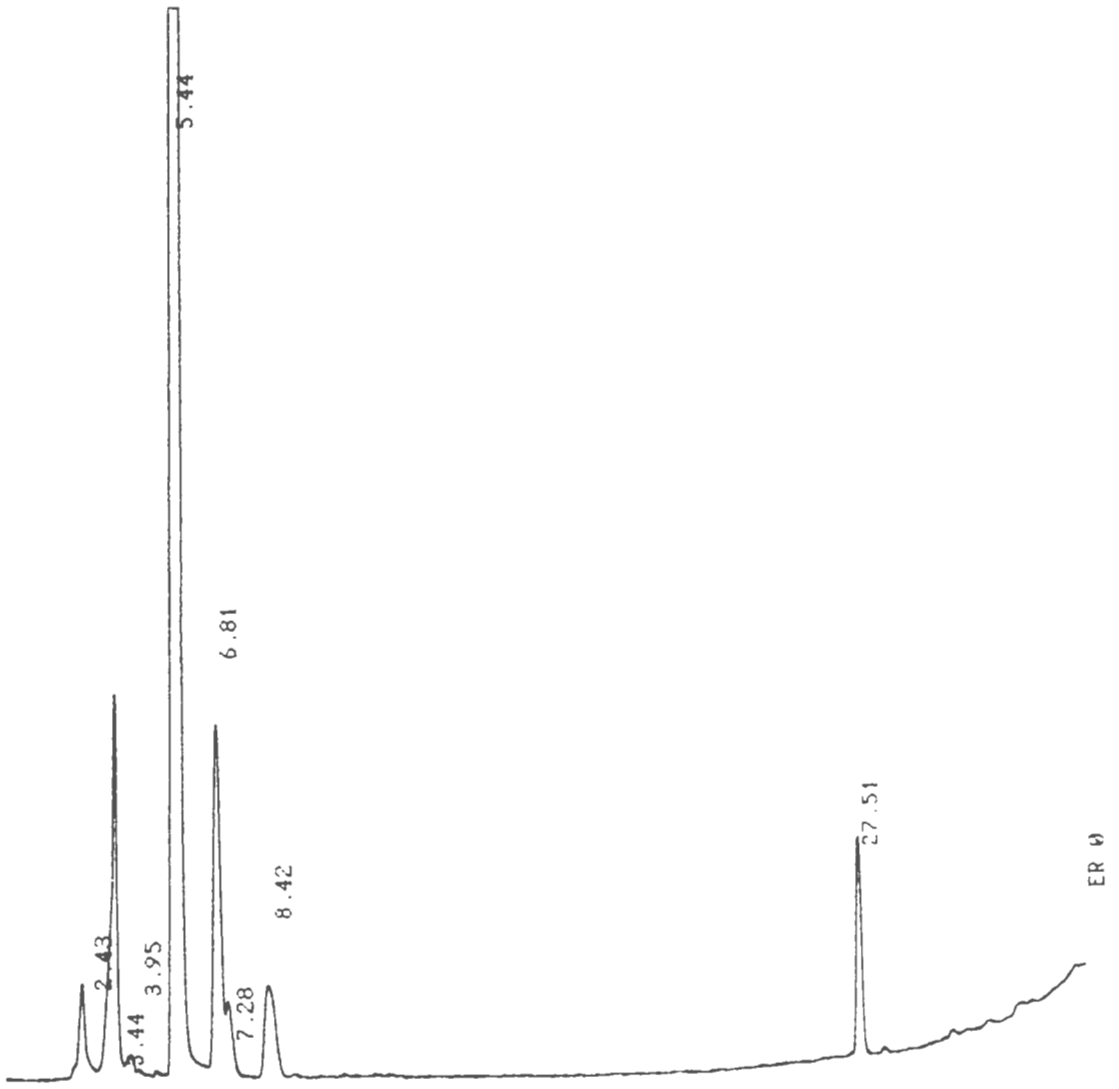


Figure 22. Chromatogram of aqueous phase speciation at 4 h in natural topsoil.

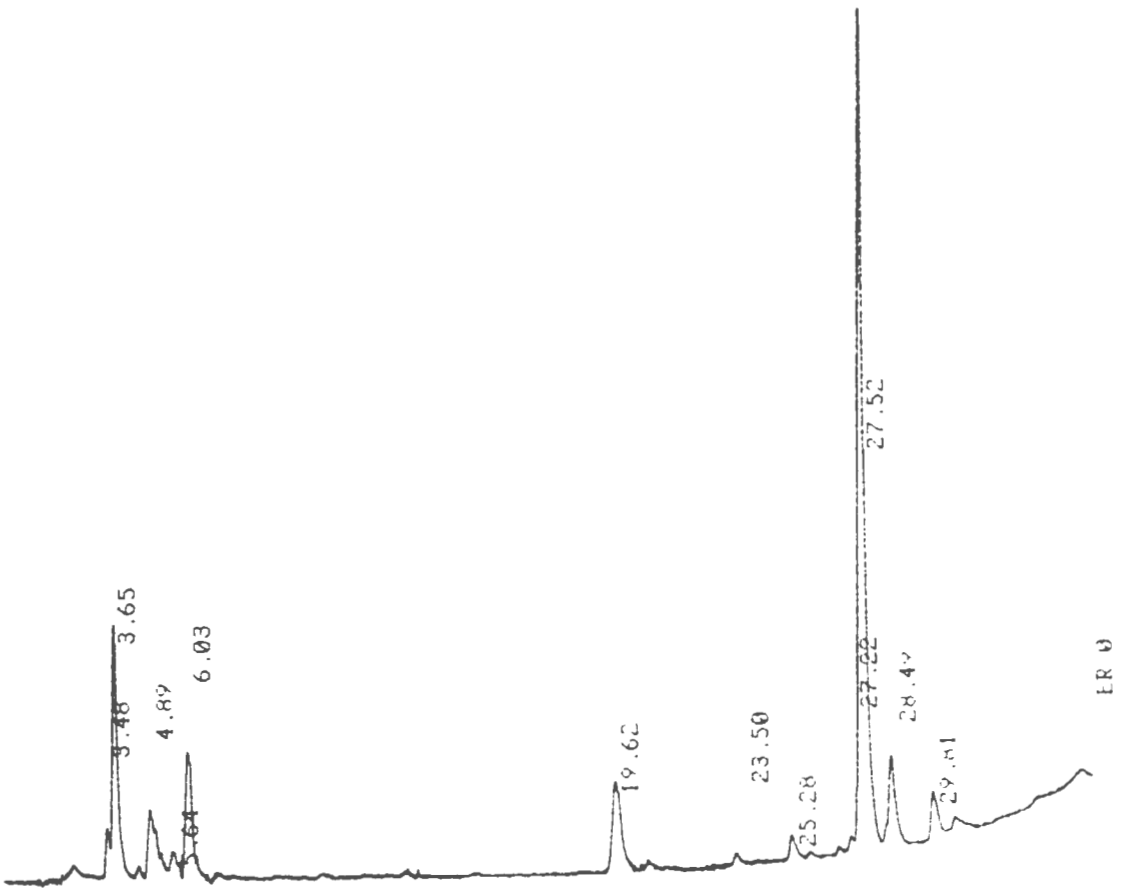


Figure 23. Chromatogram of aqueous phase speciation at 672 h in natural topsoil.

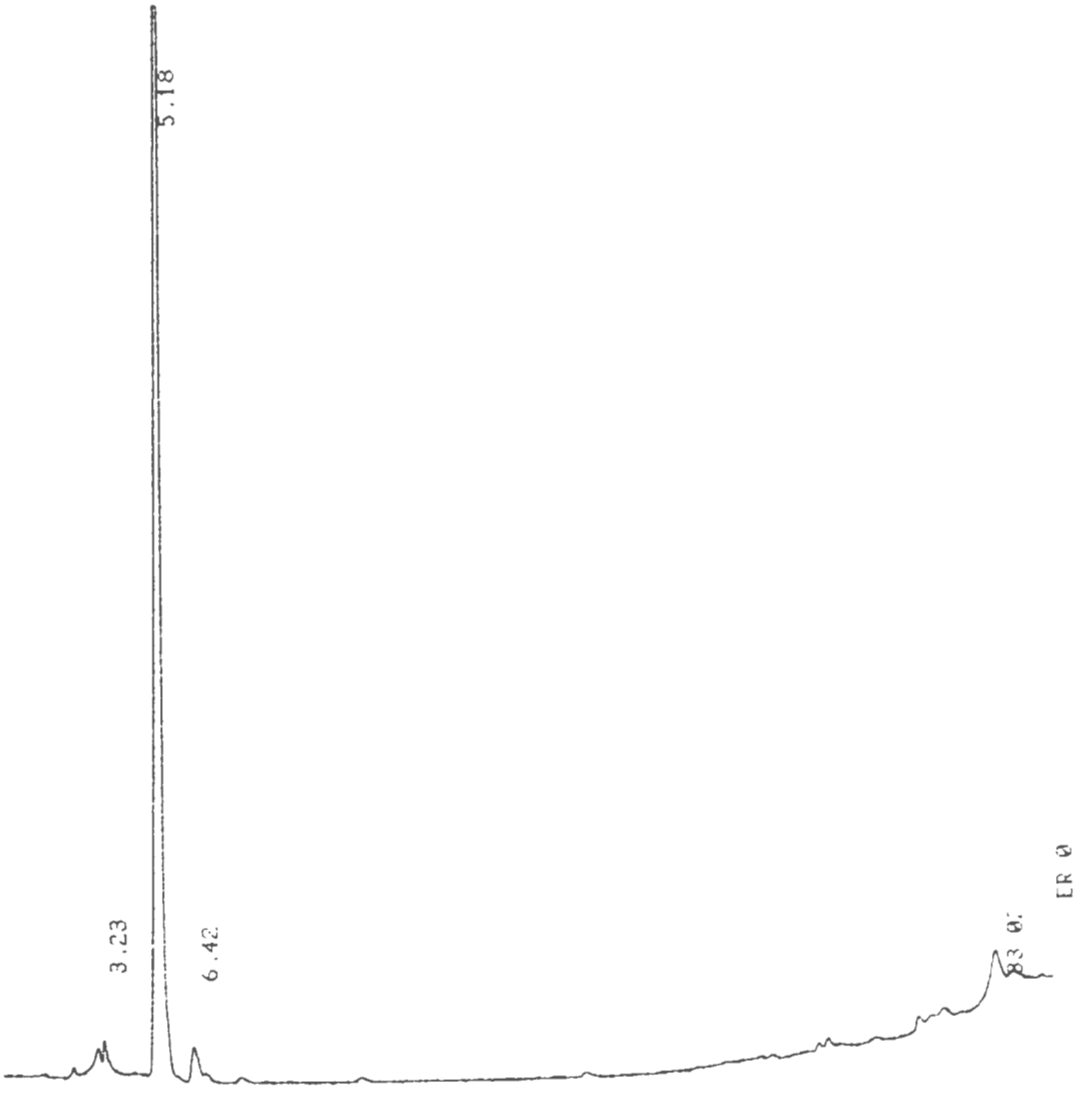


Figure 24. Chromatogram of speciation of aqueous sample from control blank at 672 h.

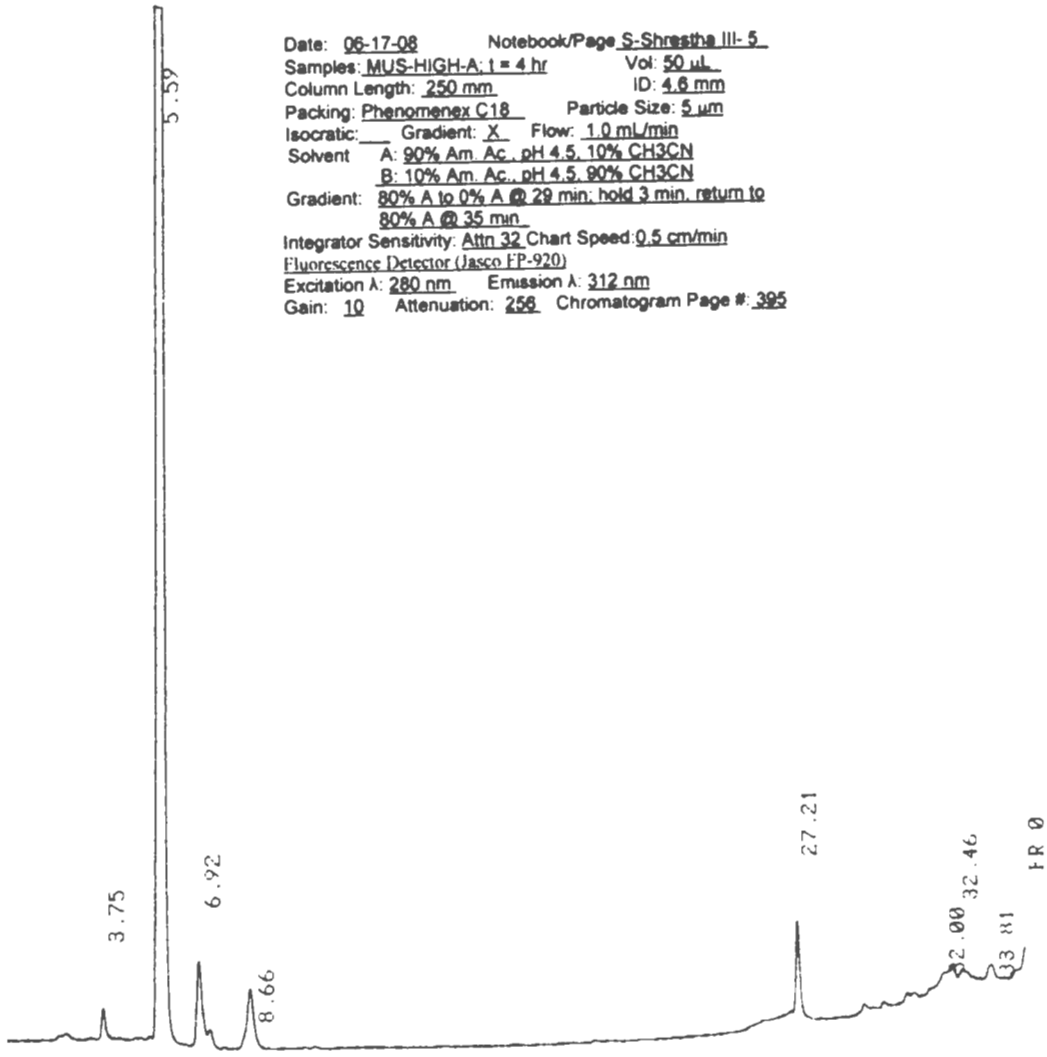


Figure 25. Chromatogram of aqueous phase speciation at 4 h in sterile topsoil.

Figure 26. Chromatogram of aqueous phase speciation at 8 h in sterile topsoil.

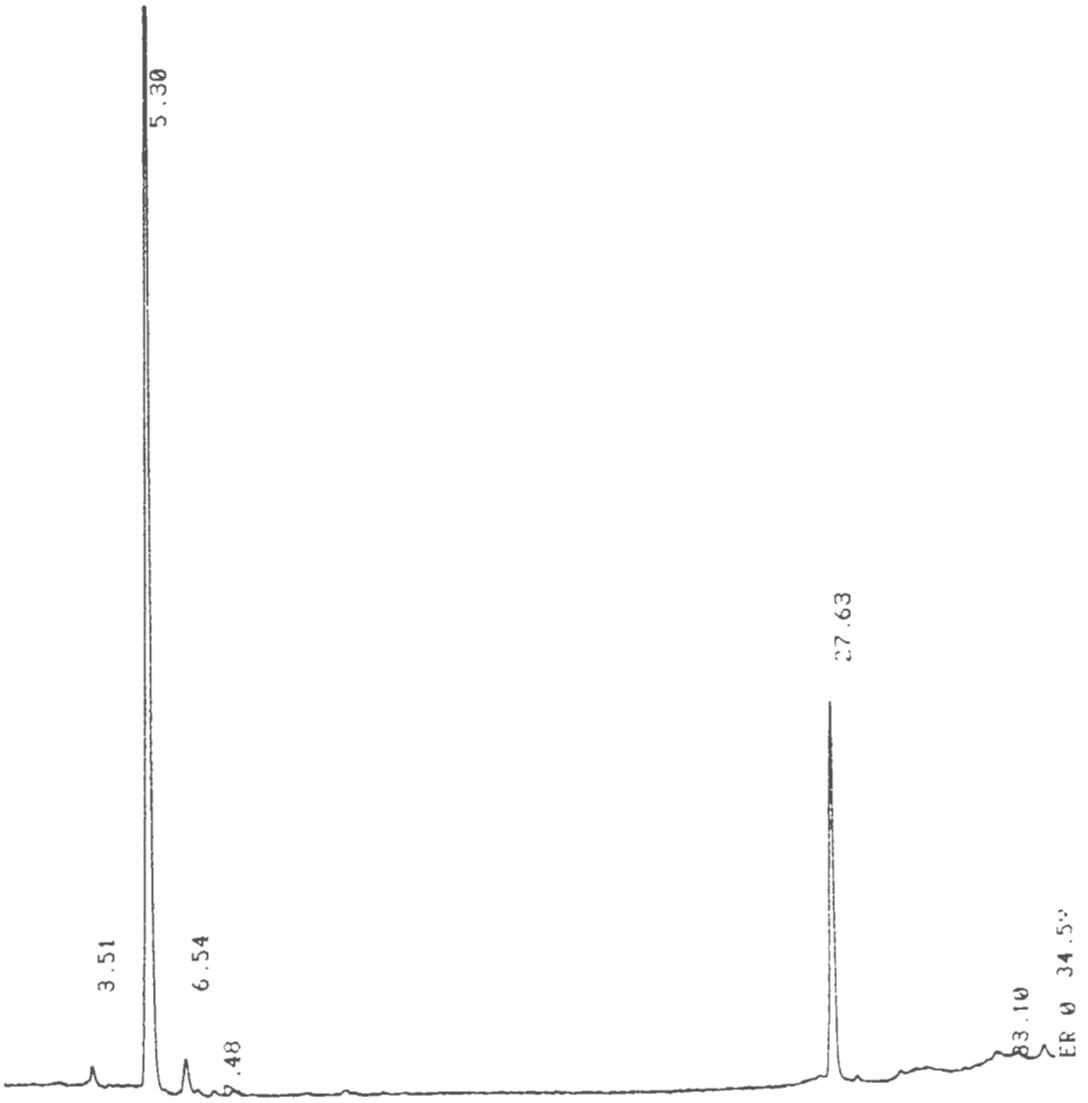


Figure 27. Chromatogram of aqueous phase speciation at 72 h in natural subsoil.

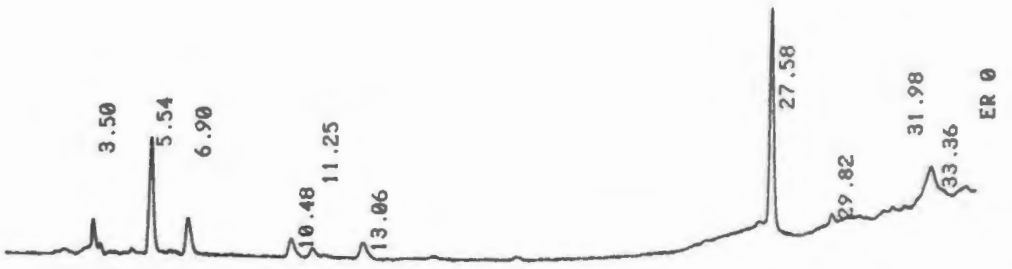


Figure 28. Chromatogram of aqueous phase speciation at 504 h in natural subsoil.

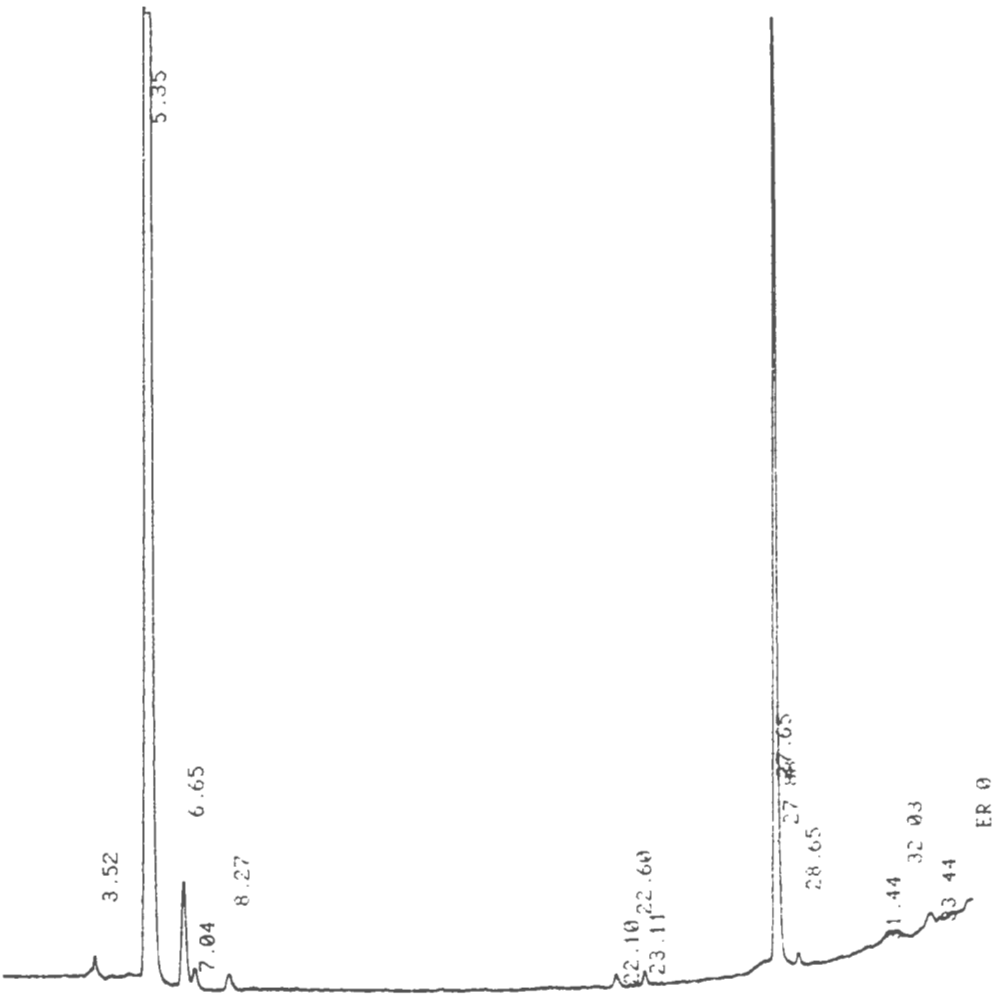


Figure 29. Chromatogram of aqueous phase speciation at 48 h in sterile subsoil.

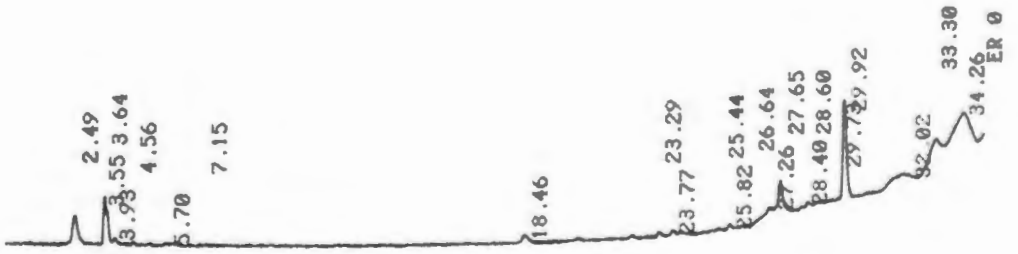


Figure 30. Chromatogram of aqueous phase speciation at 672 h in sterile subsoil.

APPENDIX III. THE EXPERIMENTAL DATA ON BATCH EXPERIMENTS WITH 17β-ESTRADIOL-3-GLUCURONIDE FOR ^{14}C

Table 8. The experimental data from liquid scintillation counting analysis of the aqueous phase through time for natural topsoil.

Treatment[†]	Time (h)									
	0	4	8	24	48	72	168	336	504	672
	^{14}C (dpm) in 100 µL									
High A	13940	9858	9053	3907	2904	2196	2102	1507	1601	1693
High B	13940	9880	8618	3654	2734	2064	1942	1458	1554	1616
High C	13940	9546	8423	3644	2748	2185	1993	1480	1615	1645
M2 A	5615	3613	2971	1208	896	679	645	455	498	505
M2 B	5615	3509	2910	1210	795	663	591	491	465	473
M2 C	5615	3470	2761	1139	831	620	591	434	467	496
M1 A	2301	1269	901	346	276	180	177	133	135	164
M1 B	2301	1244	829	326	240	166	173	130	135	216
M1 C	2301	1220	811	314	276	227	195	193	202	211
Ctrl. Blank A	331	419	344	283	325	323	346	313	303	346
Ctrl. Blank B	331	329	343	343	328	327	331	332	315	317
Ctrl. Blank C	331	288	317	331	302	311	326	323	336	289
Low A	331	198	140	42	31	22	20	22	59	27
Low B	331	188	121	35	28	45	21	ND[¶]	97	67
Low C	331	167	111	39	31	18	21	17	39	35

[†] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1};
[¶] ND = not detected

Table 9. The experimental data from liquid scintillation counting analysis of the aqueous phase through time for natural subsoil.

Treatment†	Time (h)									
	0	4	8	24	48	72	168	336	504	672
	^{14}C (dpm) in 100 µL									
High A	13940	13119	13203	12663	10718	9522	5981	3579	1882	1288
High B	13940	13258	13279	12660	11099	9691	6992	4444	2482	1647
High C	13940	13202	13361	12600	11311	9790	7008	4604	2638	1763
M2 A	5615	5139	5114	4904	4163	3458	1615	552	358	329
M2 B	5615	5217	5171	4986	4077	3360	1697	586	369	368
M2 C	5615	5186	5191	5026	4157	3422	1684	573	368	364
M1 A	2301	1921	1889	1846	1317	885	266	141	117	108
M1 B	2301	1981	1933	1815	1334	907	273	114	99	110
M1 C	2301	1927	1964	1869	1366	1014	339	135	107	119
Ctrl. Blank A	331	298	307	306	312	326	323	331	303	329
Ctrl. Blank B	331	310	315	340	312	329	326	339	302	325
Ctrl. Blank C	331	304	299	320	325	339	335	322	327	326
Low A	331	274	285	224	150	90	28	16	12	12
Low B	331	257	270	226	135	84	30	19	28	15
Low C	331	274	262	225	131	84	34	20	ND¶	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1};
¶ ND = not detected

Table 10. The experimental data from liquid scintillation counting analysis of the aqueous phase through time for sterile topsoil.

Treatment[†]	Time (h)									
	0	4	8	24	48	72	168	336	504	672
	^{14}C (dpm) in 100 µL									
High A	13940	12238	11477	9661	7118	5710	3804	3213	3126	2799
High B	13940	12799	12225	9898	6849	5049	3500	3158	3063	2892
High C	13940	12668	12165	9823	7428	4354	3070	2763	2695	2514
M2 A	5615	4706	4292	3409	2268	1522	1061	930	883	909
M2 B	5615	4767	4522	3100	1599	1158	1003	916	847	807
M2 C	5615	4860	4516	3491	2279	1568	1104	969	892	780
M1 A	2301	1701	1519	1118	615	394	350	297	283	261
M1 B	2301	1716	1563	NA[§]	NA	NA	NA	NA	NA	NA
M1 C	2301	1764	1550	1088	667	458	376	307	284	256
Ctrl. Blank A	331	304	298	318	315	286	327	296	310	310
Ctrl. Blank B	331	314	316	328	318	310	312	299	335	302
Ctrl. Blank C	331	327	324	324	315	308	307	304	314	321
Low A	331	219	185	84	51	35	30	22	22	14
Low B	331	218	189	116	57	33	30	20	22	13
Low C	331	217	191	113	53	39	38	32	27	24

[†] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1};
[§]NA= not available (the vial was broken and discontinued)

Table 11. The experimental data from liquid scintillation counting analysis of the aqueous phase through time for sterile subsoil.

Treatment[†]	Time (h)									
	0	4	8	24	48	72	168	336	504	672
	^{14}C (dpm) in 100 µL									
High A	13940	14594	14369	13823	13571	12937	10125	6280	5396	5094
High B	13940	14932	14568	14454	13920	13199	8039	3255	2883	3394
High C	13940	14167	15060	13823	11374	8678	5178	3981	3398	3354
M2 A	5615	5711	5727	5590	5276	5138	2808	2205	1758	1500
M2 B	5615	NA[§]	NA	NA	NA	NA	NA	NA	NA	NA
M2 C	5615	5931	5714	5485	5141	4603	2207	958	676	741
M1 A	2301	2031	2086	1989	1729	1485	1109	827	591	482
M1 B	2301	2243	2120	2210	2144	2065	1418	1035	898	824
M1 C	2301	2319	2173	2139	1887	1645	1231	975	787	691
Ctrl. Blank A	331	340	329	346	318	344	333	317	320	342
Ctrl. Blank B	331	340	351	379	329	345	332	347	348	374
Ctrl. Blank C	331	346	358	357	354	342	357	315	339	359
Low A	331	278	278	230	201	193	114	35	24	21
Low B	331	319	318	241	230	221	152	69	53	31
Low C	331	311	307	256	225	214	143	46	41	35

[†] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}, Low = 0.5 mg L^{-1}; [§]NA= not available (the vial was broken and discontinued)

APPENDIX IV. THE EXPERIMENTAL DATA ON MASS BALANCE ANALYSIS OF THE 'STOP' VIALS

Table 12. The experimental data from mass balance analysis of the 'stop' vial through time for natural topsoil.

Time	^{14}C (dpm)							
	Gaseous Phase	Aqueous Phase			Sorbed Phase		Total	% Mass Balance
(h)		Aqueous LSC	Water extract-ion from soil	Aqueous total	Extractable (Acetone)	Non-extractable (Combustion)		
0		184044		184044			184044	100.0
4	ND[†]	99603	18248	117852	61978	14117	193948	105.4
8	ND	65098	8188	73286	90287	23317	186890	101.5
24	ND	19796	25735	45531	134680	29836	210047	114.1
48	ND	14807	3275	18082	127388	48761	194231	105.5
72	ND	13677	3041	16718	120954	51678	189350	102.9
168	ND	9551	1170	10720	119239	56882	186841	101.5
336	ND	8816	1404	10220	113663	58320	182202	99.0
504	ND	21290	1638	22928	110231	60735	193893	105.4
672	ND	12910	702	13612	118439	55213	187265	101.8

[†]ND = not detected

Table 13. The experimental data from mass balance analysis of the 'stop' vial through time for natural subsoil.

Time	^{14}C (dpm)							
	Gaseous Phase	Aqueous Phase			Sorbed Phase		Total	% Mass Balance
(h)		Aqueous LSC	Water extraction from soil	Aqueous total	Extractable (Acetone)	Non-extractable (Combustion)		
0		184044		184044			184044	100.0
4	ND[*]	150553	25675	176228	5346	3605	185180	100.6
8	ND	144622	24427	169049	6570	4557	180177	97.9
24	ND	133616	22940	156555	13706	8514	178775	97.1
48	ND	93712	18892	112604	31542	32672	176818	96.1
72	ND	63123	16853	79975	50821	37321	168117	91.3
168	ND	19020	10990	30009	81522	53465	164997	89.7
336	ND	8123	3999	12123	113422	52123	177668	96.5
504	ND	6459	4079	10538	109990	54944	175472	95.3
672	ND	6458	3719	10177	116139	47132	173449	94.2

[*]ND = not detected

Table 14. The experimental data from mass balance analysis of the 'stop' vial through time for sterile topsoil.

Time	^{14}C (dpm)							
	Gaseous Phase	Aqueous Phase			Sorbed Phase		Total	% Mass Balance
(h)		Aqueous LSC	Water extraction from soil	Aqueous total	Extractable (Acetone)	Non-extractable (Combustion)		
0		184044		184044			184044	100.0
4	ND†	133816	16741	150557	27734	7052	185343	100.7
8	ND	115777	17996	133774	35702	9350	178825	97.2
24	ND	79952	18516	98468	62424	12235	173127	94.1
48	ND	44861	17789	62650	77637	34800	175087	95.1
72	ND	28749	21420	50169	94515	37528	182212	99.0
168	ND	23590	18836	42427	91699	42193	176318	95.8
336	ND	18871	19580	38451	97440	46389	182281	99.0
504	ND	17007	13077	30084	80695	54813	165592	90.0
672	ND	14861	13341	28202	86304	52929	167435	91.0

†ND = not detected

Table 15. The experimental data from mass balance analysis of the 'stop' vial through time for sterile subsoil.

Time	^{14}C (dpm)							
	Gaseous Phase	Aqueous Phase			Sorbed Phase		Total	% Mass Balance
(h)		Aqueous LSC	Water extraction from soil	Aqueous total	Extractable (Acetone)	Non-extractable (Combustion)		
0		184044		184044			184044	100.0
4	ND†	170286	21028	191314	2409	2079	195802	106.4
8	ND	159444	20516	179960	1954	2007	183921	99.9
24	ND	153138	21852	174990	3838	3658	182486	99.2
48	ND	134374	23323	157697	10765	4815	173277	94.1
72	ND	116865	31738	148602	29517	11210	189329	102.9
168	ND	81407	14181	95589	38200	31991	165780	90.1
336	ND	59093	13285	72378	75820	30287	178485	97.0
504	ND	45511	25179	70690	45869	49788	166347	90.4
672	ND	38268	13189	51458	66949	46442	164849	89.6

†ND = not detected

Table 16. The experimental data from high performance liquid chromatography and liquid scintillation counting for the aqueous phase speciation analysis of the 'stop' vial for natural topsoil.

Time	Aqueous Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0	184044					184044
4	84382	14962	993	2056	15458	117852
8	33341	14178	4014	4079	17609	73221
24	ND[†]	2587	4346	18109	20385	45427
48	1788	868	ND	8019	7355	18031
72	3004	2351	588	5355	5290	16587
168	3471	1626	2592	2988	ND	10676
336	3927	957	ND	1007	4330	10220
504	2729	6332	7643	6223	ND	22928
672	1035	259	1863	9265	1190	13612

[†]ND = not detected

Table 17. The experimental data from high performance liquid chromatography and liquid scintillation counting for the aqueous phase speciation analysis of the 'stop' vial for natural subsoil.

Time	Aqueous Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0	184044					184044
4	175321	ND[†]	ND	ND	907	176228
8	153516	6774	4146	818	3796	169049
24	109220	5889	32783	3341	5322	156555
48	72452	4038	27534	5327	3253	112604
72	39817	2510	21049	5305	11295	79975
168	6479	1910	4229	4774	12618	30009
336	1119	560	1616	1306	7522	12123
504	ND	4058	720	ND	5760	10538
672	ND	1445	ND	ND	8732	10177

[†]ND = not detected

Table 18. The experimental data from high performance liquid chromatography and liquid scintillation counting for the aqueous phase speciation analysis of the 'stop' vial for sterile topsoil.

Time	Aqueous Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0	184044					184044
4	779	142856	260	2855	3807	150557
8	ND[†]	96743	ND	8420	28697	133860
24	ND	80781	ND	17687	ND	98468
48	7779	15557	ND	31956	7358	62650
72	4211	10708	ND	30920	4331	50169
168	ND	2882	ND	39083	461	42427
336	ND	ND	ND	38451	ND	38451
504	ND	ND	1589	24894	3602	30084
672	ND	ND	2178	26024	ND	28202

[†]ND = not detected

Table 19. The experimental data from high performance liquid chromatography and liquid scintillation counting for the aqueous phase speciation analysis of the 'stop' vial for sterile subsoil.

Time	Aqueous Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0	184044					184044
4	184701	232	928	ND	5395	191256
8	167154	5303	6318	ND	1185	179960
24	142973	11924	8888	1325	9771	174880
48	72267	16040	40013	15778	13512	157610
72	8801	7967	61053	37892	32982	148695
168	763	6966	3915	76064	7830	95538
336	1325	816	3517	38839	27728	72225
504	ND	2917	621	47168	19922	70628
672	ND	721	ND	38271	12362	51355

[†]ND = not detected

Table 20. The experimental data from high performance liquid chromatography and liquid scintillation counting for the sorbed phase speciation analysis of the 'stop' vial for natural topsoil.

Time	Sorbed Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0						
4	ND[†]	ND	10590	32979	18409	61978
8	ND	ND	14084	53820	22383	90287
24	ND	ND	14204	120475	ND	134680
48	ND	ND	8125	81830	37433	127388
72	ND	ND	ND	120954	ND	120954
168	ND	ND	16829	95606	6803	119239
336	ND	ND	21243	80593	11826	113663
504	ND	ND	27038	83194	ND	110231
672	ND	ND	33227	63060	22152	118439

[†]ND = not detected

Table 21. The experimental data from high performance liquid chromatography and liquid scintillation counting for the sorbed phase speciation analysis of the 'stop' vial for natural subsoil.

Time	Sorbed Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0						
4	228	ND	1027	342	3750	5346
8	211	ND	2428	554	3377	6570
24	630	296	6038	1222	5519	13706
48	654	654	7354	2860	20020	31542
72	ND	ND	16804	8607	25411	50821
168	ND	ND	34876	20054	26593	81522
336	ND	ND	21434	20541	71447	113422
504	3687	ND	36254	18434	51615	109990
672	ND	ND	33874	21776	60489	116139

[†]ND = not detected

Table 22. The experimental data from high performance liquid chromatography and liquid scintillation counting for the sorbed phase speciation analysis of the 'stop' vial for sterile topsoil.

Time	Sorbed Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0						
4	330	454	ND[†]	20471	6480	27734
8	727	581	ND	31293	3100	35702
24	ND	ND	ND	51974	10450	62424
48	ND	ND	ND	66002	11635	77637
72	ND	ND	ND	84524	9990	94515
168	ND	1429	ND	66690	23580	91699
336	ND	ND	ND	74544	22896	97440
504	ND	1118	ND	56282	23295	80695
672	ND	1046	1395	73053	10810	86304

[†]ND = not detected

Table 23. The experimental data from high performance liquid chromatography and liquid scintillation counting for the sorbed phase speciation analysis of the 'stop' vial for sterile subsoil.

Time	Sorbed Phase ^{14}C (dpm)					
(h)	17β-estradiol-3-glucuronide	Estrone-3-glucuronide	17β-estradiol	Estrone	Unidentified Metabolites	Total
0						
4	ND[†]	229	592	1259	328	2409
8	42	388	430	806	288	1954
24	520	146	1249	1085	839	3838
48	530	251	4825	3514	1645	10765
72	383	638	2423	17787	8288	29517
168	461	307	692	27439	9300	38200
336	1111	ND	1944	34438	38327	75820
504	ND	ND	1867	32213	11788	45869
672	ND	ND	3134	35896	27919	66949

[†]ND = not detected

APPENDIX V. THE EXPERIMENTAL DATA ON SPECIATION ANALYSIS OF BATCH VIALS

Table 24. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol-3-glucuronide in the aqueous phase for natural topsoil.

Treatment[a]	^{14}C (dpm) from 17β-estradiol-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	6970	3997	3604	53	27	29	67	25	ND[b]	19
High B	6970	4451	3739	30	24	39	26	12	ND	8
High C	6970	4338	3668	13	69	46	23	11	11	ND
M2 A	2808	1351	1077	ND	22	12	16	ND	ND	44
M2 B	2808	1462	1080	9	13	22	ND	ND	ND	ND
M2 C	2808	1380	1022	ND	7	ND	ND	ND	ND	5
M1 A	1151	417	210	ND	31	20	88	44	28	23
M1 B	1151	494	173	ND	9	ND	ND	44	ND	ND
M1 C	1151	425	196	ND	ND	31	ND	ND	ND	ND
Ctrl. Blank A	166	203	169	141	163	161	170	153	149	172
Ctrl. Blank B	166	158	165	164	163	161	165	165	156	158
Ctrl. Blank C	166	137	156	143	151	154	161	159	167	144

[a] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; [b] ND = not detected

Table 25. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone-3-glucuronide in the aqueous phase for natural topsoil.

Treatment†	^{14}C (dpm) from estrone-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	430	440	26	74	ND¶	33	21	ND	14
High B	NM	300	318	41	16	28	ND	ND	35	ND
High C	NM	257	252	42	43	29	ND	ND	ND	ND
M2 A	NM	116	151	15	ND	ND	ND	ND	ND	7
M2 B	NM	132	135	9	5	7	35	ND	20	ND
M2 C	NM	118	122	11	ND	ND	ND	ND	ND	ND
M1 A	NM	88	59	15	6	ND	ND	ND	ND	6
M1 B	NM	72	91	13	13	40	42	21	ND	ND
M1 C	NM	78	95	ND	ND	ND	ND	ND	65	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	1
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	ND	1	ND
Ctrl. Blank C	NM	2	ND	ND	ND	1	ND	ND	1	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured;
¶ND = not detected

Table 26. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol in the aqueous phase for natural topsoil.

Treatment[†]	^{14}C (dpm) from 17 β-estradiol peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM[‡]	34	133	527	47	ND[¶]	173	180	242	262
High B	NM	29	72	413	49	14	201	165	264	225
High C	NM	54	108	460	66	12	192	193	246	269
M2 A	NM	30	77	106	ND	6	64	60	93	55
M2 B	NM	33	68	187	5	9	55	32	64	83
M2 C	NM	7	38	74	8	10	61	36	62	62
M1 A	NM	16	16	17	ND	ND	ND	ND	11	20
M1 B	NM	ND	24	11	ND	ND	ND	ND	67	ND
M1 C	NM	ND	31	18	ND	10	67	ND	ND	20
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

[†] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; [¶]ND = not detected

Table 27. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone in the aqueous phase for natural topsoil.

Treatment[†]	^{14}C (dpm) from estrone peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM[‡]	54	51	1295	999	734	487	345	482	394
High B	NM	6	40	1176	958	455	594	387	423	449
High C	NM	ND[¶]	33	1088	694	536	603	422	501	504
M2 A	NM	23	47	416	307	213	183	134	156	73
M2 B	NM	8	36	339	155	128	182	122	133	136
M2 C	NM	7	30	401	289	196	190	96	133	125
M1 A	NM	22	19	51	50	16	ND	22	28	24
M1 B	NM	9	24	77	66	43	45	ND	ND	108
M1 C	NM	ND	28	70	60	34	31	ND	36	70
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

[†] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; [‡]NM = not measured; [¶]ND = not detected

Table 28. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for unidentified metabolites in the aqueous phase for natural topsoil.

Treatment†	^{14}C (dpm) from unidentified metabolites in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	414	299	53	305	335	291	183	77	158
High B	NM	154	140	167	320	496	150	165	55	126
High C	NM	124	151	219	502	470	179	114	50	50
M2 A	NM	287	134	67	119	109	60	34	ND¶	74
M2 B	NM	120	136	61	220	166	24	92	16	18
M2 C	NM	223	169	84	112	104	45	85	39	56
M1 A	NM	92	147	90	51	54	1	1	1	9
M1 B	NM	47	103	62	32	ND	ND	ND	1	ND
M1 C	NM	107	56	69	78	39	ND	97	ND	16
Ctrl. Blank A	NM	7	3	1	ND	1	3	4	3	ND
Ctrl. Blank B	NM	7	7	8	1	3	1	1	1	1
Ctrl. Blank C	NM	5	3	23	ND	1	2	3	ND	1

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ¶ND = not detected

Table 29. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol-3-glucuronide in the aqueous phase for natural subsoil.

Treatment†	^{14}C (dpm) from 17 β-estradiol-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	6970	6356	6376	5566	4317	3944	2210	966	177	37
High B	6970	6526	6480	5658	4670	4277	2812	1430	484	82
High C	6970	6376	6471	5633	4792	4234	2766	1479	561	111
M2 A	2808	2373	2365	1946	1547	1276	396	74	31	16
M2 B	2808	2478	2458	2109	1570	1295	444	74	18	ND¶
M2 C	2808	2540	2391	NM‡	1496	1254	435	50	22	ND
M1 A	1151	961	860	622	380	215	23	18	ND	ND
M1 B	1151	975	876	676	466	213	22	ND	ND	ND
M1 C	1151	964	893	631	446	270	50	ND	ND	ND
Ctrl. Blank A	166	149	154	153	156	163	162	166	152	165
Ctrl. Blank B	166	155	158	170	156	165	163	NM	151	163
Ctrl. Blank C	166	152	150	160	163	179	168	161	164	163

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured (vial lost); ¶ND = not detected

Table 30. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone-3-glucuronide in the aqueous phase for natural subsoil.

Treatment[†]	^{14}C (dpm) from estrone-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM[‡]	94	118	192	350	115	98	125	107	84
High B	NM	70	80	123	170	110	81	165	123	89
High C	NM	173	114	59	159	154	101	153	141	102
M2 A	NM	143	91	90	83	53	42	41	27	23
M2 B	NM	117	99	45	60	118	43	29	25	20
M2 C	NM	53	113	NM	132	89	54	49	23	22
M1 A	NM	ND[¶]	48	28	32	24	7	9	18	8
M1 B	NM	ND	34	17	25	8	12	ND	22	8
M1 C	NM	ND	34	59	15	12	9	ND	22	8
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

[†] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; [‡]NM = not measured; [¶]ND = not detected

Table 31. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol in the aqueous phase for natural subsoil.

Treatment[†]	^{14}C (dpm) from 17 β-estradiol peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM[‡]	10	48	439	445	457	247	215	165	19
High B	NM	6	36	421	549	248	214	168	151	51
High C	NM	11	17	494	538	244	255	210	136	129
M2 A	NM	24	35	283	269	281	123	34	ND[¶]	ND
M2 B	NM	ND	28	252	275	123	108	21	ND	ND
M2 C	NM	ND	31	NM[‡‡]	216	240	95	24	ND	ND
M1 A	NM	ND	26	220	164	125	12	ND	11	ND
M1 B	NM	ND	22	173	143	117	21	ND	ND	ND
M1 C	NM	ND	23	186	184	127	29	26	ND	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

[†] High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; [‡]NM = not measured; [‡‡]NM= not measured (vial lost); [¶]ND = not detected

Table 32. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone in the aqueous phase for natural subsoil.

Treatment*	^{14}C (dpm) from estrone peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	ND¶	ND	29	78	54	78	98	124	111
High B	NM	ND	ND	27	72	30	53	65	109	85
High C	NM	ND	ND	23	49	37	69	90	93	90
M2 A	NM	11	ND	34	39	44	54	47	8	ND
M2 B	NM	ND	ND	26	53	46	73	38	13	ND
M2 C	NM	ND	ND	NM‡	55	69	73	73	21	6
M1 A	NM	ND	7	27	38	33	21	9	ND	ND
M1 B	NM	ND	ND	15	19	28	20	12	ND	ND
M1 C	NM	ND	7	17	38	32	29	ND	ND	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

* High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 33. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for unidentified metabolites in the aqueous phase for natural subsoil.

Treatment*	^{14}C (dpm) from unidentified metabolites in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	100	59	105	170	191	357	387	368	393
High B	NM	27	43	101	89	180	336	393	373	516
High C	NM	40	78	91	119	226	314	370	388	449
M2 A	NM	18	66	99	144	75	192	79	114	125
M2 B	NM	13	ND¶	62	81	98	180	130	128	164
M2 C	NM	ND	61	NM‡	180	58	185	90	118	154
M1 A	NM	ND	5	26	45	45	71	34	29	46
M1 B	NM	15	34	26	13	87	62	45	27	47
M1 C	NM	ND	26	42	ND	66	52	42	32	52
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

* High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 34. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17 β-estradiol-3-glucuronide in the aqueous phase for sterile topsoil.

Treatment†	^{14}C (dpm) from 17 β-estradiol-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	6970	2510	28	ND¶	ND	ND	ND	ND	ND	ND
High B	6970	2980	ND	ND	ND	ND	ND	ND	ND	ND
High C	6970	NM‡	17	ND	ND	ND	15	ND	ND	ND
M2 A	2808	ND	ND	ND	44	ND	9	ND	ND	ND
M2 B	2808	ND	ND	ND	ND	ND	ND	ND	ND	ND
M2 C	2808	20	ND	ND	NM	ND	ND	ND	ND	ND
M1 A	1151	NM	NM	ND	37	8	ND	ND	ND	ND
M1 B	1151	9	ND	NM	NM	NM	NM	NM	NM	NM
M1 C	1151	ND	ND	ND	NM	27	ND	ND	ND	ND
Ctrl. Blank A	166	152	149	159	158	143	164	148	155	155
Ctrl. Blank B	166	157	158	164	159	155	156	150	168	151
Ctrl. Blank C	166	164	162	NM	NM	154	154	152	157	161

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1};
‡NM = not measured (vial lost); ¶ND = not detected

Table 35. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone-3-glucuronide in the aqueous phase for sterile topsoil.

Treatment†	^{14}C (dpm) from estrone-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	3329	5218	4217	2843	1676	37	ND¶	ND	ND
High B	NM	3190	5835	4294	2373	1412	18	ND	ND	ND
High C	NM	NM‡‡	5768	4234	2851	183	21	ND	ND	ND
M2 A	NM	2212	1857	1464	649	289	34	ND	ND	ND
M2 B	NM	2278	2091	1417	ND	382	18	ND	ND	ND
M2 C	NM	2343	2149	1505	NM‡‡	340	19	ND	ND	ND
M1 A	NM	NM‡‡	NM‡‡	469	74	ND	25	ND	ND	ND
M1 B	NM	809	576	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M1 C	NM	842	550	481	NM‡‡	89	ND	ND	ND	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank C	NM	ND	ND	NM‡‡	NM‡‡	ND	ND	ND	ND	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured;
¶ND = not detected

Table 36. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol in the aqueous phase for sterile topsoil.

Treatment†	^{14}C (dpm) from 17β-estradiol peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	38	13	ND¶	ND	ND	ND	ND	17	ND
High B	NM	26	7	ND	ND	ND	ND	ND	ND	ND
High C	NM	NM‡‡	ND	ND	ND	ND	23	ND	45	ND
M2 A	NM	9	116	ND	ND	ND	66	ND	74	73
M2 B	NM	ND	ND	ND	ND	ND	ND	ND	38	ND
M2 C	NM	ND	ND	ND	NM‡‡	ND	ND	ND	109	91
M1 A	NM	NM‡‡	NM‡‡	ND	ND	ND	ND	ND	7	20
M1 B	NM	3	ND	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M1 C	NM	ND	ND	ND	NM‡‡	ND	ND	ND	8	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank C	NM	ND	ND	NM‡‡	NM‡‡	ND	ND	ND	ND	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 37. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone in the aqueous phase for sterile topsoil.

Treatment†	^{14}C (dpm) from estrone peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	45	136	454	656	1003	1709	1607	1494	1233
High B	NM	56	104	463	790	952	1572	1453	1361	1404
High C	NM	NM‡‡	169	437	719	1326	1164	1318	1079	1245
M2 A	NM	54	115	240	249	431	409	406	357	136
M2 B	NM	59	96	315	825	382	409	485	373	353
M2 C	NM	42	88	241	NM‡‡	320	533	485	318	283
M1 A	NM	NM‡‡	NM‡‡	70	152	134	143	149	107	111
M1 B	NM	19	55	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M1 C	NM	14	43	138	NM‡‡	123	196	154	128	128
Ctrl. Blank A	NM	ND¶	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank C	NM	ND	ND	NM‡‡	NM‡‡	ND	ND	ND	ND	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 38. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for unidentified metabolites in the aqueous phase for sterile topsoil.

Treatment†	^{14}C (dpm) from unidentified metabolites in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	197	343	78	60	176	156	ND¶	52	167
High B	NM	148	167	192	262	146	160	116	170	42
High C	NM	NM‡‡	129	241	143	668	312	64	210	12
M2 A	NM	78	58	ND	191	41	13	59	11	245
M2 B	NM	47	71	14	315	21	124	ND	35	51
M2 C	NM	25	21	ND	NM‡‡	124	ND	ND	19	ND
M1 A	NM	NM‡‡	NM‡‡	ND	35	36	ND	ND	28	ND
M1 B	NM	18	151	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M1 C	NM	26	183	ND	NM‡‡	ND	4	ND	6	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank C	NM	ND	ND	NM‡‡	NM‡‡	ND	ND	ND	ND	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 39. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol-3-glucuronide in the aqueous phase for sterile subsoil.

Treatment†	^{14}C (dpm) from 17 β-estradiol-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	6970	7013	6905	6225	5894	5266	2788	NM‡‡	NM‡‡	NM‡‡
High B	6970	7241	7166	6856	6142	5039	274	36	44	59
High C	6970	6822	7219	6359	NM‡‡	NM‡‡	NM‡‡	27	30	21
M2 A	2808	2730	2797	2618	2198	1911	40	10	10	26
M2 B	2808	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M2 C	2808	2841	2663	2391	1895	943	29	25	28	33
M1 A	1151	977	964	774	364	ND¶	ND	11	ND	ND
M1 B	1151	1054	1011	982	440	124	15	15	ND	ND
M1 C	1151	1153	988	834	465	95	ND	ND	ND	ND
Ctrl. Blank A	166	170	165	173	159	172	167	159	160	171
Ctrl. Blank B	166	170	176	190	165	172	166	NM‡‡	174	187
Ctrl. Blank C	166	173	179	179	177	165	179	158	170	180

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡‡NM = not measured (vial lost); ¶ND = not detected

Table 40. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone-3-glucuronide in the aqueous phase for sterile subsoil.

Treatment†	^{14}C (dpm) from estrone-3-glucuronide peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	76	139	179	241	289	374	NM‡‡	NM‡‡	NM‡‡
High B	NM	20	23	80	248	366	874	19	25	ND¶
High C	NM	35	48	175	NM‡‡	NM‡‡	NM‡‡	31	36	ND
M2 A	NM	29	45	51	92	182	51	8	29	ND
M2 B	NM	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M2 C	NM	7	45	77	37	69	29	14	14	11
M1 A	NM	4	42	64	85	26	ND	8	17	ND
M1 B	NM	ND	12	83	314	708	137	ND	24	14
M1 C	NM	ND	40	69	99	60	ND	8	6	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM‡‡	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 41. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for 17β-estradiol in the aqueous phase for sterile subsoil.

Treatment†	^{14}C (dpm) from 17β-estradiol peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	42	62	192	247	638	1269	NM‡‡	NM‡‡	NM‡‡
High B	NM	13	39	133	359	788	105	15	49	16
High C	NM	102	141	149	NM‡‡	NM‡‡	NM‡‡	15	36	ND¶
M2 A	NM	9	ND	77	205	237	ND	7	ND	9
M2 B	NM	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M2 C	NM	29	66	181	414	585	511	16	ND	ND
M1 A	NM	9	37	87	232	300	24	30	10	ND
M1 B	NM	ND	37	13	17	8	23	24	ND	ND
M1 C	NM	7	38	61	227	359	30	15	ND	ND
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM‡‡	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

† High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 42. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for estrone in the aqueous phase for sterile subsoil.

Treatment*	^{14}C (dpm) from estrone peak in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	5	ND¶	19	31	62	302	NM‡‡	NM‡‡	NM‡‡
High B	NM	15	ND	34	33	171	1542	1242	832	1098
High C	NM	12	9	41	NM‡‡	NM‡‡	NM‡‡	1375	1060	1220
M2 A	NM	13	ND	10	21	38	966	811	531	375
M2 B	NM	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M2 C	NM	7	ND	11	60	51	425	173	80	83
M1 A	NM	ND	ND	10	94	196	496	152	158	164
M1 B	NM	ND	ND	ND	27	61	486	248	328	337
M1 C	NM	ND	ND	14	87	213	514	362	274	242
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM‡‡	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

*High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

Table 43. The experimental data from high performance liquid chromatography and liquid scintillation counting analysis for unidentified metabolites in the aqueous phase for sterile subsoil.

Treatment*	^{14}C (dpm) from unidentified metabolites in 50 µL Injection (adjusted to batch recovery)									
	Time (h)									
	0	4	8	24	48	72	168	336	504	672
High A	NM‡	ND¶	78	296	374	126	330	NM‡‡	NM‡‡	NM‡‡
High B	NM	ND	57	124	178	128	1225	315	491	524
High C	NM	ND	113	188	NM‡‡	NM‡‡	NM‡‡	542	535	436
M2 A	NM	ND	22	39	123	156	346	266	308	339
M2 B	NM	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡	NM‡‡
M2 C	NM	ND	83	83	165	618	110	251	216	245
M1 A	NM	ND	ND	59	90	226	34	213	111	77
M1 B	NM	ND	ND	27	274	144	48	230	97	60
M1 C	NM	ND	21	91	65	130	72	101	113	103
Ctrl. Blank A	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ctrl. Blank B	NM	ND	ND	ND	ND	ND	ND	NM‡‡	ND	ND
Ctrl. Blank C	NM	ND	ND	ND	ND	ND	ND	ND	ND	ND

*High = 22.5 mg L^{-1}, M2 = 9.1 mg L^{-1}, M1 = 3.7 mg L^{-1}, Ctrl. Blank = 0.5 mg L^{-1}; ‡NM = not measured; ‡‡NM= not measured (vial lost); ¶ND = not detected

APPENDIX VI. THE C SOURCE CODE FOR PAPER 4

```
/*************************************************************************
***
** Code to inversely estimate the fate and transformation parameters
**based on the data generated from the experimental value
*************************************************************************
**/

#include <stdio.h>
#include <stdlib.h>
#include <math.h>

#include "sharefunc.h"
#include "ESSRSort.h"
#include "ESES.h"

#include "llnltyps.h"
#include "cvode.h"
#include "cvdense.h"
#include "nvector.h"
#include "dense.h"

#define Ith(v,i) N_VIth(v,i-1)
#define IJth(A,i,j) DENSE_ELEM(A,i-1,j-1)

#undef OUTPUT
#undef REFINE

#define MV 200.0

#define WT1 10000.0

int NEQ, tn, dim;

double RTOL, ATOL;
double T0, T1, Tm;

ESfcnTrsfm *trsfm;

double w1un, w2un, w3un, w4un, w5un;
double w1us, w2us, w3us, w4us, w5us;
double w1ln, w2ln, w3ln, w4ln, w5ln;
double w1ls, w2ls, w3ls, w4ls, w5ls;
double s1u, s2u, s3u;
double a1u, a2u, a3u, a4u, a5u;
double b1u, b2u, b3u, b4u, b5u;
double kd1u, kd2u, kd3u, kd4u, kd5u;
double s1l, s2l, s3l;
double a1l, a2l, a3l, a4l, a5l;
double b1l, b2l, b3l, b4l, b5l;
```

```
double kd11, kd21, kd31, kd41, kd51;

double **w_mun, **w_mus, **w_mln, **w_mls;
double **a_mun, **a_mus, **a_mln, **a_mls;
double *c_mun, *c_mus, *c_mln, *c_mls;

double transform(double x);
void fitness(double *, double *, double *);
static void difeq(integer N, real t, N_Vector y, N_Vector ydot, void
*f_data);
double square(double xxx);
double **ReadWA(const char file[], const int iRow, const int iCol);
double *ReadC(const char file[], const int iRow);

int main(int argc, char **argv) {
      int i, es, constraint, miu, lambda, gen, retry;
      unsigned int seed;
      double *ub, *lb, gamma, alpha, varphi, pf;
      double *sim_para;

      ESParameter *param;
      ESPopulation *population;
      ESStatistics *stats;

      seed = shareDefSeed;
      gamma = esDefGamma;
      alpha = esDefAlpha;
      varphi = esDefVarphi;
      retry = esDefRetry;
      pf = essrDefPf;
      es = esDefESSlash;

      constraint = 1;
      dim = 56;
      miu = 300;
      lambda = 3500;
      gen = 100000000;

      ub = NULL;
      lb = NULL;

      ub = ShareMallocM1d(dim);
      lb = ShareMallocM1d(dim);
      sim_para = ShareMallocM1d(dim);

      trsfm = (ESfcnTrsfm *)ShareMallocM1c(dim * sizeof(ESfcnTrsfm));

      for (i = 0; i < dim; i++)
            trsfm[i] = transform;

      for (i = 0; i < dim; i++) {
            lb[i] = 1e-8;
            ub[i] = 1.0;
```

```
    }

    ub[22] = 0.0;
    lb[22] = 0.0;
    ub[25] = 0.000000000001;
    lb[25] = 0.000000000001;
#ifdef OUTPUT
    sim_para = ReadC('sim2_para.txt', dim);

    for (i = 0; i < dim; i++) {
        ub[i] = sim_para[i];
        lb[i] = ub[i];
    }

#endif

#ifndef OUTPUT

#ifdef REFINE
    sim_para = ReadC('sim2_para.txt', dim);

    for (i = 0; i < dim; i++) {
        ub[i] = sim_para[i] * 3.0;
        lb[i] = sim_para[i] * 0.3;
    }
#endif

#ifndef REFINE
    lb[31] = 0.0;
    ub[31] = 0.0;
    lb[32] = 0.0;
    ub[32] = 0.0;

    lb[46] = 0.0;
    ub[46] = 0.0;
    lb[47] = 0.0;
    ub[47] = 0.0;

#endif
#endif

#endif

    NEQ = 60;
    RTOL = 1e-4;
    ATOL = 1e-4;
    T0 = 0.0;
    T1 = 0.1;
    Tm = 674;

    w_mun = ShareMallocM2d(9, 5);
    w_mus = ShareMallocM2d(9, 5);
    w_mln = ShareMallocM2d(9, 5);
```

```
w_mls = ShareMallocM2d(9, 5);

a_mun = ShareMallocM2d(9, 5);
a_mus = ShareMallocM2d(9, 5);
a_mln = ShareMallocM2d(9, 5);
a_mls = ShareMallocM2d(9, 5);



c_mun = ShareMallocM1d(9);
c_mus = ShareMallocM1d(9);
c_mln = ShareMallocM1d(9);
c_mls = ShareMallocM1d(9);

w_mun = ReadWA("w_mun.txt", 9, 5);
w_mus = ReadWA("w_mus.txt", 9, 5);
w_mln = ReadWA("w_mln.txt", 9, 5);
w_mls = ReadWA("w_mls.txt", 9, 5);

a_mun = ReadWA("a_mun.txt", 9, 5);
a_mus = ReadWA("a_mus.txt", 9, 5);
a_mln = ReadWA("a_mln.txt", 9, 5);
a_mls = ReadWA("a_mls.txt", 9, 5);

c_mun = ReadC("c_mun.txt", 9);
c_mus = ReadC("c_mus.txt", 9);
c_mln = ReadC("c_mln.txt", 9);
c_mls = ReadC("c_mls.txt", 9);

ESInitial(seed, &param, trsfm, fitness, es, constraint, dim, ub,
lb,miu, lambda, gen, gamma, alpha, varphi, retry, &population,
        &stats);

while (stats->curgen < param->gen)
      ESStep(population, param, stats, pf);

ESDeInitial(param, population, stats);

ShareFreeM1c((char *) trsfm);

ShareFreeM1d(ub);
ub = NULL;
ShareFreeM1d(lb);
lb = NULL;
ShareFreeM1d(sim_para);
sim_para = NULL;
ShareFreeM1d(c_mun);
c_mun = NULL;
ShareFreeM1d(c_mus);
c_mus = NULL;
ShareFreeM1d(c_mln);
```

```
    c_mln = NULL;
    ShareFreeM1d(c_mls);
    c_mls = NULL;

    ShareFreeM2d(w_mun, 9);
    w_mun = NULL;
    ShareFreeM2d(w_mus, 9);
    w_mus = NULL;
    ShareFreeM2d(w_mln, 9);
    w_mln = NULL;
    ShareFreeM2d(w_mls, 9);
    w_mls = NULL;
    ShareFreeM2d(a_mun, 9);
    a_mun = NULL;
    ShareFreeM2d(a_mus, 9);
    a_mus = NULL;
    ShareFreeM2d(a_mln, 9);
    a_mln = NULL;
    ShareFreeM2d(a_mls, 9);
    a_mls = NULL;

    return 0;
}

void fitness(double *x, double *f, double *g) {
    real ropt[OPT_SIZE], reltol, t, tout;
    long int iopt[OPT_SIZE];
    N_Vector y;
    real abstol;
    double sum1, sum2, sum3, sum4, sumr=0.0, aceton=0.0, combus=0.0;
    void *cvode_mem;
    int iout, flag, i, iPos = -1, ii, kk;

#ifdef OUTPUT
    FILE *mun, *mus, *mln, *mls;


    if ((mun = fopen("mun_output2.txt", "w")) == NULL) {
        printf("fopen %s failed!\n", "mun_output.txt");
        exit(-1);
    }

    if ((mus = fopen("mus_output2.txt", "w")) == NULL) {
        printf("fopen %s failed!\n", "mus_output.txt");
        exit(-1);
    }

    if ((mln = fopen("mln_output2.txt", "w")) == NULL) {
        printf("fopen %s failed!\n", "mln_output.txt");
        exit(-1);
    }

    if ((mls = fopen("mls_output2.txt", "w")) == NULL) {
        printf("fopen %s failed!\n", "mls_output.txt");
```

```
            exit(-1);
        }


#endif
        sum1 = 0.0;
        sum2 = 0.0;
        sum3 = 0.0;
        sum4 = 0.0;
        sumr = 0.0;

        w1un = (trsfm[0])(x[0]);
        w2un = (trsfm[1])(x[1]);
        w3un = (trsfm[2])(x[2]);
        w4un = (trsfm[3])(x[3]);
        w5un = (trsfm[4])(x[4]);

        w1us = (trsfm[5])(x[5]);
        w2us = (trsfm[6])(x[6]);
        w3us = (trsfm[7])(x[7]);
        w4us = (trsfm[8])(x[8]);
        w5us = (trsfm[9])(x[9]);

        w1ln = (trsfm[10])(x[10]);
        w2ln = (trsfm[11])(x[11]);
        w3ln = (trsfm[12])(x[12]);
        w4ln = (trsfm[13])(x[13]);
        w5ln = (trsfm[14])(x[14]);

        w1ls = (trsfm[15])(x[15]);
        w2ls = (trsfm[16])(x[16]);
        w3ls = (trsfm[17])(x[17]);
        w4ls = (trsfm[18])(x[18]);
        w5ls = (trsfm[19])(x[19]);

        s1u = (trsfm[20])(x[20]);
        s2u = (trsfm[21])(x[21]);
        s3u = (trsfm[22])(x[22]);

        s1l = (trsfm[23])(x[23]);
        s2l = (trsfm[24])(x[24]);
        s3l = (trsfm[25])(x[25]);

        a1u = (trsfm[26])(x[26]);
        a2u = (trsfm[27])(x[27]);
        a3u = (trsfm[28])(x[28]);
        a4u = (trsfm[29])(x[29]);
        a5u = (trsfm[30])(x[30]);

        b1u = (trsfm[31])(x[31]);
        b2u = (trsfm[32])(x[32]);
        b3u = (trsfm[33])(x[33]);
        b4u = (trsfm[34])(x[34]);
        b5u = (trsfm[35])(x[35]);
```

```
kd1u = (trsfm[36])(x[36]);
kd2u = (trsfm[37])(x[37]);
kd3u = (trsfm[38])(x[38]);
kd4u = (trsfm[39])(x[39]);
kd5u = (trsfm[40])(x[40]);

a11 = (trsfm[41])(x[41]);
a21 = (trsfm[42])(x[42]);
a31 = (trsfm[43])(x[43]);
a41 = (trsfm[44])(x[44]);
a51 = (trsfm[45])(x[45]);
b11 = (trsfm[46])(x[46]);
b21 = (trsfm[47])(x[47]);
b31 = (trsfm[48])(x[48]);
b41 = (trsfm[49])(x[49]);
b51 = (trsfm[50])(x[50]);

kd11 = (trsfm[51])(x[51]);
kd21 = (trsfm[52])(x[52]);
kd31 = (trsfm[53])(x[53]);
kd41 = (trsfm[54])(x[54]);
kd51 = (trsfm[55])(x[55]);


if (kd3u <= kd31 || kd4u <= kd41) {
      (*f) = 800000000000.0;
      g[0] = 0.0;
      return ;
}

if (kd2u <= kd21 || kd2u <= kd21) {
      (*f) = 800000000000.0;
      g[0] = 0.0;
      return ;
}

if (s1u >= s11 || s2u >= s21 || s3u >= s31) {
      (*f) = 800000000000.0;
      g[0] = 0.0;
      return ;
}

if (kd3u < kd4u || kd31 < kd41) {
      (*f) = 800000000000.0;
      g[0] = 0.0;
      return ;
}

if (kd4u <= kd5u || kd41 <= kd51) {
      (*f) = 800000000000.0;
      g[0] = 0.0;
      return ;
}
```

```
if (kd5u < kd1u || kd5u < kd2u || kd51 < kd11 || kd51 < kd21) {
      (*f) = 800000000000.0;
      g[0] = 0.0;
      return ;
}

y = N_VNew(NEQ, NULL);

for (i = 1; i <= NEQ; i++)
      Ith(y, i) = 0.0;

Ith(y, 1) = 1.0;
Ith(y, 16) = 1.0;
Ith(y, 31) = 1.0;
Ith(y, 46) = 1.0;

reltol = RTOL;
abstol = ATOL;

cvode_mem =
    CVodeMalloc(NEQ, difeq, T0, y, BDF, NEWTON, SS, &reltol,
           &abstol, NULL, NULL, FALSE, iopt, ropt, NULL);

if (cvode_mem == NULL) {
      printf("CVodeMalloc failed.\n");
      exit(1);
}

CVDense(cvode_mem, NULL, NULL);

for (iout = 1, tout = T1; tout <= Tm;  iout++, tout = iout * T1)
{
      flag = CVode(cvode_mem, tout, y, &t, NORMAL);

      if (flag != SUCCESS) {
            (*f) = 800000000000.0;
            g[0] = 0.0;
            return ;
      }

      iPos = -1;

      if (iout == 40.0)
            iPos = 0;

      if (iout == 80.0)
            iPos = 1;

      if (iout == 240.0)
            iPos = 2;

      if (iout == 480.0)
            iPos = 3;
```

```
            if (iout == 720.0)
                iPos = 4;

            if (iout == 1680.0)
                iPos = 5;

            if (iout == 3360.0)
                iPos = 6;

            if (iout == 5040.0)
                iPos = 7;

            if (iout == 6720.0)
                iPos = 8;

if (iPos == 0 || iPos == 1 || iPos == 2 || iPos == 3 || iPos == 4 ||
iPos == 5 || iPos == 6 || iPos == 7 || iPos == 8) {

aceton = Ith(y,6)+Ith(y,7)+Ith(y,8)+Ith(y,9)+Ith(y,10);
combus = Ith(y,11)+Ith(y,12)+Ith(y,13)+Ith(y,14)+Ith(y,15);

                if (iPos == 0)
                    sumr = sumr+(aceton/combus - 4.4) * WT1;

                if (iPos == 1)
                    sumr = sumr + (aceton/combus - 3.9) * WT1;

                if (iPos == 2)
                    sumr = sumr + (aceton/combus - 4.5) * WT1;

                if (iPos == 3)
                    sumr = sumr + (aceton/combus - 2.6) * WT1;

                if (iPos == 4)
                    sumr = sumr + (aceton/combus - 2.3) *WT1;

                if (iPos == 5)
                    sumr = sumr + (aceton/combus - 2.1) * WT1;

                if (iPos == 6)
                    sumr = sumr + (aceton/combus - 1.9) * WT1;

                if (iPos == 7)
                    sumr = sumr + (aceton/combus - 1.8) * WT1;

                if (iPos == 8)
                    sumr = sumr +(aceton/combus - 2.1) * WT1;


sum1 = sum1 + square(Ith(y, 1) - w_mun[iPos][0]) + square(Ith(y, 2) -
       w_mun[iPos][1]) + square(Ith(y, 3) - w_mun[iPos][2])
```

```
        + square(Ith(y, 4) - w_mun[iPos][3]) + square(Ith(y, 5) -
        w_mun[iPos][4]) + square(Ith(y, 6) - a_mun[iPos][0]) +
        square(Ith(y, 7) - a_mun[iPos][1]) + square(Ith(y, 8) -
        a_mun[iPos][2]) + square(Ith(y, 9) - a_mun[iPos][3]) +
        square(Ith(y, 10) - a_mun[iPos][4])+ square(Ith(y, 11) +
        Ith(y, 12) + Ith(y, 13) + Ith(y, 14) + Ith(y, 15) -
        c_mun[iPos]);


#ifdef OUTPUT

      fprintf(mun, "%d\t", iout);

      fprintf(mun, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 1), Ith(y, 2),
      Ith(y, 3), Ith(y, 4), Ith(y, 5));
      fprintf(mun, "%f\t %f\t %f\t %f\t %f\t\t\t", w_mun[iPos][0],
      w_mun[iPos][1], w_mun[iPos][2], w_mun[iPos][3], w_mun[iPos][4]);

      fprintf(mun, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 6), Ith(y, 7),
      Ith(y, 8), Ith(y, 9), Ith(y, 10));

      fprintf(mun, "%f\t %f\t %f\t %f\t %f\t\t\t", a_mun[iPos][0],
      a_mun[iPos][1], a_mun[iPos][2], a_mun[iPos][3], a_mun[iPos][4]);

      fprintf(mun, "%f\t %f\n", Ith(y, 11)+Ith(y, 12)+Ith(y,
      13)+Ith(y, 14)+Ith(y, 15), c_mun[iPos]);

#endif

sum2 = sum2 + square(Ith(y, 16) - w_mus[iPos][0]) + square(Ith(y, 17)
       - w_mus[iPos][1]) + square(Ith(y, 18) - w_mus[iPos][2])
       + square(Ith(y, 19) - w_mus[iPos][3]) + square(Ith(y, 20) -
       w_mus[iPos][4]) + square(Ith(y, 21) - a_mus[iPos][0]) +
       square(Ith(y, 22) - a_mus[iPos][1]) + square(Ith(y, 23) -
       a_mus[iPos][2]) + square(Ith(y, 24) - a_mus[iPos][3]) +
       square(Ith(y, 25) - a_mus[iPos][4])+ square(Ith(y, 26) +
       Ith(y, 27) + Ith(y, 28) + Ith(y, 29) + Ith(y, 30) -
       c_mus[iPos]);

#ifdef OUTPUT

      fprintf(mus, "%d\t", iout);

      fprintf(mus, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 16), Ith(y,
      17), Ith(y, 18), Ith(y, 19), Ith(y, 20));

      fprintf(mus, "%f\t %f\t %f\t %f\t %f\t\t\t", w_mus[iPos][0],
      w_mus[iPos][1], w_mus[iPos][2], w_mus[iPos][3], w_mus[iPos][4]);

      fprintf(mus, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 21), Ith(y,
      22), Ith(y, 23), Ith(y, 24), Ith(y, 25));

      fprintf(mus, "%f\t %f\t %f\t %f\t %f\t\t\t", a_mus[iPos][0],
      a_mus[iPos][1], a_mus[iPos][2], a_mus[iPos][3], a_mus[iPos][4]);
```

```
      fprintf(mus, "%f\t %f\n", Ith(y, 26) + Ith(y, 27) + Ith(y, 28) +
      Ith(y, 29) + Ith(y, 30), c_mus[iPos]);

#endif

sum3 = sum3 + square(Ith(y, 31) - w_mln[iPos][0]) + square(Ith(y, 32)
       - w_mln[iPos][1]) + square(Ith(y, 33) - w_mln[iPos][2])
       + square(Ith(y, 34) - w_mln[iPos][3]) + square(Ith(y, 35) -
       w_mln[iPos][4])+ square(Ith(y, 36) - a_mln[iPos][0]) +
       square(Ith(y, 37) - a_mln[iPos][1]) + square(Ith(y, 38) -
       a_mln[iPos][2])+ square(Ith(y, 39) - a_mln[iPos][3]) +
       square(Ith(y, 40) - a_mln[iPos][4])+ square(Ith(y, 41) +
       Ith(y, 42) + Ith(y, 43) + Ith(y, 44) + Ith(y, 45) -
       c_mln[iPos]);

#ifdef OUTPUT
      fprintf(mln, "%d\t", iout);

      fprintf(mln, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 31), Ith(y,
      32), Ith(y, 33), Ith(y, 34), Ith(y, 35));

      fprintf(mln, "%f\t %f\t %f\t %f\t %f\t\t\t", w_mln[iPos][0],
      w_mln[iPos][1], w_mln[iPos][2], w_mln[iPos][3], w_mln[iPos][4]);

      fprintf(mln, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 36), Ith(y,
      37), Ith(y, 38), Ith(y, 39), Ith(y, 40));

      fprintf(mln, "%f\t %f\t %f\t %f\t %f\t\t\t", a_mln[iPos][0],
      a_mln[iPos][1], a_mln[iPos][2], a_mln[iPos][3], a_mln[iPos][4]);

      fprintf(mln, "%f\t %f\n", Ith(y, 41) + Ith(y, 42) + Ith(y, 43) +
      Ith(y, 44) + Ith(y, 45), c_mln[iPos]);

#endif

sum4 = sum4 + square(Ith(y, 46) - w_mls[iPos][0]) + square(Ith(y, 47)
       - w_mls[iPos][1]) + square(Ith(y, 48) - w_mls[iPos][2])
       + square(Ith(y, 49) - w_mls[iPos][3]) + square(Ith(y, 50) -
       w_mls[iPos][4])+ square(Ith(y, 51) - a_mls[iPos][0]) +
       square(Ith(y, 52) - a_mls[iPos][1]) + square(Ith(y, 53) -
       a_mls[iPos][2])+ square(Ith(y, 54) - a_mls[iPos][3]) +
       square(Ith(y, 55) - a_mls[iPos][4])+ square(Ith(y, 56) +
       Ith(y, 57) + Ith(y, 58) + Ith(y, 59) + Ith(y, 60) -
       c_mls[iPos]);

#ifdef OUTPUT

      fprintf(mls, "%d\t", iout);

      fprintf(mls, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 46), Ith(y,
      47), Ith(y, 48), Ith(y, 49), Ith(y, 50));
```

```
      fprintf(mls, "%f\t %f\t %f\t %f\t %f\t\t\t", w_mls[iPos][0],
      w_mls[iPos][1], w_mls[iPos][2], w_mls[iPos][3], w_mls[iPos][4]);

      fprintf(mls, "%f\t %f\t %f\t %f\t %f\t\t", Ith(y, 51), Ith(y,
      52), Ith(y, 53), Ith(y, 54), Ith(y, 55));

      fprintf(mls, "%f\t %f\t %f\t %f\t %f\t\t\t", a_mls[iPos][0],
      a_mls[iPos][1], a_mls[iPos][2], a_mls[iPos][3], a_mls[iPos][4]);

      fprintf(mls, "%f\t %f\n", Ith(y, 56) + Ith(y, 57) + Ith(y, 58) +
      Ith(y, 59) + Ith(y, 60), c_mls[iPos]);

#endif
            }
      }

      g[0] = 0.0;
      N_VFree(y);
      CVodeFree(cvode_mem);
      (*f) = sum1 + sum2 + sum3 + sum4 + sumr;

#ifdef OUTPUT
      fclose(mun);
      fclose(mus);
      fclose(mln);
      fclose(mls);
      printf("%f\t %f\t %f\t %f\t %f\n", sum1, sum2, sum3, sum4, sum1
+ sum2 + sum3 + sum4);
      exit(0);

#endif

      return;
}

static void difeq(integer N, real t, N_Vector y, N_Vector ydot, void
*f_data) {
      double C1un, C2un, C3un, C4un, C5un;
      double S1un, S2un, S3un, S4un, S5un;
      double SS1un, SS2un, SS3un, SS4un, SS5un;

      double C1us, C2us, C3us, C4us, C5us;
      double S1us, S2us, S3us, S4us, S5us;
      double SS1us, SS2us, SS3us, SS4us, SS5us;

      double C1ln, C2ln, C3ln, C4ln, C5ln;
      double S1ln, S2ln, S3ln, S4ln, S5ln;
      double SS1ln, SS2ln, SS3ln, SS4ln, SS5ln;

      double C1ls, C2ls, C3ls, C4ls, C5ls;
      double S1ls, S2ls, S3ls, S4ls, S5ls;
      double SS1ls, SS2ls, SS3ls, SS4ls, SS5ls;

      C1un = Ith(y, 1);
```

```
C2un = Ith(y, 2);
C3un = Ith(y, 3);
C4un = Ith(y, 4);
C5un = Ith(y, 5);

S1un = Ith(y, 6);
S2un = Ith(y, 7);
S3un = Ith(y, 8);
S4un = Ith(y, 9);
S5un = Ith(y, 10);

SS1un = Ith(y, 11);
SS2un = Ith(y, 12);
SS3un = Ith(y, 13);
SS4un = Ith(y, 14);
SS5un = Ith(y, 15);

C1us = Ith(y, 16);
C2us = Ith(y, 17);
C3us = Ith(y, 18);
C4us = Ith(y, 19);
C5us = Ith(y, 20);

S1us = Ith(y, 21);
S2us = Ith(y, 22);
S3us = Ith(y, 23);
S4us = Ith(y, 24);
S5us = Ith(y, 25);

SS1us = Ith(y, 26);
SS2us = Ith(y, 27);
SS3us = Ith(y, 28);
SS4us = Ith(y, 29);
SS5us = Ith(y, 30);

C1ln = Ith(y, 31);
C2ln = Ith(y, 32);
C3ln = Ith(y, 33);
C4ln = Ith(y, 34);
C5ln = Ith(y, 35);

S1ln = Ith(y, 36);
S2ln = Ith(y, 37);
S3ln = Ith(y, 38);
S4ln = Ith(y, 39);
S5ln = Ith(y, 40);

SS1ln = Ith(y, 41);
SS2ln = Ith(y, 42);
SS3ln = Ith(y, 43);
SS4ln = Ith(y, 44);
SS5ln = Ith(y, 45);

C1ls = Ith(y, 46);
```

```
C2ls = Ith(y, 47);
C3ls = Ith(y, 48);
C4ls = Ith(y, 49);
C5ls = Ith(y, 50);

S1ls = Ith(y, 51);
S2ls = Ith(y, 52);
S3ls = Ith(y, 53);
S4ls = Ith(y, 54);
S5ls = Ith(y, 55);

SS1ls = Ith(y, 56);
SS2ls = Ith(y, 57);
SS3ls = Ith(y, 58);
SS4ls = Ith(y, 59);
SS5ls = Ith(y, 60);

Ith(ydot, 1) = -w1un * C1un - MV * a1u * (kd1u * C1un - S1un);
Ith(ydot, 2) = -w2un * C2un - MV * a2u * (kd2u * C2un - S2un);
Ith(ydot, 3) = w1un * C1un - w3un * C3un - w5un * C3un - MV *
               a4u * (kd3u * C3un - S3un);
Ith(ydot, 4) = w3un * C3un + w2un * C2un - w4un * C4un - MV *
               a3u * (kd4u * C4un - S4un);
Ith(ydot, 5) = w5un * C3un + w4un * C4un - MV * a5u * (kd5u *
               C5un - S5un);

Ith(ydot, 6) = (a1u * (kd1u * C1un - S1un) - s1u * S1un - b1u *
               S1un) * MV;
Ith(ydot, 7) = (a2u * (kd2u * C2un - S2un) + s1u * S1un - b2u *
               S2un) * MV;
Ith(ydot, 8) = (a4u * (kd3u * C3un - S3un) - s2u * S3un - b4u *
               S3un) * MV;
Ith(ydot, 9) = (a3u * (kd4u * C4un - S4un) + s2u * S3un - s3u *
               S4un - b3u * S4un) * MV;
Ith(ydot, 10) = (a5u * (kd5u * C5un - S5un)  + s3u * S4un - b5u
               * S5un) * MV;

Ith(ydot, 11) = b1u * S1un * MV;
Ith(ydot, 12) = b2u * S2un * MV;
Ith(ydot, 13) = b4u * S3un * MV;
Ith(ydot, 14) = b3u * S4un * MV;
Ith(ydot, 15) = b5u * S5un * MV;

Ith(ydot, 16) = -w1us * C1us - MV * a1u * (kd1u * C1us - S1us);
Ith(ydot, 17) = -w2us * C2us - MV * a2u * (kd2u * C2us - S2us);
Ith(ydot, 18) = w1us * C1us - MV * a4u * (kd3u * C3us - S3us);
Ith(ydot, 19) = w2us * C2us - MV * a3u * (kd4u * C4us - S4us);
Ith(ydot, 20) = - MV * a5u * (kd5u * C5us - S5us);

Ith(ydot, 21) = (a1u * (kd1u * C1us - S1us) - s1u * S1us - b1u *
               S1us) * MV;
Ith(ydot, 22) = (a2u * (kd2u * C2us - S2us) + s1u * S1us - b2u *
               S2us) * MV;
```

```
Ith(ydot, 23) = (a4u * (kd3u * C3us - S3us) - s2u * S3us - b4u *
                S3us) * MV;
Ith(ydot, 24) = (a3u * (kd4u * C4us - S4us) + s2u * S3us - s3u *
                S4us - b3u * S4us) * MV;
Ith(ydot, 25) = (a5u * (kd5u * C5us - S5us)  + s3u * S4us - b5u
                * S5us) * MV;

Ith(ydot, 26) = b1u * S1us * MV;
Ith(ydot, 27) = b2u * S2us * MV;
Ith(ydot, 28) = b4u * S3us * MV;
Ith(ydot, 29) = b3u * S4us * MV;
Ith(ydot, 30) = b5u * S5us * MV;

Ith(ydot, 31) = -w1ln * C1ln - MV * a11 * (kd11 * C1ln - S1ln);
Ith(ydot, 32) = -w2ln * C2ln - MV * a21 * (kd21 * C2ln - S2ln);
Ith(ydot, 33) = w1ln * C1ln - w3ln * C3ln - w5ln * C3ln - MV *
                a41 * (kd31 * C3ln - S3ln);
Ith(ydot, 34) = w3ln * C3ln + w2ln * C2ln - w4ln * C4ln - MV *
                a31 * (kd41 * C4ln - S4ln);
Ith(ydot, 35) = w5ln * C3ln + w4ln * C4ln - MV * a51 * (kd51 *
               C5ln - S5ln);

Ith(ydot, 36) = (a11 * (kd11 * C1ln - S1ln) - s11 * S1ln - b11 *
                S1ln) * MV;
Ith(ydot, 37) = (a21 * (kd21 * C2ln - S2ln) + s11 * S1ln - b21 *
                S2ln) * MV;
Ith(ydot, 38) = (a41 * (kd31 * C3ln - S3ln) - s21 * S3ln - b41 *
                S3ln) * MV;
Ith(ydot, 39) = (a31 * (kd41 * C4ln - S4ln) + s21 * S3ln - s31 *
                S4ln - b31 * S4ln) * MV;
Ith(ydot, 40) = (a51 * (kd51 * C5ln - S5ln)  + s31 * S4ln - b51
                * S5ln) * MV;

Ith(ydot, 41) = b11 * S1ln * MV;
Ith(ydot, 42) = b21 * S2ln * MV;
Ith(ydot, 43) = b41 * S3ln * MV;
Ith(ydot, 44) = b31 * S4ln * MV;
Ith(ydot, 45) = b51 * S5ln * MV;

Ith(ydot, 46) = -w1ls * C1ls - MV * a11 * (kd11 * C1ls - S1ls);
Ith(ydot, 47) = -w2ls * C2ls - MV * a21 * (kd21 * C2ls - S2ls);
Ith(ydot, 48) = w1ls * C1ls  - MV * a41 * (kd31 * C3ls - S3ls);
Ith(ydot, 49) = w2ls * C2ls  - MV * a31 * (kd41 * C4ls - S4ls);
Ith(ydot, 50) =  - MV * a51 * (kd51 * C5ls - S5ls);

Ith(ydot, 51) = (a11 * (kd11 * C1ls - S1ls) - s11 * S1ls - b11 *
                S1ls) * MV;
Ith(ydot, 52) = (a21 * (kd21 * C2ls - S2ls) + s11 * S1ls - b21 *
                S2ls) * MV;
Ith(ydot, 53) = (a41 * (kd31 * C3ls - S3ls) - s21 * S3ls - b41 *
                S3ls) * MV;
Ith(ydot, 54) = (a31 * (kd41 * C4ls - S4ls) + s21 * S3ls - s31 *
                S4ls - b31 * S4ls) * MV;
```

```
      Ith(ydot, 55) = (a51 * (kd51 * C51s - S51s)  + s31 * S41s - b51
                  * S51s) * MV;

      Ith(ydot, 56) = b11 * S11s * MV;
      Ith(ydot, 57) = b21 * S21s * MV;
      Ith(ydot, 58) = b41 * S31s * MV;
      Ith(ydot, 59) = b31 * S41s * MV;
      Ith(ydot, 60) = b51 * S51s * MV;
}

double transform(double x) {
      double y;

      y = x;

      return y;
}

double square(double xxx) {
      return xxx * xxx * 10000.0 * 10000.0;
}

double **ReadWA(const char file[], const int iRow, const int iCol) {
      char buf[shareDefMaxLine];
      char **sl;
      FILE *fp;
      int i = 0, n, k = 0;
      double **pData = NULL;

      if ((fp = fopen(file, "r")) == NULL) {
            printf("fopen %s failed!\n", file);
            exit(-1);
      }

      pData = ShareMallocM2d(iRow, iCol);

      while (fgets(buf, shareDefMaxLine, fp) != NULL) {
            ShareChop(buf);
            sl = ShareSplitStr(buf, "\t", &n, shareDefNullNo);

            if (n != iCol) {
                  printf("line failed: %s\n", buf);
                  exit(-1);
            }

            for (k = 0; k < iCol; k++)
                  pData[i][k] = atof(sl[k]);

            i = i + 1;
      }

      return pData;
}
```

```
double *ReadC(const char file[], const int iRow) {
      char buf[shareDefMaxLine];
      char **sl;
      FILE *fp;
      int i = 0, n, k = 0;
      double *pData = NULL;

      if ((fp = fopen(file, "r")) == NULL) {
            printf("fopen %s failed!\n", file);
            exit(-1);
      }

      pData = ShareMallocM1d(iRow);

      while (fgets(buf, shareDefMaxLine, fp) != NULL) {
            ShareChop(buf);
            sl = ShareSplitStr(buf, "\t", &n, shareDefNullNo);

            if (n > 1) {
                  printf("line failed: %s\n", buf);
                  exit(-1);
            }

            pData[i] = atof(sl[0]);
            i = i + 1;
      }

      return pData;
}

/*************************************************************************
************************
**Code ends here
*************************************************************************
************************/
```