ASSOCIATION AND BIOAVAILABILITY OF 17β-ESTRADIOL WITH SOIL AND MANURE AQUEOUS DISSOLVED AND COLLOIDAL FRACTIONS

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MASTER OF SCIENCE

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ABSTRACT

Steroidal estrogens in the environment exert toxicological effects at very low concentrations. Furthermore, dissolved and colloidal fractions of soil and manure play an important role in the environmental fate and transport of steroidal estrogens. One objective of this study was to quantify the association of the natural estrogen, 17β-estradiol (E2), with the dissolved fraction and colloidal fraction isolated from liquid swine manure (LSM), soil, and soil+LSM mixtures. The second objective of this study was to evaluate whether the E2 associated with the dissolved fraction/colloidal fraction, dissolved fraction and colloidal fraction of the various media could induce an estrogenic response. Estrogenicity was assessed using an E2 receptor (ER) competitor assay, which provided E2 equivalent concentration (EEQ) of dissolved fraction/colloidal fraction, dissolved fraction and colloidal fraction solutions created from the Soil, Soil+LSM and LSM.

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THESIS ORGANIZATION

This thesis is organized in three parts that consists of a literature review, one manuscript to be submitted to a peer-reviewed journal, and a general conclusion. The literature review includes a discussion of the general properties of estrogens, environmental sources of the hormones, and hormones as endocrine disrupting chemicals and effects on aquatic life. Additionally, a general description of in vitro bioassays and estrogen associated with soils is included. The manuscript focuses on the association of 17β-estradiol with dissolved and colloidal fractions isolated from soil and liquid swine manure and whether the 17β-estradiol associated with these fractions has the ability to induce an estrogenic response.

LITERATURE REVIEW

Estrogens

Steroid hormones are biologically active compounds that are synthesized from cholesterol (Ying et al., 2002). The most potent naturally occurring estrogen is 17β-estradiol (E2), and its common metabolites estrone (E1) and estriol (E3) are less biologically active (Lai et al., 2000). The chemical structures of E1, E2 and E3 are shown in Figure 1. The chemical differences among the three estrogens are in the functional group(s) located at the C-16 and/or C-17 position on the D-ring of the steroid ring system. Estradiol can have a hydroxyl group at C-17 that either comes out of the page (β configuration) or goes into the page (α configuration). Estrone has a carbonyl at C-17, and E3 has a hydroxyl groups at both the C-16 and C-17 position. Estrogens can be conjugated with a sulfuric acid and/or glucuroni acid moiety at the C-3 and/or C-17 position, which adds polarity to the molecule and aids in urinary elimination (Hanselman et al., 2003).

Figure 1. Chemical structures for estrone, 17β-estradiol, and estriol.

Estrogens are hydrophobic organic compounds having low volatility. Natural estrogens, (e.g. E1, E2 and E3) a have solubility of approximately 1.3 mg L^{-1} (Lai et al., 2000). Steroids have low vapor pressures that range from 2.3×10^{-10} to 6.7×10^{-15} mm Hg. The base-10 logarithm

of the octanol-water partitioning coefficients (K_{ow}) is 2.81, 3.43 and 3.94 for E3, E1 and E2 respectively. Due to these physicochemical properties the sorption of natural estrogens to soils or sediments is expected to be high (Ying et al., 2002).

Table 1. Physiochemical properties of steroidal estrogens

Property	Estrone (E1)	Estradiol (E2)	Estriol (E3)
Formula	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_2$	$C_{18}H_{24}O_3$
Molecular Weight (g/mol)	270.4	272.4	288.4
Water Solubility (mg/L)	0.8–12.4	3.9–13.3	3.2–13.3
Vapor Pressure (mm Hg)	2.3×10^{-10}	2.3×10^{-10}	6.7×10^{-15}
Log K_{ow}[†]	3.1–3.4	3.1–4.0	2.6–2.8
PK_a[‡]	10.3–10.8	10.5–10.7	10.4
Refference	Hanselman et al., 2003; Ying et al., 2002)		

[†] K_{ow}-octanol-water partition coefficient
[‡] pK_a- acid dissociation constant (K_a) at a logarithmic scale ($-\log_{10} K_a$)

Estrogens are secreted by the adrenal cortex, testis, ovarian follicles and placenta in humans and animals (Lange et al., 2002; Ying et al., 2002). Morphogenesis, behavior, and reproductive differentiation during all life stages are regulated by estrogens in vertebrates (Lange et al., 2002). Additionally, estrogens regulate the secretion of other hormones, the expression of specific genes, and coordinate diverse processes such as cell proliferation, cell differentiation, and tissue organization. Once estrogens reach the bloodstream they may remain free or bind to estrogen-binding proteins (Sheehan and Young, 1979). Free (unbound) hormones are able to diffuse into target cells through the plasma membrane, where they bind to an estrogen receptor (ER) (Gillesby and Zacharewski, 1998). The binding of hormone molecule initiates a conformational change in the receptor (Zubay et al., 1995). The intracellular responses of estrogenic steroids are directed by the estrogen receptors, a member of a large superfamily of nuclear receptors that function as ligand-activated transcription factors. Receptors are characterized by their structural and functional regions that are responsible for ligand-binding

and DNA-binding and transcription activation (Katzenellenbogen, 1996). A mammalian ER is encoded by two genes, alpha and beta (ERα and ERβ), that function both as signal transducers and transcription factors (Klinge, 2000).

Sources of Hormones in the Environment

Endogenous hormones from humans and animals have entered the environment for thousands of years. However, the potential for unusually large amounts of hormone entering the environment may be increasing due to growing global populations and the advent of intensive animal production (Lange et al., 2002; Raman et al., 2004). In last few decades E1 and E2 have started to be recognized as environmental pollutants (Desbrow et al., 1998; Routledge et al., 1998). A USA national reconnaissance study conducted from 1999 - 2000 detected estrogens in 139 streams at frequencies of 7.1, 10, and 21.4% and concentration ranges of <5 to 112, <5 to 93 and <5 to 51 ng L^{-1}, for E1, E2, and E3, respectively (Kolpin et al., 2002).

Human Sources

In the USA approximately three-fourths of human waste is treated and discharged by municipal sewage treatment plants (MWTP; Raman et al., 2004). Estrogens have been detected in influents and effluents of MWTP in different countries (Table 2). In the influent of MWTP concentrations of E1 and E2 have ranged from 11 to 140 ng L^{-1} and from < LOD (limit of detection) to 224 ng L^{-1} respectively. In MWTP effluents the concentration of E1 ranged < LOD to 82.1 and E2 have ranged < LOD to 64 ng L^{-1} (Table 2).

Table 2. Concentration of E1 and E2 in influents and effluents of MWTP

Country	Influent		Effluent		Reference
	E1	E2	E1	E2	
	ng L^{-1}				
Brazil	40	21	n.d.	n.d.	Ternes et al., 1999
Canada	n.d.	n.d.	<LOD – 48	<LOD – 64	Ternes et al., 1999
Germany	n.d.	n.d.	<LOD – 70	<0.1 – 50	Ternes et al., 1999
Italy	52	12	2.5 – 82.1	0.4 – 3.3	Baronti et al., 2000
The Netherland	11 – 140	<LOD[†] – 48	<0.4 – 47	<0.1– 5	Johnson et al., 2000, Desbrow et al., 1998
United Kingdom	57 – 81	132 – 224	1.4 – 76	2.7– 48.4	Jiang et al., 2005, Desbrow et al., 1998

[‡] Lowest observable detection (LOD)

Livestock Sources

Although estrogenic steroids are found in the effluent of MWTPs, agricultural sources are more significant (Raman et al., 2004). Animal agriculture potentially contributes more steroid hormones to the environment than human sources because (1) wastes from animal feeding operation (AFOs), are higher in natural estrogens than humans wastes, (2) untreated animal manures are normally applied directly to lands as fertilizers, and (3) sometimes, estrogens are administered exogenously as growth promoters and for regulating reproductive cycles (Carmosini and Lee, 2008).

The U.S. Environmental Protection Agency (EPA) defines AFOs as livestock operations where animals are maintained or confined for more than 45 days over a one year period and concentrated animal feeding operation (CAFOs) are AFOs that meet certain EPA regulation criteria (Environmental Protection Agency, 2012). Large amounts of manure are generated from

AFOs from the confinement of hundreds to thousands of animals (swine, cattle and poultry) into a concentrate area (Zhao et al., 2010). The manure that accumulates from CAFOs is typically stored in outdoor waste holding ponds, above or below ground pits and/or litter storage facilities. Pits and ponds contain liquid, and sludge that is typically injected into or on top of the surface of agricultural land (Carmosini and Lee, 2008).

The potential of estrogens being released into to the environment is primarily dependent on AFO manure type and manure handling. Lorenzen et al. (2004) used hormone receptor gene transcription assays to survey hormone concentrations from various AFO manure types, and found all manure samples had estrogenic activity. The highest detection was from the manure of finishing pigs (5965 ng g^{-1} dry wt.), and the lowest detections were from the manure of steers (0.43 ng g^{-1} dry wt.). Fine et al. (2003) measured E1, E2, and E3 in nursery, farrowing and finishing lagoons at a swine AFO and found farrowing lagoons had the highest estrogen concentrations. Average nursery lagoon concentrations were 30, 46, and 197 ng L^{-1} for E1, E2, and E3 respectively. Average farrowing lagoons concentrations were 19104, 2408, and 1043 ng $L^{-1}$for E1, E2 and E3 respectively. Average finishing lagoon concentrations were 7.5×10^{4}, 125, and 302 ng L^{-1} for E1, E2, and E3 respectively (Fine et al., 2003).

Estrogens in the Environment

Steroidal estrogens have been detected at elevated concentrations in soil, ground water and surface water adjacent to agricultural fields fertilized with animal manure (Chowdhury et al., 2011). Contaminants in animal wastes can enter the environment through leaking and/or overflowing lagoons or in rainfall runoff from manure-fertilized fields (Burkholder et al., 2007). Chen et. al (2010) measured the concentration of E1, E2 and E3 of surface water from an animal

feeding area in southern Taiwan. The entire flow basin produces approximately 4500 cattle, 6×10^5 pigs, 3 million laying hens, and 5 million broiler chickens annually. Four kilometers downstream the source, the average concentration of E1, E2, and E3 ranged from 320 to 398 ng L^{-1}, 31 to 84.3 ng L^{-1}, and 58.9 to 93.6 ng L^{-1}, respectively. At 4 to 15 km the average concentration of E1, E2, and E3 were lower and ranged from 46.4 to 137, 9.6 to18.5 and 13.2 to 27.2 ng L^{-1}, respectively (Chen et al., 2010).

Peterson et. al (2005) used enzyme-linked immunosorbent assays to measure estradiol activity in cave water and sediment pore water from two karst basins in central Missouri. The association between groundwater and surface runoff in this karst topography reduces the capacity of the soil to filter and/or sorb E2. The sources of E2 in this area were from CAFO's, sewage water lagoons, and septic tanks, and strong correlations were measured between E2 and fecal coliform bacteria. The average concentrations of E2 measured in unfiltered stream water and cave water from the karst area were 30 – 42 ng L^{-1} and 27.3 – 33.5 ng L^{-1}, respectively. The concentration of E2 measured in the pore water of sediments were 131.2 ± 99.2 ng E2 kg^{-1} and 60.9 ± 28.0 ng E2 kg^{-1}, respectively (Peterson et al., 2005).

Thompson et al. (2009) measured E2 in lysimeter pore water beneath three plots receiving swine manure treated with three different managements methods (i.e. hoop-barn manure, static manure pile, and liquid lagoon slurry) from a farrowing to finishing swine operation that produced approximately 10,000 swine per year. Also, these treatments were compared to a control plot that received no manure. The range of E2 measured in lysimeter effluent 61 cm below the surface was 1–245 ng L^{-1} (with an average concentration of 21 ng L^{-1}). Antecedent E2 was also detected in lysimeter water samples obtained prior to manure application, as well as in the control plot. It was suggested that one potential source of the E2

was from wildlife or domestic animal inputs. Additionally, dissolved or colloidal fractions of soil and manure could potentially lead to greater mobility of hormones (Thompson et al., 2009).

Schuh et al (2011a) used liquid chromatography tandem mass spectrometry (LC/MS/MS) to measure E2 concentrations in porewater from soil cores at four locations around a swine farm that housed 4,000 animals. The first sample location received yearly applications of liquid swine manure for four years, the second location was near a static manure pile, the third location was in a cultivated field, and the fourth location was in a grass ditch next to a control well installed by the North Dakota State Water Commission, and had not received direct manure applications. The results of this study showed that E2 detections were observed in 37% of the soil extracts and the pore water E2 concentration ranged from 0-1910 ng L^{-1}. The authors concluded that the release of E2 from the soil was more closely associated with precipitation than manure application (Schuh et al., 2011a). A similar study was conducted analyzing soil cores collected six different times in a field where liquid swine manure had been applied; once before and five times after manure application. Water extractable E2 was determined in 0.15 m increments down to the water table (0.70 to 2.00 m). The average frequency of detection was 38% and the concentrations of E2 ranged from 0.9 to 202.6 ng L^{-1}. The highest E2 concentrations were observed in the spring of the final year approximately six months after manure application. The authors concluded that soil may act as a long-term storage reservoir of E2, to be periodically released to the environment (Schuh et al., 2011b).

Hormones as Endocrine Disrupting Chemicals

An endocrine disrupting chemical (EDC) is an exogenous substance that alters the function(s) of the endocrine system and causes adverse effects in organisms or their offspring. Endocrine disrupting chemicals mimic endogenous hormones, interacting with hormone receptors, and altering the natural pattern of hormone synthesis and/or metabolism (Mills and Chichester, 2005). Endocrine disrupting chemicals include synthetic organic chemicals and naturally occurring estrogenic hormones such as, E2, E1, and E3 (Jiang et al., 2005). There have been numerous observations of endocrine disruption resulting in abnormal developmental processes, altered reproductive capability, and abnormal behaviors (Trudeau and Tyler, 2007). Two mechanisms have been proposed in which EDCs exert their effects on an organism: (i) a genomic mechanism where environmental estrogens compete with endogenous estrogens to occupy the estrogen receptor sites or initiate estrogen gene expression, and (ii) a non-genomic mechanism where circulating hormones are interfered by competitive binding with plasma proteins or by disruption of synthesis and degradation pathways (Graham and Shaw, 2011).

Effects on Aquatic Life

The aquatic environment is susceptible to pollution, because most pollutants that are released into the environment make their way in to rivers, lakes, and oceans. Research on endocrine disruption in wildlife has mostly been focused on aquatic organisms because they receive continuous exposure to EDC in their aquatic environment. Estrogenic EDCs have been reported to cause the induction of the egg yolk precursor protein vitellogenin in male fish (McGree et al., 2010; Routledge et al., 1998), gonadal changes, and intersex characteristics (Jobling et al., 1998; Tetreault et al., 2011). Additional adverse effects from EDCs include altered reproductive behavior (McGee et al., 2009; McGree et al., 2010; Saaristo et al., 2009) ,

and in some cases, reduced production of viable offspring (Sumpter, 2005; Taylor and Harrison, 1999). Biological studies have been conducted at the level of the individual so the effects of EDCs on an ecosystem are inconclusive (Sumpter, 2005). There are concerns that the effects that EDCs have on the reproduction of individuals might lead to population-level effects (Taylor and Harrison, 1999).

Reproductive Effects

Exposure to exogenous estrogens and other EDCs can induce sex reversal and or intersexuality in fish. Fish are most susceptible to endocrine disruption after hatch or in the juvenile stage. Jobling et al. (1998) conducted a survey on wild roach (*Rutilus rutilus*) inhabiting rivers upstream and downstream from MWTPs in the United Kingdom. Male roach were found to have intersex characteristics at all sample sites, and characteristics ranged from slight changes to > 50% of the gonadal tissue being ovarian. In the more severe cases male sperm ducts were absent and replaced by ovarian cavities. Male fish having oocytes and higher vitellogenin concentrations were observed downstream from the MWTP (Jobling et al., 1998). McGree et al. (2010) demonstrated that exposure to E2 concentrations ranging from 34 to 160 ng L^{-1} (average 70 ng L^{-1}) for a minimum of 83 days can reduce reproductive success of adult red shiners (*Cyprinella lutrensis*), but were able to recover once removed from the E2 exposure. The tanks that had E2-exposed males had less fertilization success and none of the fertilized eggs survived to hatching when compared to the control tanks. The exposed males had fewer numbers of external matting tubercles, and the coloration of their body, and pectoral and caudal fins were significantly different from unexposed males. Futhermore, plasma vitellogenin concentrations were significantly higher than controls. The histological investigation of the E2-exposure males

showed a reduction in seminiferous tubules and the production of sperm cells was blocked. Reproductive success dramatically changed after the exposed males were removed from the E2 spiked water, despite external matting tubercle development remaining low and vitellogenin levels remaining high (McGree et al., 2010).

Behavioral Effects

Exposure to estrogens has been shown to disrupt natural predator avoidance behaviors in fathead minnows (*Pimephales promelas*). Embryos and larvae were exposed to E1 (5, 50, and 100 ng L^{-1}), E2 (1, 10, and 28 ng L^{-1}), ethinylestradiol (EE2; 0.1, 1, and 10 ng L^{-1}) and a mixture of all three estrogens and behaviors were observed 12 days post-hatch. Estrone exposed embryos and larvae, E2 and estrogen mixture exposed larvae exhibited delayed predator avoidance behaviors, while the EE2 seemed to have no effect on either the embryos or larvae (McGee et al., 2009). McGee et al. (2009) suggested that exogenous exposure of estrogens might lead to motor dysfunction by altering the releases of the neurotransmitter dopamine. A study conducted by Saaristo et al. (2009) examined the mating behavior of male Sand goby (*Pomatoschistus minutus*) after exposure to 4 ng L^{-1} EE2 for 7 to 24 days. The exposed males spent significantly less time in active courtship, and were less successful at acquiring and defending nest site, which reduced their reproductive success (Saaristo et al., 2009). In the McGree et al. (2010) study pre-spawning courtship behaviors were suppressed in male red shiners (*Cyprinella lutrensis*) after an 83 day exposure to an average concentration of 70 E2 ng L^{-1} (McGree et al., 2010).

Immune System Disruption

Small mouth bass (*Micropterus dolomieu*) possessing both skin lesions and intersex characteristic have been observed in a large number of fish kills from the Potomac and Shenandoah Rivers boardering West Virginia and Virginia. Robertson et al. (2009) hypothesized that EDCs might be causing suppression of immune function. Their experimental results showed exposing largemouth bass (*Micropterus salmoides*) to *Edwardsiella ictaluri* bacteria and simultaneously injecting 5 mg of E2 (mean plasma E2 concentration was 2471 pg mL^{-1}) interfered with the natural regulation of immune-related hepcidin peptides. Hepcidin gene sequences hep-1 and hep-2 are primarily expressed in the liver of largemouth bass. Compared to the bacterial exposed control organisms the expression of hep-1 was reduced in the liver of the E2 exposed fish. The induction of hep-2 in bacterial exposed test organisms was blocked by E2 when compared to control organism. Hepcidin peptides may play a crucial role as the first line of defense against invading pathogens. By blocking production of hepcidin, E2 may make fish more susceptible to disease-causing bacteria (Robertson et al., 2009). Another study found that juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to E2 had significantly reduced survival rates after infected with *Yersinia Rucker* (causative bacterial agent of Yersiniosis or enteric Redmouth disease) when compared to controls exposed to bacteria alone (Wenger et al., 2011).

***In vitro* Bioassays**

Multiple EDCs are suspected of binding to estrogen receptors either as agonists or antagonists and disrupting the endocrine systems of aquatic organisms. The binding affinity of a chemical to the ER can be assessed using *in vitro* assays, which are becoming increasingly important for assaying for endocrine disruptors as human populations and intensive farming

continue to expand (Rodriguez-Mozaz et al., 2004). Estrogen receptor competitor assays, yeast estrogen screen (YES) assays and Mcolloidal fraction-7 breast cancer cell proliferation assays (E-screen) (Li et al., 2012) are other examples of *in-vitro* assays developed for the screening of endocrine disruptors. These *in vitro* assays can be performed faster and are less complex when compared to *in-vivo* assays (Kase et al., 2008). *In vitro* assays provide an efficient and cost effective way to evaluate the potential for EDCs to produce an ER estrogenic response; however, like all *in vitro* assays, they cannot provide insight into uptake, distribution and metabolism of EDC's within an organism (Cespedes et al., 2004; Petrovic et al., 2004). Despite these limitations, *in vitro* assays are useful for initial screening of the estrogenicity of environment samples (Li et al., 2012). Additionally, *in vitro* assays such as estrogen receptor competitive-binding assays (Fig. 2) have been utilized to determine the estrogen receptor affinity for a large, structurally diverse group of chemicals (Blair et al., 2000). The estrogen receptor competitive binding assays used in the current study were based on a fluorescence polarization method to quantify the potential affinity of a ligand (free E2, and E2 associated with dissolved or colloidal soil and manure fractions) to bind to an ER.

Fluorescence polarization is use to study molecular interaction by monitoring size changes of fluorescently labeled molecules and viewing molecular binding events in solutions. Fluorescence polarizer passes monochromatic light through a vertical polarizing filter, only exciting those fluorescent molecules, or tracers in a vertically polarized plane. Small fluorescent molecules tend to depolarize due to their rapid tumbling rate. When a tracer molecule binds to a larger molecule, such as an ER, the tumbling rate slows and the emitted light from the molecule's fluorescence lifetime is measured in both the horizontal and vertical planes. Free tracer molecules will have lower polarization values than when in the bound state (Figure 2).

The polarization value is dependent on the viscosity, temperature and molecular volume. Therefore, polarization is directly related to the molecular volume when the samples viscosity and temperature are held content. The polarization value is a dimensionless number that can be calculated from the ratio of light intensity (I) in the vertical (v) and horizontal (h) planes and is often expressed as millipolarization (mP). The resulting equation is: $P = (I_v-I_h)/(I_v+I_h)$.

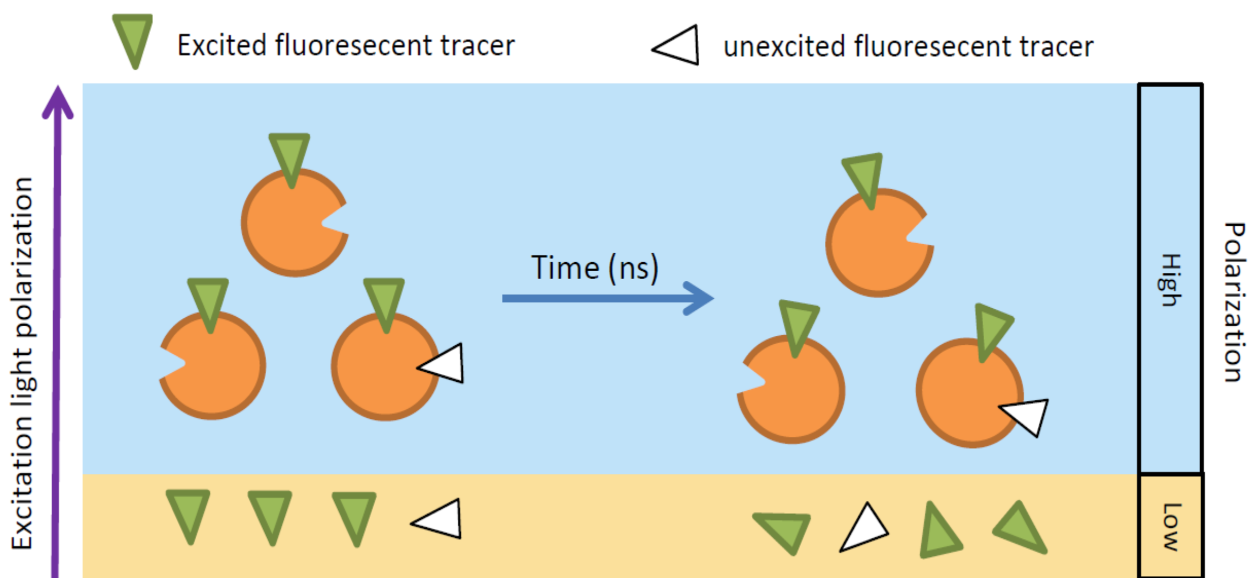

Figure 2. Schematic of fluorescence polarization differences between small and large complexes. During excited state small fluorescently labeled molecules rotate quickly and upon emission have low polarization values. When fluorescently labeled molecules bind with larger molecules (e.g. estrogen receptors) result in higher polarization values, rotate little during the excitation state (Invitrogen, 2006).

The tracer molecule in the estrogen receptor competitor assay used in the present study is called Fluormone™ ES2 (Invitrogen, Carlsbad, CA). The Fluormone™ ES2 is a florescence tagged estradiol molecule provide by the manufacture in the estrogen receptor competitor assay. An estrogen receptor/Fluormone™ ES2 complex is formed, which results in a high polarization value. If a compound in an unknown sample competes with the Fluormone™ ES2 the estrogen

receptor/ Fluormone™ ES2 complex then the polarization value will decrease. Compounds that do not compete with the tracer molecule for ER yield a high polarization. A difference in polarization after the addition of the test compound is used to determine the relative affinity of the compound for ER (Invitrogen, 2006).

Estradiol in Soils

Soil Degradation and Mineralization of Estradiol

The degradation of natural hormones in the environment is controlled through various sorption, mobility, and fate processes (Casey et al., 2005; Das et al., 2004; Fan et al., 2007; Lee et al., 2003). Sorption to particulate matter in sediments affects in hormone mobility and degradation. Fan et al. (2007) conducted column studies using agricultural soils from the Hamar soil series (sandy, mixed, frigid typic endoaquolls) and demonstrated E2 and testosterone have a rapid degradation rate with of < 1 day. Another study by Jurgens et al., 2002) found that microbial transformation of E2 to E1 in river water samples is rapid where half-lives range from of 0.2 – 9 days at 20°C. Under anaerobic condition in riverbed sediments E2 transformed to E1 over a 2 days period. The photo-degradation of E2 has an estimated half-life of 10 days in clear water exposed to 12hr of bright sunlight per day (Jurgens et al., 2002). Other, abiotic reactions can also degrade E2, for example after a 4 day reaction period 95% of the initial E2 was transformed into E1 in sterile soil slurries containing Mn oxides (Sheng et al., 2009). The soil used in Sheng et al. (2009) study were composed of 32% sand, 36 % silt and 32% clay with organic carbon content of 1.49%.

Soil Sorption and Mobility

Sorption of steroids to soil depends on the physicochemical properties of the hormone and the soil (Gineys et al., 2012). A key component in determining the environmental fate and mobility of organic pollutants is based on partitioning processes between the solid and aqueous phases. Hydrophobic partitioning plays a dominant role in the sorption of hormones to soil organic matter which is supported by a positive correlation between the K_{ow} (the octanol-water partition coefficient) and K_{oc} (the organic carbon-water partition coefficient) (Casey et al., 2008; Casey et al., 2005; Lee et al., 2003). Generally, compounds with a $\log_{10} K_{ow}$ values less than 2.5 have low sorption potentials, compounds with $\log_{10} K_{ow}$ between 2.5 and 4 have medium sorption potentials, and $\log K_{ow}$ greater than 4 have high sorption potentials (Rogers, 1996). The log Kow values for E1, E2 and E3 (Table 1) indicate they follow a hydrophobic sorption process (Casey et al., 2008; Casey et al., 2005; Lee et al., 2003). ydrophobic partitioning interactions have been suggested to be mechanistically similar to the formation of micelles (Chiou et al., 1986) where a hydrophilic exterior and a hydrophobic interior shields the non-polar molecules from the polar water environment. Piccolo (2001) suggested that humic substances from organic matter could create supramolecular structures (micelles) composed of a diversity of small organic molecules aggregated together through hydrogen bonding and hydrophobic portioning. The degree of molecular aggregation and disaggregation of these micelles is dependent on the concentration of humic materials in solution (Piccolo et al., 2001). In addition to hydrophobic interaction, hydrogen bonding may play a role in the sorption of estrogen to soil. The phenolic group of E2 and E1 are theorized to interact with the mineral faces, and the humic and fulvic acids in the soil (Yamamoto et al., 2003; Yu et al., 2004).

Dissolved and Colloidal Particles

Natural organic matter plays a significant role in the bioavailability of nutrients and chemicals by providing potential binding sites and modifying the stability in which they are bound. The organic matter of natural waters have dissolved and colloidal fractions that are typically dominated by humic substances (Wilkinson et al., 1999). The soil mineralogy, chemical properties of the sorbed molecule and its ability to resist microbial degradation are all factors that impact the sorption of dissolved fraction to soil solids (Jagadamma et al., 2012). In this current study, definition of dissolved fraction and colloidal fraction by Holbrook et al (2004) was used where colloidal fraction is defined as less than 0.45µm and greater than 1 kD, and dissolved fraction is defined as less than 1 kD.

Dissolved organic carbon can originate from natural decomposition of organic material as well as from industrial activities (Freidig et al., 1997). Soil organic carbon includes compounds such as carbohydrates, proteins and humic substance (fulvic and humic acids) (Amon and Benner, 1996). In aquatic environments, dissolved organic carbon has a wide variety of functions ranging from an energy source for microorganisms to a transport facilitator of metals and organic contaminates (Lapworth et al., 2009). Dissolved organic carbon in aquatic environments represents one of the largest active organic carbon reservoirs in the biosphere (Amon and Benner, 1996). Sorption of hydrophobic organic compounds to dissolved organic matter may significantly increase their aqueous solubility (Yamamoto et al., 2003). The aqueous solubility of hydrophobic organic chemicals is enhanced by dissolved organic carbon macromolecules (Freidig et al., 1997). This increased solubility effect of dissolved organic carbon is important for the understanding of the fate of organic compounds such as estradiol in the environment (Yamamoto et al., 2003).

Colloids in the aqueous environments have been defined as any substances that provides a molecular environment for chemicals/elements to sorb and desorb into/from the aqueous solution while gravitational settling has no effect on its vertical movement (Gustafsson and Gschwend, 1997). Colloidal particles balance the boundary between particulate matter that settles out and soluble chemical species with diameters typically ranging from 1 to 1000 nm (Wells, 1998). Natural colloids can be composed of a mixture of organic and inorganic components such as layered silicates, sesquioxides (Fe- and Al-oxyhydroxides), organic macromolecules, bacteria, and viruses (Jonge et al., 2004). Microscopic and chemical analysis has been used to identify two principal morphological structures of colloids found in natural freshwaters: (i) fibrillar, polysaccharide fraction that is likely a product of polysaccharides decompositions; and (ii) smaller branched fulvic and humic acids and aggregates (Wilkinson et al., 1999). Colloids are produced from weathering and microbiological processes and transported into natural waters such as rivers, lakes, seas, and oceans (Zhou et al., 2007). Soil colloids dispersed from soil aggregates in response to infiltrating water can lead to soil morphological soil features such as clayskins lining water-conducting pores. The source of mobile colloids in the vadose zone is considered to be the *in situ* release of water-dispersible colloids (Jonge et al., 2004). The concentration of colloids has been approximated as $10^6 – 10^8$ particles per liter in field waters (Zhou et al., 2007) and accounts for 30 – 50% of the dissolved organic carbon in seawater (Wells, 1998). Colloid composition in natural surface water is an unstable system of inputs and outputs that are continuously altered by physical, chemical and microbial actions (Buffle and Leppard, 1995). Colloids have a high sorptive capacity and can be effective sorbents of low solubility, strongly sorbing contaminants due to their high specific surface areas. The risk of contaminants leaching from the soil's solid phase increase with the

mobility and transport of colloids through the vadose zone (Jonge et al., 2004). Colloidal matter can be removed from the environment in two ways, degradation to soluble components or aggregation to sinkable macroparticles (Wells, 1998).

There is evidence that estrogen fate and transport is significantly associated with the dissolved and colloidal fractions in soil and sediments (Zitnick et al., 2011). Holbrook et al. (2004) found that 60 % of the aqueous E2 was associated with the colloidal fraction in wastewater from MWTP. Significant correlations between E2 detections and total organic carbon (0.45 µm filter dissolved organic carbon + colloidal organic carbon) under field plots that received swine manure has been reported (Thompson et al., 2009). Zitnick et al. (2011) conducted batch experiments comparing E2 sorption to soil with a supernatant composed of $CaCl_2$ or dissolved/colloidal organic carbon solutions derived from liquid swine manure. In the $CaCl_2$ solution, 100% of the E2 dissipated from the aqueous layer within 7 d via sorption and/or oxidative degradation (Fig.3). In the dissolved/colloidal organic carbon solution, however, the E2 persisted in the aqueous phase throughout the duration of the experiment (14 d). The E2 persisted in the batch experiment with liquid swine manure as a result of (1) the dissolved organic carbon/ colloidal organic carbon causing E2 to remain suspended in the aqueous phase, and/or (2) the dissolved/colloidal organic carbon obstructing the sorption and/or degradation of E2 on the soil surface (Zitnick et al., 2011).

PAPER: POTENTIAL BIOACTIVITY AND ASSOCIATION OF 17β-ESTRADIOL WITH THE DISSOLVED AND COLLOIDAL FRACTIONS OF MANURE AND SOIL

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Abstract

The dissolved (DF) and colloidal fractions (CF) of soil and manure play an important role in the environmental fate and transport of steroidal estrogens. The first objective of this study was to quantify the association of 17β-estradiol (E2) with the DF and CF isolated from liquid swine manure (LSM), Soil, and LSM and Soil mixtures (Soil+LSM). The appropriate CF and DF size fractions of the Soil, Soil+LSM, and LSM materials were obtained by first filtering through a 0.45 μm filter, which provided the combined DF and CF (DF/CF). The DF/CF from the three media were spiked with radiolabelled [^{14}C]-E2, and then ultrafiltered to isolate the CF (<0.45 μm and > 1 kDa) from the DF (<1 kDa). The average recoveries of the [^{14}C]-E2 associated with the DF were 67%-72%, 67%-79%, and 76%-78% for the Soil, Soil+LSM and LSM, respectively. For the CF that was retained on the 1 kDa filter, organic carbon (OC) and [^{14}C]-E2 were dislodged with subsequent water rinses for all three media. The Soil media was found to continue to elute organic carbon and [^{14}C]-E2 for all three water rinses while Soil+LSM and LSM did not elute these components after the first water rinse. The second objective was to evaluate whether the E2 associated with the DF/CF, DF and/or CF of the various media could still bind the ER using an E2 receptor (ER) competitor assay, which provided E2 equivalent concentrations (EEQ). The ER assay indicated that E2 in the DF of the Soil and Soil+LSM solution could still

bind the ER. Results from this study indicated that E2 preferentially associated with the DF of soil and manure, which may enhance its dissolved advective transport surface and subsurface water. Furthermore, this study indicated that E2 associated with DF solutions in the environment could potentially induce endocrine responses through its interactions with ER.

Keywords: colloid; dissolved organic carbon; estrogen; bioavailability; soil; manure

Introduction

Endocrine disrupting chemicals (EDC) mimic endogenous hormones, interacting with hormone receptors, and altering the natural pattern of hormone synthesis and/or metabolism (Mills and Chichester, 2005). Endocrine disrupting chemicals include a variety of synthetic organic chemicals and naturally occurring estrogenic hormones, such as E2, E1, and estriol (E3) (Jiang et al., 2005). Numerous reports have implicated EDCs in the cause of abnormal physiological development, altered reproductive capabilities, and abnormal behaviors of various organisms, especially in aquatic organisms (Trudeau and Tyler, 2007).

The fate and transport of estrogenic compounds are potentially influenced by their strong associations with dissolved and colloidal fractions of soil and manures (Bowman et al., 2002; Holbrook et al., 2004; Zhao et al., 2010; Zhou et al., 2007). Up to this point, we have no explanation for why E2 has been found in soil that has not received manure application. Only result documenting migration of E2 within soils would explain such findings is Thompson et al. (2009) frequently detected E2 in soil leachate and shallow ground water beneath plots that received swine manure (*Sus scrofa domesticus*) and beneath control plots that received no manure. Detections of E2 from the shallow ground water beneath these field plots were significantly correlated with total organic carbon with DF and CF size fractions ($\leq 0.45 \mu m$)

(Thompson et al., 2009). Furthermore, in wastewater 60% of the aqueous E2 was associated with the CF, indicating a preferential association of E2 to the CF (Holbrook et al., 2004). Additionally, soil batch sorption experiments revealed that E2 persistence was greater and sorption to soil decreased in the presence of liquid swine manure (Zitnick et al., 2011).

Multiple EDCs are suspected of mimicking natural hormones by binding to estrogen receptors (ERs) either as agonists or antagonists, and disrupting the endocrine systems. It is possible to assess the binding affinity of a chemical to an ER using in-vitro assays. In-vitro assays are becoming increasingly important for assessing and identifying EDCs as human populations and intensive farming practices continue to expand (Rodriguez-Mozaz et al., 2004). These in-vitro assays can be performed faster, and are less complex when compared to in-vivo assays (Kase et al., 2008). They provide an efficient and cost effective way to evaluate the potential of EDCs to produce an estrogenic response by quantifying estrogen receptor binding. Despite their restricted capacity to mimic whole animal uptake, distribution, and metabolism in an organism (Cespedes et al., 2004; Petrovic et al., 2004). In-vitro assays are effective for initial screening and provide a rough estimate of the estrogenic potential (Li et al., 2012).

Although dissolved and colloidal particles in soil/water systems are important to the fate and transport of estrogens, little is known about how these fractions influence the bioavailability of estrogenic EDCs. The objectives of this study were (i) to quantify the association of E2, a natural occurring EDC, to soil- and manure-derived dissolve and colloidal fractions; and (ii) to estimate the bioactivity of E2 associate with DF and CF. Preserved soil and LSM were used as sources of DF and CF for the filtration studies that were used to identify the relative associations of ^{14}C labeled E2 to the DF and CF. Soil and LSM samples were preserved to minimize

complications associated with E2 transformation in biologically active system. Estrogen receptor-β competitor binding assays were used to quantify the potential affinity of the ligand (either as free E2, or as E2 – associated to DF/CF) to bind to ER, which would indicate potential bioactivity of the ligand.

Materials and Methods

A schematic provides an overview of the experimental procedures and analytical methods used to achieve the first (associations of E2 with DF and/or CF) and second (bioavailability) objectives of this study (Figure 3). It should be noted that the soil and soil + LSM media used for objective 1 was 10 times the concentration of the original media which was required for TLC analysis.

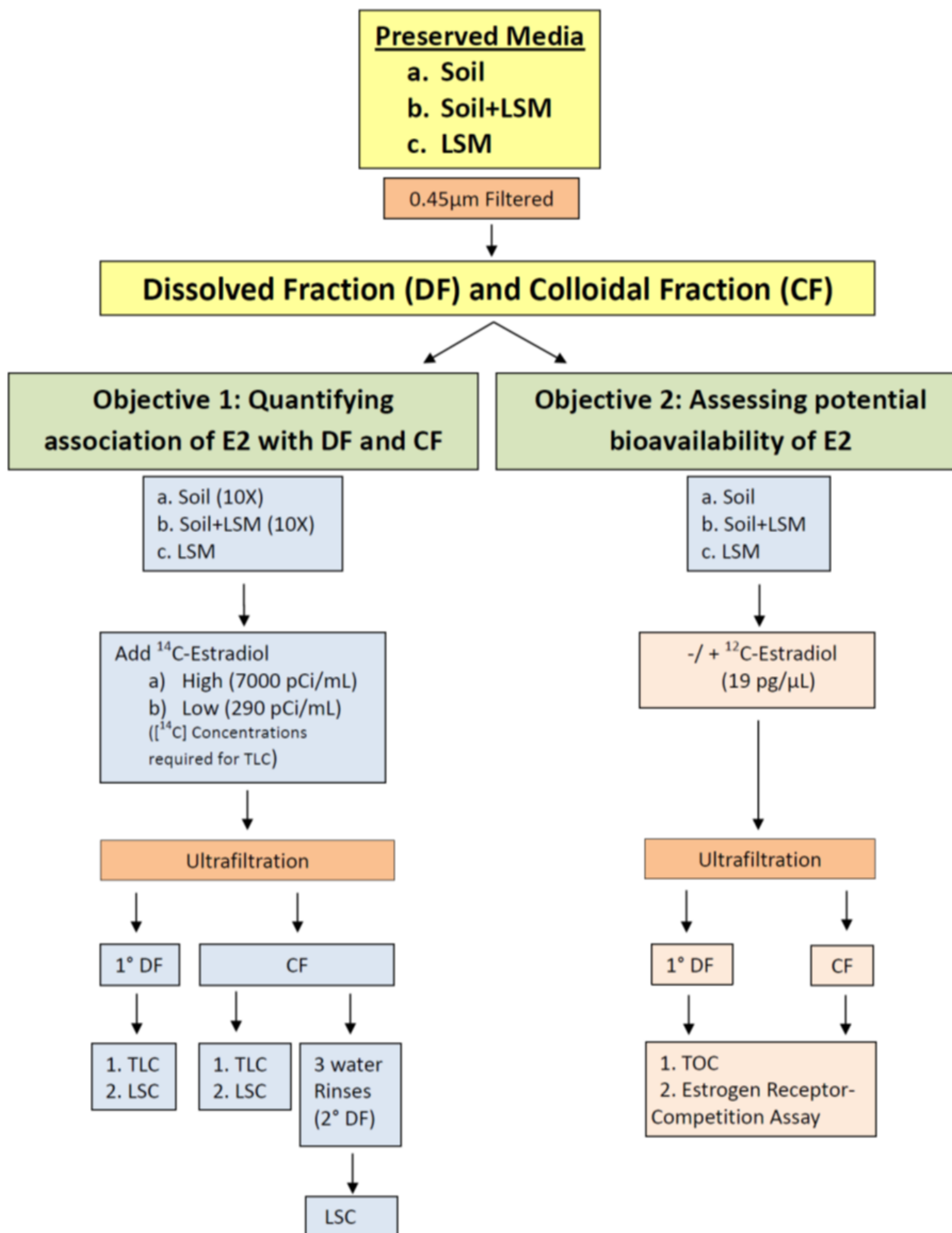

Figure 3. Flowchart of experimental procedures and analytical methods.

Soil and Liquid Swine Manure sample preparation

The LSM was obtained from a facility that housed approximately 4,000 animals, which included swine at all stages of development. The manure slurry was initially filtered using cheesecloth that was folded into several layers to a thickness of ~ 3 mm, and then filtered using filter paper (#2, Qualitative, circles, 90 mm, Whatman International Ltd, Maidstone, England) under vacuum to remove any large debris. The DF/CF fraction was obtained by filtering the LSM through a 0.45 μm membrane filter (nitrocellulose, Millipore Ltd, Tullagreen, Carrigtwoholl, Co. Cork, Ireland). The manure slurry was characterized for chemical and physical parameters by Servi-Tech Laboratories (Hastings, NE) using their lagoon analysis method and the E2 concentration was measured using LC/MS/MS (Table 3). Estradiol was quantified on the DF/CF by liquid chromatography tandem mass spectrometry (LC-MS2). Chromatography (Alliance 2695 Separation Model (Waters, Beverly, MA) and spectrometry were as described in Shappell et al. 2008 using a Waters Ultima API-US quadrupole-time-of-flight mass spectrometer in electrospray ion mode. The LSM DF/CF samples used in further analyses were preserved with 0.02% sodium azide. Sodium azide was used as a preservative because its interference with the ER competitor assay was minimum compared to other preservatives, such as mercurous chloride (0.005 mg/mL) and formaldehyde (2.5%) (data not shown).

The soil dissolved and colloidal fraction was extracted from the surface (0-15 cm) of a Ulen soil series (Sandy, mixed, frigid Aeric Calciaquolls), which was obtained near Embden, ND. A particle size distribution analysis of the soil used in this study was also performed, by

Table 3. Physical and chemical parameters of the < 0.45 µm fraction of the lagoon slurry used for the ultrafiltration studies and Estrogen Receptor Completive Assays

Parameter	Concentration
Total N (mg L^{-1})	2080
Organic N(mg L^{-1})	380
Ammonium N (mg L^{-1})	1700
Nitrate-N (mg L^{-1})	<10
Magnesium (mg L^{-1})	10
Sodium (mg L^{-1})	350
Zinc (mg L^{-1})	<1
Iron (mg L^{-1})	3
Manganese (mg L^{-1})	<1
Copper (mg L^{-1})	<1
Electrical Conductivity (dS m^{-1})	14.4
Phosphorus (mg L^{-1})	50
Potassium (mg L^{-1})	730
Sulfur (mg L^{-1})	40
Calcium (mg L^{-1})	60
Boron (mg L^{-1})	2
Total Dissolved Solids (mg L^{-1})	9220
Moisture (%)	99.3
Total Solids (%)	0.7
Organic Matter (wt. %)	57
Ash (wt. %)	43
Carbon/Nitrogen ratio	1.1
pH	8.2
17β-Estradiol (µg L^{-1})	~1.8

using the hydrometer method (Gree, G. W and Bauder, J. W., 1986). The chemical properties (soil pH, cation exchange capacity, and % organic matter) data was obtained from the Official Soil Series Descriptions (pedon 92ND073003) on the United States Department of Agriculture, Natural Resources Conservation Service website (accessed Nov 19, 2013) (Table 4).

Table 4. Physical and chemical properties of the Ap horizon of the Ulen soil series used in this study.

Horizon	Depth	Sand	Silt	Clay	OM	pH	CEC	EC
	cm	———	g kg^{-1}	———	wt. %		cmol+ kg^{-1}	ds m^{-1}
Ap	0-15	69.4 ±1.5	21.6±	8.9 ± 2.2	4.4	7.7	1.5	1.1

† OM; organic matter
‡ CEC; cation exchange capacity
§ EC; electrical conductivity

The soil was air dried, and sieved through a 2mm sieve. Air-dried soil and deionized (DI) water were mixed at a ratio of 1:2 (15 g soil: 30 ml deionized water), and then was shaken with and end-over-end tumbler at approximately 110 rpm for 30 min. The Soil+LSM media was processed the same way, except a 2% solution of LSM and deionized water (0.6 mL of LSM: 29.4 mL of deionized water) was used and 15 g of soil. The 2% LSM solution was based on the recommended application rate of LSM for soil fertilizer based on N and P needs (Hernandez and Schmitt, 2012), which was 12.2 L m^{-2} for a depth of 15 cm. The vials containing the soil + deionized water and soil + 2% LSM were then centrifuged for 20 min at 1000 × g. The supernatant was then collected, preserved with 0.2% sodium azide, and filtered through the same process as the LSM.

Table 5. pH values DF/CF, DF and CF for Soil, Soil+LSM and LSM media

Sample Size Fraction	Soil	Soil+LSM	LSM	Nanopure Water
DF/CF	7.30	7.80	8.6	6.0
1° DF (no rinse)	7.36	7.81	8.53	
CF (no rinse)	7.30	7.35	7.76	

The Soil+LSM media was processed the same way as the Soil DF/CF, except a 2% solution of LSM and DI water (0.6 mL of LSM: 29.4 mL of DI water) was used and 15 g of soil. The 2% LSM solution was based on a calculated application rate of LSM as a soil fertilizer (Hernandez and Schmitt, 2012), which was 12.2 L m^{-2} for a depth of 15 cm. The vials containing the Soil+LSM mixtures were preserved with 0.2% sodium azide, and filtered using the same process as the LSM. The pH of each media and media fraction are compiled in Table 5.

Analytical Procedures

Total organic carbon (TOC) was determined using a Total Organic Carbon Analyzer (TOC-VCPH, Shimadzu Corporation, Japan). Radioactivity was assayed by liquid scintillation counting (LSC) for 10 min using a Packard 2300 TR scintillation analyzer (Meriden, CT). Aliquots (100 µL) for LSC analysis were placed in 6 mL LSC vials with 4 mL of EcoLiteTM scintillation cocktail (MP Biomedicals, LLC, Solon, OH). Blanks were also assayed using LSC for each sample set to determine background radioactivity, which was subtracted from the sample measurements. Combustion analysis was used to measure any radioactive [^{14}C]-E2 residues remaining on the ultrafiltration disk membranes (Zitnick et al., 2011; Packard Model 307; Downers Grove, IL).

Association of ^{14}C-17β-Estradiol with Colloidal and Dissolved Fractions

Size segregation between DF and CF was based on other studies (Holbrook et al., 2004; Zitnick et al., 2011), where the CF was defined as <0.45 µm but retained by a 1 kDa ultrafiltration disk membrane, and everything that passed through a 1 kDa ultrafiltration disk membrane was considered to be the 1° DF. The 1 kDa (~0.05 µm pores) ultrafiltration disk membranes (cellulose, 25 mm, Millipore Corporation, Billerica, MA) were used with a 3 mL

ultrafiltration unit (Amicon Model 8003 stirred cell apparatus; Millipore Corporation, Bedford, MA). Samples of LSM, Soil, and Soil + LSM media were spiked with two concentrations of [^{14}C]- E2 referred to as high and low dose (Table 6).

Table 6. Initial concentration of applied [^{14}C] radioactivity

Media/Type	Ave Initial High Dose	Ave Initial Low Dose
	pci mL^{-1}	
Soil Blank	4727(±181)	338(±40)
Soil + LSM Blank	3894(±298)	251(±8)
LSM Blank	4141(±243)	266(±57)
H2O Blank	6527(±207)	1067(±47)
1% MeOH Blank	6437(±90)	746(±63)
Media/Type	Ave Initial High Dose	Ave Initial Low Dose
	pci mL^{-1}	
Soil Extract	5179(±313)	40(±42)
Soil + LSM Extract	4665(±134)	285(±42)
LSM Extract	10589(±2573)	263(±30)

†Values in parenthesis are average of three replicates ± SD

Prior to filtration, all filters were washed to remove the manufacturer's glycerin preservative according to Holbrook et al. (2005). Sample volumes of 3 mL were filtered by applying pressure (65 kPa N2) to the ultrafiltration unit for approximately 1 h or until filtered to dryness. After ultrafiltration, three 100 µL aliquots were removed from the filtrate and assayed for [^{14}C] by LSC. The stirred cell was then carefully dismantled, and using forceps, the filter was removed for [^{14}C] quantification of the CF using Packard Model 307 tissue oxidizer following air-drying for 24 h. Ultrafiltration was replicated three times for all samples. All parts of the dismantled ultrafiltration unit were rinsed with methanol then thoroughly washed with 1% solution of Liquinox liquid detergent (Alconox, Jersey City, NJ) and rinsed 3 consecutive times with deionized water between each run so there was no cross contamination between filtrates.

After the initial ultrafiltration of all three media, the experiment was repeated and this time the filters containing the CF were rinsed with three aliquots of nanopure water to assess whether the [^{14}C]-E2 could be dislodged from the colloidal particles. The effluents resulting from this portion of the experiment are referred to as 2° DF Rinse 1, 2 and 3. First, non-specific binding of radioactivity to the apparatus and filters was assessed by filtering [^{14}C]-E2 spiked solutions of nanopure water, 1% methanol:water and, 1° DF solutions from all three media (i.e. Soil, Soil+LSM and, LSM) in the same manner and also rinsed three times with aliquots of nanopure water. Each effluent from the three rinses was assayed for [^{14}C] by LSC. Capacity for ER binding of E2 in the 2° DF rinse 1, 2 and 3 from the un-spiked LSM was estimated using the estrogen receptor competitive assays. Additionally, all ultrafiltration filters after the three rinses were air-dried for 24 h, placed in combustible thimbles, and quantified for [^{14}C]-E2 residues using combustion analysis.

Reversed and normal phase TLC was conducted on DF and CF fractions of the LSM, soil and, soil+LSM. The reverse phase solvent system used in the analysis was a 1:1 solution of methanol (Honeywell Burdick and Jackson, Muskego, MI), purified water and C-18 reverse phase TLC plate (5×20 cm, 250 µm thickness; J.T. Baker Chemical Co. Phillipsburge, NJ). The normal phase solvent system used in this analysis was a 1:1:2 solution of tetrahyderofuran (Sigma-Aldrich, Inc., St. Louis, MO), ethylacetate, hexanes (EMD Chemicals Inc., Gibbstown, NJ) and Silica Gel GHL normal phase plate (5×20 cm, 250 µm thickness; Analtech Inc., Newark, DE). It was necessary to concentrate the Soil and Soil+LSM CF/DF solutions so there would be an adequate amount CF after ultrafiltration for TLC analysis. The two soil extracts were concentrated 10-fold with a centrifugal rotary evaporator (SpeedVac SC110 Savent, Holbrook, NY) subsequently [^{14}C]-E2 was added. The TLC analysis assessed whether [^{14}C]-E2 was

metabolized and provided indirect information about the association of the [^{14}C]-E2 with DF or CF. If it responded to the chromatographic separation, it behaved as a solute and if it did not respond chromatographically it was likely associated with colloids.

The 1° DF and [^{14}C]-E2 standard were spotted in separate lanes of a single TLC plates. The LSM CF could not be loaded into a syringe due to viscosity; therefore, it was transferred to the origin of the TLC plate by scraping the CF from the ultrafilter with a spatula. The CF originating for the soil and soil+LSM media were transferred to the TLC plates by adding a few drops of nanopure water (E-pure Ultrapure; Barnstead/Thermolyne, Dubuque, IA) to the filter and re-suspending the colloidal particles which were then transferred to the TLC plates with a syringe. The visible residue of CF originating from the soil and soil+LSM media was significantly less than the LSM media.

Bioactivity Assessment of Dissolved, Colloidal, and Total Fractions

Fluorescence polarization (FP) was used to assess whether [^{14}C]-E2 detected with the dissolved and colloidal fractions of soil, soil+LSM and LSM could compete with a tracer molecule for 17β-ER molecular binding sites. The tracer molecule in the ER competitor assay was Fluormone ES2 (Invitrogen, Carlsbad, CA). When the small tracer molecule binds to the estrogen receptor (large molecule), it results in a slower tumbling rate which can be measured as a high polarization value. When E2, or other estrogen receptor agonist, in the DF or CF media competes with the tracer molecule for the ER sites the polarization value decreases because free tracer yields no polarization. This shift in polarization can be quantified against a standard curve.

The commercially available in vitro PolarScreen™ ER-beta competitor assay kit (Invitrogen, Carlsbad, CA), was used to quantitate the ER affinity of the samples by FP. The

assay layout for the standards and samples were made according to the kit protocol. Each assay layout contained five controls and seven E2 standards (0.13, 0.41, 0.95, 2.72, 6.81, 27.24, and 272.4 µg L^{-1}) that were used to construct the standard curve to which all unknown samples were compared (Figure 4). Two sets of soil soil+LSM, and LSM samples were used for ER competitive assays, one spiked with unlabeled E2 (19.07 pg μL^{-1}; Sigma-Aldrich, St. Louis, MO) and one that was not spiked with unlabeled E2. Controls, standards, and unknowns were measured in triplicate wells of the microtiter plate of each assay. In order to reduce potential transformations the E2 standards and unknown solution were prepared no more than one day before the assays were performed. Initial assays were performed to test the effects of three preservatives (2.5% formaldehyde, 5 µg/mL mercurous chloride, and 0.02% sodium azide), and as a result 0.02% sodium azide was chosen as the media preservative because it had little or no effect on assay results. The limits of quantitation were exceeded when 100% concentrations were tested for most samples and therefore, the medias were diluted with nanopure water to fall along the linear portion of the standard curve (e.g. $0.2\ pg/\mu L \geq E2 \leq 10\ pg/\mu L$; Figure 4). Soil and soil+LSM media fractions (1° DF, CF and DF/CF) were diluted to 10% original strength and LSM media fractions (1° DF, CF and DF/CF) were diluted to 1% original strength. Each media CF was re-suspended in 3 mL of nanopure water by soaking the ultrafilters overnight at (4 °C) in a 20 mL glass vial. The FP of the samples was measured using a Tecan Ultra 384 microplate reader (Grödig, Austria) after daily calibration with a FP One-Step Reference Kit (Invitrogen).

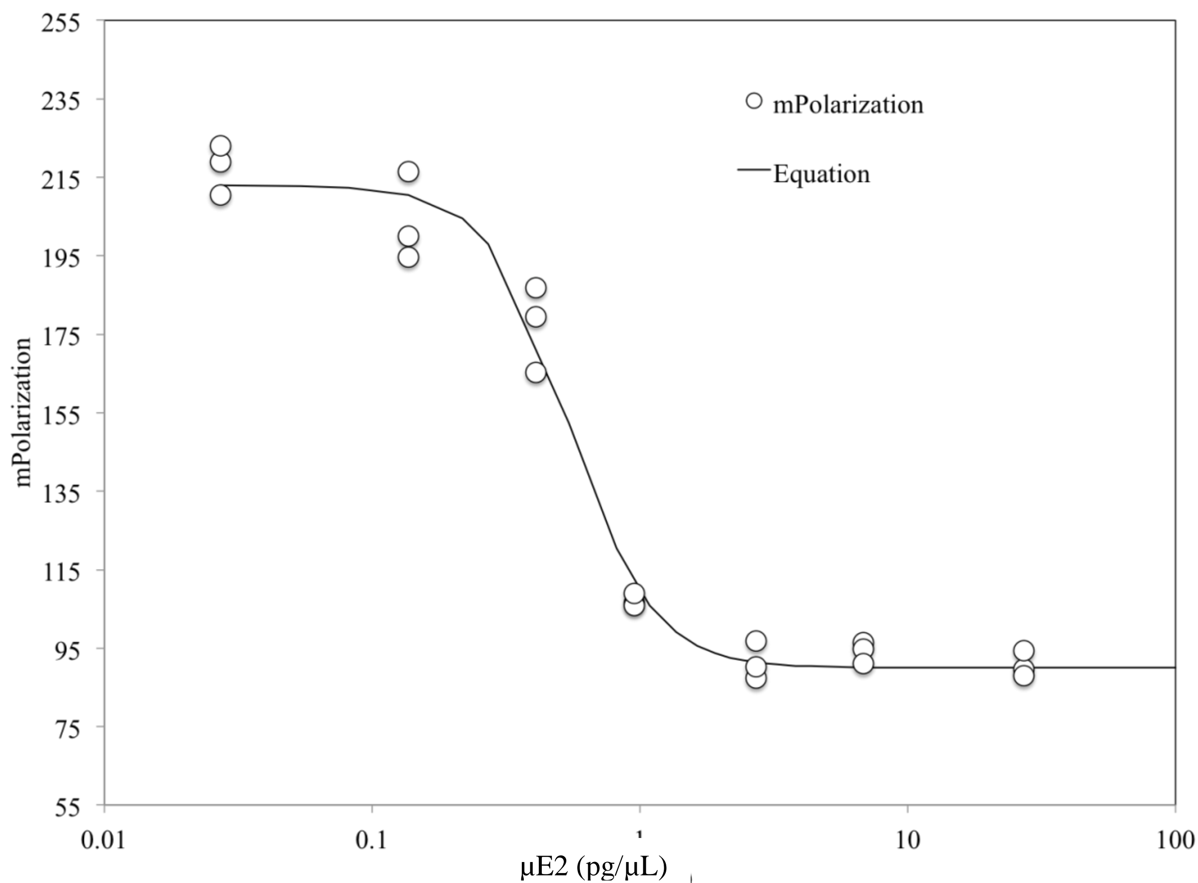

Figure 4. Calibration of estrogen receptor fluorescence polarization-based assay relating 17β-estradiol (E2) concentrations with polarization units.

Results and Discussion

Characteristics of the Media and Media Fractions

The LSM material contained much greater TOC compared to the other two materials (soil and soil+LSM). In addition, in the LSM the TOC measured in each of the three 2° DF rinses was greater than each of the respective 2° rinse of the soil and soil+LSM medias (Figure 5). The LSM has high organic content of the LSM is due to very large loads of organic solid wastes disposed of in manure storage ponds (Hart and Turner, 1965). The LSM of this study was

collected from a holding pond located at a facility that housed approximately 4,000 animals. Differences in TOC values between the LSM and soil can have important implications on the sorption of estrogens, which prefer high OC media because of their hydrophobic nature (Lee et al., 2003), and can potentially affect estrogen receptor binding.

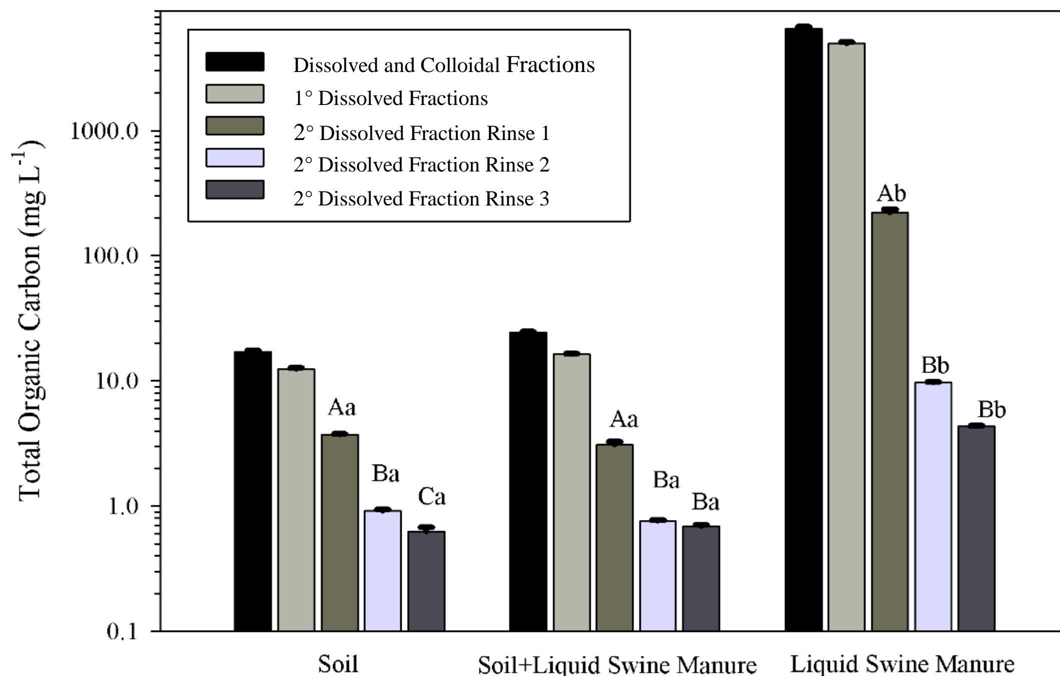

Figure 5. Total organic carbon (TOC) fractionation and dissociability. Degree of dissociation is reflected by mean ± standard deviations (error bars) of three 2° DF rinses. Identical capital letters indicate no significant difference ($p \leq 0.05$) between the different 2° rinses for a single media. Identical lower case letters indicate no significant difference ($p \leq 0.05$) for an individual 2° DF rinse across media. The data is represented on a logarithmic scale.

The TOC measurements of the 2° DF between the second and third rinse were not significantly different for LSM and soil+LSM; however all effluent rinses (1, 2 and 3) from the soil media were significantly different (Figure 5; capital letters), indicating significant OC was dislodged from the 1kDa filter after each rinse. However, comparing the different media, the soil had the least relative change in TOC between the initial filtration and all subsequent rinses,

where the percent changes in TOC values for soil, soil+LSM and LSM were 29%, 53% and 56%, respectively. This may indicate that the CF OC derived from soil is more cohesive and able to sustain the physical washing, whereas the LSM is more labile and readily dissociated after the first rinse. Although the relative amount of TOC of the soil was low compared to the LSM, its colloidal organic particles appeared to be more stable compared to LSM.

Associations of the E2 with Different Size Fractions

Apparatus Effect/Non-specific binding of radioactive E2

To identify whether the filtration apparatus or filters retained [^{14}C]-E2, filtration experiments were done using [^{14}C]-E2 dissolved in solutions of 2% MeOH, nanopure water, and 1° DF of soil, soil+LSM, and LSM. The mass recoveries for the MeOH, Water,soil, soil+LSM, and LSM, where 85(±2)%, 87(±12)%, 77(±5)%, 93(±19)%, and 78(±6)%, respectively, for the low dose; and 85(±20)%, 84(±28)%, 100(±13)%, 91(±29)%, and 89(±7)%, respectively, for the high dose. The mass recoveries were relatively high, and any incomplete recoveries may be attributed to potential binding to the apparatus. Nonetheless, there were no significant differences between the second and third 2° DF rinses for both the low and high [^{14}C]-E2 doses and for all solutions (Figure 6). No significant difference between the second and third rinse indicated that the effect of the apparatus was not significant after the first rinse.

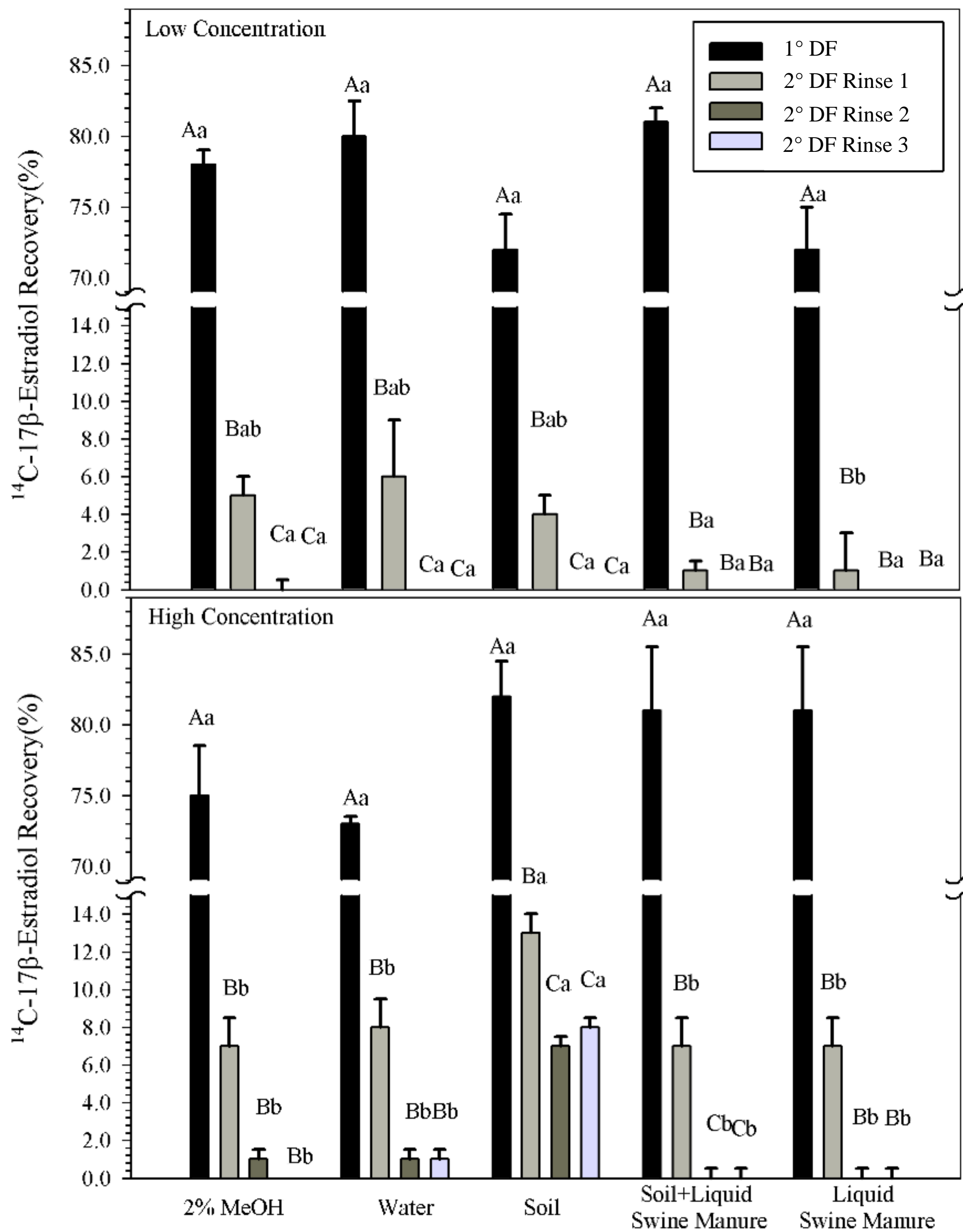

Figure 6. The percentage of applied radiolabeled 17β-estradiol ([14C]-E2) recovered non-specific binding of "blank" media solutions (MeOH=methanol; LSM=liquid swine manure) and media specific associated. Error bars are the standard deviations of three replicates. Identical capitol letters indicate no significant difference ($p \leq 0.05$) between the different effluent rinses for a single media. Identical lower case letters indicate no significant difference ($p \leq 0.05$) for an individual effluent rinse across media.

Association of 17β-Estradiol with Colloidal and Dissolved Fractions

Radiolabeled E2 was added to each of the media (<0.45μm) and then the media was ultrafiltered to separate the DF from the CF (Figure 7). It was determined that the majority of the [^{14}C]-E2 associated with the DF for all media and for both low and high doses (Figure 7). Additionally, there was no significant difference in the relative ^{14}C-E2 recovered in the DF between all three media (Figure 7), which indicated that the preference of E2 to associate with DF was not affected by the source of the organic matter (soil or manure). In similar ultrafiltration experiments using LSM as the media, Zitnick et al. (2011) also found that the majority (56 %) of ^{14}C-E2 was associated with the DF of the manure. However, Lee et al. (2011a) showed higher E2 sorption occurred in the size fraction range of <0.45 μm to > 5 kDa, and Holbrook et al. (2004) showed the majority of E2 was bound to colloidal particles <1.5 μm but > 100 kDa. The differences in the studies may reflect a number of experimental differences including size fraction differences and/or quantity and quality of the organic materials.

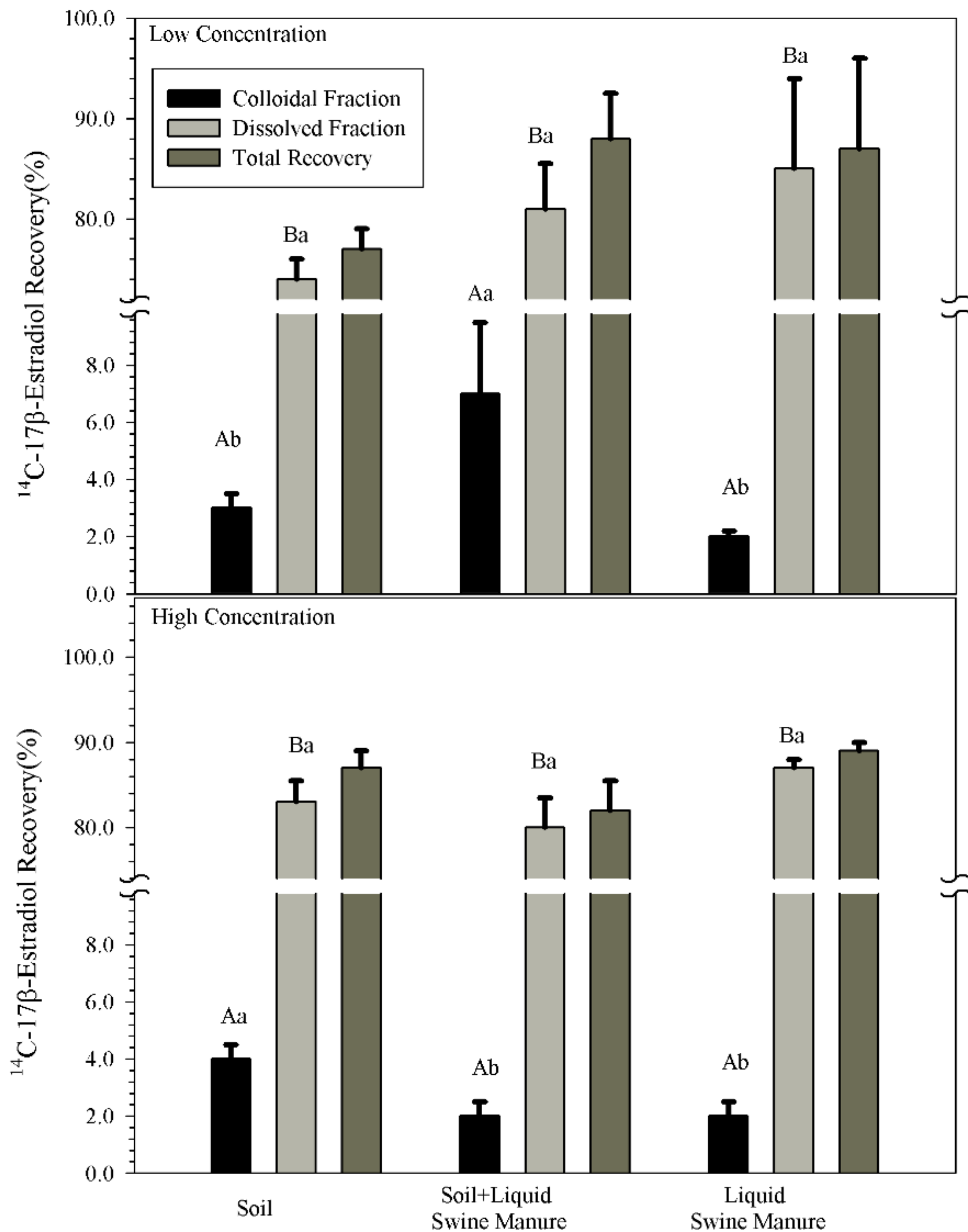

Figure 7. Average percent recoveries of radiolabeled 17β-estradiol ([^{14}C]-E2) in the colloidal (post three water rinses) and dissolved fractions for the low and high doses. Error bars are standard deviations of three replicates. Identical capitol letters indicate no significant difference ($p \leq 0.05$) within a single media. Identical lower case letters indicate no significant difference ($p \leq 0.05$) between fractions across the three media.

The Characteristic of the Association of 17β-Estradiol with Colloidal and Dissolved Fractions

The DF and CF analyzed using reverse (not shown) and normal phase TLC provided information about the formation of metabolites. It also provided information about whether the [^{14}C] was attached to a colloidal particle or present in solution as an individual molecule. If the [^{14}C]-E2 was dissolved it would be present in solution as an independent molecule and respond to chromatographic separation. For all of the media, the majority of the [^{14}C]-E2 responded to chromatographic separation in the TLC analysis with Rf values corresponding to the E2 standards (Figure 8), indicating it was indeed E2. The preferential association of [^{14}C]-E2 with DF, may indicate that DF solutions enhance the E2 solubility through hydrophobic interactions with humic substances. Humic substances have been reported to enhance the solubility of other organic contaminates in soils, such as, DDT (2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane) and p-dichlorobenzene (Chiou et al., 1986; Terashima et al., 2004). The degree of enhanced solubility of relatively water insoluble organic chemicals depends on the type of solute, its concentration, and the source of the dissolved organic matter (Chiou et al., 1986). Additionally, the preferential association of the E2 with the DF may explain the field observation by Thompson et al. (2009), where shallow ground water E2 detections were significantly correlated to the TOC values.

If the [^{14}C]-E2 is attached to a colloid, it will not respond to chromatographic separation, and remain at the origin for both reverse and normal phase TLC analysis. The majority of the [^{14}C]-E2 present in the CF remained at the origin of the TLC chromatograph for the Soil and Soil+LSM in the normal (Figure 8) and reverse phase TLC analysis (not shown). The [^{14}C]-E2 remaining at the origin of the chromatograph indicated that it was attached to a colloidal particle and could not migrate along the TLC plate. The majority of the [^{14}C]-E2 responding to the

chromatographic separation for the LSM colloidal fraction (Figure 8) may indicate that the E2 is weakly attached to LSM colloids compared to stronger binding to colloids of the Soil or Soil+LSM media. The weaker binding of the E2 to the LSM colloids was supported by the [^{14}C] recovery results from the ultrafiltration rinses (Figure 6). Compared to the Soil and Soil+LSM, the LSM media had a significantly larger difference in the amount of [^{14}C]-E2 rinsed off the CF between the initial filtration and the final third rinse.

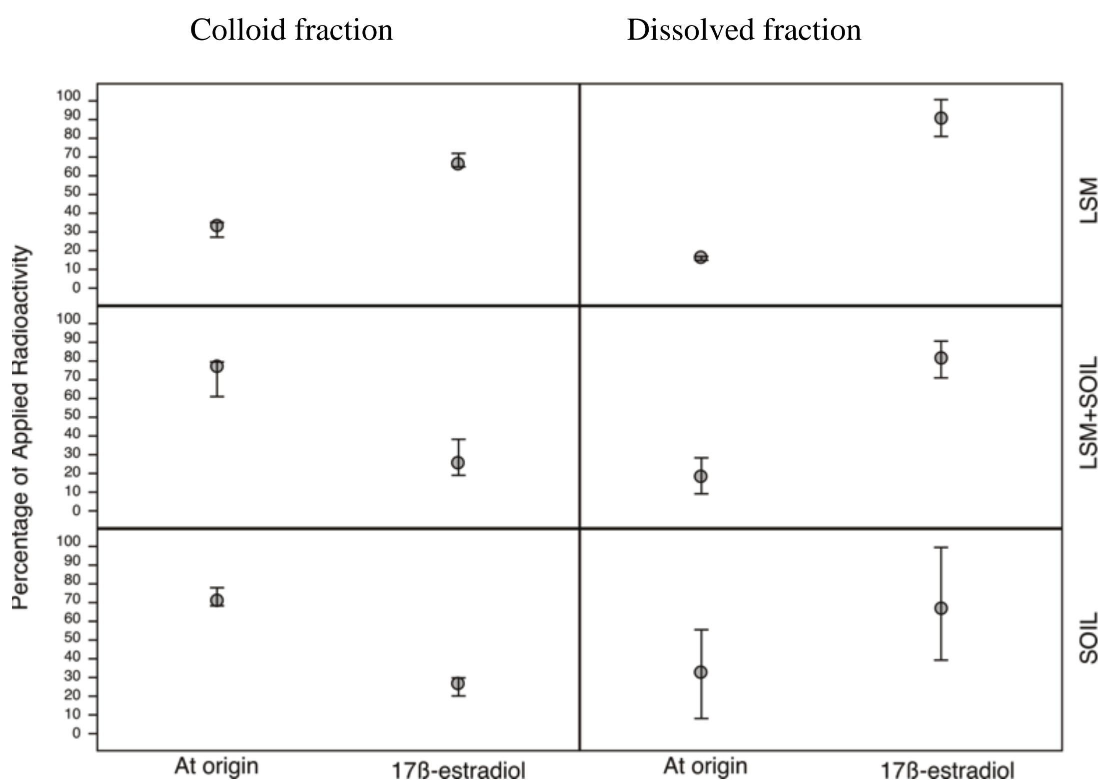

Figure 8. Percentage of applied radioactivity corresponding to the Rf values of the normal phase TLC chromatograph at the origin and E2 standard, for the CF (left column) and DF (right column).

The normal phase TLC analysis also detected a metabolite for the Soil media. The metabolite was more polar than the E2 standard, which may have been the oxidized metabolite of E2, which is E1. The average percent of [^{14}C] associated with this metabolite for the Soil was 27(±10) % (normal phase, data not shown). Abiotic oxidation of E2 to E1 occurs on mineral

manganese oxides reaction sites (Sheng et al., 2009). The observation that a polar metabolite was present only in the Soil and not LSM, suggested that the mineral fraction of the Soil may have contributed to the oxidative transformation of E2. An additional possibility is that the presence of humic substances in the LSM may reduce the transformation of E2 to E1 by protecting it from oxidation or competing with Mn-oxidation sites. It has been observed that E2 transformations to E1 is reduced as the concentration of humic acid increased (Lee et al., 2011b).

Bioactivity of 17β-Estradiol in CF and DF Solutions

The addition of E2 to the Soil and Soil+LSM total media (i.e., filtered to < 0.45 μm and containing both CF and CF size fractions) resulted in significantly higher concentrations of E2 as measured by the 17β-estrogen receptor bioassay (Figure 9). However, there was no corresponding significant increase for the LSM. This result indicated that there was significant ER interactions for the Soil and Soil+LSM, suggesting that the E2 was still bioactive in these solutions. However, for the LSM, there was interference of the LSM material with the fluorescent ligand and its displacement from the 17β-ER. This interference has been observed in other studies involving E-screen bioassays, where the addition of humic acid (<0.2 μm) reduces the estrogenicity of residual E2 in activated sludge samples (filtered to <0.45 μm) (Holbrook et al., 2004; Lee et al., 2011a). The potential bioavailability of the E2 in the LSM material was inconclusive because of the interference of the LSM material with the 17β-ER.

After filtration of the solutions with E2 added, estrogenicity measured by the bioassay was significant only for the DF solutions of the Soil and Soil+LSM, but not for any of the CF sizes. This result was consistent with the filtration studies and the results from the TLC data, where the majority of the E2 was associated with the DF (Figure 8), remained dissolved and not

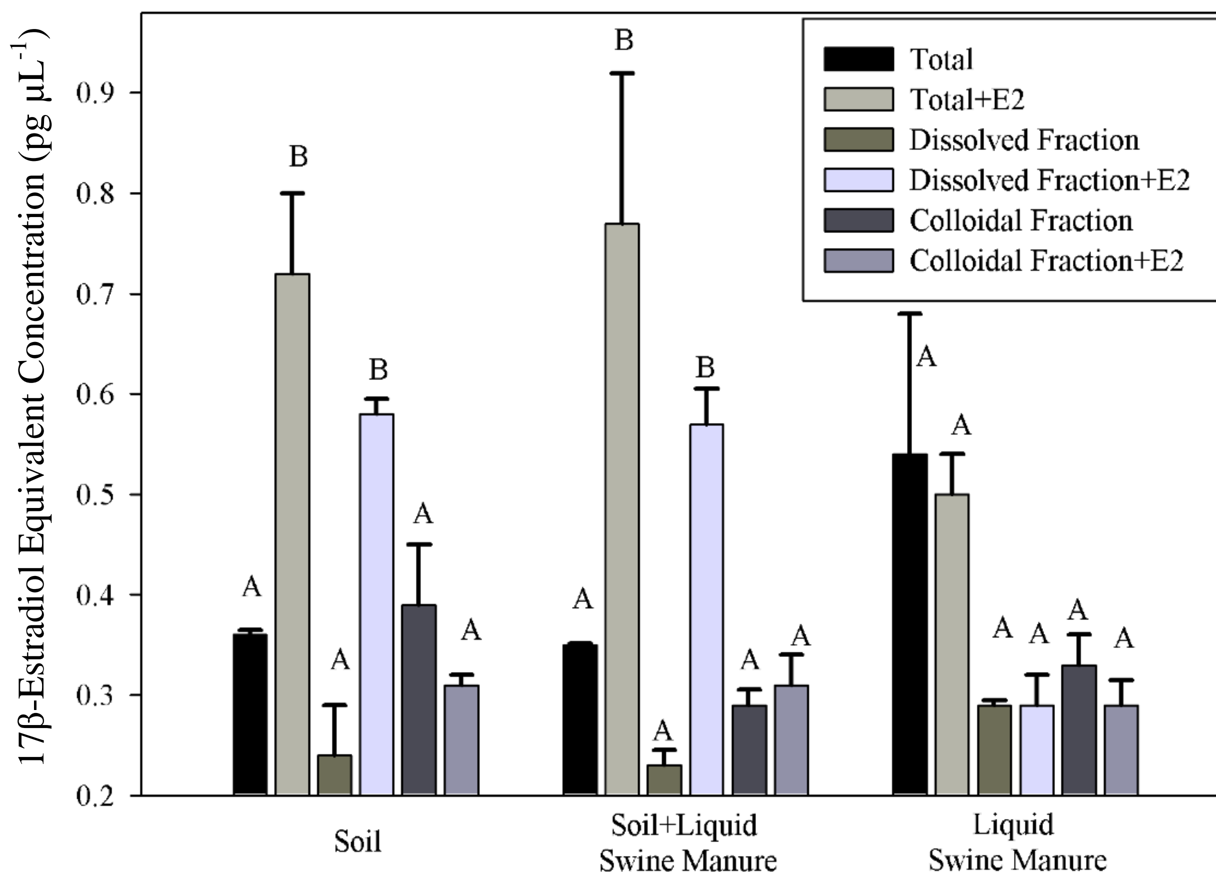

Figure 9. Mean 17β-estradiol equivalent concentrations determined by the Estrogen Receptor assay for the dissolve (DF) and colloidal (CF) fractions of the various media (LSM=liquid swine manure). Values represent assay concentrations (soil and soil+LSM diluted to 10% of their original concentration using nanopure water and LSM dilute to 1%). Identical capitol letters indicate no significant difference ($p \leq 0.05$) between antecedent and E2 spiked within a media.

attached as a particle in the DF solution (Figure 9). This indicates that the E2 associated with the colloid size fraction was unable to interact with the 17β-ER, and was not bioavailable. Holbrook et al. (2005) hypothesized that pi electron interaction and hydrogen bonding between phenolic groups of the E2 molecule and the colloidal particles could reduce the accessibility to the ER sorption sites. However, the E2 in the dissolved fraction remained potentially bioavailable.

Conclusion

17β-Estradiol preferentially associated with dissolved fractions of Soil and LSM materials, perhaps resulting from enhanced solubility of E2 in dissolved organic solutions. Physicochemical evidence indicated that much smaller amounts of E2 associated strongly to Soil and LSM colloids. Although colloids can greatly enhance hydrophobic compound mobility, the E2 associated with the CF in this study was very small compared to the DF. Furthermore, there was no significant EEQ of the CF+E2 compared to antecedent levels. In the context of manure management and understanding fate and transport of E2 in the environment, these studies indicate the Soil and manure DF may play a significant role in facilitating hormone transport. If E2 remains dissolved in the DF of Soil and LSM, it can readily transport advectively in soil water and runoff. Furthermore, the E2 found in the DF remained dissolved and could induce a potential estrogenic response. These results imply that E2 borne in manures and applied to soils will prefer the dissolved fractions, where, if they persist, will remain dissolved and can potentially remain mobile in surface and subsurface waters. If the E2 is not transformed, and is transported to a receiving water body, it can still interact with estrogen receptors and potentially cause endocrine disruption. It should be clearly understood that these studies were conducted under aseptic sterilized conditions, using preservatives to minimize complexities associated with biological transformations. It is likely that microbial activities have major effects on E2 persistence and the results of this study.

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GENERAL CONCLUSIONS

When compared to the dissolved fraction only a small portion of 17β-estradiol was associated with the colloidal fraction of the Soil and LSM materials. The higher concentrations of dissolved organic carbon may have enhanced the solubility of E2 in the dissolved fractions of the media. The ability of E2 to be dislodged from the LSM colloidal fraction appeared to also have been enhanced when compared to the Soil media. These results suggest that E2 in the DF has a high potential to transport to subsurface and surface water after manure application. This may provide insight to field studies that found frequent E2 detection in the subsurface water beneath agricultural soils (Schuh et al., 2011a; Schuh et al., 2011b; Thompson et al., 2009).

All media fractions tested demonstrated the ability to displace the fluorescent ligand (Fluormone™ ES2) from the 17β-ER, and an equivalent E2 concentration (EEQ) was calculated. Cold E2 added to the media prior to ultrafiltration increased the EEQ of Total+E2 and dissolved fraction+E2 fractions in the Soil and Soil+LSM media significantly. However, the addition of E2 did not significantly change the EEQ of the colloidal fraction when compared to the antecedent E2. In contrast, all fractions of LSM showed no significant difference regardless if E2 was added. It was hypothesized that the sorption of E2 to the colloidal fraction of the Soil and Soil+LSM and all fractions of LSM could reduce the availability of E2 to interact with the ER. Similarly, E2 sorption to colloidal fraction in the natural environment could decrease the potency of E2 and reduce the potential estrogen disruption affects to aquatic organisms.

Further Investigations

In this study, the ER binding capacity of E2 in *in vitro* assays appears to be reduced by the colloidal fraction of the Soil and Soil+LSM media potentially due to its adsorption to the interlayers of clay colloids. Further investigations of the sorption potential of smectitic clay (i.e. montomorillonite) colloids to reduce the estrogenic response would provide insight on the fate and transport of hydrophobic EDCs in the environment. The absorptive capacity of kaolinite and montmorillonite for E2, E1 and EE2 has been demonstrated in a single and multi-sorbent system (Bonin and Simpson, 2007). Experimental results of Gupta and Gardner (2005) showed that the presence of montmorillonite reduced the toxicity of poultry litter leachate but did not evaluate the estrogenicity of the samples.

Although *in vitro* assays are an effective method for providing initial screening and a rough estimate of the estrogenicity of environment samples (Li et al., 2012) they do have limitations for assessing whole organism effects. Research using freshwater mussels would provide a more accurate estimation of estrogenic impact that E2 from manure application could have on aquatic organisms. Estrogenicity of municipal effluents has been assessed by evaluating the vitellogenin level produced in the hemolymph and gonad of freshwater mussel, *Elliptio complanata* after exposure to the effluent (Gagne et al., 2001). Filter feeding organisms such freshwater mussels would be an effective model to evaluate the estrogenic potency of swine manure and clay colloids containing absorbed estrogens.

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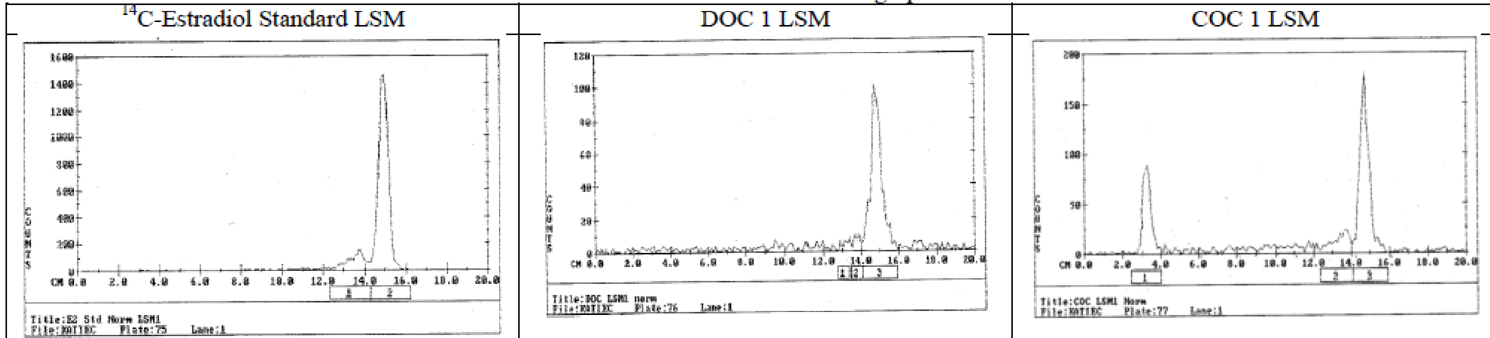
APPENDIX

Normal Phase TLC Chromatograph

^{14}C-Estradiol Standard LSM	DOC 1 LSM	COC 1 LSM

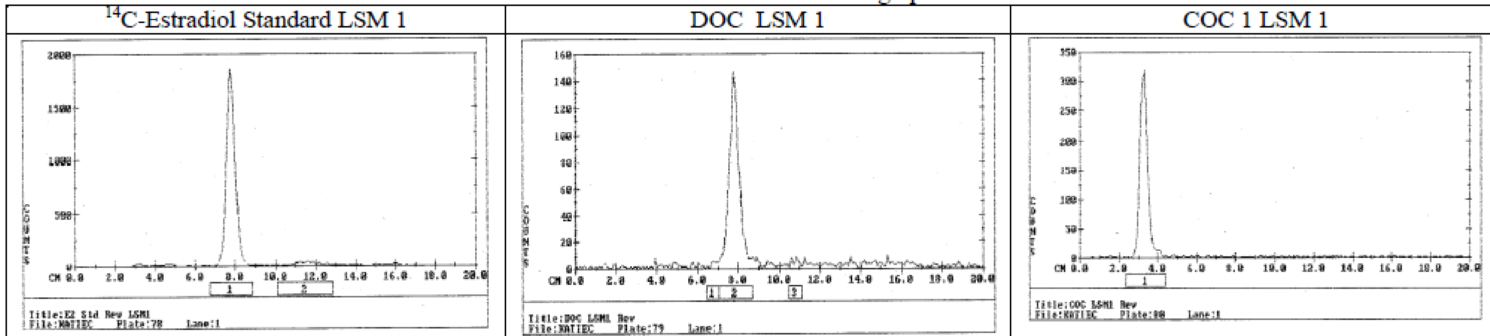


Normal Phase TLC: Liquid Swine Manure 1

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 1	3	13.8	11.88	100	0.86			
DOC1	3	13.8	11.83	100	0.86			
COC1	3	13.8	11.71	71.75	0.85	0.25	28.25	0.02

Reverse Phase TLC Chromatograph

^{14}C-Estradiol Standard LSM 1	DOC LSM 1	COC 1 LSM 1



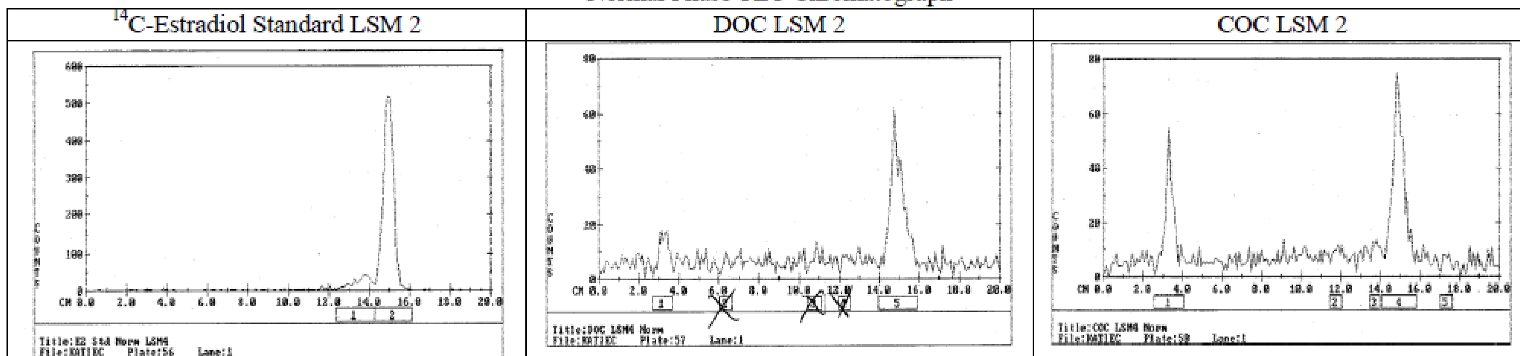
Reverse Phase TLC: Liquid Swine Manure 1

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 1	3	13.8	4.8	100	0.35			
DOC1	3	13.8	4.8	100	0.35			
COC1	3	13.8				0.2	100	0.01

Figure A1. TLC report for the Normal and Reverse phase for LSM rep 1

Normal Phase TLC Chromatograph

^{14}C-Estradiol Standard LSM 2	DOC LSM 2	COC LSM 2

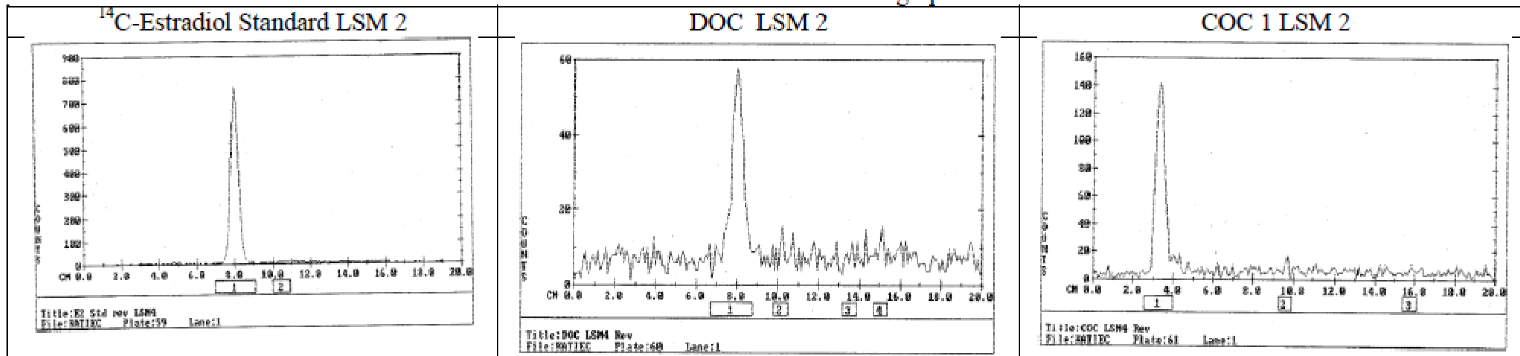


Normal Phase TLC: Liquid Swine Manure 2

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 2	3	13.9	11.92	100	0.86			
DOC2	3	13.9	11.89	81.45	0.86	0.12	18.55	0
COC2	3	13.9	11.86	66.03	0.85	0.32	33.97	0.02

Reverse Phase TLC Chromatograph

^{14}C-Estradiol Standard LSM 2	DOC LSM 2	COC 1 LSM 2

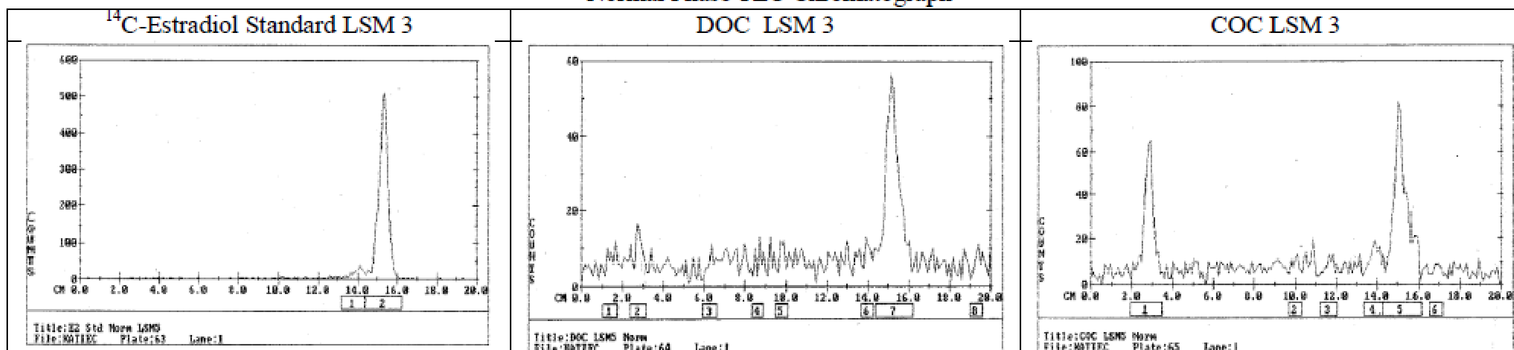


Reverse Phase TLC: Liquid Swine Manure 2

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 2	3	13.8	4.98	100	0.36			
DOC 2	3	13.8	4.89	100	0.35			
COC 2	3	13.8				0.3	100	0.02

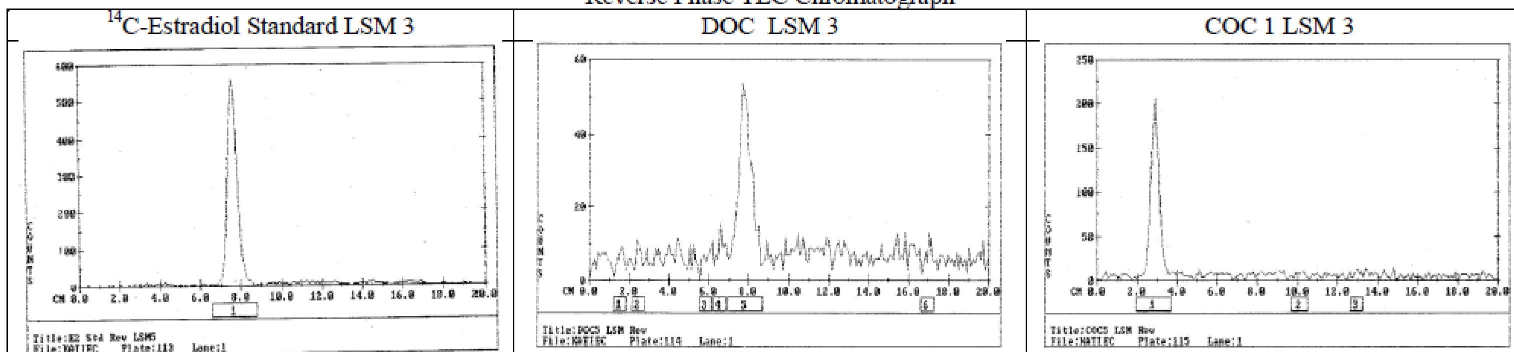
Figure A2. TLC report for the Normal and Reverse phase for LSM rep 2

Normal Phase TLC Chromatograph



Normal Phase TLC: Liquid Swine Manure 3								
Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 3	2.6	13.9	12.65	100	0.91			
DOC 3	2.6	13.9	12.61	84.18	0.91	0.11	15.82	0
COC 3	2.6	13.9	12.53	65.19	0.90	0.16	34.81	0.01

Reverse Phase TLC Chromatograph

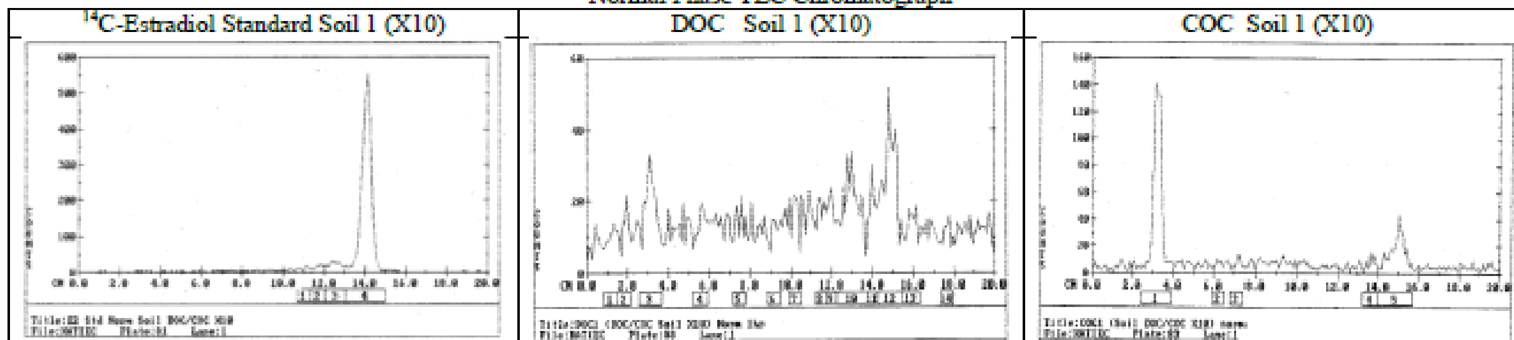


Reverse Phase TLC: Liquid Swine Manure 3								
Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 3	2.6	14.5	4.99	100	0.34			
DOC 3	2.6	14.5	5.17	100	0.36			
COC 3	2.6	14.5				0.26	100	0.02

Figure A3. TLC report for the Normal and Reverse phase for LSM rep 3

Normal Phase TLC Chromatograph

^{14}C-Estradiol Standard Soil 1 (X10)	DOC Soil 1 (X10)	COC Soil 1 (X10)

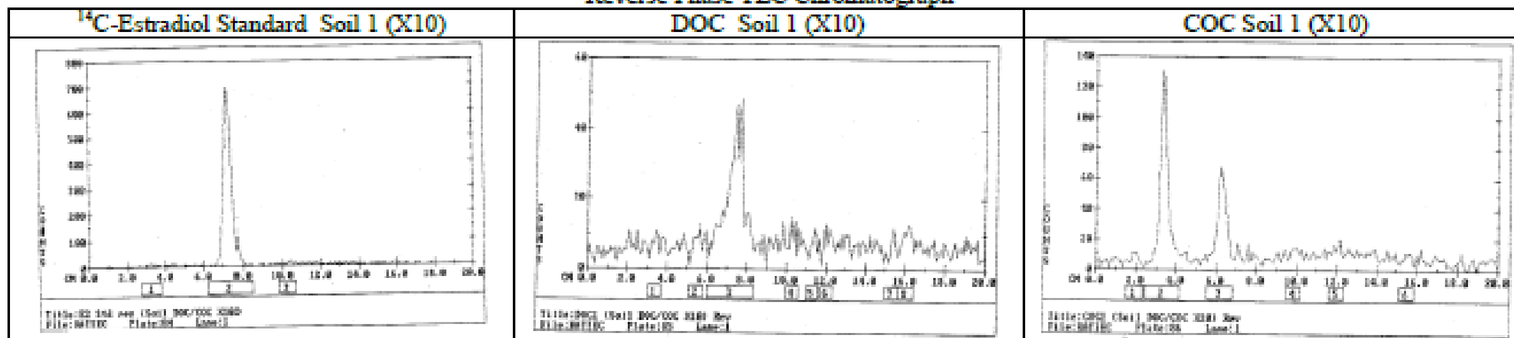


Normal Phase TLC: Soil 1 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 1	3	13.9	11.03	100	0.79			
DOC1	3	13.9	11.83	61.74	0.85	0.08	38.26	0.0
COC1	3	13.9	11.90	30.68	0.86	0.17	69.37	0.01

Reverse Phase TLC Chromatograph

^{14}C-Estradiol Standard Soil 1 (X10)	DOC Soil 1 (X10)	COC Soil 1 (X10)

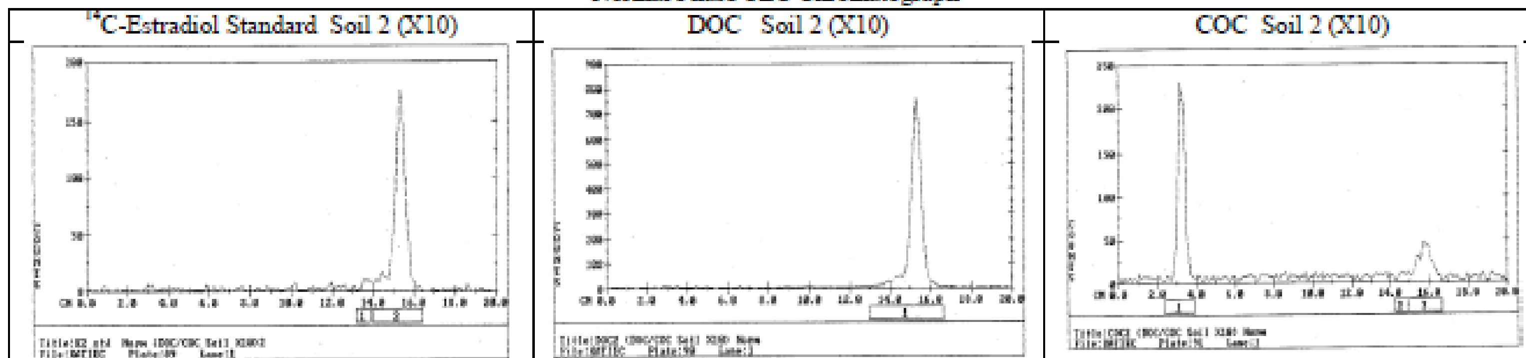


Reverse Phase TLC: Soil 1 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 1	3	13.9	4.27	100	0.31			
DOC1	3	13.9	4.26	100	0.31			
COC1	3	13.9	3.2	38.71	0.23	0.36	61.29	0.03

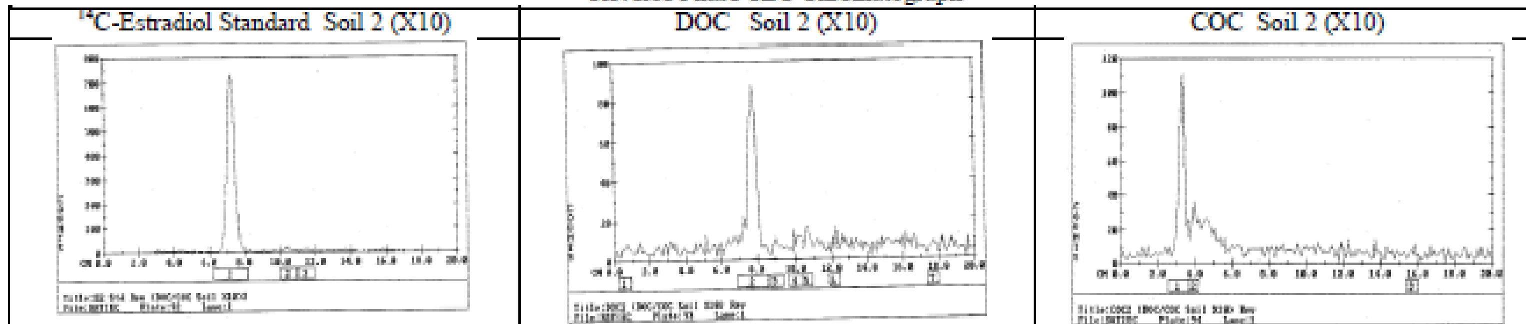
Figure A4. TLC report for the Normal and Reverse phase for Soil rep 1

Normal Phase TLC Chromatograph



Normal Phase TLC: Soil 2 (X10)								
Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 2	3	13.9	12.23	100	0.88			
DOC 2	3	13.9	12.15	100	0.87			
COC 2	3	13.9	12.69	28.71	0.91	0.21	71.29	0.02

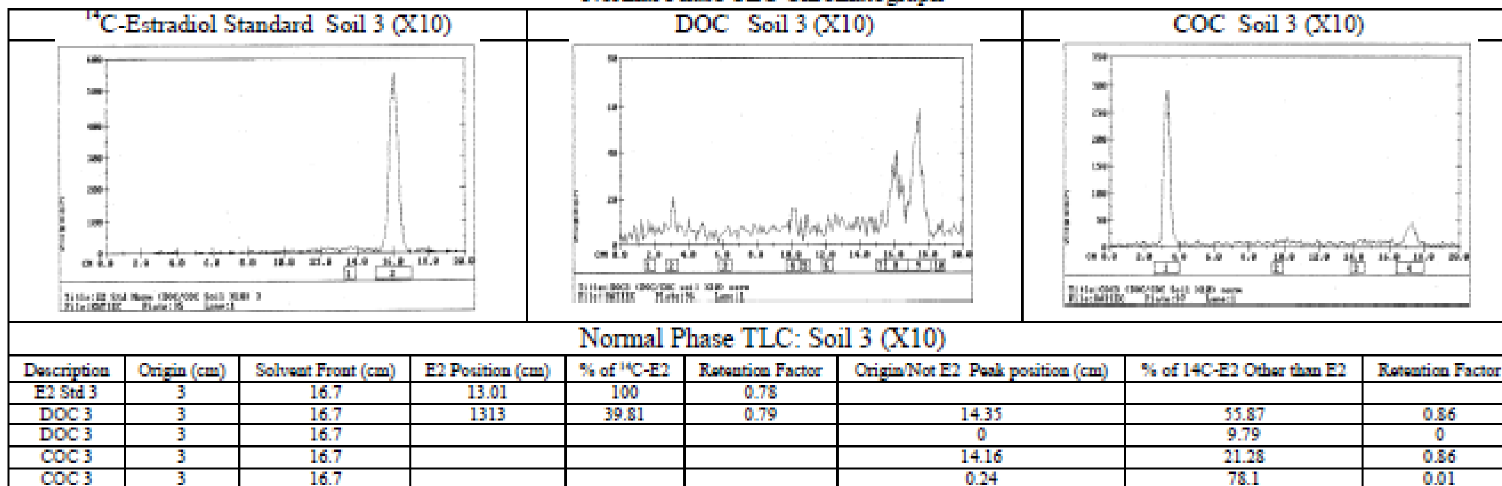
Reverse Phase TLC Chromatograph



Reverse Phase TLC: Soil 2 (X10)								
Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 2	3	13.9	4.2	100	0.30			
DOC 2	3	13.9	4.65	100	0.33			
COC 2	3	13.9				0.29	76.58	0.02
						0.9	23.42	0.06

Figure A5. TLC report for the Normal and Reverse phase for Soil rep 2

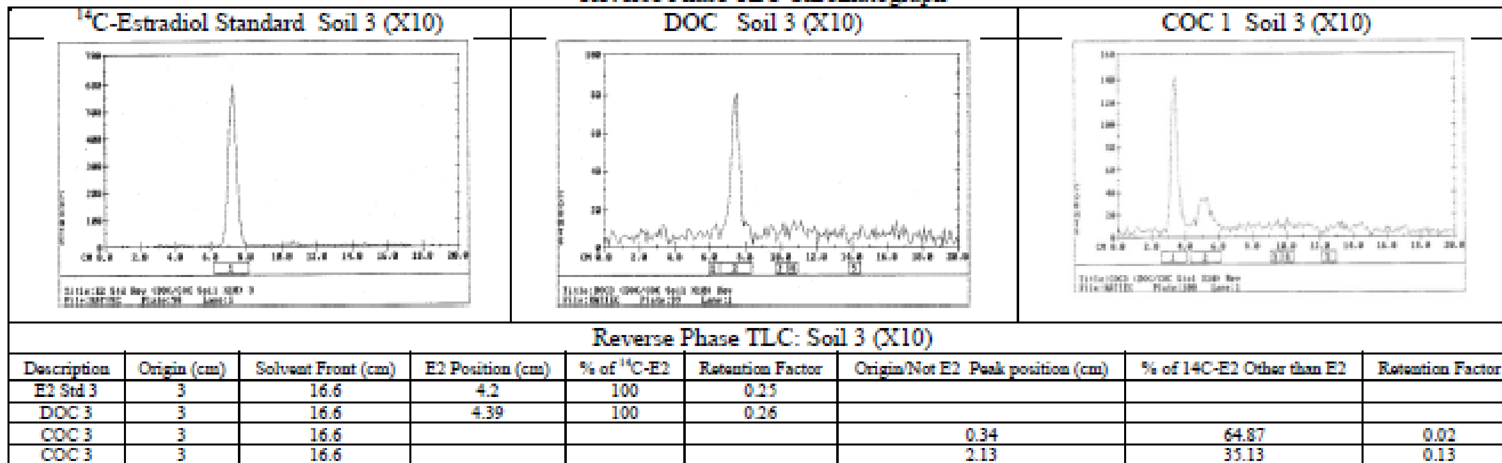
Normal Phase TLC Chromatograph



Normal Phase TLC: Soil 3 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 3	3	16.7	13.01	100	0.78			
DOC 3	3	16.7	1313	39.81	0.79	14.35	55.87	0.86
DOC 3	3	16.7				0	9.79	0
COC 3	3	16.7				14.16	21.28	0.86
COC 3	3	16.7				0.24	78.1	0.01

Reverse Phase TLC Chromatograph

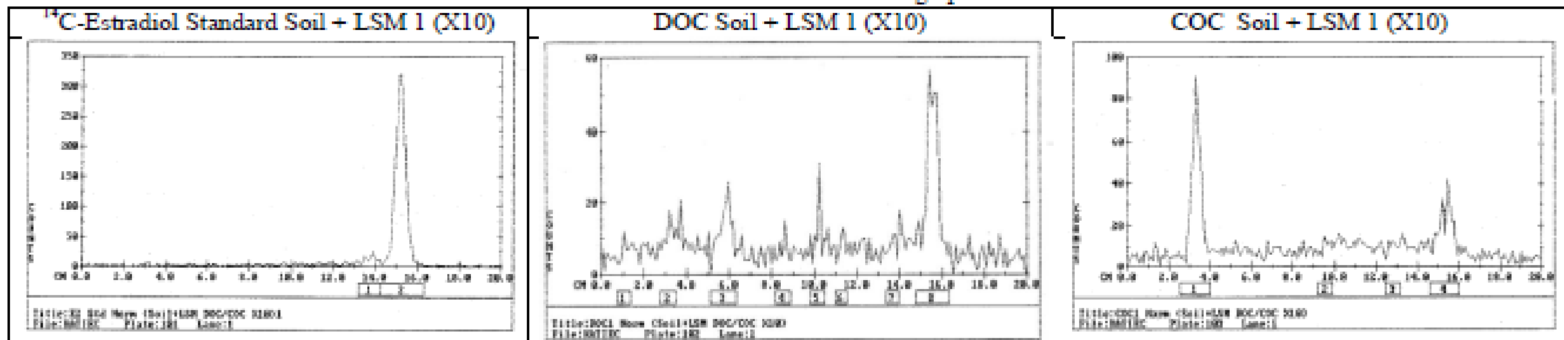


Reverse Phase TLC: Soil 3 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 3	3	16.6	4.2	100	0.25			
DOC 3	3	16.6	4.39	100	0.26			
COC 3	3	16.6				0.34	64.87	0.02
COC 3	3	16.6				2.13	35.13	0.13

Figure A6. TLC report for the Normal and Reverse phase for Soil rep 3

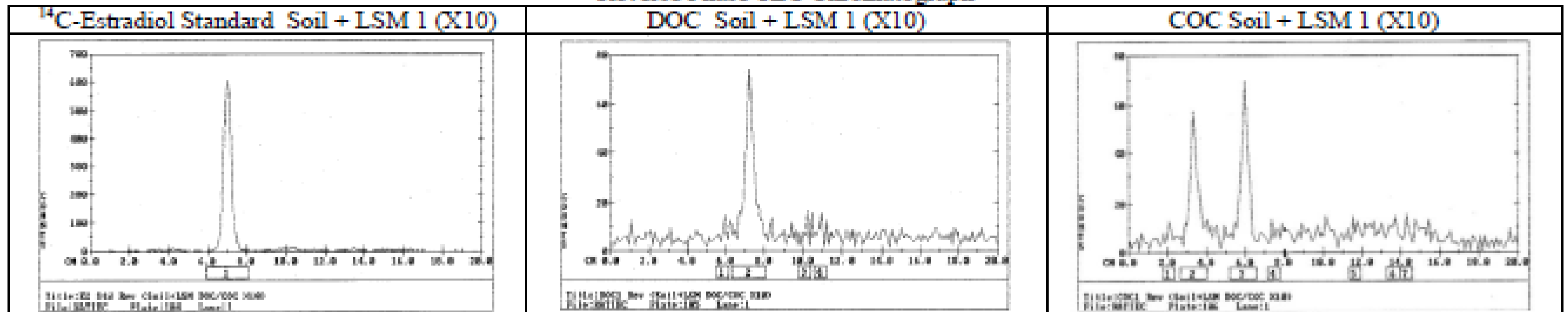
Normal Phase TLC Chromatograph



Normal Phase TLC: Soil + LSM 1 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 1	3	13.8	13.17	100	0.88			
DOC1	3	13.8	13.50	71.75	0.91	2.75	28.25	0.20
COC1	3	13.8	13.26	38.68	0.89	0.29	61.32	0.02

Reverse Phase TLC Chromatograph

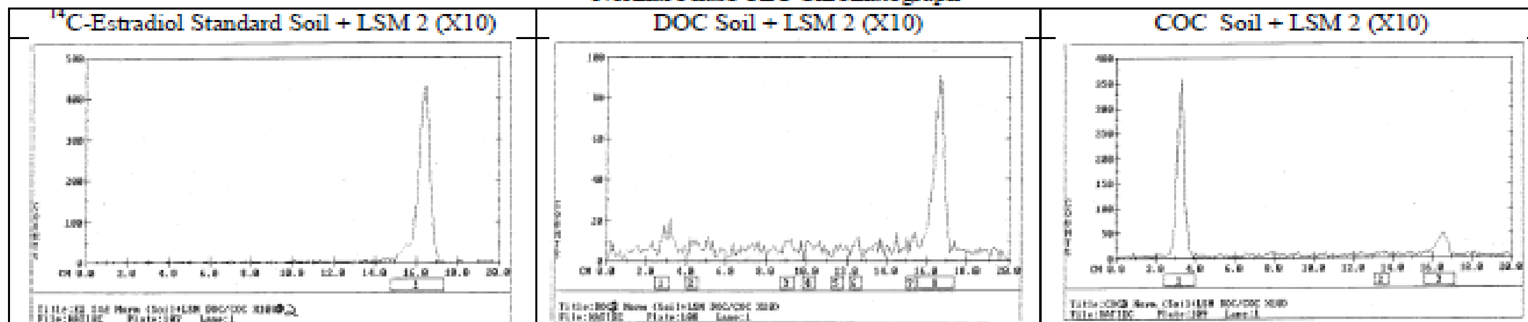


Reverse Phase TLC: Soil + LSM 1 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 1	3	13.9	3.97	100	0.29			
DOC1	3	13.9	4.17	100	0.3			
COC1	3	13.9	2.87	53.02	0.21	0.32	46.98	0.02

Figure A7. TLC report for the Normal and Reverse phase for Soil+LSM rep 1

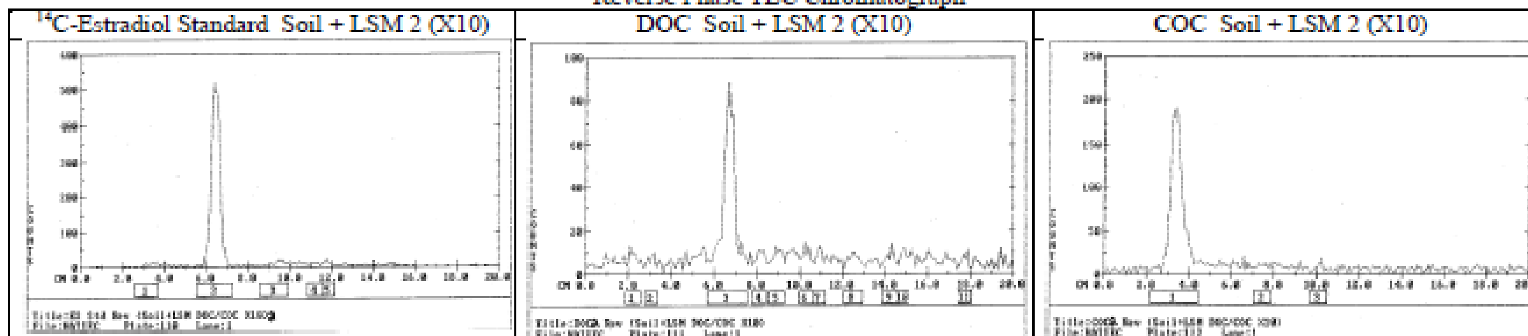
Normal Phase TLC Chromatograph



Normal Phase TLC: Soil + LSM 2 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 2	3	13.9	13.25	100	0.95			
DOC 2	3	13.9	13.51	89.45	0.97	0	10.55	0
COC 2	3	13.9	13.36	19.9	0.96	0.22	80.1	0.02

Reverse Phase TLC Chromatograph



Reverse Phase TLC: Soil + LSM 2 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 2	3	13.8	3.4	100	0.25			
DOC 2	3	13.8	3.64	100	0.26			
COC 2	3	13.8				0.36	100	0.03

Figure A8. TLC report for the Normal and Reverse phase for Soil+LSM rep 2

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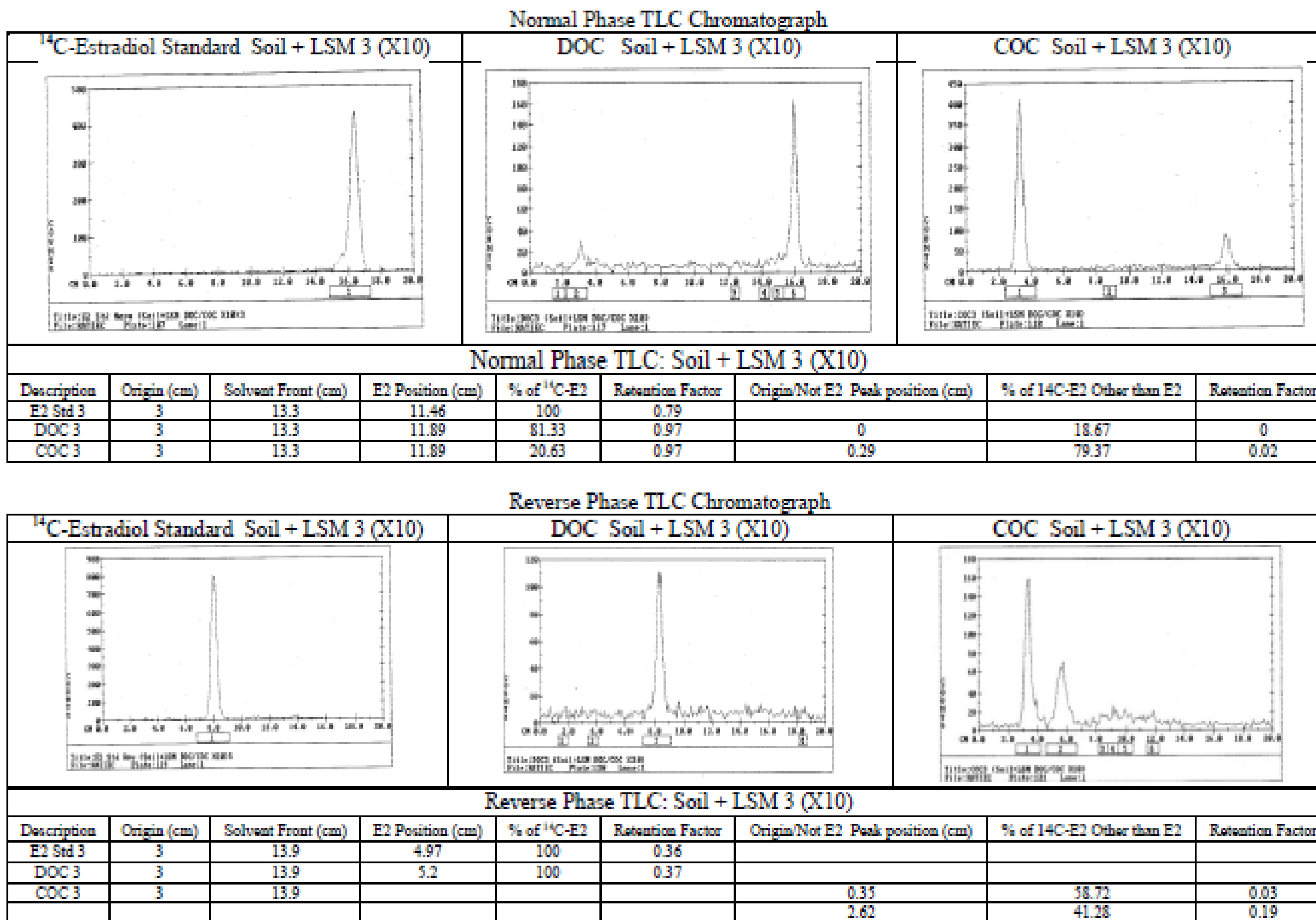

Normal Phase TLC: Soil + LSM 3 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 3	3	13.3	11.46	100	0.79			
DOC 3	3	13.3	11.89	81.33	0.97	0	18.67	0
COC 3	3	13.3	11.89	20.63	0.97	0.29	79.37	0.02

Reverse Phase TLC: Soil + LSM 3 (X10)

Description	Origin (cm)	Solvent Front (cm)	E2 Position (cm)	% of ^{14}C-E2	Retention Factor	Origin/Not E2 Peak position (cm)	% of 14C-E2 Other than E2	Retention Factor
E2 Std 3	3	13.9	4.97	100	0.36			
DOC 3	3	13.9	5.2	100	0.37			
COC 3	3	13.9				0.35	58.72	0.03
						2.62	41.28	0.19

Figure A9. TLC report for the Normal and Reverse phase for Soil+LSM rep 3