Journal of Hazardous Materials 192 (2011) 748–752

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

# Effects of field-manure applications on stratified 17β-estradiol concentrations

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ARTICLE INFO

*Article history:*
Received 21 December 2010
Received in revised form 23 May 2011
Accepted 26 May 2011
Available online 1 June 2011

*Keywords:*
Hormones
Estrogen
Estradiol
Soil
Manure
Dissipation

ABSTRACT

The occurrence of the manure-borne estrogen, 17β-estradiol (E2), was investigated in laboratory and field soils. In the laboratory, E2 was applied to soil to simulate concentrations found in swine (*Sus scrofa domestica*) manure (5000 ng $L^{-1}$). The aqueous-extracted E2 dissipated in the soil by 98% within 1 h and was not significantly different from background concentrations (18 ng $L^{-1}$) for the duration of the experiment (64 h). In the field study, soil cores were taken before and several dates after swine manure application. Equivalent porewater concentrations of water-extractable E2 were determined in 0.15-m increments down to the water table (0.70–2.00 m deep). The average frequency of detection for 168 samples was 38% (average = 40 ng $L^{-1}$ porewater equivalents). Eleven days after manure application there was no significant effect on E2 detection frequency or concentration. However, E2 concentrations significantly increased by 6 months after manure application, and appeared to be related to precipitation. Concentrations then returned to original levels by 17 months after manure application. Manure did not have an immediate effect on E2 occurrence due to the capacity of the soil to rapidly sorb E2. However, it appears that soil may act as a long-term reservoir for E2 in the environment, which may be periodically released through desorption.



## 1. Introduction

In the environment, natural estrogens excreted by humans and farm animals may affect the endocrine systems of certain organisms. 17β-Estradiol is the most potent estrogen, and can bind to hormone receptor sites with an affinity 100 times greater than its principle metabolite, estrone (E1) [1]. For example, the lowest observed adverse effect concentration of E2 for rainbow trout (*Oncorhynchus mykiss*) is between 1 and 10 ng $L^{-1}$ for sub-chronic exposures [2].

All species, sexes, and classes of farm animals eliminate natural estrogenic hormones into the environment [3]; however, the environmental significance of these releases is largely unknown. Concentrations of E2 in dairy (*Bos taurus*), swine, and poultry (*Gallus gallus domesticus*) manures vary from below detectable limits to $239 \pm 30$ μg $kg^{-1}$, $1215 \pm 275$ μg $kg^{-1}$, and $33 \pm 12$ μg $kg^{-1}$, respectively [4]. Although U.S. 2008 swine meat production ($\sim 1.0 \times 10^9$ kg) was lower than that of beef ($\sim 1.2 \times 10^{10}$ kg) and poultry ($\sim 1.6 \times 10^{10}$ kg) [5], swine manure has some of the greatest potentials to produce and contribute natural estrogenic hormones to the environment compared to other animal feeding operations [6–8].

Studies using immunoassay analysis to detect E2 have linked manure management to E2 found in field runoff [9–12], aquifer-fed karst springs [13,14], and subsurface waters [15,16]. However, if environmental samples are not first purified using solid phase extraction (SPE) or another chromatographic cleanup method, then natural organic matter can absorb to antibodies or other surfaces in the immunoassay causing interference with the analysis [17]. Studies from farmsteads and fields near manure sources that used SPE purification had much less detections of E2 and E1 compared to studies that used immunoassays alone [6,18,19].

Under common field conditions dissolved E2 is expected to dissipate rapidly in the soil as a result of sorption or degradation processes. Sorption of steroidal estrogens (e.g., E2, E1) is predomi-

*Abbreviations:* E2, 17β-estradiol; E1, estrone; LC/MS/MS, liquid chromatography–tandem mass spectrometry; AFO, animal feeding operation; MSP, manure storage pond; SPE, solid phase extraction; lnE2, natural-log transformation of E2 concentrations; $K_d$, linear sorption coefficient.

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doi:10.1016/j.jhazmat.2011.05.080

nantly a hydrophobic process. Reported log $K_{oc}$ ($\log_{10}$ transformed linear portioning coefficient ($K_d$) normalized to organic carbon) values for E2 and E1 are high, ranging between 2.99 and 3.46 [20,21]. Soil microbial activity is the predominant mechanism controlling E2 degradation [22,23]. First-order degradation rates of E2 are rapid in soil (0.0006 $h^{-1}$) [23] and in biosolids with higher microbial populations (0.252 $h^{-1}$) [24]. Manure application has also been associated with the enhanced degradation of E2 because of the microorganisms in the manure [25]. On average, field soil locations directly associated with swine manures had lower estrogenic detections, suggesting soil inoculated with fecal bacterial decreases estrogen concentrations [26].

Sufficient temporal and spatial samples that are purified for analytical analysis are necessary to determine whether manure from animal feeding operations contribute significant amounts of natural estrogens to the environment. It was hypothesized that if animal manures are a source of E2, and if swine manure slurry has the highest potential concentrations of E2, then water-extractable E2 concentrations in a field soil will respond to swine manure application. The objectives of this study were (i) to quantify dissipation of aqueous-extractable E2 in soil porewater after simulated manure application in laboratory soil columns, and (ii) to observe aqueous-extractable E2 in porewater stratified in a field soil before and after swine manure application. The majority of E2 in soil is bound; however, the E2 in the soil porewater would represent a small but proportional fraction of this bound concentration [26,27]. Furthermore, this porewater E2 is the most significant fraction from a toxicological perspective because it is the most mobile fraction (i.e., convectively transported with percolating water) and can therefore readily enter aquatic systems compared to bound E2 fractions.

## 2. Experimental

### 2.1. Laboratory experiment

A bench study was conducted to investigate factors that might control water-extractable E2 measured in a field soil that receives E2 from manure. 17β-Estradiol was purchased from Sigma (St. Louis, MO) and a 5000 ng $L^{-1}$ solution was prepared using 0.01 M $CaCl_2$ (1% EtOH) and stored at 2.5 °C. Wyndmere (coarse-loamy, mixed, superactive, frigid Aeric Calciaquoll) surface soil (0–0.15 m deep) was collected from a location near the field study area that had not received manure for at least three years. To homogenize the soil, it was air-dried, machine-ground, and sieved (2-mm maximum particle size). Grinding and sieving also exposes more sorption surfaces of the soil, and can be comparable to field soil disturbances that occur during plowing or injection of liquid manure. A mass of 105 g of soil was packed into each of twenty-seven (8 sample times × 3 replicates + 3 blank) acid-washed glass columns, measuring 3 cm in diameter and 11 cm in length. The soil was packed in 1-cm increments to obtain an approximate bulk density of 1.30 g $cm^{-3}$, which was similar to field measured values. A stainless-steel screen was fixed to the bottom of each column with Teflon tape so that the soil was held in place but any excess moisture (gravimetric water) could escape, even though the amount of solution added was determined so that none was expected to elute. Over a period of 2 min, 20 mL of the 5000 ng $L^{-1}$ E2 solution was dripped onto the surface of the soil using a burette, so that a total of 100 ng of E2 was applied to each column. Additionally, three blank columns were constructed in which no E2 was applied. Columns were covered with parafilm to prevent evaporation and set in front of a west-facing window, where they were exposed to filtered sunlight to simulate surface field soils. The surrounding room temperature was about 20 °C. Twenty-four soil columns were constructed so that at each designated time (1 h, 2 h, 4 h, 8 h, 16 h, 32 h, 48 h, 64 h) three replicate columns could be destroyed, and all the soil extracted using a 0.01 M $CaCl_2$ solution. All treatments were run in triplicate.

The 0.01 M $CaCl_2$ extraction method, SPE purification, and LC/MS/MS analysis are described by Schuh et al. [26]. A mass of 100 g of soil was placed in a 500-mL Erlenmeyer flask and 200 mL of 0.01 M $CaCl_2$ was added. The flasks were mechanically shaken for 20 min, and then set aside in a refrigerator (at 2.5 °C) for 60 min. The decant from the flask was then filtered through a pre-rinsed #2 Whatman (Maidstone, England) filter paper into 250-mL polyethylene bottles [26]. These soil extracts were then further purified using SPE, in which 100 mL of the filtrate was spiked with 1000 pg of ethanolic $d_4$-estradiol solution standard (CDN Isotopes, Pointe-Claire, Quebec, Canada). The spiked filtrate was then filtered again using a 0.45 μm disk filters (Whatman, Florham Park, NJ), and then passed through a Water HLB Oasis® SPE cartridge using a RapidTrace Workstation (Caliper Life Sciences, Hopkinton, MA). The SPE cartridge was pre-equilibrated with 3 mL of diethyl ether, 3 mL methanol, and 3 mL NanoPure (NP) water. The SPE cartridge was eluted with 1 mL of 40% methanol in NP water, 1 mL of NP water, and 1 mL of 10% methanol:2% $NH_4OH$ in NP water. The steroidal estrogens were then eluted from the SPE cartridge with 2 mL of methanol. The methanol filtrate from the final SPE elution was then blown dry on a centrifugal rotary evaporator (SpeedVac, Savant Instruments, Farmingdale, NY) and reconstituted in 100 μL of 1:1 NP water:acetonitrile. Liquid chromatography with tandem mass spectrometry (LC/MS/MS) analysis identical to the Thompson et al. [28] was then used to determine E2 and E1. The final estrogen concentrations were expressed in porewater equivalents (ng-E2 $L^{-1}$).

### 2.2. Field experiments

Field research took place in southeastern North Dakota on a field approximately 0.3 km from a swine farm where previous research had taken place [26,28,29]. At this facility, a series of enclosed barns housed approximately 2000 swine at various developmental stages in pens that ranged from nursery to finishing. The pens were constructed above slotted floors that allowed the waste material to pass through and into subfloor cisterns. Cisterns from all the pens were connected and drained monthly into an above ground manure storage pond (MSP). The approximate storage volume of the MSP was 3800 $m^3$. Once a year, liquid manure from the MSP was used to fertilizer nearby fields by injecting the manure into the upper 0.15 m of soil.

Soil cores were collected from the research field before and after MSP manure was injected. The soils in this field were predominantly Wyndmere with significant inclusions of Hecla, Garborg, and Ulen series. Taxonomic descriptions of these soils indicated that they are all coarse textured, mollisols, with periodic high water tables (i.e., there is evidence for water table within 0.16 m of the surface). Prior to the manure application for this study, no manure had been applied to this field for at least three years. Liquid manure was applied on 25 May 2006 and soil cores were collected on 9 May 2006, 5 June 2006, 14 June 2006, 13 November 2006, 24 May 2007, and 25 October 2007. Manure application rates by the producer were approximately 120 $m^3$ $ha^{-1}$, which supplied approximately 48 mm of water to the field. Manure samples were also collected (approximately 250 g) in polyethylene containers, and formalin was added to preserve the samples for E2 determination (final formaldehyde concentration in the sample was approximately 1%) [6]. The manure sample preparation, SPE purification, and LC/MS/MS analytical procedures of Thompson et al. [28] were used to determine the water extractable E2 concentrations. The SPE purification was described in Section 2.1.

Field sampling protocols, and sample processing, extraction, and purification closely followed the methods of Schuh et al. [26]. In

brief, soil cores were taken from field locations (i) where surface water would drain away from topographically high positions, and (ii) where surface water would collect in topographic depressions [30]. At each sample time, two soil cores from each topographic position were harvested, for a total of four cores. Using a steel, hollow-stemmed, hydraulic probe, soil cores (diameter = 56 mm) were removed in 1.5 m increments until the water table was reached. The depth to water table for this study ranged from 0.7 m to 2.0 m. Soil cores were contained in polyethylene sleeves that were capped, enclosed in pipe insulation, transported to the laboratory, and refrigerated (approx. 2.5 °C for 5–28 days) until extraction. Also, bore holes were backfilled with sand (≥0.15 m depth) and bentonite (0–0.15 m depth), and their locations were referenced using a global positioning system receiver for future sampling [26]. In the laboratory, cores were cut into 0.15-m increments, and extracted using the $CaCl_2$ solution as described in Section 2.1. Further purification by SPE (described in Section 2.1) and subsequent LC/MS/MS analysis were identical to Schuh et al. [26] and Thompson et al. [28]. Organic matter content [31], particle size distribution [32], bulk density, and soil moisture [33] were determined for each of the soil samples.

### 2.3. Statistical analysis

All statistical analyses were made with the software JMP (ver. 7.0.2, SAS Institute, Cary, NC) using $\alpha$ levels of 0.05, and accepting significance at probabilities ($p$) of ≤0.05. Before any statistical calculations were made, appropriate transformations (e.g., natural log) were used to obtain normal distributions for statistical variables (i.e., porewater E2 concentrations, bulk densities, and contents of organic matter, water, sand, silt, and clay). Also, all statistics were calculated using E2 concentrations expressed in porewater equivalents (ng-E2 $L^{-1}$). Analysis of covariance (ANCOVA) was employed to identify the effects of various soil physical properties on porewater concentrations of E2, while taking into consideration the covariate effects of date and depth. In the ANCOVA analysis, date and depth were assigned as discrete variables, and all other variables as continuous. If an effect was found to be significant in the ANCOVA analysis, then a student $t$-test or a Tukey–Kramer honestly significant difference (HSD) test for multiple comparisons was performed ($\alpha$ = 0.05) to determine differences in effect level. Also, Pearson's binary linear correlations were used to test for significant relationships between variables.

## 3. Results and discussion

### 3.1. Laboratory dissipation study

In the laboratory study, the average antecedent porewater E2 concentration was 18 ng $L^{-1}$. This average background level of E2 was considered the control measurement. For the treatments, the 100 ng of E2 applied in 20 mL solutions of 5000 ng E2 $L^{-1}$ dissipated to antecedent levels in the soil by 1 h and remained at this level for the duration of the experiment (Fig. 1). There were no significant differences in measured E2 concentrations between the controls and all the sampling times (Tukey's HSD at $\alpha$ = 0.05). Also, there was no observed increase of E1 with decreasing E2 concentrations, which would be a common metabolic fate for E2. These results were consistent with Fan et al. [23] who used similar soil and found that after 5 days of soil incubation, only about 2% of applied E2 was water-extractable. The lowest $K_d$ value reported for E2 is 4 L $kg^{-1}$ for streambed sediment [34]. Using this $K_d$ for the 5000 ng-E2 $L^{-1}$ applied, resulted in an estimated sorbed concentration of 20,000 ng-E2 $kg^{-1}$ soil. There were 105 g (0.105 kg) of soil in the column, which had the capacity to sorb 2100 ng of E2. Only 100 ng of total E2 was applied to the column, which meant that all of the applied E2 would be capable of being bound to the soil in the column.

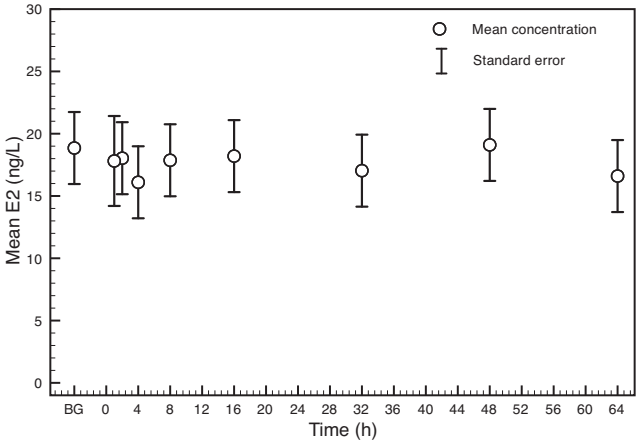


**Fig. 1.** Mean aqueous-extracted, porewater concentrations of 17%-estradiol (E2) for background (BG) and treated samples through time in laboratory dissipation study.

The bench study results indicated that the E2 concentrations measured in the soil pore water should be rapidly sorbed, indicating that much larger concentrations of E2 must be bound in the soil and not measurable in a $CaCl_2$ extraction. This suggested that perhaps a reservoir of soil-bound E2 can exist in the soil, which can potentially be released to the environment through desorption processes. Since shaking 105 g soil in 200 mL of water was the standard extraction method for all samples, desorption into water alone, without further release mechanisms, was not adequate to explain the differences in detected concentrations.

### 3.2. Effects of field manure application

The water-extractable E2 concentrations measured in the manure slurry ranged from 509 to 3767 ng $L^{-1}$. These concentrations corresponded closely to total free estrogens for swine from a survey of animal operations with manure lagoons [35]. Based on the manure application rate of 120 $m^3$ $ha^{-1}$, the amount of E2 applied per hectare was between 62.1 and 459.6 mg $ha^{-1}$.

Before statistically analyzing the porewater E2 concentrations, values were transformed using a natural-log transformation (lnE2) to obtain a normal distribution. None of the measured soil proper-

**Table 1**
Summary statistics of aqueous-extracted, porewater concentrations of 17β-estradiol (E2) measured through time in a field that received manure fertilizer on 25 May 2006.

| Date | Mean E2 (ng $L^{-1}$) | Maximum E2 (ng $L^{-1}$) | Geometric mean E2 (ng $L^{-1}$) | Number of detections | Percent detections (%) |
|---|---|---|---|---|---|
| 9-May-06 | 0.9 | 11.32 | 3.31 | 4 | 18 |
| 5-Jun-06 | 1.01 | 7.61 | 2.47 | 5 | 25 |
| 14-Jun-06 | 1.4 | 9.88 | 4.4 | 7 | 29 |
| 13-Nov-06 | 18.73 | 172.7 | 45.25 | 9 | 24 |
| 24-May-07 | 202.55 | 1298.37 | 248.07 | 13 | 50 |
| 25-Oct-07 | 16.35 | 229.63 | 13.9 | 25 | 66 |
| All dates | 40.15 | 288.25 | 13.34 | 63 | 38 |

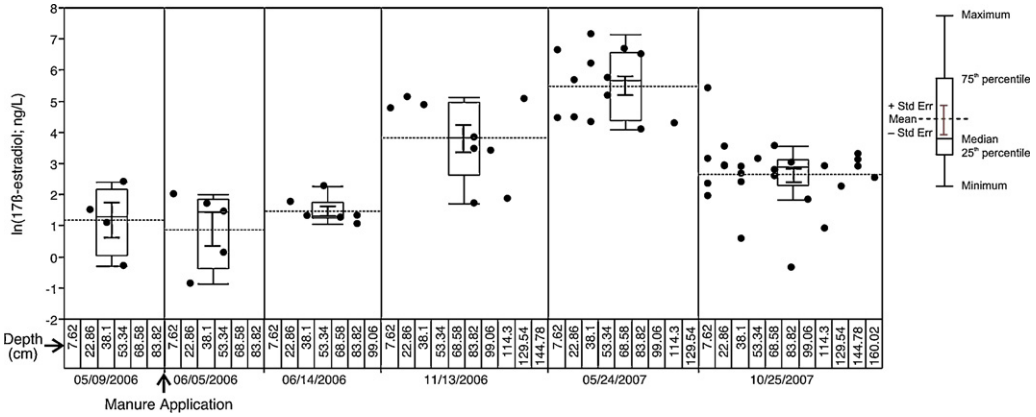


**Fig. 2.** Natural-log transformed values of aqueous-extracted, porewater concentrations of 17%-estradiol (●) measured through depth and time on a field receiving liquid manure.

ties (i.e., contents of water, organic matter, sand content, silt, and clay) were significant in explaining lnE2, which may have resulted from the narrow variations for these values. The coefficient of variations for bulk density, water content, sand, silt and clay ranged from 10% to 25% on this field.

The ANCOVA analysis indicated that "sample date" was significant ($p < 0.0001$) in explaining lnE2 values; however, "soil depth" was not. There were no statistical differences (Tukey's HSD; $p \leq 0.05$) in lnE2 values for sample dates before manure application and the two sample dates immediately after application (Fig. 2). The lnE2 concentrations increased significantly in fall 2006 (13 Nov) and spring 2007 (24 May), but returned to initial concentrations by fall 2007 (25 Oct) (Fig. 2). Furthermore, the E2 detection frequency before manure application (25 May) was similar to detection frequencies in the following three sample periods; however, detection frequencies nearly doubled in the following year (24 May and 25 October 2007) when no manure was applied (Table 1). These temporal observations suggest that stratified lnE2 concentrations and detections were not immediately associated with the application of manure, which is consistent with laboratory studies that indicate estrogenic hormones are not expected to persist in soil beyond 1–2 days because of rapid degradation and sorption [22,27,36]. However, the observations of higher E2 detection frequencies and significantly higher E2 concentrations more than six months after manure application were unexpected (Table 1 and Fig. 2). Extended persistence and mobility of E2 has been observed in other field studies, where estrogens have been detected in subsurface tile drainage [37], and in surface streams [38] months after manure application. In other field studies it was suggested that the leaching of E1 and E2 was in response to precipitation events [37,38]. Estrogenic activities have also been found to respond to hydrologic events in rivers [39] and in soil leachate [28]. In this study, a significant correlation ($p = 0.03$) between mean lnE2 concentration of the sample date and the mean rainfall between sample events was observed (Fig. 3). The correlation suggested that E2 concentrations responded more immediately to precipitation and/or hydrologic events compared to the manure application. Also in this study, only the water extractable E2 was measured, not accounting for the sorbed E2, which would be more than ten times greater than the aqueous E2 fractions [26]. Perhaps the detection of E2 and response to precipitation (Fig. 3) were related to the release of E2 bound to the soil.

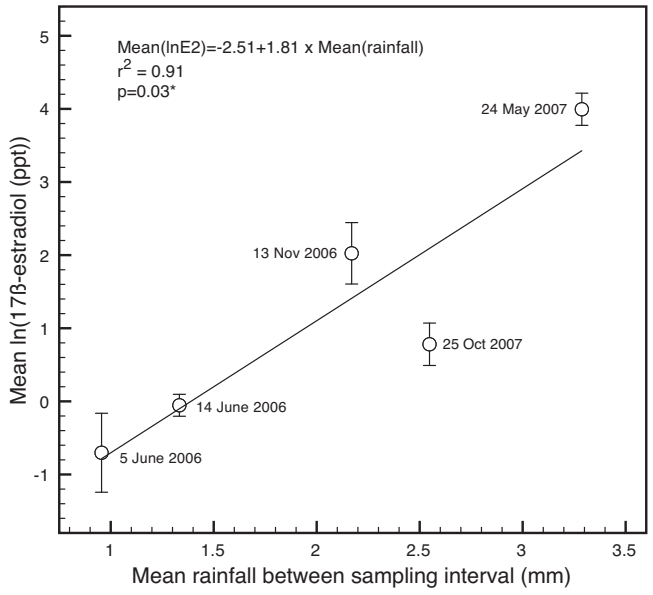


**Fig. 3.** Correlation between natural-log transformed values of aqueous-extracted, porewater concentrations of 17%-estradiol and mean rainfalls between field sample events.

## 4. Conclusion

The porewater equivalent E2 concentrations appear to follow a temporal trend, but this trend did not immediately respond to manure application. Rather porewater equivalent E2 concentrations increased 6 months after manure application, but returned to antecedent levels by 16 months after manure application. Laboratory study results were consistent with field results in that nearly all applied E2 was non-extractable by water within 1 h of application. Whether the E2 remains intact while bound to the soil is unknown. However, if the E2 does remain intact, it may act as a source that is periodically released to the environment as a result of other factors, such as precipitation.

## Acknowledgments

This manuscript is dedicated to the first author, Mary Cecilia Schuh, who tragically passed away in an accident in August 2010. This research was based upon work supported by the National Science Foundation under Grant No. 0730492. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement

or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable. The authors greatly appreciate the tremendous contributions made by, Mr. Nathan E. Derby, Mrs. Barbara K. Magelky, and Mrs. Colleen M. Pfaff.

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