Mineralization and Biotransformation of Estrone in Simulated Poultry Litter and Cow Manure Runoff Water

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Abstract

Application of animal manure on agricultural lands is one of the main sources of estrogen contamination in the environment. Poultry and cow manure contain free and conjugated forms of the natural estrogens (e.g., estrone [E1] and estradiol [E2]) that can enter surface waters during runoff events. Estrone has been identified as the major form of estrogen in the environment; therefore, this study is focused on the evaluation of the degree of mineralization and fate of E1 in a simulated poultry litter and cow manure runoff water. A time-course study was conducted using simulated runoff water that consisted of 0.5 mg cow manure or poultry litter dissolved in 1 L of water spiked with radiolabeled E1 (14C-E1). Samples were analyzed for estrogen concentrations at Day 0, 0.5, 1, 2, 3, 5, and 7 using liquid chromatography with tandem mass spectrometry. In the poultry litter simulated runoff water, E1 was biotransformed to 17β-estrone-3-sulfate (E1-3S) but was eventually mineralized to 14CO2; a total E1 mineralization of 92.2% occurred after 7 d of aerobic incubation. In contrast, the concentrations of E1 and other forms of endogenous estrogens detected in the cow manure simulated runoff water, such as E1-3S, 17α-estradiol (α-E2), and 17β-estradiol (β-E2), remained relatively constant and persisted over the 7 d of aerobic incubation. Results of this study demonstrate the differences in the fate of estrone in the simulated poultry litter and cow manure runoff water, highlighting the ability of the endogenous microbial community from poultry litter to mineralize estrogens to CO2.

Core Ideas

• A 7-d study showed mineralization and biotransformation of E1 in poultry litter runoff water.
• E1 biotransformed to E1-3S in poultry litter runoff water eventually, and mineralized to CO2.
• Total mineralization of E1 was observed at an average of 92.2% in poultry litter runoff water.
• Concentrations of E1 and other estrogens in the cow manure runoff did not change within the 7-day study.

Abbreviations: 14C-E1, radiolabeled estrone (4-14C); α-E2, 17α-estradiol; β-E2, 17β-estradiol; E1, estrone; E1-3S, 17β-estrone-3-sulfate; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LOQ, limit of quantification.

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A GRONOMIC application of animal manure into agricultural land is a common manure management practice; unfortunately, it has also been identified as one of the main sources of estrogens in the environment (Adeel et al., 2017; Hanselman et al., 2003). It is estimated that in the United States and the European Union alone, the annual estrogen excretion by farm animals is more than double that of the global human population, or about 83,000 kg yr−1 (Lange et al., 2002). This is of great concern as the number of concentrated animal feeding operations continues to increase, particularly in the United States. Estrogens in land-applied manure are stable and can eventually reach aquatic environments through surface runoff. For instance, Nichols et al. (1997) found that 17β-estradiol (β-E2) persisted for 7 d under field conditions and that simulated storm events occurring immediately after field application of poultry litter produced significantly higher β-E2 in poultry litter amended runoff compared with the control.

Discharges of estrogens into receiving waters and the occurrence of endocrine disruption in fishes, reptiles, amphibians, and other animals that have been exposed to agricultural runoffs have been studied. Even the low concentrations of estrogens commonly found in the environment (at ng L−1 levels) result in endocrine disruption of exposed fish and wildlife. For instance, a study by Irwin et al. (2001) showed higher vitellogenin levels in female painted turtles after 28 d of exposure in farm ponds affected by beef cattle runoff (with β-E2 ranging from 0.05 to 1.8 ng L−1) compared with controls. Similarly, an exposure to as low as 1 ng L−1 of β-E2 in wastewater was found sufficient to induce vitellogenin production in male trout (Hansen et al., 1998).

Poultry and cow manure contain free and conjugated forms of the natural estrogens (Andaluri et al., 2012; Gadd et al., 2010; Zheng et al., 2012); the free estrogens such as β-E2, 17α-estradiol (α-E2), estrone (E1), and estriol (E3) are classified as potent endocrine disrupting chemicals, while the conjugated forms are generally biologically inactive. Several in vitro assays such as the growth induction test in a human breast cancer cell line and binding assays with the human estrogen receptors α and β showed β-E2 to be most potent, followed by E3 and E1 (Gutendorf and Westendorf, 2001).
Due to the strong estrogenic potency of β-E2 in vitro assays, β-E2 has been the primary focus of many research and monitoring programs, while little attention has been given to E1 despite its occurrence at higher concentrations than β-E2 in the environment. Estrone is the predominant form of estrogen found in many environmental samples, including dairy manure and poultry litter (Adeel et al., 2017; Jenkins et al., 2009; Lange et al., 2002). Several studies have also shown that E1 is the main degradation product of E2 in different environmental matrices such as soil, sediment, and animal wastes (Lee and Liu, 2001; Mashmash et al., 2013; Prater, 2012; Ternes et al., 1999; Xuan et al., 2008; Zheng et al., 2008). Although E1 is less potent than β-E2, environmentally relevant levels of E1 cause feminization of male fish due to the ability of fish to convert E1 to β-E2. Recently, Ankley et al. (2017) reported that male fathead minnows exposed to 15.6 ng L\(^{-1}\) E1 (or higher) showed significant increases in the amount of β-E2 in the plasma when compared to the untreated controls. The conversion of E1 to β-E2 was attributed to the presence of 17β-hydroxysteroid dehydrogenase that stereospecifically reduces or oxidizes a keto- or hydroxy group at C17 of the steroid scaffold (Mindnich and Adamski, 2009). In addition, a dose-dependent increase in hepatic expression of vitellogenin and a significant induction in plasma vitellogenin protein were observed in male fathead minnows exposed in E1-treated water starting at the treatment level of 41.9 ng L\(^{-1}\) after 4 d of exposure (Ankley et al., 2017).

Because animal manure is a significant source of estrogen contamination in the environment, many researchers have investigated the efficiency of manure composting and anaerobic digestion in reducing and/or controlling the release of estrogens into aquatic systems (Dutta et al., 2010; Hakk et al., 2005; Jenkins et al., 2009). In a study that investigated the efficiency of a full-scale anaerobic codigestion system to reduce hormone concentrations in dairy manure, researchers found that the total concentrations of hormones (E1, α-E2, β-E2, and sulfated estrogens) did not decrease significantly after digestion but the relative composition changed primarily to E1 (Noguera-Oviedo and Aga, 2016).

A study by Hakk et al. (2005) on the fate of hormones during composting of poultry litter reported that water-extractable β-E2, which was measured by an enzyme immunoassay, was reduced by 84% after 139 d of composting. This study was limited to the fate of β-E2 and did not investigate the concentration of E1 or the changes in the overall estrogenic potential of the poultry litter during composting. A separate study demonstrated that aerated composting of poultry litter can result in more than 75% reduction in the estrogenicity of poultry litter when used in simulated runoff water, as measured using a yeast estrogen assay (Hammett et al., 2017). The concentrations of E2 in this study were below the detection limit (2.2 ng L\(^{-1}\)), and therefore it was unclear if the reduction in estrogenicity mirrored reduction in E2. Notably, a substantial increase in E1 on Day 10 was observed and resembled the increase in estrogenicity in the simulated runoff water; the increase in E1 has been attributed to microbial deconjugation of E1-conjugates (Hanselman et al., 2003; Yu et al., 2013). Nevertheless, the increase in estrogenicity cannot be completely attributed to the increase in E1 because of its low estrogenic potential compared with β-E2.

It is clear from earlier studies that E1 is an important form of estrogen in the environment. In fact, a soil microcosm study by Colucci et al. (2001) reported that while β-E2 can be oxidized to E1, both biotically and abiotically, the transformation of E1 can only occur when microbially mediated, resulting in the net accumulation of E1 in soil. This same study also showed that both β-E2 and E1 formed nonextractable, soil-bound residues that were only slowly mineralized. The transformation and mineralization of E1 in the aqueous phase of agricultural runoff, which is what is bioavailable, has not been investigated. In this study, the fate of E1 in simulated runoff water treated with either poultry litter or cow manure was investigated with the following aims: (i) to determine the mineralization and transformation of E1 in runoff water contaminated by poultry litter using \(^{14}C\)-labeled E1, and (ii) to compare the fate of E1 in runoff water contaminated by either poultry litter or cow manure under aerobic conditions.

**Materials and Methods**

**Sample Collection and Matrix Preparation**

Freshly collected poultry litter and cow manure from an organic farm in western New York were used to prepare the simulated runoff water by adding 1.0 g of either poultry litter or cow manure to 2.0 L of distilled water and mixing intermittently for approximately 16 h. The resulting mixture mimics the relative abundances of water-extractable estrogens in runoff during an initial rain event after manure surface application, as described in our earlier study (Hammett et al., 2017). Samples were stored at 4°C prior to the actual aerobic degradation experiments.

**Aerobic Degradation Study**

A 100-mL simulated runoff water sample was transferred into a 125-mL glass amber container and spiked with 40 µL of 50 ng L\(^{-1}\) E1 standard (≥99% purity, Sigma Aldrich), resulting in a final concentration of 20 µg L\(^{-1}\). The spiked sample was covered with a rubber stopper containing holes that allowed connection to dehumidified air for continuous aeration (in the dark) for 7 d (Supplemental Fig. S1). Sampling was done at 0, 5, 1, 2, 3, 5, and 7 d. The sample collected on Day 0 was not aerated and was processed and analyzed as soon as it was spiked with E1, which served as the initial concentration. There were three replicates for each sampling time, wherein three separate containers were spiked for each test condition. Separate setups were performed for poultry litter, cow manure, autoclaved (4 h at 120°C) poultry litter, and autoclaved cow manure simulated runoff water experiments.

Temperature and pH of the test mixtures were measured directly after sampling. The pH of the sample was then adjusted to 2.0 ± 0.2 to stop further microbial activity. Each sample (100 mL) was filtered using a 0.22-µm Millipore nitrocellulose membrane (Millipore Sigma). Any amount of E1 that could potentially sorb in the suspended solids and adhere to the walls of the container were extracted with 100 mL of 95:5 (v/v) water/methanol mixture. Estrogens in both the aqueous portion and suspended solids were preconcentrated and cleaned up using solid phase extraction and quantified using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) procedure previously developed in our laboratory (Tso et al., 2011). A 30-µL aliquot of 1.5 µg L\(^{-1}\) isotopically labeled estrone, E1-d4 (C/D/N Isotopes Inc.) was used as internal standard and added.
to all samples and standard solution. Quantification of estrogens in each sample was done using an internal standard method wherein the response factor was calculated from the concentration and peak areas of analyte and internal standard in the standard solution (Eq. [1]), and the concentration of analyte in the sample was then calculated using Eq. [2]:

\[
F = \frac{C_{IS}}{C_A} \times \frac{A_A}{A_{IS}}
\]  

[1]

\[
C_A = \frac{C_{IS}}{F} \times \frac{A_A}{A_{IS}}
\]  

[2]

where \(F\) is the response factor; \(C_{IS}\) is the concentration of internal standard, \(C_A\) is the concentration of the analyte, \(A_{IS}\) is the peak area of the internal standard, and \(A_A\) is the peak area of analyte.

Primary standards of \(\alpha\)-E2 and \(\beta\)-E2 were obtained from Sigma Aldrich, and \(17\beta\)-estradiol (E3) was obtained from ICN Biomedicals Inc. Estrogen conjugates were obtained from Steroloids Inc.: \(17\beta\)-estrone-3-sulfate (E1-3S), \(17\beta\)-estradiol-3-sulfate (E2-3S), \(17\beta\)-estradiol-17-sulfate (E2-17S), \(17\beta\)-estradiol-17-glucuronide (E2-17G), \(17\beta\)-estrone-3-glucuronide (E1-3G), \(17\beta\)-estradiol-3-sulfate \((17\beta\)-E2-3S), and \(17\beta\)-estradiol-3-glucuronide (E2-3G).

Mineralization Study

An additional aerobic exposure study was performed using poultry litter runoff water spiked with \(^{14}\)C-labeled E1 (American Radiolabeled Chemicals, total activity of \(5\,\mu\text{Ci}\), specific activity of \(55\,\mu\text{Ci}\,\text{mmol}^{-1}\), 0.1 mCi mL\(^{-1}\)) to quantify mineralization of E1. For this setup, each sample was connected to containers with 100 mL of 1.0 M KOH serving as \(^{14}\)CO\(_2\) traps (See Supplemental Fig. 1). Duplicate samples were collected at Day 0, 1, 3, 5, and 7 after aerobic incubation. Each sample collected was filtered, and the radioactivity in the aqueous portion, suspended solids, and in the \(^{14}\)CO\(_2\) traps were measured using a liquid scintillation counter (PerkinElmer Liquid Scintillation Analyzer, Tri-carb 2910TR). PerkinElmer Hionic-fluor was used as the liquid scintillation cocktail. Prior to aerobic exposure of the simulated poultry litter runoff water, a recovery test was performed using \(^{14}\)C-labeled NaHCO\(_3\) (American Radiolabeled Chemicals, total activity of 250 \(\mu\text{Ci}\), specific activity of \(53\,\mu\text{Ci}\,\text{mmol}^{-1}\), 5.0 mCi mL\(^{-1}\)) to ensure that at least 95% of the spiked \(^{14}\)C-radioactivity was recovered. The linearity of the liquid scintillation counter was also established using \(^{14}\)C-labeled NaHCO\(_3\).

Statistical Analysis

Differences in the concentrations of estrogens between sampling times was determined by one-way ANOVA using Bonferroni t test for pairwise comparison, and comparisons versus a control group (Day 0) was determined using SigmaPlot version 11.0 (SigmaPlot, 2008) with statistical significance set at \(p < 0.05\).

Results and Discussion

Dissipation of E1 in Poultry Litter–Treated Runoff Water

The fate of E1 in the simulated poultry litter runoff water under aerobic conditions was investigated over a 7-d exposure period. The samples were analyzed using a LC–MS/MS method validated for the simultaneous analysis of free and conjugated natural estrogens to include possible transformation products of E1 after exposure. As shown in Fig. 1, E1 partitioned between the aqueous fraction (~70%) and into the suspended solids (~30%) during the initial sampling (Day 0). All results were subtracted with the average concentration of endogenous E1 found in the poultry litter (result for matrix blank; E1 = 24.5 ± 6.5 ng L\(^{-1}\)) before spiking. A significant decrease in the total percentage E1 was observed during Day 1, with less than 10% remaining in each of the aqueous and suspended solid fractions. Starting at Day 2, the amount of E1 remaining in the sample dropped to below 0.5% of the original amount in both fractions.

Notably, E1-3S was detected (initial concentration observed 35.9 ng L\(^{-1}\)) in the samples only after spiking with E1, and only in the nonsterile incubations, indicating that E1-3S is a biotransformation product of E1 in the presence of endogenous microorganisms from the poultry litter (see Table 1). Continuous aeration of the sample resulted in an increase in E1-3S concentrations that peaked at 152.4 ng L\(^{-1}\) and subsequently decreased to concentrations below the limit of quantification on Day 7. Detection of E1-3S in the aqueous fraction, but not in the suspended solids,

![Fig. 1. Recovered amount of spiked estrone (E1) (20 μg L\(^{-1}\)) in the simulated poultry litter runoff water during a 7-d aerobic biodegradation study (error bars represent standard deviation, n = 3 for each sampling time).](image)

Table 1. Amount of 17β-estrone-3-sulfate (E1-3S) detected in simulated poultry litter runoff water during a 7-d aerobic biodegradation study (limit of quantification [LOQ] = 5.0 ng L\(^{-1}\); n = 3 for each sampling time).

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Aqueous fraction</th>
<th>Suspended solids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix blank</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Day 0</td>
<td>33.4 ± 14.0</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Day 0.5</td>
<td>152.4 ± 15.1</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Day 1</td>
<td>145.7 ± 10.6</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Day 2</td>
<td>100.6 ± 14.4</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Day 3</td>
<td>68.7 ± 6.5</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Day 5</td>
<td>22.8 ± 2.5</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Day 7</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
</tbody>
</table>
can be attributed to the lower octanol-water partition coefficient ($K_{ow}$) of E1-3S compared with E1 ($\log K_{ow}$: E1 = 3.43 (Chen et al., 2012); E1-3S = 0.95 (calculated by the Windows-based software KOWWIN) (Terko et al., 2005). Biotransformation of E1 to E1-3S is desirable since the estrogenic potency of E1-3S is several orders of magnitude lower than E1 ($E_1 = 0.024$, $E_{1-3S} = 0.000012$; values are expressed as relative potency to $\beta$-E2) (Gadd et al., 2010), and thus E1-3S is considered biologically inactive. The biotransformation of E1 to E1-3S in soil has been described in the literature (Goeppert et al., 2014, 2017) and is believed to be catalyzed by the enzyme arylsulfotransferase (Goeppert et al., 2015). Sulfotransferases are widespread in bacteria and catalyze the transfer reaction of a sulfate group to an acceptor group of numerous substrates such as steroids (Negishi et al., 2001). There was a minimal biotransformation of E1 to E1-3S observed in this study that peaked to about 1.1% at Day 0.5 in the aqueous fraction. This low transformation potential of E1 to E1-3S was also observed in previous studies (Goeppert et al., 2015, 2017).

### Mineralization of $^{14}$C-E1 in Poultry Litter–Treated Runoff Water

A similar study using $^{14}$C-labeled E1 was performed to determine if mineralization of E1 occurs during aerobic biodegradation and if it could account for the dissipation of E1 and E1-3S in the aerobic biodegradation experiments. The results of the mass balance experiment are in agreement with the LC–MS/MS results. As shown in Fig. 2, the same initial partitioning of $^{14}$C-E1 in the aqueous fraction (~70%) and suspended solids (~30%) was observed in Day 0. An increase in radioactivity was observed in the $^{14}$CO$_2$ trap over the course of exposure, indicating continued mineralization with exposure time. The samples collected at Day 5 and Day 7 showed an average radioactivity measured in the $^{14}$CO$_2$ traps of 83.5 and 92.2%, respectively, which suggest that E1 is completely biodegradable in the poultry litter and can potentially be eliminated in the environment. The total recovery of the spiked radioactivity for all sampling points was within 92.2 to 106.4% (Supplemental Table S1).

These results are in agreement with the findings of Hemmings and Hartel (2006), who reported that estrogen mineralization in breeder and boiler litter is favored with increasing water content and at optimum temperature of 25°C. In the present work, the aerobic incubation experiments were conducted at room temperature between 22 and 24°C, which is close to the optimum temperature. In addition, aeration and pH could also help to achieve high mineralization of estrogens. Previous studies demonstrated that aerated composting resulted in >75% reduction in the estrogenicity of the aqueous mixtures treated with poultry litter (Hammeter et al., 2017) and that E2 degradation by bacterial co-culture is favored at pH 6 to 9 (Li et al., 2018). The pH of the samples during the aerobic exposure remained between 6.4 and 7.6, which is within the optimal pH for estrogen biodegradation by bacterial co-culture. These experimental results highlight the usefulness of the endogenous microbial community from poultry litter to mineralize estrogen. The conditions used in our laboratory-scale study can be adapted to optimize conditions for aerated composting of animal wastes at the field scale to maximize mineralization of estrogens into CO$_2$ prior to land application.

### Cow Manure Exposure

To determine if the endogenous microbial population in cow manure will biodegrade E1 to the same extent observed when using the endogenous microbial population in poultry litter, a similar biodegradation study was conducted using simulated cow manure runoff water. Notably, the results using cow manure were quite different from that obtained when using poultry litter. Figure 3 shows the amount of E1 in each sampling time in both aqueous fraction and suspended solids. All results are normalized to the initial amount of E1 spiked into the sample (Day 0) and are subtracted with the average concentration of endogenous E1 found in the manure sample (result for matrix blank; E1 = 15.0...
± 2.0 ng L\(^{-1}\)) before spiking. Unlike in poultry litter, the amount of E1 that partitioned into the suspended solids was lower in the cow manure, with ~95% remaining in the aqueous phase and only ~5% adsorbed to the solids. This is not surprising as cow manure generally has lower dry matter content (hence lower percentage C) than poultry litter. While the characteristic of manures from the same type of animals vary considerably depending on feeds and storage practices, poultry litter contains approximately 55% dry matter content while beef cow manure contains only about 23% (Magdoff and van Es, 2009). The higher amount of organic matter in poultry litter will result in higher sorption of the estrogens. It is also noticeable that the percentage of E1 in the aqueous phase of the cow manure simulated runoff experiment remained within about 70% even after 7 d of aerobic exposure. Similarly, the amount of sorbed E1 remained at about 30% up to Day 5 and decreased slightly to about 25% at Day 7. These results suggest that biodegradation of E1 in cow manure did not readily occur, unlike in poultry litter.

Based on the data shown in Fig. 3, where no biodegradation of E1 occurs, it appears that partitioning of E1 between the aqueous phase and the suspended solids reached equilibrium on Day 2. The slower equilibration in partitioning observed in cow manure compared with poultry litter can be attributed to the relatively lower percentage dry matter content in cow manure. On the other hand, the decreasing E1 concentration in the suspended solids in poultry litter, observed starting at Day 0.5 (Fig. 1), is a result of biodegradation that played a more important role than sorption in changing E1 concentrations. Furthermore, due to the higher organic matter content in poultry litter, it can be expected that sorption of E1 to the suspended solids can occur more rapidly than in cow manure.

In the analysis of matrix blank (unspiked cow manure runoff water), occurrence of E1-3S, α-E2, and β-E2 were detected, with α-E2 found at the highest concentration (Fig. 4). This is in agreement with previous studies that reported the levels and occurrence of free and conjugated estrogens in dairy manure (Noguera-Oviedo and Aga, 2016; Gadd et al., 2010). Figure 4 summarizes the amount of E1-3S, α-E2, and β-E2 detected at each aerobic incubation time, which were detected in the aqueous fraction. No significant differences were noted on the partitioning observed in cow manure compared with poultry litter. Similarly, the amount of sorbed E1 remained at about 30% up to Day 5 and decreased slightly to about 25% at Day 7. These results suggest that biodegradation of E1 in cow manure did not readily occur, unlike in poultry litter.

The detection and persistence of these estrogens, specifically E1 and β-E2, during the 7-d aerobic incubation indicate an ecological risk considering that exposure to 15.6 ng L\(^{-1}\) E1 for 4 d (Ankley et al., 2017) and 1 ng L\(^{-1}\) of β-E2 for 5 d (Hansen et al., 1998) are sufficient to induce endocrine disruption in fish.

**Conclusion**

This study describes the transformation of E1 to E1-3S, followed by mineralization to CO\(_2\), in farm waste simulated runoff water. The high extent of mineralization (>90%) observed in the simulated poultry litter runoff water aerobic incubation together with findings from previous research (Hammett et al., 2017; Hemmings and Hartel, 2006) provides useful information for optimizing conditions during aerated composting of animal wastes, other than cattle, to eliminate estrogens before land application. In contrast, no significant degradation of E1, E1-3S, α-E2 and β-E2 in the simulated cow manure runoff water aerobic incubation was observed, suggesting persistence of estrogens from this animal waste stream.

**Supplemental Material**

The information presented in this material describes the set-up for the aerobic exposure experiments and the recovery data for each sample in the mineralization study using radiolabeled estrone.

**Conflict of Interest**

The authors declare no conflict of interest.

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