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Fate of Conjugated and Free Estrogens in Swine Manure Collected from areas Housing Piglets, Pregnant Sows and Finisher Pigs

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Abstract

Occurrence and fate of estrogens and their metabolites were studied in pig manure collected during winter and summer seasons from sites housing piglets (Nursery (Nur)-manure), pregnant and nursing sows (PS manure) and finisher pigs (FPig manure). The liquid and solid fractions of manure were analyzed for (1) total solids (TS), volatile solids (VS) and total organic carbon (TOC), (2) *E coli* and *M fujisawaense* enumeration, (3) total and individual estrogens and (4) estrogenic activity. This study showed that VS and OC values, *E coli* and *M fujisawaense* enumerations, estrogen concentrations and estrogenic activity exhibited the following pattern: Nur-manure << FPig-manure < PS-manure. The values for summer and winter Nur-manure did not differ significantly, while the values in summer PS-manure or FPig manure samples were significantly higher than values in corresponding winter samples. Although, estrogens distributed between liquid and solid fractions of manure, concentrations of free, but not conjugated, estrogens depended on manures' TOC values: an increase in TOC associated with an increase in free estrogen concentrations in liquid manure. However, an increase in TOC decreased the bacterial population in manure liquid by increasing their translocation from liquid into the in solids and ensuing bacterial stabilization. This may increase estrogens' deconjugation and/or degradation. Estrone (E1) sulfate (sE1), free E1 (fE1), E1 glucuronide (gE1) and E1 metabolites were major steroids present in Nur- and PS-manure, while fE2 and gE2 were predominant estrogens in FPig-manure. In total, the winter estrogen load in liquid was 5.1 mg/L and the load in solid was 4.93 mg/kg. Assuming that 2.3 x 10^8 kg of manure is produced in the USA per day, approximate hormone load will be 2.3 tons/day. However, manure contains microorganisms that hydrolyze estrogens, thus actual hormone load will be considerably lower.

Keywords: Manure, free estrogens, conjugated estrogens, estrogenic activity, organic carbon, E *coli*, *M fujisawaense*.

1. Introduction

Land application of animal manure, especially pig manure, is an efficient alternative to synthetic fertilizers because of its lower costs and the nutrient benefits derived by crops. Manure can also improve soil tilth, increase water-holding capacity, and promote beneficial organisms. However, manure has a key disadvantage, it contains large quantities of natural estrogens excreted by the animals housed in mega animal farms called concentrated animal feeding operations (CAFO). Therefore, estrogens present in animal-manure, either stored at the site of generation or applied to the agricultural fields (Jobling et al. 1996), may be the prime source of estrogens in freshwater sources (Combalbert et al., 2010; Combalbert and Hernandez-Raquet, 2012). Environmental estrogens and other estrogenic compounds are known as endocrine disruptors because they impair the endocrine system that has been linked to a variety of adverse effects in humans, aquatic animals and wildlife at ng/L levels (Hanselman et al., 2003; Jobling and Tylor, 2003; Snyder et al 2003; Harrision et. al., 1997; Colborn et. al., 1993; Jobling et al.,1996). In humans, endocrine disruptors have been shown to increase incidences of testicular, prostate, female breast cancer, polycystic ovaries in women and altered physical and mental development in children (Jobling and Tylor, 2003; Harrision et. al., 1997). In wildlife species, endocrine disruption include masculinization of females, feminization of males, altered sex ratios, intersexuality, and reduced fertility and fecundity (Vajda et. al., 2008; Coe et. al., 2008; Jobling et. al., 2003). Earlier studies have reported presence of estrogens and other estrogenic chemicals in rivers and lakes that may be casually associated with an increase in intersex population in wild freshwater fish and other aquatic organisms (Shore et. al., 1993; Massart et al., 2006; Jobling et. al. 2006). Chronic exposure to estrogens via drinking water may also cause or contribute to adverse human health effects (Diamanti-Kandarakis et. al., 2009). However, the potential environmental and health impact of steroid hormones originating from swine manure operations remains to be elucidated since information regarding estrogen levels, especially conjugated estrogens, in various types of animal housing areas is lacking.

It is well established that estrogens in manure are present in two forms: the free estrogens that are lipophilic and biologically active, and the conjugated estrogens (glucuronidase or sulfate conjugated) that are hydrophilic and biologically inactive. Because of conjugated estrogens' preserved lack of biological activity, free estrogens have been the focus of many earlier studies (Raman et al., 2004; Combalbert and Hernandez-Raquet, 2010). In addition, manure also contains diverse bacterial populations such as E coli, M fujisawaense, Rhodococcus zopfii, Rhodococcus equi, Novosphingobium sp., Achromobacter xylosoxidans, Ralstonia sp., etc. (Blunt et. al., 2009; Zhang et al 2008, Yu et al 2007, Weber et al 2005, Yoshimoto et al 2004, Fujii et al 2002, Yu et. al., 2007; Yoshimoto et. al., 2004; Lee and Liu, 2002). Of those listed above, E coli has been shown to deconjugate and activate conjugated estrogens, while M fujisawaense is associated with hydrolysis and deactivation of free estrogens (Legler et al 2002, Duong et al 2011, Iasur-Kruh et al 2011, Blunt et al 2009, D'Ascenzo et al 2003). Thus, E coli may increase, while M fujisawaense may decrease manure's estrogenic activity. Only a

few recent studies have looked at the fate of conjugated hormones in manure, but their results are not comparable due to differences in manure properties and methodology used.

(i) Combalbert et al. (2012) quantified conjugated hormones by analyzing free hormones after enzyme hydrolysis, while Kumar et al (2009) and Hutchins et al. (2007) have employed direct analysis using HPLC-MS/MS. Recently, we have developed and validated direct analysis of conjugated estrogens using HPLC-MS/MS and an internal standard for each hormone and metabolite to reduce matrix-related recovery variations (Singh et al 2013 and Supporting Information).

(ii) Manure is a complex suspension (Figure 1a) of liquid (consisting of water, urine and dissolved organic carbon – DOC, suspended particles containing organic carbons, and solid (soil, plant parts such as leaves, stem and flowers, animal excreta, littered food and other organic carbons). Because of this diversity, manure solids and liquid both contain hydrophobic, anionic and cationic sites. In general, fEs bind to the hydrophobic sites via hydrophobic interaction or via forming hydrogen bonds, while conjugated estrogens bind to cationic sited on soil or adsorbed proteins. Figure 1b shows possible fate of estrogens in the two manure fractions. The solid fraction may range from <10% to 80% w/v in manure samples. This may add to considerable diversity.

Therefore, the aims of the present investigation were (i) to study characterize and fate of conjugated and free estrogens in manure samples collected from facilities holding piglets (nursery (Nur)-manure), pregnant sows and litters (PS-manure) and finisher pigs (FPig-manure) during summer and winter seasons and (ii) to assess possible environmental consequences manure estrogens.

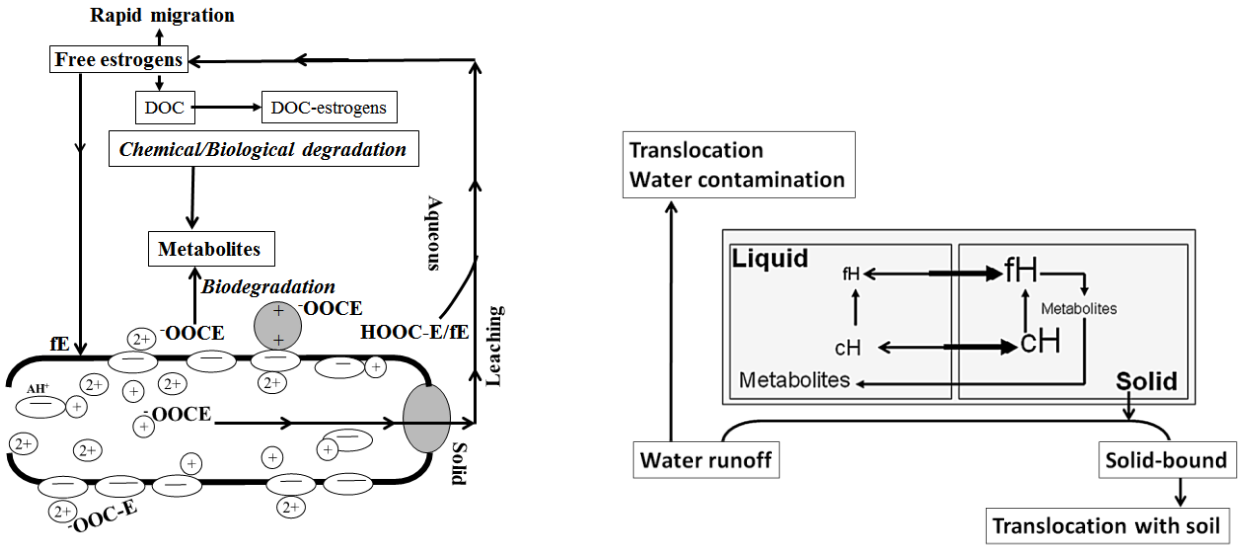

Figure-1. Composition of and estrogen fate in manure

A) Manure composition and distribution of estrogens in it. B) Fate of conjugated and free

estrogens in manure. Conjugated and free estrogens both distributed between the liquid and solid fractions of manure. In manure, conjugated estrogens may get converted into free estrogens and free estrogens are metabolized into different metabolites. The soil-bound estrogens remain with soil molecules, while soluble estrogens are translocated to different sites.

2. Materials and Methods

A detailed description of experimental design is described in the supplementary section. A brief description is provided below.

2.1. Sample Collection, Storage and Extraction (supplementary section I)

Nursery-manure (n=10), PS-manure (n=10) and FPig-manure (n=36) samples were collected from different pig farms during winter and summer seasons. Whole manure samples were divided in four aliquots (Figure S1) for manure characterization, bacterial enumeration (adjusted to 0.15M NaCl to prevent bacterial lysis), individual estrogen analysis, total estrogen analysis and estrogenic activity, respectively.

2.2. Manure Characterization

2.2.1. Determination of organic carbon

The procedure described by Perrier and Kellogg (1960) was used for analysis of organic carbon (TOC) In brief, a 0.5 g of manure solid (aliquot 1) was place in 75 ml test-tube and 10 ml of 1 N $K_2Cr_2O_7$ and 5.5 ml conc. H_2SO_4 were gradually added, swirling as the reagents were added. For blank, the all reagents were added in a test-tube without manure. For standard curve, different amounts of standard soil (known organic matter) were added into individual tubes and the same amounts of these reagents were added. The tubes were placed into a boiling water bath for 5 min., then cool in a water bath. The soil solution was thoroughly mixed. One ml of each of these solutions was transferred into a labeled 100mLvolumetric flask. A 3.3 ml aliquot of 6 N H_2SO_4 and 1.0 ml of s-diphenylcarbazide was added to each sample. The solution was mixed thoroughly. Absorbance was measured at 540 nm. For standard curve, the known percent organic matter was plotted against absorbance readings. Organic carbon was calculated using the standard curve.

2.2.2. Determination of Soluble Organic Carbon (SOC), Total Wet Manure Solid (TS), Volatile Manure Solid (VS) and Total Kjeldahl Nitrogen (TKN) (aliquot-1)

The procedures described by Gratteau et al (1968) and in a University of Wisconsin Madison Extension Publication A3769 (Peters J, 2003) were used for analysis of SOC, TMS, VMS and TKN in manure samples.

2.2.3. Determination of E coli and M fujisawaense enumeration in manure (aliquot-2)

An analytical procedure described by Wang et. al. (2005) was used to determine the bacterial enumeration. In brief, the non-acidified manure samples (prior to and after aerobic incubation) were centrifuged and the liquid and solid fractions were collected. The liquid fraction was mixed with sufficient 0.5M NaCl solution to achieve 0.15M final NaCl concentration to

prevent bacterial lysis. 0.1 The solid fraction was suspended in 0.15M NaCl (1:10 w/v), mixed thoroughly and then centrifuged. The clear supernatant containing bacterial populations were collected. Both the liquid and the solid-extracts were subjected to immuniaffinity extraction and then tested for colony formation (CFU) as described previously (Wang et al 2005).

2.3. Analysis of total estrogens and estrogenic activity (aliquot-2)

2.3.1 Manure extraction

Manure liquid and solid samples were mixed with internal standards and extracted as described in *supplementary section II.1.3*. After extraction, each manure sample yielded the following extracts: liquid ethyl acetate extract, liquid aqueous extract, solid aqueous extract, ethyl acetate wash of the solid aqueous extract and solid methanol extract.

2.3.2. Enzyme Hydrolysis conjugated estrogens

All aqueous extracts were divided in two aliquots, one for determination of estrogenic activity in non-hydrolyzed extracts and the other for determination of estrogenic activity in hydrolyzed extracts as described in *supplementary section III*.

2.3.3. Determination of estrogenic activity

The assay described by Ramamoorthy et al. (1997) for determinations of estrogenic activity using *Saccharomyces cerevisae* strain BJ3505 transformed with a yeast expression plasmid containing the CUP1 metallothionien promoter fused to the human α-estrogen receptor cDNA and a reporter plasmid containing two estrogen response elements upstream of the structural gene for b-galactosidase was used in the present study. Labelled and unlabeled acetaminophen was used at internal standards (*supplementary section III.*1.).

2.3.4. Determination of total estrogens

A total estrogen (E1+E2+E3) ELISA kit (Abraxis International) designed for samples with 10% alcohol was used in the present study. The dried samples and standards were prepared in methanol and then diluted to achieve 10% final concentration as described in the ELISA kit. The detailed method and validation is shown in *supplementary section III*.

2.4. Quantitative analysis of individual estrogens and metabolites (aliquot-4)

Acidified manure liquid and solid samples pre-mixed with ^{13}C labeled E2, gE2 and sE2 (0.25 mM) were spiked with an internal standard mixture containing deuterium labeled conjugated and free estrogens and then extracted as described by Singh et al (2013) and in the *supplementary section II.1.4*. The water extract was analyzed using HPLC-MS/MS for conjugated hormones and metabolites (*supplementary section II.1.3)* and the ethyl acetate/methanol extracts were analyzed using GC-MS/MS for free estrogens as described in the Supplementary Information. Estrogen metabolites were analyzed using a, stable-isotope coding and HPLC-Electrospray mass spectrometry (Yang et. al., 2008).

3. Results and Discussion

3.1. Manure characteristics

3.1.1. Chemical Characteristics of Manure

Table-1 shows some of the characteristics of Nur-, PS- and FPig-manure samples collected during winter and summer. Manure pH was slightly acidic and did not differ significantly in different samples collected during winter or summer. TS, VS, TOC, SOC and TKN values in Nur-manure were significantly lower than corresponding values in FPig-manure or PS-manure. VS represents the volatile organic contents in manure (Zhang et al 2003, Wright 2005 and USDA-NRCS 1996). The present study showed that Nur-manure had lowest, while PS-manure has highest organic matter (VS+TOC), possibly because of following differences.

i. The weaned piglets received controlled starter diet that were low in fibers and grains, but high in amino acids, dried skim milk, dried whey and fishmeal, while PSs and FPigs received fibrous diet that is adjusted to their specific needs (Augenstein et al. 1994, Beltranena and Patience. 1997, Dritz et. al., 1999; Tokach 2001, Masse et. al., 2003; Lammers et al 2007). During summer, there may be more opportunity for plant products to get mixed up with manure. Since, diet has been shown affect manure characteristics (Gralapp et al 2002. Croteau et al 2003), we propose that the above differences may be partially responsible for the differences in characteristics of manure samples.

ii. Piglets were housed at higher (80^{o}C) room temperature, while grown animals and pregnant sows were housed at around 70^{o}C (Dee 1999, Harmon et al 1991). Although experimental data is not available, higher ambient temperature in Nur may activate manure enzymes and microorganisms, thus reducing organic carbon. This may explain comparable summer and winter values in Nur-manure.

Unlike the Nur-manure samples, the PS- and FPig-manure samples collected in summer exhibited significantly greater TKN values vs corresponding winter samples (Table-1), possibly because TKN values increase at higher temperatures during summer months. Similar to the present study, earlier studies have also shown that the nitrogen values in manure were temperature dependent with maximal values occurring in the range 20 – 35^{o}C (Stark 1996 and Grundmann et al. 1995). However, Maag and Vinther 1999 have shown that, under high soil water conditions, formation of N_2O and loss of N via denitrification increase with temperature. The present study showed significant increase in TS contents in summer-manure vs winter-manure, thus, there should have been greater nitrogen loss during the summer, resulting in a decrease in nitrogen contents. But, thus was not the case. This may be because the fate of nitrogen in manure may, in addition to soil, moisture and temperature, also depend upon the source of N (Agehara and Warncke 2005). The higher nitrogen contents observed in summer-manure may be related to the diet animals receive during the summer time.

The present study also showed a significant increase in TOC and VS values in summer vs winter manure samples. This observation is contrary to earlier studies that have shown (i)

rapid degradation of OC in aerobic conditions (Bengtsson et. al. 2003, Burger and Jackson, 2003), (ii) rapid mineralization and CO2 release were key features of the aerobic degradation of OCs (Doelsch et. al., 2009) and (iii) rapid anaerobic degradation of OCs (Kemmitt et. al. 2006) that indicated greater loss of TOC may be occurring in summer than in winter. Thus, an increase in TOC and VS in summer-manure may be related to the animals' diet or other environmental factors. However, high carbon content of manure may affect the plant-available Nitrogen since the N mineralization rate is negatively related to the C to organic N ratio of manures (Chadwick et al 2000).

Table-1. Manure characteristics.

	Nursery (Nur)		Pregnant animals (PS)		Finisher pigs (FPig)	
	Winter	Summer	Winter	Summer	Winter	Summer
pH	7.3	6.87	6.7	6.77	6.8	6.8
TS (%)	6 ±4	6.7 ±3.5	32 ±17*	37 ±8*	67 ±26**	71 ±22**
VS (%)	13 ±4	17 ±9	57 ±15*	71 ±11*#	46 ±9*	63 ±7*#
TOC (g/kg)	9 ±2	10 ±2.3	72 ±8*	120 ±11*#	52 ±28*	86 ±17*#
F_{OC}	0.009 ±0.003	0.01 ±0.004	0.07 ±0.02	0.11 ±0.035	0.005 ±0.003	0.009 ±0.094
TKN (g/L)	4.5 ±1.1	4.1 ±1.0	11 ±3	29 ±5#	9 ±2	22 ±3#
E coli x10^6 (S)	1.2 ±0.7	1.6 ±0.4	3.1 ±0.3	6.3 ±0.8	2.4 ±0.4	6.1 ±0.6#
E coli S/L ratio.	2.05 ±0.2	1.95 ±0.2	2.4 ±0.2	2.6 ±0.3	2.3 ±0.1	2.15 ±0.1
M *fujisawaense* x 10^6 (S)	2.5 ±0.5	3.0 ±0.2	3.6 ±0.5	5.1 ±0.3#	2.1 ±0.5	6.7 ±0.4#
M *fujisawaense S/L ratio.*	1.1 ±0.1	1.1 ±0.2	1.5 ±0.3	1.2 ±0.3	1.1 ±0.4	1.3 ±0. 3

Values are mean ± SD, n=10 for piglets manure, 10 for pregnant/nursing sows and 36 for finishing pigs. Abbreviations: F_{OC}: fraction of organic carbon (OC), TS: total solid, VS: volatile solid, TOC: total OC, TKN: total Kjeldahl nitrogen. * $p<0.05$ when compares with corresponding Nur values, ** $p<0.05$ when compared with Nur and PS values and #: $p<0.05$

3.1.2. E coli and M fujisawaense Enumerations

As discussed earlier, pig manure contains bacterial populations that deconjugate cEs (example E coli) and hydrolyze and deactivation of fEs (example: M fujisawaense) (Legler et al 2002, Duong et al 2011, Iasur-Kruh et al 2011, Blunt et al 2009, D'Ascenzo et al 2003). The present study showed that both E coli and M fujisawaense bacteria were present in the liquid and solid fractions of manure from all three sites of each facility, although PS and FPig samples exhibited greater enumeration than Nur manure (Table-1). Bacterial count in winter and summer Nur-manure samples did not differ significantly, while bacterial counts in summer PS- and FPig-manure were significantly higher than in corresponding winter samples. The solid/liquid ratio of E coli in the three manure samples ranged from 1.95 to 2.4, while the ratio of M fujisawaense in the three manure samples were close to 1. This suggests that,

during summer, there may be an imbalance between conversion of cEs into fEs (an increase in estrogenic activity) and hydrolysis of fEs (decrease in estrogenic activity), resulting in higher estrogenic activity. There also appears to be a correlation between manure TOC concentrations and translocation of E coli, but not M fujisawaense, from liquid to solid, resulting in stabilization of the bacterial population (Franz et al 2008, 2011, Garzio-Hadzick et al 2010). However, differential stability of the bacterial population in solid fraction of PS-manure and FPig manure cannot be solely attributed to their TOC values since winter PS-manure solid and summer FPig manure solid had comparable TOC values but different stability of the bacteria. Further research is needed to characterize fate of manure bacterial populations relevant to estrogen metabolism in manure samples.

3.2 Total Estrogen Concentrations and Estrogenic Activity

Concentrations of total-conjugated (Σ sulfate and conjugated E1 + E2 + E3) and total-free (Σ free E1 + E2 + E3) estrogens in manure's liquid and solid fractions are shown in Figure 2. In both fractions, the estrogen concentrations showed the following pattern: PS-manure > FPig-manure > Nur-manure. The E2Eq values (a determinant of estrogenic activity) paralleled the fE concentrations in non-hydrolyzed samples (Figure 2 filled bar), but paralleled total estrogen concentrations in hydrolyzed samples (Figure 2, white bar). Concentrations of free and conjugated estrogens observed in the present study were lower than those reported by Combalbert et al. (2010) and Williams (2001), but comparable to those reported by Hanselman, et al. (2004). The inter-study differences may be due to the differences in the manure-facilities and/or analytical procedures used. Despite considerable estrogen load of pig manure, actual accumulation of estrogens may be much lower since approximately 90% of the free and conjugated hormones may degrade within 5 to 12 days in manure (Hanselman et al 2003, Lee and Leu et. al., 2002). Studies have shown that estrogen concentrations and estrogenic activity in manure may be related to the following factors:

(1) Deconjugation and activation of conjugated estrogens by *E coli* (Duong et. al., 2011; D'Ascenzo et. al., 2003)

(2) Degradation of free estrogens by manures' gram-positive (*Bacillus, Nocardia, Rhodococcus, Mycobacterium* especially *M fujisawaense*, *Nocardia, etc.*) and gram-negative bacteria (*Comamonas and Pseudomonas*) (Blunt et. al., 2009; Yu et. al., 2007; Matsouka et. al. 2005; Yoshimoto et. al., 2004; Fujii et. al. 2002)

(3) Manure's soluble and soil-bound OC contents (Quanrud et. al., 2004; Hu et. al., 2006). We propose that the different manures' bacterial population and TS, VS and TOC values may also play a key role in differential distribution of estrogen in Nur-, PS- and FPig-manures.

Figure-2 also shows that the E/E2Eq ratio was close to 1 for Nur-manure liquid or solid fractions irrespective of the season of collection and for PS- or FPig-manure liquid and solid samples collected during winter. However, the E/E2Eq ratio for PS- and FPig-manure solid fractions collected during summer were significantly lower (approximately 0.5) than that of the ratios for corresponding samples collected during winter. Studies have shown that the E/E2Eq ratio close to 1 represents that total estrogen are associated with the sample's

estrogenic activity, while the ratio <0.8 means presence of estrogenic compounds that has not been measured (Gibson et. al., 2005). The present study showed that PS- and FPig-manure solid samples collected during summer had E/E2Eq <0.8, suggesting that these samples may contain estrogenic compounds that were not measured in the present study.

Earlier studies (Yost et al 2013, Hoerger et al 2009, 2011 and Burnison et al 2002) have reported presence of phytochemicals such as daidzein (DE) daidzin (DI). Genistein (GE), genistine (GI), equol (Eq) (genistein metabolite) and formononeitin (FR) that are weakly estrogenic, contributing 0.5% of total manure estrogenic activity. In the present study, manure samples were analyzed using a HPLC-MS/MS method developed by Franke et al (2002) was modified to analyze glycosides of GE and DE. We detected (i) DE and GE in winter-Nur manure and DI, DE and GE in summer-Nur manure (Tables S2 and S3), (ii) DHDE, DI, GI, DE and GE in winter-PS and winter-FPig samples (Tables S2 and S3), and (iii) DHDE, DI, GI, Eq, DE, GE and FR in summer-PS and summer-FPig samples (Figure S3). However, it is unlikely that these weakly estrogenic phytoestrogens may be solely responsible for lower E2/E2Eq ratio in summer-PS manure samples.

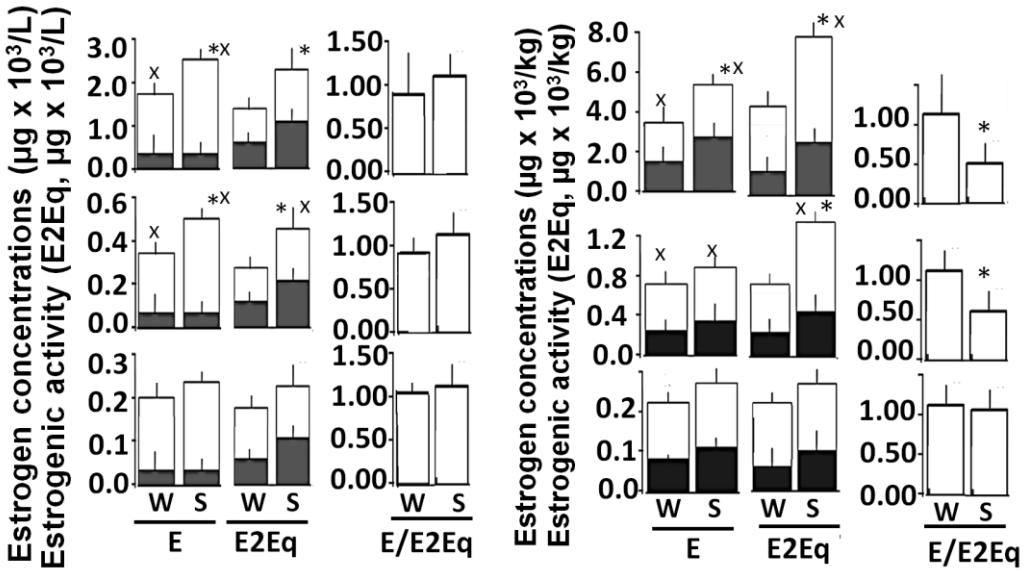

Figure-2. Total estrogen concentration and corresponding estrogenic activity in liquid and solid fractions of manure.

E: Estrogen concentrations analyzed using the HPLC-MS method, E2Eq: estrogen concentration determined using the E2 ‘s estrogenic activity, E/E2Eq ratio: contribution of estrogens in manure’s estrogenic activity, W: winter manure, S: summer manure, dark bar: free estrogens prior to manure hydrolysis, white bar: total estrogens after hydrolysis, bottom row: manures collected from nursery, middle row: manure collected from area housing finishing-pigs and upper row: manure from area housing pregnant- and nursing-sows. Values are mean ± SD, n= 10 for piglets, = 10 for pregnant/nursing sows and =36 for finishing pigs.

*: $p<0.05$ significant when compared from corresponding winter values and x: significant when compared from corresponding nursery and finishing pig values.

3.3 Concentrations of Individual Estrogen in Pig Manure

Tables S2 and S3 show concentrations of free and conjugated estrogens in manure liquid and solid samples collected in winter and summer seasons. The methods used in this study provided excellent separation of conjugated (Figure S2A) and free estrogens (Figure S2B). E1 (sE1>fE1) and its metabolites were most abundant steroids in Nur- and PS-manure, while E2 (gE2>fE2) was the prominent steroid in FPig-manure (Table 2). Consistent with the present observations, earlier studies have also shown that E1 was the key estrogen in manure from sows and piglets, while E2 and E1were key hormones in manure from finishers (Raman et. al., 2004; Sarmah e.t al., 2006). The key source of estrogens in manure may be CAFOs housing large number of animals excreting large quantities of conjugated (via urine) and free (via feces) estrogens and their metabolites. E1 (fE1, sE1 and gE1) was major estrogens present in plasma and urine samples from new-born piglets and pregnant sows, excreting about 200 µg and >5000 µg total E1/pig/day, respectively (Baldwin and Stabenfeldt, 1975; Bate and Hacker, 1982; Raeside, 1963). In pregnant sows, E1 concentration declined rapidly after parturition (Robertson and King, 1963), indicating that placenta may be the key source of E1 (Knight 1994). In piglets, E1 concentrations remain elevated for 1 to 3 months of age, followed by gradual decrease (Baldwin and Stabenfeldt, 1975; Bate and Hacker, 1982). Unlike pregnant sows and piglets, FPigs predominantly excreted gE2 and fE2 (Baldwin and Stabenfeldt, 1975) that may account for gE2 being the major hormone in FPig-manure. The differential distribution of estrogens in Nur, PS and FPig samples may be attributed to their differential estrogen excretion.

Table-2. Relative distribution of estrogens and their metabolites in pig manure.

Nursery (Nur) Manure
Nur Liquid
Winter: sE1>gE2>fE1>3-MeOE1>gE3>2-MeOE1>fE2>2-MeOE2>sE3>4-MeOE2> 16α-OHE1>4-OHE1, 4-MeOE1>2-OHE1
Summer: sE1>gE2>gE2>gE3>fE1>2-MeOE1>2-MeOE2>sE1>fE2>3-MeOE1, 4-OHE1> sE1>4-MeOE2-4-MeOE3>OHE1, 2-OHE2
Nur Solid
Winter: gE1>gE2, fE1, gE3, 2-MeOE1>fE2>fE3>sE2>sE1, sE3, 2-MeOE2>2-OHE2, 4-MeOE3>3-MeOE1>4-MeOE1>2-OHE1.
Summer: gE1>fE1>fE2>gE3>fE3>20MeOE1>2-M3OE2>sE1>sE2>fE2> 3-MeOE1>sE1>4-MeOE1> 4-MeOE2>2-OHE1, 4-OHE2
Pregnant Sow (PS) Manure
PS Lquid
Winter: sE1>fE1>4-OHE1>fE1>gE2>fE2>16α-OHE1>4-MeOE1>gE3>2-MeOE1 >2-OHE1> 4-MeOE2>3-MeOE1>2-MeOE2>sE2>sE3
Summer: sE1>fE1>gE1>4-OHE1>gE2>fE2>gE3, 2-OHE1>16α-OHE1> 2-MeOE1>sE3>sE2>2-OHE2>4-MeOE1>2-OHE1>4-MeOE2>fE3

PS Solid
Winter: sE1>4-OHE1>fE1>gE1>gE2>2-MeOE1>2-OHE1>4-MeOE1>fE2>3-MeOE1> 2-OHE2>gE3>2-MeOE2, 4-MeOE2>fE3>sE2
Summer: sE1>4-OHE1>fE1>gE1>3-MeOE1>2-MeOE1>gE2>fE2> 2-OHE2>MeOE2>gE3>fE3>40MeOE1>4MeOE2>sE2>sE1
Finisher Pig (FPig) Manure
FPeg Liquid
Winter: gE1>gE2>fE2>fE1>gE3>sE2>2-MeOE1>fE3>16α-OHE1>2-OHE2, sE3> 2-OHE1>3-MeOE1>fE3
Summer: gE1>gE2>fE2>sE1>gE3>fE1>sE3>sE2>16α-OHE1>fE3>4-OHE1>2-OHE1> 2-MeOE2>2-OHE2, 2-MeOE1, 4-MeOE1,
FPig Solid
Winter: gE1>gE2>fE2>fE>gE3>2-MeOE2>2-MeOE1>4-MeOE1>2-OHE2>4-OHE1> sE2>3-MeOE1>fE>sE3>2-OHE1>4-MeOE2
Summer: gE1>gE2>fE2>fE1>gE3>sE2>sE1>sE3>2-MeOE1>4-OHE1>2-OHE2>2-MeOE2> 2-MeOE1>2-OHE2>4-MeOE1>3-MeOE1

3.4. Estrogen Metabolites

As discussed earlier, estrogens in stored manure undergo bacterial and chemical transformation (Shrestha et al 2012) associated with (i) deconjugation of estrogens resulting in an increase in biologically active free hormones (Kumar et al 2012, Flores et al 2012), (ii) hydrolysis of free estrogens and (iii) inter-conversion between estrogens (Mansell et al 2011, Ying et al 2002), resulting in formation of different metabolites shown in Tables S2 and S3, respectively, for liquid and solid fractions of pig manure. High concentrations of estrogen metabolites, especially 2- and 4-methoxy-estrogens and 2-, 4- and 16-hydroxy-estrogens were observed in both fractions. Methoxy-estrogens were present in greater proportion than hydroxyl-estrogens. E1 metabolites were present in highest abundance in PS- and Nur-manure samples, while E3 metabolites were present in highest abundances in FPig-manure samples. The present observations are supported by earlier studies in pig manure (Baldwin and Stabenfeldt, 1975, Zhu and Conney, 1998). Since estrogen metabolites lack estrogenic activity than parent estrogens, they are generally assumed to be harmless. An overview of metabolite toxicity is shown in Figure 4. Although it is generally believed that methoxy estrogens are not estrogenic, earlier studies have shown that 4-methoxyequilenin exhibited estrogenic activity. 4-OH and 16-OH metabolites exhibit greater potency in causing DNA damage and breast cancer than the parents (Miller, 2003, 2008; Cavalieri et al., 2006; Kabat et al., 1997). Studies have shown that 2-OHEs are converted into corresponding quinines that exhibit greater genotoxicity than the parent molecule (Zhang et al., 2001). Taken together, these observations indicate that, in addition to the estrogens, their metabolites also are significant and persistent hazard that must be addressed.

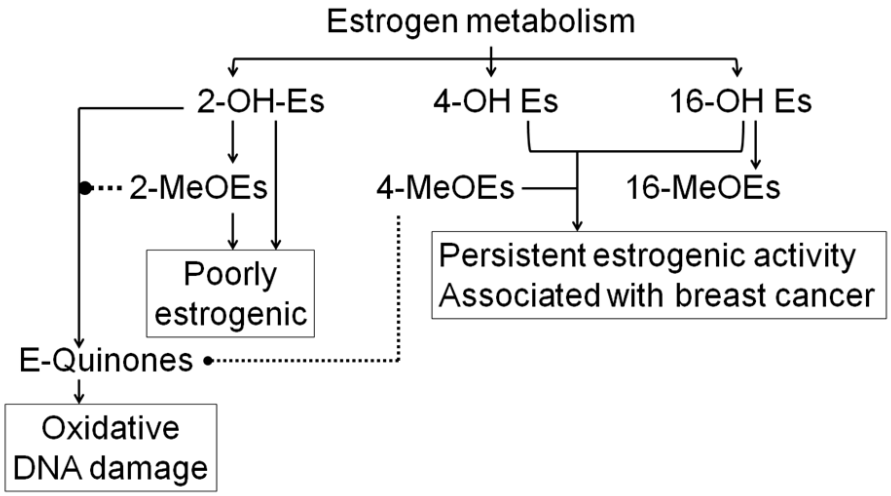

Figure-4. Estrogenic effects of and oxidative damage by estrogen metabolites present in manure.

It is proposed that 2-hydroxy-–estrogens (2-OH-Es) are converted into E-quinones that are not estrogenic but cause oxidative DNA damage. 2-Methoxy estrogens (2-MeOEs) and 4-methoxy estrogens (4-MeOEs) suppress the toxic effects of 2-OH-Es and E-quinones. 4-OH-Es and 16-OH-Es exhibit weak estrogenic activity and may be associated with breast cancer.

3.5 Distribution of estrogens between solid and liquid fractions of manure

Estrogens, depending upon their properties, may distribute between the liquid and solid fractions of manure. cEs are water soluble and contains a net negative charge due to $-COO^-$ and/or $-SO_3^-$ groups (Figure 5), while fEs are highly lipophilic (logP: >4).

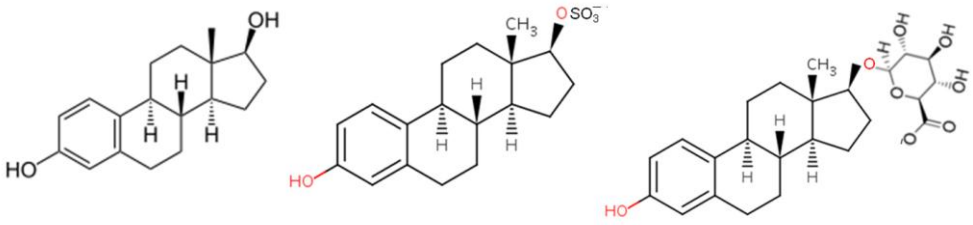

Figure-5. Structure of conjugated and free estrogens. Conjugated estrogens that exhibit net negative charge, and free estrogens that are moderately hydrophobic.

Therefore, we expected that conjugated estrogens will remain in the liquid phase and the free estrogens will translocate into the soil. However, contrary to this hypothesis, both conjugated and free estrogens bound to manure solids (Figure 6), although by via different mechanisms. Conjugated and part of free estrogens eluted with water, while the remaining free estrogens eluted with methanol. Conjugated estrogens (or metabolites) bind manure solids possibly involving H-bonds or electrostatic interactions with positively charged adsorbed ions, while the methanol-eluted fEs bind to solid via hydrophobic interactions. The $\log K_{OC}$ values, ranging from 2

to 3.3 for fEs and from 1 to 1.6 for cEs (Table 3) suggests that fEs may exhibit lower mobility then cEs in manure.

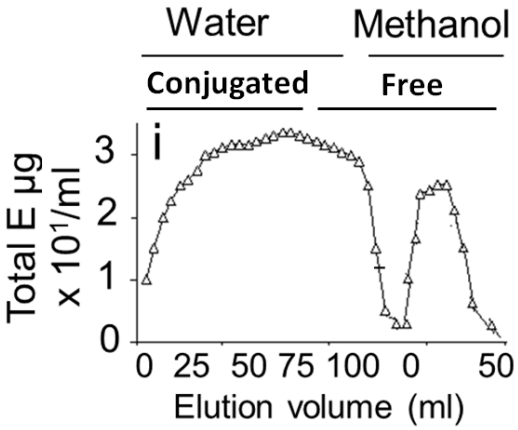

Figure-6. Elution pattern of conjugated and free estrogens from manure solid.

All of the conjugated and part of free estrogens are eluted with water. The remaining free estrogens are eluted with methanol.

In addition to the binding of estrogens to solid, fate of estrogens may also be determined by microscopic colloids in liquid that have been shown to bind estrogens (Zitnick et al. 2011). Thus, an equilibrium state may exist between hormones in liquid and solid fractions of manure (Figure-1b). Water may leach conjugated and, to some extent, free hormones from soil into the manure liquid that may contaminate the stream and underground water supply. Although mechanisms are not fully understood, hydrophobic partitioning into organic carbon domains of solids may plays a key role in sorption of lipophilic compounds (Kumar et al., 2006). Since sorption retards a chemical's leaching, it minimizes groundwater contamination. Chemicals that bind to soil particles will migrate when the soil particles move during erosion, while chemicals soluble in water or bound to dissolved organic carbon may migrate freely.

3.6 Seasonal Changes in Manure Estrogen Concentrations and Estrogenic Activity

The colder regions of the United States experiences wide fluctuations in temperature ranging from below zero with snow cover in winter to over 90^{o}C in summer. To accommodate different energy needs during winter and summer, farmers may alter the animals' diets that may alter the manures' physicochemical properties. However, very little information is available about the influence of season on manure and the fate of estrogens. The present study showed that concentrations of total (Figure-2) and/or individual estrogens (Tables S2 and S3) in Nur-manure samples collected during winter and summer did not differ significantly, while the estrogen concentrations in PS- and FPig-manure samples collected in summer were significantly greater than the concentrations in corresponding winter samples. Although causes for the differences between Nur-manure and PS- or FPig-manure are not fully understood, we propose possible involvement of the animals' diet, manure storage condition and manure characteristics in the process as discussed below.

(i) *Differences in the animals' diet:* As discussed above, piglets receive controlled diet that is

fairly consistent throughout the year, FPigs receive more stray and fresh diet in summer and high fat diet in winter and pregnant sows receive diet depending on the needs at different stages of pregnancy (van Heugten et al 2003).

(ii) Differences in manure storage and composition: The present and earlier studies have shown that Nur-manure contained lowest, PS-manure contained highest and DPig-manure contained intermediate levels of TS, VS, TOC, SOC and nutrients. PS-and FPig-manure, but not Nur-manure exhibited season-related differences. In addition, *E coli* and *M fujisawaense* populations in summer samples were significantly greater than those in winter samples.

Studies have shown that an increase in TOC may attenuate binding of estrogens to manure solid and enhance their translocation (Quanrud et. al., 2004; Hu et. al., 2006), but increases binding of *E coli* and *M fujisawaense* to manure's solid that has been shown to stabilizes the bacterial population (Franz et al 2008, 2011, Garzio-Hadzick et al 2010). Bacterial stabilization may result in (1) deconjugation and activation (in case of *E coli*) of conjugated estrogens and (2) further degradation and deactivation of free estrogens (in case of *M fujisawaense* or other related bacteria). Thus, the animal's diet and manure characteristics may determine estrogen accumulation in manure samples.

4. Public Health and Environmental Consequences

Annual excretion of estrogens by farm animals, including cattle, pigs, sheep, and chickens, has been estimated to be 39 tons in the European Union and 41 tons in the United States (Moore et al., 1982; Zhao et al., 2010). Since manure also contains large quantities of estrogen metabolites, we hypothesize a possible relationship between estrogen metabolism in manure and its estrogenic activity (Figure 7). Studies have shown that majority of estrogens excreted from animals arise from urine in conjugated form that has poor estrogenic activity. However, when manure samples are stored, conjugated estrogens may be converted to free estrogens by manure-microorganisms, resulting in gradual increase in manure's estrogenic activity. Long term storage or manure at the generation site may result in gradual decrease in estrogenic activity due to further degradation and inactivation of hormones or leaching of conjugated hormones from the storage site to stream or underground water sources. Thus, storage may reduce estrogen load at the site of production, but may increase the ecosystem pollution.

Manure solids, in addition to fEs, contain large quantities of cEs. Although cEs do not exhibit estrogenic activity, they can be hydrolyzed into free hormones, thus serving as a reservoir for free, biologically active hormones. As the hormones in manure liquid are degraded more conjugated hormones will be released from solid into the liquid fraction. In manure solid, conjugated estrogens may persist for relatively longer periods and leech with rain water. In surface water, conjugated estrogens may get converted into free estrogen resulting in an increase in surface water's estrogenicity.

Earlier studies have shown that estrogen concentration in wells ranged from <0.1 ng/ml to 30 ng/ml, depending on land application of manure (Shore et al. 1993). Relatively high levels of estrogens have also been found in surface water near the animal farms. E2 concentrations >5

ng/L have been reported in ducks and fish populations (Zhao et al., 2010). Estrogen exposure has been shown to (i) cause reproductive problem, especially in male marine animals and (ii) altered embryonic sexual development that may depress subsequent reproductive success (Shore et al., 1995). Therefore, this study suggests that steroid hormones emerging from pig farms may have severe ecological impact in the pig-farm neighborhoods.

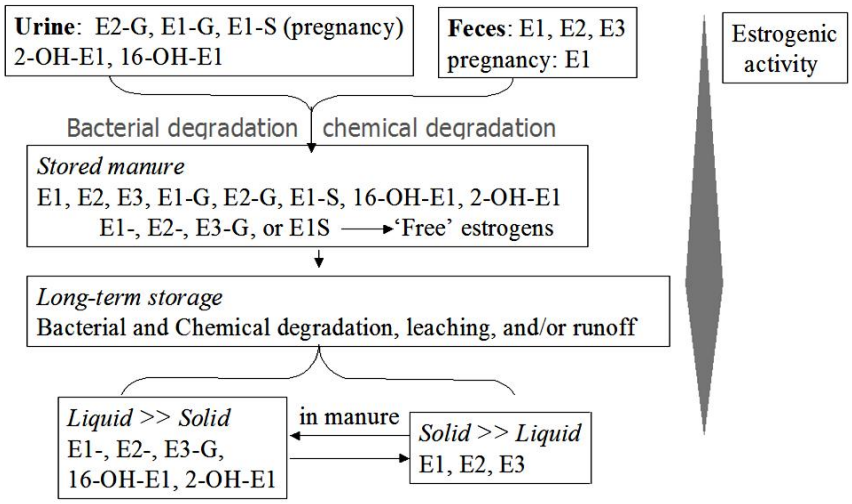

Figure-7. Metabolism and fate of estrogens in manure samples.

Urine contains conjugated, while feces contain free estrogens. In manure, bacterial populations (1) deconjugate conjugated-estrogens that increases the samples estrogenic activity and (2) degrade free estrogens resulting in decrease in estrogenic activity. If new manure is not continually added, the estrogenic activity with first increase followed by a gradual decrease. Free estrogens ne distribute in manure solid, while conjugated estrogens may remain in liquid fraction. Abbreviations: E2: estradiol, E1: estrone, E3: estriol, G: glucuronide conjugate, S: sulfate conjugate, -OH hydroxyl metabolite.

A recent study from our laboratory gas shown that anaerobic incubation of pig manure that increases the plant-available nitrogen (PAN) poorly degrades estrogens, while aerobic incubation of pig manure that accelerates estrogen degradation also degrades the PAN (Singh 2014). Since PAN is the key factor in use of manure as fertilizer, estrogen contamination presented a unique dilemma: both aerobic and anaerobic processing of manure has distinct disadvantaged and may not be ideal converting pig manure into safe organic fertilizer. Further research is needed to develop methods to convert manure into safe fertilizers.

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