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Article

Aerobic and Anaerobic Transformations in Estrogens and Nutrients in Swine Manure: Environmental Consequences

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Abstract: Pig manure is an excellent fertilizer and rich source of organic carbon and nitrogen compounds such organic nitrogen (O-N) (95% of total nitrogen) that is plant-unavailable-nitrogen (PUN) and mineralized nitrogen (about 1% of total nitrogen) such as ammonium (NH_4^+) and nitrate (NO_3) that are plant-available-nitrogen (PAN). In addition, manure also contains two forms of estrogens: (i) poorly estrogenic thus essentially nontoxic conjugated estrogens (cEs) such as estrone (cE1), estradiol (cE2) and estriol (cE3); and (ii) highly estrogenic and toxic free estrogens (fEs) such as fE2, fE1 and fE3. This study showed that aerobic processing reduced concentrations of total carbon (TC), O-N, PAN and NH_4^+/NH_3 ratio, transiently hydrolyzed cEs (cE2 > cE1 > cE3) into corresponding fEs, transiently increased estrogenic activity and potential toxicity, and rapidly degraded fEs (fE2, fE1 > fE3), thus reducing the estrogenic activity in manure. Unlike aerobic processing, anaerobic processing stabilized and increased PAN and NH_4^+/NH_3 ratio, thus increasing the manure's fertilizer value. However, anaerobic processing, relative to aerobic processing, poorly hydrolyzed cEs (reducing transient toxicity and increasing reserve toxicity potential) and poorly degraded fEs (increasing toxicity) in manure. Thus, aerobic and anaerobic environments have distinct effects on manures' PAN and estrogenic activity, presenting an interesting dilemma: anaerobic incubation that increases manures' PAN does not effectively degrade estrogens, while aerobic incubation that effectively degrades estrogens (after transiently increasing their estrogenic activity) also decreases PAN, thus making manure less profitable. New techniques are need to fully use manure as organic fertilizer.

Keywords: manure; aerobic; anaerobic; estrogens; plant available nitrogen; ammonium; ammonia; organic nitrogen

1. Introduction

The United States has approximately 1.3 million farms holding livestock and, about 100,000 of which, are considered swine Concentrated Animal Feeding Operations (CAFOs) where large number of pigs are held and raised in a small area [1]. On average, a pig produces about 3.9 kg of manure (a slurry of urine and feces)/day [2], thus about 2.3×10^8 kg of manure is generated each day by the pig industry. This is equivalent to an influx into the environment of about 3.6×10^9 kg nitrogen and approximately 3.5×10^{11} kg organic carbon each day. Compared to the smaller operations, CAFOs have limited land available for effective use/storage of the generated waste as fertilizer [3]. In addition, CAFOs do not require treatment of manure as long as it is not disposed directly into water bodies [2]. As a result, the waste load arising from CAFO may exceed the absorption capacity of the local environment, necessitating storage of large quantities of manure at the site of production [4]. The negative environmental implications of CAFO are related in significant part to the large volume of manure contaminants coming from a small area.

A serious concern regarding pig CAFO is that manure, despite being rich in nutrients, also contains high concentrations of estrogens that, even at low doses, have been proven to have long-term adverse health effects in humans and aquatic/wild animals [5,6]. The ecological hazard posed by steroidal hormones resulting from swine production is not clearly known. Mammals excrete two main forms of steroid hormones: (i) the biologically active free-estrogens (fEs) are excreted through feces (accounting for 10% of total steroid excretion); and (ii) the biologically inactive conjugated steroids (cEs) are excreted through urine [7]. Since cEs are the predominant form of estrogens found in manure [7,8], there is a presumption that manure may have poor estrogenic activity. However, earlier studies have shown that storage may increase manure's estrogenic potency due to the hydrolysis of conjugated hormones by glucuronidase and sulfatase enzymes present in manure microorganisms [9,10]. Swine manure contains aerobic and anaerobic bacteria that, under aerobic and anaerobic incubations, respectively, metabolize estrogens and convert organic nitrogen (O-N) into mineralized nitrogen (nitrate (NO_3), nitrite (NO_2), ammonium (NH_4^+), ammonia (NH_3), *etc.*) (Figure 1) [11].

However, possible effects of incubation conditions on relative alterations in estrogen concentrations, estrogenic activity and nutrient concentrations in manure are not fully understood. The aim of the present study was to establish the time-course of change in estrogen concentrations, estrogenic activity and nutrient (organic nitrogen and ammoniated nitrogen) concentrations in swine manure stored in aerobic and anaerobic conditions. Possible environmental consequences of these transformations has also been discussed.

2. Materials and Methods

Collection and processing of manure: Fresh manure samples (n = 27) were collected from swine facilities housing finishers. The samples containing 40% to 80% solid were mixed thoroughly and

divided into three aliquots. Aliquot-1 was analyzed for estrogenic activity, estrogen concentrations and nutrients; and aliquot-2 and -3 were subjected to aerobic and anaerobic incubations, respectively, as described in Supporting Information.

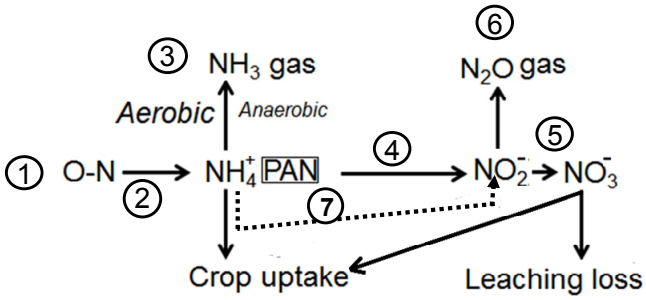

Figure 1. Biotransformation of organic nitrogen (O-N) in manure. (1) Decay of dead plants and animals release amino acids and proteins; (2) Ammonification of O-N by Saprotrophs, resulting in formation of NH_4^+ (in acidic pH); (3) In aerobic environment the pH is basic, thus NH_4^+ is converted into NH_3 gas that enters the atmosphere. In anaerobic process, the pH is acidic that favors NH_4^+; (4) Oxidative nitrification by nitrifying bacteria (*Nitrosomonas)* at acidic pH; (5) Nitrification by *Nitrobacter*; (6) Bacteria such as *Pseudomolas denitrificans convert* NO_3^- into N_2O gas; (7) Anoxic oxidation of NH_4^+. Abbreviations: NH_4^+: ammonium, PAN: plant available nitrogen, NO_3^-: nitrate, NO_2^-: nitrite, N_2O: nitrous oxide gas, NH_3: ammonia gas.

Anaerobic incubation of manure*:* 20 mL of thoroughly mixed slurry of each sample was transferred to amber conical flask fitted with gas intake tube, a gas collection port and chemically inert air-tight cap. Multiple flasks could be connected to a single gas source. The flasks' headspaces were immediately evacuated and filled with nitrogen gas. For manure sterilization, a second set of flasks were mixed with sodium azide (50 g/L) to inhibit biodegradation. Samples were analyzed at different time intervals from start of incubation. At regular intervals, the outgoing air from collecting port was passed through 20 mL 0.1 N H_2SO_4 in order to collect the volatilized NH_3. The experiment was run in a constant-temperature room at 25 °C. NH_3 volatilization was determined using a colorimetric method based on the Berthelot reaction [12].

Aerobic Incubation of manure: Aerobic incubation were prepared in a similar manner to the anaerobic experiments, except the manure liquid or slurry was constantly oxygenated using a 16-gauge Teflon connected to compressed air (breathing-air grade) and bubbled at a continuous rate. Samples were incubated at room temperature. Samples were analyzed at different time intervals from start of incubation. The procedure described above were followed.

Sample processing and analysis: Each slurry sample was mixed thoroughly and split into two equal parts (Figure S1). Part-1 was analyzed for estrogenic activity (in terms of estradiol equivalent—E2Eq), organic carbon (OC), NH_4^+-N, NH_3, NO_2, NO_3 and O-N. E2Eq was measured using the Yeast Estrogen Screen (YES) assay that was validated for pig manure (Figures S2 and S3). NH_4^+-N, NH_3, NO_2, NO_3 and O-N were analyzed using standard procedures described in supplementary section. Part-2 manure was mixed with internal standards and poured into a fritted glass column. Conjugated estrogens were eluted with 100 mL of pure water and free estrogens were eluted with 100 mL methanol [8]. The two

effluxes were concentrated to 5 mL under nitrogen and reduced pressure. Individual cEs (E2 glucuronate (E2G), E2 sulfate (E2S), E1G, E1S, E3G and E3S) were analyzed using an Applied BioSystems binary High Performance Liquid Chromatograph (HPLC) equipped with a triple-stage quadruple mass spectrometer (Figure S4A). Individual fEs (estradiol (fE2), estrone (fE1) and estriol (fE3)) were derivatized to the silyl ethers in using pyridine:bis(trimethylsilyl)-trifluoroacetamide before analysis by GC-MS (Agilent 7000 series gas chromatograph Mass Spectrometer, Agilent, Palo Alto, CA, USA) (Figure S4B) [8]. The detailed analytical procedures are described in the Supplementary section (Tables S1 and S2). Analysis of pre-incubation samples established baseline for estrogens and estrogenic activity. Changes in free estrogen concentration were assessed by subtracting basal values from the corresponding test-values (a negative value indicated decrease in concentration). Changes in cEs concentrations were determined by subtracting test-values from basal values. Data were recorded as mean ± SD using Microsoft Excel. Statistical analysis was performed using ANOVA followed by Tukey's Multiple Comparison Test or *t*-test at <0.05 significance level.

3. Results

3.1. Fresh Manure Characteristics

Manure slurry used in this study contained 40% to 80% solid contents. Total O-N, NH_4^+ and NH_3, NO_2^-, NO_3^-, total carbon (TC) and Kjeldahl nitrogen (KN) concentrations, and NH_4^+/NH_3 and C/N ratio values were 53 ± 43 (% DM), 0.5 ± 0.3 ($gN \cdot kg^{-1}$), 0.1 ± 0.08 ($gN \cdot kg^{-1}$), <MDL, 23 ± 21 ($gN \cdot kg^{-1}$), 51 ± 20 ($gC \cdot kg^{-1}$) 37 ± 29 ($gN \cdot kg^{-1}$), 5.1 ± 1.8, 17 ± 7, respectively. Free and conjugated estrogens distributed between the liquid and solid fractions of manure (Table 1). Total fE concentrations in liquid and solid fractions of manure were 33 ± 10 $\mu g \cdot L^{-1}$ and 646 ± 230 $\mu g \cdot L^{-1}$, respectively. Total cEs concentrations in liquid and solid fractions of manure were 255 ± 103 $\mu g \cdot L^{-1}$ and 355 ± 170 $\mu g \cdot L^{-1}$, respectively. This suggests that cEs were greater than fEs in liquid, while fEs were greater than cEs in solid fraction of manure analyzed in this study. Contrary to the perception, manure samples exhibit estrogenic activity.

3.2. Effects of Aerobic Digestion

Manure Carbon and Nutrients: Aerobic digestion transiently increased manure pH that peaked at day-10 and then returned to the basal level at day-30. (Figure 2). O-N concentrations decreased in biphasic manner: a rapid decrease at a rate of 0.9 ± 0.5 $gN \cdot kg^{-1} \cdot day^{-1}$ for up to day-5 was followed by a slow decrease at a rate 0.2 ± 0.1 $gN \cdot kg^{-1} \cdot day^{-1}$. Ammonium ($NH_4^+$) concentrations decreased (rate of 0.03 ± 0.02 $gN \cdot kg^{-1} \cdot day^{-1}$) gradually with minimal values of 0.1 $gN \cdot kg^{-1}$ (Figure 3iiib). Ammonia (NH_3) concentrations increased in two stages (Figure 3iiia): a rapid increase from 0.05 to 1 $gN \cdot kg^{-1}$ occurred at day-1 and the values remained unchanged for up to day-15, then the values increased further to 2 $gN \cdot kg^{-1}$ and remained elevated throughout the experiment. This resulted in a significant decrease in the NH_4^+/NH_3 ratio (Figure 4i). Nitrate (NO_3^-) levels increased rapidly and peaked at day-10, followed by a gradual decrease reaching to 75% of basal values at day-30 (Figure 4i). Nitrite (NO_2^-) was not detected in this study. A delayed decrease in TC contents and C/N ratio in manure samples reaching to 50% of basal values occurred at day-30 (Figure 4i).

Table 1. Hormone levels in manure liquid and solid samples.

	Estradiol (E2)		Estrone (E1)		Estriol (E3)		Total
	Conjugated µg/L	Free µg/L	Conjugated µg/L	Free µg/L	Conjugated µg/L	Free µg/L	µg/L
			Manure Liquid				
n = 23	43 ± 17	13 ± 11	165 ± 4 *	3 ± 7	15 ± 13 #	17 ± 12	310 ± 117
	G 78%		G 20%		G 90%		
E2Eq Ph							13 ± 12
E2Eq Ah							71 ± 22 †
			Manure Solid				
n = 23	143 ± 63	210 ± 111	229 ± 138	413 ± 211	45 ± 8 #	18 ± 13 *	973 ± 471
	G 80%		G 30%		G 90%		
E2Eq Ph							53 ± 11
E2Eq Ah							97 ± 32 †
K_D	3.3 ± 1.7	16 ± 5 *	1.4 ± 0.9	105 ± 21 *	3 ± 2	1 ± 0.8	

Values are mean ± SD; * $p < 0.05$ when compared from control values; # $p < 0.05$ when compared with E2 values; † $p < 0.05$ when Ph (prior to hydrolysis) values were compared with Ah (after hydrolysis) values; Ph: prior to hydrolysis; Ah: after hydrolysis.

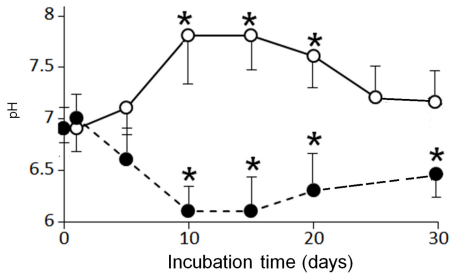

Figure 2. Effects or aerobic (solid line) and anaerobic (broken line) incubation on manure pH ($n = 27$). Values are mean ± SD. * $p < 0.05$, significant when compare with control.

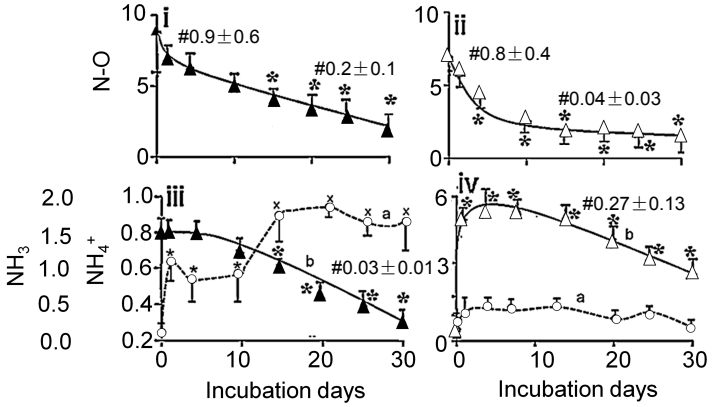

Figure 3. Effects of aerobic and anaerobic incubations on time-course of change in pH, organic nitrogen (O-N), ammonium (NH_4^+) and ammonia (NH_3) in manure samples. i-*aerobic* and ii-*anaerobic*: Time-course of change in O-N in manure samples subjected to aerobic and anaerobic incubations; iii-*aerobic* and iv-*anaerobic*: Time-course of change in NH_4^+ (plot b) and NH_3 (plot a) concentrations in manure samples subjected to aerobic or anaerobic incubations; values are mean ± SD, $n = 27$, * significant ($p < 0.05$) compared with control.

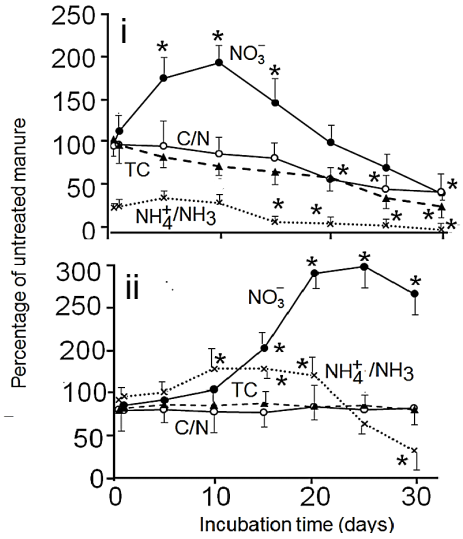

Figure 4. Effects of aerobic (i) and anaerobic (ii) incubations on time-course of change in NO_3^-, NH_4^+/NH_3 ratio, total carbon (TC) and carbon/nitrogen (C/N) ratio in manure samples. Values are mean ± SD, $n = 27$, * significant ($p < 0.05$) compared with control.

Manure Estrogens: Aerobic incubation gradually decreased conjugated estrogen concentrations (E1 = E2 > E3) with $t_{1/2}$ values ranging from 8 to 10 days (Figure 5I: cE1—plot 5, cE2—plot 6 and cE3—plot 7). Approximately 15%, 40% and 50% cE3 (Figure 5I plot 7), cE1 (Figure 5I plot 6) and cE2 (Figure 5I plot 5), respectively, remained in manure at day-30 of aerobic incubation. A transient increase (37% ± 10%, Figure 5I plot 1) in E2Eq levels and fE concentrations (fE2: 36% ± 10%, Figure 5I—plot 2; fE1: 31% ± 8%, Figure 5I—plot 3; and fE3: 11% ± 5% increase, Figure 5I plot 4) occurred in manure. The fE2 and E2Eq values peaked at day-5 and decreased gradually reaching to the control values at day-15 to day-20. As shown in Figure 5III, gEs and corresponding sEs exhibit comparable sensitivity to aerobic (a) incubation (for 30 days) in manure. A comparable change in E2Eq and fE2 values indicate that fE2 may solely account for the increase in estrogenic activity in manure samples subjected to aerobic digestion. In sterilized manure, only 10% decrease (Figure 5IV and V) in estrogen concentrations were observed. This represents chemical degradation of estrogens.

3.3. Effects of Anaerobic Digestion

Manure Carbon and Nutrients: Anaerobic incubation significantly decreased manure pH at day-10 that remained suppressed throughout the experiment (Figure 2). O-N exhibited a rapid decrease occurring for up to day-5 (rate 0.8 ± 0.4 $gN \cdot kg^{-1} \cdot day^{-1}$), followed by a slower decrease (rate 0.04 ± 0.03 $gN \cdot kg^{-1} \cdot day^{-1}$) (Figure 3ii). A rapid increase in NH_4^+ concentrations (peak value at day-5) (Figure 3ivb), a gradual increase in NH_4^+/NH_3 ratio (peak value at day-10) (Figure 4ii) no change in NH_3 concentrations (Figure 3iva) was observed. NO_3^{-1} concentrations remained unchanged for up to 10 days and then increased gradually that peaked at day-20 and remained elevated thereafter. The TC levels and C/N ratio remained unchanged throughout the experiment.

Manure Estrogens: Anaerobic digestion caused <10% (Figure 5II plot 7), 30% a (Figure 5II plot 6) and 32% (Figure 5II plot 7) decrease in cE3, cE2 and cE1 concentrations, respectively, without increasing fE concentrations and E2Eq activity levels (Figure 5II, plot 1–4). In sterilized manure, only

10% decrease in cEs occurred (Figure 5IV and V). Similar to aerobic incubation, gEs and corresponding sEs exhibit comparable sensitivity to anaerobic (an) incubation (for 30 days) in manure.

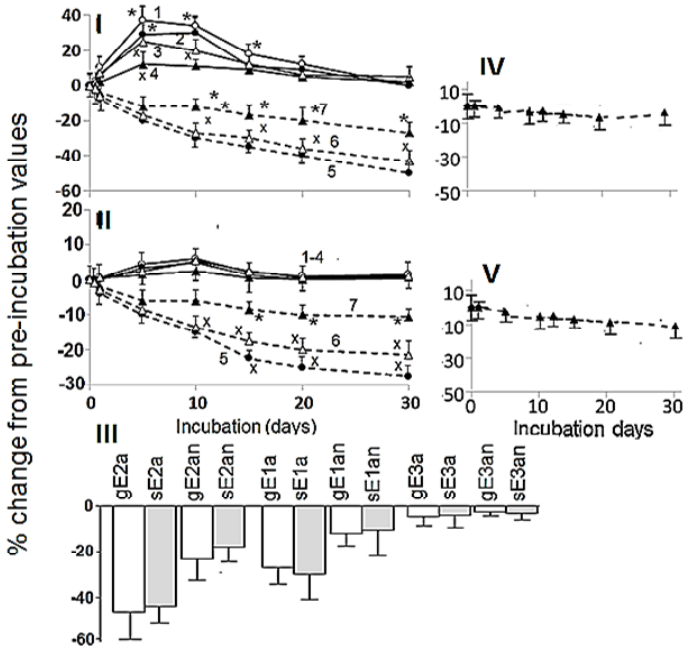

Figure 5. Effects of aerobic and anaerobic incubations on fate of conjugated and free estrogens in manure samples. I-*aerobic* and II-*anaerobic*: Plots 1 to 4, respectively, represents the time-course of change in estrogenic activity, free βestradiol (fE2), free estrone (fE1) and free estriol (fE3), while plots 5 to 7, respectively, represents conjugated βestradiol (cE2), conjugated estrone (cE1) and conjugated estriol (cE3) in manure samples incubated in aerobic (I) and anaerobic (II) environment; III: Percentage decrease in estrogen glucuronides (gE2, gE1 and gE3) and estrogen sulfates (sE2, sE1 and sE3) in manure at 30-days after aerobic (a) or anaerobic (an) incubation. Both conjugates were comparably sensitive to manure processing; IV-*aerobic* and V-*anaerobic:* Time-course of change in cEs in sterilized-manure following aerobic or anaerobic incubations; values are mean ± SD, $n = 27$, ***** significant ($p < 0.05$) compared with control and x: significant when compared with corresponding plot 1 values for free estrogens and plot 7 values for conjugated estrogens.

4. Discussion

4.1. Manure Characteristics

Swine manure contains organic carbon, O-N, mineralized nitrogen compounds such as NH_4^+, NO_2^-, NO_3^-, NH_3, nitrogen gases, soil and other nutrients such as P, S and K, although their proportions may vary depending on its source, moisture content, storage, and handling methods. The C/N ratio for fresh manure was 17:1 that concurs with the values reported earlier [13,14]. A critical C:N ratio of about 15:1 is essential for efficient aerobic transformations of carbon and nitrogen, a higher C:N ratio is associated with (1) a decrease in ammonia emission, possibly due to an increase in nitrogen immobilization [14,15] and (2) an increase in odorous volatile organic acids during anaerobic

digestion [16,17]. An earlier study has shown an inverse relationship between C/N ratio and PAN [18,19]. Thus C/N ratio may be a rough indicator of PAN in manure.

Manure contains two forms of nitrogen: (i) plant-unavailable-nitrogen (PUA) such as O-N that accounts for almost 95% of total nitrogen; and (ii) PAN such as NH_4^+ and NO_3^- that account for <1% of total nitrogen in manure. Contrarily, earlier studies have reported equal proportions of O-N and PAN (NH_4^+ and NO_3^-) in swine manure [2,20]. This discrepancy may be due to differences in the animals' diet or the manures' pH that may range from 7 to 8.4, resulting in 1% to 5% loss of NH_4^+ via volatilization as NH_3. Livestock manure has been identified as a key source of NH_3 emission around the world [21].

In addition to plant nutrients and organic carbon, swine manure also contains conjugated (cE) and free (fE) forms of estrogens originating from animal urine and feces, respectively. Total estrogen concentrations observed in the present study were comparable to those reported by Combalbert *et al*. [7], Raman *et al*. [22] and Zhang *et al*. [23], but lower than those reported by Furuichi *et al*. [24]. The following patterns of estrogen concentrations were observed:

Manure liquid: cE1G > cE2G > cE1S > cE2S, cE3G, fE2, fE3 > fE1 > cE3S
Manure Solid: fE1 > fE2 > cE1S > cE1G > cE2G > fE3, cE3G > cE2S, cE3S

Similar to the present study, earlier studies have also shown cE1 and fE1 to be the major estrogens present in swine manure samples [7,23,25]. However, there were several folds differences in estrogen concentrations in swine manure, possibly due to: (1) rapid degradation of fE2 and fE1 bound to the soil [26–28]; (2) differential hydrolysis of cEs resulting in an increasing in free estrogens [29]; and (3) potentially higher mobility of conjugated estrogens compared to free estrogens (E2 and E1), because of their high water solubility [26,28,30–33]. Based on these observations, it was expected that cEs, being water soluble estrogens, will remain in the aqueous phase and leach with water flow, while fEs, being lipophilic, remain bound to the solid particles and leach slowly. Contrarily, the present study showed that fE and cEs both bound to manure solid (although fE binding > cE binding), possibly via different mechanisms: fEs bound to soil via hydrophobic interactions and cEs bound to soil via forming hydrogen bonds.

4.2. Plant Nutrients in Aerobic and Anaerobic Environment

General considerations: This study showed that O-N accounted for most of the nitrogen present in swine manure samples. Since O-N is PUN, manure is processed aerobically or/and anaerobically to convert O-N into PAN such as NH_4^+ and NO_3^- (Figure 1). In general, this is a multi-step process described below [34–38].

1. Heterotrophic bacteria mediated ammonification and/or denitrification of amino acids convert O-N into NH_4^+. Bacteria deamination of amino acids occur via oxidative deamination or anoxic deamination via sehydratase or sulfhydrase enzymes [39].
2. Oxidative nitrification converts NH_3 or NH_4^+ to nitrites (NO_2^-) according to the following equation: $NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$. *Nitrosomonas*, *Nitrosococcus*, and *Nitrosospira* are most frequently associated with this reaction, however, *Nitrosolobus* and *Nitrosovibrio* can also autotrophically oxidize ammonia [40]. This is followed by the oxidation of

nitrites to nitrates (NO_3^-) by *Nitrobacter*, *Nitrospira*, *Nitrococcus* [41] according to the equation: $NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$.

3. Anaerobic oxidation (Anammox) of NH_4^+ to N_2 with NO_2^- as the electron acceptor [42].
   $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$.
4. Denitrification in the presence of easily available carbon as an energy and carbon source and carried out by numerous facultative bacteria such as Pseudomonas [43]. In this process, nitrates and nitrites are reduced to nitrogen gas, such as N_2O and N_2, when a proton donor (energy source such as carbon) is available. The process involve the following pathway:
   $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$.
   In this process microorganisms first reduce nitrates to nitrites and then produce nitric oxide, nitrous oxide and nitrogen gas. A source of organic carbon is an important component of the denitrification process.

This suggests that manure storage conditions may determine whether manure nitrogen will be stabilized with accumulation of PANs or nitrogen will be lost as NH_3, N_2O and N_2.

Aerobic processing of manure nutrients: Aerobic incubation (i) transiently increased pH that converted NH_4^+ into NH_3 gas, resulting in significant decrease in NH_4^+/NH_3 ratio and increase in NH_3 nitrogen loss; and (ii) rapidly increased manure NO_3^- concentrations that peaked at day-10, then decreased gradually, reaching to basal levels at day-30. This suggests that O-N was converted into NO_2^- and NO_3^-, causing transient increase in NO_3^- concentrations and severe decrease in the NH_4^+/NH_3 ratio. Comparable aerobic processing of manure nitrogen has also been reported previously [44–48]. Studies have shown that during complete nitrification, NO_3^- can be reduced to NO by nitrite reductase, which is then reduced to N_2O by nitric oxide reductase [49,50]. During incomplete nitrification, however, ammonia mono-oxygenase may oxidize NH_3 sequentially to hydroxylamine and nitroxyl by hydroxylamine oxidoreductase, which is then converted to N_2O [51]. Approximately10% of O-N may be emitted in the form of N_2O [52–54]. This suggests that Aerobic environment may reduce the fertilizer value of swine manure. Since N_2O is considered to be an important factor in ozone depletion [55] aerobic oxidation may pose serious environmental threat.

Anaerobic processing of manure nutrients: Anaerobic digestion involves anoxic degradation of organic materials by microbial organisms that leads to the formation of biogas and stabilization of PAN such as NH_4^+ and NO_3^- [56].The present study showed that anaerobic incubation converted organic nitrogen (O-N) into NH_4^+ and reduced their pH to less than 7.0, thus stabilizing NH_4^+ in manure. This was confirmed by the observation that, following a rapid increase at day-1, NH_3 concentrations did not increase further. Recently Moller [57] has shown that the organic fraction remaining after anaerobic digestion is much more recalcitrant than the input manure leading to a stabilization of the organic matter. The present study also showed that NO_2^- was not detected, but NO_3^+concentrations began to increase at day-10, peaked at day-20 (about 270% of basal) and remained elevated thereafter. The time-course of increase in NO_3^+ and decrease in NH_4^+/NH_3 ratio were comparable. The decrease in NH_4^+/NH_3 ratio may be due to conversion of NH_4^+ to NO_3^- via bacterial thermophilic anaerobic deamination that also yields corresponding fatty acid [40,58]. In field applications, however, contradictory results regarding the effects of anaerobic digestion on NH_3

volatilization has been revealed: a decrease, an increase and no change have all been reported [59–65]. This may be due to differences in experimental design, soil composition and influx nitrogen composition.

4.3. Effects of Aerobic and Anaerobic Digestions on Estrogen Concentrations and Estrogenic Activity

Aerobic incubation: The present study showed that aerobic incubation (1) degraded cEs (cE2, cE1 > cE3); and (2) transiently increased fEs concentrations (fE1, fE2 > fE3) and ensuing estrogenic activity in manure samples, possibly due to diverse groups of aerobic bacteria that activated (resulted in an increase in estrogenic activity) and/or hydrolyzed (resulted in a decrease in estrogenic activity) estrogens [66–69]. In aerobic environment, aerobic bacteria were several folds more active than anaerobic ones [70]. A recent study has isolated bacteria that transformed fE1 into fE2, resulting in further increase in manures' estrogenic activity since a transformation of fE1 to fE2 results in two folds increase in estrogenic activity [71]. In addition, estrogen glucuronates and sulfates both exhibited comparable de-conjugation of conjugated estrogens in manure in oxidative and reductive environment, although D'Ascenzo *et al.* [72] and Hutchins *et al.* [73] have shown that sulfate-conjugated estrogen are more recalcitrant to biodegradation. Taken together, these observations suggest that an increase in estrogenic activity during aerobic incubation may be due to de-conjugation of estrogens and/or transformation of E1 to E2, while, the subsequent decrease in estrogenic activity may be due to hydroxylation of estrogens by mono- and di-oxygenases and ensuing decarboxylation of the phenolic groups [74]. Chemical hydrolysis contributes about 10% of total estrogen degradation without a decrease in estrogenic activity.

Anaerobic Incubation: Unlike the effects of aerobic environment, effects of anaerobic environment on estrogen biotransformation are not fully characterized: an increased, a decrease and no change in estrogen levels have all been reported [69,75,76]. In the present study, anaerobic incubation for 30 days caused about 20% to 30% decrease in cE concentrations without any increase in fE concentrations in manure samples. These observations concur with previous studies concerning estrogen degradation in manure incubated under anaerobic conditions [77] or in anaerobic plants processing active sludge [78,79]. Furuichi *et al*. [24] have shown that fE2 degraded more rapidly than fE1 or fE3, while de-conjugation of conjugated estrogens did not occur in up-flow anaerobic sludge biological (UABS) effluent. However, Shappel *et al*. [80] have shown comparative removal (approximately 90%) of all three estrogens from swine primary lagoon. Pholchan *et al*. [81] have also shown differential degradation of E1 and E2 (E3 was not reported) in different nitrite accumulating reactors. Unlike the earlier studies, the present study showed differential degradation of conjugated (glucoronate and sulfate conjugates) estrogens in manure exposed to anaerobic environment (cE2 > cE1 > cE3).

4.4. Environmental Consequences

Swine manure has been shown to contain both fEs and cEs (glucuronide/sulfate), and cEs, in the presence of manure glucuronidase and sulfatase, release fEs. Conjugated hormones, therefore, can be considered hormone reserve. Manure also contains two types of nitrogen compounds, O-N that is PUN and NH_4^+ nitrogen that is considered PAN. Untreated manure contained mostly O-N, thus nutrition value of untreated manure is limited. The present study showed that (1) aerobic processing of manure

degraded estrogens, transiently hydrolyzed cEs into fEs, and then degraded fEs, but severely reduced PAN (fertilizer value of manure) and (2) anaerobic processing of manure poorly degraded the hormones, did not induce hydrolysis of cEs, but increased PAN, thus increasing the fertilized value of manure. This distinct effect of aerobic and anaerobic manure processing on their estrogenic activity and fertilized capacity presents an interesting dilemma: Anaerobic incubation that increases manures' PAN does not degrade estrogens, while aerobic incubation that effectively degrades estrogens also decreases PAN, thus making manure less profitable. New techniques are need to fully use manure as organic fertilizer.

5. Conclusions

This study showed that anaerobic incubation stabilized both PAN that increased the fertilizer value and estrogens that increased the toxicity of manure samples, while aerobic processing decreased PAN thus decreasing the manure's fertilizer value, but increased estrogen degradation thus in decreasing the manure's toxicity. This suggests that aerobic or anaerobic processing may not convert manure into effective and safe fertilizer. A new approach such as an aerobic-anaerobic hybrid may be needed.

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Author Contributions

Manure processing and analysis were performed by the analytical technicians. The author supervised the technical staff, provided training, performed statistical analysis of the data and prepared the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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