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Rainfall and tillage effects on transport of fecal bacteria and sex hormones 17β-estradiol and testosterone from broiler litter applications to a Georgia Piedmont Ultisol

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ABSTRACT

Poultry litter provides nutrients for crop and pasture production; however, it also contains fecal bacteria, sex hormones (17β-estradiol and testosterone) and antibiotic residues that may contaminate surface waters. Our objective was to quantify transport of fecal bacteria, estradiol, testosterone and antibiotic residues from a Cecil sandy loam managed since 1991 under no-till (NT) and conventional tillage (CT) to which either poultry litter (PL) or conventional fertilizer (CF) was applied based on the nitrogen needs of corn (*Zea mays* L) in the Southern Piedmont of NE Georgia. Simulated rainfall was applied for 60 min to 2 by 3-m field plots at a constant rate in 2004 and variable rate in 2005. Runoff was continuously measured and subsamples taken for determining flow-weighted concentrations of fecal bacteria, hormones, and antibiotic residues. Neither *Salmonella*, nor *Campylobacter*, nor antimicrobial residues were detected in litter, soil, or runoff. Differences in soil concentrations of fecal bacteria before and after rainfall simulations were observed only for *Escherichia coli* in the constant rainfall intensity experiment. Differences in flow-weighted concentrations were observed only for testosterone in both constant and variable intensity rainfall experiments, and were greatest for treatments that received poultry litter. Total loads of *E. coli* and fecal enterococci, were largest for both tillage treatments receiving poultry litter for the variable rainfall intensity. Load of testosterone was greatest for no-till plots receiving poultry litter under variable rainfall intensity. Poultry litter application rates commensurate for corn appeared to enhance only soil concentrations of *E. coli*, and runoff concentrations of testosterone above background levels.

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1. Introduction

The U.S. poultry industry generates annually millions of tons of litter consisting of feces, bedding material, and feathers. Litter serves as a nutrient source for plants, and is frequently applied to agricultural fields as fertilizer (Moore et al., 1995). Litter can also contain pathogenic bacteria *Salmonella*, *Campylobacter*, and *Clostridium perfringens* (Kelley et al., 1994; Jeffrey

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et al., 1998), fecal indicator bacteria *Escherichia coli* and fecal enterococci, and gender regulating hormones 17β-estradiol and testosterone (Shore et al., 1993) which are considered potent endocrine disrupting compounds when distributed in the environment (Lintelmann et al., 2003). Antibiotics are often used in poultry feed at sub-therapeutic concentrations to promote growth (Levy, 1998), and excreted antibiotic residues may make their way to surface water (Kolpin et al., 2002). Runoff from agricultural fields with poultry litter applications may contaminate recreational and municipal drinking waters. Jenkins et al. (2006), for example, reported concentrations of fecal indicator bacteria greater than background concentrations in storm runoff three weeks after litter application to four cropped catchments under conservation tillage in the Southern Piedmont, but observed no significant increase in levels of estradiol or testosterone.

Other researchers have reported the presence of fecal bacteria from poultry litter in runoff from cropped fields and pastures (Giddens and Barnett, 1980; Edwards and Daniels, 1994; Coyne and Blevins, 1995). Giddens and Barnett (1980) simulated rainfall on fallow soil and Coastal bermudagrass with various application rates of poultry litter, and enumerated total coliforms in runoff. Concentrations of total coliforms in runoff increased with increasing tonnage of applied litter. Currently *E. coli* and fecal enterococci are the preferred fecal indicator bacteria (USEPA, 1986), and it is likely that runoff from agricultural fields with applied broiler litter could have unacceptable concentrations of these fecal indicator bacteria, resulting in impaired recreational and drinking waters.

Estrogen concentrations have been observed to range from 14 to 65 μg kg^{-1} dry weight of litter, and testosterone concentrations have been observed to be as high as 133 μg kg^{-1} dry weight of litter (Shore et al., 1993). These naturally occurring gender regulating hormones have been detected in surface waters across the U.S. and Europe (Adler et al., 2001; Kolpin et al., 2002). Presence of these hormones in surface waters can adversely affect both aquatic life and human health (Sharpe and Skakkebaek, 1993; Toppari et al., 1996; Harrison et al., 1997; Epstein, 1997; Tyler and Routledge, 1998).

Past studies have reported on the transport of estradiol and testosterone in runoff from fields receiving poultry litter applications (Nichols et al., 1997, 1998; Finlay-Moore et al., 2000). Shore et al. (1995) reported estrogen (presumably estradiol and estrone) concentrations ranging from 4 to 23 ng l^{-1}, and testosterone concentrations ranging from 1 to 34 ng l^{-1} of pond water receiving runoff from fields to which poultry litter had been applied. Nichols et al. (1997) performed rainfall simulations and demonstrated that runoff concentrations of estradiol increased with increased application of poultry litter. In another rainfall simulation study, Nichols et al. (1998) looked at the effects of grass buffer strips on concentrations of estradiol in runoff from litter applications, and demonstrated significant decreases in estradiol concentrations with increased widths of the buffer strips.

Various forms of conservation tillage including no-till agriculture have been widely adopted in many parts of the United States (Endale et al., 2002). Conservation tillage systems accumulate residue and organic carbon at the soil surface with time, and, thus, are effective in increasing infiltration and reducing runoff and soil loss attributable to dissipated raindrop impact energy, increased aggregate stability/soil resistance and water dispersible clay (Langdale et al., 1979; Edwards et al., 1992; Bruce et al., 1995; Reeves, 1997; Shaw et al., 2002; Truman et al., 2003, 2005, 2007; Causarano et al., 2006). With conservation tillage systems poultry litter is surface applied and not incorporated. This practice may increase the risk of transported bacteria, hormones, and pharmaceutical residues during runoff events.

Concentrations of pathogens, fecal indicator bacteria and hormones have been measured in runoff generated from simulated rainfall immediately after litter applications. However, no rainfall simulation studies have investigated the effects of poultry litter application on *Salmonella*, *Campylobacter*, *C. perfringens*, *E. coli*, fecal enterococci, estradiol, testosterone and antibiotic residues in runoff from cropped fields. Furthermore, rainfall simulation studies that Giddens and Barnett (1980) and Nichols et al. (1997, 1998) reported used a constant intensity rate. Natural rainfall, however, is characterized by wide variations in temporal and spatial intensity (Bosch et al., 1999). Runoff, sediment, carbon, and herbicide losses were greater for variable intensity rainfall simulations than for constant intensity rainfall simulations (Potter et al., 2006; Truman et al., 2007).

The objective of this study was to determine the impact of tillage systems on the transport of bacterial pathogens, fecal indicator bacteria, estradiol, testosterone and antibiotics from poultry litter applications at rates commensurate with nutrient requirements of corn under no-till and conventional tillage management. Two experiments were conducted, one at a constant rainfall intensity, and the second at a variable rainfall intensity based on most frequent spring time storm rainfall intensity patterns.

2. Materials and methods

2.1. Study site and management

The study was conducted at the USDA-ARS J. Phil Campbell, Sr., Natural Resource Conservation Center, Watkinsville, GA (83°24′ W and 33°54′ N) in 2004 and 2005. The experimental design was a split-plot of three replications. Twelve 12×30.5 m^2 plots are located on nearly level (0 to 2% slope) Cecil sandy loam (fine, kaolinitic, thermic, Typic Kanhapludults). Bruce et al. (1983) described in detail the characteristics of the soil at the site. Each plot has been under either a conventional tillage or no-tillage management since 1991. Tillage system makes up the main plots. The conventional tillage (CT) management practice consisted of 30-cm deep chisel plowing, followed by disc harrowing to a depth of 20 cm, and subsequent disking to 8 cm for seeding. On no-till (NT) plots a coulter disc planter was used for planting. Subplots were two fertilizer treatments: poultry litter (PL) and conventional fertilizer (CF) as ammonium sulfate applications to supply N at 168 kg ha^{-1} (224 kg ammonia sulfate ha^{-1}). All plots had a winter cover crop of rye (*Secale cereale* L.).

Three weeks before the planned rainfall simulations glyphosate was used to kill the winter rye crop on all plots. A week before the simulations the CT plots were chisel plowed

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and harrowed. The day before the rain simulation, ammonium sulfate (224 kg ha^{-1}), phosphate (56 kg ha^{-1}), and potassium (45 kg ha^{-1}), or poultry litter (Table 1) were applied to the designated conventionally tilled plots, which were then disk harrowed. On the no-till plots mineral fertilizer or poultry litter were applied by hand to the area confined by the 2×3 m steel frame as well as a 3×5 m area surrounding the frame from which soil samples were obtained before simulating rainfall. The rate of poultry litter application was determined to match the N application of the mineral fertilizer based on the potential mineralization of N in the litter which, according to Ritz and Merka (2004), ranges between 40 and 70%.

2.2. Rain simulation and sampling

Rainfall simulation plots consisting of a stainless steel frame 2-m wide by 3-m long by 15-cm high were established on the 12 experimental plots. In April, 2004, 12 simulation plots were evaluated with a constant intensity rainfall pattern. In April, 2005, 12 rainfall simulation plots were evaluated with a variable intensity rainfall pattern. Each simulator plot had a 2% slope and consisted of a wheel track in the middle with half beds on either side. An area surrounding each 6-m^2 simulator plot was treated like the test area to allow soil and water to be splashed in all directions. This border area also received the same distribution of simulated rainfall.

The oscillating nozzle rainfall simulator (Frauenfeld and Truman, 2004; Truman et al., 2007) was placed 3 m above each 6-m^2 plot. Simulated rainfall was applied at a constant (target intensity of 53 mm h^{-1}, Ave. intensity of 56.3 mm h^{-1}) and variable intensity rainfall pattern. Determination of the variable rainfall pattern has been described (Frauenfeld and Truman, 2004; Strickland et al., 2005; Truman et al., 2007). The characteristics of the simulated storm were determined from rainfall data at Athens, GA with an actual storm programmed into the simulator computer on a 1-min basis as the variable rainfall intensity pattern. The actual pattern was like one Truman et al. (2007) reported. Total rainfall volume of the variable intensity rainfall experiment (mean = 1073 ml, cv=4%) was similar to that of the constant rainfall intensity experiment (mean=1026, cv=4%). Water for all simulations was obtained from the public water supply and passed through reverse osmosis filters and held in tanks.

Before simulating rainfall, soil samples from ten locations around each rainfall simulation plot were taken from the upper 2.5 cm with a flame sterilized soil corer, placed in sterile plastic bags, mixed thoroughly, and stored on ice until taken to the lab for analysis. Subsamples for hormone analysis were stored at – 20 °C until analyzed. Antecedent water content was determined on soil samples taken before simulating rainfall and dried at 105 °C. Thirty minutes after ending the rainfall simulation ten surface soil samples (2.5 cm depth) were taken from inside the simulation plots for microbiological, hormone and antibiotic analyses, and subsamples were dried at 105 °C to estimate final water content.

2.3. Microbiological analyses

E. coli and fecal enterococci in runoff, soil, and litter samples were measured with commercial Colilert® and Enterolert® kits (IDEXX, Atlanta, GA). These kits represent a defined substrate technology (Edberg and Edberg, 1988; Edberg et al., 1988, 1990). Both Colilert® and Enterolert® are semi-automated most probable number (MPN) methodologies. For both litter and soil, 10 g fresh weight were suspended in 90 ml of sterile phosphate buffered saline (PBS) (Clesceri et al., 1998) and shaken 100× by hand. Ten ml subsamples from appropriate ten-fold dilutions of soil and litter suspensions and runoff samples were added to 90 ml of Colilert and Enterolert substrate. The inoculated substrate was then poured into a Quanti-Tray®, sealed, and incubated for 24 h at 35.5 °C for Colilert® and 24 h at 41 °C for Enterolert®. At the end of the incubation trays were read according to the manufacturer's guidelines and a most probable number per 100 ml runoff and per g soil and litter was derived. Soil and runoff samples analyzed by Colilert® and Enterolert® methodology that resulted in no cells detected were considered to have a concentration of at most 0.5 MPN g $soil^{-1}$ or 0.5 MPN 100 ml $runoff^{-1}$.

To make cultural analysis and species confirmation for *C. perfringens*, all samples were subjected to both direct selective cultural microbial analysis as well as a non-selective enrichment technique (Craven, 2000). Twenty-five grams of soil or litter samples were delivered aseptically to one quart size plastic ziploc bag (Reynolds, Inc., USA) and diluted with 250 ml of Membrane *C. perfringens* (mCP) phosphate buffer, pH 7.2 (USEPA, 1995) and mixed by shaking and hand mixing for 1–2 min. The sample was allowed to settle briefly and replicate 100 μl aliquots were spread plated on Tryptone Sulfite Cycloserine (TSC) agar (Oxoid Ltd., Basingstoke, England, UK) and mCP agar (Oxoid, Ltd.) and incubated in an anaerobic jar (Oxoid, Ltd.) at 37 °C for 48 h. Four ml of the mixed sample was simultaneously inoculated into 11.0 ml of freshly prepared and reduced Iron Milk Medium (IMM) (USEPA, 1995) in 16×150 mm

Table 1 – Rates of poultry litter (PL) (dry weight basis), total load of *E. coli* (Ec), fecal enterococci (FE), *Salmonella* (Salmon), *Campylobacter* (Campy), *C. perfringens* (Cp), estradiol, testosterone applied to experimental plots for the 2004 constant intensity rainfall experiment and the 2005 variable intensity rainfall experiment

Experiment	Litter	Ec	FE	Salmon	Campy	Cp	Estradiol	Testosterone
	kg ha^{-1}	Log_{10} MPN ha^{-1}		Log_{10} cells ha^{-1}			mg ha^{-1}	
2004	11.2	11.6	12.4	ND[a]	ND	10.8	21.0	8.4
2005	11.2	10.7	11.8	ND	ND	9.4	9.1	1.4

[a] Not detected.

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screw capped tubes and incubated under anaerobic conditions (Anaerogen, Oxoid, Ltd.) at 37 °C for 48 h. Following IMM enrichment, a 100 μl aliquot was streaked for isolation to TSC agar and incubated as described above. Unfiltered runoff water samples were agitated to resuspend sediment and direct spread plated on TSC agar (100 μl aliquots) and mCP agar (1.0 ml aliquot) as well as inoculated into IMM for non-selective enrichment and subsequent selective agar plating.

Presumptive colonies and all IMM enrichment cultures were further analyzed by a species specific *C. perfringens* PCR reaction on either an isolated presumptive colony or an aliquot of the non-selective enrichment culture. The PCR assay was performed using primers previously reported (Kikuchi et al., 2002) with Promega Master Mix (Promega, Inc., Wisconsin, USA). *C. perfringens* species specific primers (ClPER-F 5′-AGA TGG CAT CAT CAT TCA AC3′ and ClPER-R 5′-GCA AGG GAT GTC AAG TGT-3′) were synthesized by Invitrogen (Invitrogen, Inc., California, USA). Amplifications were performed using an MJR PTC-200 thermocycler according to Kikuchi et al. (2002). *C. perfringens* 793 bp characteristic amplicons were detected by agarose gel analysis by comparing to a DNA 100 to 1000 bp ladder standard (Bio-Rad, Carlsbad, CA, USA). Suspensions of IMM enrichments (50 μl in 450 μl molecular biology grade water) and suspensions of putative *C. perfringens* colonies (in 100 μl molecular biology grade water) were boiled for 10 min. Each PCR reaction mixture contained 5 μl of each respective boiled sample as template. The detection limit of *C. perfringens* was 5 cells ml^{-1} runoff and g^{-1} soil. This limit of detection was determined to be the minimum number of viable cells that would reach a detectable number in the IMM enrichment medium. If the culture-based methods were negative but positive by the PCR method, the concentration of *C. perfringens* was recorded at the detection limit. If both culture-based and PCR methods were negative, the concentration was set at 0.5 cells ml^{-1} runoff and g^{-1} soil.

For enumerating *Campylobacter* and *Salmonella*, litter and soil samples were homogenized inside Whirl-Pak® bags by hand. Ten grams of litter and soil were each placed in duplicate 50 ml of sterile Bolton broth with antibiotics for *Campylobacter* analysis and duplicate 50 ml of double strength buffered peptone water (BPW) for *Salmonella* analysis. The Bolton slurry was incubated microaerobically (5% O_2, 10% CO_2, 85% N_2) at 42 °C for 24 h; after incubation the slurry of *Campylobacter* was streaked on Campy Line Agar® (CLA), and plates were incubated microaerobically at 42 °C for 48 h. Following incubation, typical *Campylobacter* colonies were enumerated and confirmed by microscopic wet mount and latex agglutination using a Microscreen *Campylobacter* Agglutination Kit® (Microgen Bioproducts, Camberley, Surrey, UK) for confirmation as necessary. Each of the three *Campylobacter* recovery methods described were successful in recovering *Campylobacter* from water samples spiked with 10^2 ml^{-1} *C. jejuni* (data not shown). Following incubation of the BPW slurry of *Salmonella*, 1.0 ml of the slurry was transferred to 9 ml of Tetrathionate enrichment broth (TTB) and incubated aerobically for 24 h at 37 °C, after which 0.1 ml of broth was streaked on replicate Brilliant Green Sulfa (BGS) agar and Modified Lysine Iron Agar (MLIA) plates. Typical colony forming units of *Salmonella* were confirmed by biochemical characterization (growth and H_2S production on Triple Sugar Iron agar, and lysine decarboxylase activity and H_2S production on Lysine Iron agar) and serology (Clesceri et al., 1998). From composite runoff samples, 10-ml aliquots were added to 10 ml of double strength BPW and incubated and confirmed as described above for *Salmonella* and *Campylobacter*.

2.4. Analysis of estradiol and testosterone

Estradiol and testosterone concentrations in soil, litter and unfiltered runoff were measured with a commercial competitive enzyme-linked immunosorbent assay (EIA) (Caymen Chemical Company, Ann Arbor, MI). Soil, litter, and runoff samples were stored at – 80 °C before their analysis. Hormones were extracted from 1-g subsample of air-dried and sieved (2-mm) composite soil sample and 1-g composite litter with 5 ml of ethylacetate in 15-ml glass centrifuge tubes. Tubes were secured on a reciprocal shaker and shaken at high speed for 60 min, then centrifuged at 480 ×*g* at 10 °C for 30 min. The solvent phase was removed and evaporated under a stream of ultrapure N_2. Residue was then dissolved in the manufacturer's enzyme immunoassay buffer and hormone content measured as described above. The assay procedure for runoff, soil and litter samples has been described in detail by Finlay-Moore et al. (2000). We performed the assay according to the manufacturer's protocol. Wells were washed with an automatic strip washer (AM60 Multi-Reagent Washer, Dynex Technologies, Chantilly, VA) to remove unbound reagents. Color intensity of reactions between free hormones and tracer was measured on a spectrophotometer (μQuant, Bio-Tek Instruments, Winooski, VT) with intensity being inversely proportional to free estradiol or testosterone. Limit of quantification for estradiol and testosterone were 8 and 6 pg ml^{-1}, respectively.

2.5. Analysis of antimicrobials

Runoff, chicken litter, and soil samples were analyzed with the CHARM system (CHARM Sciences, Inc., Lawrence, MA). Antimicrobials assayed for included β-lactams, Tetracyclines, Sulfonamides, and Macrolides. Runoff samples were processed for analysis as described by Meyer et al. (2000). Between 5 and 10 g subsamples of litter and soil were extracted with solvents appropriate for each antimicrobial in a weight to volume ratio of 1:2. For β-lactams, Tetracyclines, Sulfonamides, and Macrolides the following solvents were used: in 1:1 (vol:vol) ratios, 73.1 mM KH_2PO_4 (pH 6):methanol, 0.1 M HCl:methanol, 3.1 M NaCl, 20 mM $CaCl_2$:H_20, and 73.1 mM KH_2PO_4 (pH 8):methanol, respectively. Soil was homogenized in a stomacher for 2 min and litter was homogenized on a reciprocal shaker for 10 min. Solid material was allowed to settle, and several milliliters of extract were removed and centrifuged at 600 ×*g* to remove particles. The processed runoff, litter, and soil samples were then assayed for the antimicrobials as described by Meyer et al. (2000). Limit of detection of the antimicrobials was around 1 ppb (Meyer et al., 2000).

2.6. Data analysis

The load of *E. coli*, fecal enterococci, *C. perfringens*, estradiol and testosterone removed from plots for each runoff event was the product of their concentrations for each composited

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increment of runoff collected between time intervals 5 to 15, 20 to 30, 35 to 45, and 50 to 60 min and runoff volume of the respective time intervals. Flow-weighted concentrations of *E. coli*, fecal enterococci, *C. perfringens*, estradiol, and testosterone were calculated by dividing the total load of each analyte by the total amount of runoff. Before analysis of variance and regression analyses the MPN determinations for *E. coli* and fecal enterococci, and total colony forming units (cfu) of *C. perfringens* were normalized by natural log transformation. Because hormone data displayed a Poisson distribution, original values were transformed for statistical analysis using the equation $X' = (X + 0.5)^{1/2}$ (Zar, 1999). No direct statistical comparisons between the constant and variable rainfall experiments are appropriate since they were conducted in separate years. Variables such as antecedent moisture and surface residue may have altered runoff characteristics between years. Comparisons among the experiments would be confounded with the method of applying the rainfall and could result in differences that were artifacts. Analysis of variance was conducted in both constant and variable intensity rainfall experiments with significant differences ($P = 0.05$) tested between main effect means, tillage (Till), fertilization (Fert), and Till × Fert interaction (NTPL, NTCF, CTPL, CTCF) using Proc Mixed (SAS, 2004; Littell et al., 2006). Relationships between runoff volumes, and soil and runoff concentrations of the fecal bacteria and hormones were analyzed with Proc Reg (SAS, 2004).

3. Results

3.1. Poultry litter and runoff

Although the same rates of poultry litter were applied in each rain simulation study, the concentrations of *E. coli*, fecal enterococci, *C. perfringens*, and the two gender regulating hormones were greater in the litter applied in 2004 than in 2005 (Table 1). *Salmonella*, *Campylobacter*, and antibiotic residues were not detected in either of the two batches of poultry litter.

Total runoff for the 2004 constant intensity rainfall experiment showed tillage differences; whereas, no differences between main effects were observed for the 2005 variable intensity rainfall experiment (Table 2).

Table 2 – Mean total runoff volumes from conventional tillage and no-tillage treatments for constant and variable rainfall intensity experiments of 2004 and 2005, respectively

Rainfall experiment	Tillage treatments	
	Conventional tillage	No-till
	Liters	
Constant intensity	176.3[a]	35.6[b]
Variable intensity	208.7[a]	175.1[a]

Means not followed by the same letter per rainfall experiment indicate a significant difference at $P < 0.05$.

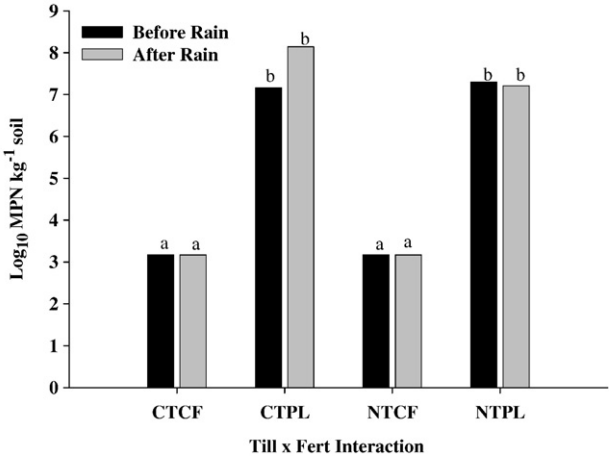

Fig. 1 – Soil concentrations of *E. coli* of the four main tillage × fertilization interactions (NTPL, NTCF, CTPL, CTCF) for before and after the constant intensity rainfall experiment of 2004. Means followed by different letters indicates a significant difference at $P \leq 0.05$.

3.2. Soil concentrations of fecal indicator bacteria and hormones before and after rain simulations

Differences in soil concentrations of the fecal bacteria before and after rain simulation were observed only for *E. coli* during the constant intensity rainfall experiment (Fig. 1). No differences in soil concentrations of *E. coli* were observed between tillage treatments that received conventional fertilizer; they were, however, less than soil concentrations of *E. coli* for the two tillage treatments that received poultry litter (Fig. 1). No differences were observed in soil concentrations of *E. coli*, fecal enterococci, or *C. perfringens* before and after the variable intensity rainfall experiment. The range of background soil concentrations of *E. coli*, fecal enterococci, and *C. perfringens* was from 2.7 to 4.1 $\log_{10}$ MPN kg^{-1} soil, from 6.3 to 8.2 $\log_{10}$ MPN kg^{-1} soil, and from 2.4 to 5.7 $\log_{10}$ cfu kg^{-1} soil, respectively. Differences in soil concentrations of estradiol and testosterone were not observed for before and after either constant or variable intensity rainfall simulations. The range of background soil concentrations of estradiol and testosterone was from <6.0 to 176.8 ng kg^{-1} of soil, and from 8.8 to 172.5 ng kg^{-1} of soil, respectively. Neither *Salmonella*, nor *Campylobacter*, nor targeted antibiotic residues were detected in soils sampled.

3.3. Runoff concentrations of fecal bacteria and hormones

Neither tillage, fertilization, nor the interaction between tillage and fertilization affected flow-weighted concentrations of *E. coli*, fecal enterococci, *C. perfringens*, and estradiol for either simulated rainfall experiment. Range of concentrations for *E. coli* was from – 0.3 to 3.7 $\log_{10}$ MPN 100 ml^{-1}, for fecal entercocci 2.3 to 5.5 $\log_{10}$ MPN 100 ml^{-1}, for *C. perfringens* 2.7 to 3.1 $\log_{10}$ cfu l^{-1}, and for estradiol 6.0 to 145 ng l^{-1}. Tillage, fertilization, and the interaction of the two main effects affected the flow-weighted concentration of testosterone in both constant and variable intensity rainfall simulations (Fig. 2). Excepting the no-till plots receiving poultry litter,

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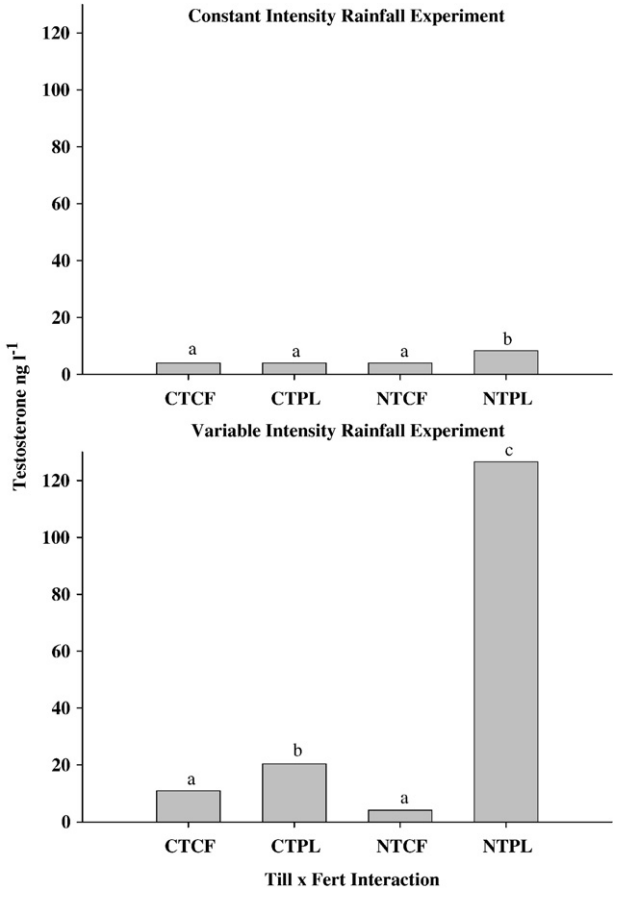

Fig. 2 – Flow-weighted concentrations of testosterone associated with the four tillage × fertilization interactions (NTPL, NTCF, CTPL, CTCF) for constant intensity and variable intensity rainfall experiments. Means followed by different letters indicates a significant difference at $P \leq 0.05$.

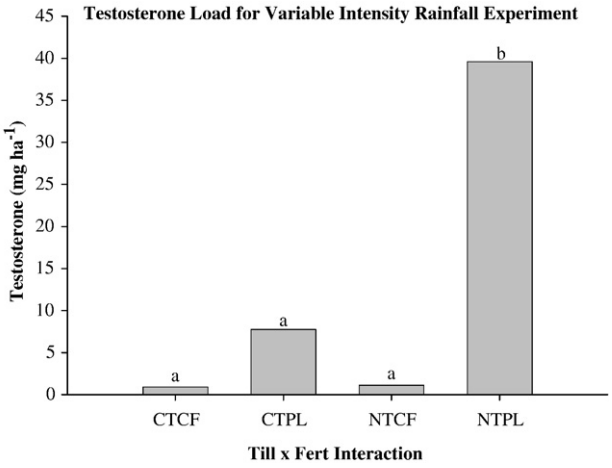

Fig. 3 – Total load of testosterone associated with the four tillage × fertilization interactions (NTPL, NTCF, CTPL, CTCF) for the variable intensity rainfall experiment. Means followed by different letters indicates a significant difference at $P \leq 0.05$.

flow-weighted concentrations of testosterone were very low for both experiments and near the limit of detection for the constant intensity rainfall experiment. Differences in flow-weighted concentrations of testosterone between the tillage and fertilization treatments of the variable intensity rainfall experiment were larger than in the constant intensity rainfall experiment. The treatment interactions in both experiments were the result of relatively greater concentrations of testosterone found in the combination of no-till and poultry litter although the effect was small in the constant intensity rainfall experiment (Fig. 2). Neither *Salmonella*, nor *Campylobacter*, nor the targeted antibiotic residues were detected in the increments of runoff sampled.

Total load of *E. coli* and fecal enterococci for both constant and variable intensity rainfall experiments ranged from 5.9 to 10.2 $\log_{10}$ MPN ha^{-1}, and from 8.1 to 12.1 $\log_{10}$ MPN ha^{-1}, respectively. Although no differences in flow-weighted concentrations of these fecal indicator bacteria were observed between tillage and tillage by fertilization interactions, fertilization affected their total loads in the variable intensity rainfall experiment ($P=0.02$) but not in the constant rainfall intensity experiment. No differences in load for *C. perfringens* (which ranged from 8.4 to 9.1 $\log_{10}$ cfu ha^{-1}) were observed between treatment effects (data not shown). The total load of testosterone for the variable intensity rainfall experiment (which ranged from 1.10 to 39.2 mg ha^{-1}) (Fig. 3) paralleled the flow-weighted testosterone concentrations; whereas, the range of testosterone loads from the constant intensity rainfall experiment was between 0.30 and 1.3 mg ha^{-1} with no differences between tillage or fertilization treatments. No differences in total load of estradiol (which ranged from 0.58 to 58.8 mg ha^{-1}) were observed between treatments for either rainfall experiments.

Significant correlation coefficients were observed between total load of estradiol and testosterone and total volume of runoff for the constant intensity rainfall experiment (Table 3). A significant correlation coefficient was also observed between the total load of fecal enterococci and total runoff volume for the variable intensity rainfall experiment (Table 3). No significant correlation coefficients were observed between the fecal bacteria and total volume of runoff for the constant intensity

Table 3 – Correlation coefficient (R) from linear regression analyses of total load of *E. coli* (EC), fecal enterococci (FE), *C. perfringens* (CP), estradiol and testosterone against total volume of runoff for each rainfall intensity experiment

Rainfall simulation experiment	Bacterium/ hormone	R	Pr>F
Constant	EC	0.06	0.89
	FE	0.39	0.33
	CP	0.03	0.94
	Estradiol	0.75	0.03
	Testosterone	0.99	0.0001
Variable	EC	0.40	0.19
	FE	0.74	0.006
	CP	0.49	0.11
	Estradiol	0.25	0.46
	Testosterone	0.07	0.84

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rainfall experiment, nor between *E. coli*, *C. perfringens*, estradiol, and testosterone for the variable intensity rainfall experiment.

4. Discussion

4.1. Broiler litter characteristics

Broiler litter for 2004 and 2005 rain simulation studies were obtained from two different commercial broiler producers. That *Salmonella* and *Campylobacter* were not detected in either batch of litter may not be unusual; Jenkins et al. (2006) reported not detecting either of these pathogens in two different batches of broiler litter, and Omeira et al. (2006) reported not detecting *Salmonella* in fresh litter from houses of different broiler operations. Failure to detect antibiotic residues in the two batches of litter does not exclude the possibility that sub-therapeutic doses of antibiotics were a part of flock management, just as the detection and enumeration of antibiotic resistant genes in gram-positive bacteria in poultry litter does not necessarily indicate that the flocks were administered antibiotics (Nandi et al., 2004).

Greater concentrations of gram-positive fecal enterococci than gram-negative *E. coli* was a pattern that has been observed before (Nandi et al., 2004; Jenkins et al., 2006; Omeira et al., 2006). Observed litter concentrations of *C. perfringens* were an order of magnitude less than that of *E. coli*, and were greater than concentrations that Omeira et al. (2006) observed in several different sources of broiler litter.

Variations in concentrations of estradiol and testosterone in poultry litter have previously been reported (Jenkins et al., 2006; Hemmings and Hartel, 2006). Rates of litter application and loads of hormones applied in this study were greater than the rates and loads that Jenkins et al. (2006) had applied to four small catchments, but substantially less than concentrations of these hormones in broiler litter that Finlay-Moore et al. (2000), Nichols et al. (1997, 1998), and Shore et al. (1993) reported. In contrast to the 21.0 and 9.4 mg estradiol ha^{-1} and 8.4 and 1.4 mg testosterone ha^{-1} we applied in 2004 and 2005, respectively, Finlay-Moore et al. (2000) applied between 50 and 180 mg estradiol ha^{-1} and between 50 and 280 mg testosterone ha^{-1} which corresponded to a litter application of 1760 and 4750 kg ha^{-1}, respectively. Nichols et al. (1998) applied a load of 234 mg estradiol ha^{-1} which corresponded to a litter application of 1760 kg ha^{-1}. These differences in litter applications and their respective differences in load of hormones applied appear to be indicative of a wide range of hormone concentrations within litters of different operations. These differences may be the result of differences in flock management or physiology of the flock itself, and not differences in mineralization of the hormones since Hemmings and Hartel (2006) reported minimal hormone degradation within poultry litter under controlled temperature and moisture conditions.

4.2. Soil concentrations of fecal bacteria and hormones

The load of *E. coli* applied with the poultry litter for the 2004 constant intensity rainfall experiment increased soil concentrations of *E. coli* above background levels as observed for the conventional fertilizer treatments. Incorporation of poultry litter with tillage, as opposed to no-till, did not affect the soil *E. coli* concentration before or after the constant intensity rainfall experiment. The load of *E. coli* applied with poultry litter for the variable intensity rainfall experiment did not enhance the soil *E. coli* population above background levels either before or after the simulated rainfall. The *E. coli* concentration of the poultry litter for the variable intensity rainfall experiment was less than the poultry litter for the constant intensity rainfall experiment, and may have been below a threshold concentration that would enhance background soil *E. coli* concentrations. The *E. coli* population associated with the poultry litter application for the variable intensity rainfall experiment may not have been as robust as the population associated with the poultry litter application for the constant intensity rainfall experiment, and may have died off more rapidly. Environmental factors such as differences in solar UV radiation, or differences in antagonistic soil microbial communities at the time of poultry litter application may have contributed to variation in *E. coli* survival. The range of background soil *E. coli* concentrations (from 2.7 to 4.1 $\log_{10}$ MPN $kg\ soil^{-1}$) both before and after the constant and variable intensity rainfall experiments was comparable to background concentrations that Jenkins et al. (2006) reported for four cropped catchments close by the current research site. The load of fecal enterococci and *C. perfringens* added to soil with each poultry litter application did not enhance the background soil concentration of these microbial communities. Background concentrations of fecal enterococci determined for the two rainfall simulations were within the range that Jenkins et al. (2006) observed for Cecil soils in a cropped watershed that had received regular poultry litter applications.

Loads of estradiol and testosterone added to the soil with the two poultry litter applications did not increase background soil concentrations of these hormones. The range of background soil concentrations of estradiol and testosterone observed were within the range and comparable to the soil estradiol and testosterone concentrations that Finlay-Moore et al. (2000) observed in hayed and grazed fields in the Southern Piedmont. Considering that the time between poultry litter applications and soil sampling before simulating rainfall was a matter of minutes for no-till treatments and only a few hours for conventional tillage treatments, the observed kinetics of abiotic transformation of estradiol to estrone and biodegradation of estrone (Colucci et al., 2001) and dissipations of testosterone (Lorenzen et al., 2005) in agricultural soil could be minimal. Furthermore, incorporation of poultry litter in top soil with conventional tillage did not decrease soil concentrations of estradiol and testosterone compared to no-till treatments. This lack of difference between conventional tillage and no-till treatments may be associated with the relatively small quantity of the load of hormone with the poultry litter application.

4.3. Runoff concentrations of fecal bacteria and hormones

Differences in soil concentrations of *E. coli* between treatments for the constant intensity rainfall experiment were not reflected in subsequent flow-weighted concentrations

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of *E. coli* in runoff. The range of flow-weighted concentrations of *E. coli* and fecal enterococci was comparable to concentrations that Jenkins et al. (2006) observed from natural storm events. The range of flow-weighted estradiol concentrations was comparable to those that Finlay-Moore et al. (2000) observed for no-litter control fields, and was within the range that Jenkins et al. (2006) observed in runoff from small watersheds during storm events. The linear correlation between total loads of estradiol and testosterone and total runoff for the constant intensity rainfall experiment may be attributable to the constancy of the rainfall intensity. The differences between constant and variable intensity rainfall experiments had no effect on the flow-weighted concentrations of *E. coli*, fecal enterococci, *C. perfringens*, or estradiol. Although no differences were observed between treatments for soil concentrations of testosterone, the differences in flow-weighted concentration of testosterone in both experiments may be attributable to the chemical characteristics of testosterone and its interactions with soil. Testosterone is more soluble than estradiol, has a smaller partitioning coefficient and thus sorbs less strongly to soil (Sangsupan et al., 2006), and is transported out of the near soil surface more readily than estradiol (Lange et al., 2002). Since the testosterone load was less for the variable intensity rainfall experiment than the constant intensity rainfall experiment (Table 1), the overall increase in flow-weighted concentration of testosterone in the variable intensity rainfall experiment may be attributable to the increased physical force associated with its high peak of rainfall intensity. Difference between conventionally tilled and no-till plots receiving poultry litter for both constant and variable intensity rainfall experiments may be attributable to the difference between the poultry litter being incorporated into soil as opposed to its remaining exposed on the soil surface. The range of flow-weighted testosterone concentrations was comparable to flow-weighted concentrations that Finlay-Moore et al. (2000) reported for no-litter control fields and flow-weighted concentrations that Jenkins et al. (2006) reported, with the exception of the relatively high flow-weighted concentration from the no-till plots receiving poultry litter and the variable rainfall intensity experiment.

Total loads of *E. coli* and fecal enterococci were within the range of *E. coli* and fecal enterococci loads associated with runoff from several rain events that Jenkins et al. (2006) reported for four small Southern Piedmont catchments. Although poultry litter did not affect flow-weighted concentrations of *E. coli* and fecal enterococci, the greater load of *E. coli* and fecal enterococci observed for both conventionally tilled and no-till plots receiving poultry litter for the variable intensity rainfall experiment indicated that runoff volume can affect the total load of a pollutant. Given that the loads of *E. coli* and fecal enterococci were comparable to loads associated with runoff from actual rain events and on a catchment-scale, we may infer that the range of the *C. perfringens* load is probably comparable to catchment-scale loads under actual rainfall conditions. With the exception of the testosterone load for the no-till plots receiving poultry litter in the variable intensity rainfall experiment, the total loads of estradiol and testosterone were within the range that Jenkins et al. (2006) reported for significant storm event runoff from a 2.7 ha catchment.

5. Summary and conclusion

Differences in concentrations of fecal bacterial communities and sex hormones between litters from different broiler operations may have complicated results. The failure to detect *Salmonella*, *Campylobacter*, and antibiotic residues is noteworthy since they may not be counted as pollutants from litter applications, at least under the experimental conditions we have reported. Both simulation methodologies accentuated differences between no-till and conventional tillage in runoff, and flow-weighted concentrations of testosterone. Higher concentrations of testosterone were found in runoff from no-till plots fertilized with poultry litter for both simulation rainfall intensities. Flow-weighted concentrations of fecal bacteria and estradiol were not effected by tillage or fertilization treatments or their interactions for either rainfall simulation. Results of these plot-scale rainfall simulations were similar to results of runoff events from catchment-scale and actual rainfall conditions indicating rainfall simulation methods are appropriate to evaluate the risk of contaminant transport in agricultural systems. The application of poultry litter at appropriate agronomic levels, depending on the litter concentrations of fecal bacteria and hormones, followed immediately by rainfall appeared to have little potential of contaminating surface waters with pathogens and endocrine disrupting hormones.

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