

## The effect of poultry manure application rate and $\text{AlCl}_3$ treatment on bacterial fecal indicators in runoff

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### ABSTRACT

Increasing costs associated with inorganic fertilizer have led to widespread use of broiler litter. Proper land application, typically limiting nutrient loss, is essential to protect surface water. This study was designed to evaluate litter-borne microbial runoff (heterotrophic plate count bacteria, staphylococci, *Escherichia coli*, enterococci, and *Clostridium perfringens*) while applying typical nutrient-control methods. Field studies were conducted in which plots with high and low litter rates, inorganic fertilizer,  $\text{AlCl}_3$ -treated litter, and controls were rained on five times using a rain generator. Overall, microbial runoff from poultry litter applied plots was consistently greater ( $2\text{--}5 \log_{10} \text{plot}^{-1}$ ) than controls. No appreciable effect on microbial runoff was noted from variable litter application rate or  $\text{AlCl}_3$  treatments, though rain event, not time, significantly affected runoff load. *C. perfringens* and staphylococci runoff were consistently associated with poultry litter application, during early rain events, while other indicators were unreliable. Large microbial runoff pulses were observed, ranging from  $10^2$  to  $10^{10}$  CFU  $\text{plot}^{-1}$ ; however, only a small fraction of litter-borne microbes were recoverable in runoff. This study indicated that microbial runoff from litter-applied plots can be substantial, and that methods intended to reduce nutrient losses do not necessarily reduce microbial runoff.

**Key words** | antibiotic resistance, indicators, litter, poultry, runoff, water

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### INTRODUCTION

The fouling of the nation's surface water supply through rain runoff on land treated with residual waste (e.g. manure, biosolids, etc.) has led to decreases in overall available fresh water (Sharpley *et al.* 1993; Kistemann *et al.* 2002; Pote *et al.* 2003). Runoff of nutrients and microbial constituents has led to eutrophication of freshwater sources and unnecessary burdens on drinking water supply purification (USEPA 2007). A recent survey of the nation's water supplies determined that nearly 50% of the surveyed rivers and streams were polluted and virtually unusable (USEPA 2007). Common pollutants ranged from nitrogen and phosphorus to pathogens and pathogen indicators (USEPA 2007). These were the first and third most common contaminants, respectively, and were thought to have resulted from

nearby agricultural practices. The US Environmental Protection Agency (USEPA) estimates that the cost of treatment alone of 1 million gallons of water is approximately \$300 (USEPA 2004); however, additional surface water fouling (sediment, known microbial contamination, etc.) can add additional costs to public water works which tend to be passed onto the consumers.

The land application of confined animal feeding operation manure is a common disposal practice, with the simultaneous advantageous use of this waste product. The concern with proper use of manure, or any fecal waste product, results from the potential presence of microbial and antibiotic-resistant pathogens and the over-application of macronutrients such as N and P (Shroeder *et al.* 2004; Adeli *et al.* 2006). However, some mitigation measures can be implemented such as application at lower P-based rates, buffer zones, soil-incorporation, and pH altering chemical

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amendments (Coyne *et al.* 1995; Shroeder *et al.* 2004; Maguire *et al.* 2006; Meals & Braun 2006; Tate *et al.* 2006; Adeli *et al.* 2011). That being said, a large-scale rain event can still result in the transport of fecally derived microbial pathogens and indicator bacteria (Curriero *et al.* 2001; Tyrell & Quinton 2003), and it is not known if any of the mitigation measures, outside of buffer zones, reduce bacterial transport or presence.

Very few studies have investigated the potential microbial runoff associated with poultry litter land application. Possible 'fecal' indicators and pathogens in poultry litter can vary from *Escherichia coli* and *Clostridium perfringens* to opportunistic pathogens such as *Staphylococcus aureus* and *Enterococcus* spp. and true pathogens such as *Salmonella* and *Campylobacter* (Kelley *et al.* 1998; Terzich *et al.* 2000; Lu *et al.* 2003). With such a wide breadth of bacterial quality, suitable poultry litter microbial indicators are lacking and the need for them has been noted in recent court cases (US Court of Appeals 2009). Jenkins *et al.* (2006), Sistani *et al.* (2009), and Brooks *et al.* (2009) have reported on various microbial runoff releases following land application of poultry litter and either natural or simulated rainfall. These three studies demonstrated that *E. coli*, enterococci, staphylococci, and *C. perfringens* can be released during rain events with successive rainfalls (up to 30 d) continually associated with microbial release. Maximum microbial runoff levels were shown to reach as high as  $10^{11}$  CFU plot<sup>-1</sup> ( $10^{15}$  CFU ha<sup>-1</sup>) which was two orders of magnitude greater than control plots. Many studies of this kind tend to focus exclusively on *E. coli*; however, the current study focused on a number of bacterial parameters to accomplish its objectives.

The current study had two objectives: (1) to quantify the runoff release of fecal indicator bacteria from litter-applied plots compared to non-applied plots; (2) to determine the effects of (a) litter at (high) N- and (low) P-based loading rates; and (b) application of AlCl<sub>3</sub>-treated litter to reduce runoff bacterial losses. AlCl<sub>3</sub> has been demonstrated to reduce P solubility and reduce NH<sub>3</sub> emissions from poultry litter when applied as a litter treatment (Do *et al.* 2005); while P-rate-based litter applications are thought to reduce nutrient load and hence runoff. It was expected that the two mitigation methods would also reduce bacterial runoff over standard N-rate applications.

## MATERIALS AND METHODS

### Experimental design

The two main study objectives and sub-objectives were addressed on an experimental runoff field with plots devoted to sub-objectives 2a (high and low litter rate) and 2b (AlCl<sub>3</sub>). All litter was applied at an N-based rate (18 Mg litter ha<sup>-1</sup>), considered a typical litter application rate for forage, except for the P-based litter treatments. The study was established as a complete randomized block design, with triplicate replications. Block treatments consisted of: N-based litter– $3.14 \times 10^2$  kg ha<sup>-1</sup> N (18 Mg litter ha<sup>-1</sup>) (N); P-based litter –  $8.51 \times 10^1$  kg ha<sup>-1</sup> N (5 Mg litter ha<sup>-1</sup>) (P); inorganic fertilizer (rate equivalent to N-based rate stated above) (F); N-based with AlCl<sub>3</sub> (10% v/v) (N + 100AlCl); N-based with AlCl<sub>3</sub> (20% v/v) (N + 200AlCl); and a no fertilizer control (C). In addition, the N levels in the P-based litter treatment were supplemented with inorganic NH<sub>4</sub>OH<sub>3</sub> fertilizer to bring the N levels to that of the N-based litter rate. N-based litter and control treatments were repeated the following year (data not shown).

Poultry litter was collected from a nearby commercial broiler operation housing approximately 25,000 broilers per house and approximately six flocks per year as previously described (Brooks *et al.* 2009). Broiler litter was collected from the houses approximately 7–14 d prior to application.

### Field plots and runoff

Plots measured  $4.3 \times 2.1$  m ( $9.03 \times 10^{-4}$  ha) and were located on the Mississippi State Agricultural and Forage Experimental Station near Mississippi State University. The study was carried out from May to August. Soil was characterized as a Marietta silt loam with characteristics of fine-loamy, siliceous, active, thermic Fluvaquentic Eutrudepts soil as previously described (Brooks *et al.* 2009). Plots were planted with common bermuda grass (*Cynodon dactylon* (L.) Pers) and individual plot integrity was maintained by soil berms on all sides. Individual manure and fertilizer treatment applications were applied via hand-held scoops in a sweeping motion to ensure even broadcast dispersal. There were five rain events which

comprised days 1–69 post application. Rain runoff was generated by a portable rain maker following a previously established design (Miller 1987). Rain was simulated at a rate of 75 mm h<sup>-1</sup> and consisted of pumped groundwater located on the site. Groundwater was filtered through a pre-filter to remove large debris followed by a 0.45 µm filter prior to reaching the rain nozzles. Rain event times averaged 30 min in length, and were dependent on plot soil moisture; 30 min was the approximate minimal time necessary to generate rain runoff. The rain maker was covered in plastic tarps to ensure droplets did not escape the plot perimeter. Upon commencement of runoff, the rain maker was allowed to proceed for approximately 5 min. Given that only one rain simulator was available, rain was delivered to each plot in sequential fashion following the layout of the randomized block design and was completed for all plots by mid afternoon.

Runoff was collected at the edge of each plot, where trays were placed at a flat level relative to the slope of the plots (~2–3%) and were placed in-line to the plots such that a portion of the berm was removed to accommodate the opening of the tray (runoff collector). Collectors were protected from inadvertent drops of water by stainless steel covers. Runoff was directed to two 2-L steam-sterilized polypropylene bottles (10- and 100-fold dilution rate bottles) via stainless steel dividers. The bottle catching runoff at the 10-fold and 100-fold dilution rates would then represent plot runoff at 1/10 and 1/100 of the total runoff, respectively. Rarely was rain runoff generated such that runoff would be collected in the 100-fold dilution rate bottle. Total rain runoff (runoff load) for the plot was calculated as

$$TR = BV \times BDR \quad (1)$$

where TR = total runoff per plot (mL), BV = bottle volume (mL), and BDR = bottle dilution rate corresponding to the dilution rate in which the runoff was collected (either 10 or 100). Samples were collected immediately after rain runoff was terminated and brought back to the laboratory (approximately 5 min) for BV measurement and microbial analyses. Ambient climate data were collected on site through the use of a HOBO H21-002 microstation logger (Onset Computer Corp., Bourne, MA).

### Microbial parameters

Runoff water from each replicate treatment and rain event was analyzed for: heterotrophic plate count bacteria (HPC), thermotolerant (TT) *E. coli* (USEPA 1103.1 2002), enterococci (USEPA 1106.1 2002), staphylococci, and *C. perfringens* (Payment & Franco 1993) using previously described methods for runoff collection and microbial analyses (Brooks et al. 2009). Briefly, aliquots and dilutions were filtered through a membrane filter (0.45 µm) and placed onto the appropriate assay media (Neogen-Accu-media; Lansing, MI) for measurement of TT *E. coli* (mTEC agar at 44.5 °C, overnight), enterococci (mEnterococcus agar at 35 °C, 48 h), and *C. perfringens* (mCP agar at 45 °C, anaerobic environment, overnight). Suspected enterococci colonies were further confirmed by transferring the membrane filter from mEnterococcus agar to bile-esculin agar and incubating a further 1 h at 35 °C. Pink to red colonies with black haloes were presumptively confirmed as enterococci. Staphylococci and HPC bacteria were assayed by standard spread plate to MSA agar (35 °C, 48 h) and R<sub>2</sub>A (35 °C for 2 h followed by 14 d at room temperature), respectively. Prior to membrane filtration, *C. perfringens* samples were heat shocked at 70 °C for 10 min. Anaerobic conditions for *C. perfringens* were established using an Anoxomat gas generation system (Mart Microbiology; Lichtenvoorde, The Netherlands) using the default anaerobic gas mixture and setting. *C. perfringens* presumptive positive samples were further confirmed by exposing colonies to NH<sub>4</sub>OH fumes and noting a pink to fuchsia colony color; positive colonies were then streak isolated to 5% sheep blood agar and noted for a double zone of hemolysis.

Soil and poultry litter samples were subjected to similar assays. Soil samples from each plot were collected at two time points, the week prior to litter application and following completion of all rain events. Soil and litter were first diluted by addition of 10 g (moist) of the sample to 95 mL of sterile physiological saline (0.85% NaCl) and blended for 30–60 s in an InterscienceBagMixer (Interscience; St Nom, France). After homogenization, samples were then subject to assays, as dictated above, following appropriate serial dilutions. A 10 g (moist) portion of each sample was subjected to moisture content analysis by heating to

104 °C for 24 h then recording dry mass. All soil or litter samples were calculated as dry kg<sup>-1</sup>.

Assay positive controls consisted of *E. coli* ATCC 25922 (American Type Culture Collection; Manassas, VA), *S. aureus* ATCC 25923 and *S. epidemidis* ATCC 12228, *Enterococcus faecalis* ATCC 19433, and *C. perfringens* ATCC 3624 for mTEC, MSA, MSA, mEnterococcus, and mCP agars, respectively.

## Statistics

Plot microbial runoff (PMR, CFU plot<sup>-1</sup>) values for each plot during each rain event were calculated as functions of the respective microbial runoff concentrations (C, CFU mL<sup>-1</sup>) multiplied by total runoff volumes (TR, mL plot<sup>-1</sup>) for each microbial assay, thus:

$$\text{PMR} = C \times \text{TR} \quad (2)$$

Equation (3) was used to determine the microbial runoff release rate (%) from plots receiving the N treatment:

$$\% \text{ release rate} = \frac{(\text{PMR}_N - \text{PMR}_C)}{\text{SC}} \times 100 \quad (3)$$

where PMR<sub>N</sub> and PMR<sub>C</sub> are the mean PMRs for the N and C plot treatments, respectively, and SC is the microbial concentration measured in either soil, poultry litter, or both prior to land application. Percentage release represents the microbial runoff load as a function of the initial microbial level present in litter, soil, or both, and represents the runoff-recovery of land-applied microbes.

Before statistical analyses, microbial values were log<sub>10</sub> transformed to achieve normal distribution. Geometric means were calculated for each set of three replicate plots per rain event. The mixed model analysis (PROC MIXED) in SAS Enterprise Guide 4.2 for a completely random design was used to analyze runoff mean values for each microbial parameter. The PROC MIXED compared manure treatment (N, P, F, N + 100AlCl<sub>3</sub>, N + 200AlCl<sub>3</sub>, and C) and rain event (runoff event), and determined the significance of their interactions. Runoff mean values were then compared to ascertain differences between PMR related to treatment and rain events. Residuals were normally distributed and the Tukey corrected least square means *t*-test was used to determine significant differences between model parameters.

Unless otherwise stated  $\alpha = 0.05$ , and all differences noted in the results and discussion as significant, were below this level. Microbial data (HPC, staphylococci, TT *E. coli*, enterococci, and *C. perfringens*) were reported in arithmetic values of the geometric means.

## RESULTS AND DISCUSSION

### Poultry litter and soil

Poultry litter bacterial levels were measured prior to land application (Table 1). Only enterococci appeared to be negatively affected by the addition of AlCl<sub>3</sub> (which produces an exothermic reaction which reduces the matrix pH to 3–4); however, a bench-scale study in which treatments were repeated, demonstrated a reduction of greater than 4 log<sub>10</sub> for all measured bacteria including the spore-forming

**Table 1** | Litter bacterial levels (geometric mean) following litter AlCl<sub>3</sub> treatment and prior to land application

	Bacterial levels CFU dry kg <sup>-1</sup>				
	HPC <sup>a</sup>	Staphylococci	Enterococci	<i>E. coli</i>	<i>C. perfringens</i>
N <sup>b</sup>	1.09E + 12	3.14E + 11	1.33E + 07	1.00E + 05	8.69E + 06
N + 100 AlCl	8.97E + 11	1.39E + 11	6.83E + 04	9.22E + 04	7.16E + 06
N + 200 AlCl	1.34E + 12	4.22E + 11	1.06E + 06	1.09E + 05	1.34E + 07

<sup>a</sup>HPC = Heterotrophic plate count bacteria.

<sup>b</sup>Treatments–N = N-manure application rate and baseline for all litter; N + 100AlCl, N + 200AlCl = N-manure application rate + AlCl<sub>3</sub> at a low (100AlCl) and high (200AlCl) rate.

*C. perfringens* (data not shown). In the attempt to mimic real world conditions, litter was only lightly homogenized prior to mixing with  $AlCl_3$ . Heterogeneous litter particle size most likely contributed to discrepancies with the  $AlCl_3$  effect and may disrupt any effect on a large (e.g. farm) scale as well; therefore processing litter to an overall smaller nominal size is most likely necessary for full effect.

Soil bacterial levels, before and after litter treatments, are represented in Figure 1. As can be seen, litter applications had little influence on soil bacterial levels collected post application. For the most part, very few litter-borne bacteria increased in soil following land application. *E. coli*, which was detected in pre-application soil, dropped to below detection limits following the five rain events indicating that the rain events acted as a wash, removing *E. coli* from the soil and litter surfaces. Typically, surface applied poultry litter microorganisms have been shown to move horizontally with frequent rain events (Heinonen-Tanski & Uusi-Kamppaa 2001; Brooks et al. 2009; Sistani et al. 2009; Adeli et al. 2011). Only *C. perfringens*, was shown to significantly increase and remain in soil as a result of litter application, most likely due to shallow vertical transport rather than regrowth, as clostridia require an

anaerobic environment for growth. In addition, given the propensity towards spore-formation, *C. perfringens* likely remained viable throughout the experiment, whereas vegetative cells may have been more likely to be inactivated due to relatively hot and dry ambient seasonal conditions over the 2.5 month period (mean temperature  $32.2^\circ C$  and natural precipitation 84.1 mm).

### Plot microbial runoff (PMR) – effect of rain events and treatment

The effect of rain events and treatments on bacterial rain runoff is shown in Figures 2 and 3, respectively. In general, all treatments demonstrated their peak runoff during the first two runoff events with decreases following thereafter, particularly from plots which received poultry litter. Table 2 shows microbial runoff release/recovery as a percentage of microbes applied in litter, or present in soil, for each runoff event, since soil cannot be discounted as a source. These data also suggested that peak losses occurred during the first two rain events (days 1–14) with percentage releases reaching their peak during that time. This held true for *C. perfringens* and staphylococci (Table 2); however,

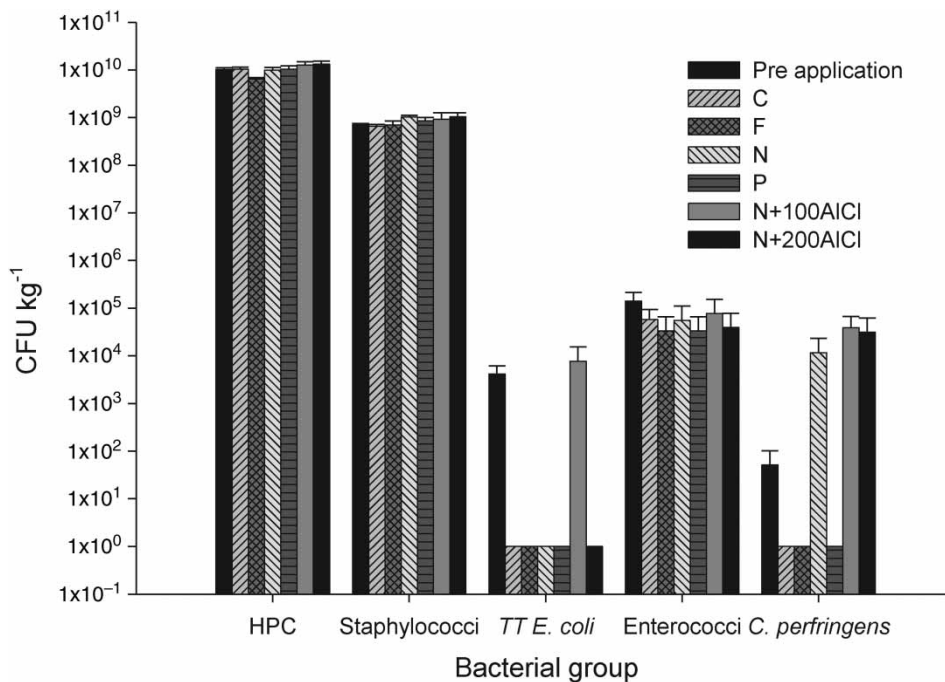
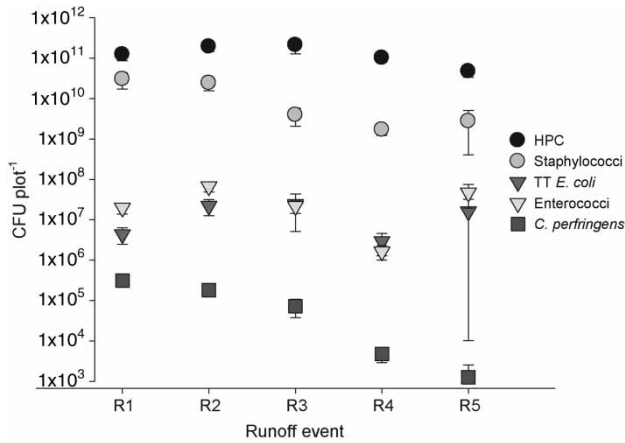


Figure 1 | Bacterial soil levels (geometric mean) per plot for pre- and post-application of litter treatments (C, F, N, P, N + 100AlCl<sub>3</sub> & N + 200AlCl<sub>3</sub>). Error bars are standard error of the mean.

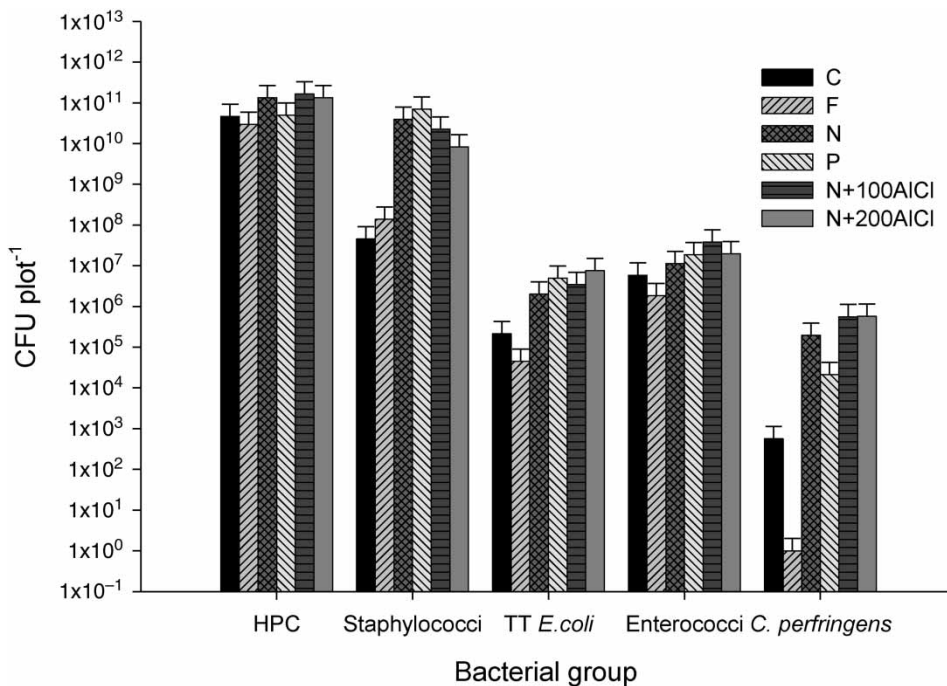


**Figure 2** | Effect of rain runoff event on bacterial runoff levels (geometric mean) for all treatments combined. Error bars are standard error of the mean.

enterococci and TT *E. coli* demonstrated possible regrowth with slight increases starting with rain event 4 (Figure 2). Relative to initial runoff (runoff event 1), it appeared that no microbial group decreased to control treatment levels, particularly if treated with the N-based litter treatment, indicating that time (>60 d) and five rain events did not exhaust all microbial sources. N, N + 100AlCl, and N + 200AlCl *C. perfringens* and staphylococci runoff levels were significantly

greater than C and F plots, on a consistent basis through runoff event 4; all other measured bacteria were not significantly greater than C and F plots even at runoff event 1.

The findings in this study may suggest microbial runoff as a function of the rain event itself, which has been suggested by others (Sistani *et al.* 2009), and not necessarily related to timing after application. This was supported as runoff load during the first rain event was similar in magnitude to a previous study (Brooks *et al.* 2009), regardless of initial rain event timing. Likewise, runoff during the first rain event was similar the following year when the N-based litter treatment was repeated, despite an 8-d difference between rain events (1 d vs. 8 d post application) (data not shown). It remains to be seen if large temporal space between land application and the first rain event would significantly decrease microbial runoff load; however, given that microbial runoff was still above control levels at 60 d post application and five rain events, it would seem as if a large temporal space between application and initial rain event would not limit microbial losses considerably. The effect of rain events was also corroborated by Thurston-Enriquez *et al.* (2005). In that study, three simulated rain events occurred within a 72 h time period



**Figure 3** | Effect of treatment on bacterial runoff levels (geometric mean) for rain event 1. Error bars are standard error of the mean.

**Table 2** | Microbial runoff source<sup>a</sup> recovery (%)<sup>b</sup> per runoff event

Microbial parameter	Runoff event				
	1	2	3	4	5
<b>Attributed to litter (%)</b>					
<i>Clostridium perfringens</i>	6.4	3.7	1.0	0.0	0.0
<i>Clostridium perfringens</i>	6.4	3.7	1.0	0.0	0.0
Staphylococci	16.7	9.7	1.8	0.5	0.1
<b>Attributed to soil + litter (%)</b>					
<i>Clostridium perfringens</i>	6.4	3.7	1.0	0.0	0.0
Staphylococci	2.5	1.5	0.3	0.1	0.0

<sup>a</sup>Source = total indicator bacterial load in soil + manure, or manure per plot.

<sup>b</sup>Release % was calculated as bacterial load present in the N-based litter treatment(N)-control (C) runoff load from each respective rain runoff event divided by the bacterial load present in the litter or soil + litter and multiplied by 100. Similar values for litter and soil + litter indicate litter was the most likely source.

following manure land application, which would be analogous to the current study's first three rain events (1–21 d). Both studies demonstrated a drop of one order of magnitude for *C. perfringens*, and an increase in enterococci and TT *E. coli* runoff loads following the first three rain events, despite the differences in timing.

Treatment effects (e.g. litter vs. no litter) were only evident during early rain events, as shown in Figure 3. Microbial runoff from plots receiving N-based poultry litter decreased from a peak at the first two rain events for HPCs, staphylococci, TT *E. coli*, enterococci, and *C. perfringens* ( $1.44 \times 10^{11}$ ,  $2.32 \times 10^{10}$ ,  $4.33 \times 10^6$ ,  $2.30 \times 10^7$ , and  $4.43 \times 10^5$ ) to the final rain event ( $5.34 \times 10^{10}$ ,  $1.63 \times 10^9$ ,  $1.34 \times 10^7$ ,  $2.44 \times 10^7$ , and  $1.52 \times 10^5$ ), respectively. These levels were significantly greater than for C and F plots, as would be expected. HPCs, staphylococci, and *C. perfringens* were released in larger runoff concentrations when litter was applied at either the N- or P-based rates compared with C and F treatments. No differences were noted between the N and P rates, a finding supported by the previous study (Brooks et al. 2009). This contradicts findings for runoff total P or N which have been shown to be correlated to manure rates (Shroeder et al. 2004). This is most likely due to greater water solubility and stability (e.g. no regrowth or inactivation, etc.) associated with P or N. Acosta-Martinez & Harmel (2006) did show an increase in soil-microbial biomass and enzyme activity at higher litter application rates

(>6.7 Mg ha<sup>-1</sup>), though this may not have an appreciable effect in runoff, as demonstrated in the current study. The additions of AlCl<sub>3</sub>, rarely affected the release of any of the measured runoff bacteria (Figure 3). Staphylococci runoff was reduced by addition of the high rate of AlCl<sub>3</sub>, either through heat-induced inactivation in the litter or inhibition of bacterial release, possibly from stronger cation bonds made with the litter surface. Enterococci runoff was slightly reduced by addition of AlCl<sub>3</sub> during runoff events 2 and 3 (data not shown).

*C. perfringens* and staphylococci runoff levels were significantly associated with litter application through runoff events 2 and 4, respectively. Staphylococci were present in applied litter at levels greater than 10<sup>12</sup> CFU g<sup>-1</sup>, and most likely explained its strong association with litter application. This sheer level of litter staphylococci may lend itself to use as an indicator of poultry litter application, though its ubiquitous nature may prohibit that, as evidenced by the large soil-staphylococci contribution to runoff (Table 2). Other studies have found similar findings associated with various poultry niches (Brooks et al. 2009; Chinivasagam et al. 2010). In addition, it is unclear if staphylococci are present in litter at consistent levels, given environmental attenuation (e.g. storage), though data provided by Terzich et al. (2000) seems to suggest these levels are typical. The more climate-tolerant and less ubiquitous *C. perfringens* only remained significantly associated with litter through the second rain event, and was most likely due to the relatively low levels present in the litter. Approximately 12% of *C. perfringens* applied in poultry litter treated plots was recovered through all runoff events, suggesting limited transport (Table 2). Typically following initial rain events, free or litter-attached bacteria do one or more of the following: (1) wash away horizontally; (2) vertically transport and facilitate tight bonds to exposed soil surfaces; (3) or inactivate. It is unlikely that clostridia present in litter were inactivated, as most were present in a spore state, as evidenced by the recovery technique in the current study. This suggests stronger attachment to soil, forage, or dense litter particles. Strong microbial attachment may inhibit or reduce eventual runoff load following the initial rain events, which are more likely to facilitate horizontal transport of loosely associated, less dense particulates and bacteria (Tyrell & Quinton 2003; Sistani et al. 2009).

Likewise, complex surfaces such as clay or litter particles could harbor small niches capable of protecting bacteria from runoff transport. Tate *et al.* (2006) suggested either high attenuation or strong bonding to account for low microbial recovery in runoff. Percentage recovery of staphylococci (Table 2), *E. coli* and enterococci (data not shown) were difficult to interpret given their presence in soil or due to regrowth potential.

This study is one of a few associated with poultry litter application and runoff where more than *E. coli* was investigated, and likewise, demonstrated that many types of bacteria can be transported horizontally from land applied with poultry litter, even more than 60 d post application. The release of known and unknown microbial entities following rain events can lead to considerable degradations in water quality in addition to transfer of zoonotic disease, which can affect nearby confined animal feeding operations or the public. To monitor these releases, unique indicators are necessary; poultry litter land application, to date, has no suitable bacterial indicator. In the current study, enterococci, *E. coli*, and HPCs failed criteria necessary for useful indicators. *E. coli* and enterococci both appeared to regrow throughout the study, as demonstrated by others (Thurston-Enriquez *et al.* 2005; Unc *et al.* 2006), and were ubiquitously present in control plots. This finding is not uncommon, as *E. coli* can grow in favorable niches and climates, despite no fecal matter being present (Rivera *et al.* 1988). Regrowth was plausible, given temperatures near 35 °C, moisture (i.e. rainfall), and an organic carbon and nutrient source (i.e. litter). The pH of the AlCl<sub>3</sub> treated litter may have been initially prohibitive to regrowth, though this was not seen by runoff events 3–5. On the other hand, staphylococci and *C. perfringens* may yield more promising results. Given its ubiquitous presence in nature, a host specific molecular marker may be necessary to further pursue *Staphylococcus* spp. as an optional litter indicator. *C. perfringens* has been recommended as an alternative ‘fecal’ indicator to *E. coli* or coliforms (Thurston-Enriquez *et al.* 2005; Brooks *et al.* 2009), though few studies have demonstrated its usefulness for poultry litter impacted environments (Brooks *et al.* 2009). *C. perfringens* appeared to be the most suitable litter indicator in the current study as it was consistently associated with poultry litter runoff, generally

not present in control plots, and decreased as rain events progressed. That being said, its presence in poultry litter may not be ubiquitous and *C. perfringens* has been commonly found in agricultural soils as well (Voidarou *et al.* 2011). Antibigrams (antibiotic resistance profiles) were investigated for individual isolates of *E. coli*, *Staphylococcus*, and *Enterococcus* (data not shown) and failed to demonstrate any significant differences between litter-applied and non-applied plots.

## CONCLUSIONS

The land application of manures is a beneficial practice, recapturing macronutrients, water, and organic matter. However, there are potential problems associated with the practice, particularly with rain-associated runoff of nutrients and microbial contaminants such as pathogenic and fecal bacteria. The purpose of this study was to measure the amount of microbial runoff associated with litter plots applied with treatments meant to limit nutrient losses. Sufficient microbial runoff from all plots was generated with all litter treatments associated with higher levels of microbial runoff, even following three rain events. It also appeared that microbial runoff was not necessarily associated with time following application, but rather with rain events. *C. perfringens* and staphylococci may prove to be suitable for tracking microbial runoff to poultry litter, at least more so than more traditional indicators such as enterococci and TT *E. coli*. Their use may warrant more investigation, and likewise warrant further studies to find a suitable indicator. The two approaches meant to mitigate nutrient runoff proved to be relatively fruitless with regards to microbial runoff. It appears that while the application of litter at lower P-based rates, while useful from a water quality-and soil-P basis, did not reduce bacterial counts in runoff. Likewise the use of AlCl<sub>3</sub>, a common approach to chemically treat litter to reduce P solubility, only affected some microbial constituents, though not consistently or significantly. Thus far grass buffers, litter storage, and composting, and soil incorporation appear to be the most useful approaches to reducing litter-borne microbial runoff.



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