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Setback distance impacts on transport and antibiotic resistance phenotypes of fecal indicators

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Abstract

Although the land application of livestock manure has numerous agronomic benefits, runoff from manured fields can degrade water quality. Setbacks instruct a minimum distance be maintained between manure application and surface waters. They are commonly used to manage nutrient contamination of surface waters; however, their utility for reducing microbial inputs remains unclear. Here we evaluated the efficacy of five setback distances in no-till wheat (*Triticum aestivum* L.) residue plots for reducing runoff fecal indicator concentrations from swine manure-amended fields. Also, since there is increasing interest in the use of water quality indicators to monitor antibiotic resistance in environmental systems, *Escherichia coli* and *Enterococcus* isolates were collected, and evaluated for resistance to 12 antibiotics. Seven of the 12 antibiotics evaluated in this study are critically important to human health and another four of the antibiotics evaluated are highly important to human health, according to World Health Organization (WHO) guidelines. Significant differences existed in amounts of indicators from pre- and post-manure application time points; however, no significant differences were observed for any of the five setback distances measured (range 4.9–23.2 m). Antibiotic-resistant *E. coli* and *Enterococcus* were isolated from pre- and post-application runoff, indicating presence of antibiotic-resistant fecal indicators in both manured and in non-manured soils, although the source manure had a higher percentage of isolates displaying resistance. It remains difficult to provide recommendations for concurrent reduction of nutrient and microbial contaminants.

1 | INTRODUCTION

As livestock production operations become larger and more concentrated cost effective, manure application becomes an increasing challenge, and there is pressure to maximize the amount of land to which manure is

Abbreviations: ARB, antibiotic-resistant bacteria; ARG, antibiotic-resistant genes; CAFOs, confined animal feeding operations; NPDES, National Pollutant Discharge Elimination System; PCR, polymerase chain reaction

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applied. One management strategy that has been widely used to address environmental and water quality issues is the implementation of setback distances (Gilley, Sindelar, & Woodbury, 2016; USEPA, 2012a). Setbacks are rules or guidelines instructing a certain minimum distance be maintained between two points of interest. They are commonly used in agricultural settings as part of mandated nutrient management plans (Henry, 2003).

Setbacks are required as part of the 1987 Water Quality Act (40 CFR 122.26) administered by the EPA National Pollutant Discharge Elimination System (NPDES) programs (USEPA, 2012a). They are used to limit contamination of surface waters from manure-borne nutrients. For Confined Animal Feeding Operations (CAFOs), NPDES requires a 30.8 m (100 ft) setback distance between manure application and any down-gradient surface waters, open tile intake structures, sinkholes, agricultural well heads, or other conduits to surface waters, with no restrictions on what is grown in this setback area. Alternatively, CAFOs may establish a 10.68 m wide (35 ft) permanent vegetated buffer strip, but this land may not be used for row crops. The loss of row-crop revenue from the 10.668 m permanent vegetative buffer strip zone is one consideration farmers must weigh when deciding on a setback option. A third option for compliance is to demonstrate that an alternative practice provides reduction equivalent to the 30.48 m (100 ft) setback.

Setbacks and vegetative buffer strips are effective at reducing transport of some manure-associated nutrients and sediment into surface waters (Bingham, Westerman, & Overcash, 1980; Dillaha, Reneau, Mostaghimi, & Lee, 1989; Gilley et al., 2016; Gilley et al., 2017; Gilley, Risse, & Eghball, 2002; USEPA, 2002). Although, in the Al-wadaey, Wortmann, Shapiro, Franti, and Eisenhauer (2010) field trials using cattle manure, setback distances did not impact measured nutrient parameters. Setbacks work by increasing the distance that contaminants need to travel to reach surface waters, slowing the flow rate of the runoff, allowing for particulate matter to settle, and allowing liquid to infiltrate into the soil (Cromley & Lorey, 2006).

In addition to nutrients, manure-impacted runoff has the potential to deliver other chemical and biological contaminants into surface waters. Bacterial contaminants include pathogens, both naturally occurring and anthropogenically influenced antibiotic-resistant bacteria, fecal indicators, and non-pathogenic environmental bacteria carrying antibiotic resistance genes. Runoff from manured soils following rain events has the potential to carry large microbial loads (Thurston-Enriquez, Gilley, & Eghball, 2005), including residual antibiotics (Ray, Chen, Knowlton, Pruden, & Xia, 2017), antibiotic-resistant fecal indicators (Durso, Miller, & Henry, 2018), and antibiotic resistance genes (Le, Maguire, & Xia, 2018).

Core Ideas

- Significant differences in microbial load were not detected at the five setback lengths.
- The recommended setback distance for nutrient control is insufficient for microbes.
- Detection of ARB/G in pre-application soils makes source attribution difficult.

The efficacy of setbacks for reducing transport of bacteria, specifically fecal indicator bacteria which the EPA recommends to assess health risk from water (USEPA, 2012b), remains unclear. Models based on laboratory studies indicate that setbacks are effective at removing some microbial contaminants (Cinque & Jayasuria, 2013; Tate, Das Gracas, Pereira, & Atwill, 2004). Lysimeter studies report that vegetative filter strips are effective at reducing the transport of fecal coliforms (Roodsari et al., 2005). Plot-based rainfall simulations demonstrated efficacy of a narrow perennial grass hedge for reducing microbial transport from manure-amended fields (Durso, Gilley, Marx, & Woodbury, 2019b). Research on microbial attachment to particulate matter in manure suggests controlling the amount of sediment reaching surface waters will also reduce transport of manure-borne bacteria (Guber, Pachepsky, Shelton, & Yu, 2007). However a 1.4-m wheat strip did not reduce runoff of microbes from broadcast beef manure (Durso, Gilley, Marx, & Thayer, 2019a), and a larger cool-season grass vegetative treatment system did not physically remove the microbes from beef feedlot runoff, or prevent them from traveling further along the treatment cell (Durso et al., 2017).

It has been proposed that setback areas containing non-manured crop residue could potentially function as an alternative to the 30.48-m cropped setback, or the 10.68-m perennial vegetative buffer to reduce discharge concentrations of contaminants, allowing for smaller manure application setback while still allowing the production of row crops and protecting water quality (Gilley et al., 2017). Recent work by Gilley et al. (2016, 2017) evaluated setback distances for nutrients from cattle and swine manure and demonstrated that a setback distance of 12.2 m was effective for reducing selected manure-borne nutrients to background levels (Gilley et al., 2016; Gilley et al., 2017). The objective of this study was to evaluate the efficacy of five setback distances ranging from 4.9 to 23.3 m for reducing the concentration of total coliforms, *Escherichia coli*, and enterococci following land application of swine manure slurry to no-till cropland containing winter wheat residues. Due to the impact of water quality on dissemination of antibiotic resistance (Durso & Millmier-Schmidt,

TABLE 1 Bacterial abundances (most probable number [MPN] ml^{-1}) of total coliform bacteria, *Escherichia coli*, and enterococci and average number of resistances for manure, pre-application runoff, and post-application runoff isolates

	Log abundance[a]			Avg. no. resistances	
Organism	Total coliforms	*E. coli*	Enterococci	*E. coli*	*Enterococcus*
Manure	6.17 (0.17)	5.56 (0.11)	5.75 (0.14)	1.7 (0.2)	5.5 (0.6)
Total pre-application runoff	6.53 (0.13)	2.85 (0.18)	4.90 (0.16)	0.1 (0.1)	2.2 (0.2)
4.9 m	6.79 (0.07)	2.90 (0.54)	4.80 (0.68)	0.0 (0.0)	1.7 (0.2)
7.9 m	6.00 (0.55)	3.49 (0.12)	4.72 (0.41)	0.2 (0.2)	2.0 (0.4)
11 m	6.65 (0.04)	2.53 (0.49)	5.17 (0.06)	0.0 (0.0)	2.2 (0.2)
17.1 m	6.52 (0.20)	2.67 (0.40)	4.87 (0.22)	0.2 (0.2)	3.2 (0.7)
23.2 m	6.76 (0.00)	2.61 (0.31)	4.97 (0.23)	0.0 (0.0)	1.3 (0.3)
Total post-application runoff	7.15 (0.09)	4.46 (0.13)	5.50 (0.05)	1.1 (0.2)	2.3 (0.2)
4.9 m	6.98 (0.25)	4.08 (0.15)	5.56 (0.12)	1.0 (0.4)	2.2 (0.6)
7.9 m	7.14 (0.08)	4.21 (0.28)	5.54 (0.08)	1.2 (0.5)	2.2 (0.2)
11 m	7.09 (0.13)	4.96 (0.20)	5.58 (0.06)	1.5 (0.6)	1.7 (0.2)
17.1 m	7.34 (0.25)	4.49 (0.32)	5.35 (0.11)	0.5 (0.5)	2.7 (0.5)
23.2 m	7.22 (0.27)	4.56 (0.38)	5.49 (0.16)	1.2 (0.5)	2.7 (0.7)

[a]Average reported with standard error in parentheses.

2018; Graham, Collington, Davies, Larsson, & Snape, 2014), and the increased interest in using water quality indicators to monitor antibiotic resistance in environmental systems, antibiotic phenotypes of fecal indicators were also assessed. The long-term goal that motivated this research study was a desire to provide manure handling recommendations to producers that combine both nutrient and bacterial best management practices.

2 | RESULTS AND DISCUSSION

2.1 | Fecal indicators

In total, runoff was collected from four replicate plots containing wheat residue, at each of five setback distances, before and after swine manure application. The number of total coliforms, *E. coli*, and enterococci in each runoff sample, along with the counts from the source manure are displayed in Table 1. A repeated measures ANOVA was used to check whether setback distance, time of collection pre- vs. post- manure application, or distance × time were significant. Only time of collection was significant ($P < .001$).

Interpretation of our microbial results is informed by nutrient setback distance work. Significant differences were observed between setback distances for nutrient removal by Gilley et al. (2016), who used the same setback distances and the same agronomic fields as this current study. One of the primary mechanisms identified for the removal of nutrients via setbacks or vegetative buffer strips is that the vegetation slows overland flow, allowing particulate matter and associated nutrients to settle before reaching surface waters (Dabney, Meyer, Harmon, Alonso, & Foster, 1995; Meyer, Dabney, & Harmon, 1995). Guber et al. (2007) suggest that controlling sediment can also limit the transport of manure-borne bacteria. Soupir, Mostaghimi, and Dillaha (2010) reported that most *E. coli* and enterococci in a plot-based runoff study surface-applied manure are attached to manure colloids. Likewise, Guzman, Fox, and Penn (2012) reported that fecal bacteria in swine manure effluent were predominantly attached to particles, not suspended free cells. Pachepsky et al. (2008) reported preferential attachment of individual *E. coli* strains to particles of different sizes.

Based on the statistically significant differences observed between setback distances in a different study using the same fields in Gilley et al. (2016), in combination with studies indicating that microbes in general–and *E. coli* and *Enterococcus* specifically–attach to particles, we hypothesized that these same setback distances used in Gilley et al. (2016) have the potential to be effective at reducing transport of fecal indicators. However, as reported above, no differences were observed for any of the measured fecal indicators at the five setback distances evaluated. There were significantly more fecal indicators isolated from the post-manure application runoff samples compared to the pre-manure application runoff samples ($P = .002$, $P < .001$, $P = .004$ for total coliforms, *E. coli*, and enterococci, respectively), confirming the contributions of land-applied manure to fecal indicator loads in runoff. There are numerous agronomic benefits to environmentally sound land application of swine manure, including long-term soil productivity, reduced need for inorganic fertilizer application, increased water infiltration into the

TABLE 2 Comparisons of proportions of *Escherichia coli* and *Enterococcus* isolates from manure and soils (pre- and post-application) resistant to a particular antibiotic

Population	Antibiotic	Proportion of isolates resistant			*P* difference	
		Manure	Pre-application runoff	Post-application runoff	Manure vs. runoff	Pre- vs. post-application
E. coli	Ampicillin[a]	0.70	0.00	0.05	<.001	.472
	Cefoxitin[b]	0.00	0.00	0.00	–	–
	Amoxicillin with CA[a]	0.00	0.00	0.00	–	–
	Ceftriaxone[a]	0.00	0.00	0.00	–	–
	Tetracycline[b]	0.70	0.10	0.50	.297	.032
	Streptomycin[a]	0.30	0.10	0.45	.429	.055
	Ciprofloxacin[a]	0.00	0.00	0.00	–	–
	Kanamycin[a]	0.00	0.00	0.05	.472	.472
	Nalidixic acid[a]	0.00	0.00	0.00	–	–
	Sulfamethoxazole with trimeth[b]	0.00	0.00	0.05	.472	.472
	Chloramphenicol[b]	0.00	0.00	0.00	–	–
	Gentamycin	0.00	0.00	0.00	–	–
Enterococcus	Ampicillin[a]	0.00	0.00	0.00	–	–
	Cefoxitin[b]	0.50	0.11	0.15	.041	.676
	Amoxicillin with CA[a]	0.00	0.05	0.00	–	.299
	Ceftriaxone[a]	0.90	0.11	0.25	<.001	.239
	Tetracycline[b]	1.00	0.00	0.00	<.001	–
	Streptomycin[a]	1.00	0.74	0.85	.1917	.382
	Ciprofloxacin[a]	0.10	0.00	0.00	.150	–
	Kanamycin[a]	0.50	0.16	0.05	.004	.267
	Nalidixic acid[a]	1.00	1.00	1.00	–	–
	Sulfamethoxazole with trimeth[b]	0.10	0.00	0.00	.150	–
	Chloramphenicol[b]	0.10	0.00	0.00	.150	–
	Gentamycin	0.30	0.00	0.05	.058	.323

Note. CA, clavulanic acid.
[a]Critically important antibiotic to human health.
[b]Highly important antibiotic to human health.

soil, and addition of organic matter and microbes to the soil (Ozlu & Kumar, 2018; Risse et al., 2006). However nonpoint source pollution from manure-impacted runoff remains an important challenge associated with the land application of manures.

Many knowledge gaps remain in predicting microbial transport in agricultural runoff (Bradford et al., 2013), and in addition to being attached to manure or soil particles, bacteria can remain suspended as free cells or bacterial aggregates or attach to lighter-weight plant fragments and residue (Guber et al., 2007). This, potentially, is what occurred in the current study. Bacterial cells are similar in size to coarse clay or silt particles, but have densities similar to water (Roodsari et al., 2005), and thus if not attached to particles, they remain suspended in the liquid portion of the runoff. Transport is also impacted by soil type, grade of the field, flow rate, preferential flow patterns, antecedent conditions, and macroecological factors such as weather (Bradford et al., 2013; Collins, Donnison, Ross, & McLeod, 2004; Guber et al., 2007; Thayer, Gilley, Durso, & Marx, 2012), all of which could have influenced the observed results. Although no differences in microbial concentrations based on setback distance were observed in this study, the observed results are not atypical, with both laboratory and field-based studies reporting limited efficacy of vegetation for reducing microbes in runoff (Durso et al., 2017, Fox, Matlock, Guzman, Sahoo, & Stunkel, 2010).

2.2 | Antibiotic resistance phenotypes

In addition to the impact of agricultural runoff on microbial water quality, there is increasing attention being paid to the role of runoff as a potential mechanism for the transport of agriculturally-associated antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) out of agricultural systems via surface or ground waters (Durso & Millmier Schmidt, 2018; Durso et al., 2018). We collected *E. coli* and *Enterococcus* isolates from pre- and post-manure application runoff for each plot, and from the source swine manure. These isolates were characterized for their phenotypic antibiotic resistance profiles using a suite of 12 drugs (Table 2). Resistance was found to 5 of the 12 antibiotics among the *E. coli* isolates, and 11 of the 12 antibiotics among the *Enterococcus* isolates (no ampicillin resistance was observed).

There was a higher proportion of resistances observed in the source manure compared to the *E. coli* and *Enterococcus* isolated from pre- ($P < .001$), and post-application runoff ($P = .091$ and $P < .001$ for *E. coli* and *Enterococcus*, respectively). These data reveal the potential for antibiotic-resistant bacteria from manure to be mobilized and transported by runoff to other sites far from the source manure. Combined with information on average number of resistances pre-and post-manure application (increase from 0.1 to 1.1 for *E. coli* isolates, no significant change from 2.2 to 2.3 for *Enterococcus* isolates) the overall contributions from manure are statistically significant for *E. coli*, but not *Enterococcus* for this data set. Although some statistical differences were noted in proportions of resistance to selected antibiotics (Table 2), the study design does not allow us to draw conclusions beyond this specific set of isolates. Nonetheless, this data set does reveal some interesting biological trends.

Manure is an important source of fecal indicators, including antibiotic-resistant fecal indicators, but it remains unclear how one can attribute the occurrence of resistance in any individual isolate to either the manure or the broader environment. Resistant *E. coli* and *Enterococcus* exist in both manure and non-manured farm soils (Durso, Wedin, Gilley, Miller, & Marx, 2016; Pérez-Valera et al., 2019; Udikovic-Kolic, Wichmann, Broderick, & Handelsman, 2014). For purposes of manure management recommendations, the question of interest is what proportion of the resistant bacteria in runoff come from the manure? We observed four instances where a resistance was not observed in pre-application runoff but was found both in the manure and the post-application runoff (of 16 bacteria/drug positives total). These instances strongly imply manure was the source of the antibiotic-resistant isolate. In six instances the bacteria/drug resistance combination was found in manure, pre-, and post-application runoff, but was identified in a higher proportion of manure samples, compared to pre-application runoff. And in three instances the bacteria/drug combination was found only in the pre- or post- application runoff, but not in the source manure. These data points highlight the difficulty of attributing manure as the source of any individual antibiotic-resistant isolate, since the resistance phenotypes were also observed in the runoff before manure was applied. This inability to determine if an isolate originated from a recent manure application presents challenges for designing environmental antibiotic resistance surveillance studies, evaluating the impact of best management practices for reducing antibiotic-resistant bacteria and their genes, and providing recommendations to producers for how they can minimize the impact of manure-borne antibiotic resistance.

2.3 | Conclusions

The current study examined if setbacks would also be effective for reducing transport of microbial contaminants. Although differences had been observed for nutrients at the five setback distances (range 4.9–23.2 m) evaluated, significant differences between monitored microbial groups were not observed. Antibiotic-resistant *E. coli* and *Enterococcus* were isolated from the manure, pre- and post-application runoff, indicating presence of antibiotic-resistant fecal indicators in both manure and in non-manured soils. Source attribution of ARB remains difficult. Manure samples had a higher proportion of isolates displaying antibiotic resistance compared to runoff samples.

3 | MATERIALS AND METHODS

3.1 | Study site

Experiments were performed 28.97 km east of Lincoln, NE, at the University of Nebraska Rogers Memorial Farm. The study plots were in a winter wheat, soybean [*Glycine max* (L.) Merr.], corn (*Zea mays* L.), and grain sorghum [*Sorghum bicolor* (L.) Moench] rotation, with winter wheat harvested from the study site in July of the previous year. The wheat residue was left on the plots as part of a long-term no-till management strategy and provided a 100% surface soil coverage (7.73 Mg ha^{-1}). The site consisted of Aksarben clay loam (fine, smectitic, mesic Typic Argiudoll) belonging to hydrologic group C. Plots were sloped, with a mean gradient of 4.9%.

3.2 | Runoff simulation and manure application

Twenty plots were established for a set of rainfall simulation experiments (four replicate plots each of $n = 5$ setback lengths), and assigned treatments using a randomized block design (Gilley et al., 2017). The plots were 3.7 m wide, placed perpendicular to the slope, with lengths of 4.9, 7.9, 11.0, 17.1, or 23.2 m. Sections of sheet metal were placed along the top and sides of each plot, and diverted runoff into a collection trough. Rainfall was applied to the plots and setback areas using a portable rainfall simulator (Schulz & Yevjevich, 1970) at a rate of approximately 55 mm h^{-1}. Rain gauges placed along the outer edge of each plot were used to monitor rainfall intensity and a stage recorder was mounted in the collection trough to measure flow rate. Due to the labor-intensive nature of running the rainfall simulator, experiments were run on two plots per week, for 10 wk. Replications one and two were performed the first 5 wk, and replications three and four were performed the second 5 wk. On the first day of each week, rainfall was applied until steady-state runoff conditions were established, after which runoff was collected for microbiological analysis. Steady-state runoff conditions were previously described in (Gilley et al., 2016; Lim, Edwards, Workman, Larson, & Dunn, 1998). On the following day, the rainfall application was again applied until steady-state conditions were reached. On Day 3, swine manure slurry that had been collected that week from a commercial wean-to-finish operation was hand applied to the upper 4.9 m of the plots. It was applied at 151 kg N ha^{-1} yr^{-1}, the estimated annual requirement for corn. No rainfall was applied on Day 3. On Day 4 the rainfall simulation was run again, with post-application runoff collected for microbiological analysis. Although runoff tests were staggered over 10 wk across the 20 plots, each plot had the same sampling and experimental treatment.

3.3 | Sample collection, processing, and analysis

Runoff samples ($n = 19$ pre-application, $n = 20$ post-application) were collected in 1-L plastic bottles, packed into a cooler with ice packs, transported to the lab within 2 h of collection, and processed on the same day as collection. Due to a transport error, the fourth rep of the 4.9-m plot was lost. Since manure is the primary mechanism by which bacteria from the animal are introduced into the environment, this study focused on the resistance of fecal-associated bacteria. Total coliforms, *E. coli* and enterococci, counts were quantified using the EPA-approved Quanti-Tray system (IDEXX Laboratories) where necessary samples were serially diluted using phosphate-buffered saline. All samples were incubated for 24 h. Total coliform and *E. coli* assays were incubated at 37 °C, and enterococci trays were incubated at 42 °C. Samples were also plated on CHROMagar *E. coli* (CHROMagar), and M-enterococcus agar (BD Difco), for isolation of *E. coli* and *Enterococcus* sp., respectively. Three typical colonies from each sample/media combination were struck for isolation and confirmation. *E. coli* was confirmed using the *uidA* polymerase chain reaction (PCR) (Lasalde, Rodriguez, Smith, & Toranzos, 2005), and *Enterococcus* was confirmed on Enterococcosel agar plates (BD Difco). Confirmed isolates were stored as glycerol stocks at −80 °C, until further characterization.

Weekly manure samples were also collected and processed as above. Disk diffusion assays (Table 2) were performed according to Clinical Laboratory Standards Institute guidelines for *E. coli* and *Enterococcus* (CLSI, 2012). Frozen isolates were grown up in 5 ml TSB and incubated at 37 °C for 27 h or at 42 °C for 48 h for *E. coli* and *Enterococcus*, respectively. After incubation, a BioMate3 spectrophotometer (Thermo Fisher Scientific) was used to adjust the optical density of each isolate to o.d. = 0.300. Adjusted broth tubes were applied to two identical petri dishes containing Mueller–Hinton Agar

(Becton, Dickinson and Company) by swabbing with a sterile cotton swab to the entire plate in three directions, separated by 60 degrees. Discs were applied using a BBL Sensi-Disc 6-disc Dispenser System (Becton, Dickinson and Company) and plates were incubated using the same temperature and time conditions as the broth tube growth. Zones of clearing were measured using the Flash and Grow Automated Colony Counter (Neutec Group Inc.) with the supplemental zone measuring software module.

3.4 | Statistical analysis

Microbial abundance data was log transformed and analyzed using PROC MIXED in SAS version 9.2 (SAS Institute) with a repeated measures design (Field Plot was the unit of observation, and Runoff Day was the repeated effect). A compound symmetry covariance structure provided the best fit for the model. Main effects (Runoff Date and Distance from manure application) and the two-way interaction were evaluated for significance. For *E. coli* and *Enterococcus* isolates, a two-sample test of proportional differences in resistance compared (a) runoff isolates (pre- and post-manure application) and (b) all runoff isolates to manure isolates. A probability level (P value) of $P < .05$ was used to reject the null hypothesis.

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CONFLICT OF INTEREST

No conflict of interest is declared.

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