# Rainfall Timing and Frequency Influence on Leaching of *Escherichia coli* RS2G through Soil Following Manure Application

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## ABSTRACT

**The time between swine (*Sus scrofa*) manure application to soil as a crop fertilizer, the first rainfall event, and the frequency of rainfall events should influence leaching potential of fecal pathogens. Soil microcosms were inoculated in the lab with a swine manure isolate of *Escherichia coli*, strain RS2G, expressing green fluorescent protein, to examine how timing and frequency of rainfall events influences RS2G leaching and survival in soil. Liquid swine manure inoculated with RS2G was applied to intact soil cores (20 cm in diameter × 30 cm long) 4, 8, or 16 d before the first rainfall event (50.8 mm over a 4-h period), and each core received one to three rainfall events. Manure application methods (no-till surface-broadcast, broadcast and incorporated, and tilled before broadcast) had no affect on leaching, although there was greater survival in soils when the manure had been incorporated. Most of the RS2G in the leachate appeared following the first rainfall event and RS2G leaching decreased with increasing time between manure application and the first rainfall, although leachates contained as much as 3.4 to 4.5 log colony forming units (CFU) 100 mL$^{-1}$ of RS2G when the first rainfall occurred 16 d after manure application. With increasing frequency of rainfalls there was a decrease in subsequent concentrations of RS2G in the leachate. There was no correlation between leachate RS2G and total coliforms or fecal streptococci concentrations. Soil RS2G numbers were 1 to 10% of the inoculum regardless of the length of time between manure application and the first rainfall. RS2G leaching was mostly influenced by the time between manure application and first rainfall event, and significant leaching and survival in soil was possible even if the first rain occurred 16 d after manure application.**

Animal production operations generate large quantities of manure. In Iowa alone it is estimated that approximately 14.5 million swine produce more than 22 million Mg of liquid manure per year (Iowa State University Extension, 1999). Generally, swine manure is stored in pits beneath farm buildings or outside in earthen lagoons or concrete basins before land application. This storage process does not necessarily treat the manure to reduce its bacterial content and thus large numbers of bacteria, including potential human and animal pathogens, are applied to soil along with the manure (Pell, 1997). Contamination of surface and subsurface water by microorganisms from animal manures is of great concern. One of the main concerns is the survival of microorganisms following land application since their survival presents a potential health hazard because it increases the likelihood that they can serve as nonpoint sources for contaminating surface or subsurface waters.

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Unlike human sewage sludge, animal manures are not treated to reduce the pathogen content before land application. Strategies for applying swine manure to soil generally incorporate best management practices that are designed to limit nitrogen to amounts that crops can readily use, which may or may not reflect the amount of manure that a soil can accommodate to successfully reduce contamination of water by pathogenic bacteria. The greatest risk of contamination by pathogenic bacteria occurs when high-intensity and/or long-duration rainfall causes runoff or subsurface drainage on agricultural land that recently received manure (Joy et al., 1998). Contamination of well water is of particular concern since it can expose humans and farm animals to pathogenic bacteria.

The ability of manure-derived pathogenic bacteria to survive in soil following manure application increases the probability of water contamination after rainfall or irrigation events (Joy et al., 1998). Pathogen survival will initially reflect the ability to tolerate the sudden change in habitat and subsequent ability to tolerate possibly adverse environmental conditions such as extremes in temperature or desiccation. Soil moisture is one of the more important factors influencing pathogen survival, and survival is more likely when soils are moist (Crane and Moore, 1986; Entry et al., 2000b; Gagliardi and Karns, 2000; Mubiru et al., 2000). Soil temperature also influences survival since warm temperatures along with drying decrease survival rates (Entry et al., 2000b; Kemp et al., 1992; Sjogren, 1994) while cooler temperatures promote survival (Kibbey et al., 1978; Ogden et al., 2001). Nutrient availability will also influence survival, and manure undoubtedly provides a supply of potentially utilizable nutrients that support the survival and growth of bacteria, at least for a while, once they are introduced into soil. Several recent studies have shown that *Escherichia coli* O157:H7 can survive for extended periods of time in manure-amended and unamended soils (Fenlon et al., 2000; Gagliardi and Karns, 2000; Gagliardi and Karns, 2002) and that vegetation promotes survival (Bolton et al., 1999; Fenlon et al., 2000; Gagliardi and Karns, 2002; Sjogren, 1995).

Survival may also require colonization of specific microhabitats, such as within the dispersible clay fraction, which protects pathogenic organisms from abiotic stresses (Recorbet et al., 1995). Survival in soil is probably a dynamic process were the majority of cells may die off quickly once introduced into the soil environment, but a subpopulation may be better suited for survival and this subpopulation may die off more slowly possibly because of colonization of favorable sites or

**Abbreviations:** CFU, colony forming units; RS2G, *E. coli* strain RS2 expressing the green fluorescent protein.

because of its physiological properties (Ogden et al., 2001).

Movement of bacteria through soil to ground water will in part depend on soil type, climatic and soil properties, manure properties and the method and amount applied, and the amount and type of vegetation (Entry et al., 2000a, b; McMurry et al., 1998). The timing and frequency of rainfall following manure application to soil will strongly influence both vertical and lateral movement of pathogenic bacteria through soil. The potential for ground water contamination depends on the depth of soil to the water table as well as the properties of the soil. Preferential water movement, due to the presence of old root channels, insect and animal burrows, and natural soil features, is probably the primary means by which bacteria move through soil (Abu-Ashour et al., 1998; McMurry et al., 1998; Smith et al., 1985). Tillage disrupts soil structure and pores and can reduce fecal coliform (McMurry et al., 1998) and *E. coli* O157:H7 (Gagliardi and Karns, 2000) transport compared with transport through similar intact soils. Rain can promote survival of pathogenic bacteria by keeping the soil moist, and it can redistribute bacteria through the profile to more or less favorable sites in addition to potentially contaminating ground water.

Most often bacterial transport is investigated following one or more simulated rainfalls immediately following manure application and less is known on how periodic and/or intermittent rainfalls influence pathogen transport or survival in soil (Stoddard et al., 1998). In the present study, we evaluated the effect of time between manure application and the first rainfall and the frequency of rainfall events on the leaching of a swine manure *E. coli* isolate through intact soil cores and on its survival in those cores. Since many Iowa soils have had an extensive history of manure application, we chose to examine the fate of a green fluorescent protein-marked *E. coli* strain that we isolated from swine manure to facilitate discrimination between manure-derived and soil-derived pathogenic bacteria. The *E. coli* was isolated from the source of manure that we used in these studies. Additionally, we evaluated the role of different manure application procedures on *E. coli* leaching and survival in soil.

## MATERIALS AND METHODS

### Isolation and Preliminary Characterization of *Escherichia coli* RS2G

We isolated several probable *E. coli* from swine manure obtained from Iowa State University's Swine Nutrition Management and Research Center (SNMRC) near Ames, IA by plating aliquots of dilutions onto m-Coliblue24 (Hach Company, Loveland, CO). Probable *E. coli* isolates were repeatedly streaked for purity and subjected to a variety of biochemical tests to obtain a presumptive identification, and one isolate, strain RS1, was chosen for use. A rifampicin-resistant derivative of RS1 was obtained by plating an aliquot of an RS1 culture onto a Luria–Bertani agar (Gibco/BRL, Gaithersburg, MD) plate amended with 25 μg $mL^{-1}$ rifampicin; this strain was designated as RS2. The sequence of a nearly full-length polymerase chain reaction (PCR) amplified copy of the 16S rDNA gene (1351 bases) of strain RS2 showed 99% sequence identity to *E. coli* following a BLAST search of GenBank. The identity of RS2 as an *E. coli* was further verified by its ability to ferment lactose and produce indole, its lack of urease activity, and by its β-glucuronidase and B-galactosidase activities, all of which are traits typically associated with *E. coli* (Leclerc et al., 2001; Rompre et al., 2002). We transferred plasmid $pP_{nptII}$-*gfp* (Stiner and Halverson, 2002), which contains a constitutively expressed green fluorescent protein gene (*gfp*) fused to the neomycin phosphotransferase promoter, and a kanamycin-resistance gene, into strain RS2; this strain was designated RS2G.

### Amplification of 16S rDNA gene

Genomic DNA was extracted using a QIAamp DNA mini kit (QIAGEN, Valencia, CA) and the DNA was eluted from the spin column with water. For PCR amplification of a nearly full-length 16S rDNA gene, we used domain bacteria primers 27F (5′-AGAGTTTGATCMTGGCTC-3′; M = A or C) that corresponds to positions 8 to 25 in the *E. coli* numbering system (Weisburg et al., 1991) and 1387R (5′-GGGCGGWG TGTACAAGGC-3′; W = A or T) that corresponds to positions 1387 to 1404 (Marchesi et al., 1998). The PCR amplification was performed using reaction mixtures (final volume, 50 μL) containing 100 ng of sample DNA, 2 U of Recombinant Taq DNA polymerase (Gibco/BRL), 1X PCR buffer, 1.5 m*M* $MgCl_2$, each deoxynucleotide triphosphate at a concentration of 0.2 m*M*, and each primer at a concentration of 2 μ*M*. The thermocycling program used was as follows: initial denaturation at 94°C for 1 min; 35 cycles consisting of a denaturation at 95°C for 30 s, an annealing temperature of 45°C for 30 s, and an extension step at 72°C for 1 min; and a final extension step at 72°C for 5 min. The product obtained from the amplification reaction was purified using a QIAquick gel-extraction kit (QIAGEN). The amplified product was sequenced using primers 27F, 1387R, and 533F (5′-GTGCCAGCMGCCGCG GTAA-3′; M = A or C). The sequences were determined by using an Applied Biosystems Model 377 Prism DNA sequencer (PerkinElmer, Wellesley, MA) at the Iowa State University DNA Sequencing and Synthesis Facility. For the nearly full-length 16S rDNA sequence, we queried the BLAST-nt search program (Altschul et al., 1990) of the GenBank database maintained by the National Center for Biotechnology Information (Bethesda, MD).

### Selective Media and Enumeration of Bacteria

Leachate samples were serially diluted in saline-phosphate buffer and plated onto Luria–Bertani agar supplemented with 100 μg $mL^{-1}$ kanamycin and 10 μg $mL^{-1}$ rifampicin, respectively, for enumeration of RS2G. The membrane filtration technique was used to enumerate fecal streptococci by plating onto KFS agar (Difco, Detroit, MI), and *E. coli* and total (non-*E. coli*) coliforms by plating on m-Coliblue24 (Hach Company) using the procedures provided by the manufacturer. Plates were inverted and incubated at 37°C for 24 h. RS2G colony counts were confirmed by placing the plates in a long-wavelength UV light box and counting only those colonies that fluoresced green. Green fluorescent protein fluoresces green when excited with ultraviolet light (365 nm) or optimally when excited at 488 nm and the emission optimum is 510 nm. All red and light red colonies growing on KFS agar were counted and assumed to be presumptive fecal streptococci. All light red to red and light blue to blue colonies growing on m-Coliblue24 were counted and assumed to be presumptive total coliforms (non-*E. coli*) and *E. coli*, respectively. Soil

samples (10 g) were resuspended in sterile buffered saline, sonicated in an ultrasonic cleaning bath for 5 min, and then shaken for 15 min on a gyratory shaker before enumeration of bacteria. The number of colony forming units (CFU) per sample was $\log_{10}$–transformed and expressed as log CFU 100 $mL^{-1}$ or total log CFU in the leachate or soil sample. For purposes of statistical analysis, in samples that had no colonies at our detection limit we assigned a value that was 10-fold less than the detection limits, which was 10 CFU $mL^{-1}$ leachate and 100 CFU $g^{-1}$ of soil for RS2G and 100 CFU $mL^{-1}$ of leachate for total coliforms and fecal streptococci.

### Inoculum Preparation

All soil cores were inoculated with manure spiked with *E. coli* isolate RS2G grown overnight in Luria–Bertani broth (Gibco/BRL) on a gyratory shaker (200 rpm) at room temperature. The culture was pelleted by centrifugation ($5000 \times g$) and resuspended to the original volume in sterile distilled water. Fresh manure was obtained from Iowa State University's Swine Nutrition Management and Research Center near Ames, IA. For each core that received manure, 15 mL of inoculum was mixed with 152 mL of liquid manure slurry, which resulted in each core receiving $8.02 \times 10^9$ CFU ($4.8 \times 10^7$ CFU $mL^{-1}$) of RS2G with 100% of the population expressing green fluorescent protein as determined by visual inspection of the colonies.

### Soil Cores and Experimental Treatments

Thirty soil cores were collected from the Kelly Farm located near the Iowa State University Agronomy and Agricultural Engineering Research Center near Ames, IA. The soil was Clarion loam (fine-loamy, mixed, superactive, mesic Typic Hapludoll) (bulk density = 1.42 g $cm^{-3}$, pH = 6.8, and 3.5% organic matter) and was previously cropped under an annual corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] rotation. Soil cores were extracted in late summer 2000, before the soybean harvest, using a Giddings probe. The 20-cm-wide × 30-cm-long cores were extracted in a 38-cm-long autoclaved galvanized tube that had been sharpened on the down-facing edge; this provided a holding area above the soil surface for the liquid manure and simulated rain. Autoclaved screens were installed on the bottom of each core to prevent soil loss. Wax was poured into crevices and spaces visible between the soil and the wall of the galvanized tube. The cores were then arranged in a random block design in a leachate collection apparatus comprised of 25-cm-diameter autoclaved funnels and a guide table that held the cores upright over the funnel. The cores were kept at room temperature (approximately 21°C) and normal building relative humidity (approximately 60%). The cores were saturated to field capacity by placing them in buckets of water for 48 h and allowing saturation to take place from bottom to top, and then the soil water was allowed to drain for 2 d. Three manure application methods were simulated in this study. The manure was poured directly over the soil column to simulate a no-till broadcast manure application. The top 2.5 cm of the soil column was disrupted with a sterile spatula before the manure application to simulate a surface-tilled broadcast manure application. The manure was poured directly over the soil column and then the top 2.5 cm of soil was disturbed with a sterile spatula to simulate an incorporated broadcast manure application. Manure was applied to cores once at the beginning of the experiment at a rate consistent with best management practices for optimal nitrogen utilization in a corn–soybean rotation in these soils.

### Rainfall and Leachate Collection

We chose to simulate intense convective rains (thunderstorms), which are common rainfall events in Iowa during spring and early summer (April–June). A rainfall rate of 50.8 mm over a 4-h period was simulated by applying five 330-mL aliquots of sterile distilled water at hourly intervals (total of 1650 mL) to each core surface. A subset of cores received one rainfall event on either the 4th, 8th, or 16th day after manure application. A second subset of cores received two rainfall events on the 8th and 16th day after manure application. A third subset of cores received three rainfall events on Days 4, 8, and 16 following manure application. A fourth set of cores did not receive any manure application, but they did have three simulated rainfall events on Days 4, 8, and 16. After each rain, leachate was collected at the bottom of each soil core in sterile plastic sample bottles until the cores stopped draining. Leachate samples were stored at 4°C for no longer than 24 h before enumeration of bacteria. At the end of the experiment, we assessed RS2G survival in the soil and its distribution throughout the core as well as soil moisture content at each depth. Soil was sampled at 0- to 2.54-, 0- to 10-, 10- to 20-, and 20- to 30-cm depths. Three 1-cm-diameter subcores were taken from each depth and each subcore was homogenized in a plastic bag by hand before determining RS2G concentrations.

### Statistical Analysis

Statistical analyses were performed by using JMP Version 4.04 (SAS Institute, 2001). For the leachate analyses, a separate two-factor analysis was performed on the leachate volume and RS2G, total coliforms, and fecal streptococci concentrations. In these analyses manure application method and date of the rain event were subplot treatment factors. For the RS2G survival in soil analyses, a separate two-factor analysis was performed on each set of cores that were rained on at different times after manure application. In these analyses manure application method and soil depth were subplot treatment factors. For each analysis, a Student's *t* test ($P = 0.05$) was calculated by JMP software for comparisons among treatment means. For comparisons between RS2G and total coliform concentrations within a sample we performed a paired *t* test analysis. Unless stated otherwise, values reflect the mean ± SE of three replications.

## RESULTS

### Characterization of RS2G

Green fluorescence and rifampicin and kanamycin resistance were stable since all of these traits were maintained in 98 ± 2% (mean ± SE; $n = 3$) of the RS2G population following cultivation over 100 generations in the absence of antibiotic selection. Constitutive expression of green fluorescent protein did not reduce the fitness of RS2G since culture doubling times in Luria broth and survival in manure and soil were comparable with those observed for the nongreen fluorescent parent RS2 (data not shown). In a series of preliminary studies, we examined the survival properties of RS2G in manure and in soil that was spiked with manure over a three-week period. Population sizes of RS2G in the manure did not change during the first three days after inoculation of the manure. Survival of RS2G in manure-treated soils did not change during the first eight days and there-

after there was a consistent decline in RS2G culturability and by the 16th day it had decreased to 10% of the concentration on the 8th day. There were no indigenous green fluorescent colonies in manure and soil samples in uninoculated samples.

## Effect of Time Before First Rain on RS2G Leaching

RS2G was mixed with the manure before it was applied to the cores. We varied the length of time before applying 1650 mL of water (equivalent to 50.8 mm of rain) to each core and collecting the leachate to evaluate the mobility of bacteria through the soil cores following a rain event. The volume of liquid manure (167 mL) applied to each core was insufficient to produce any leachate. The volume of leachate collected was not affected by the length of time before the first rainfall event, but it was affected by the manure application procedure (Table 1). Less leachate was collected from cores in which the manure was incorporated into soil than from cores in which the surface was disturbed before manure application (tilled) or undisturbed (no-till). The manure solids created a visible manure layer over the surface of both the tilled and no-till treated columns but not over the columns in which the manure was incorporated into the soil surface.

The RS2G concentrations in the leachate were affected by the length of time before the first rainfall event and not by manure application procedure (Table 1). In general, there was a 10- to 316-fold reduction in RS2G concentrations in the leachate if the length of time before the first rainfall increased from 4 to 8 d. Increasing the length of time before the first rainfall from 8 to 16 d resulted in a 0- to- 50-fold further reduction in RS2G leachate concentrations. Based on a paired *t* test, there were 8- to 79-fold significantly ($P = 0.0005$) greater RS2G than total coliform concentrations in the leachate following a rain on Day 4, no significant differences ($P = 0.38$) between RS2G and total coliform concentrations following a rain on Day 8, and 4- to 32-fold significantly lower ($P = 0.0002$) RS2G than total coliform concentrations following a rain on Day 16. The total coliform concentrations in the leachate from manure-treated cores were generally comparable with those in the leachate obtained from the control cores (Table 2). There were 10- to 32-fold fewer fecal streptococci in the leachate if the length of time before the first rainfall event was increased from 4 to 8 d (Table 1), and increasing the length of time before the first rainfall event to 16 d did not result in any consistent change in fecal streptococci concentrations.

## Effect of Multiple Rains on RS2G Leaching

Regardless of the manure application procedure, leachate concentrations of RS2G after the first rainfall event were equivalent (Table 2). A second rainfall event on the 8th day after manure application resulted in a 16- to 50-fold decrease in the RS2G leachate concentration (Table 2), and the second rainfall contributed only 1 to 2% of the total RS2G cells that were transported through the core following the two rainfall events. The leachate RS2G concentrations following a third rainfall event on the 16th day after manure application were equivalent to the amount that leached following the second rainfall event on the 8th day and contributed only 1 to 2% of the total RS2G cells present in the leachate after the three rainfall events. Given multiple rainfall events, most of the RS2G that was leached had leached after the first rainfall event (compare data in Tables 2 and 3). In cores (controls) that did not receive manure, the indigenous soil *E. coli* concentrations in the leachate were comparable with the RS2G concentrations in the leachate from manure-treated cores (Table 2).

There was no statistically significant difference in the leachate concentrations of total coliforms collected following each rainfall event among the various manure application procedures and for the nonmanured control cores (Table 2), except there was a significantly lower total coliform concentration in leachates collected from the no-till cores following the first rainfall event. In general, in the manure-treated cores, there was a significant decrease in the fecal streptococci concentration following the second rainfall event on the 8th day compared with the first rainfall event on the 4th day after manure application (data not shown). There was no significant difference in the leachate fecal streptococci concentrations collected following rains on the 8th and 16th days after manure application (data not shown).

After two equivalent rain events, if the length of time before the first rainfall event increased from 4 to 8 d there was a substantial (8- to 100-fold) decrease in the RS2G concentration in the leachate (compare Experiments 1 and 3 in Table 3). However, there was only a

**Table 1. Effect of time between manure application and the first rainfall event on leachate concentrations of *E. coli* RS2G and other indicator bacteria.**

| | No-till | | | | Tilled | | | | Incorporated | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time† | Leachate‡ | RS2G§ | TC§ | FS§ | Leachate | RS2G | TC | FS | Leachate | RS2G | TC | FS |
| d | mL | log CFU | | | mL | log CFU | | | mL | log CFU | | |
| 4 | 1315a | 7.9a | 6.0ab | 5.3a | 1079a | 7.6a | 6.7a | 5.7a | 851a | 7.6a | 6.4a | 5.6a |
| 8 | 872b | 5.4b | 5.4b | 3.8b | 1038a | 6.6b | 6.3a | 4.7b | 1020a | 5.7b | 6.4a | 4.1b |
| 16 | 1355a | 5.9b | 6.5a | 4.8a | 1132a | 4.9c | 6.3a | 4.3b | 683a | 4.2c | 5.7a | 3.8b |

† The length of time between manure application and a 50.8-mm (1650-mL) rain event.
‡ For the leachate collected, there was a significant ($P \leq 0.02$) effect of manure application procedure on the volume collected.
§ Total coliforms (TC) and fecal streptococci (FS). For RS2G and FS concentrations in the leachate there was a significant ($P \leq 0.0001$) effect of time between manure application and the first rainfall. Means ($n = 3$) with the same letter in each column are not significantly different at $P = 0.05$ level using a Student's *t* test for comparison of treatment means.

slight, if any, decrease in total coliform and only a 4- to 16-fold decrease in leachate fecal streptococci concentrations if the length of time before the first rain event increased from 4 to 8 d. There were more RS2G, total coliforms, and fecal streptococci leached from cores following the second rainfall event on Day 16 than following the first rainfall event on Day 8 (compare Day 8 in Table 1 and Experiment 3 in Table 3). This is in contrast to what was observed when the first rainfall event occurred 4 d after manure application and the second rainfall occurred 8 d after manure application (compare the results from Day 4 in Table 2 and Experiment 2 in Table 3). Taken together, these results suggest there is a dynamic interplay between the length of time between manure application and first rainfall event and the leachability of manure-derived bacteria following a second rainfall event.

## Predicting Leachability of RS2G

A regression analysis (Fig. 1) was performed with leachate concentrations of RS2G measured from cores that experienced a single rainfall event on the 4th, 8th, or 16th day after manure application and from cores that experienced three rainfall events after manure application. Linear regression parameters for *E. coli* RS2G concentrations in the leachate from cores that received one rainfall event at various days were $r^2 = 0.81$; $P < 0.0001$; slope = −0.218; $y$ intercept = 7.4 log CFU 100 mL$^{-1}$; $x$ intercept = 34 d; $x$ intercept, 95% lower confidence limit = 30 d; and $x$ intercept, 95% upper confidence limit = 40 d. Linear regression parameters for *E. coli* RS2G concentrations in leachate over the 16-d period for cores that received three rainfalls were $r^2 = 0.57$; $P$ value < 0.0001; slope = −0.207; $y$ intercept = 7.13 log CFU 100 mL$^{-1}$; $x$ intercept = 56 d; $x$ intercept, 95% lower confidence limit = 43 d; and $x$ intercept, 95% upper confidence limit = 84 d. Although other models may be better than the linear regression model for predicting the leachability of RS2G the linear regression models suggest that RS2G would continue to leach through the soils for about 34 to 56 d after manure application, depending on the timing before and frequency of rainfall events.

## RS2G Survival in Soil

Upon completion of the simulated rainfall events, we evaluated the survival of RS2G in soil at various depths in cores where the first rainfall occurred 4, 8, or 16 d after manure application, receiving three, two, or one rainfall events, respectively (Tables 4 and 5). RS2G population sizes were highest 0 to 10 cm below the core surface and lowest 20 to 30 cm below the core surface (Table 4). In general, 25 to 63% of the RS2G population was recovered from the top 2.5 cm of the core, which included the manure layer (data not shown). There was greater RS2G survival when the manure was incorporated into the soil, followed by the tilled and then the no-till treatments (Table 4). Interestingly, there was no decrease in the concentration of RS2G in the soil with an increase in time between manure application and the

**Table 2. Effect of three consecutive rainfall events on leaching of RS2G and other indicator bacteria.**

| | No-till | | | | Tilled | | | | Incorporated | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time† | Leachate‡ | RS2G§ | TC§ | FS§ | Leachate | RS2G | TC | FS | Leachate | RS2G | TC | FS | Leachate | *E. coli*¶ | TC | FS |
| d | mL | log CFU | | | mL | log CFU | | | mL | log CFU | | | mL | log CFU | | |
| 4 | 1315a | 7.9a | 6.0a | 5.3a | 1079a | 7.6a | 6.7a | 5.7a | 851c | 7.6a | 6.4a | 5.6a | 1323a | – | 6.1b | 6.1a |
| 8 | 1233a | 6.2b | 5.4a | 4.3b | 1002b | 6.4b | 6.1a | 4.7b | 1342a | 6.3b | 6.0a | 4.6b | 1318a | 6.6a | 6.8a | 6.3a |
| 16 | 1222a | 6.2b | 5.7a | 4.8ab | 1210a | 6.2b | 5.8a | 4.0b | 1035b | 6.0b | 6.2a | 4.4b | 1148a | 6.0b | 6.2ab | 6.0a |

† Number of days after the manure was applied to the core when the rain event occurred. Each rainfall event consisted of 50.8 mm (1650 mL).
‡ There was a significant effect of the day of rain event ($P < 0.0013$) and manure application procedure effect ($P < 0.018$) on the volume of leachate collected.
§ Total coliforms (TC) and fecal streptococci (FS). For RS2G concentrations there was a significant day of rain effect ($P < 0.0001$), for the TC there was no significant manure application procedure or day of rain effect, and for the FS concentrations there was both a day of rain effect ($P < 0.005$) (counts in the leachates from the control were higher than in leachate collected from manure treated cores on Days 8 and 16) and manure application procedure effect ($P < 0.0004$). Means ($n = 3$) with the same letter in each column are not significantly different ($P \geq 0.05$) based on a Student's $t$ test for comparing treatment means.
¶ Values for the control reflect the indigenous *E. coli* population and not the RS2G population.

**Table 3. Cumulative effect of multiple rainfall events on leaching of RS2G and other indicator bacteria.†**

| Manure application procedure | Time between manure application and rainfall events | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Days 4 and 8 (Experiment 1)‡ | | | | Days 4, 8, and 16 (Experiment 2)§ | | | | Days 8 and 16 (Experiment 3)¶ | | | |
| | Leachate | RS2G | TC | FS | Leachate | RS2G | TC | FS | Leachate | RS2G | TC | FS |
| | mL | log CFU | | | mL | log CFU | | | mL | log CFU | | |
| No-till | 2548ab | 7.9a | 6.4b | 5.7b | 3770a | 7.9a | 6.9a | 6.2b | 1910a | 5.9b | 6.2a | 4.5a |
| Tilled | 2848a | 7.5a | 7.3a | 5.9b | 4058a | 7.5a | 7.5a | 6.0b | 2033a | 6.6a | 6.8a | 5.3a |
| Incorporated | 2193b | 7.6a | 7.0ab | 6.2ab | 3225b | 7.6a | 7.3a | 6.6ab | 2122a | 6.2ab | 6.9a | 5.1a |
| Control | 2642a | NA# | 7.1a | 6.7a | 3789a | NA | 7.5a | 7.1a | ND†† | ND | ND | ND |

† Total coliforms (TC) and fecal streptococci (FS). For each comparison (experiment) a separate one-way analysis of variance was performed on total leachate volume and RS2G, TC, and FS concentrations. Means ($n$ = 3) with the same letter in each column are not significantly different ($P \geq 0.05$) based on a Student's $t$ test for comparing treatment means.
‡ For Experiment 1, manure application procedure was not significant ($P \geq 0.05$) for RS2G, TC, and FC concentrations, but it was significant ($P = 0.03$)) for total leachate volume.
§ For Experiment 2, manure application procedure was significant ($P \leq 0.02$) for leachate volume and FC concentrations.
¶ For Experiment 3, manure application procedure was not significant ($P > 0.05$) for any comparison.
# Not applicable.
†† Not determined.

first rainfall event (Tables 4 and 5). Increasing the time before the first rain event decreased the concentration of RS2G in the leachate, even though there was no decrease in the concentration of RS2G in the soil cores (Table 5). Most of the total RS2G in the leachate was due to the cells that leached through cores following the first rainfall event after manure application (Table 2 and data not shown). Despite the differences in time between manure application and the first rainfall event and the number of rainfall events a core experienced, there was no difference in the grand total of RS2G recovered even though there were differences in the amount of RS2G collected in the leachates (Table 5). This suggests that most of the RS2G that was applied to the soil was retained by the soil and this retention of RS2G creates a reservoir of RS2G cells in the soils that are potentially leachable. The total amount of RS2G recovered from soil and leachate was 1 to 3% of the inoculum with the tilled and no-till treatments and 10 to 13% of the inoculum with the incorporated manure application.

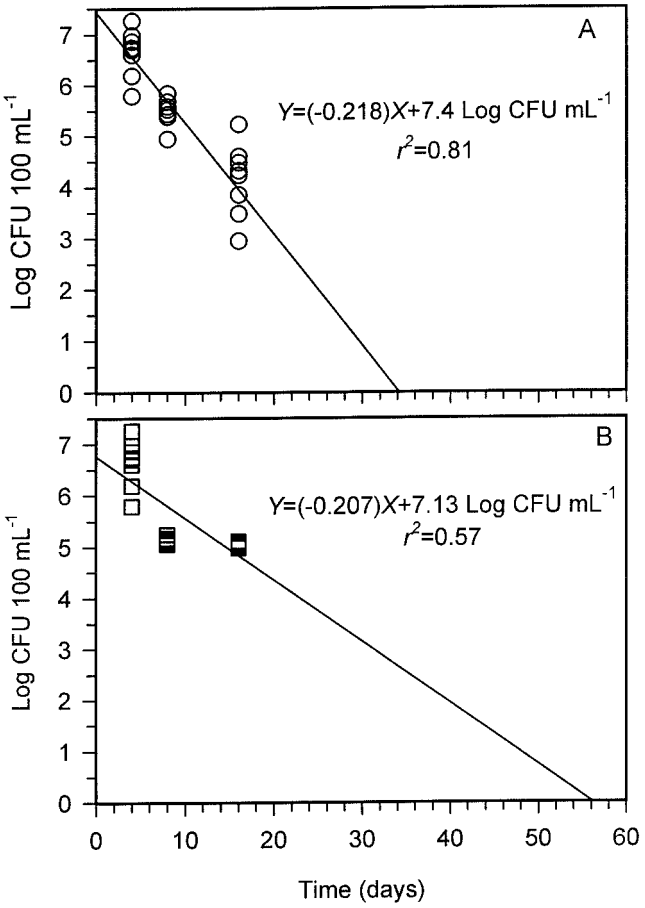


**Fig. 1. Concentrations of *E. coli* RS2G recovered from soil core leachate samples after one or more 50.8-mm rainfall events at different times following manure application. (*A*) The ○ symbols indicate samples obtained from cores that received a rainfall event on the 4th, 8th, or 16th day after manure application. (*B*) The □ symbols indicate samples obtained from cores that received three rainfall events on the 4th, 8th, and 16th day after manure application. Since there was no statistically significant effect of manure application on RS2G concentrations in the leachate, the three replicate cores for each manure application procedure were pooled to generate a total of nine samples per rainfall event.**

## DISCUSSION

Our results show that a significant number of *E. coli* RS2G cells can still be leached through the upper portion of the soil profile after the manure has resided for extended periods on the soil surface before the first rain event (Fig. 1 and Table 1). Subsequent rain events can result in additional leaching of RS2G cells (Fig. 1, Tables 2 and 3) at levels that are substantially lower than were leached following the first rain event. We did not detect a significant difference in RS2G leachability resulting from different manure application procedures (no-till broadcast, broadcast on tilled soil, and incorporation following broadcast). The amount of rainfall that we used (50.8 mm) is an average rate for storm events in the spring or early summer for the area studied. The intermittent wetting and drying of the soil throughout the study did not influence the survival of RS2G in the soil (Tables 4 and 5). It is likely that the available moisture remaining in the soil between rainfall events was adequate for bacterial survival, although unlike a recent report demonstrating growth of *E. coli* O157:H7 in wet soils (Gagliardi and Karns, 2000) there did not appear to be any growth of RS2G in the soil. Incorporation of the manure into the soil increased *E. coli* RS2G

**Table 4. Survival of RS2G at various depths within cores that experienced various lengths of time after manure application before the first rainfall and number of rainfall events.†**

| Manure application procedure | Total RS2G recovered | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Days 4, 8, and 16 (Experiment 2) | | | Days 8 and 16 (Experiment 3) | | | Day 16 (Experiment 4) | | |
| | Depth (cm) | | | | | | | | |
| | 0–10 | 10–20 | 20–30 | 0–10 | 10–20 | 20–30 | 0–10 | 10–20 | 20–30 |
| | log CFU | | | | | | | | |
| No-till | 7.46b | 6.18a | 5.69b | 7.62b | 5.84b | 5.69b | 8.17b | 6.26ab | 6.09b |
| Tilled | 7.50b | 6.64a | 6.70a | 8.16ab | 6.91a | 6.14a | 8.19b | 5.78b | 5.58c |
| Incorporated | 8.77a | 6.84a | 6.55a | 8.87a | 7.20a | 6.34a | 8.69a | 7.15a | 7.26a |

† For all experiments there was a significant manure application method and depth effect ($P \leq 0.009$). Means with the same letter in a column are not significantly different at $P = 0.05$ based on a Student's $t$ test. The log CFU/depth values were derived by multiplying the CFU g soil × g dry weight of soil at that depth increment.

presumably because it protected *E. coli* RS2G from environmental stresses, such as desiccation and UV exposure, which are more severe at the soil surface than in protected sites within the soil fabric. The liquid manure solids may have created a protective layer over the soil surface thereby protecting RS2G from desiccation in the tilled and no-till treatments.

Following a rain event, percolating water transports RS2G cells through the core, which redistributes RS2G in the soil profile, potentially to become residents of these newly colonized habitats. Most of the RS2G cells were found near the soil surface and fewer were found throughout the remaining portions of the core. Our observation that RS2G cells can survive for extended periods (16 d) before the first rain event clearly indicates that either the nutrients provided by the manure facilitate this long-term survival, that *E. coli* RS2G is able to compete with both the indigenous soil microbiota and the manure microbiota for nutrients and microhabitats in the soil, or that *E. coli* RS2G or other manure bacteria produce toxic compounds that decrease the competitive ability of indigenous soil bacteria and consequently increase the survival capabilities of RS2G. We only examined the culturable RS2G cells and it is possible that we underestimated the total number of RS2G cells in the leachate that were injured yet viable, but not culturable (Rompre et al., 2002; Roszak and Colwell, 1987). Leachates collected from the no-manure treated cores also had relatively high concentrations of the indicator organisms. We evaluated the concentrations of total coliforms and fecal streptococci in our leachate samples to have an alternative assessment of the mobility of bacteria through the soil cores. It is reasonable to assume that the fecal streptococci concentrations in the leachate obtained from manure-treated cores should equal or exceed their concentrations in the leachate from the control (no-manure) cores. Yet, this was not the case. In general, there were fewer fecal streptococci in the leachate from manure-treated cores than from the no-manure treated cores (Tables 2 and 3), suggesting that manure treatments either killed or prevented the leaching of soil fecal streptococci. This decrease could be due to death of soil fecal streptococci because they are susceptible and/or sensitive to toxic compounds present in the manure or they are unable to compete for nutrients or microsites with manure bacteria introduced into the soil. The dynamic nature of changes in the population structure of fecal streptococci has also been reported in runoff water containing fresh cattle feces (Doran and Linn, 1979).

There was no difference among the manure application procedures and the control cores in the total coliform concentrations in leachate collected from those cores, although there were discrepancies between the RS2G and total coliform concentrations in the leachates from the manure-treated cores. Similarly, leachates collected from cores in which the manure was spiked with RS2G should have total coliform counts that equal or

**Table 5. Total recovery of RS2G from soil cores and leachate.**

| Rainfall events | Manure application | Recovery† | | |
|---|---|---|---|---|
| | | Soil‡ | Leachate§ | Total¶ |
| d | | log CFU | | |
| 4, 8, and 16 (Experiment 2) | no-till | 7.556 ± 0.281b | 7.927 ± 0.094a | 8.108 ± 0.061b |
| | tilled | 7.622 ± 0.130b | 7.493 ± 0.246a | 7.870 ± 0.158b |
| | incorporated | 8.778 ± 0.329a | 7.571 ± 0.482a | 8.812 ± 0.337a |
| 8 and 16 (Experiment 3) | no-till | 7.632 ± 0.266b | 5.881 ± 0.266a | 7.640 ± 0.288b |
| | tilled | 8.293 ± 0.253ab | 6.572 ± 0.070b | 8.304 ± 0.248ab |
| | incorporated | 8.889 ± 0.110a | 6.206 ± 0.447a | 8.891 ± 0.108a |
| 16 (Experiment 4) | no-till | 8.175 ± 0.294a | 5.900 ± 0.216a | 8.178 ± 0.293a |
| | tilled | 8.192 ± 0.192a | 4.882 ± 0.241b | 8.193 ± 0.027a |
| | incorporated | 8.842 ± 0.283a | 4.235 ± 0.420b | 8.842 ± 0.283a |

† Values followed by the same letter within a column are not significantly different based on a Student's $t$ test for comparison of treatment means.
‡ For the soil cores, the effect of number of rain events on recovery of RS2G was not significant, but the method of manure application was significant ($P < 0.0001$).
§ For the leachate, there was a significant effect of number of rain events ($P < 0.0001$) on recovery of RS2G, but the method of manure application had no significant effect on recovery.
¶ For the total recovered, there was no significant effect of number of rain events on RS2G recovery, although the method of manure application ($P < 0.0005$) had a significant effect on recovery.

exceed those for RS2G. Yet, this was clearly not the case when the time before the first rain event following manure application increased from 4 to 16 d (Table 1). These results could be explained by a poor ability of RS2G to grow on the indicator medium when they are stressed or that over time there is a decrease in RS2G concentrations that occurs concurrently with an increase in either indigenous soil total coliforms or preferential survival of non-RS2G total coliforms present in the manure. These results suggest that RS2G enumerations based on the use of the total coliform indicator medium would underestimate the true RS2G concentrations. Similar discrepancies between indicator media and selective media for enumeration of specific *E. coli* populations have been reported previously (Gagliardi and Karns, 2002).

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