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DIEOFF AND RELEASE OF FECAL PATHOGENS FROM ANIMAL MANURE

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***Abstract*.** Contamination of surface waters with fecal pathogens has been linked to many outbreaks of human illness worldwide. The objective of this study is to evaluate the environmental factors that influence pathogen mortality in exposed manure and their initial migration from manure to the surface water. A 13 mm thickness of manure was held at constant environmental conditions for more than 6 weeks. Concentrations of fecal coliforms (FC), *E. coli*, and fecal streptococci (FS) were measured on multiple occasions during this period. A factorial design of 3 temperatures (4, 27, and 41 C) and 3 manure moisture contents (79, 69, and 50% wet basis). All treatments had high bacterial concentrations, initially 7.7×10^3 to 7.9×10^5 cfu/g for FC and 7.0×10^4 to 8.8×10^6 cfu/g for FS. In most cases, those concentrations increased over the 42 to 47 day experimental period. In general, 27 C appeared to provide the best conditions for bacterial growth, whereas 41 C demonstrated the low growth or slight dieoff under otherwise-similar conditions. This study demonstrates that fecal bacteria are resilient under a wide range of natural conditions; even after cattle leave a feedlot, microbial populations are still likely to be high.

Keywords. Manure, fecal pathogens, mortality, release, water, moisture, temperature.

Introduction

Pathogens in dairy cow manure include protozoans, bacteria and some enteric viruses. There have been many cases of contamination related to cow manure; *e.g.*, *Escherichia coli* (*E. coli*) *O157:H7* in hamburgers, *Salomonella ssp.* in ice cream and eggs, and *Cryptosporidium parvum* in Milwaukee water supply (Pell, 1997).

Protozoans *Cryptospordium parvum (C. parvum)* and *Giardia spp.* can cause severe diarrhea in both animals and humans. *C. parvum* was first identified in 1907 but not considered pathogenic until 1975 (Ross, 1990). Even though *Giardia* was identified in the 17th century, the taxonomy of this genus is not well understood (Pell, 1997). *C. parvum* is considered the most difficult to control because it is not affected by traditional chlorination of water treatment (Craik *et al.*, 2001; Driedger *et al*., 2001).

Outbreaks of bacteria contamination such as *L. monocytogenes, Salmonella spp., M. paratuberculosis* and *E. coli O157:H7* have been linked with cattle (Mubiru *et al*., 2000; Pell, 1997; Wang *et al.,* 1996; Kearney *et al.,* 1993). *L. monocytogenes* and *M. paratuberculosis* are widely dispersed in the environment because of their ability to grow under a wide range of conditions. *E. coli O157:H7* has been considered as an important agent of food-borne disease with worldwide distribution since it was first identified in 1982 (Hancock *et al.*, 1997).

Now there are sensitive DNA gene probes that can be used to detect those bacterial pathogens even at a low level in some cases (Dargatz *et al.,* 1997; Droffiner and Brinton, 1995). Biosensors for rapid bacterial detection have been developed also (Monis and Saint, 2001; Yang *et al*., 1998; Ye *et al*., 1997; Deshpande and Rocco, 1994). However, these gene probe methods require careful sample handling and the use of sophisticated laboratories to ensure complete DNA extraction without contamination.

In water quality control and resource management, indicator organisms are used instead of the actual pathogens (Mubiru *et al.*, 2000; Tate *et al.*, 2000). The reasons for such practice include:

1 Indicator bacteria are usually present in greater numbers than pathogens,

2 Indicator bacteria are easier to isolate, and

3 Indicator bacteria are much safer to work with than that of pathogens.

Total coliforms, fecal coliforms, *E. coli*, and fecal streptococci are commonly used indicator bacteria. The sensitivity of each group for determining fecal pollution varies (Thelin and Gifford, 1983).

Payment *et al.* (2000) checked total coliforms, fecal coliforms, and *Clostridium perfringens* against *Giardia lamblia*, *Cryptospridium*, and human enteric viruses in Saint Lawrence River in Canada. They found significant correlations between the bacterial indicators and pathogens. There are studies showing that these groups of indicator bacteria may not be well correlated with presence of *C. parvum* (Brush, 1997). However, Hsu *et al.* (2001) found that while parasites were concentrated with cartridge filters, the significant correlation was observed between *Giardia* concentrations and the levels of heterotrophic bacteria, total coliforms, fecal coliforms and Enterococcus. No significant relationship was found between *Cryptosporidium* oocysts and indicator microorganisms besides fecal coliforms. When parasites were concentrated with membrane filters, a significant correlation was observed between *Giardia* concentrations and the levels of herotrophic bacteria and fecal coliforms. No significant relationship was found between *Cryptosporidium* and any indicator microorganism.

Pathogens in Manure

Cryptospordium parvum (C. parvum) and *Giardia spp.* are two pathogenic protozoans of concern found in cow manure. Both have complicated life cycles and change forms several times. *C. parvum* oocysts and *Giardia* cysts can resist many environment pressures, which enables them to remain viable in the environment for at least a year (Craik *et al.*, 2001; Driedger *et al*., 2001; Pell, 1997).

Pathogenic bacteria *L. monocytogenes, Salmonella spp., M. paratuberculosis* and *E. coli O157:H7* are of great concern in North America. All of these organisms can be transmitted from animals to humans directly or though food and water supplies. *E. coli O157:H7* is a verotoxin-producing strain of *E. coli*. They can cause serious symptoms and survive under adverse conditions. *L. monocytogenes* and *M. paratuberculosis* are widely dispersed in the environment (Mubiru *et al*., 2000; Pell, 1997; Wang *et al.,* 1996; Kearney *et al.,* 1993). Fecal bacteria concentration in cow manure can vary widely depending on the cow age, health conditions, diet, housing types etc., as well as the age of manure.

Similar to the protozoans, viruses are unable to replicate outside of their host, and therefore, their numbers never increase once they are released into the environment. Many types of viruses have been found in dairy cattle manure (Table 1). The stability of different viruses varies depending on whether the virus particle is adsorbed to or embedded within suspended solids (Pesaro *et al*., 1995); environmental temperature and pH; and the presence of bacteria that can inactivate viruses (Deng and Cliver, 1995).

Table 1. Viruses excreted by dairy cattle (Dinter and Morein, 1990).

Zoonotic Virus	**Environmentally stable Virus**
Vaccinia	Adenovirus
Pseudorablies[1]	Parvovirus
Bovine popular stomatitis[1]	Acute respiratory and enteric disease
Bovine rotavirus[1]	Bovine rotavirus
Bovine respiratory virus[2]	Astrovirus
Vesicular stomatitis	Bovine enterovirus
Rift Valley Fever virus[3]	Rift Valley Fever virus
Rabies	

[1] Possible, but not common.
[2] Humans are likely reservoirs of infection.
[3] Primarily found in Africa.

Survival of Pathogens

Doyle *et al.* (1975) reported that fecal coliforms and fecal streptococci in fresh manure were 4.67×10^7 and 2.70×10^7/g dry solid, respectively. Larsen *et al*. (1994) reported that the average concentration of fecal bacteria was 5.6×10^5/g with a standard error of 2.4×10^5/g. McMurry *et al.* (1998) found fecal coliform concentrations averaging 1.3×10^7/g and ranging from 0.1×10^7 to 4.3×10^7/g in 6 samples of undercage poultry manure.

Crane (1988) observed bacteria growth in cow manure: an initial “after growth” for 3 to 6 days following manure storage and a “delayed regrowth” where bacterial densities began to increase slowly following 10 days of manure storage. Howell *et al.* (1996) also found that fecal coliforms and fecal streptococci increased for the first 3 days after dairy manure was added to different soil (sand, silt and clay) at different temperature (25°C, 35 °C). Mubiru *et al.* (2000) found both *E. coli* O157:H7 and non-pathogenic *E. coli* strains initially regrew in Kentucky Zanesville soil.

Generally, survival of bacteria in manure is affected by the source, pH, dry matter content, age, and chemical composition of the manure as well as by microbial characteristics. Environmental conditions play important roles for bacterial survival. The amount and availability of nutrients and carbon in soil will also affect pathogen survival (Entry *et al.*, 2000; Mubiru *et al.*, 2000). Davenport *et al.* (1976) suggested that bacterial survival is inversely related to temperature below 15 °C and found that maximum survival under natural conditions occurs in 0 °C water under ice cover. Crane *et al*. (1980) found that the mortality rate of enteric pathogens is initially very high. However, Zhai *et al*. (1995) concluded that two to three months is required in most cases to reduce pathogens to negligible numbers after manure has been applied to soil.

Crane (1988) suggested minimal die-off of bacteria was expected under typical storage temperatures of 5 °C to 15 °C. Howell *et al.* (1996) found that the mortality rates of fecal coliforms declined as temperature decreased. Mortality rates significantly increased from 4 °C, to 25 °C and 35 °C. Different pathogens have different mortality rates at the same condition. The addition of urine to stored feces caused increased die-off of fecal coliforms while enhancing the survival of fecal streptococcus (Crane 1988). Extremely hot (>28 °C) soil temperatures combined with drying effectively decreased survival rates (Sjogren, 1994; Reddy *et al*., 1981). Entry *et al.* (2000) found that mortality of total coliform bacteria and fecal coliform bacteria at different soil depths correlated with increasing temperature. Other studies have shown that temperature affects fecal bacteria survival in water, soil, and sediment (Faust, 1982; Gerba *et al*., 1975).

Stoddard *et al.* (1998) observed fine soil particles increase *E. coli* survival. Soils of fine texture and high organic matter content have been observed to support microbial populations three times larger than coarse textured soils. Howell *et al.* (1996) found that the mortality rates of fecal coliforms declined as sediment particle-size shrank. The mortality rates of fecal coliforms exceeded that of fecal streptococci. This is in contrast with observations by Sheer *et al.* (1992) that fecal streptococci survive longer than fecal coliforms in fine sediment and fecal coliforms survive longer in coarse sediment. Davies and Bavor (2000) reported the adsorption of bacteria to fine particles protected them from predators. The concentrations of bacteria in sediments were generally higher than the water column concentrations by several orders of magnitude. Thermotolerant coliforms and enterococci declined in concentration with time in a period of 28 days.

Among the physical and chemical properties in soil, soil-water content is one of the major factors determining fecal bacteria survival. Greater survival is associated with moist soils and periods of high rainfall. Survival of pathogenic bacteria in soil increases when the soil is moist. Entry *et al.* (2000) found that mortality of total coliform bacteria and fecal coliform bacteria at different soil depths correlated with decreasing moisture and increasing temperature in a curvilinear relationship. Mubiru *et al.* (2000) also found the matric potential in the individual soils was a significant factor. The availability of water in soil is found to be the overriding factor in *E. coli* survival. Powelson and Mills (2001) studied transport of *E. coli* in air-water interfaces in unsaturated porous media. They found that the presence of air-water interfaces reduced bacteria transport.

Himathongkham *et al.* (1999) reported survival times for *E. coli O157:H7* as 70, 56, and 49 days at 5, 22, and 37 °C, respectively. They found an exponential linear destruction of *E. coli O157:H7* and *Salmonella typhimurium* in cattle manure and manure slurry stored at different temperature (4, 20 and 37 °C). The main effects of time as well as temperature were pronounced with the most rapid destruction at 37 °C.

Mubiru *et al.* (2000) had conducted experiments to compare the mortality rate of *E. coli O157:H7* with that of a non-pathogenic *E. coli* strain in two typical Kentucky soils with different physical and chemical characteristics. Both strains appeared to have similar survival patterns in the individual soils: both initially regrew in the Zanesville soil, whereas die-off in Pope soil was apparent in the first week. Entry *et al.* (2000) found pathogen survival time in the upper soil varies from 4 to 160 days. Many pathogenic organisms can live in the soil for months.

Several regions of plant roots are known to produce compounds that may leak from root cells or be actively pumped by metabolic processes and which may inhibit certain microorganisms. In addition, the enhanced development of populations of bacteria (*Pseudomonas*) in the rhizosphere with antibiotic activity may also account for coliform die-off. On the other hand, anaerobic conditions were reported to prolong fecal coliform survival in natural waters (Gray, 1989). Ottova *et al.* (1997) studied fecal coliform removal in constructed wetlands. They suggested that root excretions of certain aquatic plants (*e.g.*, *Scirpus lacustris* or *Phragmites australis*) can kill fecal indicators (*E. coli*) and pathogenic (*Salmonella*) bacteria.

Perkins and Hunter (2000) concluded the following factors may influence the apparent bacterial removal efficiency of a reed bed: the rate of effluent flow; die-off rate; rate of removal by filtration and sedimentation; rate of addition from transient animal sources; and the rate of predation. These factors may be interrelated. Effluent flow rate may influence contact time with bed soil and vegetation and, hence, the opportunity for predation, filtration and sedimentation.

Pathogen Release from Manure

In most of the bacteria-release studies, rain simulators were used to simulate rains at different intensities (Brush, 1997). Kress and Gifford (1984) found that rainfall intensity had little effect on peak fecal coliform release from fecal deposits that were 2 or 10 days old. However, the effect was significant at age 20 days. The highest intensity gave the lowest peak counts. They suggested that the rainfall intensity effect might be related to the dryness of the fecal deposits.

Thelin and Gifford (1983) concluded that fecal deposits of 5 day or less of age released fecal coliform concentrations into the water on the order of millions per 100 ml. Fecal deposits as old as 30 day of age produced concentrations on the order of 4.0×10^4/100 ml. Equilibrium in fecal coliform release was reached after no more than 10 min of rainfall. The voided fecal coliforms pass through retardation, maximum population, and death phases. The retardation phase lasted about 1 day, the maximum population phase about 2 days, and the death phase continued beyond 30 days for an unknown period. The age of manure affected the length of time to reach equilibrium release of fecal coliforms. Increasing age extended the time to thoroughly wet a fecal deposit.

Larsen *et al*. (1994) monitored release of fecal coliforms from dairy cow manure and subsequently through a vegetative buffer plot. They found about 17% of fecal coliforms were released from 2.5-cm deep manure after 30 min of 5 or 10 cm/h simulated rainfall, and only 5% or less were found in runoff after passing through 1.37-m (or greater) vegetative buffer plots. Fecal bacteria may also be transported via subsurface water flow through soils. McMurry *et al.* (1998) found drainage water below 32.5-cm deep undisturbed soil blocks consistently exceeded

2.0×10^5 fecal coliforms per 100 mL and reached as high as 3.3×10^7 fecal coliforms per 100 mL. Well-structured soils had greater concentrations of fecal coliforms in leachate.

Brush (1997) studied release of *C. parvum* oocysts and other manure constituents, and correlated *C. parvum* release with each of the other measured constituents. He found that fecal coliforms and streptococci concentrations in the runoff water samples were spiky, with both major and minor peaks, and a low r^2 value for linear regressions of coliform and streptococcus populations against C. *parvum* oocyst concentrations. He also suggested that the majority of oocysts are not associated with the liquid portion of the feces, but with the same portion of the fecal solids as much of the NH_4-N and PO_4-P.

Objectives

These studies indicate that many factors influence the production, survival, and release of pathogens from animal manure. The overall goal of this study was to assess the influence of environment on survival of fecal bacteria in animal manure.

Materials and Methods

About 1 kg of fresh diary cow manure samples from the Kansas State Univeristy Animal Production Facility, about 5 minute drive from the campus, were collected just after the cow excreted on the ground and placed in two layers of trash bags in a plastic container for transportation. All samples for each experiment were taken from a single cow pie. Dry mass of the manure was obtained by drying fresh samples at 103-105 °C for about 3 hours, and used with initial mass to estimate initial manure moisture content. The average moisture content of the fresh manure was found to be around 80%.

Two experiments were run using different manure: Experiment 1 ran 47 days and Experiment 2 ran 42 days. Manure samples were collected from polystyrene dishes and alternately from one of the three replicates of each treatment after 5, 6, 7, 8, 9, 14, 19, 26, and 47 days in Experiment 1 and after 1, 2, 3, 7, 9, 14, 21, and 42 days in Experiment 2. Fresh manure was distributed into 27 polystyrene dishes (Fisher brand, Extra-Deep vented dishes, 100 mm dia. $\times$ 25 mm deep) with a manure depth of 13 mm. Nine dishes each were placed into a refrigerator, an open humidified box, and an oven. Three levels of air temperature and manure moisture content were arranged in a factorial design. Temperature was maintained at around 4±1 °C (refrigerator), 27±1 °C (room temperature, humidified box), 41±2 °C (oven). The moisture content was maintained at about 79±1% (initial water content), 69±2%, and 50±4%. Each treatment had 3 replicate dishes.

In 4 °C and 41 °C treatments, dishes maintained at 79% moisture content were covered throughout the experiment; the others were uncovered until they reached the desired moisture content. All dishes under room temperature were uncovered. Once or twice a day, each dish was weighed. Changes in weight were assumed to be due to evaporation of water. Distilled water was sprayed directly onto the manure as needed to maintain moisture content in the desired range.

At room temperature, manure samples ranging from 0.170 to 1.663 g were weighed in aluminum weighing dishes by an analytical balance (0.1 mg accuracy). About 10 mL distilled water was added into the aluminum dish, and the manure mixture was transferred into 1000-mL beakers. The aluminum dish was rinsed with distilled water at least three times to ensure that there was no manure left in the dish. A total of 500±10 mL of distilled water was added to each beaker. The mixture was allowed to settle for about 30 minutes, and then about 50 mL of

supernatant was collected and sent to the Biology Department lab for analysis. Water sample preparation was done within two hours of collecting manure samples for analysis.

Each water sample was diluted into three different concentrations using 0.01 mL, 0.1 mL, and either 1 mL or 10 mL of the original water sample. Then, the diluted water samples were filtered using 0.45 μm gridded sterile filter paper. The filter papers were put into different media: media mFC (Difco Brand, BD, Franklin Lakes, NJ) for fecal coliforms and KF (Difco Brand, BD, Franklin Lakes, NJ) for fecal streptococci. mFC plates were incubated at 44.5 °C for 24 hours and KF plates were incubated at 37 °C for 48 hours. After the mFC plates had been counted, the filters were transferred to MUG (4-methylumbelliferyl-β-D glucuronide Difco Brand, BD, Franklin Lakes, NJ) plates and incubated for 4-6 hours at 37 °C for *E. coli* enumeration. The microbial counts of each sample that was not too numerous to count were averaged and converted to colonies per gram of manure sample (at given moisture content).

Results and Discussion

After 6 weeks in both Experiments 1 (Figs. 1 and 2) and 2 (Figs. 2 and 3), a high percentage of the initial bacteria populations were detected in almost all dishes (Figs. 1 to 4). Final concentrations exceeded 10^5 cfu/g in a total 11 of 15 treatments for FC and 12 of 15 treatments for FS. Bacteria concentrations exceeded 10^9 cfu/g in some cases for both types of bacteria.

Experiment 1 clearly demonstrated the influence of temperature on fecal bacteria mortality. Initial concentrations were 7.7×10^3 cfu/g for FC and 7.0×10^4 cfu/g for FS (Figs. 1 and 2). After 47 days, average FC concentrations increased by 1.2 log units at 4 C and decreased by about 3.5 log units at 41 C (Fig. 1, Table 2). Similarly, average FS concentrations increased by about 3.8 log units at 4 C and decreased by about 1.2 log units at 41 C (Fig. 2, Table 2). For both types of bacteria, colonies increased at 4 C and decreased at 41 C. Differences between samples at different moisture contents were 1.9 log units or less for both FC and FS.

Table 2. Change in bacteria concentration ($\log_{10}$ difference from initial) after 47 days (Exp. 1).

	Temperature		
Moisture Content	4 °C	27 °C	41 °C
	Fecal Coliforms		
79%	+2.4	-	-3.7
69%	+0.5	-	-3.5
50%	+1.0	-	-3.4
	Fecal Streptococci		
79%	+3.9	-	-1.9
69%	+3.1	-	-0.5
50%	+4.5	-	-1.2

Experiment 2 indicated that both temperature and moisture content influenced fecal bacteria mortality. Initial concentrations were 7.9×10^5 cfu/g for FC and 8.8×10^6 cfu/g for FS (Figs. 3 and 4). The greatest growth of FC bacteria occurred at 27 C, where concentrations increased by 3.5 log units after 42 days (Fig. 3, Table 3). Growth of FC bacteria at 42 days was less both at higher and lower temperatures. Concentrations increased by 0.5 log units for 4 C and 2.0 log units for 41 C. However, in these treatments, the samples with higher moisture content (79%)

average an increase of 2.6 log units whereas the lower moisture contents (50 and 69%) averaged an increase of 0.3 log units.

Table 3. Change in bacteria concentration (log_{10} difference from initial) after 42 days (Exp. 2).

	Temperature		
Moisture Content	4 °C	27 °C	41 °C
	Fecal Coliforms		
79%	+2.5	+3.4	+2.8
69%	-0.3	+3.6	-0.8
50%	+0.7	+3.5	+0.5
	Fecal Streptococci		
79%	+1.6	-0.2	-0.6
69%	+1.3	+2.7	-1.4
50%	-0.4	-0.6	+2.1

Table 4. Change in bacteria concentration (log_{10} difference from initial) after 3 days (Exp. 2).

	Temperature		
Moisture Content	4 °C	27 °C	41 °C
	Fecal Coliforms		
79%	+1.9	+2.6	+3.2
69%	+1.6	+3.6	+3.6
50%	+0.3*	+3.5	+3.7
	Fecal Streptococci		
79%	+1.4	+1.5	+1.0
69%	-0.8	+1.9	+2.4
50%	+1.3*	+1.5	+1.9

*After 7 days.

In most of the treatments, there was an increase in bacterial numbers for the first several days under controlled conditions (Figs. 1 to 4). Table 4 summarizes this effect for the first 3 days of Experiment 2. For FC, the number of bacteria in manure increased in all dishes by about 3 log units (or more) for 27 and 41 C treatments at all moisture contents. The 4 C treatment also increased but by a lesser amount. FS showed a similar trend, though increases were less (Table 4). This agreed with Crane (1988), who found bacteria numbers increased after manure was stored for 3 to 6 days, and defined this process as an initial “after growth” period. He also found a slower “delayed regrowth” period following 10 days of manure storage, which can also be seen in our data (Figs. 1 to 4).

The ratio of fecal coliforms and fecal streptococci varied widely. The initial ratio was found to be around 0.09 (fecal coliforms/fecal streptococci) in Experiment 2 and 0.11 in Experiment 1. This

compares well with the established values of 0.1 to 0.6 for domestic animal (Howell *et al.*, 1996). However, the ratios changed with time, reaching as low as 0.02 and more than 400 at different conditions.

Conclusions

This study demonstrates that fecal bacteria are resilient under a wide range of natural conditions; even after cattle leave a feedlot, microbial populations are still likely to be high.

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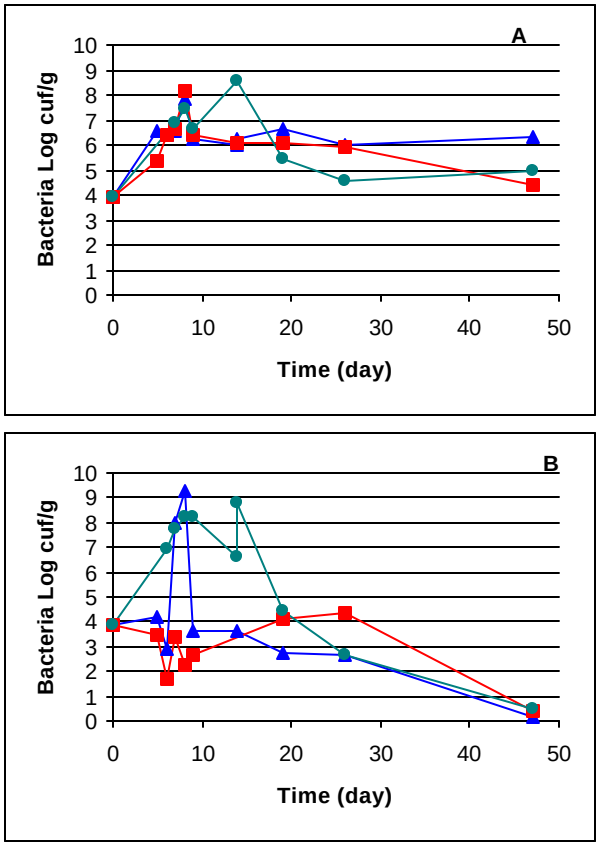

Figure 1—Fecal coliform bacterial counts (cfu/g) in manure held at **(A)** 4°C and **(B)** 41°C; and moisture contents of 79% ( ), 69% (■), and 50% (●) at discrete times over a 42 day period (Experiment 1).

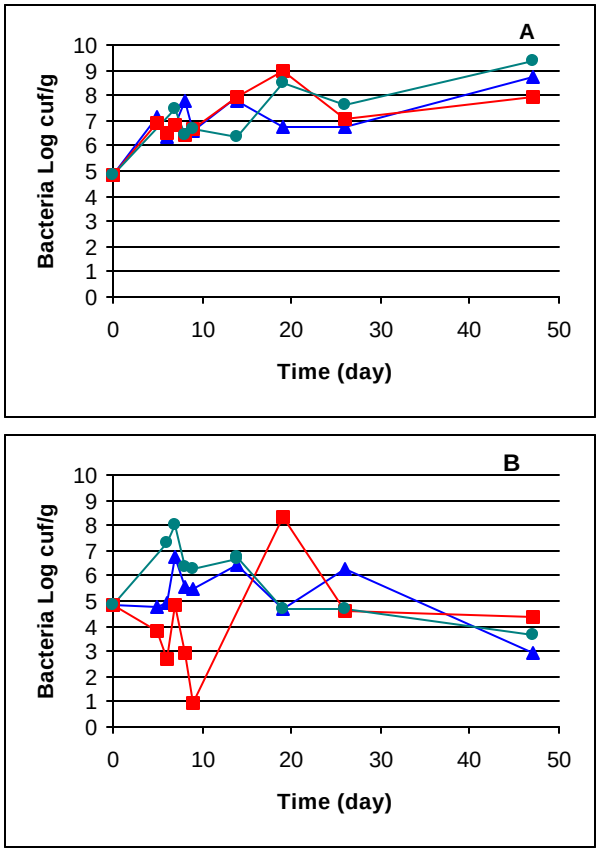

Figure 2—Fecal streptococci bacterial counts (cfu/g) in manure held at **(A)** 4°C and **(B)** 41°C; and moisture contents of 79% ( ), 69% (■), and 50% (●) at discrete times over a 42 day period (Experiment 1).

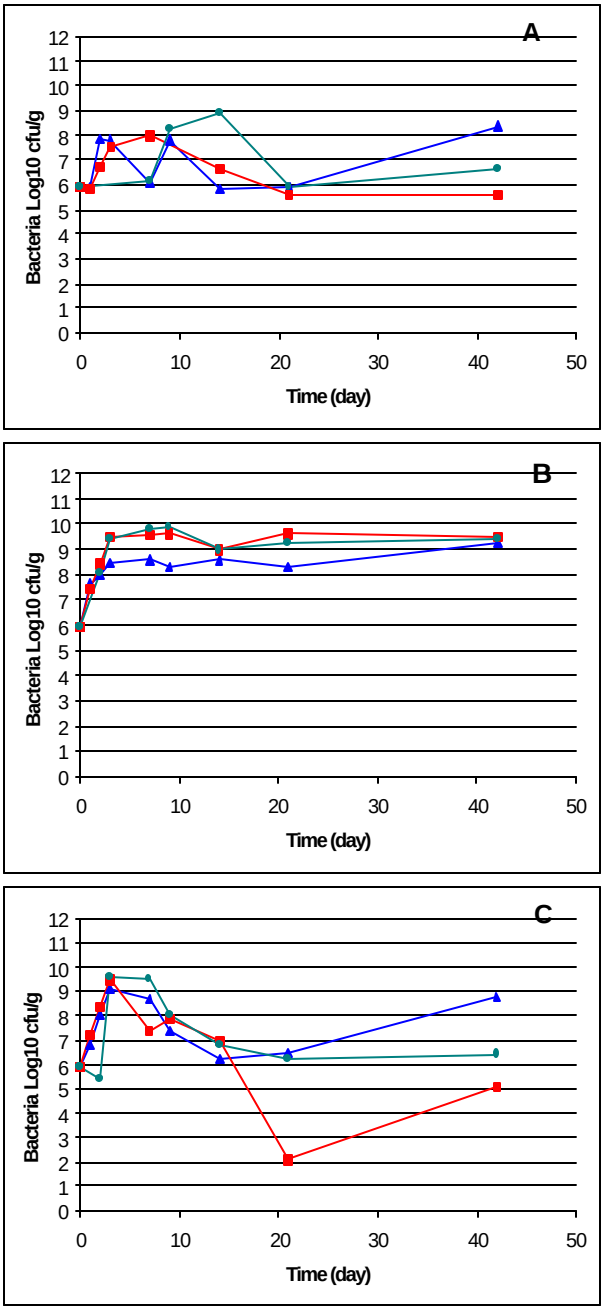

Figure 3—Fecal coliform bacterial counts (cfu/g) in manure held at **(A)** 4°C, **(B)** 27°C and **(C)** 41°C and moisture contents of 79% ( ), 69% (■), and 50% (●) at discrete times over a 42 day period (Experiment 2).

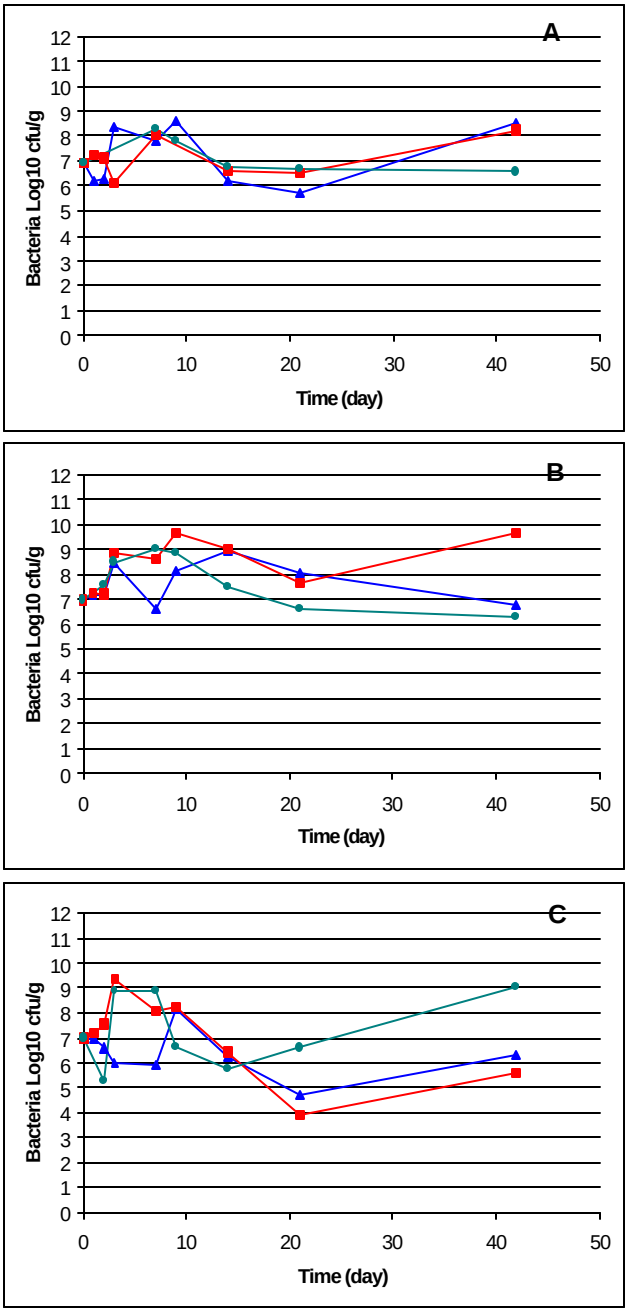

Figure 4—Fecal streptococci bacterial counts (cfu/g) in manure held at **(A)** 4°C, **(B)** 27°C and **(C)** 41°C and moisture contents of 79% ( ), 69% (■), and 50% (●) at discrete times over a 42 day period (Experiment 2).