LOSS OF BIOACTIVE PHOSPHORUS AND ENTERIC BACTERIA IN RUNOFF FROM DAIRY MANURE APPLIED TO SOD


Information on the concurrent release and interactions between manure-borne phosphorus (P) and enteric bacteria to runoff from a live or dead grass sod is limited. A study of simulated runoff and an enzyme-based fractionation of runoff P forms from dairy manure applied on grass-covered soil in runoff boxes was conducted to compare the detachment and potential edge-of-field movement of manure P, Escherichia coli, and enterococci in runoff. Concentrations and mass loads of bioactive P forms and bacteria in runoff were log-normally distributed over time during all simulations. Although P and enteric bacteria were simultaneously released to runoff, high correlations were found predominantly between water turbidity, concentrations of bacteria, and phosphohydrolase-labile P, a fraction associated with particulate manure. Delayed bacteria and particulate P concentrations and mass loads indicated live leaf and bacterial surface interactions that impeded their release to runoff. Resultant deviations in linearity between manure water-extractable P and bacteria distributions and the significant correlation between bacteria and the phosphohydrolase-labile P fraction suggested that manure-borne E. coli were released in association with manure particulates that contained organic P. The state of the grass cover determined the asymmetry of bacteria and bioactive P distributions. Given the micrometer size range of suspended particles, losses of colloidal particulate P and colloid-associated bacteria may extend well beyond the immediate vicinity of the deposited manure. (Soil Science 2008;173:511-521)

Key words: Bioactive phosphorus, organic phosphorus, colloid-associated transport, Escherichia coli, Enterococcus, fecal coliforms.

ANIMAL manure is applied to rangeland and hay fields to improve nutrient status and physical properties of the underlying soil, and eventually forage production and forage quality. However, surface-applied manure on grassland is susceptible to loss in runoff, depending on factors that include slope, compaction, soil hydraulic properties, and plant factors such as plant architecture, plant density, state of the grass, and other hydrological characteristics of the sward. Many studies of manure phosphorus (P) transport in runoff and transport of manure-borne organisms have been conducted to assess their impact on aquatic environments (Coyne et al., 1998; Atwill et al., 2002; Meals and Braun, 2006; Green et al., 2007). Of the limited available literature on the concurrent release and transport of both manure-borne pollutants, there is no consensus on whether those mechanisms have similar quantitative effects on P and microorganism transport. Shelton and coworkers (2003) showed similarities in infiltration of manure-borne P and Escherichia coli. A significant relationship was observed between the transport of manure-borne total P (TP) and E. coli from
land-applied manure (Stout et al., 2005). The correlation in TP and fecal coliform runoff concentrations have been attributed to the transport of P in bacterial cells, as the total microbial biomass is usually not less than 30% of manure mass on a dry mass basis. Phosphorus-containing biomolecules in living tissues such as adenosine 5'-triphosphate have been shown to be a proxy for total microbial biomass in soil or marine sediments. Other mechanisms, that is, bacterial polyphosphate inclusion bodies and binding to cell wall sites could also contribute to the observed correlation. This implies that different forms of P may differ in their correlations with enteric bacteria, and the ability of different forms of P to reveal manure-borne bacteria presence and transport mechanisms may be different.

Adhesion and colonization of natural and synthetic polymeric surfaces (McElldowney and Fletcher, 1987) have been reported to be essential in the dissolution of minerals and cycling of nutrients or the formation of biofilms (Lovley and Philips, 1986; Geesey and White, 1990; Hall-Stoodley et al., 2004), although bacteria may move as free-living organisms (Muirhead et al., 2005, 2006). Bacteria were observed to adhere to and colonize carbon-rich aggregates to use particulate organic matter in aquatic ecosystems (Knoll et al., 2001; Kiorboe et al., 2002). As manure is composed of partly digested feed materials that are primarily organic carbon in nature, E. coli and enterococci may be physically attached to organic P-containing manure colloids that serve both as an attachment surface for colonization and a substrate for their nourishment and energetic requirements. Therefore, based on our knowledge of manure constituents and bacterial interactions, this study was conducted to compare the distributions of different forms of manure P with concentrations of two major fecal contamination indicators, E. coli and enterococci, and derive insights into mechanisms of detachment of these two classes of manure-borne organic pollutants and their release to runoff from manure applied to sod.

MATERIALS AND METHODS

Rainfall Simulations

An application of freshly deposited dairy manure was made to replicated sod boxes, and simulated rainfall was applied to study the relationships between bacteria and manure P release to runoff. Details of the rainfall simulator and runoff box setup were given in a previous work (Green et al., 2007; Guber et al., 2007). In summary, runoff boxes (200 cm x 41 cm x 10 cm, length/width/height [L/W/H]) were made from commercial lumber and were divided into five sections lengthwise, with four blades installed at 25, 50, 100, and 150 cm from the top of the box (Guber et al., 2007). A 5-cm layer of commercial tall fescue (F. arundinacea Schreb.) sod was placed in each box. The top 25-cm section of the sod runoff boxes (25 cm x 41 cm x 10 cm, L/W/H) received dairy manure at the rate of 11.7 kg m⁻² and had a channel mounted at its lower end to collect all the surface runoff into calibrated 4-L jugs. In this study, we reported only runoff data and experimental results from manure applied to this section of six soil-grass boxes. Before initiation of the rainfall simulation, the grass was clipped to a uniform height of 7.5 cm, and clippings were removed. Three experimental simulations were made with live grass. Another three simulations were made with dead grass when watering was withheld and the grass was left to wilt. Plant dry matter averaged 223 ± 77 g m⁻² and 176 ± 122 g m⁻² in live grass and dead grass boxes, respectively. The three replicated soil-grass boxes of each grass state (i.e., live or dead) were placed on an incline to have a 4% slope inside the simulator frame area for each grass treatment in separate simulations.

Water samples were collected after runoff initiation, and at time increments of 10 min, up to 90 min after rainfall initiation. Rainwater, made from deionized water and reagent-grade chemicals to simulate local rain composition, was applied using a solenoid-controlled variable-intensity rainfall simulator (2.8 x 2.8 x 3 m, L/W/H). The simulator was equipped with a single TeeJet™ 1/2 HH SS50WSQ nozzle (Spraying Systems Co., Wheaton, IL) positioned 3 m above the soil surface (Green et al., 2007). Constant rainfall intensity of 32.4 ± 0.6 mm h⁻¹ was maintained for 90 min. The rain intensity has a mean recurrence interval of 5 years for a 30-min period in central Maryland, according to the precipitation frequency Atlas 14 of the US National Oceanic and Atmospheric Administration, Vol. 2 (http://hdsc.nws.noaa.gov/hdsc/pfds/index.html, verified on December 16, 2007).

Suspended Solids and Turbidity Analyses

Six samples of the dairy manure (100 g) were oven dried at 60 °C to determine dry matter. Turbidity of diluted dairy manure (1:10, vol/vol)
and runoff samples was determined using a nephelometer equipped with a tungsten incandescent bulb as the light source (model 2020, LaMotte Company, Chestertown, MD; the mention of a trade or manufacturer name does not imply an endorsement, recommendation, or exclusion by the USDA-Agricultural Research Service). Detection of scattered light by suspended particulates was made at 90 degrees. The nephelometer was calibrated for raw manure suspensions containing up to 40 g L⁻¹ to relate total suspended particulate matter concentrations to turbidity expressed in nephelometric turbidity units (NTU). Particle size distributions were measured using an LA-920 laser-scattering particle size analyzer (Horiba Instruments Inc., Irvine, CA) as described in Pachepsky et al. (2008).

**Runoff Phosphorus Fractionation**

Various forms of bioactive P present in runoff samples were determined according to the procedure developed by Dao (2003; Dao et al., 2006; Green et al., 2007). In brief, four P fractions were differentiated; that is, manure water-extractable phosphate-P in runoff (WEP), ligand-exchangeable inorganic phosphate-P (EEP), and ligand-exchangeable phosphate-P (EEP), and the all-inclusive total bioactive P (WEP + EEP + EDTA-PHP), and the all-inclusive total bioactive P (WEP + EEP + EDTA-PHP) in runoff, which were determined as follows.

Manure WEP in runoff was measured in duplicate aliquots (7 mL) after centrifugation of the runoff samples at 10,000g for 15 min before phosphate-P analysis. Identical runoff aliquots were combined with a 5-nmol L⁻¹ solution of EDTA (1:10, wt/vol) and agitated at 250 r.p.m. for 1 h to determine the EEP fraction in the runoff. The supernatant phosphate-P concentrations were measured after centrifugation at 10,000g.

Total bioactive P (WEP + EEP + EDTA-PHP) was determined after EEP measurements. An aliquot (0.5 unit mL⁻¹) of a stock of *Aspergillus fuscum* phosphohydrolases that was prepared in our laboratory (Dao et al., 2007; Dao and Hoang, 2008) was added to replace the volume removed from individual rainfall simulations. Because the

Acid-digest TP was determined in duplicate 15-mL aliquots of the runoff using a modified potassium persulfate-sulfuric acid digestion procedure. The mixture was brought to a boil at 180 °C for 0.5 h in a heating block and at 350 °C for another 0.5 h. The digest was diluted to a final known volume for P analysis. Extracted and total P concentrations were determined using the phosphomolybdate-ascorbic acid method and a semiautomated ion-analyzer (Bran-Luebbe, Buffalo Grove, IL).

**Enterococcus and E. coli Enumeration**

Fecal coliform bacteria were enumerated according to the protocols described previously (Guber et al., 2007). In summary, enterococci in water and manure samples were enumerated by plating a 50-µL exponential spiral onto culture plates containing selective membrane *Enterococcus* agar (Becton, Dickinson and Co., Franklin Lakes, NJ) using an Autoplate 4000 spiral-plater (Spiral Biotech, Gaithersburg, MD). Red-colored colony-forming units (CFU) that developed after 48 h of incubation at 37 °C were counted with a Q-Counter colony counter (Spiral Biotech). Similarly, numbers of presumptive *E. coli* CFU in manure and runoff water aliquots were determined by plating 50 µL of exponential spirals on MacConkey agar. Red-colored CFU that developed after overnight incubation at 44.5 °C were counted. Although red-colored CFU only indicated the presence of lactose-fermenting Gram-negative rods, we found that more than 99% of presumptive *E. coli* determined with this procedure were actually *E. coli* in previous studies with fresh dairy manure slurries. For the sake of brevity, presumptive *E. coli* was referred hereafter to *E. coli* in this study. The number of enterococci and *E. coli* in manure was determined by plating as above after dilution with sterile water (1:10 vol/vol).

**Statistical Analysis**

The distributions of concentrations and mass loads of bacteria, WEP, EEP, and EDTA-PHP forms in runoff were fitted to the log-normal distribution function as described in Dao and Cavigelli (2003). Briefly, the flux density distribution was described by four parameters that included (i) the amplitude or concentration max or mass load max, (ii) time of occurrence of concentration max or load max, (iii) the distribution width at half-concentration max or half-mass load max, and (iv) the asymmetry at half-concentration max or half-mass load max. The root mean square error (RMSE) for the fitted distribution was computed in addition to the coefficient of determination (r²) to determine the goodness of fit of the log-normal equation to the concentration or mass flux density data.

Linear regression equations were fitted to paired chemical and bacterial concentrations from individual rainfall simulations. Because the
concentrations of interest were decreasing up to two orders of magnitude during the rain simulations, the log-log plots were used to compress the scales and quantify these binary correlations. Line comparisons were made using Proc GLM of the Statistical Analysis System (SAS, Cary, NC). Individual and common regression analyses were calculated to evaluate the homogeneity of residual variances and slopes of regression lines, and detect significant differences in slope and intercept of the regressions for the grass treatments at the 0.05 level of probability.

RESULTS

Manure Particulates, Bacteria, and Phosphorus Released to Runoff

Table 1 summarizes concentrations of E. coli, enterococci, and TP in rainwater, manure, and background runoff samples obtained before manure application. Background bacteria concentrations were four to six orders of magnitude lower than those in the stored manure from the USDA-ARS Beltsville dairy facility. The total suspended solids concentration of the manure averaged 80 g L⁻¹, and the suspension pH was 8.0 in a 1:10 manure dilution.

Differences in water turbidity were observed between the two grass treatments, although the turbidity of simulated runoff was highly variable, particularly during the simulations with live grass (Fig. 1A). The estimated particulate load in runoff from the dead grass cover was significantly greater throughout the simulation when runoff volume was taken into account (Fig. 1B). The log-normally distributed turbidity load peaked earlier in the dead than the live grass treatment, at 5.5 and 10.5 min after rain initiation, respectively. Water turbidity decreased but the runoff did not completely clarify at the latter stage of the simulation (Figs. 2A, B). Runoff samples contained suspended particulates ranging in size between 0.1 and 100 μm. The predominant fraction, between 1 and 50 μm, was still present in runoff samples collected toward the end of the 90-min simulation period.

Overall, concentrations and loads of bacteria peaked early during the simulation then declined in the runoff over time; their release to runoff was also described by the log-normal density distribution (Fig. 3A). Enterococci concentrations followed the same release pattern but were about one tenth of those of E. coli (data not shown). The state of the grass cover can alter the amplitude (i.e., concentration_max and mass load_max) of the release of both bacteria to runoff as the dead

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<td><strong>Background E. coli, enterococci, and TP concentrations measured in simulated rainfall water, runoff before manure application, and dairy manure</strong></td>
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<tr>
<td><strong>E. coli</strong></td>
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<td><strong>CFU m L⁻¹</strong></td>
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<td>Rainwater</td>
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<td>(1.28 ± 0.20) x 10⁸</td>
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grass cover delivered peak concentration or mass load sooner than live grass cover. As a result, the total mass loadings to runoff during the 90-min simulation averaged $9.8 \times 10^6$ and $20 \times 10^6$ E. coli CFU and $0.4 \times 10^6$ and $2.3 \times 10^6$ enterococci CFU for the live and dead grass treatments, respectively (Figs. 2, 3A; enterococci data not shown).

Manure WEP concentration fluxes and mass loadings to runoff displayed distribution patterns similar to those of enteric bacteria (Fig. 3B). A notably greater precision, that is, lower RMSE, was attained with bioactive P measurements than those for bacteria. The pattern of manure WEP release to runoff was in agreement with previous results (Dao and Cavigelli, 2003; Green et al., 2007); soluble P forms desorption and release from cattle manure and manure-amended soils have been observed to follow the log-normal distribution. The state of the grass cover again affected the timing of peak concentrations and the mass loads of any of the bioactive P forms delivered to runoff (Figs. 3B, C). For example, the log-normal distribution parameter “b” or time of occurrence of the PHP concentration max (i.e., $7.0 \pm 0.4$ and $5.6 \pm 0.1$) and the asymmetry parameter “d” (i.e., $2.1 \pm 0.4$ and $3.4 \pm 0.5$) for the live grass treatment were greater than those of the dead grass treatment, reflecting the delayed release.

Relationships Between P Forms and Bacterial Releases

General relationships between P forms and bacterial concentrations were found in the majority of cases and are shown in Figs. 4A–C. Although both types of manure pollutants were initially desorbed together, a process of mass

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**Fig. 2.** Changes in particle size (means and positive S.D.) distributions and associated probability of occurrence of suspended material in runoff and a dairy manure suspension spread on live (A) and dead (B) tall fescue (*F. arundinacea* Schreb.) sod over 90 min of simulated rain applied at 3.2 cm h $^{-1}$. Solid and dashed lines are representations of the log-normal distribution function fitted to means (±S.D.) of three simulations for each grass treatment.
Fig. 3. Distribution of concentrations and mass loads of *E. coli* (A), manure WEP (B), and PHP (C) in runoff from grass-applied dairy manure over 90 min of simulated rain applied at 3.2 cm h⁻¹. Solid and dashed lines are representations of the log-normal distribution function fitted to means (+S.D.) of three simulations for each grass treatment.

Segregation occurred during the release to runoff, resulting in deviations in linearity of P forms-bacteria concentration relationships.

**Manure WEP in Runoff**

Manure WEP was generally not well correlated to either *E. coli* or enterococci concentrations, that is, pooled $r^2$ of 0.64 and 0.72, respectively (Fig. 4A). The grass treatments more often influenced the release of *E. coli* and manure WEP in solution than that of enterococci. Manure WEP in runoff had the lowest correlation with water turbidity from the live grass treatment (Table 2); the curvilinear relationship would suggest a delayed release...
Manure WEP in runoff was better correlated with *E. coli* release from the dead grass treatment; that is, \( r^2 = 0.89 \). The improved correlation could be attributed to high runoff volume of bacteria and particulate P forms at the early stage of the simulation compared with the unimpeded very rapid release of manure WEP to runoff.
and reduced bacterial interaction with dead grass biomass. Enteric bacteria can vary considerably in terms of size, morphology, and cell membrane surface topography and physicochemistry that leads to differences in the sorption characteristics onto grass leaf surfaces and the apparent transport of these two bacteria (Beveridge and Graham, 1991; Celico et al., 2004).

**Bacteria and Manure TP and Organic PHP in Runoff**

Improvements in the goodness of fit were more evident between manure total bioactive P or TP in runoff and bacteria because mean RMSE of all individual regressions were lower for bacteria-TP than bacteria-WEP, that is, 0.081 versus 0.123, respectively (Fig. 4B); the total bioactive P pool included all manure inorganic WEP, EEP, and organic PHP in the runoff water. Therefore, these results suggested that a portion of the correlation was attributable to manure organic P-containing particulate materials in the runoff. In fact, the net enzyme-hydrolyzable organic P fraction (PHP) had the highest and direct correlation to both E. coli and enterococci releases (Fig. 4C). The PHP fraction was a unique and discrete fraction that is associated with particulate manure P, unlike the all-inclusive total bioactive P fraction.

**DISCUSSION**

Differences in the release kinetics of the two bacteria were previously attributed to the distribution of these organisms between readily soluble, suspended, and large particulate components of manure. Guber and coworkers (2007) observed similarities in bromide (Br) and E. coli release kinetics. They showed that E. coli, like Br, were present in the liquid phase of manure and was released as the liquid manure fraction was diluted and displaced by the rainwater. However, the liquid fraction was later found to contain micrometer-sized colloids. Enterococci were present in substantial numbers, being adsorbed onto the manure solid phase and therefore occurred in lower numbers in the runoff. Nola and coworkers (2008) reported that Enterococcus faecalis were preferentially adsorbed to soil surfaces, and that E. coli and E. faecalis sorption appeared to be a competitive process when both bacteria were present.

Soil and manure P exist as inorganic and organic forms (Cosgrove, 1980; Dao, 2004). As the proportion of inorganic to organic P fractions can vary across manure sources or soils, the correlation of bacterial movement to that of TP would not adequately describe the mechanisms of transport of either type of manure-borne pollutants. Over the course of the simulation, all manure P forms and bacteria, and manure soluble and particulates concurrently were released to runoff upon the breakup and dispersion of the manure mass by raindrops’ impact. Analogous log-normal distributions were found to describe the distributions of inorganic and organic P forms and bacterial concentrations and loads in runoff for most simulation conditions.
As a discrete measure of enzyme-labile organic P species, the manure enzyme-labile PHP pool was distinct from the WEP fraction and showed robust correlations to the releases of E. coli and enterococci. These organic P forms generally are found complexed with metallic polyvalent cations or are sorbed on suspended colloids and coarser particles (Dao, 2003). They are often regarded as unreactive but seem to play a more biologically active role in the movement of manure-borne pollutants than usually assumed (Sundershwar et al., 2003; Dao, 2006; Green et al., 2007). The two complementary correlations observed between PHP and runoff turbidity and bacteria-turbidity might suggest an association of bacteria with suspended particulates in the runoff. These observations were not in agreement with other work on the state of coliforms from cattle feces released to runoff; manure-borne E. coli cells were shown to be transported in runoff primarily as individual unattached cells in a biphasic water-organic solvent system based on buoyancy-density differences (Muirhead et al., 2005, 2006). The discrepancy may partially be attributed to differences in age of the manure slurry. As manure gets old with storage, bacterial adhesion and attachment to manure particulates increase and bacteria would be released to runoff while associated with colloids. Furthermore, had the bacteria been released and moved as unattached single cells in the solution phase of runoff, there would have been a close relationship between bacteria and WEP fractions because they were released together at the initiation of the runoff event. Procedurally, manure WEP in the runoff solution phase was defined as water-soluble phosphate concentrations that are measured after centrifugation at 10,000g. According to Stokes law governing particle sedimentation, centrifugation at 10,000g removed particles larger than approximately 0.1 μm from the solution phase, and the supernatant contained water-soluble phosphate species. This runoff treatment step in the determination of WEP removed more colloids than the more common procedure of measuring P after filtration through a porous membrane with 0.45-μm openings (Dao et al., 2006). Therefore, the results imply that a size or mass segregation process occurred during the initial release of manure-borne pollutants in addition to the biochemical relationships.

The differential effect of the state of the grass cover on manure WEP and on chemical and bacterial distribution in runoff provided additional support to the particulate-associated release of E. coli and enterococci to runoff. The retardation of particulate loading to runoff, arising from interactions of live grass surfaces with manure particulates and bacterial cells, resulted in large deviations from linearity of the WEP-bacteria concentration relationships. This is in contrast to the improved correlation between these two variables, when water velocity and particulate release and movement were unimpeded by less reactive dead grass dry matter. Dead grass blades were not as turgid as live ones and thus might have had less interaction with suspended solids and dissolved manure components in the runoff. Therefore, the similarities between the distributions of both manure-borne pollutants and manure particulates suggested that mechanistically, E. coli and, to a lesser extent, enterococci, were released along with organic P-containing colloids or larger manure particulates during the dispersal of the manure layer upon raindrops’ impact. In the end, the experimental results suggested the need to further quantify manure-borne E. coli and enterococci released to runoff while associated with manure organic P particulates. Bacterial cell surfaces undergo constant interplay with the external environment in response to changes in pH, electrolytes, or water potential gradients. Adhesion or the need for cell attachment to particulate matter may be dictated by the need for carbon-rich substrates for nourishment and the energy value of the substrate such as that stored in an O-P ester bond.

**SUMMARY AND CONCLUSIONS**

The differential effect of live versus dead grass covers had on the release of the two enteric bacteria and manure P fractions pointed to interactions between grass surfaces and contaminants, resulting in a mass segregation process. The similarities in total bioactive P, more specifically enzyme-labile PHP, and the bacteria may have serious implications for the management of grassland-applied manure at the edge of fields and riparian zones of streams near livestock facilities or animal stocking areas. Raindrops’ impact energy must be attenuated by a well-maintained live vegetative cover. Live leaf blade’s surface roughness and structural features such as stomata and trichomes may play an important role in impeding the release of bacteria and particulate-associated P forms to runoff. Given the continuous size range of
suspended particles detached from applied manure in runoff, submillimeter solids may not be effectively filtered to reduce lateral transport of colloid-associated manure-borne organic P and fecal coliforms. Flocculation agents may also be needed to mitigate the transport of manure-borne constituents that are released to the waterphase in the 0.1- to 50-μm range.

REFERENCES


