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Decay of Bacterial Pathogens, Fecal Indicators, and Real-Time Quantitative PCR Genetic Markers in Manure-Amended Soils▽†

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This study examined persistence and decay of bacterial pathogens, fecal indicator bacteria (FIB), and emerging real-time quantitative PCR (qPCR) genetic markers for rapid detection of fecal pollution in manure-amended agricultural soils. Known concentrations of transformed green fluorescent protein-expressing *Escherichia coli* O157:H7/pZs and red fluorescent protein-expressing *Salmonella enterica* serovar Typhimurium/pDs were added to laboratory-scale manure-amended soil microcosms with moisture contents of 60% or 80% field capacity and incubated at temperatures of −20°C, 10°C, or 25°C for 120 days. A two-stage first-order decay model was used to determine stage 1 and stage 2 first-order decay rate coefficients and transition times for each organism and qPCR genetic marker in each treatment. Genetic markers for FIB (*Enterococcus* spp., *E. coli*, and *Bacteroidales*) exhibited decay rate coefficients similar to that of *E. coli* O157:H7/pZs but not of *S. enterica* serovar Typhimurium/pDs and persisted at detectable levels longer than both pathogens. Concentrations of these two bacterial pathogens, their counterpart qPCR genetic markers (*stx1* and *ttrRSBCA*, respectively), and FIB genetic markers were also correlated ($r = 0.528$ to 0.745). This suggests that these qPCR genetic markers may be reliable conservative surrogates for monitoring fecal pollution from manure-amended land. Host-associated qPCR genetic markers for microbial source tracking decayed rapidly to nondetectable concentrations, long before FIB, *Salmonella enterica* serovar Typhimurium/pDs, and *E. coli* O157:H7/pZs. Although good indicators of point source or recent nonpoint source fecal contamination events, these host-associated qPCR genetic markers may not be reliable indicators of nonpoint source fecal contamination events that occur weeks following manure application on land.

Cultivation-based methods for fecal indicator bacteria (FIB) such as *Escherichia coli* and *Enterococcus* spp. have long been used to indicate potential public health risks associated with water impacted by human and other animal feces (53). FIB cultivation methods are simple to perform and inexpensive. However, these methods require 18 to 24 h following sampling to generate test results; this allows potential exposure of the public to fecal pathogens in the interim. Regulatory agencies, business owners, and other stakeholders have expressed interest in more rapid and specific methods to identify water quality impairment.

Emerging real-time quantitative PCR (qPCR) methods designed to estimate the concentration of fecal pollution by targeting genomic DNA (gDNA) from FIB such as *Bacteroidales*, *Enterococcus* spp., and *E. coli* are now available and can generate test results in just a few hours after sampling (10, 16, 48). Some of these genetic markers can be correlated to public health risk and may soon be incorporated by the U.S. Environmental Protection Agency into water quality standards in the United States (16, 59). These genetic markers may also detect viable but nonculturable (VBNC) cells that are not detected by conventional cultivation approaches but may still pose a public health risk (38). *Salmonella enterica* serovar Typhimurium and *E. coli* O157:H7 are two examples of bacterial pathogens that may enter VBNC states (8, 62). In addition, host-associated qPCR genetic markers are available and are reported to discriminate between human, cattle, swine, and other animal sources (4, 23, 25, 32, 35, 43–45), providing fecal pollution source information that may lead to better-informed remedial actions to improve water quality.

Several of the emerging FIB and host-associated qPCR assays target gDNA from obligate anaerobic bacteria, primarily *Bacteroidales*. These organisms offer improved host specificity for development of host-associated qPCR assays and comprise a large fraction of the bacterial community in feces. However, obligate anaerobes may not persist in aerobic environments as well as facultative bacteria such as *E. coli* and *Enterococcus* spp. Potential differences in the decay of these genetic markers could lead to different risk assessment interpretations and remediation outcomes depending on which marker is used. Therefore, many researchers have begun to explore the utility and limitations of emerging qPCR-based methods. Studies include exploration of qPCR genetic marker decay rates in different water types (2, 3, 36, 61) and beach sands (65), as well as genetic marker density and distribution in feces and sewage (11, 23, 40, 46, 47, 49, 58).

At present, very little is known about the persistence of host-associated and FIB qPCR and PCR genetic markers in manure-amended soils and correlations to cultivation-based measurements of FIB and manure pathogens. This is a critical

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knowledge gap; runoff from agricultural lands fertilized with livestock manures is one of the leading causes of water quality impairments in the United States (55). Decay of obligate anaerobes may occur rapidly following manure application to land. In contrast, manure pathogens, such as *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7, may persist in manure-amended soils for periods of more than a year, posing long-term risk to nearby water quality (18, 21, 66). Nondetection of a livestock-associated genetic marker in an agricultural watershed impaired by excessive fecal coliform bacteria may lead an investigator to the conclusion that the potential livestock source is not contributing significantly to impairment of the water body. However, it may be the case that the livestock-associated genetic marker simply did not persist as long as the fecal coliform bacteria on a manure-amended landscape prior to runoff. Improved knowledge of the fate of qPCR genetic markers in agricultural soils will increase understanding of their utility in watersheds impacted by nonpoint source manure runoff. Watershed managers will better understand how to appropriately interpret qPCR data in context with traditional cultivation-based measurement data.

The objective of this study was to investigate the utility of qPCR genetic markers as surrogates for cultivation-based measurements of viable bacterial pathogens and FIB in agricultural soils amended with manures at agronomic (nutrient-limited) rates. Promising qPCR genetic marker surrogates are (i) detectable and persist as long as or longer than bacterial pathogens and/or FIB as measured by cultivation-based approaches; (ii) decay in a manner characteristically similar to, and at rates similar to, FIB and bacterial pathogens; and (iii) return concentrations that can be correlated to their counterpart cultivation-based measurements. Of interest in this study were qPCR genetic markers for FIB (*Enterococcus* spp., *E. coli*, and *Bacteroidales*), qPCR genetic markers for bacterial pathogens (enterohemorrhagic *E. coli* and *Salmonella* spp.), and host-associated qPCR and PCR genetic markers for pigs and cattle. Cultivable bacterial pathogens and FIB investigated in this study included *E. coli* O157:H7, *S. enterica* serovar Typhimurium, *E. coli*, and *Enterococcus* spp. To address this objective, cultivable FIB, cultivable pathogens, and qPCR genetic markers were challenged in different manure and soil matrices under variable environmental stress (i.e., temperature and moisture content). A two-stage first-order decay model was used to determine best-fit decay rate coefficients for each bacterium or qPCR genetic marker, as influenced by environmental stressors. Statistical approaches were used to test for relationships between cultivable pathogens, cultivable FIB, and qPCR genetic markers. The results of this study highlight strengths and limitations regarding the use of genetic markers as indicators of risk and for identifying potential host sources of fecal pollution in watersheds impaired by nonpoint source manure runoff.

MATERIALS AND METHODS

Soils and manures. Coshocton silt loam from the USDA-ARS's North Appalachian Experimental Watershed near Coshocton, OH, and silty, clayey loam from the Animal Sciences Research and Education Center at Purdue University were used for this study. Soils were extracted from the top 12 in. of land that had not received manures in greater than 5 years, composited by site location, passed through a number 4 sieve, and stored at 4°C for up to 6 months prior to use. Soil properties were measured at the Cornell University Nutrient Analysis Laboratory, Ithaca, NY (see Table S1 in the supplemental material).

Liquid swine manure from a concentrated animal feeding operation (CAFO) in Northeast Ohio was collected from a manure spreader at five different time intervals, encompassing the period of field application, and composited into a single sample prior to use in this study. Beef cattle manure solids from the North Appalachian Experimental Watershed were sampled at five random locations within a broadcast spreader at the time of field application and composited prior to use in this study. Manures were shipped overnight at 4°C and retained at −20°C for up to 6 months prior to use. Physical and chemical properties of the manures were measured by the USDA-ARS at the North Appalachian Experimental Watershed (see Table S2 in the supplemental material).

Reporter bacteria. *E. coli* O157:H7 (ATCC 43894) was made chemically competent by using a $CaCl_2$ protocol modified from Sambrook et al. (41) and transformed using the pZsGreen vector plasmid DNA (Clontech, Mountain View, CA), encoding the *Zoanthus* sp. green fluorescent protein (GFP). Transformed cells (*E. coli* O157:H7/pZs) were cultured overnight at 37°C on Luria-Bertani Miller (LB) agar supplemented with 50 μg/ml ampicillin (Sigma-Aldrich, St. Louis, MO) and 200 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (LB-Amp-IPTG) (Fisher Scientific, Pittsburgh, PA). These cells fluoresced green when illuminated with UV light (302 nm) in a fluorescence analysis cabinet (Spectroline model CM-10). Clones were verified to be *E. coli* O157:H7 by qPCR, as described below.

S. enterica serovar Typhimurium (ATCC 35664) was made electrocompetent using a protocol modified from the Lindlow Laboratory (University of California Berkeley, CA). Briefly, an individual colony from a tryptic soy agar plate was inoculated into 50 ml LB broth and grown on an orbital shaker (300 rpm) at 37°C until the cells had an A_{600} of 0.75. The cell suspension (25 ml) was transferred to prechilled 50-ml centrifuge bottles, incubated for 15 min on ice, and then harvested by centrifugation (6,000 × *g*, 4°C, 3 min). The cell pellet was washed twice with 20 ml ice-cold 10% caliber glycerol (Hoefer, Holliston, MA) and once with 10 ml ice-cold 10% glycerol and then resuspended in 300 μl ice-cold 10% glycerol. Forty-microliter aliquots of cells were added to prechilled microcentrifuge tubes and iced until transformation. Electrocompetent *S. enterica* serovar Typhimurium cells were transformed using the pDsRed-Express vector (Clontech) encoding the *Discosoma* sp. red fluorescence protein (RFP) using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA) at 2.5 kV, 200 Ω, 25 μF, and 4.8 to 5.0 ms, as previously described (34). Resuscitated cells were plated onto LB-Amp-IPTG and incubated overnight at 37°C. Cells that express the pDsRed-Express vector (*S. enterica* serovar Typhimurium/pDs) fluoresce red when exposed to UV light (302 nm). Clones were verified to be *S. enterica* serovar Typhimurium by qPCR as described below.

DNA extraction. gDNA from 1.8 ml overnight cultures of bacterial strains was extracted using the MoBio Ultraclean microbial DNA isolation kit (MoBio, Carlsbad, CA). Cells were lysed by bead milling at 6.0 m/s for 30 s using the FastPrep-24 bead beater (MP Biomedicals, Solon, OH). DNA concentrations were determined using the Quant-iT double-stranded DNA (dsDNA) BR assay kit (PicoGreen) with the Qubit fluorimeter (Invitrogen, Carlsbad, CA).

The MoBio PowerSoil DNA extraction kit (MoBio) was used to extract quantitative DNA (qDNA) from soil. Lysis was by bead milling at 6.0 m/s for 30 s using the FastPrep-24 bead beater. Each sample received 2 ng salmon testis gDNA (catalog number D-1626; Sigma Aldrich) prior to lysis, which served as an exogenous extraction and amplification control. The expected concentration of salmon testis gDNA in each extract was 20 pg/μl, assuming 100% recovery and no PCR inhibition. Actual recovery of salmon testis gDNA was measured for each sample using either the Sketa or Sketa22 qPCR as described below and described the combined effects of PCR inhibition and extraction inefficiency. Extraction blanks ($n = 20$) were used to test for the presence of extraneous DNA contamination introduced during laboratory procedures. DNA extracts were stored at −20°C until processing by endpoint PCR or qPCR.

Endpoint and qPCR. Primers and probes are listed in Table 1. Endpoint PCR assays were performed on a DNA Engine Tetrad2 peltier thermal cycler (Bio-Rad, Hercules, CA) for 35 cycles. Each 25-μl reaction mixture contained 12.5 μl of 2× HotStarTaq Plus master mix (Qiagen, Valencia, CA), 12.5 pmol forward and reverse primers, 5.0 μl template DNA, and 5.0 μl PCR-grade water. Reaction products were electrophoresed on precast E-Gel 96, 2% agarose GP with the E-Gel 96 high-range DNA marker ladder (Invitrogen) as per the manufacturer's instructions. Gels were viewed under UV light for verification of presence of the gene target of interest. No template controls (NTCs) and positive controls (PCs) were included with each instrument run for quality control.

qPCR assays were performed with either a Roche LightCycler 480 (Roche, Basel, Switzerland) or a 7900 HT fast real-time sequence detector (Applied Biosystems). For Roche LightCycler experiments, reaction mixtures (25 μl)

TABLE 1. Endpoint PCR and qPCR oligonucleotides and amplification conditions

Target and assay name	Primer and probe sequences (5′ to 3′)[a]	Locus[b]	Platform[c]	Reference
Oncorhynchus keta, Sketa	F, GGTTTCCGCAGCTGGG; R, CCGAGCCGTCCTGGTCTA; [6-FAM]-AGTCGCAGGCGGCCACCGT-[NFQ-MGB]	ITS region 2	A	16
Oncorhynchus keta, Sketa22	F, GGTTTCCGCAGCTGGG; R, CCGAGCCGTCCTGGTC; [6-FAM]-AGTCGCAGGCGGCCACCGT-[TAMRA]	ITS region 2	B	17
Salmonella spp., *ttrRSBCA*	F, CTCACCAGGAGATTACAACATGG; R, AGCTCAGACCA AAAGTGACCATC; [6-FAM]-CACCGACGGCGAGACCGA CTTT-[NFQ-MGB]	*ttrRSBCA*	A	31
E. coli O157:H7, stx_1	F, GACTGCAAAGACGTATGTAGATTCG; R, ATCTATCCC TCTGACATCAACTGC; [6-FAM]-TGAATGTCATTCGCTC TGCAATAGGTACTC-[NFQ-MGB]	stx_1	A	20
E. coli O157:H7, *eaeA*	F, GTAAGTTACACTATAAAAGCACCGTCG; R, TCTGTGT GGATGGTAATAAATTTTTG; [VIC]-AAATGGACATAGC ATCAGCATAATAGGCTTGCT-[NFQ-MGB]	*eaeA*	A	20
Campylobacter spp., Camp2	F, CACGTGCTACAATGGCATAT; R, GGCTTCATGCTCTC GAGTT; [6-FAM]-CAGAGAACAATCCGAACTGGGACA-[NFQ-MGB]	16S rRNA gene	A	30
Enterococcus spp., Entero1	F, AGAAATTCCAAACGAACTTG; R, CAGTGCTCTACCTC CATCATT; [6-FAM]-TGGTTCTCTCCGAAATAGCTTTAG GGCTA-[TAMRA]	23S rRNA gene	A	29
Bacteroidales, GenBac3	F, GGGGTTCTGAGAGGAAGGT; R, CCGTCATCCTTCACG CTACT; [6FAM]-CAATATTCCTCACTGCTGCCTCCCGTA-[NFQ-MGB]	16S rRNA gene	A	10
E. coli, EPA-EC23S	F, GGTAGAGCACTGTTTTGGCA; R, TGTCTCCCGTGATA ACTTTCTC; [6-FAM]-TCATCCCGACTTACCAACCCG-[TAMRA]	23S rRNA gene	C	6
Cattle feces, CowM2	F, CGGCCAAATACTCCTGATCGT; R, GCTTGTTGCGTTCC TTGAGATAAT; [6-FAM]-AGGCACCTATGTCCTTTACCT CATCAACTACAGACA-[NFQ-MGB]	HDIG domain protein	A	44
Cattle feces, CowM3	F, CCTCTAATGGAAAATGGATGGTATCT; R, CCATACTTC GCCTGCTAATACCTT; [6-FAM]-TTATGCATTGAGCATC GAGGCC-[TAMRA]	HD superfamily hydrolase	A	44
Swine feces, PigBac1	F, GCATGAATTTAGCTTGCTAAATTTGAT; R, ACCTCATA CGGTATTAATCCGC; [VIC]-TCCACGGGATAGCC-[TAMRA]	16S rRNA gene	D	33
Swine feces, PF163	F, GCGGATTAATACCGTATGA; R, CAATCGGAGTTCTT CGTG	16S rRNA gene	E	9

[a] F, forward; R, reverse; 6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; NFQ-MGB, nonfluorescent quencher minor groove binder.
[b] ITS, internal transcribed spacer.
[c] Platforms and master mix included: A, Roche LightCycler 480 with Roche ProbeMaster; B, ABI 7900 with ABI FastMix; C, ABI 7900 with ABI gene expression mix; D, ABI 7900 with ABI universal master mix; E, Bio-Rad DNA Engine Tetrad2 with HotStarTaq Plus master mix.

contained 12.5 μl of 2× LightCycler 480 Probe Master (Roche), 12.5 pmol forward and reverse primers, 5.0 pmol of fluorogenic probe, 5.0 μl template DNA, and 5.0 μl PCR-grade water. For the 7900 HT fast real-time sequence detector, either TaqMan fast universal master mix, universal PCR master mix, or gene expression master mix reagents were used for all amplifications (Table 1). Regardless of instrument platform, all qPCRs were performed in duplicate and began with a hold at 95°C for 10 min, followed by 45 cycles of primer/probe-dependent cycling parameters, as previously described (Table 1). Three NTCs and three positive controls containing a known concentration of gDNA were included on each reaction plate. Six replicate standard curves were generated for each qPCR assay spanning from 10 to 10^8 copies of the gene of interest; average standard curves were used to estimate gene copy numbers and target DNA concentrations in each soil sample. gDNA extracted from cultured organisms used to generate standard curves included *Enterococcus faecalis* (ATCC 29212), *S. enterica* serovar Typhimurium (ATCC 35664), and *E. coli* O157:H7 (ATCC 43894). Purified gDNA supplied by a manufacturer included *Bacteroides fragilis* (ATCC 25285D), *Campylobacter* spp. (ATCC 43446D-5), and salmon testis (Sigma Aldrich). Calibration curve equations and performance characteristics of the 10 qPCR assays are shown in Table S3 in the supplemental material. The range of quantification (ROQ) spanned the entire range of standard concentrations tested for all qPCR assays. Precision of measurements across the defined ROQ for all assays was less than 2.62% coefficient of variation (CV); amplification efficiencies ranged from 92.1% to 99.6%.

Soil inoculation. For each soil type, 25 g (dry weight) of soil was placed into each of 300 Seward Circulator 400 filtered bags (Seward, Bohemia, NY). Swine manure slurry (0.51 ml) was added to each bag of Ohio soil; 0.26 g beef manure slurry was added to each bag of Indiana soil. Manure amendment was based on the annual nitrogen agronomic rate for corn. The application rate was equivalent to 160 lb nitrogen/acre and based on a potential yield of 8,780 kg · ha^{-1} corn (Ohio State University Extension Services Fertilizer Recommendations). Each bag of manure-amended soil was brought up to 1 ml less than 60% or 80% of the field capacity (FC) by adding sterile water. One milliliter of cell suspension containing approximately 2.5×10^7 cells each of *E. coli* O157:H7/pZs and *S. enterica* serovar Typhimurium/pDs was added to each bag, resulting in approximately 10^6 cells per gram (dry weight) of soil for each cell type. A second set of 150 Seward Circulator 400 filtered bags was prepared for each soil and manure type in the same manner, except that they were not amended with *E. coli* O157:H7/pZs or *S. enterica* serovar Typhimurium/pDs. Negative controls were autoclaved twice for 20 min at 121°C and 15 lb/in^2 and were not amended with manure or transformed bacterial pathogens. The manure-amended soil mixtures and control soils were homogenized by stomaching for 30 s in a Stomacher 400 circulator (Seward) at 230 rpm for 45 s prior to bags being sealed shut with paper clips. These soil microcosms were incubated in the dark at −20°C, 10°C, or 25°C. Bags were weighed daily and readjusted to 60% or 80% FC with sterile water if necessary.

Sampling and pathogen, FIB, and qPCR genetic marker enumeration. For each manure-amended soil, triplicate bags were sacrificed on days 0, 3, 6, 10, 14, 25, 41, 55, and 120. Negative controls were tested on days 0, 28, and 56; triplicate bags for FIB were sacrificed on days 0, 14, 41, 55, and 120. Upon sacrification, 225 ml of buffered peptone water (Difco, Lawrence, KS) was added to each bag and mixed thoroughly by using a Stomacher 400 circulator (230 rpm, 45 s). DNA was extracted from 250 μl homogenized soil solutions using the MoBio PowerSoil kit as described previously. Eluted template DNA was screened for genetic markers for *S. enterica* serovar Typhimurium, *E. coli* 0157:H7, *Campylobacter*

spp., FIB, and host-associated genetic markers using qPCR or endpoint PCR (Table 1). Another 1 ml of the homogenized soil solution was serially diluted in washing buffer (modified phosphate-buffered saline [PBS] plus 0.15 M NaCl and 0.05% Tween 20, pH 7.4), plated in triplicate on LB-Amp-IPTG, and incubated at 35°C for 18 to 24 h. On days 25 and 55, 10% of these dilutions were also plated in triplicate onto CHROMagar *Salmonella* (DRG International, Mountainside, NJ), CHROMagar O157 (DRG International), and MacConkey sorbitol agar (Fischer Scientific) amended with cefixime and tellurite (Invitrogen) (CT-SMAC) and incubated at 35°C for 18 to 24 h. After incubation, LB-Amp-IPTG plates were viewed with a UV lamp (302 nm) in a fluorescence analysis cabinet. Red colonies (*S. enterica* serovar Typhimurium/pDs) and green colonies (*E. coli* O157:H7/pZs) were counted using a Science Ware electronic colony counter (Bel-Art Products, Pequannock, NJ). Putative *Salmonella* sp. and *E. coli* O157 colonies were also counted on CHROMagar *Salmonella*, CHROMagar O157, and CT-SMAC agars. DNA was extracted from putative colonies and tested by qPCR for *ttrRSBCA* or stx_1 as described above. *E. coli* O157 and *Salmonella* spp. measured on CHROMagar and CT-SMAC were within the same order of magnitude and less than *E. coli* O157:H7/pZs and *S. enterica* serovar Typhimurium/pDs as measured on LB-Amp-IPTG, suggesting plasmid stability (data not shown). From the sample homogenates without transformed bacterial pathogens and the negative controls, 1.1 ml was added to 108.9 ml of sterile water and serially diluted to measure concentrations of *Enterococcus* spp. and *E. coli* using the Enterolert kit (IDEXX, Westbrook, ME) and the Colisure kit (IDEXX), respectively, as per the manufacturer's instructions.

Initial concentrations in manure-amended soils. Initial concentrations of viable FIB and qPCR genetic markers in the raw manure and amended soils are reported in Table S4 in the supplemental material. *Campylobacter* spp. were detected in low concentrations in both raw manures but were diluted to concentrations below the limits of detection in the manure-soil mixtures. *E. coli* and PigBac1 were similarly diluted to a level that was below the limits of detection in the swine manure-amended soils. Neither cultivable *E. coli* O157, cultivable *Salmonella* spp., *stx1*, nor *ttrRSBCA* was detected in the raw manures or soils prior to being inoculated with *E. coli* O157:H7/pZs and *S. enterica* serovar Typhimurium/pDs.

Data analysis. Recovery of sample DNA in each DNA extraction was determined by dividing the measured concentration of salmon testis gDNA (C_{Sketa}) with either the Sketa or Sketa22 qPCR assay by the expected concentration (20 ng · μl^{-1}):

$$\%\ \text{recovery} = \frac{C_{\text{Sketa}}}{20\ \text{ng}\cdot\mu\text{l}^{-1}} \times 100\% \quad (1)$$

Measured concentrations were normalized for DNA loss during extraction and/or for potential PCR inhibition by dividing the measured concentration of the target of interest by the percent recovery calculated in equation 1. Normalized DNA concentrations were adjusted to a soil dry weight basis by multiplying by a factor of 10, accounting for 10-fold dilution during sample processing (25 g of soil with 225 ml of buffered peptone water).

A two-stage die-off model was used to estimate decay rate coefficients of FIB and qPCR genetic markers (7). This model combines two first-order decay equations, one for each decay phase:

$$N_t = N_0 e^{-k_1 t} \text{ for } t < t' \quad (2)$$

$$N_t = N_0 e^{-k_1 t'} e^{-k_2 (t - t')} \text{ for } t \geq t' \quad (3)$$

where N_t is the cell concentration at any time t, N_0 is the initial cell concentration on day 0, k_1 and k_2 are first-order decay rate coefficients for stages one and two of inactivation, respectively, and t' is the transition time (time when stage one of inactivation ends). This model accounts for a high initial decay rate followed by a slower, extended decay rate. Simple first-order exponential decay (equation 2 only) was selected as the preferred model when insufficient data existed between the last data point and t' to generate reliable estimates of t' and k_2, or when best-fit k_1 and k_2 were not significantly different. Best-fit decay rate coefficients and transition times were determined using nonlinear regression in GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Prior to determining best-fit coefficients, normality in the distribution of the data was determined using the Anderson-Darling test (MiniTab 16; MiniTab Inc., State College, PA).

Multifactor analysis of variance (ANOVA) was performed to assess the influence of temperature, soil moisture content, and soil-manure combination on best-fit decay rate coefficients and transition times using MiniTab 16. Each of these factors was analyzed independently and in different combinations to determine combined interaction effects. Two-way ANOVA tests were performed to identify whether differences existed between organisms and/or genetic markers for each soil type. All calculations were performed using the general linear model (GLM) procedure in MiniTab 16. Specific differences among the levels within a factor group were determined using Tukey's *post hoc* multiple comparison test. For each test, the level of significance (α) was taken as 0.05.

RESULTS

DNA extraction and PCR amplification quality control. Recovery of salmon testis gDNA from sample extractions ranged from 1.8% to 52.4% (mean = 23.3%). Two DNA extracts were removed from the data set due to lack of recovery of the Sketa or Sketa22 genetic marker. NTCs and extraction blank controls indicated the absence of extraneous DNA molecules in 99.2% of all amplifications (5 false positives of 609 extraction blanks and NTCs).

Persistence of cultivable bacteria and qPCR genetic markers. One characteristic of reliable qPCR genetic marker surrogates is that they will persist at detectable concentrations under environmental stress for as long as, or longer than, bacterial pathogens and/or FIB as measured by cultivation-based approaches. Toward this end, the persistence of cultivable bacteria and qPCR genetic markers was monitored in the soil microcosms for 120 days. Enterococci could be cultured from beef manure-amended soil for >120 days regardless of temperature and moisture content but for only 41 to 56 days from swine manure-amended soils. *E. coli* could be recovered for 41 to 120 days from beef manure-amended soils at 10°C and 41 to 56 days at 25°C but could not be cultured from swine manure-amended soils. *E. coli* O157:H7/pZs was recoverable for >120 days following challenge by all conditions except for swine manure-amended soils at 25°C and a moisture content of 80% FC (55 to 120 days). *S. enterica* serovar Typhimurium/pDs was cultivable from swine manure-amended soils for 55 to 120 days at 10°C and 25°C but only for 25 to 55 days from beef manure-amended soils at these temperatures. Reported survival times of cultivable bacterial pathogens and FIB in field scale studies are similar to those reported in this work (12, 39).

All genetic markers, except for PF163, could be detected in manure-amended soils for the entire 120-day study period when retained at −20°C. At temperatures of 10° and 25°C, GenBac3 could be detected at 120 days, exceeding the 1 to >30 days previously reported for *Bacteroidales* 16S rRNA genes in environmental waters (3, 36, 43). Entero1, EPA-EC23S, stx_1, and *ttrRSBCA* were also detectable at 120 days of incubation in both soil-manure combinations incubated at 10°C and 25°C. The results described above suggest that these qPCR genetic markers will persist in manure-amended landscapes long enough to be potentially useful surrogates to cultivable FIB for indentifying nonpoint source fecal pollution in agricultural watersheds. In contrast, host-associated genetic markers did not persist. PigBac1 could not be detected in any samples. CowM3 decayed to nondetectable levels within 41 to 55 days at 10°C and 3 to 6 days at 25°C. PF163 persisted for only 0 to 3 days at 10°C and 25°C. Rapid decline of host-associated genetic markers below analytical limits of detection is not entirely surprising; these markers can be as much as 10,000-fold less abundant in manure than FIB (44). The utility of these host-associated markers may be limited to recent contamination events only.

Decay of cultivable bacteria and qPCR genetic markers. A two-stage, first-order decay model was used to describe decay

of cultivable bacteria and qPCR genetic markers. Figure 1 shows an example of the fit of this model to the data of one of 12 experimental conditions: beef manure-amended soils at 25°C and moisture content of 80% FC. Cultivable organisms and qPCR genetic markers shown in Fig. 1 include *S. enterica* serovar Typhimurium/pDs and *ttrRSBCA* (Fig. 1A), *E. coli* O157:H7/pZs and stx_1 (Fig. 1B), and EPA-EC23S, Entero1, and GenBac3 (Fig. 1 C). As can be seen in Fig. 1, a second, slower stage of inactivation was required to describe the decay of these organisms and qPCR genetic markers at extended times. Table 2 reports the best-fit stage one and stage two decay rate coefficients (k_1 and k_2) and transition times (t') for *E. coli* O157:H7/pZs, stx_1, *S. enterica* serovar Typhimurium/pDs, *ttrRSBCA*, Entero1, EPA-EC23S, GenBac3, and CowM3 for experiments at 10°C and 25°C. Decay of these bacteria or genetic markers at −20°C was not observed, consistent with prior reports (1, 13, 51, 64). First-stage decay rate coefficients of *E. coli* O157:H7/pZs and *S. enterica* serovar Typhimurium/pZs in this study were similar to those previously reported for wild types of these pathogens in manure-amended soils summarized previously (54). Although not previously available for manure-amended soils, first-stage decay rate coefficients (k_1) of GenBac3 in the soils of this study were similar to those previously reported for *Bacteroidales* in river water (−0.06 day^{-1} to −2.37 day^{-1}) (3, 39, 64). Likewise, first-stage decay rate coefficients (k_1) for Entero1 were similar to those previously reported for river water (−0.046 day^{-1} to −0.344 day^{-1}) (60). Not reported in Table 2 are cultivable *E. coli* or enterococci. At −20°C, cultivable *E. coli* in beef manure-amended soil exhibited simple (monophasic) first-order decay, with a decay rate coefficient of -0.020 ± 0.0089 ($R^2 = 0.34$) for a moisture content of 60% FC and -0.031 ± 0.0064 ($R^2 = 0.72$) for 80% FC. Although *E. coli* could be cultivated from manure-amended soils at 10°C and 25°C for both moisture contents for up to 56 days, the data were too variable for accurate prediction of decay rate coefficients and t'.

Different matrices exerted important effects on best-fit decay rate coefficients and transition times of cultivable bacteria and qPCR genetic markers ($P < 0.029$). In general, the magnitude of best-fit stage 1 and 2 decay rate coefficients (k_1 and k_2) were greater, and transition times (t') were shorter for beef manure-amended soils than for swine manure-amended soils; exceptions were k_1 for EPA-EC23S and t' for GenBac3. Increasing moisture content from 60% to 80% field capacity reduced the magnitude of k_1 ($P = 0.053$) but had no observable effect on k_2 or t'. Increasing temperature from 10°C to 25°C increased the magnitude of k_1 ($P < 0.001$) and resulted in shorter t' ($P = 0.006$) but did not significantly affect k_2 ($P = 0.224$). This result is consistent with other reported literature (18, 22, 42).

A second characteristic of reliable qPCR genetic marker surrogates is that they will decay under environmental stress at rates similar to, or slower than, cultivable FIB and bacterial pathogens. Table 3 reports P values based on two-way ANOVA and Tukey's *post hoc* test to determine significant differences between best-fit k_1, k_2, and t' of qPCR genetic markers and cultivable FIB and pathogens at 10°C and 25°C. Best-fit k_1 and k_2 of *S. enterica* serovar Typhimurium/pDs were significantly greater and t' was significantly less than the best-fit coefficients for *ttrRSBCA* ($P < 0.0003$), indicating possible entrance of this organism into a VBNC state during the study

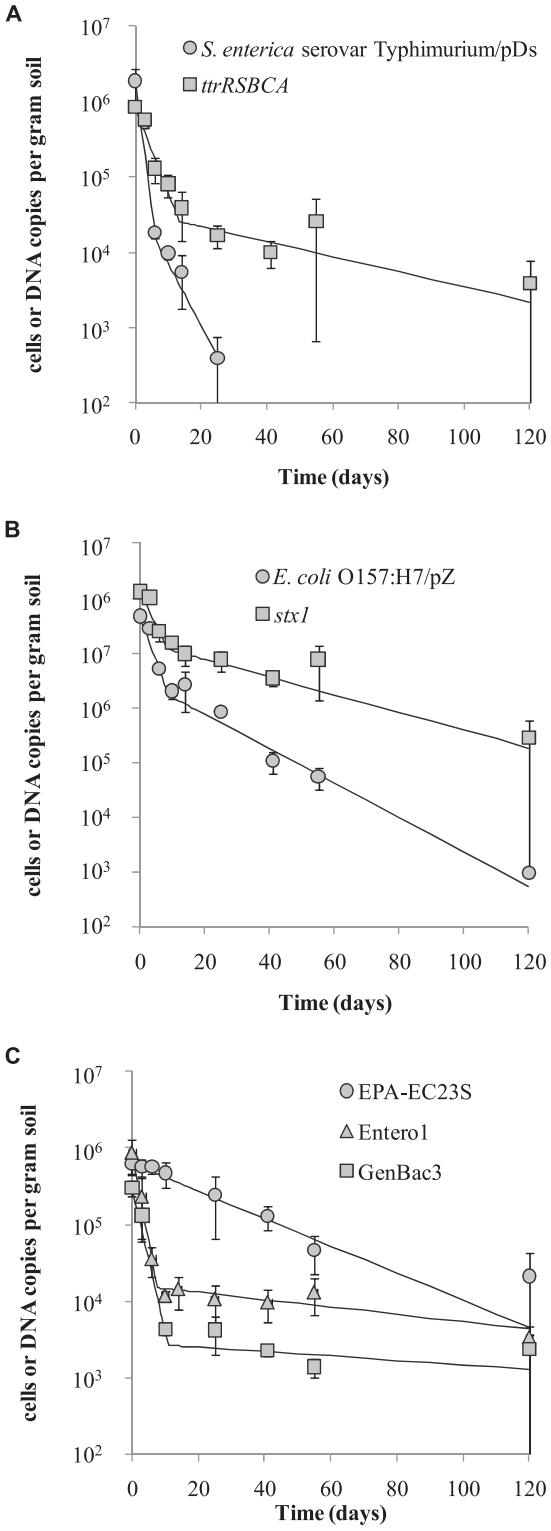

FIG. 1. Measured decay of bacterial pathogens and qPCR genetic markers from beef manure-amended soils at 25°C with 80% FC for *S. enterica* serovar Typhimurium/pDs and *ttrRSBCA* (A), *E. coli* O157:H7/pZs and stx_1 (B), and EPA-EC23S, Entero1, and GenBac3 (C) qPCR genetic markers. Plotted with the data are the best-fit-modeled biphasic decay curves. In some cases, such as for EPA-EC23S, single-phase exponential decay was the selected model because either k_1 and k_2 were not significantly different or there were insufficient data between the predicted t' and the time at which the target was no longer detectable to provide an accurate prediction of t' and k_2.

TABLE 2. Decay rate coefficients and transition times for viable bacteria and qPCR genetic markers

Organism or genetic marker	Variable	Result[a]							
		Swine manure-amended soil				Beef manure-amended soil			
		10°C		25°C		10°C		25°C	
		60% FC	80% FC	60% FC	80% FC	60% FC	80% FC	60% FC	80% FC
E. coli O157:H7/pZ	t'	20.1	19.5	11.0	16.2	19.0	19.0	9.5	9.0
	k_1	0.22	0.19	0.40	0.28	0.17	0.15	0.33	0.37
	k_2	0.045	0.050	0.061	0.070	0.039	0.037	0.076	0.072
	R^2	0.99	0.98	0.97	0.96	0.96	0.97	0.97	0.98
stx$_1$	t'	ND	ND	7.0	12.4	17.0	16.1	7.5	8.7
	k_1	0.048	0.050	0.25	0.090	0.13	0.09	0.31	0.28
	k_2	ND	ND	0.033	0.039	0.032	0.049	0.038	0.037
	R^2	0.93	0.91	0.86	0.90	0.86	0.97	0.90	0.94
S. enterica serovar Typhimurium/pDs	t'	21.0	17.3	5.35	14.0	ND	ND	6.37	6.45
	k_1	0.39	0.27	0.84	0.33	0.26	0.18	0.81	0.76
	k_2	0.041	0.13	0.090	0.065	ND	ND	0.14	0.19
	R^2	0.98	0.98	0.97	0.91	0.87	0.96	0.96	0.96
ttrRSBCA	t'	26.2	35.7	37.8	46.6	33.3	32.5	9.3	13.5
	k_1	0.15	0.10	0.11	0.072	0.13	0.10	0.34	0.26
	k_2	0.028	0.031	0.012	0.012	0.022	0.039	0.040	0.023
	R^2	0.96	0.93	0.86	0.90	0.89	0.98	0.85	0.90
Entero1	t'	ND	ND	ND	ND	8.64	16.9	7.09	7.65
	k_1	0.037	0.043	0.035	0.041	0.42	0.23	0.65	0.53
	k_2	ND	ND	ND	ND	0.030	0.018	0.013	0.011
	R^2	0.68	0.62	0.51	0.79	0.87	0.93	0.90	0.90
EPA-EC23S	t'	16.9	10.9	10.7	10.7	ND	ND	ND	ND
	k_1	0.23	0.20	0.26	0.16	0.014	0.012	0.026	0.041
	k_2	0.033	0.029	0.035	0.048	ND	ND	ND	ND
	R^2	0.85	0.85	0.86	0.87	0.53	0.84	0.32	0.60
GenBac3	t'	32.4	ND	16.2	15.6	16.4	18.7	15.4	11.0
	k_1	0.068	0.046	0.090	0.076	0.23	0.23	0.35	0.43
	k_2	0.005	ND	0.008	0.005	0.027	0.017	0.006	0.007
	R^2	0.79	0.82	0.64	0.51	0.92	0.96	0.89	0.95
CowM3	t'	NA	NA	NA	NA	ND	ND	ND	ND
	k_1	NA	NA	NA	NA	0.19	0.085	BDL	BDL
	k_2	NA	NA	NA	NA	ND	ND	ND	ND
	R^2	NA	NA	NA	NA	0.84	0.52	BDL	BDL

[a] FC, field capacity; BDL, below detection limits; ND, not determined, single-phase exponential decay best fits the data; NA, not applicable. Data for decay rate coefficients (k_1, k_2) are measured in days^{-1}, and transition times (t') are measured in days.

period. In contrast, best-fit k_1, k_2, and t' of *E. coli* O157:H7/pZs were not significantly different from those of *stx*$_1$ and EPA-EC23S qPCR genetic markers ($P = 0.587$ to 1.000). This result indicates that *E. coli* O157:H7/pZs remained cultivable and supports plasmid stability of these transformants during the study period.

Best-fit k_1 and t' of *E. coli* O157:H7/pZs were not significantly different from those of Entero1, GenBac3, and EPA-EC23S. However, k_2 for *E. coli* O157:H7/pZs was significantly greater than that of Entero1 and GenBac3 ($P < 0.04$), indicating that at protracted times following manure application to soils, these qPCR genetic markers may be conservative surrogates. In contrast, best-fit k_1 and k_2 of Entero1, GenBac3, and EPA-EC23S were significantly less than those of *S. enterica* serovar Typhimurium/pDs ($P < 0.011$). This result is consistent with other studies that report that these qPCR genetic markers do not necessarily decay at the same rates as bacterial pathogens in water environments (15, 19, 26, 52). Best-fit k_1 and k_2 of Entero1, GenBac3, and EPA-EC23S were similar to those of *ttrRSBCA*, but the transition times were greater ($P < 0.058$).

Culture-based versus qPCR measurements of bacteria. Cultivation-based measurements of FIB and pathogens remain the gold standard. Measurements of reliable qPCR genetic marker surrogates will yield predictable relationships with measurements of their cultivation-based counterparts over variable environmental stress and time. Log-transformed concentrations of bacteria as measured by cultivation (x axis) are plotted against log-transformed concentrations as measured by qPCR genetic marker (y axis) in Fig. 2 for all measurements from soil microcosms maintained at 10°C and 25°C, from 0 to 120 days of incubation. Linear regression analysis indicates that the relationships between paired measurements of viable *E. coli* O157:H7/pZs versus *stx*$_1$ (Fig. 2A) and *S. enterica* serovar Typhimurium/pDs versus *ttrRSBCA* (Fig. 2B) were highly predict-

TABLE 3. *P* values based on Tukey's *post hoc* test to determine significant differences between best-fit model coefficients of viable cells and qPCR genetic markers

Organism or genetic marker	Variable	Result[a]						
		S. enterica serovar Typhimurium/pDs	stx_1	*ttrRSBCA*	Entero1	GenBac3	EPA-EC23S	CowM3
E. coli O157:H7/pZ	t'	0.962	1.000	**0.002**	0.999	0.942	0.747	NA
	k_1	0.127	0.900	0.907	1.000	0.990	0.635	0.999
	k_2	**0.002**	0.587	0.140	**0.039**	**0.003**	0.992	NA
S. enterica serovar Typhimurium/pDs	t'		1.000	**0.000**	1.000	0.449	0.997	NA
	k_1		**0.003**	**0.003**	0.080	**0.011**	**0.001**	0.287
	k_2		**0.000**	**0.000**	**0.000**	**0.000**	**0.002**	NA
stx_1	t'			**0.001**	1.000	0.770	0.958	NA
	k_1			1.000	0.957	1.000	1.000	1.000
	k_2			0.998	0.703	0.405	0.995	NA
ttrRSBCA	t'				**0.006**	0.058	**0.000**	NA
	k_1				0.961	1.000	1.000	1.000
	k_2				0.938	0.739	0.869	NA
Entero1	t'					0.829	0.988	NA
	k_1					0.998	0.760	1.000
	k_2					1.000	0.472	NA
GenBac3	t'						0.250	NA
	k_1						0.990	1.000
	k_2						0.194	NA
EPA-EC23S	t'							NA
	k_1							1.000
	k_2							NA

[a] NA, not applicable; biphasic decay was not detected for the CowM3 biomarker. Values in bold indicate significant differences in the response variables.

able ($R^2 > 0.85$, $r > 0.94$). Correlations were also observed between *E. coli* O157:H7/pZs and Entero1 ($r = 0.745$), EPA-EC23S ($r = 0.704$), and GenBac3 ($r = 0.737$) and between *S. enterica* serovar Typhimurium and Entero1 ($r = 0.693$), EPA-EC23S ($r = 0.558$), and GenBac3 ($r = 0.685$).

Poor correlations were observed between viable enterococci versus Entero1 (Fig. 2C) and viable *E. coli* versus EPA-EC23S (Fig. 2D). Variability in linear correlation coefficient values has been observed in other research efforts focusing on culture and qPCR measurements of FIB in biosolids (57) and ambient waters (24, 63). Variability may be attributed to dead cells, VBNC cells, extracellular free DNA, and/or other, more specific causes. For instance, correlations for *E. coli* O157:H7/pZs with the EPA-EC23S genetic marker were stronger than for fecal indicator *E. coli* and EPA-EC23S due to the greater prevalence of *E. coli* O157:H7/pZs in the manure-amended soils. *E. coli* O157:H7 lacks β-glucuronidase activity and therefore could not be detected by fluorescence-based FIB methods that rely on cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUG). EPA-EC23S detects all *E. coli* strains, including *E. coli* O157:H7. Although correlations between enterococci and Entero1 have been derived in prior studies for ambient surface waters (63), unpredictable correlations were observed in this study. This may be caused by transitioning of *Enterococcus* spp. into and out of a VBNC state (28). Potential VBNC cells are further evidenced by decay rate coefficients for cultivable enterococci that were not significantly different than zero for any combination of temperature and moisture content, whereas Entero1 exhibited k_1 and k_2 of 0.011 to 0.65 day^{-1}. Strong correlations for viable pathogens and their counterpart qPCR genetic markers indicate that dead cells, VBNC cells, and/or extracellular free DNA do not always confound culture/qPCR relationships.

DISCUSSION

qPCR approaches have emerged to resolve challenges associated with real-time monitoring of public beaches, identifying potential threats to public health posed by pathogenic microorganisms in drinking water sources, and allocating sources of FIB in impaired waters. Prior studies regarding the behavior of qPCR genetic markers versus cultivable FIB and pathogens have focused largely on surface waters. Although reasonable for point sources of pollution, nonpoint source runoff from manure-amended lands may be the largest source of FIB and pathogens in agricultural watersheds. Variability in the decay of these markers on the landscape prior to runoff may significantly affect their utility in these watersheds.

The objective of this study was to investigate qPCR genetic markers as useful surrogates for cultivation-based measurements of viable FIB and pathogens in manure-amended agricultural soils. qPCR genetic markers for FIB (Entero1, EPA-EC23S, and GenBac3) and bacterial pathogens (stx_1 and *ttrRSBCA*) persisted at detectable levels for as long as, or longer than, cultivable bacterial pathogens (*E. coli* O157:H7/pZs and *S. enterica* serovar Typhimurium/pDs) and FIB (*E.*

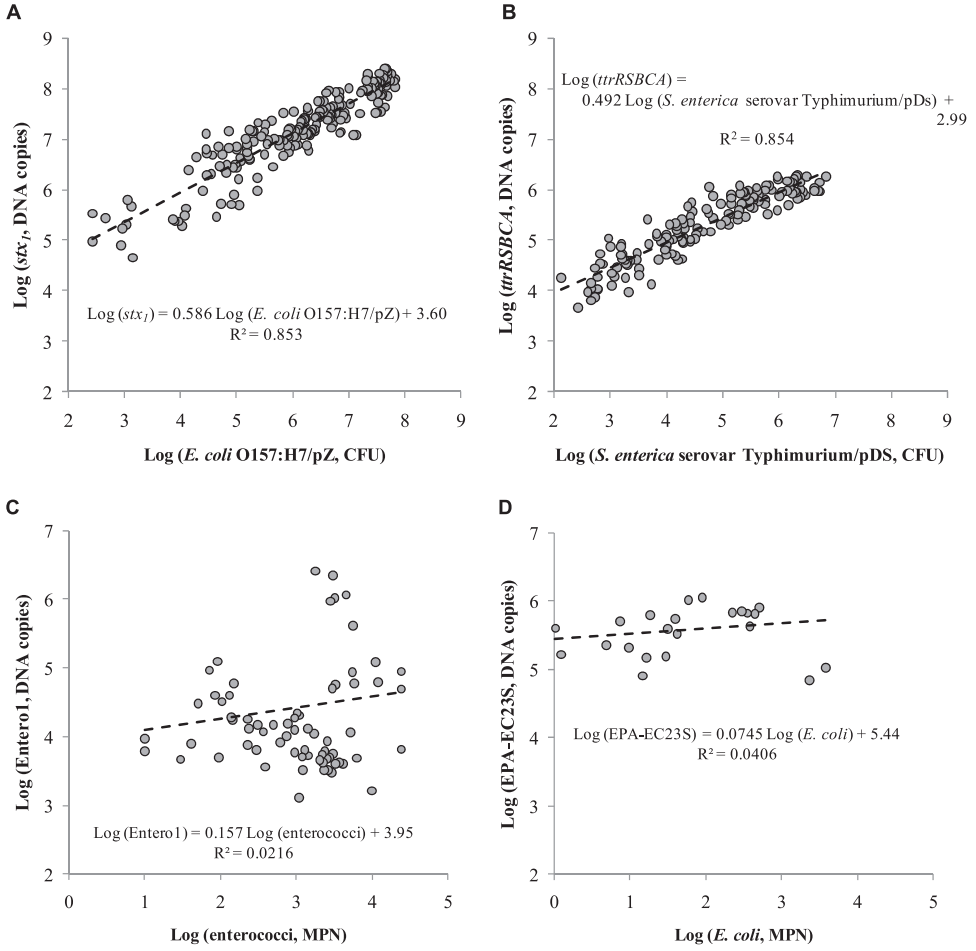

FIG. 2. CFU or most probable numbers of cultivable cells per gram of soil plotted against the number of copies of qPCR genetic markers per gram of soil for *E. coli* O157:H7/pZs and stx_1 (A), *S. enterica* serovar Typhimurium/pDs (B), enterococci and Entero1 (C), and *E. coli* and EPA-EC23S (D).

coli and enterococci). Few significant differences in decay rate coefficients of these qPCR genetic markers and cultivable microorganisms were observed; where differences were observed, decay rate coefficients and transition times of the qPCR genetic markers were conservative. Measurements of qPCR genetic markers could be correlated to cultivable bacterial pathogens (*E. coli* O157:H7/pZs and *S. enterica* serovar Typhimurium/pDs), further evidencing their utility as surrogates for cultivation-based measurements. These results were expected; qPCR can detect DNA of cultivable cells, VBNC cells, nonviable intact cells, and extracellular free DNA. It can be argued that the detection of both cultivable and VNBC cells makes qPCR a preferred (conservative) method of detection since VNBC cells have also been shown to cause illness (5). However, qPCR may suffer from sample concentration issues, PCR inhibition, and other factors that lead to unfavorable method performance (e.g., sensitivity). These nuances can be especially important when monitoring for organisms with low infectious doses, such as *E. coli* O157:H7 (5 to 10 cells).

On the other hand, host-associated PCR and qPCR genetic markers (PF163, PigBac1, CowM2, and CowM3) for microbial source tracking in impaired waters did not persist in manure-amended soils as long as cultivable FIB, pathogens, or qPCR biomarkers for FIB and bacterial pathogens. Thus, utility of these genetic markers may be limited to point source or recent nonpoint source fecal contamination. Use of these markers in agricultural watersheds in which manure is applied to land may lead to a high rate of false-negative results; runoff of FIB and bacterial pathogens may continue from manure-amended soils long after these markers decay to nondetectable levels. Misinterpretation of such false-negative results can potentially lead watershed restoration efforts astray.

Accurate predictions of the decay of fecal microorganisms in this study required the use of a biphasic decay model. Such models are often required to describe long-term behavior of manure microorganisms in soils and surface waters (12, 39). Easton et al. (12) offer the following two possible explanations: (i) Microorganisms may die off at a rapid rate until the carrying capacity of the environment is approached. The slower, second phase of decay may occur when organisms are better supported by the nutrients present. (ii) Microorganisms may be self-regulating their populations via quorum sensing (50). Velz (56) offers a third explanation: two subpopulations with different decay rates may be present. For example, You et al. (66) noted

that antibiotic-resistant strains of *S. enterica* serovar Newport persisted for longer periods of time than their nonresistant counterparts in manure-amended soils. A fourth explanation may be that organisms enter a viable but not cultivable (VBNC) state, resulting in the appearance of an initial rapid decay, until stressed cells alter their physiology, followed by slower decay of more resilient organisms that remain cultivable, or as cells transition into and out of the VBNC state (8, 12, 14, 27). Other potential sources of this behavior may include predation, biofilm formation, or the acquisition of genetic materials by a subpopulation of fecal bacteria from the native soil microbial community that favor their survival over counterparts that do not similarly adapt.

In this study, k_2 was not affected by temperature or moisture content but only by the matrix. This may be a clue to the mechanisms that lead to extended persistence; for instance, acquisition of antibiotic resistance genes is not likely to affect thermal resilience in late stages of decay. However, the alteration of cell physiology that occurs as microbes transition to more resilient states (e.g., VBNC) may improve thermal resistance and tolerance to desiccation. This may increase the importance of matrix effects, such as nutrient status or presence of predators. Quorum sensing may play a role in the induction and resuscitation of VBNC bacteria (37). Further studies using different soil and manure combinations, and considering other stressors that may impact the decay of these markers in manure-amended soils, are needed to better identify the extent to which these potential processes may affect bacterial survival and to elucidate the specific conditions that lead to extended survival. In any event, the results of this study suggest that qPCR analysis may, in some instances, be a more rapid and less time-consuming alternative to culture-based approaches, especially for developing data for modeling studies.

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