**Decay Rates of Zoonotic Pathogens and Viral Surrogates in Soils Amended with Biosolids
and Manures and Comparison of qPCR And Culture Derived Rates**

B.N. Roberts¹, R.H. Bailey², M.R. McLaughlin¹, and J.P. Brooks¹

¹Agriculture Research Services, United States Department of Agriculture, Mississippi State, MS
39762

²Pathobiology and Population Medicine Department, College of Veterinary Medicine,
Mississippi State University, Mississippi State, MS 39762

Key words: *Campylobacter*, *Clostridium perfringens*, *E. coli* O157:H7, land application,
Listeria, MS2, ØX174, *Salmonella*, waste, manure, biosolids

Running title: Microbial decay in waste-amended soil

Correspondence

Brandy Roberts, Agriculture Research Services, United States Department of Agriculture, POB
5367, Mississippi State, MS 39762, USA. E-mail: bnh3@msstate.edu

Approved for publication as Journal Article No. J-XXXXXX of the Mississippi Agricultural and Forestry Experiment
Station, Mississippi State University. Mention of a trade name, proprietary product, or specific equipment does not
constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products
that may be suitable. This work was prepared by employees of the U.S. Government as part of their official duties
and is in the public domain and may be used without further permission.

ABSTRACT

Aims: The purpose of this study was to establish inactivation constants of foodborne pathogens and coliphage in clay and sandy soils to be used in quantitative microbial risk analysis and compare cultural and qPCR methods in determining if inactivation rates differ among certain bacteria.

Methods and Results: *Salmonella enterica*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Clostridium perfringens*, were seeded together with MS2 and ØX174 phages, into three waste matrices (Class B biosolids, swine lagoon effluent, cattle manure), and phosphate buffered saline (PBS) as a control, and applied to two soil types (sandy loam, clay loam) using two application practices (incorporated, surface applied). *S. enterica* survived longer and at higher levels in cattle manure than in other matrices. Culturally, *C. jejuni* and *L. monocytogenes* were below detection limits (~ 200 CFU g⁻¹) by days 7 and 28, respectively, but molecularly, both were detectable 28+ days. *Clostridium perfringens* persisted at higher levels in biosolids and swine lagoon effluent, regardless of waste, soil type, or application practice. Phage survival was longer for MS2 in biosolids than in other waste matrices, while ØX174 did not differ; neither survived longer than 90 days.

Conclusions: Class B biosolids and cattle manure sustained pathogens longest. Higher solid content waste-residual as opposed to liquid waste may provide suitable matrices capable of protecting pathogens.

Significance and Impact of the Study: This study provides new and more conclusive decay rate data that will be useful for assessment of public and occupational health risks.

50

51 **1. INTRODUCTION**

52 Quantitative microbial risk assessment (QMRA) defines potential individual and population
53 health risks associated with microbial pathogens. QMRA models rely on important inputs such
54 as pathogen levels, transport, decay rates, and dose response parameters (Brooks et al., 2012;
55 Haas et al., 1999). Fresh food crop outbreaks have often left more questions regarding pathogen
56 presence, transport, and inactivation once in the field (Brooks et al., 2012; Eileen R. Choffnes et
57 al., 2012). Fecal contamination of crops can occur naturally by wild and feral animals or by
58 insect movements from feces to food crops (Moore and Gross, 2010). Contamination can occur
59 unintentionally from accidental release of manure from animal feeding operations, or as an
60 unintended consequence following land application of biosolids or manure. Land application of
61 biosolids and manure poses a growing concern for environmental and human health risks due to
62 the zoonotic microbial pathogen loads found in these waste products and to the increasing
63 volumes of these waste residuals as the size and scope of animal feeding operations increases to
64 keep pace with worldwide food demand. Although much is known about potential threats from
65 foodborne microbial pathogens, knowledge gaps exist and must be filled to understand the
66 survival and fate of these pathogens when introduced into the environment.

67 Any untreated fecal waste potentially harbors pathogens (Gerba and Smith, 2005;
68 Hutchison et al., 2005). Common foodborne bacterial pathogens which can be isolated from
69 both human and animal wastes are *Campylobacter jejuni*, *Salmonella enterica*, *Listeria*
70 *monocytogenes*, *Escherichia coli* O157:H7 and *Clostridium perfringens*, all of which have been
71 implicated in foodborne outbreaks (Moore and Gross, 2010; Scallan et al., 2011). Additionally,

viral pathogen loads can be extremely high in feces of infected hosts. Because viruses are often host specific, the main environmental source for human enteric viral transmission is biosolids or untreated sewage (USDA-NASS., 2011). Common viruses that are found in biosolids are norovirus, adenovirus, enterovirus, hepatitis A and E, and rotavirus (USEPA, 1995; Viau et al., 2011; Wong et al., 2010). Use of animal and human viruses in decay studies is limited by the need for animal cell-culture models and by difficulty in virus propagation, hence the use of surrogate phage models. MS2 phage is routinely used as an indicator of adenoviruses (USEPA, 1995), rotavirus (Bhattarai et al., 2011), norovirus (D'Souza and Su, 2010), and enteric viruses in residual wastes (Katz and Margolin, 2007). The somatic DNA phage, ØX174, is a surrogate for adenovirus (Berger et al., 2010) and an ideal contrast to MS2, which is a male specific, RNA coliphage. In the present study, ØX174 and MS2 were used to estimate viral decay rates in waste residuals applied to sandy loam and clay loam soils.

Pathogens may survive in the environment for days to months (Holley et al., 2006; Inglis et al., 2010; Islam et al., 2004; Scallan et al., 2011; USEPA, 1995; You et al., 2006), but site- and waste-specific survival rates for these pathogens are not well defined. Several studies have reported decay rates for microbes associated with residuals applied to soils (Table 1) (Holley et al., 2006; Lang et al., 2007; Lang and Smith, 2007; Rogers et al., 2011; Straub et al., 1992; Toth et al., 2011), but most studies were limited to a single pathogen and soil type or multiple pathogens and a single waste residual. Thus far, no studies have compared microbial decay rates in biosolids and manure.

One aspect of QMRA that is not yet fully understood is the use of molecular data in current models. In low nutrient and high stress environments, many pathogens can enter a reduced metabolic state called “viable but non-culturable” (VBNC) (Freestone and Lyte, 2010;

Hutchison et al., 2005; Olsen et al., 2000). When VBNC bacteria cannot be isolated using standard culture methods and enrichment processes, quantitative polymerase chain reaction (qPCR) can bypass this limitation and allow quantifiable pathogen-specific detection. Both culture and molecular analysis have benefits and limitation; a comparison of the detection of pathogens provides insight into whether the derived decay rates of foodborne pathogens are statistically different. Because of the inhibitory properties of humic acid in soil and the small quantities analyzed it has been concluded that culture analysis may be more sensitive in some samples (Colinon et al., 2013). The present study reports results of microcosm experiments which investigated multiple variables (waste residual type, soil type, pathogen, and residual management), to: 1) understand the effects of soil type, waste type, and residual management which influence microbial decay in waste-amended soils; and 2) compare cultivation-dependent and -independent (i.e. qPCR) methods of determining microbial decay rates.

2. METHODS

2.1 Study Design

The experimental design was laid out as factorial (4x2x2x2) with four residuals (Class B biosolids, cattle manure, swine lagoon effluent, and phosphate buffered saline [PBS] control) applied to two types of southeastern soils (Stough and Leeper), using two application management practices (surface-applied residual incorporated and not incorporated), with two pathogen levels (concentrated pathogen-spiked cocktail and sterile PBS). Pathogen-spiked cocktails comprised *S. enterica*, *E. coli* O157:H7, *L monocytogenes*, *C. jejuni*, *C. perfringens*, MS2, and ØX174. Treatments were replicated in triplicate and assayed at 11 time points (0, 7, 14, 21, 28, 60, 90, 120, 150, 180, 210 d).

2.2 Soil Preparation

Soils were collected from the Mississippi State University R. R. Foil Plant Science Research Center (North Farm). Stough (sandy loam) soil is taxonomically classified as coarse-loamy, siliceous, semi-active, thermic Fragiaquic Paleudults, and Leeper (clay loam) soil as fine, smectitic, nonacid, thermic Vertic Epiaquepts. Each soil was homogenized by passage through a #10 (2.54 cm) nominal sized sieve. Residual waste (swine effluent, cattle manure, and Class B biosolids) were collected from local farms or municipalities. Swine effluent and cattle manure were collected from a commercial pig rearing facility and local independent rancher, respectively. Class B biosolids were collected from a regional municipal wastewater treatment plant. Residual wastes were collected 2-3 days prior to study commencement. Moisture content was assessed by weighing 10 g of soil, heating at 104°C for 48 h, reweighing the soil, and calculating the difference as % moisture. Stough fine sandy loam consisted of 64% sand, 27 % silt, 10% clay, and pH 5, and Leeper silty clay loam was 20% sand, 49% silt, 31% clay, and pH 7 (Abdelwhab et al., 2011).

2.3 Inoculant Preparation

Salmonella enterica (ATCC 14028), *E. coli* O157:H7 (ATCC 43895), and *Listeria monocytogenes* (ATCC 51722) cells were prepared by growing each bacterial culture to exponential phase (approximately 6 h) in tryptic soy broth (TSB) in 50-mL centrifuge tubes at 35°C with shaking at 200 rpm. *Campylobacter jejuni* (ATCC 33560) was prepared by growing to exponential phase (approximately 24 h) in *Campylobacter* Enrichment Broth (CEB) in 50-mL centrifuge tubes at 42°C in microaerophilic conditions. *Clostridium perfringens* (ATCC 3624) was inoculated in TSB and grown overnight at 44.5°C, anaerobically, followed by inoculation

into Duncan sporulation media (1:10 v:v) and sporulated at 35°C for 2 weeks. Each culture was pelleted by centrifugation at $5000 \times g$ for 30 min. Supernatants were decanted, pellets were re-suspended in an equal volume of sterile PBS, and the process was repeated for a total of three times. Final washed cells were suspended in 25 mL PBS for titer determinations (10^8 to 10^9 mL⁻¹) and storage at 4°C. All cells were used within 3-7 days of preparation and were quantified prior to inoculation into wastes.

2.4 Bacteriophage Preparation

MS2 (ATCC 15597-B1) and ØX174 (ATCC 13706-p1) coliphages were propagated in *E. coli* (ATCC 15597) and *E. coli* (ATCC 13706), respectively, to 10^6 plaque forming units (PFU) mL⁻¹ according to (Brooks et al., 2005).

2.5 Soil Microcosms

Dry soil (150 g per microcosm) was placed in 355-mL Styrofoam cups. Waste residuals were applied at a rate of 10% based on the dry weight of soil (15 g or mL depending on the waste matrix), to mimic the top soil surface layer (0-10 cm) following a land application event. Prior to waste application, each of the three wastes and PBS was seeded with bacteria and phages to final levels of approximately 10^6 g⁻¹ of soil for each microbe. For comparison of farm management, wastes were applied evenly on the soil surface (surface application), or using a wooden stick, waste was mixed in the top 3 – 5 cm of soil (incorporated application). Plastic lids were placed over each microcosm to reduce water evaporation. A non-seeded control set of microcosms were prepared for each time point, for each waste and PBS, to account for natural bacteria and phage levels.

Microcosms were randomly placed in three controlled temperature chambers and incubated continuously at 30°C and 20°C for 14 h and 10 h cycles (day : night), respectively, to simulate summer growing season conditions in the southeastern US. Maintaining water moisture of 25% was achieved by adding sterile deionized water weekly. Microcosms were weighed when soil moisture was at desired levels and brought to that weight each week. At each time point, each representative microcosm was sacrificed for analysis of bacterial and viral levels. The top 5 – 7 cm of each microcosm was mixed thoroughly with wooden tongue depressors prior to culture and molecular assays.

2.6 Cultivation-dependent Enumeration

Prior to microbial analyses, 10 g of each sample was suspended in 95 mL of sterile physiological saline in a stomacher bag and stomached for 30 s prior to serial dilution and plating. Enumeration of *S. enterica* and *E. coli* O157:H7, was achieved by direct plating to Hektoen Enteric agar (BD-Difco) and Cefixime Tellurite Sorbitol MacConkey (CTSMAC) agar, respectively, and incubated overnight at 35°C. *C. jejuni* was enumerated by direct plating to Preston agar (Neogen-Accumedia) containing 5% horse blood (Hema-Resources; Aurora, OR) and incubated at 42°C under microaerophilic conditions for 48h. *L. monocytogenes* was direct plated to Oxford agar (Neogen) and incubated for 24 - 48 h at 35°C. *C. perfringens* was enumerated by membrane filtration on mCP media and incubating at 44.5°C overnight. When pathogens could no longer be isolated by direct plating, 1 g of representative microcosm sample was added to 10 mL of the respective enrichment broth and incubated 24 – 48 h and plated on their respective media and incubation temperatures. *S. enterica*, *E. coli* O157, *L. monocytogenes*, and *C. jejuni* were enriched using RVR10 (Neogen), ECMUG (Neogen), Fraser (Neogen), and Campy Enrichment Broth (Neogen) and 5% Sheep Blood TSB, respectively according to previous

research (McLaughlin et al., 2009). Representative colonies of each isolated bacteria was confirmed via PCR.

Coliphages, MS2 and ØX174, were enumerated by plaque assays using their respective log- phase *E. coli* propagation hosts ($\sim 10^6$ - 10^7 CFU ml⁻¹). Phages were enumerated by adding 0.1 ml of serial diluted sample with 0.1 ml of fresh exponential growth phase *E. coli* in TSB to 5.0 ml of soft TSA (0.75% agar) which had been melted and maintained at 50°C in a water-bath. Soft agar mixtures were poured onto full strength TSA and plates were incubated overnight at 35°C. Counts of PFU were made within 12 – 16 h.

2.8 Quantitative PCR

Immediately following sample collection, 1 g of each microcosm was homogenized and frozen archived at -80°C. DNA was extracted from 0.25 g of each sample from days 0, 7, 14, 28 and 60 d using the Qiagen QiAmp DNA stool mini kit (Cat No. 51504) (Qiagen, Victoria, Australia) following the manufacturer's recommended procedure. Reaction mixtures included 2 µl DNA extract (diluted 1:10 or 1:100), 12.5 µl of the ABI syber green master mix (Applied Biosystems, Foster City, CA), 1.0 µl of each associated forward and reverse primer (10 µM), and 9.5 µl PCR H₂O for each quantitative PCR (qPCR). Sample inhibition and qPCR optimizations for each assay were performed prior to study commencement or were based on previously published work (Brooks et al., 2014). Dilution was suitable for most samples to remove the effect of inhibition. For samples that contained clay loam soil, 0.5 µl polyvinylpyrrolidone was added per reaction to reduce inhibition associated with clay soils (Koonjul et al., 1999). *S. enterica*, *C. jejuni*, *E. coli* O157:H7, and *L. monocytogenes* were all quantified using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). All qPCR reactions were performed using the same

conditions: 95°C (10 min), 40 repetitions of 95°C (15 s) and 60°C (1 min), and melt-curve analysis at 95°C (15 s), 60°C (30 s), and 95°C (15 s). Positive, negative, and spike (inhibition) controls were included in every qPCR plate. Each reaction was set up in duplicate and amplification signals were quantified with a PCR-based standard curve and confirmed using melt-curve analysis. Standard control DNA was extracted from pure colony cultures and PCR-amplified, purified, quantified, and diluted from $\sim 10^5$ to 1 genomic unit per μl ($\text{GU } \mu\text{l}^{-1}$). Standard curves were required to have an r^2 of 0.98. An acceptable positive was only considered positive if both duplicates had Ct values less than the negative control and spike samples were within $\pm 25\%$ of the positive control. Table 2 lists the genetic markers, primer sequences and sizes of each target analyzed.

2.9 Inactivation Rates

Inactivation rates were calculated using a first order survival curve determined by $\text{Log}_{10}(N_t/N_0) = kt + b$; where N_t is the titer (CFU, PFU, or GU g^{-1}) at time t (d), N_0 is the original titer (CFU, PFU, or GU g^{-1}) at $t=0$ d, k is the inactivation rate based on the slope of decay of each microorganism, and b is the y-intercept for each organism-waste-soil treatment combination.

2.10 Statistical Analysis

SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC) was used for all statistical analyses. Geometric means were calculated prior to analysis. Prior to analysis all non-seeded microcosm bacterial or phage levels were subtracted from seeded levels per time point. This would account for any natural populations within each respective waste-residual. When enrichment detection was used for bacterial detection, the detection limit of 100 CFU g^{-1} was used for statistical purposes. A mixed model analysis was performed for all cultivated bacteria and phages and

qPCR assayed bacteria (*S. enterica*, *E. coli* O157:H7, *L. monocytogenes*, and *C. jejuni*) to determine the effect of waste residual, soil type, farm management and their interactions (waste residual * soil and waste residual * management) on inactivation rates (k values). This was conducted for both cultivation-dependent and –independent assays. Waste residuals were analyzed separately to determine if soil or management contributed to each microbial parameter’s persistence. Means were compared with difference of least squares means. A standard ANOVA was performed to compare calculated inactivation rates associated with waste residual, soil type, management and assay. Differences in phage enumeration between waste residuals, soil types and management practices were compared in paired t-tests. Differences were considered statistically significant at a probability level of $p \leq 0.05$.

3. RESULTS

3.1 Effect of waste, soil, time, and management

In general at least one of the test variables: waste-residual, soil type, or time significantly affected the decay rates of all bacteria and phage tested ($p < 0.05$), whether assayed via cultivation or qPCR (Table 3). Additionally, the interactions of waste-residual*soil, waste-residual*management, and waste-residual*soil*management significantly affected inactivation rates for nearly all bacteria and phage tested ($p < 0.05$). Table 3 provides a summary of all significant effects and mean inactivation rates, averaged across all experiments.

3.1.1 Effect of Waste Residual

Overall, waste-residual significantly affected all tested organisms’ inactivation rates (i.e. k-rates), though differences in k-rates were minor within *C. jejuni* inactivation rates. *C. perfringens* inactivation was negatively affected (e.g. more inactivation or less persistence) by

the presence of cattle waste compared with all other matrices, nearly 1 order of magnitude difference. *S. enterica* inactivation rates were positively affected (e.g. less inactivation or longer persistence) in biosolids and cattle waste-residuals by nearly 2-fold compared with swine effluent and PBS control ($p<0.05$); there was no difference between cattle and biosolids ($p<0.05$). Generally, *S. enterica* persisted longer ($p<0.0001$) in cattle manure and biosolids than in other wastes with mean k -values ranging from -0.0369 to -0.0757, while the liquid matrices were greater than -0.1362. *L. monocytogenes* inactivation rates were also significantly affected by biosolids and cattle waste-residuals ($p<0.05$); inactivation rates averaged -0.1835 to -0.2584 for solid wastes, while liquid matrices averaged -0.4579, approximately a 2-fold increase in inactivation. Both phage, in general, were inactivated more readily by cattle than biosolids wastes, while both liquid matrices were nearly the same (data not shown). Non-cultivated *E. coli* O157 and *C. jejuni* were significantly affected by ($p<0.05$) waste-residual. Overall, solid wastes negatively affected inactivation rates associated with *C. jejuni* and *E. coli* O157, and though not significant, also appeared to similarly affect cultivation-independent *L. monocytogenes* and *S. enterica* (Table 3).

3.1.2 Effect of Soil Type and Management

Soil type had fewer effects on cultivated organisms. For instance, sandy loam soil, in general, negatively affected inactivation rates more so than clay loam for cultivated *L. monocytogenes*, OX174 and MS2; on the contrary, *C. jejuni* was negatively affected by clay loam soil (Table 3). Clay loam soil was also found to negatively impact *C. perfringens* inactivation rates ($p<0.05$) by nearly 3-fold. Management affected cultivated *S. enterica*, *C. perfringens*, *E. coli* O157, and MS2 ($p<0.05$). *E. coli* O157 inactivation was negatively affected

by incorporating wastes into soil by approximately $0.1 \log d^{-1}$, while conversely *Salmonella* inactivation rates were negatively affected by surface-applied ($p < 0.05$).

The effect of soil type and management on non-cultivated bacterial inactivation rates demonstrated that clay loam soil had a negative impact ($p < 0.05$) on *Campylobacter jejuni* and *E. coli* O157, while no effect was noted for *L. monocytogenes* or *S. enterica*. Incorporating waste-residuals into soil appeared to negatively impact non-cultivated *S. enterica* inactivation rates by 2-fold, while not incorporating waste-residuals negatively impacted inactivation rates by $0.01 \log d^{-1}$ for *L. monocytogenes* (Table 3). *C. jejuni* rates were negatively impacted by surface-applied by approximately $0.01 \log d^{-1}$, while *E. coli* O157 was not affected.

3.1.3 Effect of Main Effect Interactions

The interaction of waste-residual*soil, waste-residual*management, and waste-residual*soil*management significantly affected most tested organisms (Table 3). Table 4 shows individual permutations of organism*waste-residual*soil*management. *E. coli* O157 inactivation rates in biosolids with either soil type were more negative by at least $0.1 \log d^{-1}$ compared with cattle manure in clay loam soil, with the exception when cattle manure was incorporated into clay loam soil ($k = -0.6772$). Similarly, cattle manure*clay loam soil had a more negative effect on *C. perfringens* inactivation rates, often times by $-0.1 \log d^{-1}$ compared to liquid x soil. Waste-residual*management negatively affected inactivation of *Salmonella* ($p < 0.05$) within liquid matrices more than the solid wastes with the exception of clay loam soil*incorporated.

3.2 Cultivation-dependent vs. -independent

292 *C. jejuni*, *E. coli* O157, *L. monocytogenes*, and *S. enterica* inactivation rates were all
293 affected by assay type. Almost exclusively, cultivation-based inactivation rates were more
294 negative than cultivation-independent-based rates ($p < 0.05$) (Table 3 and 4). Cultivation-based
295 inactivation rates were negatively affected by nearly 2 fold, from -0.07 to -0.13 for
296 *Campylobacter*. *E. coli* O157 and *S. enterica* inactivation rates behaved similarly, while *Listeria*
297 *monocytogenes* k-rates were most affected with a mean of -0.33 for cultivation-based
298 inactivation rates to $-0.04 \log d^{-1}$ for cultivation-independent inactivation rates.

299 Ratios of cultivation-dependent to -independent data ($CFU g^{-1} / GU g^{-1}$) for *S. enterica*, *E.*
300 *coli* O157, and *L. monocytogenes* provide a comparison of these two assay types (Table 5). *S.*
301 *enterica* was very similar for both cultivation-dependent and -independent detection for the first
302 2 weeks. For instance, cattle manure in sandy loam had similar detection with ratios close to 1:1
303 (CFU:GU). At d14, cultivation detection was at or below detection limits in biosolids, but the
304 cultivation-independent techniques were still able to quantify *S. enterica* markers. For example,
305 *Salmonella* was detected at a 1:10 ratio when surface applied via cattle manure to clay loam soil.
306 However, *S. enterica* (cattle manure) was detected through cultivation-enrichment for a longer
307 time period. Cultivation-dependent data for *E. coli* O157 and *L. monocytogenes* were favored at
308 a ratio of 15 to 1 for initial levels, when waste was surface applied to sandy loam soil. By day 60
309 the molecular markers were no longer detected for *E. coli* but cultivation data indicated *E. coli*
310 was still viable through enrichment methods (10:1). *L. monocytogenes* was not present through
311 cultivation-dependent detection but molecular markers were detected (1:100).

312 4. DISCUSSION

According to USDA-AMS (2000), manure can be applied to food crops with a stipulated post-application harvest delay of 90 to 120 days. Overall, based on the current study, the effect of waste-residual was the most likely of the tested variables to influence inactivation rates (e.g. k constants), followed closely by soil-type, and management. This makes intuitive sense, given the highly complex nature of waste-residuals and influence of feed, animal type, antibiotics, and municipality (Hutchison et al., 2005; Brooks et al., 2012). Additionally, the presence of relatively high solid material such as cattle manure and biosolids would inherently bias towards certain organisms. This was not always the case in the current study. The effects of each variable were not “globally” constant, nor predictable, with rates, organism and soil-type dependent. Simply viewing the mean effect of solid waste-residuals (i.e. cattle manure and biosolids), the current data shows positively affected inactivation rates for cultivated *S. enterica* and *L. monocytogenes*. Meanwhile *E. coli* O157:H7 and *C. perfringens* inactivation rates were negatively affected by cattle manure. Variability in the effect of solid material has been shown in previous studies by Hutchison et al. (2005) with liquid and solid dairy manure selecting for salmonellae and *L. monocytogenes*, respectively. Though the current study did not compare liquid and solid wastes from the same species, *Salmonella* k-rates were slower by 1 order of magnitude in solid wastes. For the most part limited inactivation of *Clostridium perfringens*, based on waste type was expected, owing to its ability to form spores capable of enduring environmental changes that vegetative cells cannot. This was the case in most permutations, with the exception of cattle manure surface-applied on clay loam, $k=-0.1758$, whereas other combinations had at least 1 order of magnitude slower k-rates than most bacteria or phage.

Many of the interactions were organism and experimental-variable specific. The interaction of organism*waste-residual*soil-type or *management significantly affected

inactivation rates. For example, results of the current study suggest that if *S. enterica* were present at high levels in land-applied manure, it may persist longer in soil than the recommended waiting period for harvesting food crops. This may only be reasonably plausible if assuming ideal conditions of moisture and organic matter (e.g. high solid wastes) are present; if a field is dry and conditions are unfavorable, it's reasonable to assume most pathogens begin rapid die off (Hutchison et al., 2005; Brooks et al., 2012). Inactivation rates established in the current study were, however, based on initially high levels (10^6 CFU g⁻¹) of pathogens, a caveat of laboratory studies (Hutchison et al., 2004). *S. enterica* may remain viable beyond harvest delays, assuming cattle manure surface applied to sandy loam soil at starting levels of 1.6×10^2 - 2.5×10^3 CFU g⁻¹ (Hutchison et al., 2005; Volkova et al., 2011). Applying the current k-rates, indicates *S. enterica* may remain viable beyond the recommended post-application harvest delay. Longer persistence of *S. enterica* in cattle manure than in other residuals may be due to a lack of chemical or physical attenuating factors present in other high solid content wastes, such as biosolids. Biosolids are commonly lime stabilized to control pathogen levels and vector attraction (e.g. insects)(Bean et al., 2007; Hansen et al., 2007), though the increased pH (Bean et al., 2007) and the reduced moisture (Opara et al., 1992) did not increase inactivation. On the other hand, liquid matrices increased inactivation by nearly 1 order of magnitude (log d⁻¹) in the case of cultivated *S. enterica*; this trend held true for most bacteria/waste combinations.

On the other hand, inactivation of the two phage indicators was more unpredictable, with biosolids and sandy loam soil positively affecting MS2 inactivation while negatively affecting ØX174 inactivation. This may be due to differences in surface receptor attachment in either matrix. Decay rates of MS2 phage in biosolids, in the present study, are in agreement with those of Wei et al. (2010), who reported that MS2 had a higher affinity for biosolids than swine or

cattle manure potentially due to increased iron oxide. The presence of a suitable *E. coli* host in biosolids (Yang and Griffiths, 2013) may also partially offset phage degradation. Straub et al. (1992) reported that MS2 was not affected by clay or sandy soils. In the present study, inactivation rates of MS2 phage in all wastes were much slower when applied to sandy loam than to clay soil. It is possible that phage adherence to clay particles (Sobsey et al., 1980) made it more difficult to detect MS2 in the present study.

The effect of waste incorporation was not an obvious predictor of inactivation rates. For example, *S. enterica* inactivation rates were decreased by waste-incorporation in certain scenarios, while *E. coli* O157 rates were increased. It was expected that all waste incorporation would decrease inactivation. However, the effect of management was also an interactive effect coupled with waste-residual and soil-type. Several mechanisms may explain the effect of application method on *S. enterica* survival. For instance *S. enterica* has been shown to be more recalcitrant to the effects of desiccation (Koseki et al., 2015). Incorporating the waste reduces desiccation of the bacteria allowing for prolonged survival, but also promotes interaction with soil microbes, which may explain the negative effect of incorporation. Others, like *L. monocytogenes*, were generally not affected by management practice, possibly owing to the thick peptidoglycan wall surrounding Gram + bacterial cells, which can resist desiccation. Additionally, *Listeria* spp. are known to be present in many soil environments (Linke et al., 2014). Study limitations such as the lack of wind air desiccation and UV light on the microcosms may have influenced the variable effect of waste-residual (Hutchison et al. 2005).

Cultivation-dependent and -independent methods are both useful for enumerating microorganisms in environmental samples. Cultivation-dependent is cost-effective, simple, and can be used to analyze large sample aliquots; however the time from sample to results is longer

and not all organisms can be cultivated. Cultivation-independent methods via qPCR overcome these limitations but increased costs, ability to analyze only small sample aliquots, and the caveat that genetic markers can persist longer than viable pathogens which leads to positive assays for inactivated bacteria and viruses. Quantitative PCR methods are subject to issues distinguishing viable from inactive cells, although some chemistries are providing this distinction, with caveats, via propidium monoazide (Kim and Ko, 2012).

Notwithstanding these limitations, many laboratories are turning towards qPCR for pathogen detection, which ultimately will affect the way inactivation rates are determined, interpreted, and utilized. Overall, in the current study, inactivation rates yielded by qPCR were slower than comparable cultivated rates. Klein et al. (2011) noted that qPCR data for microorganisms yielded slower decay rates than cultivation data, which corroborates findings in the present study. For example, mean inactivation rates for *S. enterica* were significantly different ($p < 0.05$) for cultivation-independent ($k = -0.06$) and –dependent ($k = -0.010$); *E. coli* O157 fared similarly $k = -0.07$ and -0.13 , respectively. The biggest effect of assay was on *C. jejuni* inactivation rates with cultivation-independent ($k = -0.03$) and –dependent ($k = -0.68$). Additionally, the effect of time influenced the effectiveness of each respective method. As seen in Table 6, the closer a ratio approaches 1.0 at a specific time point, the more corroborative the results were. Typically, the ratios approach $\pm 25\%$ of each other as time approaches 0, though many combinations held larger discrepancies at early time points as well, most likely owing to difficulty in either cultivation-dependent or –independent methods. Additionally, multiple copies of a gene within a cell can lead to over-inflated qPCR values, though aside from the *Campylobacter* primer pair, no other primer target was associated with multiple genomic copies.

The effect of assay was also dependent on combinations of experimental variables. The presence of clay loam soil and solid waste-residuals selected for more negative inactivation rates via qPCR, whereas that effect may not have been evident with cultivation (Table 4). For instance, the cultivation-independent k-rates for *E. coli* O157 were -0.026 and -0.1, respectively for cattle*sandy loam*non incorporation and cattle*clay loam*non incorporation. Comparable cultivation-dependent k-rates were -0.02 and -0.03, respectively. In other instances, k-rates were similar between both methodologies, such as for *S. enterica*, often times nearly identical or within 2-fold (Table 4). The discrepancy in k-rates varied from 2-fold to 10-fold depending on the scenario, and may be influenced by a combination of ease of cultivation, thus accurately depicting viable cells, but most likely was a result of detection of VBNC DNA. The detection of VBNC DNA most likely under represented true inactivation rates. In some instances, cultivation was the problem, such as with *C. jejuni*, in which cultivation-independent approaches most likely yielded the most accurate k-rate, though most likely facilitated by the detection of VBNC DNA. *C. jejuni* is difficult to detect in a complex population (ACMF, 2010) and can be more readily detected using cultivation-independent methods (McLaughlin et al., 2009).

In summary, this study looked at the effect of waste residuals on pathogenic bacteria and viruses under various microcosm-simulated scenarios. This study compared the effect of: waste residual; soil type and management practice; model interactions of each; and detection methods on inactivation rates, k-rates. The current study investigated the effect of each of these variables and determined interactions to be very situation specific.

For most bacterial and phage inactivation constants, one or both solid wastes positively influenced inactivation rates, though there were exceptions. On the contrary, the presence of solid waste led to more negative inactivation rates when determined via cultivation-independent

methodologies. Soil type and management practice had variable effects on microbe inactivation rate, with clay loam soil negatively selecting for cultivation-independent k-rates, while incorporating wastes did not positively influence inactivation rates. The inactivation rates determined from cultivation-dependent and –independent analyses weren't always in agreement, particularly as microcosm time progressed beyond 14 days and nuances of each method were apparent. When practical, the complementary strengths and weaknesses of both methods suggest the use of cultivation-dependent and –independent methods in environmental studies. At the very least, the appropriate of use of either approach should be employed based on pathogen specific study objectives (e.g. qPCR for detection of *C. jejuni*). Additionally, it's more apparent that interpreting results from a study utilizing only one method must be more heavily scrutinized to determine if the inactivation rates are applied correctly. For instance, application of qPCR-derived k-rates may be more appropriate for recalcitrant but difficult to cultivate bacteria such as *Listeria*, especially beyond 30 days. Whereas, *S. enterica* k-rates may be more represented by cultivation-dependent methods, even up to 60 days. Perhaps the application of CFU:GU ratios, such as those presented in Table 5, can be used as corrective factors if only one methodology is utilized, though the implication of the current study suggest that specific organism/soil/waste/environmental combinations will affect each ratio. The advantages offered by each detection method can aid in the selection of more appropriate inactivation constants and improved risk models for pathogens in the environment. Differences in microbial decay rates determined by cultivation-dependent and –independent methods in this study suggest that more research is needed to reconcile these approaches and develop improved microbial survival data for more accurate risk assessments.

Acknowledgements

450 This research was funded by Agriculture and Food Research Initiative Grant 3556 from the
451 USDA National Institute of Food and Agriculture. We appreciate our cooperators for allowing
452 us to collect cattle manure, swine effluent and municipal biosolids from their facilities. In
453 addition, a special thanks to Cindy Smith and Renotta Smith, for technical assistance with the
454 research.
455

REFERENCES

- Advisory Committee on the Microbiological Safety of Food (ACMSF). The Isolation Of *Campylobacter* Spp From Food And Environmental Samples. Advisory Committee On The Microbiological Safety Of Food, 2010.
- Abdelwhab EM, Grund C, Aly MM, Beer M, Harder TC, Hafez HM. Multiple dose vaccination with heterologous H5N2 vaccine: immune response and protection against variant clade 2.2.1 highly pathogenic avian influenza H5N1 in broiler breeder chickens. *Vaccine* 2011; 29: 6219-25.
- Bean CL, Hansen JJ, Margolin AB, Balkin H, Batzer G, Widmer G. Class B alkaline stabilization to achieve pathogen inactivation. *Int J Environ Res Public Health* 2007; 4: 53-60.
- Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P, et al. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ Microbiol* 2010; 12: 2385-97.
- Bhattarai R, Kalita PK, Yatsu S, Howard HR, Svendsen NG. Evaluation of compost blankets for erosion control from disturbed lands. *J Environ Manage* 2011; 92: 803-12.
- Brooks JP, McLaughlin MR, Gerba CP, Pepper IL. Land application of manure and Class B biosolids: an occupational and public quantitative microbial risk assessment. *J Environ Qual* 2012; 41: 2009-23.
- Brooks JP, Tanner BD, Josephson KL, Gerba CP, Haas CN, Pepper IL. A national study on the residential impact of biological aerosols from the land application of biosolids. *J Appl Microbiol* 2005; 99: 310-22.
- Colinon C, Deredjian A, Hien E, Brothier E, Bouziri L, Cournoyer B, et al. Detection and enumeration of *Pseudomonas aeruginosa* in soil and manure assessed by an *ecfX* qPCR assay. *J Appl Microbiol* 2013; 114: 1734-49.
- D'Souza DH, Su X. Efficacy of chemical treatments against murine norovirus, feline calicivirus, and MS2 bacteriophage. *Foodborne Pathog Dis* 2010; 7: 319-26.
- Choffnes ER, Relman DA, Olsen L, Hutton R, Rapporteurs AM. IMPROVING FOOD SAFETY THROUGH A ONE HEALTH APPROACH. National Academy of Sciences, Washington, D.C., 2012.
- Fakruddin M, Mannan KS, Andrews S. Viable but Nonculturable Bacteria: Food Safety and Public Health Perspective. *ISRN Microbiol* 2013; 2013: 703813.
- Freestone P, Lyte M. Stress and microbial endocrinology: prospects for ruminant nutrition. *Animal* 2010; 4: 1248-57.
- Gerba CP, Smith JE, Jr. Sources of pathogenic microorganisms and their fate during land application of wastes. *J Environ Qual* 2005; 34: 42-8.
- Haas CN, Rose JB, Gerba CP. Quantitative Microbial Risk Assessment. New York, NY: John Wiley & Sons, 1999.
- Hansen JJ, Warden PS, Margolin AB. Inactivation of adenovirus type 5, rotavirus Wa and male specific coliphage (MS2) in biosolids by lime stabilization. *Int J Environ Res Public Health* 2007; 4: 61-7.
- Holley RA, Arrus KM, Ominski KH, Tenuta M, Blank G. *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. *J Environ Qual* 2006; 35: 1170-80.

Hutchison ML, Walters LD, Avery SM, Munro F, Moore A. Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. *Appl Environ Microbiol* 2005; 71: 1231-6.

Hutchison ML, Walters LD, Avery SM, Synge BA, Moore A. Levels of zoonotic agents in British livestock manures. *Lett Appl Microbiol* 2004; 39: 207-14.

Inglis GD, McAllister TA, Larney FJ, Topp E. Prolonged survival of *Campylobacter* species in bovine manure compost. *Appl Environ Microbiol* 2010; 76: 1110-9.

Islam M, Morgan J, Doyle MP, Phatak SC, Millner P, Jiang X. Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Appl Environ Microbiol* 2004; 70: 2497-502.

Katz BD, Margolin AB. Inactivation of hepatitis A HM-175/18f, reovirus T1 Lang and MS2 during alkaline stabilization of human biosolids. *J Appl Microbiol* 2007; 103: 2225-33.

Kim SY, Ko G. Using propidium monoazide to distinguish between viable and nonviable bacteria, MS2 and murine norovirus. *Lett Appl Microbiol* 2012; 55: 182-8.

Klein M, Brown L, Ashbolt NJ, Stuetz RM, Roser DJ. Inactivation of indicators and pathogens in cattle feedlot manures and compost as determined by molecular and culture assays. *FEMS Microbiol Ecol* 2011; 77: 200-10.

Koonjul PK, Brandt WF, Farrant JM, Lindsey GG. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nucleic Acids Res* 1999; 27: 915-6.

Koseki S, Nakamura N, Shiina T. Comparison of desiccation tolerance among *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella enterica*, and *Cronobacter sakazakii* in powdered infant formula. *J Food Prot* 2015; 78: 104-10.

Lang NL, Bellett-Travers MD, Smith SR. Field investigations on the survival of *Escherichia coli* and presence of other enteric micro-organisms in biosolids-amended agricultural soil. *J Appl Microbiol* 2007; 103: 1868-82.

Lang NL, Smith SR. Influence of soil type, moisture content and biosolids application on the fate of *Escherichia coli* in agricultural soil under controlled laboratory conditions. *J Appl Microbiol* 2007; 103: 2122-31.

Linke K, Ruckerl I, Brugger K, Karpiskova, R, Walland J, Muri-Klinger S, Tichy A., Wagner M, and Stessl B. Reservoirs of *Listeria* species in three environmental ecosystems. *Appl Environ Microbiol* 2014; 80: 5583-5592.

Locatelli A, Spor A, Jolivet C, Piveteau P, Hartmann A. Biotic and abiotic soil properties influence survival of *Listeria monocytogenes* in soil. *PLoS One* 2013; 8: e75969.

McLaughlin MR, Brooks JP, Adeli A. Characterization of selected nutrients and bacteria from anaerobic swine manure lagoons on sow, nursery, and finisher farms in the Mid-South USA. *J Environ Qual* 2009; 38: 2422-30.

Moore JC, Gross EA. Update on emerging infections: news from the Centers for Disease Control and Prevention. Surveillance for foodborne disease outbreaks-United States, 2006. *Ann Emerg Med* 2010; 55: 47-9.

Olsen SJ, MacKinnon LC, Goulding JS, Bean NH, Slutsker L. Surveillance for foodborne-disease outbreaks--United States, 1993-1997. *MMWR CDC Surveill Summ* 2000; 49: 1-62.

Opara OO, Carr LE, Russek-Cohen E, Tate CR, Mallinson ET, Miller RG, et al. Correlation of water activity and other environmental conditions with repeated detection of *Salmonella* contamination on poultry farms. *Avian Dis* 1992; 36: 664-71.

Rogers SW, Donnelly M, Peed L, Kelty CA, Mondal S, Zhong Z, et al. Decay of bacterial pathogens, fecal indicators, and real-time quantitative PCR genetic markers in manure-amended soils. *Appl Environ Microbiol* 2011; 77: 4839-48.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis* 2011; 17: 7-15.

Sobsey MD, Dean CH, Knuckles ME, Wagner RA. Interactions and survival of enteric viruses in soil materials. *Appl Environ Microbiol* 1980; 40: 92-101.

Straub TM, Pepper IL, Gerba CP. Persistence of viruses in desert soils amended with anaerobically digested sewage sludge. *Appl Environ Microbiol* 1992; 58: 636-41.

Toth JD, Aceto HW, Rankin SC, Dou Z. Survival characteristics of *Salmonella enterica* serovar Newport in the dairy farm environment. *J Dairy Sci* 2011; 94: 5238-46.

USDA-AMS. National Organic Program. 7 CFR Part 205 USDA-AMS, Washington, D.C., 2000.

USDA-NASS. Census of Agriculture. 2012, 2011.

USEPA. A guide to the biosolids risk assessments for the EPA Part 503 Rule. 1995.

Viau E, Bibby K, Paez-Rubio T, Peccia J. Toward a consensus view on the infectious risks associated with land application of sewage sludge. *Environ Sci Technol* 2011; 45: 5459-69.

Volkova VV, Wills RW, Hubbard SA, Magee DL, Byrd JA, Bailey RH. Risk factors associated with detection of *Salmonella* in broiler litter at the time of new flock placement. *Zoonoses Public Health* 2011; 58: 158-68.

Wei J, Jin Y, Sims T, Kniel KE. Manure- and biosolids-resident murine norovirus 1 attachment to and internalization by Romaine lettuce. *Appl Environ Microbiol* 2010; 76: 578-83.

Wong K, Onan BM, Xagorarakis I. Quantification of enteric viruses, pathogen indicators, and *Salmonella* bacteria in class B anaerobically digested biosolids by culture and molecular methods. *Appl Environ Microbiol* 2010; 76: 6441-8.

Yang Y, Griffiths MW. Comparative persistence of subgroups of F-specific RNA phages in river water. *Appl Environ Microbiol* 2013; 79: 4564-7.

You Y, Rankin SC, Aceto HW, Benson CE, Toth JD, Dou Z. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Appl Environ Microbiol* 2006; 72: 5777-83.

Table 1

Table 1 Recently published decay rates (k-rates) involving common indicator bacteria and phage as well as select pathogens under specific moisture, waste, soil, and management practices.

Pathogen	k-rate (log d ⁻¹)	Waste	Moisture	Soil	Management	Temp (°C)	Reference
Coliforms	-0.1	cattle manure (compost)	51-57%	na	na	25-34	Klein et al. 2011
	-0.14	cattle manure (stockpile)	51-57%	na	na	33-39	Klein et al. 2011
<i>E. coli</i>	-0.05	biosolids (DMAD moist)	32%	silty clay	incorporated	15	Lang et al. 2007
	-0.05	biosolids (DMAD moist)	21.30%	sandy loam	incorporated	15	Lang et al. 2007
	-0.005	biosolids (DMAD air-dried)	8.60%	silty clay	incorporated	15	Lang et al. 2007
	-0.01	biosolids (DMAD air-dried)	6.80%	sandy loam	incorporated	15	Lang et al. 2007
	-0.041	cattle manure (compost)	51-57%	na	na	25-34	Klein et al. 2011
	-0.082	cattle manure (compost)	51-57%	na	na	25-34	Klein et al. 2011
<i>E. coli</i> (EPA-EC23S)	-0.26	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.16	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.026	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.041	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.4	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.28	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.33	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.37	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.629	biosolids	30%	clay loam	incorporated	27	Straub et al. 1992
MS2 phage	-0.652	biosolids	30%	sandy	incorporated	27	Straub et al. 1992
	-0.84	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
<i>S. enterica</i> serovar Typhimurium/pDs	-0.33	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.81	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.76	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
<i>Salmonella</i> (6 strains)	-0.0908	swine lagoon effluent	FC 80%	sandy	surface	25-4	Holley et al. 2006
	-0.0682	swine lagoon effluent	FC 80%	sandy	incorporated	25-4	Holley et al. 2006
	-0.0601	swine lagoon effluent	FC 80%	clay loam	surface	25-4	Holley et al. 2006
	-0.0584	swine lagoon effluent	FC 80%	clay loam	incorporated	25-4	Holley et al. 2006
	-0.1373	swine lagoon effluent	FC 60%	sandy	surface	25-4	Holley et al. 2006
	-0.1358	swine lagoon effluent	FC 60%	sandy	incorporated	25-4	Holley et al. 2006
	-0.1109	swine lagoon effluent	FC 60%	clay	surface	25-4	Holley et al. 2006
	-0.1105	swine lagoon effluent	FC 60%	clay	incorporated	25-4	Holley et al. 2006
	-0.0697	cattle manure	FC 90%	sandy silt loam	incorporated	24	Toth et al. 2011
<i>Salmonella</i> Newport <i>Salmonella</i> spp.	-0.11	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011

(ttrRSBCA)							
-0.072	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011	
-0.34	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011	
-0.26	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011	

Table 1 continued: Published decay rates

Pathogen	k-rate (log d ⁻¹)	Waste	Moisture	Soil	Management	Temp (°C)	Reference
Coliforms	-0.1	cattle manure (compost)	51-57%	na	na	25-34	Klein et al. 2011
	-0.14	cattle manure (stockpile)	51-57%	na	na	33-39	Klein et al. 2011
<i>E. coli</i>	-0.05	biosolids (DMAD moist)	32%	silty clay	incorporated	15	Lang et al. 2007
	-0.05	biosolids (DMAD moist)	21.30%	sandy loam	incorporated	15	Lang et al. 2007
	-0.005	biosolids (DMAD air-dried)	8.60%	silty clay	incorporated	15	Lang et al. 2007
	-0.01	biosolids (DMAD air-dried)	6.80%	sandy loam	incorporated	15	Lang et al. 2007
	-0.041	cattle manure (compost)	51-57%	na	na	25-34	Klein et al. 2011
	-0.082	cattle manure (compost)	51-57%	na	na	25-34	Klein et al. 2011
<i>E. coli</i> (EPA-EC23S)	-0.26	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.16	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.026	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.041	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.4	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.28	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.33	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.37	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
MS2 phage	-0.629	biosolids	30%	clay loam	incorporated	27	Straub et al. 1992
	-0.652	biosolids	30%	sandy	incorporated	27	Straub et al. 1992
<i>S. enterica</i> serovar Typhimurium/pDs	-0.84	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.33	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.81	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.76	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
<i>Salmonella</i> (6 strains)	-0.0908	swine lagoon effluent	FC 80%	sandy	surface	25-4	Holley et al. 2006
	-0.0682	swine lagoon effluent	FC 80%	sandy	incorporated	25-4	Holley et al. 2006
	-0.0601	swine lagoon effluent	FC 80%	clay loam	surface	25-4	Holley et al. 2006
	-0.0584	swine lagoon effluent	FC 80%	clay loam	incorporated	25-4	Holley et al. 2006
	-0.1373	swine lagoon effluent	FC 60%	sandy	surface	25-4	Holley et al. 2006
	-0.1358	swine lagoon effluent	FC 60%	sandy	incorporated	25-4	Holley et al. 2006
	-0.1109	swine lagoon effluent	FC 60%	clay	surface	25-4	Holley et al. 2006
	-0.1105	swine lagoon effluent	FC 60%	clay	incorporated	25-4	Holley et al. 2006
<i>Salmonella</i> Newport	-0.0697	cattle manure	FC 90%	sandy silt loam	incorporated	24	Toth et al. 2011

<i>Salmonella</i> spp. (ttrRSBCA)	-0.11	swine lagoon effluent	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.072	swine lagoon effluent	FC 80%	clay loam	incorporated	25	Rogers et al. 2011
	-0.34	cattle manure	FC 60%	clay loam	incorporated	25	Rogers et al. 2011
	-0.26	cattle manure	FC 80%	clay loam	incorporated	25	Rogers et al. 2011

Table 2

Table 2 Quantitative PCR primer sequences associated with each bacterial targets

Target	Locus	Primer sequence (5' to 3')	Primer size	References
<i>Campylobacter jejuni</i>	campF2	CACGTGCTACAATGGCATAT	109 bp	(Wong et al., 2010)
	campR2	GGCTTCATGCTCTCGAGTT		
<i>E. coli</i> O157	vt2 (stx2) - f	TGTTGGCTGGGTTCGTTAATACGG	121 bp	(Lu et al., 2003)
	vt2 (stx2) - r	TCCGTTGTCATGGAAACCGTTGTC		
<i>Listeria monocytogenes</i>	hlyQF	CATGGCACCCAGCATCT	64 bp	(Meng et al., 1997)
	hlyQR	ATCCGCGTGTTTCTTTTCGA		
<i>Salmonella enterica</i>	spaQvicF	GCAATTACAGGAACAGACGCT	100 bp	(Scallan et al., 2011a)
	spaQvicR	CCTGACGCCCCGTAAGAGA		

Table 3

Table 3: Significance (p values) of main effects and interactions by bacterium in mixed model analysis of inactivation rates, with mean inactivation rates ($\log d^{-1}$) for each variable averaged across all experiments, below the main effect.

Effect	<i>C. jejuni</i>		<i>C. perfringens</i> ¹	<i>E. coli</i> O157		<i>L. monocytogenes</i>		<i>S. enterica</i>	
	Cult-Dep ²	Cult-Ind	Cult-Dep	Cult-Dep	Cult-Ind	Cult-Dep	Cult-Ind	Cult-Dep	Cult-Ind
Waste-residual	0.0439	<.0001	0.0012	<.0001	0.0001	<.0001	0.0259	<.0001	0.0059
<u>Bio</u> ³	<u>-0.6732</u>	<u>-0.03444</u>	<u>-0.0076</u>	<u>-0.0809</u>	<u>-0.1008</u>	<u>-0.2584</u>	<u>-0.0442</u>	<u>-0.0757</u>	<u>-0.07788</u>
<u>Cat</u>	<u>-0.6887</u>	<u>-0.03844</u>	<u>-0.0477</u>	<u>-0.196</u>	<u>-0.0729</u>	<u>-0.1835</u>	<u>-0.0389</u>	<u>-0.0369</u>	<u>-0.0626</u>
<u>Swi</u>	<u>-0.681</u>	<u>-0.02429</u>	<u>-0.0059</u>	<u>-0.1282</u>	<u>-0.0549</u>	<u>-0.5321</u>	<u>-0.0469</u>	<u>-0.1583</u>	<u>-0.0651</u>
<u>PBS</u>	<u>-0.6431</u>	<u>-0.02084</u>	<u>-0.0084</u>	<u>-0.1208</u>	<u>-0.0559</u>	<u>-0.3837</u>	<u>-0.0322</u>	<u>-0.1362</u>	<u>-0.0481</u>
Soil	<.0001	<.0001	0.0369	0.3619	0.0006	<.0001	0.9493	0.0646	0.3514
<u>San</u> ⁴	<u>-0.6279</u>	<u>-0.0226</u>	<u>-0.0089</u>	<u>-0.149</u>	<u>-0.0579</u>	<u>-0.5321</u>	<u>-0.0407</u>	<u>-0.1142</u>	<u>-0.0608</u>
<u>Cla</u>	<u>-0.7152</u>	<u>-0.0364</u>	<u>-0.0259</u>	<u>-0.1139</u>	<u>-0.0844</u>	<u>-0.1298</u>	<u>-0.0405</u>	<u>-0.0894</u>	<u>-0.066</u>
Management	0.7288	0.0216	0.0045	<.0001	0.577	0.1773	<.0001	0.0049	<.0001
<u>Inc</u> ⁵	<u>-0.6695</u>	<u>-0.0313</u>	<u>-0.0054</u>	<u>-0.1823</u>	<u>-0.0731</u>	<u>-0.3589</u>	<u>-0.0321</u>	<u>-0.0822</u>	<u>-0.0808</u>
<u>Sur</u>	<u>-0.6735</u>	<u>-0.0278</u>	<u>-0.0294</u>	<u>-0.0806</u>	<u>-0.0692</u>	<u>-0.32</u>	<u>-0.0491</u>	<u>-0.1214</u>	<u>-0.046</u>
Residual*Soil	0.0003	0.0004	0.0002	<.0001	0.0185	<.0001	0.0562	0.575	0.0012
Residual*Manage	0.0271	<.0001	0.0009	<.0001	0.0002	0.001	0.0427	0.0077	0.2404
Residual*Soil*Manage	<.0001	<.0001	0.0003	<.0001	0.0027	0.0002	<.0001	0.194	0.5988

¹qPCR was not performed for *C. perfringens* detection.

²Cult-Dep = cultivation-dependent; Cult-Ind = cultivation independent

³Mean inactivation, k-rates, ($\log d^{-1}$). Bio = biosolids, Cat = cattle manure, Swi = swine effluent, and PBS = PBS control.

⁴Mean inactivation, k-rates, ($\log d^{-1}$). San = sandy loam soil, Cla = clay loam soil.

⁵Mean inactivation, k-rates, ($\log d^{-1}$). Inc = incorporated, Sur = surface-applied.

Table 4: Inactivation rates (k-rates) derived via cultivation-dependent and -independent methods

		Decay Rates ($\log_{10}(N_t/N_0)/t$)													
Soil Type and Management Practice	Residual Applied	<i>C. jejuni</i> ¹		<i>C. perfringens</i>		<i>E. coli</i> O157 ¹		<i>L. monocytogenes</i> ¹		<i>S. enterica</i> ¹		MS2 phage ¹		ØX174 phage ¹	
		Cult-Dep ¹²	Cult-Ind	Cult-Dep	Cult-Ind	Cult-Dep	Cult-Ind	Cult-Dep	Cult-Ind	Cult-Dep	Cult-Ind	Cult-Dep	Cult-Ind	Cult-Dep	Cult-Ind
Sandy loam															
Surface-applied	Biosolids	-0.5857 ²	-0.0287 ⁶	-0.0245 ⁸	ND	<u>-0.1261</u> ⁵	-0.1512 ⁵	-0.2522 ⁵	-0.0459 ⁶	<u>-0.0760</u> ⁶	-0.0767 ⁵	-0.0827 ⁶	ND	-0.1219 ⁵	ND
	Cattle	-0.5666 ²	-0.0153 ⁶	-0.0046 ¹⁰	ND	<u>-0.0216</u> ¹⁰	-0.026 ⁶	-0.4188 ³	-0.035 ⁶	<u>-0.0225</u> ¹¹	-0.026 ⁶	-0.2116 ⁴	ND	-0.0709 ⁶	ND
	Swine	-0.6032 ²	-0.004 ⁶	-0.009 ¹¹	ND	-0.2266 ⁴	-0.0311 ⁶	-1.049 ²	-0.0362 ⁶	-0.1941 ⁵	-0.0311 ⁶	-0.1803 ³	ND	-0.1381 ³	ND
	PBS	-0.5329 ²	-0.0001 ⁶	-0.0121 ⁹	ND	-0.1737 ³	-0.0371 ⁶	-0.4949 ³	-0.0472 ⁶	-0.1855 ⁵	-0.0371 ⁶	-0.15 ³	ND	-0.2829 ³	ND
Incorporated	Biosolids	-0.6525 ²	-0.0299 ⁶	-0.0012 ⁸	ND	-0.1148 ⁵	-0.0605 ⁵	-0.4859 ³	-0.0278 ⁶	<u>-0.0968</u> ⁵	-0.1115 ⁵	-0.0629 ⁶	ND	-0.262 ⁴	ND
	Cattle	-0.6758 ²	-0.0354 ⁶	-0.0036 ¹¹	ND	<u>-0.0544</u> ⁷	-0.08 ⁵	-0.0641 ⁷	-0.0537 ⁶	<u>-0.0475</u> ⁸	-0.0829 ⁵	-0.2638 ⁴	ND	-0.0719 ⁵	ND
	Swine	-0.7696 ²	-0.0338 ⁶	-0.0089 ¹¹	ND	<u>-0.24</u> ⁴	-0.0256 ⁵	-0.8402 ²	-0.055 ⁶	-0.161 ³	-0.0644 ⁵	-0.0982 ⁴	ND	-0.1806 ⁵	ND
	PBS	-0.6365 ²	-0.0033 ⁶	-0.0075 ¹¹	ND	-0.2471 ⁴	-0.0511 ⁵	-0.7872 ²	-0.0248 ⁶	-0.1302 ⁵	-0.0567 ⁵	-0.182 ³	ND	-0.6566 ²	ND
Clay loam															
Surface-applied	Biosolids	-0.802 ²	-0.0600 ⁶	-0.0045 ¹¹	ND	<u>-0.026</u> ⁷	-0.1 ⁵	-0.1157 ⁵	-0.0698 ⁶	-0.0655 ⁷	-0.0488 ⁵	-0.1029 ⁴	ND	-0.1617 ⁴	ND
	Cattle	-0.8352 ²	-0.0359 ⁶	-0.1758 ³	ND	<u>-0.0308</u> ⁷	-0.0997 ⁵	-0.0814 ⁵	-0.0427 ⁶	<u>-0.0422</u> ⁸	-0.0456 ⁶	-0.8324 ²	ND	-0.218 ³	ND
	Swine	-0.7656 ²	-0.0407 ⁶	0.0053 ¹¹	ND	<u>-0.0278</u> ⁷	-0.08 ⁵	<u>-0.0518</u> ⁵	-0.0812 ⁵	-0.2079 ⁵	-0.0767 ⁵	-0.4512 ²	ND	-0.1039 ⁴	ND
	PBS	-0.6967 ²	-0.0371 ⁶	-0.01 ³	ND	<u>-0.0237</u> ⁷	-0.0282 ⁵	-0.0958 ⁵	-0.0345 ⁵	-0.1775 ⁵	-0.026 ⁵	-0.199 ³	ND	-0.038 ⁶	ND
Incorporated	Biosolids	-0.6525 ²	-0.0191 ³	-0.0003 ¹¹	ND	<u>-0.0568</u> ⁷	-0.0918 ⁵	-0.1798 ⁵	-0.0334 ⁶	<u>-0.0648</u> ⁷	-0.0705 ⁵	-0.1089 ⁵	ND	-0.1954 ⁴	ND
	Cattle	-0.6772 ²	-0.0671 ⁵	-0.007 ¹¹	ND	-0.6772 ⁷	-0.0861 ⁵	-0.1698 ⁵	-0.0242 ⁶	<u>-0.0354</u> ⁹	-0.0957 ⁵	-0.6691 ²	ND	-0.1925 ⁴	ND
	Swine	-0.5856 ²	-0.0186 ⁶	-0.011 ¹¹	ND	<u>-0.0304</u> ⁷	-0.0823 ⁵	-0.1873 ⁵	-0.0154 ⁶	<u>-0.0702</u> ⁷	-0.0881 ⁵	-0.5472 ²	ND	-0.1326 ⁴	ND
	PBS	-0.7064 ²	-0.0130 ⁶	-0.0041 ¹¹	ND	<u>-0.0385</u> ⁷	-0.1069 ⁵	-0.157 ⁵	-0.0022 ⁶	<u>-0.0515</u> ⁸	-0.0725 ⁵	-0.5107 ²	ND	-0.0507 ⁶	ND

¹Inactivation rates derived by cultural (left) and qPCR (right) methods using CFU or GU g⁻¹ data from 0 to final time point with detection limits, unless specified. The underlined rates are cultivated data which indicate bacterial inactivation rates that are slower than comparable cultivation-independent rates. ² Detection limits reached at day 7; ³ at day 14; ⁴ at day 21; ⁵ at day 28; ⁶ at day 60; ⁷ at day 90; ⁸ at day 120; ⁹ at day 150; ¹⁰ at day 180; ¹¹ at day 210. ¹²Cult-Dep = cultivation-dependent; Cult-Ind = cultivation independent

Table 5

Table 5 Ratio of cultivation-dependent (CFU g⁻¹) to -independent (GU g⁻¹) for *E. coli* O157, *L. monocytogenes*, and *S. enterica*

Soil Type and Management Practice	Residual Applied	<i>E. coli</i> O157				<i>L. monocytogenes</i>				<i>S. enterica</i>			
		0	7	14	30	0	7	14	30	0	7	14	30
Sandy loam													
Surface- applied	Biosolids	1.4407	1.5446	1.3239	0.6439	1.5471	0.6202	0.9200	<0.0001	0.8486	1.0162	0.7372	0.6866
	Swine	1.5980	1.4276	1.3353	<0.0001	1.7949	<0.0001	<0.0001	<0.0001	0.9484	1.1092	1.0678	0.4801
	Cattle	1.4167	1.1838	1.4241	1.6726	1.7774	0.8967	0.4450	<0.0001	0.8284	0.9896	1.1599	1.1078
	PBS	1.6437	1.4395	1.1197	<0.0001	1.5563	1.0589	0.0000	<0.0001	0.9809	1.1438	0.9900	0.5394
Incorporated	Biosolids	1.2140	1.1558	0.7111	0.8577	2.2659	0.4214	0.1981	<0.0001	0.7563	0.9206	0.5677	0.6732
	Swine	1.0828	0.9680	0.6769	<0.0001	1.4914	0.3533	<0.0001	<0.0001	0.6662	0.9210	0.3901	0.0000
	Cattle	1.0474	1.2504	1.2432	1.4079	1.8213	1.7863	1.7615	1.3683	0.8798	1.1260	1.2487	1.2509
	PBS	1.1013	0.6739	0.3199	<0.0001	1.6433	0.4491	<0.0001	<0.0001	0.8382	0.6440	0.4435	0.3064
Clay loam													
Surface- applied	Biosolids	1.2341	0.9652	0.9497	1.6771	1.0447	1.0308	0.8346	0.5545	1.0403	0.6179	0.6161	0.1387
	Swine	1.2807	1.4663	1.5907	1.4384	0.9409	1.0146	1.1931	1.2475	1.0955	0.8512	0.1721	0.2160
	Cattle	1.2730	1.9237	2.0552	1.7867	1.0720	1.7420	1.0140	1.1671	1.1235	1.1931	1.2030	0.7032
	PBS	1.5719	1.3347	1.5460	1.5120	1.5076	1.2688	1.1566	0.8399	1.2655	1.0730	0.9339	1.6117
Incorporated	Biosolids	1.3251	1.1261	0.9621	1.5435	2.4406	0.6484	0.6392	7.8575	0.9544	1.0246	0.8106	0.6442
	Swine	1.2846	1.2166	1.3751	1.6713	2.6752	1.0846	0.7920	1.0122	1.3074	1.0977	0.9926	1.0407
	Cattle	1.2186	1.2495	1.8742	1.5457	2.4286	1.5004	1.5115	2.7298	1.3351	1.1790	1.3902	2.2471
	PBS	1.0817	1.1264	1.1740	1.2051	2.4230	1.3857	1.0989	1.6692	1.0466	1.2237	1.1336	0.7830

No shading = ratios of cultivation-dependent: -independent are within 1.0 ± 0.25 limits. Dark gray shading indicates cultivation data provided higher bacterial counts than cultivation-independent detection. Light gray shading indicates cultivation-independent data provided higher bacterial counts than cultivation.

Pathogen Decay in Microcosms Representing Land Application

Land application of "Organic" Wastes: Class B biosolids, Swine effluent, & Cattle manure