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Decay of enteric microorganisms in biosolids-amended soil under wheat (*Triticum aestivum*) cultivation

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Running title: Pathogen decay in biosolids-amended soil

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ABSTRACT

There is a growing need for better assessment of health risks associated with land-applied biosolids. This study investigated *in-situ* decay of seeded human adenovirus (HAdV), *Salmonella enterica*, *Escherichia coli*, and bacteriophage (MS2) in biosolids-amended soil under wheat cultivation. The biosolids seeded with microorganisms were placed in decay chambers which were then placed in the topsoil (10 cm depth) at three different sites. Sites were selected in arid wheat-growing regions of Australia with loamy-sand soil type (Western Australia) and sandy soil (South Australia). Seeded *E. coli* and *S. enterica* had a relatively short decay time ($T_{90} = 4-56$ days) in biosolids-amended soil compared to un-amended soil ($T_{90} = 8-83$ days). The decreasing soil moisture over the wheat-growing season significantly ($P<0.05$) influenced survival time of both bacteria and MS2 at Western Australia (Moora) and South Australia (Mt Compass) sites, particularly in the un-amended soils. Increasing soil temperature also significantly ($P<0.05$) influenced the decay of MS2 at these sites. In this study, no noticeable decline in HAdV numbers (PCR detectable units) was observed in both biosolids-amended and the un-amended soils at all three sites. The HAdV decay time ($T_{90} = >180$ days) in biosolids-amended and un-amended soils was significantly higher than MS2 ($T_{90} = 22-108$ days). The results of this study suggest that adenovirus could survive for a longer period of time (>180 days) during the winter in biosolids-amended soil. The stability of adenovirus suggests that consideration towards biosolids amendment frequency, time, rates and appropriate withholding periods are necessary for risk mitigation.

Keywords: Human adenovirus, *Salmonella enterica*, MS2, pathogen decay, biosolids-amended soil.
1. Introduction

Recycling of biosolids as a low-grade fertilizer and soil amendment, to improve the chemical and physical properties of soil, is increasingly favored over un-sustainable landfill disposal options worldwide. In Australia, over 330,000 tonnes per year of dry solids are produced and are predominantly used for agricultural purposes (ANZBP 2013). There is a recognized pathway for transfer of enteric pathogens such as hepatitis A virus, adenovirus, *S. enterica*, *Campylobacter* spp., *E. coli* O157:H7, *Cryptosporidium* and *Giardia*, to humans from biosolids-amended soil, through contamination of water and food chain (Gerba and Smith 2005, Pepper et al. 2006, Sidhu and Toze 2009).

Enteric pathogens may accumulate in the soil under favorable conditions depending on the frequency and rate of biosolids application (Gerba and Smith, 2005; Viau and Peccia, 2009). Therefore, land application of biosolids is controlled via regulations that set out acceptable levels for pathogens and heavy metals in biosolids prior to the release onto land, along with other management practices to reduce human health risks and environmental contamination (DEC 2012; EU 2003; US-EPA 2003). Under the United States Environmental Protection Agency (US-EPA) regulations, biosolids which meet Class A or B classifications at the wastewater treatment plant are allowed for un-restricted and restricted land application, respectively. Similarly, under the European Union and Australian guidelines, treatment-based standards and monitoring for fecal indicators and pathogens in the finished product are required for risk mitigation (DEC 2012; EU 2002, US-EPA 2003).

Fecal coliform numbers in the stabilized biosolids can be high, up to $10^5$ g$^{-1}$ dry weight (Davies et al. 1999, LeClerc et al. 2001, Vasseur et al. 1996, Zaleski et al. 2005). Furthermore, a significant number of enteric viruses such as adenovirus, polyomavirus ($10^4$ g$^{-1}$
along with bacterial and protozoan pathogens may also be present in biosolids (Bofill-Mas et al. 2006, Chauret et al. 1999, Sidhu and Toze 2009). In the USA, Class B biosolids containing $<2 \times 10^6$ MPN g$^{-1}$ fecal coliforms are commonly applied to agricultural land (Pepper et al. 2006). Enteric pathogens such as *E. coli*, *S. enterica*, *Shigella*, *Campylobacter*, *Cryptosporidium*, *Giardia*, norovirus and enteroviruses may be present in class biosolids (NIOSH 2002). Surviving enteric pathogens can subsequently become incorporated into soil during the land-application of biosolids, potentially leading to contamination of food crops (Bonjoch et al. 2009, Gantzer et al. 2001, Sidhu and Toze 2009). Surface run-off from biosolids-amended soil during storm events could also lead to the contamination of surface water, groundwater, and food chain (Esseilli et al. 2012; Lapen et al. 2008). Limiting access or withholding period to biosolids amended soil is often used as a management practice to limit the risk of transmission of diseases to humans and environmental contamination. An improved understanding of the persistence of enteric pathogens in biosolids-amended soils is essential to provide a sound scientific basis for risk management practices such as amendment rates, frequency of application and duration of withholding periods.

Natural decay of enteric pathogens occurs in biosolids-amended soil (Lang et al. 2007, Zaleski et al. 2005). A number of environmental and soil-specific factors such as temperature, UV radiation, soil moisture, pH, nutrients and antagonistic activity of soil autochthonous microorganisms are known to influence decay of pathogens (Hussong et al. 1985, Sidhu et al. 2001). A number of studies have explored the fate of enteric pathogens in biosolids-amended soils (Gibbs et al. 1997, Horswell et al. 2007, Horswell et al. 2010, Lang et al. 2007). However, there is a wide variation in the reported survival times of enteric microorganisms in biosolids-amended soil. General survival times for bacteria in soil are reported to be 2 to 12 months, viruses 3 to 6 months, protozoa 2 to 10 days and helminthes 2 to 7 years (Gerba and
The survival potential of pathogens under different climatic conditions and biosolids application rates is difficult to accurately predict due to limited understanding of the decay mechanisms and complex interactions between environmental and soil specific factors which influence decay of enteric pathogens.

An improved understanding of the factors influencing persistence of enteric pathogens in biosolids-amended soil could assist in better risk assessment. For an improved risk assessment, quantitative data on pathogens numbers in wastewater, removal during wastewater treatment and the fate of pathogens in land-applied biosolids is required. However, limited experimentally derived (in-situ) quantitative data is available on the decay of enteric pathogens especially enteric viruses in biosolids-amended soils.

Monitoring the fate of a wide range of pathogenic microorganisms in biosolids-amended soil is impractical due to methodological difficulties and most of them are intermittently present and often in low numbers. A more sensible approach could be to assess the fate of microorganisms which are expected to behave similar to pathogens of concern in biosolids amended soil. Monitoring for *Salmonella* enterica in biosolids is required under US EPA Part 503 rule to determine the bio-safety of finished product. Bacteriophage such as MS2 are suggested as suitable indicators of enteric viruses in wastewater matrix due to their similar structure, morphology and size (Sidhu and Toze 2009). Adenovirus are present in wastewater in high numbers ranging from $10^3$ to $10^6 \text{ L}^{-1}$, are thermostable and resistant to UV radiation (Bofill-Mass et. al. 2006). In the present study, the survival potential of representative above mentioned pathogens and indicator microorganisms were assessed in agricultural land amended with biosolids during the cultivation of wheat. Specific objectives of the study were: (1) to examine the decay times of human adenovirus, *S. enterica*...
(pathogenic bacteria), *E. coli* (indicator bacteria), and bacteriophage MS2 (surrogate virus) in biosolids-amended agricultural soil; (2) to determine any association of microorganism decay with changes in soil temperature and soil moisture in the field.

2. Materials and methods

2.1 Site description

Three field sites were selected in dry temperate cropping regions of Australia to determine the decay times of the selected pathogens. Sites A (30° 50’24.07”S, 116° 05’18.37”E) and B (30° 50’9.31”S, 116° 05’44.53”E) were located at Moora in Western Australia (WA) and Site C (35° 21’39.68”S, 138° 32’47.67”E) was located at Mt Compass in South Australia (SA). The field experiments were conducted during the wheat growing season (May to December). Moora is 175 km north-east of Perth, WA, with an annual average rainfall of 460 mm. Topography was undulating with medium slope and soil type was a loamy-sand. Mt Compass is 69 km south of Adelaide, SA, with an annual average rainfall of 700 mm. Topography was undulating with gentle slope and soil type was a sandy soil. Soil and biosolids characteristics for each site are presented in Table 1.

2.2 Plot establishment

The decay experiments were undertaken using two treatments: biosolids-amended soil (treatment) and un-amended soil (control). Two types of plots (each 10 m$^2$) of either biosolids-amended or un-amended soil were established in triplicate ($n = 6$ plots) using a randomised-block design ($n = 3$ replications for the treatment and for the control). Biosolids application rates are generally determined from the soil nitrogen loading requirements (DEC et al. 2012). However, following initial experimentation (Site A in 2006), it was determined that biosolids application rates needed to be increased in line with those used in the
chambers, thus application rates for subsequent trials at Sites B and C were increased to 25:75 biosolids to soil.

At Sites A and B, anaerobically-digested dewatered biosolids cake from the Beenyup (Perth, WA) wastewater treatment plant (containing *E. coli* at $10^6$ cfu g$^{-1}$) were applied to the treatment plots at 6 dry tonnes ha$^{-1}$ (80% moisture) according to the normal district practice and 19 dry tonnes ha$^{-1}$ (81.5% moisture) higher than the normal district practice, respectively. Biosolids were incorporated into the top 10 cm of soil using a disc-seeder. Wheat (*Triticum aestivum* cv. Calingiri) was sown to all plots at 60 kg ha$^{-1}$, 18 cm width and 2.5 cm depth within 2 hours. At Site C, tertiary treated (stockpiled) biosolids from the Bolivar (Adelaide, SA) sewage treatment plant (containing *E. coli* at $10^2$ g$^{-1}$) were applied to the top 10 cm soil using a rotary hoe to three plots at 28 dry tonnes ha$^{-1}$ (34.3% moisture). Wheat (*Triticum aestivum* cv. Clearfield Janz) was sown at 60 kg ha$^{-1}$ rate, 25 cm width and 2.5 cm depth within 2 hours of application of biosolids. Soils from the treatment and control plots were tested at the beginning of the study for the presence of *E. coli*. In the biosolids-amended soil, namely ‘site samples’, *E. coli* was present as $3 \times 10^6$ colony forming units (cfu) g$^{-1}$ at Site B and $2 \times 10^3$ log cfu g$^{-1}$ at Site C. Background levels of *E. coli* numbers in the un-amended soil were nil at Site B and $3 \times 10^3$ cfu g$^{-1}$ at Site C at the time of study.

In addition, an experiment was conducted at Site B to determine whether the sentinel chambers reasonably predict soil ambient conditions, and whether the decay times of *E. coli* in the chambers were similar to that of *E. coli* in the outside soil. *E. coli* was chosen as the target bacteria as the biosolids-amended soil was found to contain reasonably high ($10^6$ g$^{-1}$) numbers. At each sampling time, in addition to the retrieval of sentinel chambers soil samples (10g) were also collected.
2.3 Microorganisms used in the study

The microorganisms tested were: *Escherichia coli* (ACM 1803), *Salmonella enterica* serovar *Typhimurium* (ATCC 13311), bacteriophage MS2 (ATCC 15597-B1) and human HAdV (serotype 41). *E. coli* and *S. typhimurium* were cultured in 100 mL nutrient broth (Oxoid) in a shaking platform incubator overnight at 37°C. Prior to inoculation, overnight cultures were washed in phosphate buffer as described in Gordon and Toze (2003) and rested in phosphate buffer for 24 hours prior to seeding to acclimatize cultures to low nutrient environment. The final suspension was determined to have a cell count of more than $1 \times 10^6$ cfu mL$^{-1}$. MS2 was cultured in tryptone yeast extract broth (Oxoid) with an *E. coli* HS(pFamp)R (ATCC 700881) in a shaking incubator overnight at 37°C. MS2 was purified using centrifugation at 6000 rpm for 10 minutes to pellet the *E. coli* cell debris followed by filtration through a 0.2 µm membrane. Purified MS2 suspension was stored at 4°C in phage buffer until required. The final suspension was determined to have a final virus count of more than $1 \times 10^7$ plaque forming units (pfu) mL$^{-1}$. HAdV serotype 41 was sourced from the Pathology Centre, WA. The virus was cultured in cell lines (African Green Monkey Kidney cells) and then harvested from the lawns. The stock was stored in a -80°C freezer until required. The number of infective viral particles in the viral suspensions was determined by the Pathology Centre through the MPN method in fresh cell culture lawns.

2.4 Modified sentinel chambers

The survival experiments were carried out using modified sentinel chambers (Jenkins et al. 1999) placed into each of the plots. Briefly, sentinel chambers were constructed using 3.5 mL Microsep™ centrifugal devices with a membrane pore size of 0.2 µm (PALL Life Sciences, New York USA) and Eppendorf Lid-Bac membrane lids (Eppendorf, Germany) with 0.2 µm
pore size membranes to close the top of the columns (Figure 1). The pore size of the Microsep™ membrane and the Eppendorf lids were sufficiently large to allow exchange of gases and moisture without the loss of bacteria and viruses from the sentinel chambers. Initially three chambers were constructed and tested for leakage, after addition of 500 µL of distilled water the lid was firmly pressed in place. Each chamber was then observed for any leakage after inverting 3-4 times. This set up was found to work well without leakage and was subsequently used in this study. There are some limitations of sentinel chambers such as smaller sample size and micro-climatic conditions of moisture and oxygen may be different from the soil ambient conditions. However, their use for pathogen decay studies outweighed these limitations.

Collected soil samples (2 Kg) from each site were sieved (<2mm) and then split into two equal portions. One portion was amended with biosolids to a final ratio of 1:4 (i.e. 20% biosolids to 80% soil) for Site A, then increased to 1:3 (i.e. 25% biosolids to 75% soil) at Sites B and C (thus 25 times the normal rate). Anaerobically-digested dewatered biosolids cake were collected from the Beenyup wastewater treatment plant (Perth, WA) were used in sentinel chambers for Sites A and B, whereas, tertiary treated (stockpiled) biosolids were collected from the Bolivar sewage treatment plant (Adelaide, SA) for use in sentinel chambers for Site C. This higher ratio of biosolids to soil (used inside the chambers) was expected to reflect conditions inside biosolids clumps, where the highest numbers and most persistence of pathogens would be expected to occur. The second portion was left un-amended with no addition of biosolids. Each portion was then inoculated with the washed *E. coli*, *S. enterica* and MS2. HAdV was seeded into separate chambers due to the different sample analysis methods (i.e. PCR). The amended and un-amended soils were then used to fill the sentinel chambers to provide an approximate final number of $10^7$ cfu g$^{-1}$ of *E. coli* and
S. enterica, $10^7$ pfu g$^{-1}$ of MS2, and $10^3$ PCR detectable units (pdu) g$^{-1}$ of HAdV. Sufficient numbers of chambers were prepared so that destructive sampling of individual chambers could occur throughout the experimental duration for both treatments. In addition to the chambers seeded with the microorganisms, sufficient chambers were prepared to test the moisture content at each sample event. Once constructed, the chambers were placed vertically in the topsoil (0-10 cm) of each plot within 6 hours of construction.

Samples (in sentinel chambers) were collected at Time 0 and then every second week until week 4. Sampling frequency was then reduced to monthly intervals up to a maximum of 7 months or until target microorganisms fell below the detection limit. At each sampling event, 3 sentinel chambers were randomly selected from each of the 3 blocks in each treatment. Topsoil from the biosolids-amended plots, namely ‘site samples’, was also taken in triplicate to compare any changes in E. coli numbers in the soil outside the chambers with the decay of the E. coli seeded into the sentinel chambers. All collected chambers and soil samples were transported on ice to the CSIRO Microbiology Laboratory, Floreat, WA and processed within 24 hours of collection. Collected samples from Mt Compass site in South Australia were shipped on ice via overnight courier for processing at Floreat, WA.

At each site, daily air temperature and relative humidity were recorded every 20 mins using a Tinytag Plus 2 (Gemini Data Loggers (UK) Ltd). Soil temperature and soil moisture were recorded at hourly intervals using a Watermark Monitor (Irrometer Company Riverside, CA USA). Rainfall was recorded every 20 mins with a tipping bucket rain gauge (Davis Instruments Corp, Hayward CA USA) and Tinytag data logger (Gemini Data Loggers (UK) Ltd). Soil moisture was determined at each sample event for field soil samples and chambers samples by oven-drying ($105^\circ$C for >24hrs).
2.5 Quantification of E. coli, S. enterica and MS2

All analyses for each microorganism were performed in triplicate. Contents from the sample chambers (approximately 2 to 5 g) were transferred into pre-weighed sterile tubes and net weights were obtained. Sterile P-buffer (pH 7.2) was added (30 mL) and samples were vortexed for 2 mins, left to settle for 10 mins, then vortexed again for 1 min. A 1 mL sample of the resulting supernatant was then collected without disturbing the pellet. A serial tenfold dilution was made in the P-buffer from the supernatant for the detection of E. coli, S. enterica and MS2.

The numbers of E. coli, S. enterica and MS2 at each time interval were detected by direct culture. E. coli and S. enterica was detected by spread plating 100 µL of appropriate dilutions onto the selective agar plates as outlined in Sidhu et al., (2008). E. coli was plated on Chromocult™ coliform agar (Merck) and S. enterica was plated on xylose lysine deoxychlorate agar (BBL). Inoculated plates were incubated overnight at 37ºC and then typical colonies were counted to determine the average number of cfu mL\(^{-1}\) of final suspension. The detection limit with this methodology was 3 cfu mL\(^{-1}\). The cfu per gram were then calculated from the original weight of the samples processed. The quantification of F-specific bacteriophage MS2 was carried out by standard double layer agar method using E. coli HS(pFamp)R (ATCC 700881) as the host bacteria (Havelaar and Hogeboom 1984). Clear plaques were counted to determine the average pfu mL\(^{-1}\) after overnight incubation at 37ºC. Pathogen counts were normalized from the raw data by transformation into log\(_{10}\) cfu/pfu/pdu g\(^{-1}\) using the log conversion formula 

\[
\text{Count} = \log_{10} \left[ ((\text{Count} \times 10^{\text{Dilution}} \times 10^{\text{volume}} \times \text{plated}) \times \text{mL phosphate buffer}) / (\text{soil weight}) \right] + 1.
\]
2.6 Quantification of HAdV

The quantification of HAdV in biosolids was performed by determining the PCR detectable copies of genomic DNA. Soil samples from each chamber (1 g) were weighed out, added to 5 mL Star Buffer (Roche), vortexed for 2 mins and stored overnight at 4ºC. Samples were vortexed again for 2 mins, then centrifuged at 2500 rpm for 10 mins at 4ºC. HAdV DNA was extracted from 200 µL of supernatant using a QIAamp DNA Stool Mini (Qiagen) as per manufacturer instructions and stored at -80ºC prior to analysis. To increase the DNA yields, the initial cell lysis step was carried out at 90°C for 10 min, rather than 70°C for 5 min. All analyses for virus quantification were performed using Real Time PCR in triplicate. Quantitative PCR reactions were performed on a BioRad iQ5, using iQ Supermix kit (Bio-Rad). HAdV were detected using previously published primer set (Heim et al. 2003). Bovine serum albumin (Sigma) was added to each PCR reaction (0.3µg µL⁻¹) to reduce PCR inhibition and improve detection (Kreader 1996). Thermal cycling conditions for the detection of HAdV were as outlined in Sidhu et al. (2010). Briefly, initial incubation at 95ºC for 8 min, then 55 cycles at 95ºC for 30 sec, 55ºC for 20 sec, and then 72ºC for 20 sec. The final cycle had an extension time of 5 min at 72ºC. Standards for qPCR were prepared from the plasmids as outlined in Sidhu et al. (2013).

2.7. PCR limit of detection, reproducibility, and evaluation of PCR inhibition

The qPCR limit of the detection and reproducibility of assay was done as outline (Sidhu et al., 2013). Briefly, known gene copies (i.e., 10⁶ to 10⁶) of adenoviruses seeded in MilliQ water were tested in triplicates with the qPCR. The reproducibility of the qPCR was assessed by determining intra-assay repeatability and inter-assay reproducibility. Extracted DNA from biosolids amended samples (n=8) from all three sites was serially diluted in MilliQ water.
The threshold cycle ($C_T$) values from undiluted and 10-fold diluted samples was compared to
determine the PCR inhibition.

2.8 Adenovirus recovery efficiency.

The effectiveness of the virus extraction procedure from biosolids was determined by seeding
known numbers of adenovirus into biosolids-amended and un-amended soil samples
collected from all sites. Briefly, one g biosolids-amended and un-amended soil samples
(n=6) collected from timescale (T0 to T8) at Sites A (n=3), Site B (n=3), and Site C (n=3)
were spiked with 500 $\mu$L$^{-1}$ of adenovirus ($1 \times 10^7$ PDU mL$^{-1}$). Seeded biosolids samples were
mixed well and DNA was extracted with QIAamp DNA Stool Mini (Qiagen) as mentioned
previously after addition of Star Buffer. In parallel, MilliQ water sample (1mL) was seeded
with same number of adenovirus and DNA was extracted and PDU mL$^{-1}$ were used to
determine recovery efficiency after seeding from the biosolids. All samples were tested
separately for the presence of indigenous adenovirus the detected numbers were subtracted
from the final numbers prior to determining recovery efficiency which was calculated as:

$$\% \text{ Recovery} = \frac{\text{Recovered PDU of adenovirus from the biosolids or soil sample}}{\text{seeded PDU of adenovirus in biosolids or soil sample}} \times 100.$$ 

2.9 Statistical analysis

The microbial numbers in each replicate from each sampling event were converted to $\log_{10}$
values so that a generalized linear statistical model could be applied to the data. The counts
from Time 0 were removed from all field data prior to any statistical analyses as it was
observed that some variability in numbers relating to clumping and un-clumping of
microorganisms may have occurred between Time 0 and day 14 (first sampling event) that
might have had a major effect on the reliability of the statistical analysis. Associated standard
deviations, trendlines and logarithmic transformations were performed in Origin® 6.1 (OriginLab Corporation 1991-2000).

For each individual microorganism, the generalized linear model of analysis of variance (ANOVA) fitting linear and quadratic terms of sampling date was applied to identify significant sources of variation affecting final pathogen counts (log$_{10}$ Count) within individual sites or across sites. These variation sources comprised the fixed effects of site (Site A, Site B, Site C), treatment (biosolids-amended soil vs. un-amended soil), interaction between site and treatment, linear and quadratic terms of a covariate - sampling date (sdate) and the interactions between sampling date with site and treatment, plus nested effect of the chamber within the plot of each treatment of individual site. The least-square effects of all fixed factors were then produced. Overall regression coefficients of linear and quadratic terms of sampling date were used as the indication of average first and second orders of pathogen decay rate across treatments and sites. Specific pathogen decay rate within a treatment was represented by the regression coefficient of the sampling date within the treatment of individual sites. All analyses were performed using SAS version 9.1 (SAS Institute, 2005). Based on the estimated linear (denoted as ‘a’) and quadratic (denoted as ‘b’) decay rates for either ‘biosolids-amended’ or ‘un-amended’ soil, the decay time for one-log$_{10}$ (90%) reduction of pathogen count ($T_{90}$ values) was then estimated using the quadratic equation: $T_{90} = \frac{-b + \sqrt{b^2 - 4a}}{2a}$.

The generalised linear model of ANOVA was also applied to compare the decay rate of *E. coli* inside the chambers with that outside the chambers at Site B. In this case, additional fixed effects in the model included status (i.e., inside vs outside) that was nested in the
treatment*plot, linear and quadratic decay rates that were nested in the status*plot*treatment combinations.

To determine any relationship between changes in soil temperatures or changes in soil moisture with the decay patterns of individual enteric microorganism, the correlations were calculated in Microsoft® Excel using the CORRE function and the significances were determined using Student *t*-tests for each experimental site. The critical *P*-value for the test was set as 0.05. A one-tailed Student *t*-test was also applied to determine any significant difference between soil moisture levels in the chambers with that in the topsoil (outside chambers).

### 3. Results

#### 3.1. Climatic parameters

The observed climatic conditions, soil moisture and temperature during the decay experiments are summarized in Tables 1 and 2. Mean daily soil temperatures ranged from 12 to 16°C across all sites (Table 1). The cumulative rainfall over the duration of the experiment was 262 mm and 275 mm at Moora (Sites A and B, respectively) and 328 mm at Mt Compass (Site C). The mean soil moisture content inside the sentinel chambers was higher in the biosolids-amended soil (21 to 26%) than the un-amended soil 13%. The same trend was observed in the soil moisture outside the chambers, 8-17% in the biosolids-amended soil and 7-14% in the un-amended soil. In general, soil moisture was higher in the chambers than in the topsoil outside the chambers (Table 2).

#### 3.2. Adenovirus detection limits, PCR inhibition and recovery rates
The lowest numbers of gene copies that were detected consistently in replicate assays was 10 or less adenovirus PDU mL\(^{-1}\). The mean \(C_T\) values from the un-diluted and 10-fold diluted samples were compared and were found to be comparable (4±1). Therefore, undiluted samples were used for the qPCR. Adenovirus recovery from biosolids amended and soil samples returned mean recovery efficiencies of 22-78% from the biosolids-amended soil and 34-63% from the un-amended soil (Table 4).

### 3.3. Comparative E. coli survival in decay chambers and soil

In order to determine if the moisture and temperature inside the chamber were similar to the ambient soil conditions (outside the chambers), moisture patterns inside chambers and in the soil outside were compared at Site B. Due to smaller mass of the decay chambers, the temperature inside the chambers was assumed to be at equilibrium with the soil ambient temperature once placed in the soil. The changes in the moisture content inside the chambers and topsoil at Site B were significantly \((P<0.001)\) correlated, thus demonstrating that moisture exchange occurred between the surrounding soil through the chamber membranes. A comparative decay pattern of *E. coli* inside the chambers and in topsoil is presented in Figure 2. The *E. coli* numbers inside the chambers were higher than the *E. coli* numbers outside the chambers due to seeding of the *E. coli* into the biosolids placed in the chambers (Sites B and C); however, the changes in *E. coli* inside the chambers was significantly correlated \((P<0.05)\) to the decay patterns of *E. coli* outside the chambers (topsoil) at sites B and C.

### 3.4. Enteric microorganism decay in decay chambers

All seeded microorganisms, except HAdV, generally showed linear patterns of decay (Figures 3 and 4). The majority of the target microorganisms in the biosolids-amended soil
had shorter decay times ($T_{90}$) than those in the un-amended soil (Table 3). The systematic evaluation for the influence of types of microorganism, site location, treatment and related soil type during the wheat growing season showed that all factors significantly ($P<0.01$) affected decay rates (Table 4).

Bacteriophage MS2 numbers declined by $>6 \log_{10}$ during the duration of the experiment across all three sites (Figure 4). In general, decay times ($T_{90}$) for MS2 were less than 36 days, except at Sites A and B (Moora, WA) in the un-amended soil where estimated decay times were 108 and 90 days respectively (Table 3). The decay times for MS2 in biosolids-amended soil ($T_{90} = 22-36$ days) were generally less than in the un-amended soil ($T_{90} = 29-108$ days). The MS2 decay in biosolids-amended and un-amended soil was statistically significant ($P<0.0001$) (Table 4).

In comparison to MS2, much slower reduction in HAdV numbers was observed at the three field sites over the duration of the experiment (Figure 4). At Site B, there was little change in HAdV numbers over the duration of experiment and hence no $T_{90}$ value was achieved. At Sites A and C some decline in HAdV numbers was observed which resulted in $T_{90}$ values of $>180$ days (Table 3). Similar decay rates were observed for both biosolids-amended and un-amended soil at Site A, Moora 2006 ($P>0.05$, see sdate*treatment, sdate$^{2}$*treatment, and sdate$^{3}$*treatment in Table 4), but highly and significantly different decay rates between biosolids-amended and un-amended soil were found at Site C, Mount Compass in 2008 (Table 4, $P<0.0001$).

3.5. Influence of moisture and temperature on the decay of microorganisms
At all three sites, soil temperatures increased from winter through to summer (Figures 5 and 6). Decreasing soil moisture over the duration of the experiments significantly ($P<0.05$) influenced decay of seeded enteric microorganisms at Sites B and C, particularly in the un-amended soils (Figures 5 and 6). Similarly, increasing soil temperature also significantly ($P<0.05$) influenced MS2 decay patterns at Sites B and C (Figure 6), as well as *E. coli* decay patterns at Site B in the biosolids-amended and un-amended soil (Figure 5).

When decay of MS2 over time was plotted with ambient soil moisture and temperature at Site C, an interesting pattern was observed where MS2 numbers only declined by 2 to 3-log$_{10}$ over the first 120 to 150 days of the experiment and then rapidly declined to below 1-log$_{10}$ during the next 30 days (Figures 6). The rapid decline in the MS2 numbers coincided with the increasing soil temperatures (above 20°C) and reduced rainfall which resulted in a decline in soil moisture content.

### 4. Discussion

Natural decay of enteric pathogens does occur over time in biosolids-amended soil however, improved health risk assessments of land-applied biosolids is required to protect public health and ensure continued beneficial use of biosolids in agriculture. One of the major hurdles in health risk assessment is the limited availability of experimentally driven data on the survival potential of enteric pathogens in biosolids-amended soils. The present study was focused on investigating the survival potential of selected enteric pathogens and indicators in soil amended with biosolids during cultivation of wheat.

Decay chambers, as used in this study, make it possible to monitor decay over time in environmental sampling for pathogens often found in low numbers such as adenovirus and *S.*
enterica. However, the ability of chambers to replicate soil ambient conditions inside the chambers could be the major source of under estimation or over estimation of the decay time of pathogens. The observed similarity between ambient soil moisture conditions and decay chambers suggested that moisture and gaseous exchange occurred well between the soil and chambers during the duration of the experiment. The significant correlation ($P<0.05$) between the changes in E. coli inside the chambers to the decay patterns of E. coli outside the chambers (topsoil) at sites B and C suggest that decay chambers could be used for in situ monitoring of enteric pathogen decay in soil.

The observed one log$_{10}$ reduction time ($T_{90}$) of E. coli and S. enterica seeded into the biosolids-amended soil varied from 4-56 days which is in agreement with previously reported decay rates for animal manure-amended soils (Franz et al. 2005; Semenov et al. 2009; Nyberg et al. 2010). Longer survival times for bacteria in biosolids-amended soils at cooler temperatures have been reported in the literature (Gantzer et al. 2001; Zeleski et al. 2005; Horswell et al. 2010). E. coli O157:H7 and S. enterica serovar Typhimurium reduction times ($T_{90}$) between 15-90 days have been reported in soils amended with animal manures (Nyberg et al. 2010). Conversely, decay times between 8 to 15 days for S. enterica in biosolids applied soils in a pine plantation have been reported in a study from New Zealand (Horswell et al. 2010). Similarly, in soils irrigated with farm effluent in Victoria (Australia), one-log$_{10}$ reduction times were reported as 15 days for E. coli and 10 days for Salmonella (Chandler and Craven 1980). The reported variation in the decay rates of bacterial pathogens in the literature is most likely due to climatic and site specific factors. As biological and chemical properties of soil such as temperature, pH, water holding capacity, oxidation-reduction potential, presence of a rhizosphere and microbial interactions are also known to influence the survival of pathogens (Fenlon et al. 2000).
In the biosolids-amended soils, higher decay rates of seeded *E. coli* and *S. enterica* were observed compared to un-amended soils (Table 3). The higher decay rate of enteric pathogens in biosolids-amended soil is potentially due to enhanced antagonistic activity of autochthonous microorganisms as a result of higher availability of nutrients and better moisture retention (Sidhu et al. 2001). Comparatively higher microbial activity has been reported to occur in biosolids-amended soils compared to soils amended with chemical fertilizers (Zerghi et al. 2010). The observed shorter decay times of *E. coli* and *S. enteric* in the biosolids-amended soils indicates that the application of biosolids to agricultural land may have had a positive influence on the natural attenuation of pathogens when introduced to the soil. This inference, that the addition of biosolids to soil increases the decay times of enteric microorganisms, was also found by Ingham et al., (2004) who reported that *E. coli* decreased more rapidly in manure-fertilized soils. *E. coli* O157:H7 have been reported to inactivate more rapidly in non-autoclaved soil than autoclaved soil due to the antimicrobial activities of autochthonous microorganisms in manures and soil (Jiang et al. 2002). Conversely, in another study, the presence of animal manures actually enhanced the survival of *E. coli* (in no-till soil) which was hypothesized to be due to an enhanced micro-site habitat and the addition of nitrogen (Gagliardi and Karns 2000).

HAdV have been reported in high numbers in wastewater worldwide (Bofill-Mas et al. 2006; Sidhu et al. 2009) and are becoming highly important process-indicators in the drinking water industry as they are among the most thermally stable of viruses and are resistant to ultraviolet (UV) light (Gerba et al. 2002). In this study, no notable decay of HAdV tested by PCR was observed in both the biosolids-amended and the un-amended soils ($T_{90}$ of >180 d) at three sites. The observed high stability of HAdV is in agreement with previously reported high
environmental stability of adenovirus (Bofill-Mas et al. 2006; Wei et al. 2009). Temperature is one of the major factors known to influence the decay of enteric virus in the soil, with higher survival times expected at temperatures below 20 ºC (Santamaria and Toranzos 2003; Wei et al. 2009). In addition, adhesion of viruses to solid matrix is also reported to support prolonged survival by protecting them against thermal and proteolytic enzyme decay (Wei et al. 2009). Prolonged stability of HAdV observed in this study could be potentially due to the favorable survival conditions in the environment such as low soil temperature across all three sites (12-18ºC) combined with favorable moisture, lack of UV exposure and adhesion to the solid matrix. The results of this study suggest that HAdV may be stable for a longer period of time in biosolids-amended soils under low temperature and favorable moisture content. However, further investigations are required to establish if higher decay of adenovirus occurs during the summer months and the infectivity status of surviving HAdV in the biosolids-amended soil.

A careful approach must be adopted while interpreting risks from HAdV survival data, since PCR based techniques are known to be very sensitive and specific in the detection of virus genome; however, there can be a difference between the loss of infectivity and the complete degradation of viral genome (Charles et al. 2009, de Roda Husman et al. 2009). In this study, the PCR method was used for the quantification of HAdV which tend to over estimated virus numbers due to the detection of infectious and non-infectious virus particles (Sidhu et al. 2010). Conversely, cell culture based assays can detect infectious virus particles, however, cell infectivity is influenced by a number of variables such as the duration of exposure to host cells, the age of the cells, non specific plaques in the presence of multiple virus and the cell toxicity resulting from environmental samples (Rodriguez et al. 2009). The qPCR results from this study provide a conservative estimate of stability of HAdV. From a risk assessment
point of view, an overestimation of risk is considered preferable rather than an
underestimation obtained using cell culture-based methodologies until methodologies are
advanced enough to accurately determine infective virus numbers in biosolids.

Somatic coliphages, F-RNA specific bacteriophages and *Bacteroides fragilis* phage, are
relatively abundant in the wastewater sludge, and are potentially suitable indicators of enteric
viruses in biosolids (Mignotte-Cadiergues et al., 2002; Guzman et al. 2007; Sidhu and Toze
2009). In this study, survival potential of MS2 was compared with HAdV with an aim to
determine if MS2 could be a useful indicator for predicting enteric virus behaviour in
biosolids-amended soils. The decay rate of MS2 was much higher ($T_{90} = 22-108$ days)
compared to HAdV ($T_{90} > 180$ days) across all three sites. Similarly, in another study,
poliovirus has been reported to survive for longer than MS2 in soil amended with biosolids
(Straub et al. 1993). Although the decay times for MS2 and HAdV are not directly
comparable due to the use of qPCR for the quantification of the latter and culture based assay
for former; however, a one log$_{10}$ reduction time of 108 days for MS2 suggests that prolonged
survival of bacteriophage, and possibly even long survival time for enteric viruses is expected
in biosolids-amended soil during the winter. The results suggest that bacteriophage MS2
might not be a useful indicator for prediction of survival potential of adenovirus. Further
studies are required to ascertain the usefulness of MS2 and other bacteriophage as indicators
of enteric virus presence and behaviour in biosolids-amended soil.

Survival of different microorganisms in the environment has been reported to be influenced
by their ability to withstand adverse environmental conditions (Lasobras et al. 1999, Moce-
Llivina et al. 2003, Schwarz et al. 2014, Sidhu et al. 2008). In the present study, decay times
were observed to be influenced by the type of microorganism, with the faster decay of *E. coli*
and *S. enterica* (bacteria) compared to MS2 and HAdV (viruses). Consequently, different survival times are expected for other bacterial, viral and protozoan pathogens. Further research work is required to determine survival potential of other pathogens of concern such as *Campylobacter* spp., *Cryptosporidium*, *Giardia*, norovirus and rotavirus.

The influence of soil type and/or site on the decay of enteric microorganisms was found to be statistically non-significant. This suggests that other factors such as temperature, moisture content and nutrient are more likely to influence pathogen decay in the biosolids-amended soils than soil type. Increasing soil temperatures were found to significantly (*P*<0.05) influence MS2 (Figure 4) and *E. coli* (Figure 2) decay in the biosolids-amended and un-amended soils. Rapid decline in bacterial numbers has also been reported to occur with increasing soil temperatures (i.e. 15 to 25°C), along with declining soil moisture (i.e. rainfall to <15 mm) (Chandler and Craven 1980, Cools et al. 2001, Holley et al. 2006, Horswell et al. 2007, Unc and Goss 2006). In this study, a slower initial decay of MS2 was observed across all sites up to approximately 150 days followed by a rapid decline to below one-log₁₀ after 180 days (Figure 6). It is noteworthy, that this coincided with an increase in the soil temperatures across all sites along with fewer rainfall events which resulted in low soil moisture. F-specific bacteriophage (MS2) has been reported to be sensitive to temperatures over 25°C (Guzman et al. 2007, Lasabras et al. 1999, Moce-Llivina et al. 2003). Consequently, it is possible that the rapid decline observed in the MS2 numbers after 180 days was due to higher temperatures and lower soil moisture content. In the present study, declining soil moisture was also found to significantly (*P*<0.05) increase the decay rate of most enteric microorganisms, particularly in the un-amended soils.
In this study, higher starting numbers of seeded enteric pathogens at the beginning of each experiment, coupled with higher biosolids application rates, served as a worse-case risk scenario. The observed maximum time before the bacteria and bacteriophage fell below detection was well within the time frame of the wheat growing cycle of 4 to 6 months. Therefore, bacterial pathogens are unlikely to present health risks from biosolids-amended soils. Since microorganisms studied in this project demonstrated high stability in particular adenovirus, recommendations for the best management practices based on findings include consideration towards biosolids amendment frequency, time and rates. The existing exclusion periods of public access to biosolids-amended soil, or with holding periods prior to crop harvesting and animal grazing should as outline under West Australian biosolids managements (DEC 2012) should stay.

5. Conclusions

- Bacterial pathogens present the lowest potential health risks compared to enteric virus due to faster decay rates in biosolids-amended soils;
- Adenovirus could be expected to survive for a longer period (>180 days) in the biosolids-amended soil during the cultivation of cereals in winter when ambient environmental conditions are favorable for enteric virus survival;
- Bacteriophage MS2 may not be a suitable indicator for the assessment of survival potential of adenovirus and other enteric viruses;
- An increase in soil temperature and soil moisture was correlated to the decay of enteric bacteria whereas the decay of MS2 was correlated to soil temperature only;
- The influence of soil type and/or site on the decay of enteric microorganisms was found to be statistically non-significant; and
• The existing exclusion periods of public access to biosolids-amended soil, or with holding periods prior to crop harvesting and animal grazing should stay as part of risk management strategy;

6. Acknowledgements

The authors wish to acknowledge the financial support of the Water Corporation of Western Australia, Water Quality Research Australia (WQRA), Victorian Department for Human Services and the CSIRO Water for a Healthy Country Flagship Program.

7. References


Guzman, C., Jofre, J., Montemayer, M., Lucena, F., 2007. Occurrence and levels of indicators and selected pathogens in different sludges and biosolids. Journal of Applied Microbiology 103(6), 2420-2429.


Table 1. Climatic conditions, soil and biosolids characteristics over the experimental period (May to December) at three study sites.

<table>
<thead>
<tr>
<th>Source</th>
<th>Site A Moora 2006</th>
<th>Site B Moora 2008</th>
<th>Site C Mt Compass 2008</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SOIL:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil pH</td>
<td>5.3</td>
<td>4.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Mean daily air temp. (ºC)</td>
<td>16 (± 6.4)</td>
<td>14 (± 3.6)</td>
<td>12 (± 3.7)</td>
</tr>
<tr>
<td>Mean relative humidity (%)</td>
<td>63 (± 12.2)</td>
<td>73 (± 14.6)</td>
<td>62 (± 22.7)</td>
</tr>
<tr>
<td>Mean soil temp. (ºC)</td>
<td>18 (± 6.5)</td>
<td>17 (± 5.4)</td>
<td>12 (± 3.6)</td>
</tr>
<tr>
<td>Minimum soil temp. (ºC)</td>
<td>9 (± 6.5)</td>
<td>12 (± 1.9)</td>
<td>5 (± 3.8)</td>
</tr>
<tr>
<td>Maximum soil temp. (ºC)</td>
<td>34 (± 6.5)</td>
<td>26 (± 1.9)</td>
<td>25 (± 3.8)</td>
</tr>
<tr>
<td><strong>BIOSOLIDS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biosolids pH</td>
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<td>7.9</td>
<td>6.7</td>
</tr>
<tr>
<td>Total solids (%)</td>
<td>20</td>
<td>19</td>
<td>66</td>
</tr>
</tbody>
</table>

Table 2. Recorded soil moisture content inside and outside sentinel chambers across all three sites.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Soil moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site A</td>
</tr>
<tr>
<td></td>
<td>Un-amended</td>
</tr>
<tr>
<td>Moisture Inside chambers</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>NT</td>
</tr>
<tr>
<td>Maximum</td>
<td>NT</td>
</tr>
<tr>
<td>Mean moisture</td>
<td>NT</td>
</tr>
<tr>
<td>Moisture outside chambers</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>NT</td>
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<tr>
<td>Maximum</td>
<td>NT</td>
</tr>
<tr>
<td>Mean</td>
<td>NT</td>
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<tr>
<td>Cumulative rainfall (mm)</td>
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</table>

NT = Not tested
Table 3. Time ($T_{90}$) for a one log$_{10}$ reduction to occur for enteric microorganisms in sentinel chambers in soil at three field sites.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Site A Biosolids</th>
<th>Site A Un-amended</th>
<th>Site B Biosolids</th>
<th>Site B Un-amended</th>
<th>Site C Biosolids</th>
<th>Site C Un-amended</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E. coli$</td>
<td>5</td>
<td>12</td>
<td>56</td>
<td>83</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>$E. coli$ outside chambers</td>
<td>NT</td>
<td>NT</td>
<td>29</td>
<td>21</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>$S. enterica$</td>
<td>4</td>
<td>21</td>
<td>12</td>
<td>25</td>
<td>37</td>
<td>57</td>
</tr>
<tr>
<td>MS2</td>
<td>36</td>
<td>108</td>
<td>29</td>
<td>90</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>&gt;180</td>
<td>&gt;180</td>
<td>&gt;180</td>
<td>&gt;180</td>
<td>&gt;180</td>
<td>&gt;180</td>
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</table>

NT = Not tested

Table 4. Recovery rates of seeded adenovirus in biosolids-amended and un-amended soil.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Seeded virus numbers (PDU mL$^{-1}$)</th>
<th>Recovered virus number (PDU mL$^{-1}$)</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
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<tr>
<td>Water</td>
<td>4.40 X 10$^4$</td>
<td>4.40 X 10$^4$</td>
<td>100</td>
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<tr>
<td>Moora</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site A - Amended</td>
<td>3.17 X 10$^5$</td>
<td>6.94 X 10$^4$</td>
<td>22</td>
</tr>
<tr>
<td>Site A - Unamed</td>
<td>2.10 X 10$^5$</td>
<td>1.33 X 10$^5$</td>
<td>63</td>
</tr>
<tr>
<td>Site B - Amended</td>
<td>5.06 X 10$^2$</td>
<td>3.96 X 10$^2$</td>
<td>78</td>
</tr>
<tr>
<td>Site B - Unamed</td>
<td>5.44 X 10$^4$</td>
<td>3.27 X 10$^4$</td>
<td>60</td>
</tr>
<tr>
<td>Mt Compass</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Site C - Amended</td>
<td>1.20 X 10$^5$</td>
<td>4.36 X 10$^4$</td>
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<td>Site C - Unamed</td>
<td>2.40 X 10$^5$</td>
<td>8.12 X 10$^4$</td>
<td>34</td>
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</table>
Figure 1. A commercial 3.5 mL Microsep™ centrifugal device (35 mm x 10 mm) used as a sentinel chamber and; (bottom right) filled with the sample contents of soil, biosolids and laboratory-cultured microorganisms.
Figure 2. Decay patterns of *E. coli* with SE bars, outside chambers (soil) and inside chambers in biosolids-amended soil and at Site B (Moora 2008).
Figure 3. Decay patterns of *E. coli* and *S. enterica* with SE bars across three sites in biosolids-amended soil and un-amended soil.
Figure 4. Decay patterns of human adenovirus (HAdV) and MS2 with SE bars across three sites in biosolids-amended soil and un-amended soil.
Figure 5: Decay patterns of *E. coli* and *S. enterica* at Site B (Moora 2008) with SE bars in biosolids-amended (black symbols) and un-amended (clear symbols) soil in chambers, with changes in soil moisture in both soil types (inside chambers) and soil temperature.
Figure 6. Decay patterns of MS2 and adenovirus at Site C (Mount Compass 2008) with SE bars in biosolids-amended (black symbols) and un-amended (clear symbols) soil in chambers, with changes in soil moisture in both soil types (inside chambers).
Table 4. ANOVA results of individual factors influencing decay of enteric microorganisms in biosolids-amended soil and un-amended soil chambers.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>E. coli</th>
<th>E. coli (site)</th>
<th>S. enterica</th>
<th>MS2</th>
<th>Adenovirus</th>
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<tbody>
<tr>
<td></td>
<td>Mean square</td>
<td>P-value</td>
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<td><strong>Moora 2006</strong></td>
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<tr>
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<tr>
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<td>110.87</td>
<td>&lt;.0001</td>
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<td>sdate²</td>
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<td>&lt;.0001</td>
<td>41.08</td>
<td>&lt;.0001</td>
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<td>sdate³</td>
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<td>&lt;.0001</td>
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<td>&lt;.0001</td>
<td>0.60</td>
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<td>sdate³*treatment</td>
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<td>64.23</td>
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<td>52.96</td>
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<tr>
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<td>0.60</td>
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<tr>
<td>R-square</td>
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<td>0.91</td>
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</tr>
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</tr>
<tr>
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<tr>
<td>sdate</td>
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<td>12.13</td>
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<td>24.78</td>
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<td>0.91</td>
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<tr>
<td><strong>Mt Compass 2008</strong></td>
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<tr>
<td>Treatment</td>
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<td>0.9092</td>
<td>0.11</td>
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</tr>
<tr>
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<td>&lt;.0001</td>
<td>0.16</td>
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</tr>
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<td>0.744</td>
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<td>0.5045</td>
<td>0.01</td>
</tr>
<tr>
<td>plot (treatment)</td>
<td>2.76</td>
<td>0.181</td>
<td>1.97</td>
<td>0.0068</td>
<td>2.35</td>
</tr>
<tr>
<td>Error</td>
<td>1.76</td>
<td></td>
<td>0.54</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>R-square</td>
<td>0.82</td>
<td></td>
<td>0.90</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>
Treatment is biosolids-amended vs un-amended soil. sdate, sdate^2 and sdate^3 refer to the overall first, second and third order of decay rates across treatments respectively. sdate*treatment, sdate^2*treatment sdate^3*treatment refer to first, second and third order of decay rate comparison between two treatments (biosolids vs nil biosolids soil). plot(treatment) refers to the plot nested in the treatment. Highly significant P-values are in bold (<.0001).
Highlight of this paper

- *E. coli* and *Salmonella enterica* decayed faster in biosolids amended soil than un-amended soil.

- Human adenovirus and other enteric virus are expected to survive for a longer period of time at low ambient temperature.

- MS2 may not be a suitable indicator for assessment of survival potential of adenovirus

- Longer survival time for adenovirus suggests that appropriate withholding periods are necessary for risk mitigation