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ORIGINAL ARTICLE

Inactivation of hepatitis A HM-175/18f, reovirus T1 Lang and MS2 during alkaline stabilization of human biosolids

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Abstract

Aim: To compare the inactivation rates of male-specific bacteriophage-2 (MS2), hepatitis A HM-175/18f (HM-175) and reovirus T1 Lang (T1 L) during alkaline stabilization of wastewater residues.

Methods and Results: A bench scale alkaline stabilization model was used to evaluate the inactivation of MS2 seeded into raw sludge simultaneously with HM-175 or T1 L. Stabilization was performed in triplicate at 28 and 4°C for both viral combinations. During stabilization at 28 and 4°C, MS2 and T1 L concentrations were similar at each time point (t = 0·1, 2, 12 and 24 h). MS2 and HM-175 concentrations were also similar at each time point during stabilization at 28°C. At 4°C, MS2 and HM-175 concentrations were not similar at the first two time points (t = 0·1 and 2 h), but were similar at later time points (t = 12 and 24 h).

Conclusions: The inactivation rates of T1 L at 4°C and both T1 L and HM-175 at 28°C were similar to the inactivation rate of MS2 at all time points. At 4°C, MS2 was inactivated at a faster rate during the first two time points (t = 0·1 and 2 h) than HM-175, but was inactivated similarly at later time points (t = 12 and 24 h).

Significance and Impact of the Study: Phages, such as MS2, would be ideal indicators for the presence of enteric viruses in wastewater residues because of their ubiquity, nonpathogenic nature, low cost and time associated with their detection. The findings of this study suggest that MS2 could serve as an indicator for monitoring the persistence of enteric viruses, such as HM-175 and T1 L, during alkaline stabilization performed at moderate temperatures (28°C), but may not serve as an indicator for HM-175 at reduced temperature (4°C). The utility of MS2 as an indicator of viral persistence during biosolids treatment should be further evaluated, as the increased efficiency and frequency of pathogen monitoring associated with their use may reduce the potential public health risk associated with biosolids, facilitating a greater acceptance for their land application.

Introduction

Biosolids are a complex mixture of beneficial components, pathogens and pollutants derived from the treatment of wastewater residues. When properly treated, biosolids can be used as an amendment to improve the chemical and physical properties of soil (EPA 2000b). Transmission of enteric viruses known to be present in wastewater residues, such as hepatitis A virus (HAV), reoviruses (REOs), adenoviruses and noroviruses, via biosolids (EPA 2003) is of particular concern. As a result of their small size, enteric viruses easily infiltrate soils, where upon favourable pH and moisture conditions, they can migrate through the subsurface environment, contaminate groundwater (Borchardt *et al.* 2003) and penetrate the roots of edible plants (Oron *et al.* 1995), thereby posing a

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potential public health threat. Most data regarding the potential of enteric viruses to contaminate groundwater and their persistence during treatment of wastewater residues come from experimentation with their current indicator, enteroviruses (EVs), a subpopulation of the enteric virus group.

EVs, polioviruses in particular, were originally selected as the indicator for the fate of enteric viruses during wastewater treatment processes because of their ubiquity in the environment, overall resistance to chemical and physical treatments and their relative ease of detection by standard plaque assay methods (EPA 2000b). Recent studies have shown the level of detectable polioviruses, once thought to be the most numerous of the EVs, is low in comparison with more host restrictive EVs, not easily detected by the plaque assay method specified under the CFR part 503 (Sedmak *et al.* 2003). The reduction of polioviruses in the environment and limited ability to detect nonpoliovirus EVs using the CFR part 503 methods suggest that EVs may no longer be an appropriate indicator for the presence and persistence of enteric viruses in wastewater residues intended for land application as biosolids.

Surveillance studies have shown that monthly sewage samples and clinical cases of viral illness often demonstrate a similar picture of EV activity (Sedmak *et al.* 2003). Clinical cases of enteric viral disease should therefore correlate to an increase in the offending agents' presence within wastewater residues. Noroviruses, coronaviruses and HAV were not primary concerns during the development of the pathogen standards governing biosolids, but have since been documented as causative agents of serious and highly communicable illnesses. The recent surge in clinical cases caused by these enteric viruses emphasizes the need to determine whether the current indicator, EVs, can accurately predict their presence in biosolids and persistence during treatment (Wang *et al.* 2005; Colomba *et al.* 2006; Fiaccadori *et al.* 2006).

HAV, the most common cause of infectious hepatitis in the world, infects approx. 140 000 people in the US each year with an annual cost of between $332 and $580 million (Berge *et al.* 2000). HAV demonstrates a high level of resistance to inactivation by chemicals (Li *et al.* 2002), heat (Sobsey *et al.* 1986) and has survived for longer periods of time than EVs in experimentally contaminated freshwater, seawater, wastewater and soils (Sobsey *et al.* 1988). The ubiquity of HAV in biosolids has not been well characterized as wild-type virus rarely exhibits cytopathic effects (CPE) in cell culture and replicates slowly (Graff *et al.* 1993) making it unlikely to be detected by the CFR part 503 method (EPA 2003). Recent studies utilizing molecular methods of detection indicate that HAV levels can exceed those of EVs in waters contaminated by sewage (Gersberg *et al.* 2006). These data suggest that HAV may be prevalent in wastewater residues and persist in treated biosolids following the inactivation of EVs.

REOs are capable of causing asymptomatic or debilitating illness in a wide range of hosts including reptiles, plants and mammals. Mammalian REOs commonly infect livestock, which serve as the primary reservoir for human infection and contribute to the high frequency by which REOs are isolated from water (Sattar and Springthorpe 1999). REO infections in humans are often asymptomatic, mimicking commonly encountered minor respiratory and gastrointestinal illnesses (Jackson and Muldoon 1973), but have been suggested to infrequently cause serious infections in children (Lerner *et al.* 1962; Tyler *et al.* 1998). REOs shed by infected humans are commonly isolated from secondary sewage effluent (Irving and Smith 1981) and raw sewage (Havelaar *et al.* 1993), where they have been shown to be more resistant to temperature (Ward and Ashley 1978) and chemicals inactivation than EVs. These data suggest that REOs, like HAV are likely to be present in wastewater residues and may persist in treated biosolids following the inactivation of EVs.

Phages have received significant evaluation as indicators of enteric virus presence and persistence following treatment of source water (Cole *et al.* 2003), drinking water (Shin and Sobsey 2003) and wastewater (Havelaar *et al.* 1993). Male-specific bacteriophages (MSB) are nonpathogenic, ubiquitous in wastewater at sufficient concentrations for detection (Calci *et al.* 1998), enumerated by rapid and inexpensive plaque assays and have been suggested as useful indicators for determining the fate of human viruses in sludge (Lasobras *et al.* 1999; Moce-Llivina *et al.* 2003). Therefore, phages may serve as more efficient indicators than EVs for determining the fate of enteric viruses, such as HM-175 and T1 L, during treatment of wastewater residues intended for land application as biosolids.

The goal of this research was to compare the inactivation rate of MS2 with that of HM-175 and T1 L during alkaline stabilization in order to evaluate the potential of MS2 to serve as an indicator for the inactivation of these enteric viruses.

Materials and methods

Viral propagation

Escherichia coli HS (pFamp) R was grown at 37°C for 3 h in tryptic soy broth (TSB) (Difco) supplemented with 1% (w/v) $MgCl_2$ and 1% (w/v) streptomycin/ampicillin. MS2, selected as the representative MSB based on its use in previous studies utilizing alkaline stabilization (Bean

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et al. 2007; Hansen *et al.* 2007), was added to a final concentration of 9·0 $\log_{10}$ plaque forming units per litre (PFU per litre) and incubated at 37°C for 12–18 h. Following incubation, chloroform was added at a ratio of 20 ml l^{-1} culture, refrigerated at 4°C for 1 h and then centrifuged for 10 min at 4°C and 10 000 ***g***. The resulting pellet was discarded and supernate was stored at 4°C.

T1 L was propagated within Buffalo Green Monkey Kidney Cells (BGM) in a closed system at 37°C. Briefly, tissue culture flasks were seeded to a multiplicity of infection (MOI) of 0·96 and incubated at 37°C for 90 min. Following incubation, BGM growth medium, 43% L-15 medium, 27% Eagles Minimal Essential Medium (MEM), 2% L-glutamine, 4% $NaHCO_3$ (Sigma-Aldrich) and 24% (all w/v) Hepes (Fisher Scientific International) supplemented with 5% foetal bovine serum (FBS) (JRH Biosciences), 1% nonessential amino acids (Gibco, Invitrogen) and 1% antibiotic/antimycotic (all v/v) (Gibco) was added and flasks were incubated at 37°C for 6 days. Flasks were examined for CPE and observed once, freeze-thawed three times and lysates were collected and stored at −80°C.

HM-175 was propagated within Foetal Rhesus Monkey Kidney Cells (FRhK-4) in a closed system at 37°C. Briefly, tissue culture flasks were seeded to a MOI of 0·01 and incubated at 37°C for 90 min. Following incubation, growth medium identical to that used for the BGM cells, except supplemented with 12% (v/v) FBS, was added. Flasks were incubated at 37°C for 12 days, then freeze-thawed three times after CPE was observed. Chloroform was added at a ratio of 20 ml l^{-1} lysate, centrifuged for 10 min at 4°C and 10 000 ***g***, and supernate was retained and stored at −80°C.

Viral enumeration

MS2 plaque assays were performed using a modified double agar overlay technique (Adams 1959). Briefly, *E. coli* HS (pFamp) R was grown at 37°C for 3 h in TSB broth as previously described. Two hundred microlitre of *E. coli* HS (pFamp) R and 100 μl of serially diluted sample were added to a 7·5% (w/v) agarose tube and poured over a tryptic soy agar (TSA) (Difco) plate impregnated with 1% (v/v) streptomycin/ampicillin. TSA plates were incubated, inverted at 37°C for 24 h and then examined for plaques.

Enumeration of T1 L was performed using a modified rotavirus plaque assay previously described by Smith *et al.* (1979). Briefly, 25 cm^2 tissue culture flasks (T-25) containing BGM cells were rinsed twice with serum-free MEM, inoculated with 100 μl of serially diluted sample and incubated at 37°C for 90 min. Flasks were overlaid with 10-ml MEM supplemented with 0·01 g l^{-1} trypsin (Gibco) and 0·6% (w/v) agarose and then incubated and inverted at 37°C for 4 days. Following incubation cells were fixed with 10% (v/v) formaldehyde and incubated at 37°C for 24 h. The following day each flask was rinsed gently with warm water, shaken lightly to remove the overlay, stained with 0·1% (w/v) crystal violet and examined for plaques.

Enumeration of HM-175 was performed using a modified version of an HAV plaque assay described by Cromeans *et al.* (1987). Briefly, T-25 flasks containing FRhK-4 cells were rinsed twice with serum-free MEM, inoculated with 100 μl of serially diluted sample and incubated at 37°C for 90 min. Flasks were overlaid with 5-ml MEM supplemented with 0·6% agarose and incubated at 37°C for 7 days. A second 5-ml MEM overlay containing 50-μl neutral red (Sigma-Aldrich) was added to the flasks on the 7th day. Flasks were re-incubated at 37°C for 3 days and then examined for plaques.

Sample collection

Sludge samples were obtained from a Massachusetts wastewater treatment plant on the same day in early spring. Sludge temperature upon sampling was not provided by the treatment plant. Once in receipt, sludge samples were stored at 4°C and analysed for total solids (TS) prior to alkaline stabilization (APHA 1998).

Alkaline stabilization

Enteric viruses have been documented to persist for long periods of time in different matrices and under various treatment conditions. Previous experiments utilizing alkaline stabilization have demonstrated that large reductions in enteric viruses can occur within 12 h of seeding (Sattar *et al.* 1976; Hansen *et al.* 2007). A bench scale alkaline stabilization model, adhering to EPA guidelines for the generation of class B biosolids, was constructed similar to these previous studies in order to evaluate the inactivation of HM-175, T1 L and MS2 at 0·1, 2, 12 and 24 h. This system was challenged three times with each of the two viral combinations at both 4 and 28°C representing temperature conditions under which alkaline stabilization may be preformed in enclosed and open-air facilities throughout the year.

Briefly, 100 ml of sludge was placed in ten beakers representing four test time points, 0·1, 2, 12 and 24 h, and six controls. An 11th beaker containing 100 ml of phosphate-buffered saline (PBS) was also prepared. All beakers were brought to the indicated temperatures for each experiment and adjusted to a pH of 7 under continuous mixing using either 2 mol l^{-1} NaOH or 1 mol l^{-1} HCl. Test beakers were elevated to a pH of 12 by addition of 8% (w/v) $Ca(OH)_2$ and virus added as such: 1 ml,

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6 $\log_{10}$ PFU of both HM-175 and MS2 or 1 ml, 6 $\log_{10}$ PFU of both T1 L and MS2. The seven control beakers (PBS, 0, 0·1, 2, 12 and 24 h control) were spiked with approximately the same viral concentration and combination as the stabilization beakers.

The 0-h beaker and a 100-ml sludge aliquot taken before seeding were immediately concentrated to determine seeded viral load and endogenous viral concentration in unseeded sludge. The PBS control, intended to demonstrate the reduction of seeded virus based on temperature alone, was serially diluted and assayed at 0 and 24 h.

All stabilization beakers were maintained at a pH of 12 for 2 h and then reduced to and maintained at a pH of 11·5 for the remaining 22 h. All control beakers were maintained at a pH of 7 throughout the experiment. All pH adjustments were made using either 2 mol l^{-1} NaOH or 1 mol l^{-1} HCl as needed. At the indicated time point, stabilization beakers were neutralized to a pH of 7 and both the stabilization and the corresponding control beaker concentrated.

Viral recovery from sludge

Enteric viruses were recovered from sludge using a modified version of the procedures specified in the CFR Part 503 (Berg *et al.* 1984). Modifications to the procedure included elimination of sludge conditioning prior to concentration and chloroform decontamination of the concentrated eluates. Chloroform was added to concentrated sludge eluates to achieve a final concentration of 10% (v/v), centrifuged for 10 min at 4°C and 10 000 ***g*** and supernate was removed and stored at −80°C.

Phage was recovered by adding volumes of 3% (w/v) beef extract to 10-ml sludge in order to achieve a final concentration of 1% (v/v) beef extract in each sample. The pH was elevated to 9·5 with 2 mol l^{-1} NaOH and samples were centrifuged for 10 min at 22°C and 2500 ***g***. The supernate was removed, adjusted to a pH of 7 with 1 mol l^{-1} HCl and stored at 4°C.

Viral concentrations in stored samples were determined by plaque assay performed in triplicate as described earlier. The presence of ≥1 PFU at any dilution in the test samples was considered significant and would correlate to a detection limit of 20 PFU HM-175 or T1 L and 60 PFU MS2 in a single sample.

Statistical analyses

Viral concentrations were square root transformed and analysed by two-way ANOVA using SYSTAT 11.0. A general linear model was constructed to evaluate the following null hypothesis: (i) alkaline stabilization data significantly varies from trial to trial in the same matrix, (ii) alkaline stabilization does not have a significant effect on the inactivation of MS2, T1 L and HM-175 and (iii) inactivation by alkaline stabilization is not significantly affected by the amount of time that elapses during experimentation. Lastly, a Tukey's Honestly Significantly Different Test (T-HSD) was used to detect any significant difference in the inactivation of phage and enteric virus at each time point during alkaline stabilization. Significance was noted as a P-value ≤ 0·05.

Results

Viral loss, separate of alkaline stabilization, was determined in stabilized samples by comparing the known concentration of seeded virus with the concentration of virus recovered from the 0-h control. The expected average loss was determined to be 1·0, 0·36 and 1·17 $\log_{10}$ total PFU for MS2, T1 L and HM-175, respectively. The average loss in the absence of stabilization was subtracted from the total average loss in the stabilized samples in order to determine the level of viral inactivation occurring as a result of stabilization alone (Table 1).

Endogenous viral concentrations in sludge samples prior to alkaline stabilization yielded no detectable FRhK-4 lytic virus (0/6 samples), 13 to 2600 total PFU of BGM lytic virus (4/6 samples) and 900 to 350 000 total PFU (12/12 samples) of *E. coli* HS (pFamp) R lytic phage. Plaques were not purified to confirm the identity of the lytic virus.

T1 L and MS2 at 28°C

MS2 was below detectable limits following 0·1 h of stabilization during all three trials at 28°C. T1 L seeded into the same sludge was below detectable limits following 12 h of stabilization in the first two trials and 2 h in the third (Table 2). Temperature alone did not significantly reduce viral concentration over 24 h nor was there a significant reduction (≥2 $\log_{10}$ total PFU) of virus within

Table 1 Average viral reduction caused by alkaline stabilization alone

Organism	Average viral reduction ($\log_{10}$ total PFU ± SE)*	
	4°C stabilization	28°C stabilization
MS2	4·99 ± 1·79	4·92 ± 0·04
	4·19 ± 0·63	4·80 ± 0·76
T1 L	5·29 ± 0·89	5·95 ± 0·59
HM-175	3·95 ± 2·13	5·10 ± 1·07

*Average viral loss because of experimental conditions subtracted from the average total viral reduction during three trials of stabilization ± SE.

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Table 2 MS2 and T1 L reduction during alkaline stabilization at 28°C

Time (h)	Average viral concentration ($\log_{10}$ total PFU ± SE)*						
	MS2 PBS control	T1 L PBS control	T1 L sludge control	MS2 sludge control	T1 L sludge stabilization	MS2 sludge stabilization	Significant§
0	6·98 ± 0·03	6·20 ± 0·16	6·31 ± 0·04	5·92 ± 0·04	6·31 ± 0·04	5·92 ± 0·04	
0·1	–†	–	6·28 ± 0·03	5·88 ± 0·06	1·70 ± 0·29	0	No
2	–	–	6·18 ± 0·09	5·71 ± 0·05	0·52 ± 0·26	0	No
12	–	–	6·12 ± 0·17	5·68 ± 0·06	0	0	No
24	6·76 ± 0·08	6·04 ± 0·06	6·01 ± 0·12	4·75 ± 0·62	0	0	No
Reduction‡	0·22 ± 0·11	0·15 ± 0·22	0·30 ± 0·46	1·18 ± 0·83	6·31 ± 0·59	5·92 ± 0·04	No

*Average concentration of target virus ± SE from three trials under the following conditions: PBS control (virus spiked PBS, pH 7), sludge control (virus spiked in 4% TS sludge, pH 7), sludge stabilization (virus spiked into 4% TS sludge, treated with 8% (w/v) $Ca(OH)_2$, pH 12 for 2 h then 11·5 an additional 22 h).
†Sample not tested (–).
‡Average total reduction of target virus, including loss because of experimental conditions, during three trials of stabilization ± SE.
§Significant difference between square root transformed viral concentrations of the test beakers at each time point determined by T-HSD, $P \le 0{\cdot}05$.

the sludge controls. Statistical analyses revealed no statistically significant difference between each trial ($P = 0{\cdot}707$) and that stabilization alone ($P < 0{\cdot}001$) and stabilization over a 24 h period ($P < 0{\cdot}001$) had a significant effect on viral concentration. T-HSD demonstrated no statistically significant difference ($P > 0{\cdot}05$) between MS2 and T1 L concentrations at each time point (Table 2).

T1 L and MS2 at 4°C

MS2 was below detectable limits following 2 h of stabilization during the first two trials at 4°C and 12 h in the third. T1 L seeded into the same sludge was below detectable limits following 12 h of stabilization during the first two trials and 24 h in the third (Table 3). Temperature alone did not significantly reduce viral concentration over 24 h nor was there a significant reduction (≥2 $\log_{10}$ total PFU) of virus within the sludge controls. Statistical analyses revealed no statistically significant difference between each trials ($P = 0{\cdot}335$) and that stabilization alone ($P < 0{\cdot}001$) and stabilization over a 24 h period ($P \le 0{\cdot}001$) has a significant effect on viral concentration. T-HSD demonstrated no statistically significant difference ($P > 0{\cdot}05$) between MS2 and T1 L concentrations at each time point (Table 3).

HM-175 and MS2 at 28°C

MS2 was below detectable limits following 0·1 h of stabilization during the first trial at 28°C and 2 h in the second and third. HAV was below detectable limits following 2 h

Table 3 MS2 and T1 L reduction during alkaline stabilization at 4°C

Time (h)	Average viral concentration ($\log_{10}$ total PFU ± SE)*						
	MS2 PBS control	T1 L PBS control	T1 L sludge control	MS2 sludge control	T1 L sludge stabilization	MS2 sludge stabilization	Significant§
0	6·78 ± 0·09	6·0 ± 0·18	5·65 ± 0·15	5·99 ± 0·11	5·65 ± 0·15	5·99 ± 0·11	
0·1	–†	–	5·57 ± 0·14	5·79 ± 0·10	2·43 ± 0·62	1·63 ± 0·33	No
2	–	–	5·46 ± 0·13	5·64 ± 0·18	1·42 ± 0·12	0·75 ± 0·75	No
12	–	–	5·33 ± 0·08	5·11 ± 0·69	0	0·59 ± 0·59	No
24	6·72 ± 0·06	5·96 ± 0·18	5·29 ± 0·21	4·89 ± 0·57	0	0	No
Reduction‡	0·06 ± 0·14	0·04 ± 0·36	0·37 ± 0·71	1·10 ± 1·65	5·65 ± 0·89	5·99 ± 1·79	No

*Average concentration of target virus ± SE from three trials under the following conditions: PBS control (virus spiked PBS, pH 7), sludge control (virus spiked in 4% TS sludge, pH 7), sludge stabilization (virus spiked into 4% TS sludge, treated with 8% (w/v) $Ca(OH)_2$, pH 12 for 2 h then 11·5 an additional 22 h).
†Sample not tested (–).
‡Average total reduction of target virus, including loss because of experimental conditions, during three trials of stabilization ± SE.
§Significant difference between square root transformed viral concentrations of the test beakers at each time point determined by T-HSD, $P \le 0{\cdot}05$.

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Table 4 MS2 and HM-175 reduction during alkaline stabilization at 28°C

	Average viral concentration ($\log_{10}$ total PFU ± SE)*						
Time (h)	MS2 PBS control	HM-175 PBS control	HM-175 sludge control	MS2 sludge control	HM-175 sludge stabilization	MS2 sludge stabilization	Significant§
0	6·91 ± 0·10	6·89 ± 0·06	6·27 ± 0·07	5·80 ± 0·08	6·27 ± 0·07	5·80 ± 0·08	
0·1	–†	–	6·25 ± 0·09	5·71 ± 0·10	5·49 ± 0·52	1·36 ± 0·68	No
2	–	–	6·19 ± 0·10	4·54 ± 0·58	0·48 ± 0·48	0	No
12	–	–	6·18 ± 0·10	4·12 ± 0·54	0	0	No
24	6·75 ± 0·10	6·76 ± 0·05	5·69 ± 0·16	3·47 ± 0·18	0	0	No
Reduction‡	0·17 ± 0·19	0·13 ± 0·11	0·58 ± 0·52	2·33 ± 1·48	6·27 ± 1·07	5·80 ± 0·76	No

*Average concentration of target virus ± SE from three trials under the following conditions: PBS control (virus spiked PBS, pH 7), sludge control (virus spiked in 4% TS sludge, pH 7), sludge stabilization (virus spiked into 4% TS sludge, treated with 8% (w/v) $Ca(OH)_2$, pH 12 for 2 h then 11·5 an additional 22 h).
†Sample not tested (–).
‡Average total reduction of target virus, including loss because of experimental conditions, during three trials of stabilization ± SE.
§Significant difference between square root transformed viral concentrations of the test beakers at each time point determined by T-HSD, $P \le 0·05$.

of stabilization in the first and third trial and 12 h in the second (Table 4). Temperature alone did not significantly reduce viral concentration over 24 h, but there was a significant reduction (≥2 $\log_{10}$ total PFU) of MS2 within the sludge controls caused by either pH variation or anti-viral agents. Statistical analyses revealed a significant difference between each trial ($P \le 0·001$) and that stabilization alone ($P < 0·001$) and stabilization over a 24 h period ($P < 0·001$) has a significant effect on viral concentration. T-HSD demonstrated no statistically significant difference ($P > 0·05$) in MS2 and HM-175 concentrations at each time point (Table 4).

HM-175 and MS2 at 4°C

MS2 was below detectable limits following 24 h of stabilization during all three trials at 4°C. During the first trial, HM-175 was still detectable following 24 h of stabilization, but was below detectable limits following 24 h in the second and third (Table 5). Temperature alone did not significantly reduce viral concentration over 24 h nor was there a significant reduction (≥2 $\log_{10}$ total PFU) of virus within the sludge controls. Statistical analyses revealed a significant difference between each trial ($P < 0·001$) and that stabilization alone ($P < 0·001$) and stabilization over a 24 h period ($P < 0·001$) has a significant effect on viral concentration. T-HSD demonstrated a statistically significant difference ($P < 0·05$) in MS2 and HM-175 concentrations at the 0·1 and 2 h time points (Table 5).

Discussion

Monitoring the presence and viability of enteric viruses capable of transmission via treated and untreated waste-

Table 5 MS2 and HM-175 reduction during alkaline stabilization at 4°C

	Average viral concentration ($\log_{10}$ total PFU ± SE)*						
Time (h)	MS2 PBS control	HM-175 PBS control	HM-175 sludge control	MS2 sludge control	HM-175 sludge stabilization	MS2 sludge stabilization	Significant§
0	6·27 ± 0·15	6·82 ± 0·03	6·32 ± 0·06	5·19 ± 0·07	6·32 ± 0·06	5·19 ± 0·07	
0·1	–†	–	6·32 ± 0·02	5·13 ± 0·08	5·44 ± 0·31	2·48 ± 0·05	Yes
2	–	–	6·20 ± 0·06	3·81 ± 0·02	5·22 ± 0·39	2·21 ± 0·21	Yes
12	–	–	6·13 ± 0·08	3·67 ± 0·06	4·26 ± 0·18	1·85 ± 0·29	No
24	6·20 ± 0·17	6·66 ± 0·05	5·97 ± 0·10	3·50 ± 0·13	1·20 ± 1·20	0	No
Reduction‡	0·07 ± 0·32	0·16 ± 0·08	0·35 ± 0·42	1·68 ± 0·37	5·12 ± 2·13	5·19 ± 0·63	No

*Average concentration of target virus ± SE from three trials under the following conditions: PBS control (virus spiked PBS, pH 7), sludge control (virus spiked in 4% TS sludge, pH 7), sludge stabilization (virus spiked into 4% TS sludge, treated with 8% (w/v) $Ca(OH)_2$, pH 12 for 2 h then 11·5 an additional 22 h).
†Sample not tested (–).
‡Average total reduction of target virus, including loss because of experimental conditions, during three trials of stabilization ± SE.
§Significant difference between square root transformed viral concentrations of the test beakers at each time point determined by T-HSD, $P \le 0·05$.

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water residues is impractical because of the associated cost, labour and technology. EVs, specifically polioviruses, were ideal indicators for the presence of enteric viruses when the CFR Part 503 regulations were first promulgated in 1993, especially those viruses not easily detected or quantified by available methods. Advancements in environmental virology have made it possible to more readily detect enteric viruses in the environment as well as evaluate their ubiquity and levels of resistance to traditional treatment methodologies. Numerous studies support the hypothesis that EVs are no longer the most ubiquitous viruses in the various matrices that they were once thought to be (Irving and Smith 1981; Havelaar *et al.* 1993; Sedmak *et al.* 2003), nor are EVs the most difficult to inactivate (Ward and Ashley 1978; Sobsey *et al.* 1986; Tree *et al.* 2003). Alternatively, phages such as MS2 are present at higher concentrations in wastewater residuals than anthropogenic viruses (Calci *et al.* 1998), detected by cost-efficient and rapid assays and have been found less susceptible to chemical inactivation than EVs (Tree *et al.* 2003). This study sought to compare the inactivation rates of MS2, T1 L and HM-175, during alkaline stabilization of wastewater residues and evaluate the potential of MS2 to serve as an indicator for the persistence of these enteric viruses in treated biosolids.

Alkaline stabilization can be used to generate class B biosolids by maintaining wastewater solids at a pH of 12 for 2 h without strict time or temperature regulations that are required to achieve class A standards (EPA 2000a). Pathogen inactivation under these conditions comes from the hydroxide alkalinity of the liming material added with further reduction in contaminants via flocculation or flotation (Grabow *et al.* 1978). Previous studies have shown that alkaline stabilization performed under class B conditions for 24 h at 4 and 28°C significantly reduces the level of seeded poliovirus (Sattar *et al.* 1976), adenovirus, rotavirus and MS2 (Hansen *et al.* 2007). Our findings, utilizing a bench scale model, also demonstrate that 24 h of alkaline stabilization is an effective treatment for the reduction of MS2, as well as T1 L and HM-175 (Table 1).

Previous studies by Hansen *et al.* (2007) suggests that MS2 could serve as an indicator for the presence and persistence of rotavirus Wa and adenovirus 5 during alkaline stabilization as they appeared to be inactivated at a similar rate. By comparing the concentrations of virus at each time point during stabilization, allowing one to generalize the inactivation rates as similar or dissimilar, the study also found MS2 to have a similar inactivation rate to T1 L and HM-175 following alkaline stabilization at 28°C as well as T1 L at 4°C. These results suggest that MS2 could serve as an indicator for the presence of T1 L and HM-175 following alkaline stabilization performed within enclosed facilities at moderate temperatures. Alternatively, it is questionable whether MS2 is inactivated similarly to HM-175 at 4°C as there was large variation during the three trials leading to a significant difference in their inactivation at 0·1 and 2 h. The variation between trials may have been the result of inadequate pH maintenance or phage-specific anti-viral components within the sludge affecting both the test and the control samples. Regardless of the cause of variation, MS2 cannot be suggested as an adequate indicator for the presence of HM-175 during alkaline stabilization at reduced temperatures.

Although these data support the use of MS2 as an indicator for the presence of the aforementioned viruses under moderate temperatures, more extensive studies are needed to provide additional support for the use of phage, such as MS2, as an indicator for the presence and persistence of enteric viruses during alkaline stabilization. Studies utilizing both seeded and endogenous enteric viruses, in various TS matrices may address potential issues contributing to the variability seen in this study and provide insight into the ability of bench scale alkaline stabilization to correlate with conditions that may occur under full-scale operations.

Studies utilizing both DNA and RNA viruses and phages have not demonstrated a significant difference in their inactivation during alkaline stabilization and thermal treatment processes. Comparable reductions between adenoviruses and polioviruses (Bean *et al.* 2007; Hansen *et al.* 2007), and F^+ RNA and DNA phages (Nappier *et al.* 2006) suggests that the effectiveness of treatment is less dependant on the nucleic acid of a virus and more so on the composition of its capsid. There is limited data correlating the inactivation rates of non-denovirus DNA viruses, such as polyomaviruses, to F^+ RNA phages, such as MS2, and would be useful in further supporting MS2 as an indicator for both enteric RNA and DNA virus inactivation during biosolids treatment processes.

The effect of alkaline stabilization on the capsid proteins of the enteric viruses used in this study is not known. Lack of virus detection via plaque assay would normally indicate a lack of infectivity; however, this assumption may be biased depending on the cell line used. It is possible that viral attachment proteins are rendered inoperable for a particular cell line following alkaline stabilization, but retained for other cell lines. Therefore, a lack of plaque formation using one cell line does not indicate a complete loss of infectivity. Alternative methods of viral detection and infectivity determination should be evaluated to confirm that viral reductions seen during alkaline stabilization correlate to a loss in infectivity.

If future studies conclude, as this study suggests, that there is good correlation between the inactivation of the enteric virus and phage during biosolid treatment

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processes, utilization of phage as a process control or indicator should be seriously considered. Incorporation of phage as an indicator for enteric virus persistence during treatment of biosolids will allow for increased efficiency and frequency of pathogen monitoring, reducing the potential public health risk associated with biosolids. Decreased risk and increased awareness should then facilitate a greater acceptance for the land application of biosolids.

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