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Inactivation of Hepatitis A virus HM-175/18f, Reovirus type 1 Lang and Bacteriophage MS2 during alkaline stabilization of biosolids

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ABSTRACT

A bench scale model was used to evaluate the inactivation of male-specific bacteriophage-2, hepatitis A HM-175/18f and reovirus type 1 Lang during alkaline stabilization of raw biosolids at both 28˚C and 4˚C. Male-specific bacteriophage-2 was inactivated at a similar rate compared to reovirus type 1 Lang at each time point evaluated (t = 0.1, 2, 12 and 24 hours) during 28˚C and 4˚C trials, and at a similar rate to hepatitis A HM-175/18f at 28˚C. At 4˚C male specific bacteriophage-2 was not inactivated similarly to hepatitis A HM-175/18f at the first two sampling time points (t = 0.1 and 2 hours) but was inactivated similarly following those time points. These data suggest male-specific bacteriophage-2 could serve as an indicator organism for the inactivation of reovirus type 1 Lang and hepatitis A HM-175/18f during alkaline stabilization at 28˚C and 4˚C.

INTRODUCTION

Under the Code of Federal Regulations part 503 (CFR Part 503), which governs the use and testing requirements of biosolids, enteroviruses are used to represent enteric virus persistence during and following treatment. Enteroviruses, a sub-population of enteric viruses, were chosen as the representative indicator organism of enteric viruses due to similar resistances to treatment processes (8). The standard method for the detection and enumeration of enteroviruses in sludge and treated biosolids is organic flocculation (11) followed by plaque assay (8). Detection and enumeration of enteroviruses in sludge by this method is lengthy and complicated by the presence of inorganic and organic substances that may be toxic to tissue culture during plaque assay (10).

Studies have shown that if sewage is sampled monthly and sewage isolates compared to clinical cases, qualitatively a similar picture of enterovirus activity is evident in the clinical setting (16). Therefore, clinical cases of enteric virus disease should also correlate with presence in sludge and biosolids. Enteric viruses such as hepatitis A virus (HAV) infect approximately 140,000 people a year in the U.S. (2) and are known to be more resistant to both disinfection process and environmental pressures than enteroviruses (13). Relying on enteroviruses as the indictor of enteric viruses such as HAV may pose a health risk, allowing them to persist in land applied biosolids after enteroviruses are no longer detectable.

Phages, somatic and male-specific, have received significant evaluation as indicators of enteric virus presence and persistence following treatment in source water (5), drinking water (17) and waste water (9). Male-specific bacteriophages (MSB) have been suggested as useful indicator organisms for determining the fate of human viruses in sludge (12, 14). The number of MSB, such as male-specific phage-2 (MS2), found in wastewater and human feces are sufficient for detection and can be enumerated by inexpensive and rapid plaque assay methods (4).

Therefore MSB, such as MS2, should be evaluated for their potential to serve as indicator organisms for the inactivation of enteric viruses during biosolid treatment processes.

MATERIALS AND METHODS

Viral propagation. MS2 (ATCC 15597-B1) was propagated using an *Escherichia coli* HS(pFamp)R host (ATCC 700891) harboring a conjugative plasmid and displaying streptomycin and ampicillin resistance (7). REO T1L (ATCC VR-230) was propagated using Buffalo Green Monkey Kidney Cells (BGM) grown to confluency in a closed system at 37˚C. BGM Growth media consisted of Eagles Minimal Essential Media (MEM) and Leibowitz media (L-15) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B). Tissue culture flasks were seeded at a multiplicity of infection (MOI) of approximately 0.96 and incubated for 6 days at 37˚C. Once CPE was evident, flasks were freeze-thawed three times, lysates aliquoted into 1.5 mL cryovials and stored at - 80˚C. HAV HM-175/18f (ATCC VR-1402) was propagated using Fetal Rhesus Monkey Kidney Cells (FRhK-4) grown to confluency in a closed system at 37˚C. FRhK-4 growth media consisted of MEM and L-15 supplemented with 12% FBS, and 1% antibiotic/antimycotic. Tissue culture flasks were seeded with virus at a MOI of approximately 0.01 and incubated at 37˚C for 12 days. Once CPE was evident, flasks were freeze-thawed three times as previously described and lysates chloroform extracted by placing 20 µL of chloroform per 1 mL of sample in a conical centrifuge tube and centrifuging at 10,000 x g, 4˚C, for 10 minutes. Extracted supernatants were then aliquoted into 1.5 mL cryovials and stored at -80˚C.

Viral enumeration. MS2 was enumerated by double agar overly plaque assay (1). REO T1L enumeration was performed using a method to enumerate rotaviruses (18). 25^2 cm cell culture flasks containing a confluent monolayer of BGM cells were rinsed twice with serum-free MEM, inoculated with 100 μL of serially diluted sample and incubated at 37°C for 90 minutes. Following incubation, flasks were filled with 10 mL of a 2X MEM agarose overlay supplemented with 0.01 mg/mL trypsin (Gibco) and incubated inverted for 4 days at 37°C. On the 4th day cells were fixed by adding 2 mL of 10% formaldehyde in normal saline and incubating at 37°C for 24 hours. The following day each culture flask was rinsed gently with warm water, shaken lightly to remove the overlay and stained with 1 mL of 0.1% crystal violet. Enumeration of HAV HM-175/18f was performed using a plaque assay adapted from a previously described method (6). 25^2 cm cell culture flasks containing a confluent monolayer of 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 minutes. Following incubation, flasks were filled with 5 mL of an agarose overlay and incubated for 7 days at 37°C. On the 7th day a second 5 mL overlay containing 50 μl of neutral red (Sigma) was added to the culture flasks, allowed to set, and incubated for 3 days at 37°C.

Alkaline stabilization. A bench scale model for alkaline stabilization was constructed according to methodology established in prior studies (3, 15). The system was challenged three times for each of the two viral combinations at 4˚C and 28˚C. 100 mL of a 4% total solid (TS) sludge was placed into a series of 10 beakers containing a magnetic stir bar. The beakers represented four test time points 0.1, 2, 12, and 24 hours, and six controls. One control beaker was used to determine any background phage or enteric virus in the sludge, and 5 others (0, 0.1, 2, 12, and 24) represented each time point. An 11th beaker of 100 mL 1x PBS was also included as a temperature control. All beakers were brought to the indicated temperatures for the experiment and adjusted to pH 7 under continuous mixing by magnetic stir bar. Test beakers were elevated to pH 12 by addition of 8% Ca $(OH)_{2.}$ Once pH 12 was reached and maintained virus was added as such: 1 mL 10^6 PFU/mL of HAV HM-175/18f and 1 mL 10^6 PFU/mL MS2, or 1 mL 10^6 PFU/mL REO T1L and 1 mL 10^6 PFU/mL MS2. All control beakers (temperature control, 0, 0.1, 2, 12 and 24 hour control) were spiked with approximately the same concentration and combination of viruses. The beakers used to detect endogenous virus and seeded viral concentration at time point 0, were immediately concentrated for enteric virus and bacteriophage. A pH 12 was maintained for 2 hours in the test beakers, then reduced to 11.5 by addition of 1M HCL for the remaining two time points. The pH of the control beakers was maintained at 7 with the addition of either 2M NaOH or 1M HCL throughout the experiment. At the indicated time, both test and control beakers were brought to a pH of 7 and the entire sample concentrated. Concentrations of both enteric virus and bacteriophage were determined by plaque assays performed in triplicate.

Viral recovery from sludge. MS2 was recovered from seeded sludge by mixing 5 mL of sludge from the stabilization experiment with 10 mL of a 3% beef extract in a 50 mL conical centrifuge tube. The pH of the sample was elevated to 9.5 with 2M NaOH then centrifuged at 2,500 x g for 10 minutes at 22˚C to pellet solids. The supernatant was transferred to a new 50 mL conical centrifuge tube, adjusted to a pH of 7 with 1M HCL and refrigerated at 4˚C until use. HAV HM-175/18f and REO T1L were recovered from seeded sludge using a modified version of the

USEPA method EPA/600/4-84/013. Modification to the procedure included eliminating sludge conditioning prior to concentration and further decontamination of eluents by adding 1 mL of chloroform per 10 mL of eluent and centrifuging at 10,000 x g for 10 minutes at 4˚C. Decontaminated eluents were then stored at - 80˚C until use.

Statistical analyses. Data from the alkaline stabilization experiments were square root transformed to insure a normal distribution then analyzed using a two way ANOVA in the statistical software program SYSTAT 11.0. A general linear model was constructed to evaluate the following null hypothesis: 1) alkaline stabilization data significantly varies from trial to trial in the same matrix, 2) alkaline stabilization does not have a significant effect on the inactivation of MS2, REO T1L and HAV HM-175/18f, 3) the effect of alkaline stabilization is not significantly affected by the amount of time that elapses during experimentation. A Tukey's Honestly Significantly Different test (Tukey HSD) was performed in order to determine if there was a significant difference in the inactivation of phage and enteric virus at specific time points during alkaline stabilization.

RESULTS

Viral loss as a result of the recovery method and virucidal components (microbial and chemical) of the sludge was evaluated prior to alkaline stabilization. An average loss of 0.5 logs (79%), 1.5 logs (97%) and 0.5 logs (57%) was demonstrated for MS2, REO T1L and HAV HM-175/18f respectively. These data were used to normalize the total viral reduction detected and determine how much viral inactivation was occurring as a result of alkaline stabilization alone (B. D. Katz and A. B. Margolin, unpublished data).

REO T1L and MS2 at 28˚C. MS2 seeded into a 4% TS sludge at 28˚C was below detectable limits following 0.1 hours of alkaline stabilization in all three trials corresponding to a total reduction of ≥ 5 logs (0.5 logs due to recovery loss). REO T1L seeded into the same sludge sample was below detectable limits following 12 hours of alkaline stabilization in the first two trials and 2 hours in the third trial corresponding to a total reduction of ≥ 6 log (0.5 logs due to recovery loss) (Figure 1). Statistical analyses revealed no statistically significant difference between each trial ($p = 0.707$), that stabilization alone causes a significant inactivation of virus in each trial ($p = 0.000$) and time combined with alkaline stabilization has a significant effect on viral concentration ($p = 0.000$). Tukey HSD demonstrated no statistically significant difference in viral concentration ($p > 0.05$) at all time points evaluated (0.1, 2, 12 and 24 hrs).

REOT1L and MS2 at 4˚C. MS2 seeded into a 4% TS sludge at 4˚C was below detectable limits following 2 hours of alkaline stabilization during the first two trials and 12 hours in the third trial corresponding to a total reduction of ≥ 5 logs (0.5 logs due to recovery loss). REO T1L seeded into the same sludge sample was below detectable limits following 12 hours of alkaline stabilization in the first two trials and 24 hours in the third trial corresponding to a total reduction of ≥ 5 logs in all trials (0.5 logs due to recovery loss) (Figure 2). Statistical analyses revealed no statistically significant difference between each trial ($p= 0.335$), that alkaline stabilization alone causes a significant inactivation of virus in each trial ($p= 0.000$) and time combined with alkaline stabilization has a significant effect on viral concentration ($p= 0.001$). Tukey HSD demonstrated no statistically significant difference in viral concentration ($p > 0.05$) at all time points evaluated.

HAV HM-175/18f and MS2 at 28˚C. MS2 seeded into a 4% TS sludge at 28˚C was below detectable limits following 0.1 hours of alkaline stabilization during the first trial and 2 hours in the second and third trial corresponding to a total reduction of ≥ 5 logs (0.5 logs due to recovery loss). HAV HM-175/18f was below detectable limits following 2 hours of alkaline stabilization in the first and third trial and 12 hours in the second trial corresponding to a total reduction of ≥ 6 logs (1.5 logs due to recovery loss) (Figure 3). Statistical analyses revealed a statistically significant difference between each alkaline stabilization trial ($p = 0.001$), that alkaline stabilization alone causes a significant inactivation of virus in each trial ($p = 0.000$) and time combined with alkaline stabilization has a significant effect on viral concentration ($p = 0.000$). Tukey HSD demonstrated no statistically significant difference in viral concentration ($p > 0.05$) at all time points evaluated.

HAV HM-175/18f and MS2 at 4˚C. MS2 seeded into a 4% TS sludge and alkaline stabilized for 24 hours at 4˚C was below detectable limits following 24 hours of alkaline stabilization during all three trials corresponding to a total reduction of ≥ 5 logs (0.5 logs due to recovery loss). HAV HM-175/18f was below detectable limits following 24 hours of alkaline stabilization in the second and third trial corresponding to a total reduction of ≥ 6 logs (1.5 logs due to recovery loss). During the first trial HAV HM-175/18f was only reduced by 3 total logs (1.5 logs due to recovery loss) following 24 hours of alkaline stabilization (Figure 4). Statistical analyses revealed a statistically significant difference between each alkaline stabilization trial ($p = 0.000$), alkaline stabilization alone causes a significant inactivation of virus in each trial ($p = 0.000$) and time combined with alkaline stabilization has a significant effect on viral concentration ($p = 0.000$). Tukey HSD demonstrated a statistically significant difference in viral concentration ($p < 0.05$) at the 0.1 and 2 hour time points.

DISCUSSION

When the CFR Part 503 regulations were established and enteroviruses were chosen as the indicator organism for the presence of enteric virus, data regarding the survival of HAV HM-175/18f, REO T1L, adenovirus 5 and rotavirus Wa during alkaline stabilization was unavailable. The research presented here, upon comparison to that presented in the literature, demonstrates that HAV HM-175/18f and REO T1L has the ability to persist longer than poliovirus, which was one of the original viruses evaluated during the formation of the CFR 503, following alkaline stabilization in a 4% biosolid matrix (3). Additional research also suggests that Adenovirus 5 and Rotavirus Wa persist longer in alkaline stabilized biosolids that are intended for land application than poliovirus (J. J. Brabants and A. B. Margolin, submitted for publication). Data from both of these studies may indicate that the original risks associated with the land application of alkaline stabilized biosolids might have been underestimated. Additional studies evaluating these viruses in biosolids produced by other mechanisms, such as anerobic digestion or composting, should also be performed to determine if they persist longer in these matricies. Ultimately, if it is determined that these viruses and others which could not previously be evaluated, demonstrate greater persistence then those viruses originally used for the development of the CFR 503, further risk analysis should be undertaken to determine if there is a greater risk from virus exposure associated with the practice of land application then was originally determined.

In addition, the research described here and by Brabants and Margolin demonstrates preliminary data that under prescribed conditions, MS2 or other male specific phages, may serve as an indicator organism for the inactivation of HM-175/18f, REO T1L, adenovirus 5 and rotavirus Wa during alkaline stabilization. Monitoring for these viruses in biosolids that are produced by alkaline stabilization is costly, labor intensive and slow, making monitoring an impractical approach. However, the preliminary data described here and by Brabants and Margolin does demonstrate the possibility of using bacteriophage as a means to evaluate the presence of these viruses. MS2 or other phages, such as somatic phages, need to be evaluated further to determine if they can be used as an indicator organism to accurately predict the inactivation of different enteric viruses that are present and inactivated during different biosolids processes, such as alkaline stabilization, composting and anerobic digestion. Assays that detect phage are rapid, relatively easy to perform and are much less expensive then those used for the detection of the anthropogenic virus themselves. If additional studies demonstrate, as this study did, that there is a good correlation between the inactivation of the human pathogen and the phage, eventually utilization of phage as an indicator organism can be evaluated for routine monitoring of processed biosolids.

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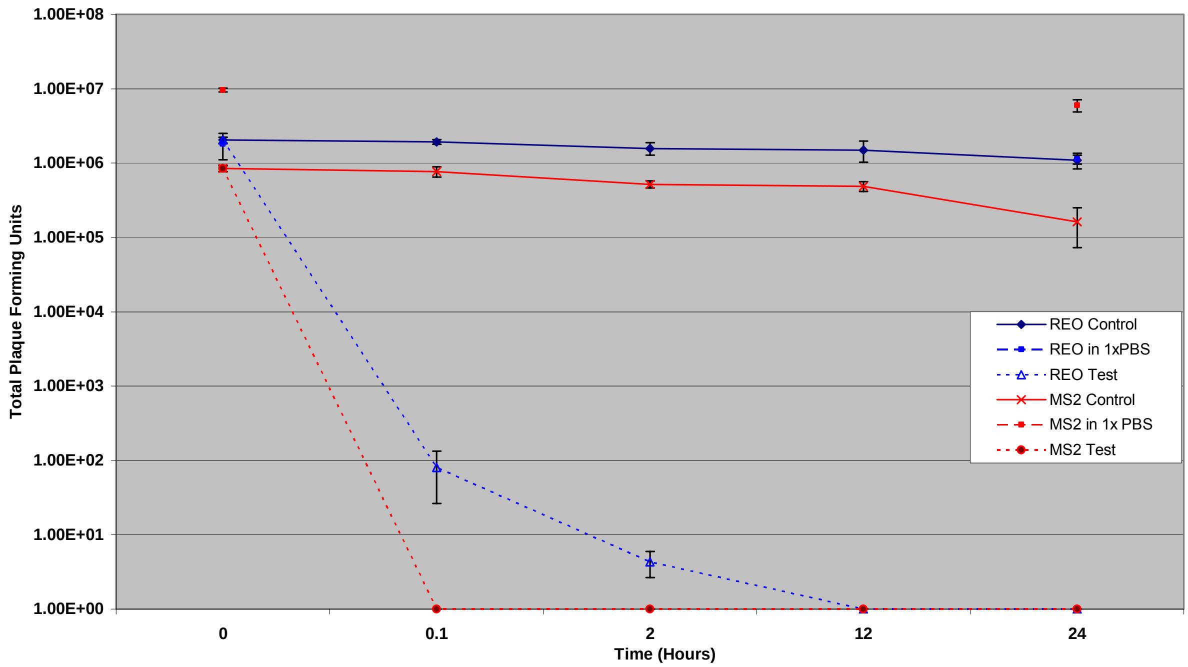

Figure 1: Average Total Reduction of MS2 and REO T1L Following Three Trials of Alkaline Stabilization Performed for 24 Hours in a 4% TS Sludge at 28˚C- REO T1L seeded to a concentration of 2.04 x 10^6 PFU was below detectable limits following 12 hours of alkaline stabilization (REO test). MS2 seeded into the same sludge sample to a concentration of 8.48 x 10^5 PFU was below detectable limits following 0.1 hours of alkaline stabilization (MS2 test). Error bars represent standard error

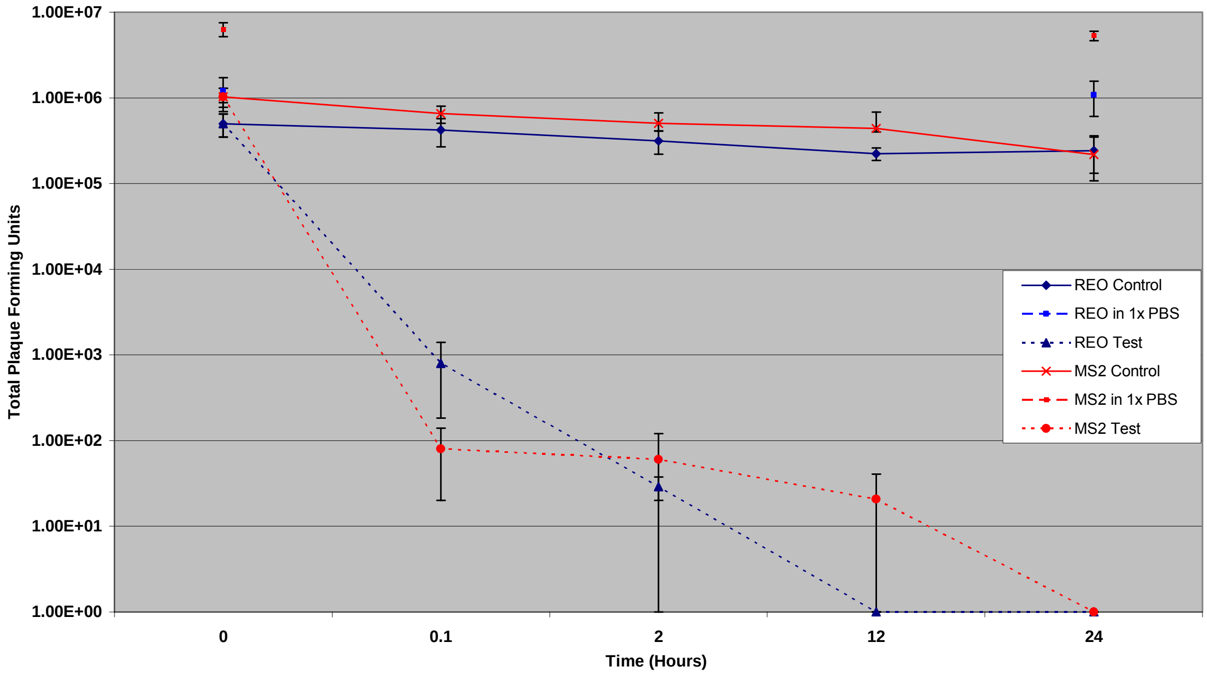

Figure 2: Average Total Reduction of MS2 and REO T1L Following Three Trials of Alkaline Stabilization Performed for 24 Hours in a 4% TS Sludge at 4˚C- REO T1L seeded to a concentration of 5.0x 10^5 PFU was below detectable limits following 2 hours of alkaline stabilization (REO test). MS2 seeded to a concentration of 1.03x 10^6 PFU in the same sludge sample was below detectable limits following 12 hours of alkaline stabilization (MS2 test). Error bars represent standard error.

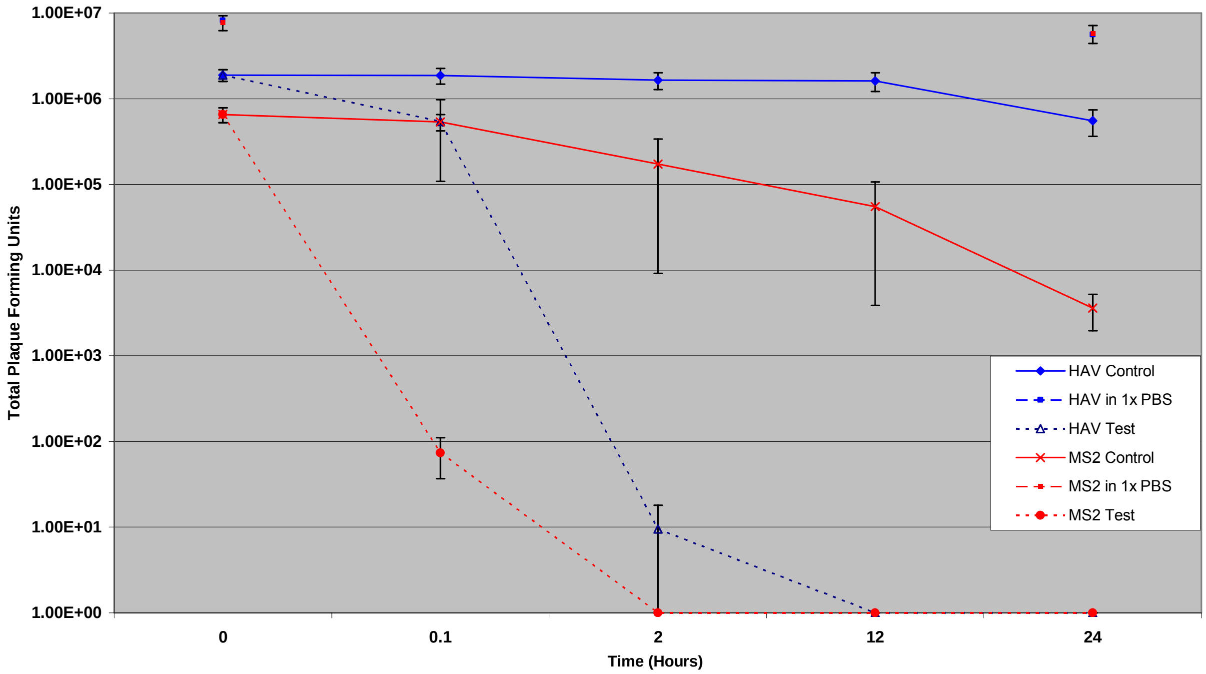

Figure 3: Average Total Reduction of MS2 and HAV HM-175/18f Following Three Trials of Alkaline Stabilization Performed for 24 Hours in a 4% TS Sludge at 28˚C- HAV HM-175/18f seeded to a concentration of 1.9 x 10^6 PFU was below detectable limits following 12 hours of alkaline stabilization (HAV test). MS2 seeded into the same sludge to a concentration of 6.5 x 10^5 PFU was below detectable limits following 2 hours of alkaline stabilization (MS2 test). Error bars represent standard error.

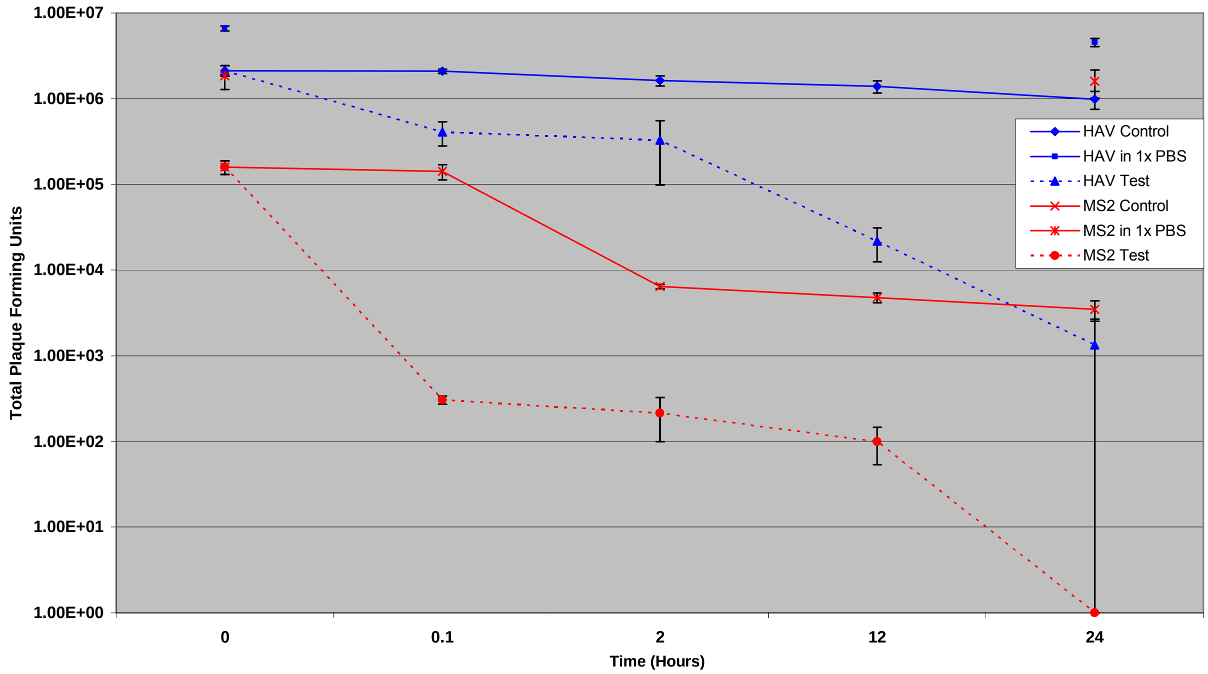

Figure 4: Average Total Reduction of MS2 and HAV HM-175/18f Following Three Trials of Alkaline Stabilization Performed for 24 Hours in a 4% TS Sludge at 4˚C- HAV HM-175/18f seeded to a concentration of 2.1 x 10^6 PFU was was still at detectable limits (1.3x 10^3 PFU) following 24 hours of alkaline stabilization (HAV test). MS2 seeded into the same sludge at a concentration of 1.59 x 10^5 PFU was below detectable limits following 24 hours of alkaline stabilization (MS2 test). Error bars represent standard error.