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Survivability, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater

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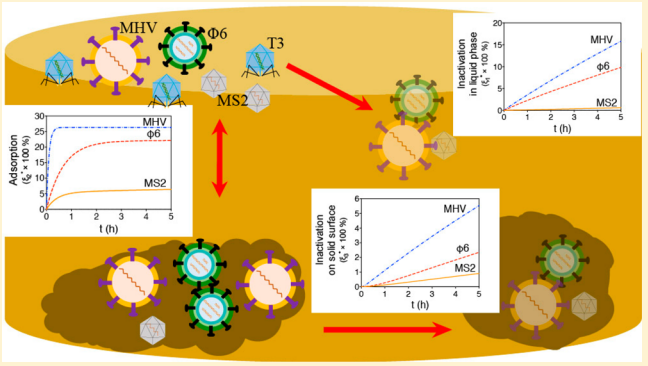
Supporting Information

ABSTRACT: Many of the devastating pandemics and outbreaks of the 20th and 21st centuries have involved enveloped viruses, including influenza, HIV, SARS, MERS, and Ebola. However, little is known about the presence and fate of enveloped viruses in municipal wastewater. Here, we compared the survival and partitioning behavior of two model enveloped viruses (MHV and $\phi6$) and two nonenveloped bacteriophages (MS2 and T3) in raw wastewater samples. We showed that MHV and $\phi6$ remained infective on the time scale of days. Up to 26% of the two enveloped viruses adsorbed to the solid fraction of wastewater compared to 6% of the two nonenveloped viruses. Based on this partitioning behavior, we assessed and optimized methods for recovering enveloped viruses from wastewater. Our optimized ultrafiltration method resulted in mean recoveries (±SD) of 25.1% (±3.6%) and 18.2% (±9.5%) for the enveloped MHV and $\phi6$, respectively, and mean recoveries of 55.6% (±16.7%) and 85.5% (±24.5%) for the nonenveloped MS2 and T3, respectively. A maximum of 3.7% of MHV and 2% of MS2 could be recovered from the solids. These results shed light on the environmental fate of an important group of viruses and the presented methods will enable future research on enveloped viruses in water environments.



INTRODUCTION

Recent severe disease outbreaks caused by enveloped viruses, such as Ebola, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and avian influenza H5N1 have heightened fears of an imminent deadly viral pandemic. The major transmission routes of these viruses involved direct person-to-person contact or indirect contact with contaminated objects.[1,2] Human enveloped viruses are often presumed to exist in low concentrations in human excrement and undergo rapid inactivation in aqueous environments; however, several lines of evidence suggest these assumptions are not always correct. The genes of coronaviruses and avian influenzas have been detected in the feces of infected individuals,[3−9] and some enveloped viruses were measured in wastewater biosolid residuals.[10] Likewise, some enveloped viruses can survive for days to weeks in pasteurized wastewater.[11−13] A review of virus T_{90} values (i.e., time to reach 90% inactivation) suggests that avian influenza viruses survive just as long, if not longer, than nonenveloped enteric viruses in some aqueous environments.[14] Based on this information, it is therefore feasible that sewage and fecal-contaminated water could serve as vectors for certain enveloped viruses. Indeed, a SARS coronavirus outbreak in an apartment complex in Hong Kong was attributed to the transport of viruses in wastewater to the air ducts.[15]

The vast majority of studies on the presence and fate of viruses in human waste and municipal wastewater have focused on nonenveloped enteric viruses (e.g., adenoviruses, polioviruses, enteroviruses, noroviruses and rotaviruses).[16−21] These viruses replicate in human gut tissues and transmit diseases primarily via the fecal-oral route. Due to the major role of water and food in the transmission of enteric viruses, there are a number of established methods for nonenveloped enteric virus detection in complex environmental matrices. Enveloped viruses differ structurally from nonenveloped viruses due to the presence of a lipid bilayer membrane outside the viral protein capsid, which contains proteins or glycoproteins. The different functional groups on the outer surface of enveloped viruses compared to nonenveloped viruses likely impact their survival and partitioning behavior in aqueous environments.[22−24] Likewise, methods to concentrate and recover nonenveloped enteric viruses from wastewater and other environmental matrices may not be suitable for enveloped viruses. For example, lipid layers are sensitive to the detergents and organic solvents[25,26] that are commonly used to extract and purify nonenveloped enteric viruses.

To address the paucity of data on the fate and recovery of enveloped viruses in wastewater matrices, we studied the survival and partitioning behavior of the human enveloped virus

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Table 1. Characteristics of Tested Viruses

virus	structure	family/genus	genome type	genome size (Kb)	particle size (nm)
MHV	enveloped	Coronaviridae/*Coronavirus*	(+)ssRNA	32	100
ϕ6	enveloped	Cystoviridae/*Cystovirus*	segmented dsRNA	13.5	80
MS2	nonenveloped	Leviviridae/*Levivirus*	(+)ssRNA	3.6	25
T3	nonenveloped	Podoviridae/T7-like viruses	dsDNA	38.2	50 × 20 (tail)

surrogates, murine hepatitis virus (MHV) and *Pseudomonas* phage ϕ6, in pasteurized and unpasteurized wastewater. We compared the inactivation kinetics and liquid−solid partitioning of the two enveloped viruses with two nonenveloped virus surrogates, *Enterobacteria* phage MS2 and T3. Furthermore, we systematically tested the effectiveness of three virus recovery methods—initially developed for using on enteric viruses—for extracting and concentrating enveloped viruses from both liquid and solid fractions in wastewater. Finally, we proposed an optimized ultrafiltration method for detecting both enveloped and nonenveloped viruses.

MATERIALS AND METHODS

Wastewater Samples. Wastewater samples were collected from the Ann Arbor Wastewater Treatment plant, an activated sludge treatment plant serving roughly 115 000 people with an average flow rate of 19 million gallons per day (MGD). Grab samples were collected after wastewater equalization, screening, and grit removal chambers, and just before the primary settling tanks. All samples were collected and sealed in sterile plastic bottles and then immediately transported on ice to laboratories at the University of Michigan where they were stored at 4 °C and analyzed within 24 h. Wastewater pH, total suspended solids (TSS), volatile suspended solids (VSS), and total chemical oxygen demand (COD) were measured with standard methods.[27]

Virus Strains and Methods. We chose to study MHV strain A59 and *Pseudomonas* phage ϕ6 because they are common surrogates for human enveloped viruses (Table 1).[11,13,28] We also studied two nonenveloped *Enterobacteria* phages MS2 and T3 to allow for direct comparisons between enveloped and nonenveloped virus inactivation, partitioning, and recovery.[29−31]

MHV strain A59, and its supporting cell lines L2 and DBT, were kindly provided by Dr. Leibowitz's lab at Texas A&M Health Science Center College of Medicine. L2 and DBT cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% newborn calf serum, 1% L-glutamine, and 1% penicillin/streptomycin, and incubated at 37 °C with 5% CO_2. MHV stocks were propagated in DBT and titered by plaque assay on L2 according to a published protocol.[32] After amplification, MHV stocks were centrifuged at 3000*g* for 10 min, and then filtered through a 0.22 μm poly(ether sulfone) (PES) membrane (Millipore, USA), in order to remove cell debris and aggregated viruses. The MHV stocks (~10^6 PFU mL^{-1}) were stored at −80 °C.

ϕ6 and its bacterial host *Pseudomonas syringae* were kindly provided by Dr. Linsey Marr's lab at Virginia Tech. *P. syringae* was grown in Luria−Bertani (LB) medium containing 5 g L^{-1} NaCl at 26 °C. To propagate ϕ6 stocks, soft LB-agar (0.7% agar) layers were removed from the double-layer plates, and dissolved in 3 mL of LB medium.[33] The recovered viruses were purified with centrifugation at 3000*g* for 10 min at 4 °C and filtration through 0.22 μm PES membranes. The ϕ6 stocks (~10^{10} PFU mL^{-1}) were stored at 4 °C.

MS2 (ATCC 15597-B1) and T3 (recovered from ATCC 11303-B4), and their corresponding *Escherichia coli* hosts ATCC 15597 and ATCC 11303, respectively, were purchased from American Type Culture Collection (ATCC). The MS2 and T3 were propagated and assayed in their *E. coli* hosts based on published methods.[34,35] The viruses were purified with an Econo Fast Protein Liquid Chromatography system (Bio-Rad) equipped with a HiPrep Sephacryl S-400 HR column (GE). The collected viral fraction was concentrated with 100 kDa Amicon ultracentrifugal filters (Millipore), and filtered through a 0.22 μm PES membrane filter. The final MS2 and T3 stocks (~10^{11} PFU mL^{-1}) were stored in phosphate buffer (5 mM NaH_2PO_4 and 10 mM NaCl, pH 7.5) at 4 °C.

Survivability Experiments. Virus surrogates were spiked into 30 mL samples of unpasteurized and pasteurized wastewater to final concentrations of 3 × 10^4 PFU mL^{-1} for MHV and 5—8 × 10^5 PFU mL^{-1} for ϕ6, MS2 and T3; the lower MHV concentrations were due to the lower MHV stock concentrations. Wastewater was pasteurized by heating to 70 °C for 3 h; this treatment is consistent with previous studies involving enveloped virus survival in pasteurized wastewater.[11,13] Wastewater samples were quickly mixed after viruses were added, titered for the initial virus concentrations, and then incubated at 25 or 10 °C to mimic typical summer and winter wastewater temperatures. Aliquots of wastewater were removed at specific incubation times and infective virus concentrations were enumerated with plaque assays. The wastewater samples were diluted at least 10-fold to minimize wastewater effects on the host cells. Replicate experiments (n = 3) were conducted in wastewater samples collected on different days to incorporate potential impacts of wastewater variation on virus survivability.

Partitioning Experiments. To evaluate the kinetics and extent of virus sorption to wastewater solids, the virus surrogates were spiked into 30 mL samples of untreated wastewater and wastewater with solids removed via centrifugation at 30 000*g* for 10 min. (i.e., solids-removed samples). This centrifugation treatment, which was previously shown to remove solids less than 0.3 μm in diameter,[36] consistently removed 85—95% of the TSS in our wastewater samples (SI Table S2). Samples were spiked to achieve final virus concentrations of 5 × 10^4 PFU mL^{-1} for MHV, and 6—8 × 10^5 PFU mL^{-1} for ϕ6, MS2, and T3—these were low enough to be feasible concentrations present in wastewater (<10^6 PFU mL^{-1}) and high enough that more than 99% loss could be quantified with plaque assays. The spiked samples were stirred and then incubated at 4 °C; this temperature is at the low-end of mean municipal wastewater temperatures in the U.S. (3—27 °C)[37] and was selected to minimize virus inactivation through the duration of the experiment. At various incubation times, aliquots of the untreated and solids-removed samples were centrifuged at 30 000*g* for 10 min, and the centrates were assayed for infective viruses.

Virus inactivation and sorption kinetics in wastewater batch reactors were analyzed with an approach proposed by Grant et al. that accounts for virus sorption and desorption from

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sorbents, as well as inactivation in the liquid and solid fractions.[38] In our system, the solids-containing samples were the untreated wastewater influent and the solids-free samples were wastewater samples with solids removed via centrifugation. Virus inactivation in the wastewater liquid was assumed to be equal to virus inactivation in the solids-removed sample, and to follow first-order kinetics:

$$\ln C_l^* = -k_1 t \tag{1}$$

where, C_l^* is the nondimensional concentration of infective viruses measured in the solids-removed wastewater samples ($C_l/C_{l,0}$), t is the incubation time in hours, and k_1 (h^{-1}) is the first-order virus inactivation constant in the solids-removed wastewater.

In a wastewater sample spiked with viruses, the nondimensional concentration of infective viruses in the wastewater liquid $C_{l,ww}^*$ is related to the fraction of viruses inactivated in the liquid phase (ξ_1^*), and the fraction reversibly adsorbed to wastewater solids (ξ_2^*):

$$C_{l,ww}^* = 1 - \xi_1^* - \xi_2^* \tag{2}$$

The change of the viral fraction in the liquid and solid phases with time can be described with the following set of differential equations:

$$\frac{d\xi_1^*}{d\tau} = 1 - \xi_1^* - \xi_2^* \tag{3a}$$

$$\frac{d\xi_2^*}{d\tau} = N_b\left[1 - \frac{n_{sro}^*}{N_f - 1} - \xi_1^* - \xi_2^*\left(\frac{N_f}{N_f - 1}\right) + \xi_3^*\left(\frac{N_s}{N_f - 1}\right)\right] \tag{3b}$$

$$\frac{d\xi_3^*}{d\tau} = N_i[n_{sro}^* + \xi_2^* - N_s\xi_3^*] \tag{3c}$$

where, ξ_3^* is the fraction of viruses inactivated on the solid surface; τ is the nondimensional time, equal to $k_1 t$; n_{sro}^* is the initial amount of viruses reversibly adsorbed to solids (assumed zero in the study); $N_b = k_2/k_1$, where k_2 (h^{-1}) is the rate constant for reversible virus adsorption; $N_s = (k_3+k_4)/k_3$, where k_3 (h^{-1}) is the rate constant for virus inactivation at the solid surface and k_4 (h^{-1}) is the rate constant for the conversion of reversibly adsorbed viruses to an irreversibly adsorbed state; $N_i = k_3/k_1$; $N_f = [(k_2 W/k_{-2} V)+1]$, where k_{-2} (g L^{-1} h^{-1}) is the rate constant for virus desorption from solid phase to liquid phase, W (g) is the mass of solids, and V (L) is the liquid volume. At time zero ($\tau = 0$), $\xi_1^* = \xi_2^* = \xi_3^* = 0$.

The relationship between $C_{l,ww}^*$ and incubation time t was solved from numerical simulations of the above differential equation system with the fourth order Runge−Kutta algorithm in MATLAB2015. An extensive description of the equation derivations, simplifications, and parameter calculations can be found in ref 38.

Virus Recovery Methods. Virus recovery methods were tested with wastewater that had been spiked with one enveloped virus (MHV) and one nonenveloped virus (MS2). Three approaches for separating and concentrating viruses from the liquid fraction of municipal wastewater, including polyethylene glycol (PEG) precipitation,[39,40] ultracentrifugation,[19] and ultrafiltration,[18,41] were selected based on their previous application in recovering viruses from wastewater. Published enteric virus methods that involved steps likely to inactivate the enveloped viruses (e.g., pH adjustment outside 6−8 range,[42−44] organic solvent extractions,[25,26] etc.) were avoided. The best-performing method for MHV and MS2 was then further validated with the enveloped virus ϕ6 and nonenveloped virus T3. In the first set of experiments, MHV and MS2 were spiked in wastewater samples to final concentrations of 8×10^3 PFU mL^{-1} and 5×10^5 PFU mL^{-1}, respectively. Samples were then briefly mixed and incubated at 4 °C for 1 h before they were treated with the extraction/concentration techniques; the 1 h incubation time was selected based on the results from the partitioning experiments. In each experiment, samples were concentrated 100 × , and infective viruses in the concentrates were measured with plaque assays. Virus recovery was calculated based on the following relationship:

$$\text{Virus recovery}(\%) = \frac{C_{con}\cdot V_{con}}{C_s\cdot V_s} \times 100\% \tag{4}$$

where ($C_s \cdot V_s$) equals the number of infective viruses in the spike and ($C_{con} \cdot V_{con}$) is the number of infective viruses measured in the concentrate.

Polyethylene Glycol (PEG) Precipitation Method. Following incubation with the spiked viruses, wastewater samples (250 mL) were centrifuged at 2500*g* for 5 min at 4 °C to remove large solids. The centrate was collected and mixed with 8% (w/v) of PEG 8000 and 0.5 M of NaCl. The mixture was incubated for 2 h at 4 °C, and then centrifuged at 10 000*g* for 30 min at 4 °C. The PEG pellet was resuspended in 2.5 mL phosphate buffered saline (PBS, pH 7.4; Life Technologies) and assayed for infective viruses.

Ultracentrifugation Method. Following incubation with the viruses, wastewater samples (60 mL) were centrifuged at 100 000*g* for 1 h at 4 °C using a Sorvall WX Ultra centrifuge (Thermo Scientific, Germany; SureSpin 630 (36 mL) rotor, P/N 79368; SureSpin swinging bucket, P/N 79388). The pellet was resuspened in 8 mL of 0.25 M glycine buffer (pH 9.5) and allowed to sit on ice for 30 min. After neutralizing the solution pH with 16 mL PBS, the solids were removed by centrifugation at 10 000*g* for 15 min at 4 °C. The supernatant was collected and centrifuged again at 100 000*g* for 1 h at 4 °C to pellet the viruses. The final virus pellet was dissolved in 600 μL PBS.

Ultrafiltration Method. Following incubation with the spiked viruses, solids in the wastewater samples (250 mL) were removed by either centrifuging at 30 000*g* for 10 min at 4 °C, or by centrifugation at 2500*g* for 5 min at 4 °C followed by filtration through 0.22 μm PES membrane filters. After the large solids had been removed, the samples were concentrated with Centricon centrifugal filters (Millipore) to a final volume of 2.5 mL. Recoveries from centrifugal filters with 10 kDa and 100 kDa cut-offs were compared. Centrifugal filter reuse was tested by first washing used filters with 100 mL of 0.5 M NaOH and then storing the regenerated filters in 70% ethanol. The reused filters were rinsed with 100 mL of Milli-Q water prior to use.

In an attempt to recover viruses associated with wastewater solids, the solids collected in the centrifugation step prior to ultrafiltration were mixed with different elution buffers, including PBS, 0.05 M glycine buffer (pH 8.5), 0.05 M glycine buffer (pH 9.5), 0.05 M glycine buffer (pH 10.5), 3% beef extract (pH 7.5), 3% beef extract (pH 9.5), and 3% beef extract with 0.5 M sodium chloride (pH 9.5). Suspensions were set on ice for 30 min and gently shaken every 10 min. The solutions

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were centrifuged at 10 000*g* for 15 min at 4 °C and the resulting centrate was neutralized with PBS (pH 7.4), and then titered for infective viruses.

Statistical Analyses. Nonparametric *t* tests were applied to two groups of experimental data to assess statistical significance. Two-tailed *P* values were calculated, and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Comparison of Virus Survival in Wastewater. Inactivation of the two enveloped viruses (MHV and ϕ6) and nonenveloped virus MS2 in unpasteurized and pasteurized wastewater at 10 and 25 °C followed first-order kinetics (Figure 1; SI Table S3), with inactivation proceeding faster for the enveloped viruses. In unpasteurized wastewater at 25 °C, the T_{90} (±SD) values for MHV and ϕ6 were 13 (±1) and 7 (±0.4) hours, respectively, and 121 (±36) hours for MS2 (SI Table S3). The nonenveloped T3 virus survived much longer than the other virus surrogates with no significant decrease in infectivity observed within the 48 h experiments for both temperatures (Figure 1). This is consistent with long survival times reported for tailed phages in adverse conditions.[45] The inactivation kinetics of the enveloped viruses were significantly ($P < 0.0001$) slower in wastewater at 10 °C compared to 25 °C (SI Figure S4), with T_{90} (±SD) values of 36 (±5) and 28 (±2) hours for MHV and ϕ6 at 10 °C, respectively (SI Table S3). Like T3, MS2 inactivation was not statistically different at the two temperatures ($P = 0.1813$) within the tested time scale (SI Figure S4).

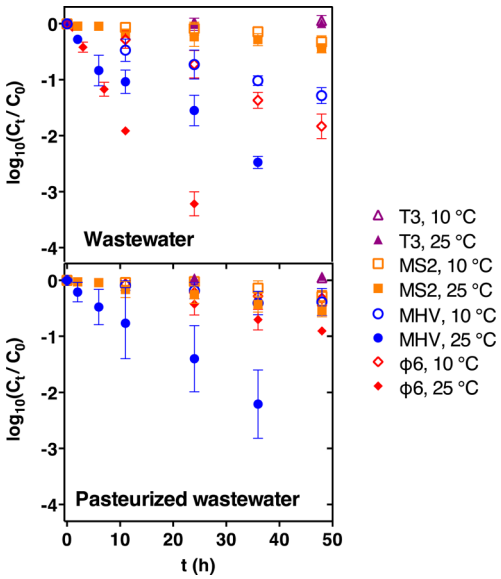

Figure 1. Virus survival in wastewater and pasteurized wastewater at 10 and 25 °C. Viruses were spiked into wastewater to final concentrations of 3×10^4 PFU mL^{-1} for MHV and 5—8 $\times 10^5$ PFU mL^{-1} for MS2, T3 and ϕ6. Error bars represent the standard deviations of replicates from wastewater samples collected on different days ($n = 3$). SI Table S3 summarizes corresponding rate constants and estimated T_{90} values.

Inactivation kinetics of the enveloped viruses MHV, ϕ6, and Ebolavirus in pasteurized or gamma-irradiated wastewater have been reported previously.[11−13] In our experiments, the two enveloped viruses lost infectivity at a significantly slower rate in pasteurized wastewater compared to unpasteurized wastewater, except for the case of MHV at 25 °C (Figure 1; SI Table S3). The most pronounced effect occurred with ϕ6, which had a first-order inactivation rate constant (±SD) of 0.317 (±0.022) h^{-1} in unpasteurized wastewater and 0.044 (±0.004) h^{-1} in pasteurized wastewater at 25 °C. A statistically significant difference in the inactivation kinetics of the nonenveloped viruses was not observed in pasteurized wastewater and unpasteurized wastewater; this may be due to the fact that our experiments were stopped before 90% of the nonenveloped viruses were inactivated. Discrepancies in inactivation kinetics in sterilized and nonsterilized wastewater have been reported previously for nonenveloped viruses,[46] and may be due to bacterial extracellular enzyme activity and protozoan or metazoan predation.[47,48] Overall, the results suggest that unpasteurized wastewater samples should be employed for survivability tests when feasible.

Wastewater residence times in sewage systems are typically less than 24 h. Although ϕ6 and MHV had T_{90} values of 7—13 h in unpasteurized wastewater at 25 °C, the T_{90} values increase to 28—36 h at 10 °C. Human enveloped viruses excreted in feces may therefore reach wastewater treatment plants in an infective state, especially in cool climates. Local outbreaks and global pandemics of enveloped viruses excreted in feces or urine are therefore relevant for wastewater utilities.

Comparison of Virus Partitioning in Wastewater. The measured concentrations of infective MHV and ϕ6 in the solids-removed wastewater samples immediately after spiking, mixing, and centrifuging, were consistently lower than the theoretical concentrations based on the amount of viruses spiked into the sample (SI Figure S1). Approximately 47% of the spiked MHV and 77% of the spiked ϕ6 were recovered in the centrate of the solids-removed wastewater. This is compared to a nearly 100% recovery of the nonenveloped viruses MS2 and T3. Nearly all of the MHV was recovered when it was spiked into PBS and centrifuged in the same manner (SI Figure S1). This suggests that a fraction of the enveloped viruses (53% MHV and 23% ϕ6) were rapidly inactivated in the solids-removed wastewater. A pronounced initial decrease in infective virus concentration was previously observed when Ebola virus was added to pasteurized wastewater.[12] In those experiments, the number of infective Ebola viruses decreased rapidly over the first 24 h (~2-log loss) and then stabilized at a much slower inactivation rate over the subsequent 7 days. Similar biphasic inactivation kinetics have also been observed with nonenveloped viruses, which were attributed to subpopulations of viruses with varied susceptibilities to solution chemistry or temperature.[38] In our partitioning experiments, we chose to normalize measured concentrations in the wastewater and solids-removed wastewater samples over time to concentrations measured in solids-removed samples immediately after they were spiked with viruses, mixed, and centrifuged. We felt this approach was justified because the behaviors of the persistent subpopulations are of most interest for real wastewater systems.

MHV, ϕ6, and MS2 concentrations decreased significantly over a three-day period in the solids-removed wastewater samples (Figure 2) and the resulting rate constants were assumed to equal virus inactivation rates in the liquid fraction of wastewater (eq 1, k_1).[38] When the viruses were spiked in wastewater samples containing solids, the normalized MHV and ϕ6 concentrations in the wastewater liquid phase (in centrate after centrifugation) decreased rapidly in the first hour, and then eventually decreased at the same rate as virus

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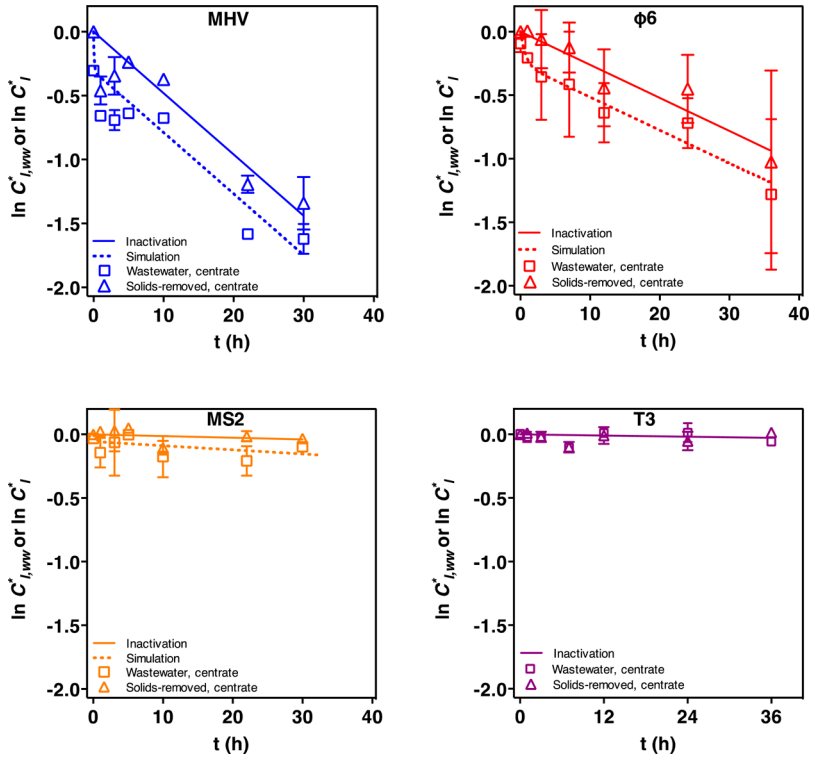

Figure 2. Adsorption and inactivation kinetics and model simulations for enveloped viruses (MHV and $\phi 6$) and nonenveloped viruses (MS2 and T3) in 4 °C wastewater. Viruses were spiked into wastewater and solids-removed wastewater samples to final concentrations of 5×10^4 PFU mL^{-1} for MHV, and 6—8×10^5 PFU mL^{-1} for MS2, T3 and $\phi 6$. C_l^* and $C_{l,ww}^*$ are nondimensional concentrations of infective viruses in the solids-removed sample centrates and wastewater sample centrates, respectively. Both values were normalized to the initial measured virus concentration in the solids-removed sample centrates. No significant decline in T3 infectivity was observed within 36 h. Error bars represent the range of data from duplicate experiments conducted in wastewater samples collected on different days (n = 2).

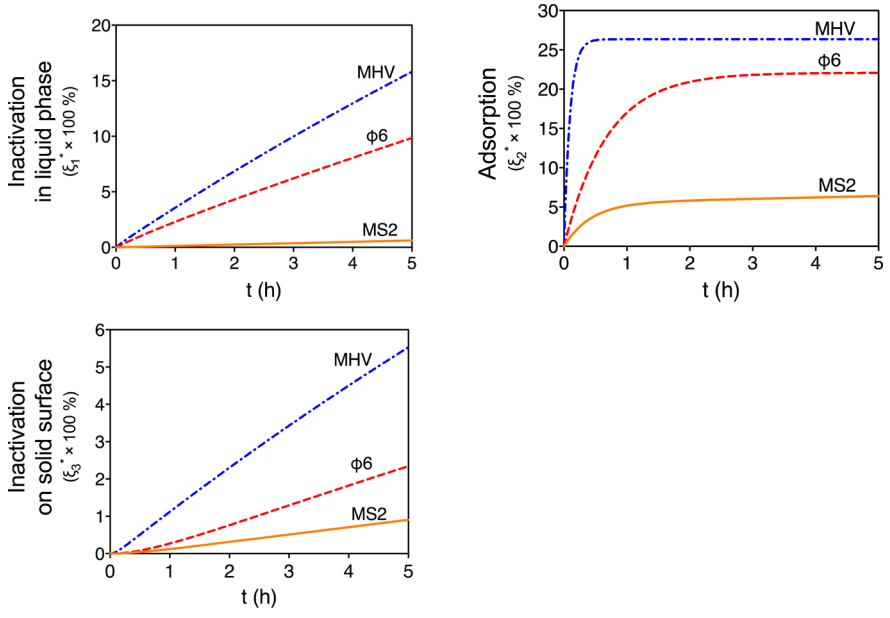

Figure 3. Models for adsorption and inactivation kinetics of enveloped viruses (MHV and $\phi 6$) and nonenveloped viruses (MS2) in 4 °C wastewater. ξ_1^* represents the fraction of viruses inactivated in liquid fraction of wastewater; ξ_2^* represents the fraction of viruses reversibly adsorbed to wastewater solids; ξ_3^* represents the fraction of viruses inactivated on the solid surface.

inactivation in the solids-removed sample (Figure 2). The MS2 concentration in the wastewater liquid phase decreased rapidly at first, and then slowed to a rate that was faster than MS2 inactivation in the solids-removed sample (Figure 2). No significant decay of T3 was observed in the solids-removed wastewater samples or the liquid phase of wastewater samples.

Based on these results, the MHV and $\phi 6$ sorption kinetics can be best described by a noninstantaneous quasi-equilibrium adsorption model in which the virus sorption to wastewater

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solids does not occur instantaneously and the inactivation rates in the wastewater solid and liquid phases are equal (SI Table S4). A similar model was used to describe bacteriophage λ sorption kinetics with sand.[38] In comparison, MS2 behavior is best described by the noninstantaneous quasi-equilibrium adsorption and surface sink model. In this model, virus inactivation is faster in the solid phase than in the liquid phase (SI Table S4); a similar model was proposed for the interaction of bacteriophage MS2 and PRD1 with sediments.[49] Bacteriophage T3 could not be modeled due to the nonsignificant decreases in infective viruses measured over the experiment time scale.

These models predict that 26% of MHV, 22% of ϕ6, and 6% of MS2 adsorbed to wastewater solids at equilibrium (Figure 3; SI Table S4). Although the T3 virus kinetics could not be modeled, < 5% of the spiked T3 had partitioned to the wastewater solids at the end of the 36 h experiment; this suggests that like MS2, T3 partitions overwhelmingly to the liquid fraction of wastewater (Figure 2). The equilibrium percentages reported here are not representative for all wastewaters because wastewater solids concentrations vary widely. It should be noted that our wastewater solid concentrations were typical for medium-strength municipal wastewaters[37] (SI Table S1) with an average TSS value of 235 mg L^{-1}.

The partitioning results for MS2 and T3 are consistent with an early observation that wastewater solids are poor at absorbing enteric viruses.[50] Wastewater solids tend to be negatively charged, as is MS2 (isoelectric point = 3.9). The isoelectric point for T3 has not been reported, but the similar T2 and T4 viruses have isoelectric points <6.[51] A study on the adsorption of four nonenveloped viruses to various solid surfaces demonstrated that long-ranged electrostatic interactions and hydrophobic effects between the virus capsid proteins and the sorbent surfaces dictated adsorption, with short-ranged van der Waals and steric interactions playing less important roles.[52] Similar work has not been conducted for enveloped viruses, and the impact that the surface phospholipids and various membrane proteins have on partitioning remains elusive.

Despite the poor sorption of nonenveloped enteric viruses to wastewater solids, some enteric viruses have been observed in primary settled solids in high concentrations.[36,53] In such cases, the viruses were likely released into wastewater within or strongly associated with fecal solids and never reached equilibrium between the liquid and solid fractions. When excreted in watery diarrhea or urine, the viruses would more likely reach equilibrium. Our results suggest that if allowed to reach equilibrium, enveloped viruses more strongly associate with wastewater solids than nonenveloped viruses. Consequently, enveloped viruses would be removed to a greater extent than nonenveloped viruses in primary wastewater treatment. More enveloped and nonenveloped viruses will need to be tested to confirm the results obtained with the two enveloped and two nonenveloped model viruses.

In addition to relaying information on virus partitioning between solid and liquid phases at equilibrium, the models also predicted the amount of time it takes for the viruses to reach equilibrium. This information is important for virus recovery experiments, where viruses are spiked into an environmental sample and then extracted and quantified with various techniques. If the spiked viruses are extracted too soon, results may be biased due to the spiked viruses in liquid phase. In water with soils and clays, nonenveloped virus adsorption is assumed to reach equilibrium within an hour.[54] Our models estimated that the viruses in wastewater reached 90% of equilibrium concentrations after 0.3—1.5 h, and 99% of equilibrium concentrations after 0.4—2.9 h (Figure 3; SI Table S4). Based on these results, we allowed samples to equilibrate for at least 1 h before extraction methods were tested.

Virus Recovery from Wastewater. According to the simulation results of virus partitioning, greater than 70% of the infective model enveloped viruses were associated with wastewater liquids at equilibrium. We therefore focused primarily on the wastewater liquid fraction in our virus recovery experiments. Of the three methods we tested, the ultrafiltration method and the PEG precipitation methods involved an initial step to remove wastewater solids and then focused on recovering the viruses in the liquid phase. Ultracentrifugation method, on the other hand, involved pelleting all of the wastewater solids and colloids and then extracting the viruses from the pellet.

The enveloped MHV recoveries were consistently lower than the nonenveloped MS2 recoveries when the PEG precipitation and ultrafiltration methods were applied (Figure 4); this was

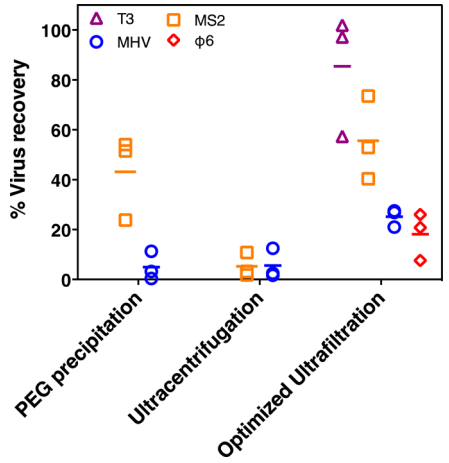

Figure 4. Recoveries for enveloped and nonenveloped viruses from wastewater with PEG precipitation, ultracentrifugation, and optimized ultrafiltration method. Viruses were spiked into wastewater samples to final concentrations of 8×10^3 PFU mL^{-1} for MHV, and $2—5 \times 10^5$ PFU mL^{-1} for MS2, T3 and ϕ6.

not unexpected given that MHV partitioned to solids to a greater extent than the MS2. Low mean recoveries (<6%) were achieved for both MS2 and MHV with the ultracentrifugation method (Figure 4). The ultrafiltration method resulted in significantly higher MHV recoveries than the PEG precipitation ($P = 0.0065$) and the ultracentrifugation ($P = 0.0084$) methods. MS2 recoveries with the ultrafiltration method were significantly higher than ultracentrifugation ($p = 0.0074$), but not significantly different than PEG precipitation ($P = 0.4137$) method (Figure 4).

Additional experiments suggested that incubation with PEG caused a major drop in infective MHV. The T_{90} for MHV in wastewater with PEG was 16 h compared to 40 h in wastewater without PEG (SI Figure S2). The enveloped influenza viruses were previously recovered from surface waters with the PEG method,[55] but recoveries were very low (0.2%—0.6%). The low recoveries for MHV and influenza with PEG may be due to

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disruption of their lipid bilayers.[56] Meanwhile, the MS2 recovery obtained here with the PEG method (43.1 ± 16.8%) was comparable to the recovery of nonenveloped *Echovirus* 7 from raw wastewater (78.5 ± 11.0%).[57] These results suggest that PEG precipitation method, which is effective at recovering infective nonenveloped viruses from water samples, is not optimal for recovering infective enveloped viruses.

In the ultracentrifugation method, the initial centrifugation (100 000*g* for 1 h) step did not effectively pellet bacteriophage MS2, and 63% of the spiked MS2 was detected in the centrate. Comparatively, only 1% of the spiked MHV was detected in the centrate. Previously, the ultracentrifugation method was successful at recovering rotavirus genes from raw wastewater (47% mean recovery), but the infectivity state of the recovered viruses was not tested.[19] Our low recovery of infective MHV viruses in the pellet may be due to virus inactivation by the large ultracentrifuge forces.[58] Taken together, this suggests that pelleting wastewater solids with ultracentrifugation may be effective at recovering enveloped viruses genes for qPCR detection, but not appropriate when infective viruses are desired.

Additional experiments were conducted to optimize recoveries with the ultrafiltration method (description in SI Figure S3). The optimized method involves prefiltering 250 mL of wastewater through a 0.22 μm PES membrane to remove solids, followed by concentration of the filtrate with 10 kDa centrifugal filters to a final volume of 2.5 mL. Using this method, we achieved mean virus recoveries of 25.1% for MHV, 18.2% for ϕ6, 55.6% for MS2, and 85.5% for T3 (Figure 4). Ultrafiltration has been successfully applied for recovering nonenveloped enteric viruses from wastewater, such as polioviruses, adenoviruses, noroviruses, and enteroviruses.[18,41] Here, we have demonstrated that the method can also be optimized for recovering enveloped viruses. In future work, we will test hollow fiber ultrafilters and tangential flow ultrafiltration to potentially increase wastewater sample volumes that can be processed, and thus decrease the detection limits of infective enveloped viruses in wastewater.

Environmental Implications. Our results shed light on the behavior of enveloped viruses in wastewater and provide guidance on how to recover infective enveloped viruses from raw wastewater. Although the two model enveloped viruses were more rapidly inactivated in wastewater, they did survive long enough to be of concern for wastewater treatment facilities, stormwater overflow events, and wastewater intrusion in drinking water. The results presented here will be particularly important during potential future avian influenza or coronavirus outbreaks in humans, as some strains of these viruses can be excreted in feces. Future work should examine additional enveloped viruses to elucidate the specific virus characteristics that contribute to their survival times and enhanced partitioning to solids.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b00876.

A description of the ultrafiltration method optimization experiments; description of experiments on recovering MHV from wastewater solids; figure of virus recoveries in wastewater before and after centrifugation, figure of virus inactivation in the presence of PEG; figure of virus recoveries with different extraction methods; illustration showing statistical differences in the inactivation rates of viruses in pasteurized and unpasteurized wastewater; table of wastewater characteristics; table of TSS and VSS removal by centrifugation; table of virus inactivation rates in wastewater; table of sorption simulation results (PDF)

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Notes

The authors declare no competing financial interest.

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