

Norovirus Removal and Particle Association in a Waste Stabilization Pond

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The presence of norovirus (NoV) genogroup I (GI) and II (GII) was evaluated using real-time reverse transcription polymerase chain reaction (rRT-PCR) in the influent, two midtreatment locations, and final effluent of a three-pond serial waste stabilization pond system from December 2005 through June 2006. Additionally, influent and effluent samples were filtered through a cascade of three membrane filters with sequentially smaller pores to determine the size range of particles with which GI and GII were associated. NoV GI and GII removal occurs primarily in the third pond. Viruses were found on large settleable particles (retained on a 180 μm filter), on smaller suspended particles (retained on a 0.45 μm filter), on colloidal particles (retained on a positively charged 0.45 μm filter), and in the final filtrate. Both GI and GII in influent samples were found to be dominantly associated with particles smaller than 180 μm , thereby suggesting that particle settling is not the main virus removal mechanism in the waste stabilization pond system. On average, NoV detected in filtered effluent samples were associated with particles between 0.45 and 180 μm in diameter (47 and 67% of detected GI and GII, respectively). The presence of NoV GI and GII in the final filtrate of influent and effluent samples shows that positively charged membrane filters often used for viral concentration methods are not capable of trapping all viruses present in wastewater samples.

Introduction

Diarrheal disease is responsible for 1.4 million preventable deaths worldwide each year, causing human suffering and significant economic costs to society (1, 2). Approximately half of all gastroenteritis (GE) outbreaks studied and almost all (73–95%) epidemic GE cases classified as nonbacterial are due to norovirus (NoV) (3). In a study done by the U.S. Centers for Disease Control and Prevention of 90 NoV outbreaks, transmission was attributed to contaminated

oysters (10%), other contaminated food (37%), person-to-person contact (20%), contaminated water (6%), and undetermined sources (27%) (4). Since contaminated water can be linked either directly or indirectly to several of these categories, it is important to understand NoV removal in wastewater treatment, as discharged treated or untreated wastewater can impact water used for irrigation, shellfish farming, recreation, and drinking (5).

NoV are genetically diverse, with genogroups I (GI) and II (GII) being most important for human disease (3, 6). NoV GI and GII have been detected in raw and treated wastewater and downstream in rivers, canals, seawater, and shellfish, in addition to a wide range of foodborne outbreaks (7–15). The lack of a straightforward, culture-based method has limited studies on NoV survival in the environment or on infectivity of detected NoV. However, the shellfish-implicated outbreaks of NoV gastroenteritis imply that infective viruses can survive wastewater treatment and subsequent environmental stresses (16–18). Using molecular detection methods, NoV concentrations have been shown to be reduced during wastewater treatment, ranging from no removal to nearly 6 log removal on occasion. The lack of obvious trends by treatment type or conditions necessitates further investigation into the mechanisms that contribute to NoV removal in wastewater treatment to better protect human health (7, 9, 12, 19).

Viral survival in the aquatic environment, whether in wastewater treatment, a pond, a stream, or on crops irrigated with contaminated water, depends on a wide array of factors, including temperature, solar radiation, adsorption, enzymatic destruction, and predation by bacteria and protozoa (20). Pinpointing the location of viral removal in a given wastewater treatment technology can help to confirm which removal mechanisms are important. Likewise, an understanding of the size of particles with which viruses are associated in raw wastewater can support evidence for certain removal mechanisms and has implications for virus transport upon discharge to the environment (21).

While there are many types of wastewater treatment technologies, waste stabilization pond (WSP) systems are appropriate to study. These systems are widely used where electricity and system operation and maintenance costs are high relative to land costs, such as in rural areas worldwide or in some urban areas in developing countries. As developing countries invest in infrastructure to improve public health and environmental protection to meet the United Nations Millennium Development Goals (22), WSP systems are likely to be an important technology for adoption.

The objectives of this paper are (1) to identify the location of removal of NoV in a WSP system and (2) to investigate the NoV–particle association in the WSP system. A cascade filtration scheme was used to separate total particles into those that had the potential to settle during the expected residence time of the WSP system, suspended particles that were not expected to settle, and fine colloidal particles. NoV GI and GII concentrations in unfiltered samples and filtered subsamples were estimated separately using real-time reverse transcription polymerase chain reaction (rRT-PCR).

Materials and Methods

Waste Stabilization Pond and Sample Collection. This study evaluates the location of removal of NoV in a three-pond gravity-fed WSP system, which receives municipal wastewater in northwest France and discharges treated effluent to an estuary. The system was built in 1985 for 1200 inhabitant equivalents and is currently serving 1800 people at an average flow rate of 300 m^3 per day (23). The system was designed

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to have a residence time of several weeks, though during the study period it was likely much shorter because of accumulated settled sludge, which had not been evacuated.

Grab samples were taken from the influent, pond 1, pond 2, and pond 3 (final effluent) at least biweekly from December 2005 through June 2006 (Supporting Information, Figure S1). Samples were immediately placed on ice and were transported to the laboratory within four hours of collection.

System Performance Indicators and Weather Data.

System performance indicators were measured by an external private laboratory according to standard procedures. Biochemical oxygen demand (BOD₅) and chemical oxygen demand (COD) measurements were performed on each sample upon arrival according to standard methods (24). Daily rainfall measurements were reported by two nearby weather stations, Lanvéoc (20 km SW) and Guipavas (20 km NW), and were averaged to represent rainfall at Daoulas (Météo France). The measured daily solar energy was likewise obtained from a weather station at Brest-Guipavas (Météo France). Total suspended solids (TSS) was measured twice according to standard methods (24). Conductivity and pH were measured once onsite using a portable meter.

Determination of Virus–Particle Association. We conducted a “cascade” filtration study to determine the size of the particles with which NoV GI and GII are associated. A preliminary study on two influent samples taken on January 3 and 10, 2006 was conducted to choose the filter sizes to use for subsequent samples. Forty milliliters of the first sample were filtered first through a 180 μm nylon net filter (Millipore) and then through a 100 μm nylon net filter (Millipore), a 20 μm nylon net filter (Millipore), a 0.45 μm HA filter (Millipore), and finally a 0.45 μm positively charged Zeta Plus filter (CUNO). Each filter was retained for viral detection. On the basis of NoV GI and GII quantities found from this first filtration experiment, the 100 μm filter was eliminated from the series. Next, 40 mL of the second sample were filtered through the 180 μm , 20 μm , 0.45 μm uncharged, and 0.45 μm positively charged filters with the final filtrate being retained and analyzed for NoV GI and GII concentrations as well. The results of this filtration experiment resulted in eliminating the 20 μm filter from the series for subsequent filtration experiments.

For our reported filtration data, a subset of six weeks of influent and effluent samples from February through March 2006 were filtered through the 180 μm , 0.45 μm uncharged, and 0.45 μm positively charged filters. Forty milliliters of each sample were filtered through the series of filters with each filter and the final filtrates being retained for NoV GI and GII detection. Samples were processed within 24 h of arrival. A schematic diagram of the filtration setup is presented in Figure S2.

Viral Concentration, Extraction, and Nucleic Acid Purification. As previously described by da Silva et al. (19), viruses in liquid samples (influent, pond 1 effluent, pond 2 effluent, pond 3 effluent, and cascade filtrate samples) were concentrated and RNA was purified according to a method adapted from Atmar et al. (25). Samples were processed within one week of sampling. Briefly, 40 mL samples were spiked with mengovirus for use as an extraction control (Costafreda et al.) to a final concentration of 250 tissue culture-infective doses per milliliter (26). Ten milliliters of a 50% polyethylene glycol (PEG) 6000 solution (Sigma, St. Quentin, France) were added, and samples were rocked overnight at 4 °C. The samples were centrifuged for 1.5 h at 1500g, and the pellet of precipitated viruses was suspended in 1 mL of distilled, sterilized water. Viruses precipitated by PEG were then digested with proteinase K (Amresco, Solon, OH) for 30 min at 56 °C to liberate nucleic acid. The nucleic acid was extracted with phenol–chloroform–water and chloroform–isoamyl alcohol. The aqueous phase was retained and precipitated

with ethanol. After resuspension in water, cetyltrimethylammonium bromide and sodium chloride were added to final concentrations of 1.4% (wt/wt) and 0.11 M, respectively, and were incubated at room temperature for 15 min. The mixture was then pelleted, was resuspended in 1 M NaCl, and again was precipitated in ethanol. The final pellet was resuspended in 100 μL of distilled, sterilized water containing 1 μL of RNase inhibitor (Invitrogen, France). Purified nucleic acids were stored at –20 °C.

Filters were likewise spiked with the same amount of mengovirus prior to extraction. Viruses were eluted from each filter using 1 mL 0.1 M glycine buffer (in 1.5 M sodium chloride adjusted with concentrated sodium hydroxide to a final pH of 12) by repeated rinsing and gentle scraping using a 1 mL pipet, followed by direct proteinase K digestion and then purification as performed for PEG-concentrated samples.

Quantification Standards. The NoV GI and GII quantification standards were long RNA fragments similar to corresponding viral genomes and were kindly provided by Robert Atmar (Baylor College of Medicine, Houston, TX). The pCRII TOPO (Invitrogen) vector was used to create vectors corresponding to nucleotides 146–6935 of the Norwalk GI.1 prototype strain and nucleotides 4191–5863 of the Houston GII.4 strain. Vectors were transformed in *Escherichia coli*, and transformant clones were screened. Plasmids were then extracted, linearized, and in vitro transcribed using the Promega riboprobe system. After DNase treatment, RNA standards were purified and subsequently were quantified by optical density at 260 nm (OD₂₆₀).

Primers, Probes, and Amplification Conditions. The NoV GI and mengovirus primers and probes are described in da Silva et al. (19). NoV GII primers and probes are described in Loisy et al. (27). *rRT*-PCR was carried out with an MX3000 (Stratagene, France) as previously described using the Platinum Quantitative RT-PCR ThermoScript One-Step system (Invitrogen, France) (27). Five microliters of RNA extract were combined with 20 μL of a mixture containing 1 \times ThermoScript reaction buffer, a 200 nM probe, 200 nM of each primer, 0.5 μM Rox reference dye 50X, 0.5 μL of ThermoScript Plus/Platinum *Taq* enzyme mixture, and 2 U RNase inhibitors (Applied Biosystems, France). RT was carried out for 30 min at 50 °C, denaturation was carried out for 5 min at 95 °C, and 45 cycles of PCR amplification were carried out, entailing 15 s of denaturation at 95 °C followed by 1 min of annealing and extension at 60 °C.

Sample RNA was analyzed for mengovirus, GI, and GII at 1/10 and 1/100 dilutions of the nucleic acid extract, as pure nucleic acid contained too many inhibitors to yield an *rRT*-PCR signal. The cycle threshold (C_T) for each positive sample was compared to the corresponding standard curve and the viral titer was estimated, accounting for the dilution factor used. A negative amplification control (distilled, sterilized water) and positive amplification controls (GI and GII reference strains) were included in each amplification series. To verify that an acceptable extraction had been achieved, a standard mengovirus RNA was included in each *rRT*-PCR plate and the C_T was compared to the C_T of the detected sample mengovirus as previously described (19). If less than 1% of spiked mengovirus was detected, the extraction was considered unacceptable and the sample was not included.

Results

Throughout the Results section, data is presented as mean values with standard deviations. Statistical analyses were conducted using a 95% confidence interval ($p < 0.05$) for hypotheses testing.

Extraction Efficiency. The extraction efficiency was verified for each sample. The average extraction efficiency for unfiltered samples was $117 \pm 96\%$ with a median value of 92%. Extraction efficiencies for filtered samples were

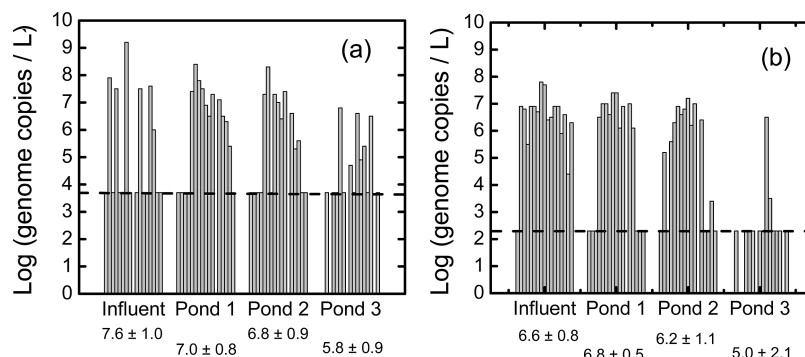


FIGURE 1. Quantities of norovirus (a) GI and (b) GII for samples taken from the influent, effluent from pond 1, and effluent from pond 3 (final effluent) of the waste stabilization pond system. Influent and effluent titers were previously reported in ref 19. In each location category, sequential bars represent 17 chronological samples taken from November 2005 through June 2006. Blank bars indicate that the individual sample had persistently poor extraction efficiency and could not be analyzed. Horizontal dashed lines represent the corresponding detection limits for NoV GI and GII. The average concentration (\pm standard deviation) of positive samples in each location is indicated below the x-axis.

similar. Filtered influent samples had a mean extraction efficiency of $107 \pm 96\%$ with a median of 74%. Extraction efficiency for filtered effluent samples was $140 \pm 143\%$ with a median of 84%. The maximum extraction efficiency observed was 477%, and the minimum cutoff for failed extraction was 1%.

System Performance. Though operating over-capacity and with a reduced residence time, the WSP system was still operating effectively with average BOD5 and COD percent removals of 85 ± 8 and 75 ± 10 , respectively, during the study period. The TSS contents of the influent, pond 1, pond 2, and pond 3 (final effluent) samples (indicated as average \pm standard deviations of two measurements) were 186 ± 100 , 71 ± 14 , 104 ± 28 , and 58 ± 9 mg/L, respectively (measured on January 4 and 10, 2006). The pH values of the influent and pond 1, pond 2, and pond 3 effluents were 8.0, 7.8, 8.0, and 7.7, respectively (measured once on November 30, 2005). The conductivities of the pond samples were 940, 1470, 1530, and $1580 \mu\text{S}/\text{cm}$, respectively (likewise measured once on November 30, 2005).

NoV Removal Location. Influent and effluent NoV concentrations were previously reported in da Silva et al. (19) and are presented here along with concentrations in ponds 1 and 2 to demonstrate removal location in this pond system (Figure 1). NoV GI and GII were detectable at all locations in the pond system and followed similar patterns of presence within the three ponds over the study period. Pond 1 and pond 2 NoV concentrations were the same order of magnitude as positive influent concentrations for GI and GII for most weeks studied. Though GI influent concentrations are sporadic, pond 1 and pond 2 concentrations are more steady, with nearly every sample positive for GI. Titrers dropped off dramatically in pond 3, the final effluent of the system, for both GI and GII. NoV broke through the system at extremely high concentrations of the same magnitude as influent concentrations at six weeks for GI and at two weeks for GII. These high titer effluent samples appeared in a sporadic fashion without an apparent linear relationship with the influent, pond 1, or pond 2 concentrations. To illustrate the lack of association between heavy rainfall events and NoV breakthrough, daily rainfall is plotted along with effluent NoV concentration in Figure 2.

Analysis of Location and Concentrations of Norovirus. The location and concentration of NoV GI and GII in the pond system were analyzed with the most appropriate statistical techniques (details in Supporting Information). We concluded from the Shapiro–Wilk test that the GI data is normally distributed ($p = 0.6771$) while the GII data is not ($p < 0.0001$). The latter result necessitates the use of nonparametric statistical techniques. Samples that were

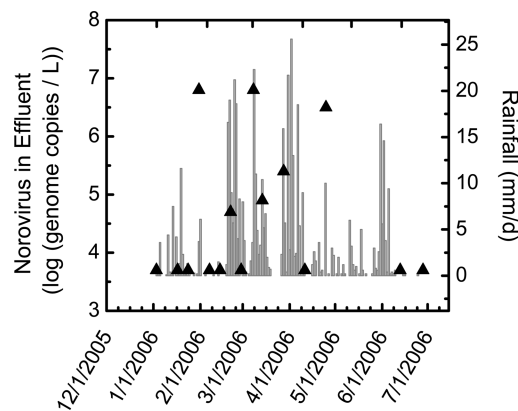


FIGURE 2. Quantities of NoV (GI and GII combined) in the effluent of a waste stabilization pond (\blacktriangle) plotted against daily rainfall (bars) for the period of December 2005 to July 2006. The NoV cumulative detection limit was 3.7 log (genome copies/L).

below the detection limit for GI or GII (“negative” samples) were not included in the analyses, which amounted to nearly half of the total sample population.

The mean concentration of NoV GI was highest in the influent and sequentially decreased through the pond system. The analysis of variance (ANOVA) F-test confirmed that the mean values of GI in the ponds are significantly different ($p = 0.024$), indicating that there are differences in removal among the ponds for GI (Supporting Information Table S1). The Duncan test, a multiple comparison of means procedure, demonstrated that the mean effluent GI concentration is significantly different than those found at the other three locations. The nonparametric Kruskal–Wallis test determined that concentrations of GII in the four ponds were not significantly different.

NoV–Particle Association. Two initial influent samples were filtered and analyzed for NoV GI and GII to determine serial filters that would be used for a more extensive filtration experiment (Supporting Information Table S2). The first sample contained GI on particles between 0.45 and $20 \mu\text{m}$ in size and GII on particles in all size categories except 100 – $180 \mu\text{m}$. Since there were no NoV detected on the $100 \mu\text{m}$ filter, it was eliminated from the subsequent serial filtration. The second sample contained no GI. GII was detected in each size category but not in the final filtrate, with the majority on particles between 0.45 and $20 \mu\text{m}$. For subsequent filtration experiments, the following filters were retained: $180 \mu\text{m}$ (to avoid subsequent filter clogging and to represent very large particles), uncharged $0.45 \mu\text{m}$ (to trap

TABLE 1. Quantities of Norovirus GI and GII Retained on Filters and in Unfiltered Samples^a

date	influent					unfiltered	effluent					unfiltered
	filtered subsamples ^b						filtered subsamples ^b					
	(a) 180 μm nylon	(b) 0.45 μm HA	(c) 0.45 μm (+)	(d) filtrate	Σ		(a) 180 μm nylon	(b) 0.45 μm HA	(c) 0.45 μm (+)	(d) filtrate	Σ	
GI												
2/14	—	—	—	4.4	4.4	—	—	—	—	—	—	4.7
2/21	—	—	—	4.9	4.9	<i>i</i>	—	6.1	—	—	6.1	—
2/28	—	—	—	—	—	—	—	6.3	—	—	6.3	6.6
3/07	—	—	4.9	4.5	5.0	7.5	—	—	—	—	—	4.9
3/14	—	6.3	4.5	—	6.3	—	—	6.4	5.9	—	6.5	5.4
3/28	—	—	5.1	—	5.1	—	5.9	5	4.2	—	6.0	<i>i</i>
GII												
2/14	5.8	7.2	4.8	6.4	7.3	7.8	—	—	—	—	—	6.8
2/21	6.2	6.3	—	5.4	6.6	7.7	—	—	3.4	4.5	4.5	—
2/28	5.6	6.9	5.2	3.9	6.9	6.4	—	5.6	4.2	—	5.6	—
3/07	4.3	4.9	—	—	5.0	6.5	—	—	—	—	—	6.5
3/14	5.8	—	—	3.8	5.8	6.9	3.7	5.6	4.5	3.6	5.6	3.5
3/28	5.3	6.6	4.5	3.9	6.6	6.9	—	4.3	4.6	2.8	4.8	—

^a Log (genome copies per liter). Dashed lines indicate that the sample was at or below limits of detection, which were 3.7 and 2.3 log (genome copies per liter) for GI and GII, respectively. Cells marked “*i*” indicate the sample was not analyzable because of poor extraction efficiency. ^b Samples were sequentially filtered through (a) 180 μm nylon net filter (Millipore), (b) 0.45 μm HA filter (Millipore), and (c) 0.45 μm (+) charged Zeta Plus filter (CUNO). The final filtrate was retained and analyzed (d).

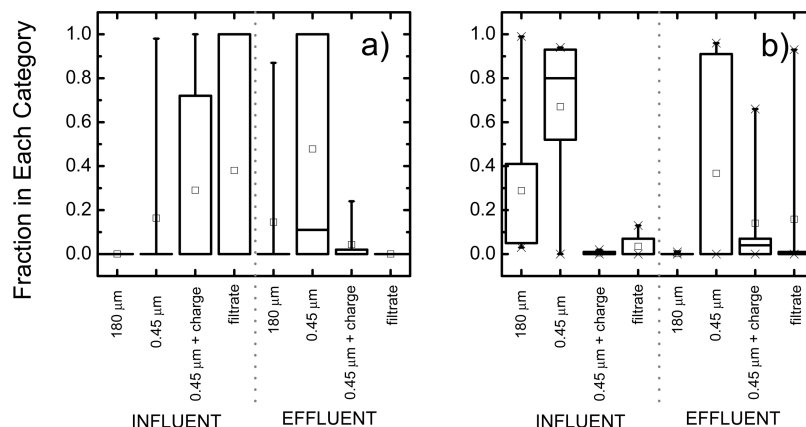


FIGURE 3. Box plots of fractions of norovirus (a) GI and (b) GII found on each filter for six filtered influent and six filtered effluent samples. Midline bars are medians and means are indicated by □. Outlined boxes and their whiskers (x) indicate the first and third quartiles and minimum and maximum values of the data, respectively.

all particles between 0.45 and 180 μm), and positively charged 0.45 μm (to trap colloidal particles smaller than 0.45 μm).

Table 1 presents the quantities of NoV GI and GII retained on each serial filter (180 μm , 0.45 μm HA, and 0.45 μm positively charged) and in the subsequent filtrate for six influent and six effluent samples with quantities of GI and GII in subsamples summed to show the total number of viruses detected in cascade filtration. The titers detected for the same samples by PEG concentration of an unfiltered aliquot are also provided for comparison of viral concentration method. GI and GII were trapped on serial filters of each pore size and in the filtrate for both influent and effluent samples. Seven of the eight samples, which were found to be negative by PEG concentration, had detectable viruses by cascade filtration. Filtered samples were positive for both GI and GII simultaneously with the exception of the influent sample taken on 2/28/06 and the effluent sample on 3/07/06. The former was only positive for GII and the latter was negative for both GI and GII after filtration. For 11 samples, the cascade filtration yielded higher total titers than the PEG concentration method, whereas the inverse was true for eight samples. For four samples, the two methods resulted in similar concentrations (within one order of magnitude).

Analysis of Concentrations of Noroviruses on Filtered Subsamples. After normalizing the quantity of NoV GI or GII found on a given filter by the total quantity detected by filtration, the fractions of NoV GI or GII found on each filter for each sample date were plotted (Figure 3). There was no filter category where viruses were not detected. The box plot illustrates the high variability of percent of GI and GII found in each filter category for influent and effluent samples. In most samples, the mean for GI or GII found on the 0.45 μm filter is higher than the other filter categories (except for GI in filtered influent samples).

Examining each category specifically, different trends are noted. Filtered influent samples did not contain any GI on the 180 μm filter, and filtered effluent samples contained no GI in the final filtrate, for all six samples. The GI detected on filtered influent samples were fairly evenly distributed between the 0.45 μm uncharged filter, the subsequent 0.45 μm positively charged filter, and in the final filtrate (average of 16, 29, and 38% of detected viruses, respectively). (Percentages do not sum to 100 because they represent averages of percentages found in each category for each sample rather than percentage of an overall total.) GII in filtered influent samples were found on filters in each category

with an average of 29% of the detected GII on the 180 μm filter, 67% on the subsequent 0.45 μm uncharged filter, 0.5% on the subsequent 0.45 μm filter, and 3.5% in the final filtrate. Filtered effluent samples contained an average of 15, 47, 4, and 0% of the detected GI and 0.2, 37, 14, and 16% of the detected GII on the sequential 180 μm filter, 0.45 μm uncharged filter, 0.45 μm charged filter, and filtrates, respectively.

To compare whether individual categories represented significantly different fractions of detected GI or GII, multiple comparisons of means analyses were conducted. According to the Shapiro–Wilk test, the GI and GII filtration data is not normally distributed ($p < 0.001$ for influent and effluent samples for both GI and GII), thus requiring nonparametric analysis of the data with the Kruskal–Wallis test (Supporting Information Table S3). Groupings for fractions of GII in filtered influent samples were significantly different ($p = 0.0098$) and nearly significant for fractions of GI in the filtered effluent ($p = 0.0568$). The other two groupings (fractions of GII in filtered effluent and fractions of GI in filtered influent samples) did not demonstrate significant differences of viruses retained on the various filters.

Pairwise comparisons of the filter categories demonstrating significant differences (GII in filtered influents) were conducted with the Wilcoxon rank–sum test (Supporting Information Table S4). The results for GII indicate that the mean fractions of GII found in filtered influent samples on the 180 μm filter are significantly different than the fractions found on the 0.45 μm positively charged filter ($p = 0.0037$), which had a smaller mean fraction of detected GII. Likewise, the 0.45 μm uncharged filter contained significantly more GII than the 0.45 μm positively charged filter ($p = 0.0278$) and the filtrate ($p = 0.0450$).

Discussion

Extraction Efficiency. Extraction efficiency was used as an overall check on the effectiveness of the extraction methods. Similar extraction efficiencies were observed regardless of the sample type. High variability is observed likely because of the relatively low titer of mengovirus spiked into the samples in order to conserve stocks.

NoV Removal Occurs in Final Pond. The analysis of means indicates pond 3 had a significantly lower mean concentration of GI than the other locations. The mean concentration of GII in pond 3 is lower than in the other locations, but was not found to be significantly different, which is likely due to the sensitivity of the Kruskal–Wallis test to small sample sizes. Taken together, most of the GI and GII NoV removal occurs in the final pond for this wastewater system. Adsorption of viruses to settling particles is likely not a major removal mechanism in this treatment system since pond 1, which allows for most of the sedimentation in WSP systems, does not cause a drop in NoV GI or GII concentrations. Likewise, since each of the ponds has roughly the same volume, dilution is not likely to be a major factor in the decreased concentrations in pond 3.

NoV Breakthrough Events Appear Independent of Rainfall Events. In a study of a NoV outbreak linked to oyster consumption, heavy rainfall (150 mm) resulted in failure of the sewage treatment system (on the basis of *E. coli* effluent criteria) and likely was responsible for the observed NoV contamination (28). In this study, effluents (pond 3) are sporadically positive for GI or GII, though pond 2 has fairly constant, elevated concentrations of each. The lack of coincidence of rainfall with NoV in the effluent (illustrated in Figure 2) suggests that significant NoV breakthrough events observed may not have been directly due to heavy rainfall events, though rainfall could have contributed in part to washout of NoV. While these results indicate that NoV breakthrough can occur independent of rainfall events,

wastewater treatment systems nonetheless need to be designed to manage hydraulic shocks, particularly as climate change forecasts predict more variable and extreme rainfall patterns.

NoV–Particle Association. Particles retained on the 180 μm filter represent particles that are likely to settle during the residence time of each pond (several days) on the basis of calculated settling times for particles of density 1.04 g cm^{-3} using Stokes Law. Particles between 0.45 and 180 μm may settle depending on the extent of mixing in the pond system. Particles trapped on the positively charged 0.45 μm filter and in the final filtrate represent nonsettling suspended colloidal particles.

The cascade filtration experiment reveals important insights about GI and GII particle association in both influent and effluent samples. The largest particles (retained on the 180 μm filter) did not contain the majority of GI or GII in influent samples (0% and 29% on average, respectively) (Figure 3). GI in influent samples were found in roughly similar fractions on the uncharged 0.45 μm filter, on the positively charged 0.45 μm filter, and in the filtrate. Most of the GII detected in filtered influents (67% on average) were on particles measuring 0.45–180 μm in size. This average fraction was shown to be significantly higher than the average fractions found on the 0.45 μm positively charged filters and in the final filtrates.

Filtered effluents indicated there were more GI and GII on 0.45–180 μm sized particles than on larger particles. The average fraction of GI in the effluent retained on the 0.45 μm filters was higher, although not significantly, than in the final filtrates. GI is very unlikely to be removed by settling since there was a very small portion of GI associated with the two largest size categories in influent samples. For GII, some settling may have occurred from the influent to effluent since there is a decrease in average fractions found in the two largest size categories; however, this decrease cannot account for the log orders of physical removal of GII by the WSP system.

Viruses in WSP systems are known to be associated with particles, which appear to confer protection from many inactivation mechanisms (28–31). A review of current literature on this subject has been conducted by Templeton et al. (32). Gerba et al. (33) employed filters of pore sizes 8.0, 5.0, 3.0, 1.2, 0.8, 0.65, 0.45, 0.30, and 0.22 μm to filter a wastewater sample from a trickling filter system and found coliphages in each category with slightly more on particles larger than 8 μm and smaller than 0.45 μm . Hejkal et al. (34) found 72.0 and 96.6% of detected enteroviruses in influent and unchlorinated effluent samples from an activated sludge system associated with particles smaller than 0.3 μm in diameter. It is apparent from these studies and the data presented here that viruses in wastewater are associated with particles of all sizes, yet tend to associate most strongly with suspended particles that may transport long distances upon release to the environment.

NoV Are Not Entirely Retained on Positively Charged Filters. The presence of GI and GII in the filtrate after cascade filtration shows that even the positively charged 0.45 μm filter does not trap all viruses. This behavior is likely attributable to the high concentration of dissolved organic matter, which instantaneously reverses the charge of the membrane surface. Thus, viral concentration methods that rely on positively charged membranes to retain all viruses by electrostatic attraction are not fully effective for prefiltered wastewater samples.

Particle Association and Removal Mechanisms. Taken together, the results presented in this study suggest that settling is not a primary mechanism of NoV GI or GII removal and that the majority of removal occurs in pond 3. Studies examining bacteriophages have shown that viral removal in

waste stabilization ponds is dominated by solar disinfection, though predation by higher trophic levels and adsorption to particles followed by settling may play additional roles (31, 35–39). Davies-Colley et al. (36) and Kohn et al. (40) demonstrated the importance of photooxidation in WSP systems for F-specific RNA and MS2 bacteriophages, respectively.

The NoV removal indicated by the data in this study represents physical removal or destruction to the point that the target RNA fragment is no longer detectable. Since inactivated viruses can still be detected by rRT-PCR, the extent of solar inactivation of NoV in WSP systems may be much more significant than indicated by the data in this study. The fact that NoV GI and GII are found in this study to be associated with particles in the raw and treated wastewater has important implications for future studies regarding removal mechanisms of NoV in WSP systems. This is primarily because adsorbed particles may alternatively protect viruses from inactivation by shielding, or enhance inactivation by photosensitization of adsorbed macromolecules (36, 41). Further research is needed to isolate the dominant removal mechanism for NoV in order to optimize WSP design when its removal is a key objective. Given the notable burden of diarrheal diseases, and NoV gastroenteritis specifically, increasing the understanding of NoV removal in wastewater treatment will advance prevention of exposure and spread of disease.

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Supporting Information Available

Description of statistical tools employed. Diagram of sampling locations within the waste stabilization pond system (Figure S1). Diagram of the cascade filtration system involving three filters employed to evaluate norovirus–particle association in six influent and effluent samples (Figure S2). Table summarizing statistical analysis of concentrations of GI and GII at four locations in the WSP system (Table S1). Table of initial filtration analysis of two influent WSP samples showing quantities of norovirus GI and GII retained on five filters of sequential pore sizes (Table S2). Table of statistical significance of comparison of fractions of norovirus GI and GII retained on each filter (Table S3). This information is available free of charge via the Internet at <http://pubs.acs.org>.

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