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ORIGINAL PAPER

Effects of pH Variability on Peracetic Acid Reduction of Human Norovirus GI, GII RNA, and Infectivity Plus RNA Reduction of Selected Surrogates

Nathan Dunkin[1] · Caroline Coulter[1] · ShihChi Weng[2] · Joseph G. Jacangelo[1,2,3] · Kellogg J. Schwab[1,2]

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Abstract

With increasing interest in peracetic acid (PAA) as a disinfectant in water treatment processes, this study determined PAA treatment effects on human noroviruses (hNoVs) genotype I (GI) and genotype II (GII) as well as effects on bacteriophage MS2 and murine norovirus (MNV) in relation to pH. Across all pH conditions, PAA achieved between 0.2 and 2.5 $\log_{10}$ reduction of hNoVs over 120 min contact time in buffer solution as measured by reverse transcription-qPCR (RT-qPCR). The PAA treatments produced similar RT-qPCR reductions of MS2 and MNV, in the range of 0.2–2.7 $\log_{10}$. Infectivity assays achieved > 4 $\log_{10}$ reduction of both MS2 and MNV in buffer solution after 120 min contact time. Comparing PAA activity across varying pH, disinfection at pH 8.5, in general, resulted in less reduction of infectivity and molecular signals compared to pH conditions of 6.5 and 7.5. This difference was most pronounced for reductions in infectivity of MNV and MS2, with as much as 2.7 $\log_{10}$ less reduction at pH 8.5 relative to lower pH conditions. This study revealed that PAA was an effective disinfectant for treatment of hNoV GI and GII, MS2 and MNV, with greatest virus reduction observed for MS2 and MNV infectivity. RT-qPCR reductions of MS2 and MNV were lower than concurrent MS2 and MNV infectivity reductions, suggesting that observed hNoV RT-qPCR reductions may underestimate reductions in hNoV infectivity achieved by PAA. Although virus disinfection by PAA occurred at all evaluated pH levels, PAA is most effective at pH 6.5–7.5.

Keywords Peracetic acid · pH · Human norovirus · MS2 bacteriophage · Mouse norovirus · Disinfection

Introduction

Human noroviruses (hNoVs) are enteric viruses transmitted by the fecal-oral route and are the leading cause of acute gastroenteritis outbreaks and sporadic cases across all age groups worldwide (Ahmed et al. 2014). In the United States, hNoVs cause 21 million illnesses and 71,000 hospitalizations each year (Lopman et al. 2011).

Contaminated water is an important mode of transmission of hNoVs, thus disinfection processes at drinking water treatment plants (DWTP) and wastewater treatment plants (WWTP) are essential in reducing human exposure to hNoVs. Chlorination is the disinfectant most commonly used in DWTPs and WWTPs for removing viruses and other microorganisms (Springthorpe and Sattar 2007). Recently, alternative disinfectants have garnered interest due to increased awareness of harmful disinfection byproducts (DBPs) formed during chlorination processes, such as trihalomethanes and haloacetic acids (Cowman and Singer 1996; Watson et al. 2012). Peracetic acid (PAA) is

Nathan Dunkin and Caroline Coulter have contributed equally to this work.

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✉ Kellogg J. Schwab
kschwab1@jhu.edu

[1] Department of Environmental Health and Engineering, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA

[2] JHU/Stantec Alliance, Johns Hopkins University, Baltimore, MD, USA

[3] MWH-Stantec, Pasadena, CA, USA

an effective disinfectant that is growing in popularity in the US with applications in the food-processing, beverage, and pharmaceutical industries (Dunkin et al. 2017a, b; Kitis 2004). PAA is effective at room temperature and has a long shelf life and reasonable cost (Luukkonen and Pehkonen 2017). Further, disinfection with PAA is favorable as in many instances it does not require posttreatment neutralization and it primarily forms DPBs that are carboxylic acids which have lower mutagenicity, carcinogenicity, and genotoxicity as compared to halogenated DBPs formed during chlorination processes (Crebelli et al. 2005; Luukkonen and Pehkonen 2017).

It has been demonstrated that PAA is an effective disinfectant for treatment of coliform bacteria, *Clostridium perfringens*, and protozoa such as *Cryptosporidium*, and *Giardia* (Briancesco et al. 2005; Veschetti et al. 2003). In experimental settings, PAA has also been shown to efficiently reduce the infectivity of bacteriophage viruses and some mammalian enteric viruses, including feline calicivirus (FCV) and murine norovirus (MNV) (Dunkin et al. 2017a, b; Fraisse et al. 2011; Zonta et al. 2016). There are limited data, however, on reduction of hNoVs by PAA. With growing interest in PAA as a wastewater disinfectant (Luukkonen and Pehkonen 2017), there is need to better understand the efficacy of PAA against hNoVs under a range of conditions relevant for wastewater treatment operations.

Developing an understanding of hNoV susceptibility to water treatment processes is critical for protecting public health. Analysis of hNoVs is challenging, however, since there is a lack of straightforward methods for cultivating hNoVs in the laboratory. While recent research indicates that this hurdle may eventually be overcome (Ettayebi et al. 2016), current infectivity measurements for hNoVs require human volunteer studies, which are costly and entail extensive regulatory hurdles. Molecular detection of viral RNA by reverse transcription-qPCR (RT-qPCR) is commonly employed for analysis of hNoVs in disinfectant and persistence studies (Cromeans et al. 2014; Knight et al. 2016), although RT-qPCR detection has been shown to underestimate viral infectivity reductions when assessing other cultivable viruses such as MNV (Dunkin et al. 2017a, b; Knight et al. 2016). As a result, researchers have relied on surrogate infectivity measurements of cultivable surrogate viruses such as MNV and MS2 to inform our understanding of hNoV behavior (Bae and Schwab 2008; Dunkin et al. 2017a, b). Nevertheless, the relevance of surrogate virus infectivity to hNoV risk in disinfection studies continues to be questioned (Richards 2012). Therefore, to assess hNoV reductions in disinfection experiments where resources are not available for human volunteer studies, the best practice is to interpret hNoV RT-qPCR signals in light of surrogate infectivity and RT-qPCR data generated under identical experimental conditions. Determination of the known infectivity–molecular relationship for surrogate viruses moderates the interpretation of hNoV RT-qPCR data by allowing researchers to understand differences between infectivity and RT-qPCR signals under a given set of experimental conditions, thus providing better information on hNoV risk and persistence during disinfection studies than if molecular analyses were performed in isolation.

With increasing use of PAA as a disinfectant at treatment plants, researchers have examined disinfection activity of PAA under varying conditions (Dunkin et al. 2017a; Kitis 2004; Luukkonen and Pehkonen 2017) and have described how water quality impacts viricidal activity of PAA (Dunkin et al. 2017b). Additionally, pH was found to affect PAA disinfection activity with respect to inactivation of coliform bacteria, with decreased disinfection activity in alkaline pH conditions (pH > 8) (Baldry and French 1989; Sagripanti and Bonifacino 1996; SanchezRuiz et al. 1995) where the non-biocidal form of PAA (i.e., the dissociated acid) predominates. Previous research has also indicated that PAA inactivation of bacteriophages is similarly affected by pH (Baldry and French 1989; Baldry et al. 1991; Sagripanti and Bonifacino 1996; SanchezRuiz et al. 1995). However, there are little data available informing researchers and engineers on the effect of pH variability on PAA reduction of mammalian enteric viruses such as hNoVs, including assessments of PAA efficacy at doses and pH levels that are relevant for water treatment processes. Effects of pH on PAA viral reduction are therefore of interest in this study.

The objective of the current study was to assess PAA reduction of hNoV GI and GII in laboratory buffers as determined by RT-qPCR at varying pH: 6.5, 7.5, and 8.5. PAA disinfection experiments were also concurrently performed for cultivable surrogate viruses MNV and MS2 in laboratory buffers, for which infectivity and RT-qPCR reductions were measured. Although experiments in laboratory grade water may not be directly applicable for wastewater (Dunkin et al. 2017b), the PAA doses chosen for this study were reflective of PAA concentrations used in water treatment processes. pH values between pH 6.5 and 8.5 assessed in this work reflect the pH range of most wastewaters where PAA may be used (Mandal 2014; Mukherjee et al. 1968). PAA disinfection was evaluated in terms of concentration–time (CT), a unit expression for concentration of disinfectant × contact time that is used in management of water treatment operations. Values of CT allow direct comparison of disinfectant effectiveness among different viruses as well as direct comparison of disinfectant activity at different pH conditions.

Materials and Methods

Human Noroviruses

De-identified stool samples were collected from two hNoV-infected patients at an affiliated hospital under

protocols approved by the Johns Hopkins Institutional Review Board. Stool samples were immediately transferred to 4 °C upon collection. The two noroviruses were identified as GI.3 and GII.2 after sequencing analysis (data not shown).

Stool samples were diluted to 10% w/v in Dulbecco's phosphate-buffered saline (DPBS) and subsequently sonicated (Branson B2510-DTH, Danbury, CT) at 40 kHz for 3 min. Each stool sample was then mixed with an equal volume of Vertrel XF (DuPont, Wilmington, DE) and subsequently homogenized (OMNI international, Inc., Marietta, GA) at 20,000 rpm for 3 min on ice. The emulsified mixtures were centrifuged for 15 min at 4000 × *g* and 4 °C, and the recovered supernatants were filtered through 0.45 micron pore-size low-protein-binding membrane filters (Millex PVDF, Millipore, Billerica, MA). Following 0.45 micron filtration, hNoVs were concentrated and further purified by ultracentrifugation on a sucrose cushion (Hwang et al. 2014). Briefly, hNoV stocks were laid over sterile-filtered 20% sucrose solution in an Ultra-Clear centrifuge tube (Beckman, Brea, CA) and centrifuged at 95,000 × *g* for 3 h at 4 °C. After centrifugation, the sucrose and media were aspirated and the procedure repeated using the same ultracentrifuge tube with an additional portion of filtered hNoV stock in order to further concentrate the virus. The virus pellets were then resuspended in 560 µL of DPBS and stored at − 80 °C.

MNV and MS2 Preparation

MNV stocks were propagated in RAW 267.4 cells and processed as previously described (Bae and Schwab 2008). Following initial processing, MNV stocks were further purified and concentrated by ultracentrifugation on a sucrose cushion as described above. The virus pellet was then resuspended in 600 µL of DPBS, portioned, and stored at − 80 °C.

The MS2 coliphage (ATCC 16,696-B1) stocks were propagated as previously described (Bae and Schwab 2008). MS2 stocks were further concentrated and purified using a 100,000-Da ultra-membrane filter (Amicon Ultra; Millipore Corp., Bedford, MD) to increase virus titers and remove soluble and low molecular weight components from the supernatant. The final retentate was washed three times with DBPS, portioned, and then stored at − 80 °C. In order to ensure monodispersed MS2 virus after ultrafiltration, an aliquot of MS2 stock was diluted in DI water to approximately 10^{10} PFU/mL and analyzed by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, UK), and results indicated that MS2 remained monodispersed after Amicon filtration (data not shown). All labware for virus stock preparation and storage was made chlorine demand-free (CDF) by acid washing prior to use.

Infectivity Plaque Assays

Enumeration of infective MNV and MS2 viral particles was performed using standard ten-fold dilution plaque assays. For MNV, the plaque assay using RAW 267.4 cell monolayers, previously described by Bae and Schwab (Bae and Schwab 2008), was performed. To confirm that cytotoxic effects were not experienced by cells, positive controls of MNV virus stock and negative controls using a phosphate buffer (PB) solution were included with each experiment. Cell monolayers showed expected virus propagation and cell growth in the positive and negative controls, respectively, and no non-viral cytopathic effects were observed in the quenched samples. For MS2 bacteriophage, the double agar layer (DAL) method with *Escherichia. coli* Famp (ATCC 700,891) bacterial host (USEPA 2001) was followed.

Molecular Analysis

Molecular analysis of all viruses was performed using RT-qPCR. Experimental samples were first treated with RNase ONE™ Ribonuclease (Promega, Madison, WI) at a concentration of one unit per 10 µL of sample to remove exogenous, non-encapsulated viral RNA. RNase reaction buffer (1 × final concentration) was added and the mixture was incubated at 37 °C for 15 min. Following RNase treatment, nucleic acid was extracted from experimental samples using a MagNA Pure LC 2.0 (Roche, Indianapolis, IN) instrument with the Total Nucleic Acid High-Performance Isolation Kit (Roche, Indianapolis, IN). Input volumes of 200 µL of sample and elution volumes of 100 µL were used. RNA extraction recovery efficiencies were validated using known concentrations of bacteriophage MS2 as control samples (data not shown).

Following RNA extraction, RT-qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers and probes used for hNoV GI and hNoV GII were as previously described (Kageyama et al. 2003). MS2 primer and probes as well as RT-qPCR protocols were followed as previously described (Bae and Schwab 2008; Gibson et al. 2011) with slight modifications. Briefly, the QuantiTect Probe RT-PCR Kit (Qiagen, Germantown, MD) was used with the following thermocycling conditions for all targets: 50 °C for 30 min, 95 °C for 15 min, and 45 cycles at 95 °C for 15 s and 60 °C for 60 s. Cycle threshold data corresponding to the viral RNA concentrations were obtained. Well-characterized and archived hNoV GI and GII RNA were used as RT-qPCR controls.

Experimental Procedure

PB solutions used in disinfection experiments were prepared at three pH values (6.5, 7.5, and 8.5) in CDF glassware by combining deionized CDF water and sodium phosphate

monobasic ($NaH_2PO_4 \cdot H_2O$; Sigma-Aldrich) and dibasic salts ($Na_2HPO_4 \cdot 2H_2O$; Sigma-Aldrich). Each PB solution was prepared at a concentration of 0.01 M. All viral reduction experiments were conducted at 23–25 °C using sterile, CDF 125 mL Erlenmeyer flasks containing an initial volume of 95 mL of water matrix, 5 mL of disinfectant, and 1 mL of virus cocktail. Viruses employed in this study were hNoV GI.3, hNoV GII.2, MNV, and MS2. Sucrose cushion purified and concentrated hNoVs stocks were seeded to 0.0001% final stool concentration (corresponding to approximately 5×10^3 RT-qPCR units/mL for hNoV GI and 1×10^5 RT-qPCR units/mL for hNoV GII) in the experimental water matrices to minimize impacts of stool organics on disinfection experiments. Surrogate virus concentrations in the final volume were approximately 1×10^5 PFU/mL. Flasks were continuously mixed throughout the experiment. After the water matrix and viruses were added to the flask, 5 mL of PAA, adjusted to achieve the final desired concentration, was added. Two PAA doses (1.5 and 10 mg/L) were selected for evaluation. All experiments were performed in duplicate except for MS2 infectivity experiments ($n = 4$). Performance of experiments in duplicate allowed for maximizing in the study the number of evaluated conditions using the limited amount of available hNoV while allowing trends in PAA efficacy to be observed across multiple doses and pH conditions.

Disinfectant residuals were measured at each sampling time point using methods previously described (Dunkin et al. 2017b). At each sampling time point, 4 mL was removed and immediately quenched with sodium thiosulfate (600 µg/mL) and catalase (2.5 mg/L) to ensure quenching of residual PAA and hydrogen peroxide prior to viral analysis. Quenched samples were then portioned and stored at − 80 °C.

Kinetic Modeling and Statistical Analyses

Disinfectant decay rate constants (k') for each experiment were calculated by regressing measured residuals using the least-squares method. First-order kinetics were assumed according to the equation:

$$C = C_0 \cdot e^{-k't}, \tag{1}$$

where k' = first-order disinfectant decay rate constant, C = observed disinfectant residual (mg/L), C_0 = initial disinfectant dose (mg/L), and t = time from start of experiment to time of sample (min).

Viral reductions were determined by calculating the negative $\log_{10}$ of the ratio of remaining organisms to initial organisms (N/N_0) at sample times and fit by a series of well-validated inactivation models derived from the generalized inactivation rate:

$$r_d = \frac{dN}{dt} = -kC^n m N^x t^{m-1}, \tag{2}$$

where r_d = inactivation rate, n, m, and x = dimensionless model parameters, k = inactivation rate constant, N = viral density (PFU or gene copies/mL), C = disinfectant concentration (mg/L), and T = contact time (min).

To account for the effect of disinfect decay throughout an experiment, Eq. (1) can be substituted into Eq. (2) and the resulting expression can be integrated to derive the specialized inactivation models employed in this study, which included the Chick–Watson (Watson 1908),

$$\log\left(\frac{N}{N_0}\right) = -\frac{k}{k'n}(C_0^n - C_t^n); \tag{3}$$

the incomplete gamma function (IGF) Hom (Haas and Joffe 1994),

$$\log\left(\frac{N}{N_0}\right) = -\frac{kmC_0^n}{(k'n)^m} \cdot \gamma(m, nk't); \tag{4}$$

the Power Law (Haas et al. 1995),

$$\log\left(\frac{N}{N_0}\right) = -\frac{\log\left[1 + (x-1) \times k/k'n(C_0^n - C_t^n) \times N_0^{x-1}\right]}{(x-1)}; \tag{5}$$

and the Hom Power Law (Anotai 1996),

$$\log\left(\frac{N}{N_0}\right) = -\frac{\log\left[1 + (x-1) \times kmC_0^n/(k'n)^m \times \gamma(m, nk't) \times N_0^{x-1}\right]}{(x-1)}. \tag{6}$$

These models were fit to experimental data using the least-squares method. Model fit was assessed by comparing the residual sum of squared errors (SSE). Partial F-tests were performed to assess significance of additional parameters in more highly parametrized models (<=0.05). Model errors were checked for normality using the Shapiro–Wilk statistical test. Modeling and statistical analysis were performed in Microsoft Excel and R (http://www.r-project.org).

Results

Virus Reduction Modeling

Temporal profiles of MS2 and MNV gene copy and infectivity reductions by a low dose of PAA (1.5 mg/L) at varying pH are shown in Fig. 1. Reductions by a high PAA dose (10 mg/L) are shown in Fig. 2. Temporal profiles of hNoV GI and GII gene copy reductions observed after low (1.5 mg/L) and high PAA (10 mg/L) doses are shown in Figs. 3 and 4, respectively. Control experiments were performed for each of the four viruses—MS2, MNV, hNoV GI, and hNoV GII—in PB (i.e., with no PAA) at each of the three experimental pH levels investigated in this study. Across all pH conditions, the control experiments resulted

in less than 0.5 $\log_{10}$ reduction in MS2 and MNV infectivity assays measurements and less than 0.4 $\log_{10}$ reduction of gene copies for all viruses after 120 min (data not shown).

Kinetic inactivation models accounting for PAA demand and residual decay were utilized in this study and fit to viral gene copy and infectivity reduction data. Data were segmented by virus, virus assay type (i.e., infectivity or RT-qPCR), and pH. For each viral reduction scenario, the best-fit model was determined and each best-fit model is presented in Table 1 with associated parameters. Modeling results for non-selected models along with Shapiro–Wilk test statistics for all model residuals are provided in Supporting Information Tables S1 and S2. As shown in Table 1, the IGF Hom model provided the best fit for 11 of the 18 scenarios. The Hom Power Law was found to be the best-fit model for six of the seven remaining scenarios, with the Power Law providing the best fit for one scenario (Table 1). For all scenarios, the best-fit model was used to plot observed gene copy or infectivity reductions against model-predicted reductions shown in Fig. 5.

Reduction of Surrogate Viruses

PAA resulted in high infectivity reductions for both MS2 and MNV in all experiments, with observed reductions between 1.7 $\log_{10}$ and 5.3 $\log_{10}$ over 120 min as shown in Figs. 1 and 2. MNV was more susceptible to PAA disinfection compared to MS2, particularly at the high PAA dose where MNV infectivity reductions were greater than 4 $\log_{10}$ after 15 min of contact time for all pH conditions. In contrast, MS2 infectivity was reduced by up to 1.6 $\log_{10}$ after 15 min (Fig. 2). At the low PAA dose, MNV infectivity in all experiments was reduced by greater than 4.5 $\log_{10}$ after 60 min, while MS2 infectivity reductions were between 1.0

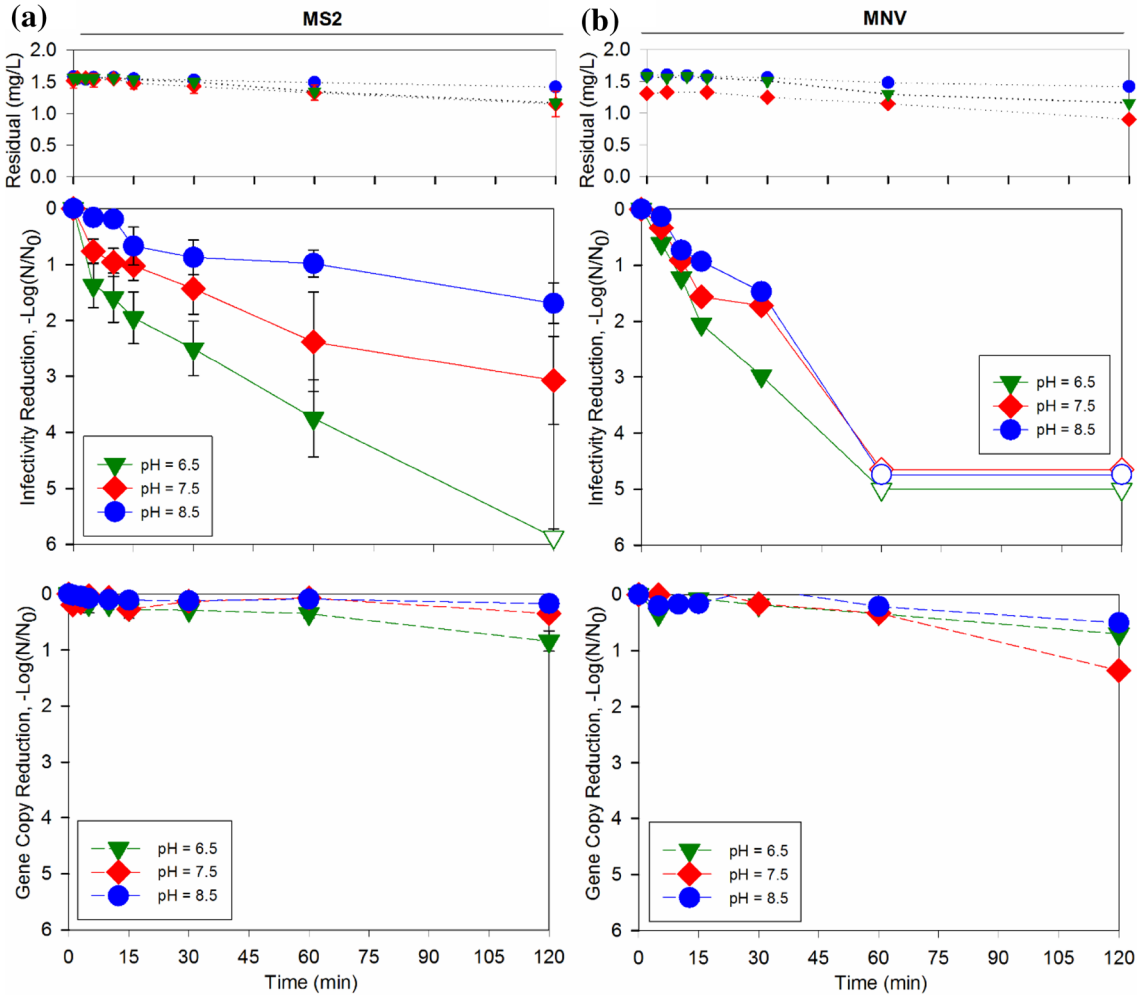

Fig. 1 Infectivity and gene copy reduction of MS2 (**a**) and MNV (**b**) in 0.01 M phosphate buffer (pH 6.5, 7.5, or 8.5) treated with PAA at a low dose of 1.5 mg/L and varying pH. Hollow symbols with no shading represent viral concentrations below the sensitivity limit of the assay. Error bars represent standard error of replicates

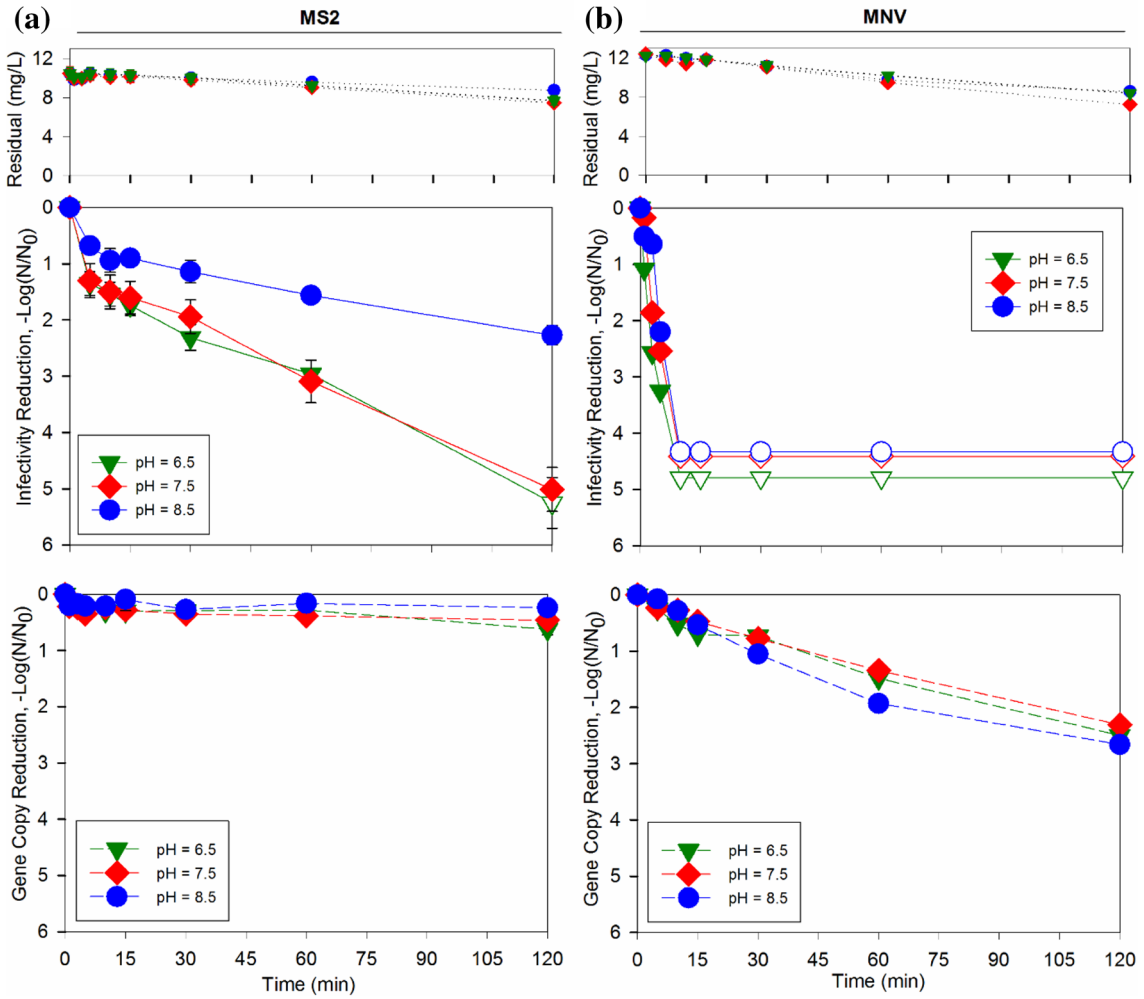

Fig. 2 Infectivity and gene copy reduction of MS2 (**a**) and MNV (**b**) in 0.01 M phosphate buffer (pH 6.5, 7.5, or 8.5) treated with PAA at a high dose of 10 mg/L and varying pH. Hollow symbols with no shading represent viral concentrations below the sensitivity limit of the assay. Error bars represent standard error of replicates

and 3.8 $\log_{10}$ at 60 min (Fig. 1). Lower gene copy reductions than infectivity reductions were observed for both MNV and MS2 at the two PAA doses (1.5 mg/L and 10 mg/L, Figs. 1, 2). Compared with MS2, MNV exhibited greater gene copy reductions, up to 2.7 $\log_{10}$ after 120 min at the 10 mg/L PAA dose (Fig. 2).

For further comparisons, CT values using integrated residuals accounting for PAA decay were determined for each virus and values were plotted against infectivity or nucleic acid reduction (Fig. 6). Plots are segmented by experimental pH, and each plot allows for direct comparisons of MNV and MS2 reductions as well as direct comparisons of virus assay type. Plots in Fig. 6 show that across experimental pH conditions, trends in susceptibilities to PAA among the four viruses were similar, with MNV and MS2 infectivity showing the greatest reductions. MNV and MS2 infectivity reductions were as high as 3.3–3.8 $\log_{10}$ at CT values less than 150 mg-min/L. Gene copy reductions of MS2 were minimal, with less than 0.4 $\log_{10}$ reduction observed at a CT value of 590 mg-min/L. MNV RNA was slightly more susceptible with gene copy reductions of 1.9 $\log_{10}$ observed at a CT value of 678 mg-min/L.

Reduction of Human Norovirus GI and GII

Temporal profiles of gene copy reductions for hNoV GI and GII in Figs. 3 and 4 show that molecular reductions by PAA were low for hNoV GI and GII. For the 1.5 mg/L PAA dose, between 0.2 and 1.2 $\log_{10}$ gene copy reduction was observed after 120 min of contact time (Fig. 3) across all pH values. Overall gene copy reductions of hNoVs were low relative to the >4 $\log_{10}$ infectivity reductions observed for MNV and MS2. At the 10 mg/L PAA dose, hNoV gene copy reductions were between 0.6 and 2.5

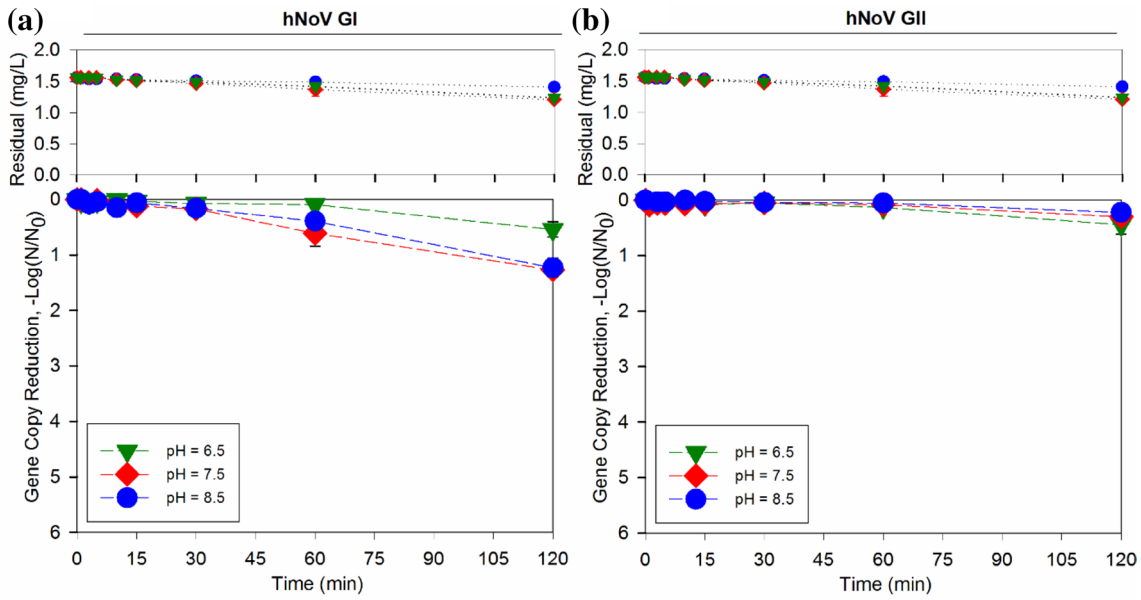

Fig. 3 Gene copy reduction of human norovirus (hNoV) GI (**a**) and GII (**b**) in 0.01 M phosphate buffer (pH 6.5, 7.5, or 8.5) treated with PAA at a dose of 1.5 mg/L and varying pH. Error bars represent standard error of replicates

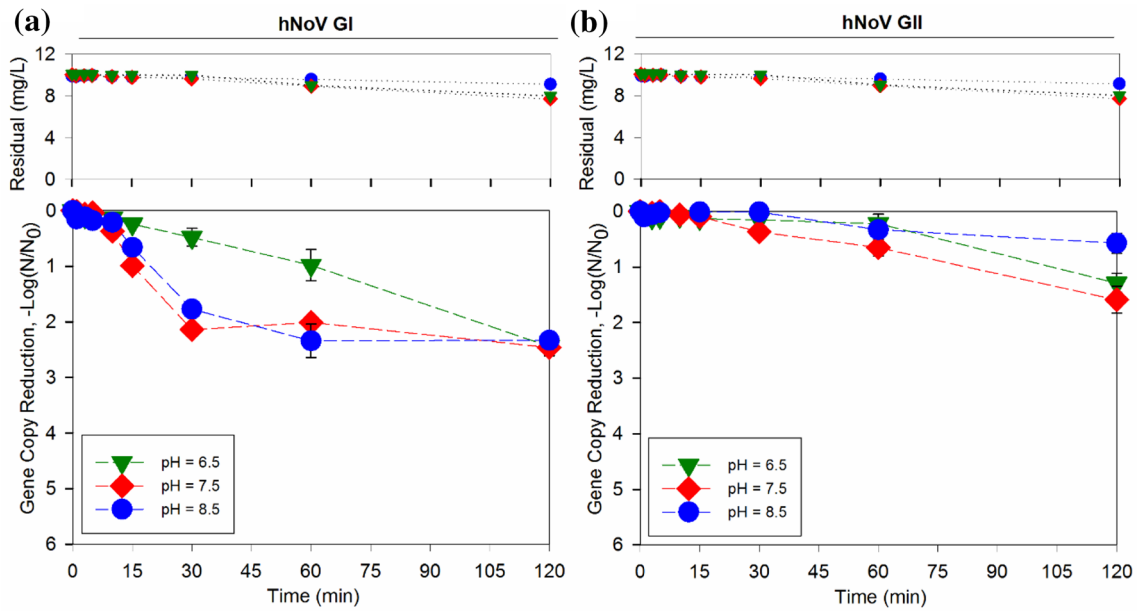

Fig. 4 Gene copy reduction of human norovirus (hNoV) GI (**a**) and GII (**b**) in 0.01 M phosphate buffer (pH 6.5, 7.5, or 8.5) treated with PAA at a dose of 10 mg/L and varying pH. Error bars represent standard error of replicates

$\log_{10}$ reduction, in a range similar to that observed for MNV gene copy reductions, which were between 0.5 and 2.7 $\log_{10}$. With similar ranges of RT-qPCR reductions observed for hNoVs and surrogates, and much greater reductions observed for infectivity surrogates, gene copy reductions may not be a robust predictor of reductions in infectivity as they may underestimate magnitude of infectivity reductions.

The gene copy reduction data show that hNoV GI is more susceptible than hNoV GII to PAA disinfection at both PAA doses. Up to 1.3 $\log_{10}$ reduction of hNoV GI was observed at the low PAA dose, compared to only 0.4 $\log_{10}$ reduction of

Table 1 Summary of best-fit model parameters for gene copy reduction of MS2, MNV, human norovirus GI, and human norovirus GII by PAA in water

pH	Virus	Assay	Best model	k'	k	n	m	x	σ	SSE
6.5	MS2	Infectivity	Hom Power Law	0.0015	1.31E+00	1.00E−12	0.407	1.01	0.20	0.17
6.5	MS2	RT-qPCR	Hom Power Law	0.0015	1.52E+00	1.00E−12	0.385	0.83	0.09	0.13
7.5	MS2	Infectivity	Hom Power Law	0.0027	1.12E+01	0.124	0.294	0.82	0.15	0.26
7.5	MS2	RT-qPCR	Hom Power Law	0.0027	3.60E+01	0.537	0.142	0.53	0.07	0.09
8.5	MS2	Infectivity	Hom Power Law	0.0024	6.10E−02	0.245	0.553	1.12	0.14	0.26
8.5	MS2	RT-qPCR	IGF Hom	0.0024	6.27E−02	0.367	0.096	–	0.04	0.03
6.5	MNV	Infectivity	Hom Power Law	0.0032	4.54E−05	2.133	2.057	1.57	0.08	0.05
6.5	MNV	RT-qPCR	IGF Hom	0.0032	1.52E−02	0.640	0.743	–	0.13	0.22
7.5	MNV	Infectivity	Hom Power Law	0.0044	1.28E−08	3.138	3.249	2.09	0.22	0.35
7.5	MNV	RT-qPCR	IGF Hom	0.0044	9.05E−05	0.139	2.009	–	0.31	0.08
8.5	MNV	Infectivity	Hom Power Law	0.003	1.75E+03	2.46E−07	0.388	0.21	0.17	0.22
8.5	MNV	RT-qPCR	IGF Hom	0.003	6.24E−03	0.930	0.818	–	0.14	0.24
6.5	hNoV GI	RT-qPCR	IGF Hom	0.0006	8.78E−04	0.871	1.245	–	0.05	0.05
7.5	hNoV GI	RT-qPCR	IGF Hom	0.0021	4.18E−02	0.581	0.611	–	0.30	1.58
8.5	hNoV GI	RT-qPCR	Hom Power Law	0.0018	8.11E−06	1.431	1.763	1.73	0.19	0.59
6.5	hNoV GII	RT-qPCR	Hom Power Law	0.0006	3.12E−01	1E−12	1.11	0.56	0.07	0.10
7.5	hNoV GII	RT-qPCR	Hom Power Law	0.0021	8.90E−05	0.838	1.46	1.19	0.04	0.04
8.5	hNoV GII	RT-qPCR	Power Law	0.0018	4.44E−03	0.564	–	0.95	0.04	0.03

MNV murine norovirus, *hNoV* human norovirus, *σ* standard deviation of the errors, *SSE* sum of squares of the errors

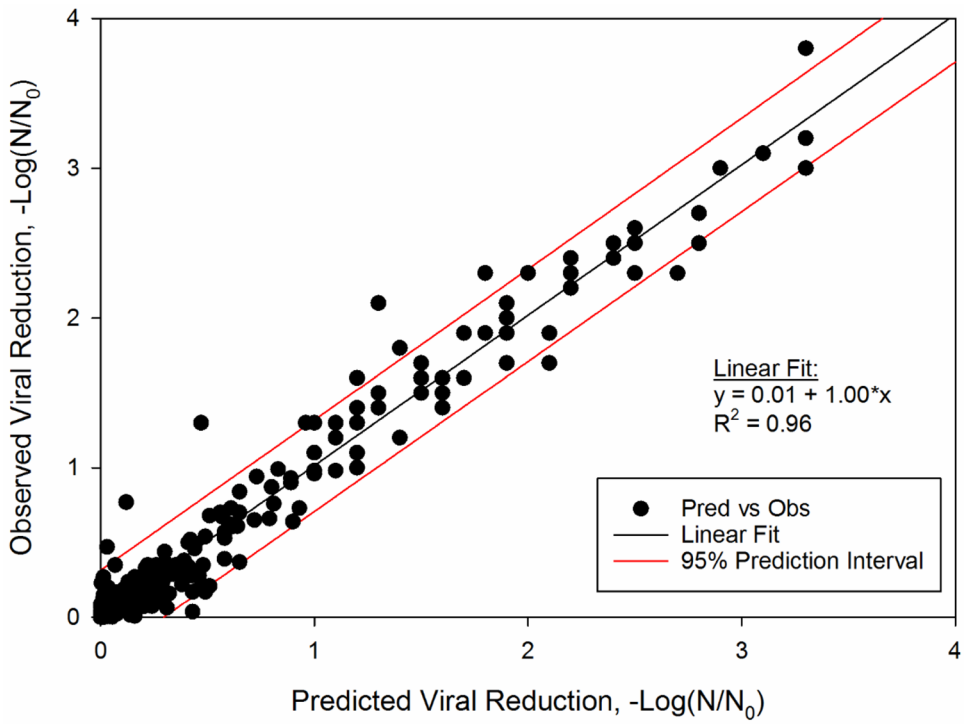

Fig. 5 Observed gene copy reduction versus model-predicted gene copy reduction for human norovirus GI, human norovirus GII, MNV, and MS2 in 0.01 M phosphate buffer

hNoV GII (Fig. 3). At the high PAA dose, hNoV GI showed reductions of 2.3–2.5 $\log_{10}$, while hNoV GII showed reductions of 0.6–1.6 $\log_{10}$ (Fig. 4). At pH 7.5, model-predicted CT values required for 1 and 2 $\log_{10}$ reduction of hNoV GII were 562 and 1000 mg-min/L, respectively, while for hNoV GI the values were 189 and 559 mg-min/L, respectively (Table 2).

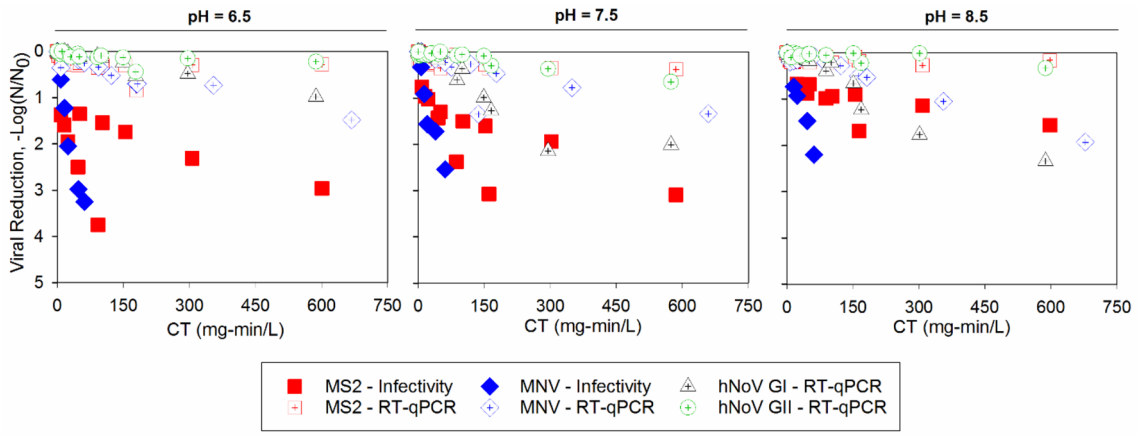

Fig. 6 Comparison of integrated PAA CT values at varying pH for reduction of MS2, MNV, human norovirus (hNoV) GI, and hNoV GII in water by infectivity (MS2 and MNV only) and RT-qPCR

Effect of pH on PAA Disinfection

Viral reductions were observed under three pH conditions—6.5, 7.5, and 8.5—as seen in temporal plots provided in Figs. 1–4, where line color indicates the relevant pH conditions. The data presented in Figs. 1–4 indicate a trend of lowered PAA viricidal efficacy at elevated pH conditions. For example, MS2 infectivity reductions after 120 min of contact time at pH 8.5 were 1.7 and 2.3 $\log_{10}$ for initial PAA doses of 1.5 mg/L and 10 mg/L, respectively, while reductions at pH 6.5 were 5.9 and 5.3 $\log_{10}$, respectively (Figs. 1, 2). Similar to MS2 infectivity results, MNV infectivity at the 1.5 mg/L PAA dose showed the least reduction at pH 8.5, where observed reduction was 1.5 $\log_{10}$ after 30 min. After 30 min of contact time at pH 6.5 and 7.5, MNV infectivity reductions were 3.0 and 1.7 $\log_{10}$, respectively (Fig. 1). For MNV treated with the high PAA dose (10 mg/L), infectivity reductions were similar across all pH values with each showing a sharp reduction of >4 $\log_{10}$ after 15 min, though the rate of reduction was slightly greater at pH 6.5 relative to other pH conditions (Fig. 2). MNV and MS2 nucleic acid reductions were low relative to infectivity reductions, though at the low PAA dose (1.5 mg/L), reductions were lowest at pH 8.5, similar to the patterns observed for MS2 and MNV infectivity (Figs. 1, 2).

hNoV GI and GII gene copy reductions at the low PAA dose (1.5 mg/L) were minimal, with 0.2–1.3 $\log_{10}$ reduction observed after 120 min of exposure. For hNoV GI, the pattern of decreased reduction at elevated pH was marginal (Fig. 3). For hNoV GII, gene copy reductions were slightly lower at pH 8.5, although the difference was minimal with differences of less than 0.2 $\log_{10}$ observed between pH

Table 2 Model-predicted PAA CT values required for 1, 2, 3, and 4-log10 reductions of MS2, MNV, human norovirus GI, and human norovirus GII as measured by RT-qPCR and by infectivity (surrogates only) in 0.01 M phosphate buffer at pH 6.5, 7.5, and 8.5

pH	Reduction	CT value (mg-min/L)					
		MS2		MNV		hNoV GI	hNoV GII
		Infectivity	RT-qPCR	Infectivity	RT-qPCR	RT-qPCR	RT-qPCR
6.5	1-$\log_{10}$	18	1572	12	323	442	728
	2-$\log_{10}$	98	3365	25	935	766	950
	3-$\log_{10}$	265	3822	49	1553	1054	1028
	4-$\log_{10}$	530	3904	94	2175	1152	1056
7.5	1-$\log_{10}$	30	*N.O.*	18	323	189	562
	2-$\log_{10}$	164	*N.O.*	40	449	559	1000
	3-$\log_{10}$	344	*N.O.*	88	579	1177	2100
	4-$\log_{10}$	508	*N.O.*	202	667	1838	2954
8.5	1-$\log_{10}$	130	*N.O.*	33	419	419	1268
	2-$\log_{10}$	549	*N.O.*	48	848	848	3245
	3-$\log_{10}$	1262	*N.O.*	*N.O.*	1396	1396	4239
	4-$\log_{10}$	2027	*N.O.*	*N.O.*	1377	1377	4913

conditions. At the high PAA dose (10 mg/L), hNoV GII experiments at pH 8.5 exhibited the least reduction, with gene copy reduction at pH 6.5 and 7.5 also not significantly different at 120 min (Fig. 4). Dissimilarly, for hNoV GI at the 10 mg/L PAA dose, pH 7.5 and 8.5 gave greater reduction than pH 6.5 for the first 60 min of exposure, although all pH conditions in this experiment achieved 2.3–2.5 $\log_{10}$ reduction after 120 min (Fig. 4).

Comparisons across pH conditions are reported in the CT plots shown in Fig. 6. The largest viral reductions were observed for MNV and MS2 infectivity among all experiments. Comparing MNV and MS2 infectivity across pH in these CT plots reveals the trend of lowest reduction occurring at pH 8.5, with a 2.2 $\log_{10}$ reduction of MNV at a CT of 61 mg-min/L and a 1.6 $\log_{10}$ reduction of MS2 at 598 mg-min/L CT (Fig. 6).

Discussion

Increased use of PAA in wastewater treatment settings necessitates in-depth characterization of PAA disinfection. In this work, PAA achieved a decrease in virus activity for surrogate viruses MNV and MS2 and also achieved a decrease in nucleic acid copy number for hNoVs, which previously have not been widely evaluated in disinfection studies employing PAA. In this work, to further assess the viricidal activity of PAA, pH effects on PAA disinfection were also assessed. Previous studies showed pH effects on PAA disinfection of bacteria and bacteriophage (Baldry and French 1989; SanchezRuiz et al. 1995), and our research similarly found that pH affects removal of hNoVs, MNV, and MS2, with generally lower virus removal at elevated pH.

Virus Reduction Models

Reduction models which considered disinfectant demand and decay were fit to viral reduction data for each experimental scenario in the study, and the plot of observed gene copy or infectivity reduction against predicted reduction is shown in Fig. 5. The plot shows that the selected models for each scenario effectively represented experimental data. The majority of virus—assay—pH scenarios (11 of 18 scenarios) showed best fit with the IGF Hom model (Table 1), while the Hom Power Law was the best-fit model for six of the seven remaining scenarios (Table 1). The IGF Hom model is able to account for virus tailing in experimental data which likely allowed this model to fit most closely with the majority of experimental data sets. The fourth parameter contained only in the Hom Power Law likely also contributed to this model's ability to effectively represent reduction data.

Surrogate Virus Reduction by PAA

hNoV surrogates MNV and MS2 were used in disinfection experiments along with hNoV GI and GII in this study. While hNoVs were only analyzed by RT-qPCR due to the lack of a cell culture system, MNV and MS2 were analyzed by both RT-qPCR and infectivity assays to elucidate the surrogate virus infectivity–molecular relationship, which can then be used to guide interpretation of hNoV RT-qPCR reduction measurements.

Both surrogate viruses showed large infectivity reductions of up to 5 $\log_{10}$ upon treatment with PAA (Figs. 1, 2). Previous studies evaluating either MS2 or MNV have found both viruses to be sensitive to PAA (Baldry et al. 1991; Fraisse et al. 2011; Zonta et al. 2016). In the current study, MS2 was more resistant to disinfection than MNV, which is in agreement with some studies of PAA water treatment as well as studies of other disinfectants such as sodium chlorite and monochloramine in which MS2 and MNV disinfection were compared (Dunkin et al. 2017a; Park et al. 2007). Mechanisms dictating the differences between MS2 and MNV reduction are not fully known, but researchers have found that disinfectants can affect capsids and genomes in a variety of ways, and, among similar viruses, even minor differences in capsid structure and genome cause vast differences in disinfection susceptibility (Wigginton et al. 2012). MS2 and MNV have differences in the surface isoelectric charges associated with their capsids, with MS2 having overall more negative charge (lower isoelectric point) than MNV (Mayer et al. 2015), and in some research, a lower isoelectric point of a virus has been found to correlate with lower virus removal or greater stability (Mayer et al. 2008, 2015).

Gene copy measurements of MS2 and MNV by RT-qPCR showed significantly less reduction than infectivity measurements under the same treatments, supporting previous findings that show an underestimation of infectivity reduction in corresponding viral RT-qPCR measurements (Hewitt et al. 2009; Knight et al. 2016). Further, while gene copy reductions were low relative to infectivity reductions for these viruses, measurements of gene copy reductions showed the same trends as infectivity, where MNV exhibited greater susceptibility to PAA at both high and low doses compared to MS2 (Figs. 1, 2). Still, even with low to moderate reductions as measured by RT-qPCR, PAA showed substantial infectivity reduction for surrogate viruses MNV and MS2.

PAA Reduction of hNoVs and Comparison to Surrogate Viruses

Gene copy reductions of hNoVs in this study were in ranges similar to those of the surrogate viruses (Figs. 1, 2, 3, 4), although greater gene copy reductions for hNoV GI were observed compared to hNoV GII at both PAA doses (Figs. 3,

4). Best-fit models were used to predict CT values for designated reductions of each virus (Table 2), and these predicted CT values also underline the differences in PAA efficacy against hNoV GI versus GII. At pH 7.5, for hNoV GI, predicted PAA CT values for 1 and 2 $\log_{10}$ RT-qPCR reductions were 189 and 559 mg-min/L, respectively. In contrast, CT values for 1 and 2 $\log_{10}$ reductions of hNoV GII at pH 7.5 were 562 and 1000 mg-min/L, respectively (Table 2).

Although some research has reported conflicting results upon comparing persistence of hNoV GI and GII (Butot et al. 2008), several studies have shown hNoV GII to be more persistent than GI upon treatment with chlorine, alcohols, high pressure, chloramines, and PAA (Cromeans et al. 2014; Dunkin et al. 2017a; Park et al. 2007; Wigginton et al. 2012). The variation in susceptibility between norovirus genogroups observed in this study is thus in agreement with evidence in the literature and suggests that for hNoVs, viral genogroup status should be considered as an important variable in treatment and persistence studies.

Differences in reduction between hNoV GI and GII are of importance for comparisons with viral surrogates. Our molecular reduction data showed that sensitivity to PAA was comparable for hNoV GI and MNV, while both MS2 and hNoV GII were more resistant (Figs. 1–4). This pattern was also observed in the plots of CT versus $\log_{10}$ reduction that allow comparisons among viruses at a single pH (Fig. 6). For MS2 and hNoV GII, gene copy reductions were comparable, as both showed less than 0.9 $\log_{10}$ at CT values up to 600 mg-min/L under all pH conditions (Fig. 6). Gene copy reductions for hNoV GI were 1.2–2.3 $\log_{10}$ at CT values between 500 and 600 mg-min/L CT, while MNV gene copy reductions were similar with reductions of 1.3–1.9 $\log_{10}$ at CT values between 600 and 700 mg-min/L. In summary, hNoV GII and MS2 exhibited the greatest resistance to PAA disinfection as measured by RT-qPCR, with large reductions in MS2 infectivity also observed. hNoV GI and MNV exhibited moderate reductions as assessed by RT-qPCR, though measurements of MNV infectivity were highly susceptible to PAA treatment during the 120-min disinfection period.

Previous research comparing hNoVs with surrogate viruses by RT-qPCR also found hNoV GI and MNV to be more susceptible to PAA, while hNoV GII and MS2 were more persistent during PAA treatment (Dunkin et al. 2017a). Disinfection studies employing free chlorine have suggested that MNV and hNoV GII exhibited similar reductions in response to disinfection (Kitajima et al. 2010; Tung et al. 2013), though similarities may be attributable to the use of free chlorine in these experiments, which is a non-specific oxidant that may have quickly oxidized both hNoV GII and MNV nucleic acid. In summary, PAA caused a moderate removal of hNoV gene copies, with hNoV GI reduction similar to that of surrogate virus MNV, while hNoV GII showed behavior more similar to MS2. These data support previous research showing that closely related viruses can have differing responses to disinfectants (Wigginton et al. 2012) and further support the importance of considering genogroup when evaluating hNoV persistence.

Although a hNoV culturing method suitable for environmental studies is not currently available, assessment of surrogate virus infectivity reductions in relation to RT-qPCR measurements can inform interpretation of hNoV RT-qPCR reductions. In this study, similar gene copy reductions were observed for MNV and hNoV GI, with reductions up to 1.4 $\log_{10}$ and 2.7 $\log_{10}$ for low (1.5 mg/L) and high (10 mg/L) PAA doses, respectively (Figs. 1–4). This suggests that PAA may cause greater reductions in viral infectivity for hNoV GI, as were observed for MNV. Infectivity reductions for MNV were greater than 4 $\log_{10}$ after 15 min of contact time at the high PAA dose (10 mg/L) (Fig. 2). Similarly, comparable gene copy reductions were observed for MS2 and hNoV GII, up to 0.8 $\log_{10}$ and 1.6 $\log_{10}$ at the low (1.5 mg/L) and high (10 mg/L) PAA doses, respectively (Figs. 1–4). This indicates that a reduction in hNoV GII infectivity may occur with PAA treatment as was observed with MS2 infectivity measurements (Fig. 2).

Effect of pH on PAA Disinfection

With increasing interest in PAA for wastewater disinfection (Kitis 2004; Luukkonen and Pehkonen 2017), there is a need to determine optimal disinfectant conditions for PAA since source water quality may vary greatly. While assessing PAA inactivation of noroviruses in this work, we sought to determine whether variation in pH affects the viricidal efficacy of PAA.

Viral reduction by PAA was observed at each of the pH conditions evaluated in this study: 6.5, 7.5, and 8.5 (Figs. 1–4). For the elevated pH conditions of 8.5, however, viral reduction was generally lower and rates of reduction across the 120 min exposure time also tended to be lower compared to pH 6.5 and 7.5. This was particularly true for infectivity assays, which at lower pH conditions showed viral reductions of greater than 4 $\log_{10}$ after 120 min contact time (Figs. 1, 2), while gene copy assays with lower reduction across experiments show smaller differences across pH conditions (between 0.1 and 0.7 $\log_{10}$) (Figs. 1–4). In previous studies that have addressed PAA disinfection efficacy under varying pH conditions, it was shown that disinfectant efficacy of PAA for *E. coli* and other fecal coliform bacteria is decreased above pH 8 (Baldry and French 1989; McFadden et al. 2017; SanchezRuiz et al. 1995). Inactivation of MS2 and other bacteriophages also showed less reduction in infectivity above pH 8 (Baldry and French 1989). The decreased activity of PAA measured at pH values greater than 8 may be in part attributable to the known pK_a of the acid, 8.2, above which the ionized form of the acid

($CH_3CO_3^-$) predominates (Kitis 2004). The ionized form of PAA is thought to hydrolize to peracetate and acetate, which provide less disinfection activity (McFadden et al. 2017). It is reasonable, then, that in the current study viral reduction was also lower at pH 8.5 relative to that at pH 6.5 and 7.5. In addition, at the higher pH, viruses that have a net negative charge at pH 8.5 due to their protein capsid composition will be more repelled from the predominating PAA species peracetate and acetate (Mayer et al. 2015), further preventing inactivation. This may explain why pH effects were not identical across viruses. Notably, for hNoV GI, nucleic acid reductions were not lowest at pH 8.5; in fact, reductions were most decreased at pH 6.5 for this virus. This pattern may be related to amino acid composition of hNoV GI causing positive charges to predominate the capsids at pH 6.5, which would attract any ionic PAA species. Contrastingly, MS2, which has a relatively low isoelectric point and overall negatively charged capsids at pH levels of interest (6.5–8.5) (Mayer et al. 2015), showed inactivation that was more strongly reduced by elevated pH (pH 8.5) compared to other viruses in this study. Overall PAA water treatment at a pH between 6.5 and 7.5 appears to be best for achieving viral reduction, with virus infectivity reductions particularly strong under these pH conditions.

Conclusions

With growing interest in PAA as a wastewater disinfectant, there is a need to understand its efficacy against hNoVs under a range of conditions relevant to water treatment. In this study, we observed that PAA efficiently reduced MS2 and MNV infectivity, with lower reductions observed for MS2, MNV, hNoV GI, and GII as measured by RT-qPCR. Through application of best-fit models, CT values for 1–2 $\log_{10}$ infectivity reduction of MS2 and MNV at pH 6.5–7.5 were found to be 12–164 mg-min/L.

Nucleic acid reductions of hNoVs were measured by RT-qPCR and found to be in a range similar to RT-qPCR reductions of surrogate viruses. In particular, gene copy reductions of MS2 were comparable to those of hNoV GII and in the range of 0.2–1.8 $\log_{10}$. MNV gene copy reductions showed similarity to those of hNoV GI, with MNV and hNoV GI gene copy reductions in the range of 0.5–2.7 $\log_{10}$.

Since PAA more efficiently reduced MS2 and MNV infectivity compared to MS2 and MNV gene copies, the moderate reductions of hNoV gene copies measured by RT-qPCR suggest that these measurements may underestimate reductions in infectivity of hNoVs during treatment with PAA. Furthermore, these data also indicate that hNoV disinfection studies performed using RT-qPCR measurements have likely underestimated hNoV infectivity reductions.

Additionally, the effect of pH on PAA viricidal efficacy was investigated. Although PAA achieved viral reductions at pH 6.5, 7.5, and 8.5 for all viruses, reductions were generally lowest at pH 8.5. PAA exhibits the greatest viricidal efficacy at pH 6.5–7.5, with particularly strong reduction of MNV and MS2 infectivity under these pH conditions. These results add to the growing knowledge base of PAA and may assist wastewater treatment managers and operators in making the best decisions for targeting hNoV and its surrogates.

Future research examining PAA pH variation in wastewater matrixes will further improve the understanding of this disinfectant in real-world settings. Also, development of a culturing method for infectivity analyses of hNoVs will allow for more accurate determination of the response of hNoVs to PAA treatments. Still, we have shown that experimental analyses of MS2 and MNV infectivity and RT-qPCR in conjunction with hNoV RT-qPCR measurements allow for effectively assessing the response of hNoVs and similar viruses to PAA treatment and provide insight for future hNoV disinfection studies.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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