Environmental Science & Technology

ARTICLE

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Impact of Virus Aggregation on Inactivation by Peracetic Acid and Implications for Other Disinfectants

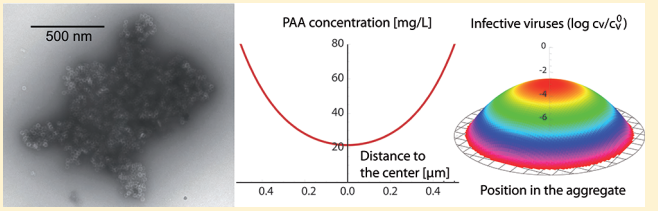
Michael J. Mattle,[†] Benoît Crouzy,[‡] Moritz Brennecke,[†] Krista R. Wigginton,[†,§] Paolo Perona,[‡] and Tamar Kohn*[,†]

[†]Laboratory of Environmental Chemistry, School of Architecture, Civil and Environmental Engineering (ENAC), École Polytechnique Fédérale de Lausanne (EPFL)

[‡]Applied Hydroeconomics and Alpine Environmental Dynamics Lab, School of Architecture, Civil and Environmental Engineering (ENAC), École Polytechnique Fédérale de Lausanne (EPFL)

Supporting Information

ABSTRACT: Viruses in wastewater and natural environments are often present as aggregates. The disinfectant dose required for their inactivation, however, is typically determined with dispersed viruses. This study investigates how aggregation affects virus inactivation by chemical disinfectants. Bacteriophage MS2 was aggregated by lowering the solution pH, and aggregates were inactivated by peracetic acid (PAA). Aggregates were redispersed before enumeration to obtain the residual number of individual infectious viruses. In contrast to enumerating whole aggregates, this approach allowed an assessment of disinfection efficiency which remains applicable even if the aggregates disperse in post-treatment environments. Inactivation kinetics were determined as a function of aggregate size (dispersed, 0.55 and 0.90 μm radius) and PAA concentration (5−103 mg/L). Aggregation reduced the apparent inactivation rate constants 2−6 fold. The larger the aggregate and the higher the PAA concentration, the more pronounced the inhibitory effect of aggregation on disinfection. A reaction-diffusion based model was developed to interpret the experimental results, and to predict inactivation rates for additional aggregate sizes and disinfectants. The model showed that the inhibitory effect of aggregation arises from consumption of the disinfectant within the aggregate, but that diffusion of the disinfectant into the aggregates is not a rate-limiting factor. Aggregation therefore has a large inhibitory effect if highly reactive disinfectants are used, whereas inactivation by mild disinfectants is less affected. Our results suggest that mild disinfectants should be used for the treatment of water containing viral aggregates.



INTRODUCTION

Virus removal and inactivation present a major challenge for drinking water treatment. Due to their small size (18−120 nm diameter) and relative resistance to common disinfectants, viruses can penetrate traditional water treatment systems.[1] To meet the drinking water treatment goals defined by the U.S. Environmental Protection Agency, 4 $\log_{10}$ (or 99.99%) of enteric viruses must be removed or inactivated during the production of drinking water from surface waters or groundwater under direct influence of surface water. This can be achieved by either combining disinfection with filtration, or by disinfection alone.[2]

The efficiency of water disinfection cannot be measured in real-time due to the fact that infective virus titer measurements can take several days for some human viruses and are not available for others. Consequently, the applied disinfectant doses are typically based on inactivation data reported in the literature. Inactivation studies are usually performed using a population of dispersed viruses; however, viruses in wastewater and surface waters are often present as aggregates.[3,4] For example, Hejkal et al.[5] reported that ultrasonication of fecal particles lead to an increase in virus titer, thus indicating the presence of aggregated or particle-associated viruses. Aggregates of viruses enveloped by cell debris have also been reported.[6]

Berg et al.[7] first proposed that viral aggregation could cause a decrease in inactivation rates. Several later studies showed important differences in inactivation kinetics between dispersed and aggregated viruses when treated with chemical disinfectants.[3,8−10] Each of these studies reported a tailing-off of the inactivation curve, which was rationalized by the presence and protective effect of aggregates. In contrast, in at least two studies consistent first-order inactivation kinetics were observed for aggregated viruses.[3,11] Cell-associated viruses, which have similar disinfection kinetics as viruses derived from feces,[12] also showed slower inactivation kinetics than dispersed viruses and occasionally exhibited the tailing-off effect.[13,14] No conclusive explanation could be given for this behavior, however, as the reduction could be due to viral aggregation, protection by the cellular structure, or both.

Received: May 13, 2011
Accepted: August 5, 2011
Revised: August 4, 2011
Published: August 05, 2011

It is thus evident that applying disinfectant doses based on results obtained with dispersed viruses may largely underestimate the doses needed to achieve a required level of inactivation in the presence of aggregates. The detrimental effects of using inadequate disinfectant doses were observed during early polio vaccination campaigns: several patients died of paralytic polio upon inoculation with insufficiently inactivated viruses. One hypothesis for the cause of the outbreak was that some viruses were protected from formaldehyde inactivation due to their assembly with tissue fragments or cell debris.[15] Alternatively, the incomplete inactivation process may have been a result of the protective effect of viral aggregates, as poliovirus has been shown to be released from its host in aggregated form.[3]

There are several potential reasons for viral aggregates to be more resistant to inactivation than dispersed viruses. For example, all viruses within an aggregate, which represents a single infectious unit, need to be inactivated for the aggregate to be inactivated. The inactivation of an aggregate will therefore take longer than the inactivation of a single virus.[7] Additionally, viruses in the core of an aggregate may be protected from inactivation[12,16] due to limited diffusion or consumption of the disinfectant during passage through the aggregate.

Most studies to date have focused on the disinfection of viral aggregates as a whole.[3,8,17,18] To the authors' knowledge, no experimental data exists that captures the disinfection of single viral particles within an aggregate. One can reconstruct the fate of a whole viral aggregate only once the inactivation behavior of single viruses within an aggregate is known. The observed reduction in disinfection efficiency can then be interpreted within the framework of the different inhibiting factors stated in the previous paragraph.

The aim of this study was to experimentally assess the effect of aggregation on virus inactivation by a chemical disinfectant, and to establish a model framework to explain the observed disinfection kinetics. A novelty of this work is the focus on the fate of single viruses, rather than entire aggregates, during the disinfection process. This approach allows an assessment of the residual infectious units, which remains accurate even if the aggregates disperse in post-treatment environments. Inactivation kinetics were investigated as a function of aggregate size and disinfection concentration. Based on these results, a reaction-diffusion model was developed. The model was further used to predict the impact of aggregation on disinfection kinetics for varying aggregation sizes and different disinfectants.

EXPERIMENTAL SECTION

In brief, dispersed and aggregated samples of bacteriophage MS2 were inactivated by peracetic acid (PAA), and inactivation kinetics were monitored and subsequently modeled. MS2 samples were aggregated, exposed to PAA, redispersed, and finally enumerated. Experiments were performed in 15 mM phosphate buffer solutions containing 15 mM chloride and varied concentrations of PAA. Aggregates of different sizes were produced by adjusting the solution pH to values between 3.0 and 5.0. To differentiate between pH effects and true aggregation effects, pH control experiments were conducted at higher phosphate concentrations (400 mM), which prevented aggregation.

Chemicals and Organisms. The chemicals, organisms and culturing methods used are described in the Supporting Information.

Aggregate Size Measurements. Experimental details relating to aggregate size measurements by dynamic light scattering (DLS) and transmission electron microscopy (TEM) are described in the Supporting Information.

Viral Aggregation and Dispersal. Aggregates were produced by lowering the solution pH below the viral isoelectric point (pI) of 3.9.[19] They were then disinfected and finally redispersed by increasing the pH above 7.0 before enumeration. Aggregation was induced at a virus concentration of 1×10^{11} plaque forming units (pfu)/mL in a 15 mM phosphate and 15 mM chloride buffer at pH values of 3.0, 3.6, and 5.0, giving rise to large viral aggregates (0.90 μm radius averaged over the duration of the experiment), intermediate aggregates (0.55 μm), and dispersed viruses, respectively. Prior to use, all buffers were filtered with a cellulose nitrate membrane filter (0.1 μm pore size; Whatman GmbH, Dassel Germany). Aggregate formation was monitored in real-time by continuous DLS-measurements.

High phosphate concentrations prevented virus aggregation, even at low pH. A set of control experiments was thus performed in 400 mM phosphate and 15 mM chloride, to investigate the effect of pH on our experimental system without the confounding effect of simultaneous aggregation.

Following treatment with the disinfectant, viruses were redispersed by raising the solution pH above 7.0. This was accomplished by the addition of low concentration phosphate buffered saline (PBS: 5 mM phosphate, 10 mM NaCl, pH 7.5) for experiments conducted in solutions with low phosphate concentrations, and with high concentration phosphate buffered saline (HPBS: 150 mM phosphate, 15 mM chloride at pH 7.5) for experiments conducted in solutions with high phosphate concentrations.

Control experiments were conducted in the absence of disinfectant to study the dispersion efficiency by increasing the pH, as well as the stability of viruses at pH 3.0. A 0.3 $\log_{10}$ loss in infectivity was observed in samples that were aggregated in a pH 3.0 solution and then redispersed. Another 0.3 $\log_{10}$ loss was observed over the time frame of the experiment.

Inactivation by Peracetic Acid. PAA's disinfection efficiency is relatively pH independent;[20] it is considered a good alternative to chlorine for wastewater disinfection.[21,22] All experiments were performed in phosphate buffers containing 15 mM chloride at 22 °C. Chloride was essential for PAA disinfection; in the absence of chloride, PAA did not inactivate MS2.

Solutions containing 15 mM phosphate and 15 mM chloride were spiked with EDTA to a final concentration of 0.4 μM in order to complex trace metals. This prevented inactivation by hydroxyl radicals generated by Fenton-like reactions with hydrogen peroxide present in the peracetic acid solution.[23] Aliquots of the prepared buffer solution were subsequently spiked with a freshly prepared PAA stock solution (5 g/L) to desired concentrations. The pH was then readjusted using HCl or NaOH.

PAA-free buffer solution (500 μL) was placed into a 1 mL cuvette (Brand GmbH, Wertheim, Germany) and introduced into the DLS. Virus stock in PBS (10 μL, 5×10^{12} pfu/mL) was then added to the cuvette and the sample was gently mixed. DLS measurements to determine the viral aggregate size were collected every two minutes throughout the experiment. The viruses were allowed to aggregate for one hour. This step was not included in pH 5.0 experiments as no aggregation was observed. Disinfection was then initiated with the addition of 500 μL of the PAA buffer solution and the samples were gently mixed. The final PAA concentrations ranged between 5 and 103 mg/L.

Sample aliquots (10 μL) were periodically extracted and diluted in 120 μL PBS. The remaining PAA was quenched by the addition of 120 μL of a 350 mg/L sodium thiosulfate solution. Diluted samples were plated to enumerate infective virus immediately after the experiments. Selected experiments were performed in duplicate and good reproducibility was observed.

The pH control experiments using 100 mg/L PAA were conducted as described above, except that the buffer contained 400 mM phosphate and 20 μM EDTA. For enumeration, samples were diluted using HPBS.

PAA concentrations were measured in duplicate before and after each experiment using a spectrophotometric method[24] which selectively measures PAA and avoids interference from hydrogen peroxide. In each experiment, PAA concentrations remained within 5% of their initial value.

Data Analysis. MS2 inactivation followed an exponential decay and could be approximated by a pseudofirst-order model

$$\partial_t c_v = -k_{app} c_v \quad (1)$$

where c_v is the concentration of infective viruses, and k_{app} is the apparent inactivation rate constant. The values of k_{app} and their associated 95% confidence intervals were determined by a pseudo-first-order fit.

RESULTS AND DISCUSSION

MS2 Aggregation. In order to evaluate the impact of aggregation on inactivation, two experimental setups had to be defined: one in which the viruses were aggregated, and one in which they were dispersed. Viral aggregation depends on the surface properties and the concentration of the virus,[25] as well as the composition of the solution in which they are stored.[26] Most viruses are negatively charged at environmentally relevant pH. In order to aggregate, the repulsive electrostatic forces between the negatively charged viruses must be sufficiently reduced or shielded such that the viral particles can approach each other closely, allowing attractive interactions (e.g., van der Waals forces, hydrophobic interactions) to dominate. This can be achieved by decreasing the pH to values close to or below the viral pI.[27−30] Alternatively, aggregation can be influenced by the presence of mono- and divalent cations,[29,31] but the effect of ions on aggregation is not fully understood. Several studies demonstrated that high ionic strength hindered aggregation of polio-, reo- and adenovirus.[25,29,32] The opposite effect was observed for Qβ phage, which exhibited more aggregation at higher ionic strength.[27] Similarly, coxsackievirus B5, GA, and SP, aggregated at neutral pH and high sucrose or salt concentrations.[11,27]

Contrary to other work,[31] the addition of cations (Ca^{2+}) did not cause MS2 aggregation in our experiments. We therefore manipulated the solution pH to induce aggregation. MS2 is negatively charged at pH values above its pI of 3.9, and the individual viruses repel each other. Correspondingly, our experimental data (Figure 1a) showed that at pH 5.0, viruses were in a dispersed state. However, if the pH was lowered below the pI, the negative surface charge decreased and the viruses readily aggregated (Figure 1a, 0 to 60 min). The rate at which aggregates formed was dependent on the pH: after one hour at pH 3.0, the radius of the aggregates was roughly 1.0 μm, whereas at pH 3.6, the radius was approximately 0.75 μm. Repulsive forces thus seemed to be less important at pH 3.0 than at pH 3.6.

The growth rate and size of aggregates decreased after the PAA was introduced to the system (Figure 1a, after 60 min). The lower growth rate can be explained by the fact that the addition of PAA led to a 2-fold dilution of the virus concentration. The lower virus concentration resulted in a reduced probability of viruses to collide, thereby decreasing the aggregate growth rate. The drop in aggregate size is attributed to partial aggregate break up by shear stress caused by the addition of PAA and subsequent mixing. Note that the addition of PAA-free buffer solution had the same effect (data not shown).

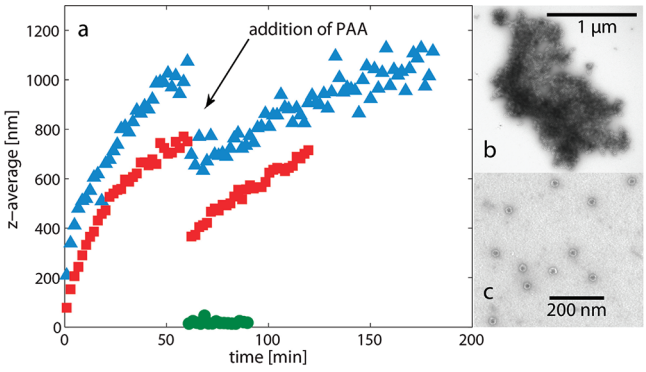


Figure 1. (a) z-average radius values measured in parallel to disinfection experiments (40 mg/L PAA) for pH values 3.0 (blue triangle), 3.6 (red square), and 5.0 (green circle) in 15 mM phosphate and 15 mM chloride (initial MS2 concentration of 10^{11} and 5×10^{10} pfu/mL before and after addition of PAA, respectively). The viruses were left to aggregate for one hour before PAA was added. The measurement for the experiment at pH 5.0 started after one hour, since no aggregation occurred at this pH. (b) TEM image of a viral aggregate (pH 3.0). (c) TEM image of a dispersed virus sample (pH 5.0). Individual viral particles can be distinguished by their dark RNA center and lighter colored protein coat.

TEM images confirmed the presence of aggregated viruses in samples at pH 3.0 (Figure 1b) and dispersed viruses at pH 5.0 (Figure 1c). Though the average radius determined from TEM images was generally smaller than the radius determined by DLS, aggregate growth over time was observed with both techniques. It should be noted that significant size variations were observed and aggregates were not spherical (Figure 1b). Aggregates furthermore exhibited some regions that were very dense and other regions with void spaces. These potentially unstable aggregate regions may explain why the aggregates partially broke apart upon addition of PAA.

Langlet et al.[27] observed that MS2's pI increased from 3.1 to 3.9 when the ionic strength increased from 1 to 100 mM. Our experimental data, however, showed that higher ionic strength inhibited aggregation down to lower pH values: at pH 3 and 380 mM ionic strength, no aggregation was observed (Supporting Information, Figure S1). This behavior is in agreement with either a decrease in pI or with a weakening of nonelectrostatic attractive forces with increasing ionic strength. A reduction in pI can be rationalized in several ways. First, the viral pI is in part determined by the amino acids that make up the viral capsid. By increasing the ionic strength, the pK_a values of these amino acids decrease due to the reduced activity of the deprotonated acid. Hence, the increase in ionic strength reduces the pI of the virus. Furthermore, it has been postulated that phosphate can adsorb onto the MS2 surface, thereby creating more negative charges on the viral surface and thus leading to a pI as low as 2.2.[33] Finally, these adsorbed phosphate ions could cause conformational changes of the capsid proteins,[34] which could also influence the pI and the aggregation behavior. An explanation involving nonelectrostatic effects was offered by

Penrod et al.,[35] who reported a lower than expected attachment of MS2 to negatively charged quartz particles at high ionic strength. This finding could not be explained by electrostatic interactions, and was instead attributed to shielding of attractive van der Waals forces.

Effect of pH and Phosphate Concentration on Inactivation by PAA. Although disinfection by PAA is generally considered to have a small dependence on pH,[20] it was necessary to determine the influence of the different pH values used in the aggregation experiments on the inactivation kinetics of MS2. This was especially important as our experiments with MS2 aggregates were conducted at low pH, for which no PAA disinfection data was found in the literature. The pH effect was studied at high phosphate concentrations (400 mM) to avoid aggregation (Supporting Information, Figure S1) and allow direct comparison between dispersed virus samples. Compared to pH 5.0, k_{app} increased by a factor of 1.35 and 1.60 for pH 3.6 and pH 3.0, respectively (Supporting Information, Figure S2). Inactivation by PAA was thus not completely pH independent at the pH values studied.

High phosphate concentrations further decreased the inactivation rate: at pH 5, k_{app} was reduced by a factor of 5.1 in high phosphate compared to experiments conducted at the same pH in 15 mM phosphate. As discussed above, the presence of chloride was necessary to inactivate MS2. This indicates either that a reactive species causing MS2 inactivation is formed by a reaction between PAA and chloride, or that the interaction of chloride ions with MS2 makes the phage more susceptible to attack by PAA. In the former case, the protective effect of phosphate may arise from its role as a radical quencher: as reported by Booth and Lester,[36] the interaction of PAA with chloride and bromide in the presence of organic or mineral contaminants leads to the formation of free halide radicals. In our system, it is thus conceivable that chloride radicals are generated which act as the inactivating species. In the presence of high phosphate concentrations, these radicals would be increasingly quenched and their bulk concentrations available for MS2 inactivation lowered. In the latter case, in which MS2-chloride interactions enhance susceptibility to PAA, phosphate could increasingly compete with chloride for MS2 interaction sites, and thereby protect the viral proteins from attack by PAA.

The effect of high phosphate concentration on inactivation is likely comparable at pH 3.0 and 5.0, as the same phosphate species ($H_2PO_4^-$) dominates under both experimental conditions. Hence, we expect the ratio of k_{app} at pH 3.0 and pH 5.0 to be independent of the phosphate concentration in the absence of confounding aggregation effects. The pH effect on inactivation observed at high phosphate concentrations should therefore also apply to low phosphate concentrations.

Effect of Aggregation on Disinfection by PAA. The collected data on the average aggregate radius and the pH effects (Figures 1 and Supporting Information Figure S2) allowed us to assess the consequences of aggregation on disinfection. To do so, aggregated and dispersed viruses were exposed to 40 mg/L of PAA, and their inactivation kinetics were compared (Figure 2).

The rate of virus disinfection clearly decreased with increasing aggregate size. At pH 3.0, where the aggregate size was largest, the disinfection process was slowest ($k_{app} = 0.05 \pm 0.002\ min^{-1}$). The intermediate aggregates formed at pH 3.6 exhibited an intermediate k_{app} ($0.17 \pm 0.02\ min^{-1}$). At pH 5.0, at which no aggregates formed, inactivation was fastest ($k_{app} = 0.31 \pm 0.04\ min^{-1}$). The differences in k_{app} could not be attributed to the differences in solution pH. In fact, pH affects inactivation in the opposite manner,

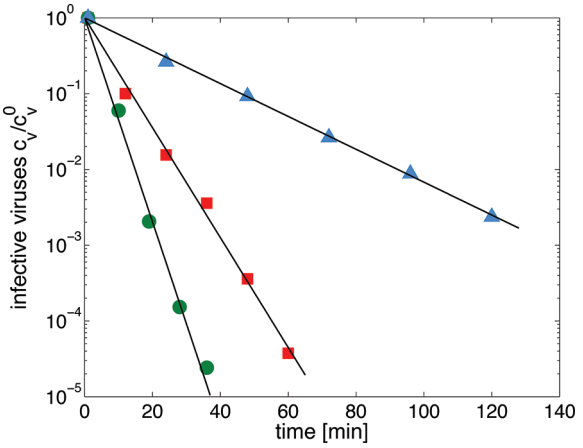


Figure 2. Inactivation at pH 5.0 (green circle; dispersed viruses), pH 3.6 (red square) and pH 3.0 (blue triangle) (average aggregation size 0.56 ± 0.10 and $0.89 \pm 0.13\ \mu m$, respectively) in 15 mM phosphate and 15 mM chloride and at an initial MS2 concentration of 5×10^{10} pfu/mL. The solid lines represent first-order fits. The corresponding PAA concentrations were 40, 38, and 38 mg/L, respectively. The resulting k_{app} values were $0.31 \pm 0.04\ min^{-1}$ ($R^2 = 0.994$), $0.17 \pm 0.02\ min^{-1}$ ($R^2 = 0.995$) and $0.05 \pm 0.002\ min^{-1}$ ($R^2 = 0.999$).

with faster inactivation occurring at lower pH (Supporting Information Figure S2). This implies that the differences in inactivation shown in Figure 2 would be even more pronounced if the effect of pH were accounted for.

All inactivation experiments, even those performed with aggregates, showed pseudo-first-order inactivation kinetics throughout the entire course of the experiment (Figure 2). The adherence to pseudo-first-order disinfection kinetics may appear as a contrast to previous studies, which showed a tailing-off in the inactivation curve after an initial linear phase.[3,8−10] However, these previous studies did not redisperse the aggregates before enumeration, and therefore measured inactivation of the whole aggregate. In contrast, this work presents inactivation curves that describe the fate of the individual viruses within the aggregate. Our findings are thus not contradictory to previous work, but rather offer new insight into the disinfection process within aggregates.

Effect of PAA Concentration. Thus far, only a single disinfectant concentration has been discussed. These findings may change if different disinfectant concentrations are used. The dependence of k_{app} on the disinfectant concentration can be expressed by the relationship

$$k_{app} = k_1 c_d^{\alpha} \quad (2)$$

where α indicates the order of the reaction in PAA, k_1 is the α^{th} order inactivation rate constant, and c_d the disinfectant concentration. As a first approximation, a linear relationship ($\alpha = 1$) between disinfectant concentration and k_{app} can often be assumed. In practice, however, deviations from this assumption have been observed. Saturation at higher disinfectant concentrations has been reported most commonly ($\alpha < 1$), as was described in the inactivation of polio[26] and coxsackievirus[11] by chlorine. To address if such sublinearity occurred in our experiments, k_{app} values were determined for dispersed viruses at different PAA concentrations. At pH 5.0, an α of 0.82 ± 0.03, and a k_1 of $0.013 \pm 0.001\ min^{-1} \cdot (L/mg)^{0.82}$ were observed (Figure 3). k_{app} thus did not increase linearly with PAA concentration, but saturated at higher PAA concentrations. This

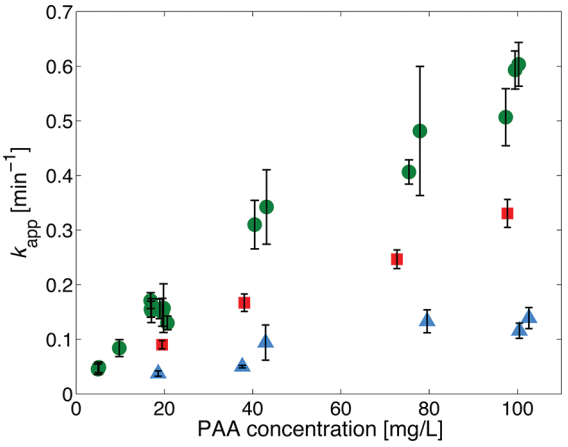


Figure 3. k_{app} (determined for an initial MS2 concentration of 5×10^{10} pfu/mL, in 15 mM phosphate and 15 mM chloride) and the associated 95% confidence intervals versus PAA concentration for experiments performed at pH 5.0 (green circle), 3.6 (red square) and 3.0 (blue triangle). The relationship at pH 5.0 between k_{app} and PAA concentration was best approximated by $k_{app} = 0.013\ \text{min}^{-1}\ (\text{L/mg})^{0.82} \cdot c_d^{0.82}$ ($R^2 = 0.980$).

relationship between k_{app} and c_d is in agreement with the data presented by Koivunen and Heinonen-Tanski for the inactivation of MS2 by PAA.[37] Their data follow this relationship for k_{app} within their given confidence intervals, even though their PAA concentrations were never higher than 15 mg/L and the pH was 6.6, slightly higher than the pH in our experiments.

The k_{app} values for the aggregated samples are also shown in Figure 3 (see Supporting Information Table S1 for aggregate radii considered). It can be seen that over the whole spectrum of PAA concentrations used, the k_{app} values obtained in dispersed samples were consistently higher than k_{app} values obtained for intermediate aggregates, which were always higher than k_{app} values for large aggregates. The differences between the k_{app} values obtained for the three different aggregate sizes increased with increasing PAA concentrations. Hence, k_{app} increased more rapidly for dispersed than for aggregated viruses. This phenomenon was more pronounced for larger aggregates than for smaller ones. For large aggregates (pH 3.0), k_{app} reached a plateau around 80 mg/L PAA. This behavior was not observed with smaller aggregates or with dispersed viruses. This not only suggests that large aggregates reduce k_{app} compared to dispersed viruses, but also that increasing the disinfectant concentration is less effective at enhancing inactivation in aggregated viruses than in dispersed viruses.

Overall, our results confirmed that aggregation inhibited inactivation by PAA, and that the inhibition was aggregate size-dependent. The physicochemical causes underlying this finding, however, are not readily apparent and could be manifold. The diffusion of PAA into relatively loose aggregates of less than a micrometer in radius is rather fast (within seconds). Furthermore, the MS2 capsid contains pores of 1−2 nm, while the diameter of PAA was estimated to be around 0.5 nm.[38] PAA may thus penetrate through these viral pores.[39] These factors indicate that the disinfection process within the aggregate should not be diffusion-limited. However, aggregation may block the viral pores, and viruses may deform due to aggregation, reducing the void spaces between viruses. Furthermore, adsorption of PAA onto the viral proteins could reduce the local PAA concentration, and could further constrict the void spaces and pores. Finally, on its way into the core of an aggregate, the PAA is consumed due to reaction with viruses, which effectively reduces the disinfectant concentration within the aggregate.

Model Development. Based on our experimental findings, we developed a model to capture the effect of aggregation on the disinfection kinetics, similar to the one developed by Stewart and Raquepas[40] who studied disinfection within a biofilm. This model needed to couple two processes: the inactivation of the viruses by a given local concentration of PAA and the propagation of the disinfectant into the viral aggregates.

Based on experiments with dispersed viruses (eqs 1 and 2), we determined that inactivation kinetics follow the relationship

$$\partial_t c_v = -k_{app} c_v = -k_1 c_d^{\alpha} c_v \quad (3)$$

where c_v and c_d are the local concentration of infective viruses and disinfectant, respectively. The relation is first order with respect to c_v while, as mentioned above, we determined from experiments without aggregation the order in c_d is $\alpha \approx 0.82$ Finally, we were also able to experimentally obtain the value of k_1 and its dependence on pH.

The propagation of disinfectant within the aggregates was modeled using a reaction-diffusion equation[41]

$$\partial_t c_d = D\nabla^2 c_d - k_2 c_d \quad (4)$$

where D denotes the diffusion constant and k_2 denotes the rate at which PAA molecules are removed from solution by either reaction with the viruses or adsorption onto the viruses. It should be noted that k_1 and k_2 are different due to the fact that PAA may react with viral amino acids or nucleotides without causing inactivation. The system of eqs 3 and 4 forms a so-called master-slave system, eq 4 being the master equation whose solution will serve as an input for eq. 3, the slave equation.

For the sake of simplicity, we assumed that all aggregates are spherical with a radius R, which corresponded to the average radius determined over the whole disinfection period (Supporting Information, Table S1). However, the model could readily be extended to include the distribution of the aggregate sizes if such data were available.

To supply eqs 3 and 4 with appropriate boundary conditions, we first imposed a constant concentration c_v^0 of infective viruses at the start of the disinfection process

$$c_v(r, t = 0) = c_v^0 \text{ for } r \le R \quad (5)$$

Note that we made use of the spherical symmetry of the problem and introduced r, the distance from the center of the aggregate. Equations 3 and 4 have to be solved for $r \in [0,R]$. We further imposed that the PAA concentration c_d^0 at the boundary of the aggregates remains constant over the duration of the experiment

$$c_d(R, t) = c_d^0 \quad (6)$$

Neglecting the depletion of PAA in the solution close to the boundary of aggregates is justified as long as molecular diffusion is fast enough to counteract the rate of absorption by the aggregates. We verified that this assumption holds for the size and the concentration of aggregates we measured in the experiments.

The master-slave system 3 and 4, together with the boundary conditions 5 and 6, can be solved analytically. Nevertheless, we chose to introduce a sound physical simplification: the diffusion coefficient of PAA in water is $D = 1.1 \times 10^{-9} \text{m}^2\text{s}^{-1}$;[42] it would therefore take $\sim 10^{-3} s$ for the PAA concentration to reach an equilibrium over the size of the largest aggregates (diffusion goes as $(Dt)^{1/2}$). The equilibrium was thus reached before the first

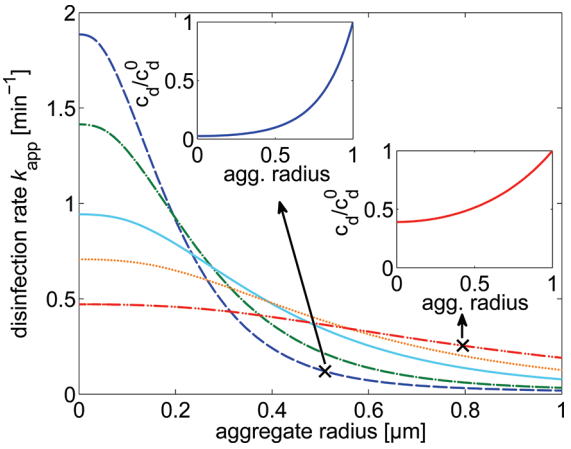


Figure 4. Model results obtained by changing k_1 and k_2 by a factor β (β = 2.0 (blue dash), β = 1.5 (green dash dot), β = 1.0 (light blue line), β = 0.75 (orange dots), β = 0.5 (red dash two dots)) and reported as k_{app} versus aggregate radius. The insets represent the disinfectant gradients within the aggregate of β = 2.0 (blue line), R = 0.5 μm and β = 0.5 (red line), R = 0.8 μm. Initial model parameters corresponded to those determined experimentally at pH 3.0 and 100 mg/L PAA.

measurement was taken (1 min). This simplification is still valid if the diffusion constant is reduced by a factor 10^5 (this factor would apply if the pores were reduced by aggregation to a diameter of 0.6 nm[39]). In other words, the characteristic time scale for virus inactivation is not related to the time of disinfectant penetration. Viruses within an aggregate are thus subject to a disinfectant concentration that is constant in time, but dependent on the position within the aggregate (Figure 4, insets). We can therefore safely neglect the time derivative in the master eq 4 and compute the equilibrium concentration of disinfectant within the aggregate $c_d(r)$. As a result, we obtain

$$c_d(r) = c_d^0 \frac{I_0(\sqrt{k_2 r/D})}{I_0(\sqrt{k_2 R/D})} \quad (7)$$

where I_0 is the modified Bessel function.[43] We want to stress that the simplification is consistent with solving the full master-slave system 3, 4 and inserting realistic values for the diffusion coefficient and the size of the aggregates. The infective virus concentration $c_v(r,t)$ is then obtained solving the slave eq 3 using the solution 7 as input for the right-hand side

$$\frac{c_v(r,t)}{c_v^0} = e^{-k_1 \left(c_d^0 \frac{I_0(\sqrt{k_2 r/D})}{I_0(\sqrt{k_2 R/D})}\right)^{\alpha} t} \quad (8)$$

In the framework of our model, the slower inactivation rate results from the negative gradient of PAA concentration (see eq 7) toward the center of the aggregates (r = 0), which allows the innermost viruses to survive longer than in a dispersed state.

Finally, in order to make a comparison with the experimental data we have to compute the average concentration of infective viruses within the aggregate ($\overline{c_v}(t)$)

$$\overline{c_v}(t) = \frac{3}{R^3}\int_0^R r^2 c_v(r,t)\,dr \quad (9)$$

since our experimental system only allowed us to measure average virus concentrations. Using this equation, we fitted the experimental data for various aggregate sizes R and disinfectant concentrations c_d^0. The only unknown parameter we needed to tune was the ratio between the rate constant k_2 and the diffusion coefficient D. We found that the best agreement with the data from the experiments at pH 3.0 and 3.6 was obtained using the same value, $(k_2/D)^{1/2}$ = 5.3 μm^{-1}.

Nearly all modeled values were within the confidence intervals of the k_{app} determined experimentally (Supporting Information Figure S3). The R^2 value was considerably higher for the experimental set at pH 3.6 (0.98) than at pH 3.0 (0.81). The difficulty to measure the low k_{app} at pH 3.0 together with the observation of a more marked deviation from the idealized spherical shape for larger aggregates may explain the poorer agreement for pH 3.0. Deviations from the spherical shape will result in shorter paths from the boundary of an aggregate to its center. As a result, the effective radius (regarding the penetration of disinfectant) will deviate from the one measured by DLS.

In the model we implicitly made the assumption that viruses consume or adsorb disinfectant at the same rate over the whole duration of the experiment (k_2 is independent of t): the process hence continues at the same rate even after a large fraction of viruses is inactivated. This assumption is based on the fact that inactivated viruses still contain moieties that can be oxidized. A reduction in k_2 would reduce $(k_2/D)^{1/2}$ over time and cause an acceleration of disinfection. The observed close adherence to pseudo-first-order inactivation kinetics of aggregated viruses suggests that changes in $(k_2/D)^{1/2}$ can be safely neglected in our system.

Prediction of Inactivation Behavior for Other Aggregate Sizes, PAA Concentrations and Different Disinfectants. As discussed above, aggregation modifies the concentration of disinfectant within the aggregates via consumption by or adsorption to the virus, and hence lowers the inactivation rates. Our model was employed to determine the variation of k_{app} as a function of the average aggregate radius R for various disinfectant concentrations c_d^0 (Supporting Information, Figure S4). It was found that k_{app} is only affected by aggregates with R > 0.2 μm. Furthermore, as also shown by our experimental data (Figure 3), the increase in disinfection efficiency from larger PAA concentrations is less pronounced in the presence of aggregates. The relation $k_{app} \propto (c_d^0)^{\alpha}$ (eq 2) valid for viruses in the dispersed form has to be replaced by a local relation when aggregates are present, $k_{app}(r) \propto c_d(r)^{\alpha}$. The resulting k_{app} increases more slowly with c_d^0 due to the presence of an area in the vicinity of the core of the aggregates that is less affected by the increase of disinfectant.

The concentration profile $c_d(r)$ within an aggregate can in turn lead to counterintuitive effects when comparing disinfectants: we find that a disinfectant can be stronger when applied to dispersed viruses but less effective in the presence of aggregates due to its high reactivity (large k_1 and k_2). This is illustrated in Figure 4 where we compare the effect of proportionally changing both values for k_1 and k_2 from their value for PAA by a factor β. For simplicity we consider that the reaction order of the disinfectant does not change (α = 0.82). The most reactive disinfectant (blue curve, β = 2) has the smallest k_{app} as soon as significant aggregation takes place (>0.3 μm). The insets in Figure 4 show that the decrease of disinfectant concentration from the boundary of the aggregates is much faster for more reactive disinfectants and explain the decrease in the inactivation rate. Figure 4 is based on relatively small changes in k_1 and k_2; however, in reality they vary by several orders of magnitude. For example, Shang et al.[44] found k_1 values of 61.8 and 0.00636 min^{-1}·L/mg for the disinfection of MS2 by chlorine and chloramines, respectively.

When these values are applied to our model, hardly any inactivation of aggregated viruses occurs for chlorine, as its reactivity is so high that it cannot penetrate far into the aggregates. On the other hand, the aggregates would have very little effect on chloramine as it is subject to very low consumption on its way to the core. Especially for the case of chlorine, additional experiments need to be performed to verify the assumption of a time-independent k_2. Nevertheless, our model predictions are consistent with findings by Sobsey et al.,[12] who observed a 13-fold increase in the chlorine dose needed to disinfect cell-associated versus dispersed hepatitis A virus; for treatment with monochloramine, however, similar doses could be used for cell-associated and dispersed viruses.

Finally, in order to obtain quantitative results for viruses other than MS2, we would have to determine the value of $(k_2/D)^{1/2}$, since the diffusion coefficient D may change in aggregates of other viruses, especially if their size and shape vary strongly from that of MS2. This issue will be addressed in future work, where different combinations of viruses and disinfectants will be experimentally tested. Nevertheless, we expect our main conclusion to hold, that the presence of aggregates considerably reduces the inactivation rates in water treatment, especially if very reactive disinfectants are used.

Our data show that disinfection of aggregates is slower than disinfection of dispersed viruses. To achieve a 4 $\log_{10}$ inactivation of viruses in an aggregate, one has to apply considerably longer disinfection times than those determined for dispersed viruses at the same disinfectant dose. The change in inactivation rate depends on both the size of the aggregates and on the reactivity of the disinfectant used. If aggregates are present, a good disinfection approach may be to apply disinfectants with low reactivity as aggregation has little effect on them.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Descriptions of the chemicals and microorganisms used; experimental details for DLS and TEM measurements; a table of experimental conditions, corresponding aggregate size and k_{app}; figures showing the inhibition of aggregation by high phosphate concentrations, the effect of pH on k_{app}, a comparison of modeled and measured k_{app}, and k_{app} as a function of aggregate size at different PAA concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +41 (0)21 693 0891; fax: +41 (0)21 693 8070; e-mail: tamar.kohn@epfl.ch.

Present Addresses

[§]Waterborne Pathogens Research Lab, Department of Civil and Environmental Engineering, University of Maryland, College Park, MD 20742.

■ ACKNOWLEDGMENT

This study was funded by the Swiss National Science Foundation (project No. 118077, 131918 and PP0P2-128545/1). KRW was supported by US-NSF international postdoctoral fellowship No. 0905713. We thank Davide Demurtas for help with the TEM images.

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