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Emerging Investigators Series: Virus Mitigation by Coagulation: Recent Discoveries and Future Directions

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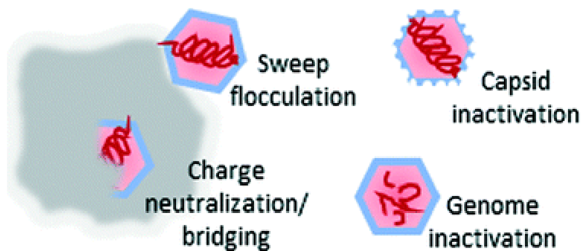
Abstract
Waterborne viruses are widespread and persistent in the environment. Coagulation is an effective process for mitigating viruses in drinking water. This review examines recent studies of virus mitigation by coagulation processes in the context of the latest scientific advances. Virus sorption is impacted by electrostatic forces, as well as the hydrophobic effect, steric hindrance, hydrodynamics and interactions with the water matrix. Organic

matter in the water may hinder or enhance sorption, depending on virus structure and environmental factors. In addition to physical separation in flocs, coagulation processes have been shown to inactivate viruses. This review evaluates reports of virus inactivation due to coagulation processes from both a process and experimental perspective. The use of bacteriophages as surrogates for human viruses is discussed, and future research needs relevant to virus coagulation are identified.



Water impact

Viruses are ubiquitous contaminants in both surface and groundwaters. Mitigation of viruses by coagulation is an effective method to pre-treat water for disinfection or membrane filtration and thus reduce energy or chemical disinfection requirements. Understanding virus sorption and inactivation by coagulation has broader implications for water/wastewater treatment, as well as virus fate and persistence in the environment.

1. Introduction

Waterborne viruses account for an estimated 30 to 40% of infectious diarrhea in the U.S.[1] Associated with both acute gastric and respiratory diseases and chronic conditions,[2] some viruses can persist for several months in the environment[3] and travel up to 100 m in groundwater.[4] Several families/genera of waterborne viruses are included on the U.S. Environmental Protection Agency's (EPA) Contaminant Candidate List (both CCL 3 and draft CCL 4) for drinking water, indicating the need for further research into occurrence and treatment.[5] Likewise, the World Health Organization's (WHO) *Guidelines for Drinking Water Quality*[6] cites eight virus categories that are of concern for drinking water, all of which have high persistence and infectivity relative to other pathogens. Although many viruses are only moderately tolerant of conventional water treatment,[6,7] adenoviruses show high resistance to emerging treatment technologies such as UV disinfection.[8] Coagulation can be used to reduce virus loads and minimize the required dose for disinfection. Coagulation is also an effective pre-treatment for virus removal by filtration systems.[9–15]

This review assesses current research of virus reduction by coagulation. Future avenues for research are discussed in light of evidence of the forces influencing virus sorption, as well as recent findings of virus inactivation in coagulation processes. Three coagulation processes are considered: conventional chemical coagulation, enhanced coagulation and electrocoagulation. In conventional chemical coagulation, metal hydrolytes are formed by dissolution of a metal salt in water. Aluminum and iron salts such as $Al_2(SO_4)_3$ and $FeCl_3$ are commonly used in water treatment,[16] although novel coagulants like polyaluminum chlorides (PACls) have gained particular attention for virus mitigation.[9,10,17–22] Polymeric iron coagulants have also been developed,[23,24] but these coagulants have not been evaluated for virus mitigation.

The EPA's Disinfectants and Disinfection Byproduct Rule (DBPR) promotes enhanced coagulation prior to disinfection of drinking water to prevent the formation of potentially carcinogenic disinfection byproducts.[25] Enhanced coagulation uses high doses of chemical coagulant and/or pH adjustment for effective removal of humic acids, fulvic acids and other dissolved and suspended organic material (collectively called

natural organic matter, NOM). For this reason, enhanced coagulation has been evaluated for virus mitigation in waters with high NOM concentrations.[12,26,27]

Electrocoagulation is the *in situ* production of coagulant by electro-oxidation of a sacrificial electrode. Both iron and aluminum sacrificial electrodes have been tested for virus mitigation.[14,28–30] Some researchers consider aluminum electrodes preferable to iron electrodes, because iron is released as soluble ferrous ions and may not be fully oxidized to form insoluble coagulant.[28,29,31] While the coagulant hydrolytes formed by iron electrocoagulation have been characterized,[32,33] less is known about the species formed during aluminum coagulation.

In all variations of coagulation, metal cations are introduced in water to form hydrolyte species. These hydrolytes destabilize colloids by overcoming the repulsive forces between colloidal particles. Colloidal destabilization commonly occurs by 1) neutralizing surface charges or forming bridges between particles to allow incorporation into a developing floc (charge neutralization or inter-particle bridging mechanism) or 2) by sorption of the colloid to a pre-formed floc (sweep flocculation mechanism). The resulting flocs may then be separated by gravity and/or filtration. Colloids are thus physically removed from the water matrix. Waterborne viruses are typically less than 100 nm in diameter,[4] making them among the smallest colloids removable by coagulation. Physical removal is usually considered the primary mechanism for virus mitigation due to coagulation, although recent research shows that coagulation can also render viruses noninfectious (inactivation mechanism).

This review examines recent studies of virus mitigation by coagulation processes in the context of the latest scientific advances in understanding virus sorption and inactivation. To begin, the forces influencing virus sorption and subsequent physical removal are described, including the role of electrostatic forces, the hydrophobic effect, steric hindrance, hydrodynamics, and cation bridging. Consideration is given to discussion of environmental matrix effects, *e.g.*, the influence of organic matter and divalent cations. Next, the phenomenon of virus inactivation during coagulation processes is addressed, including approaches and challenges to quantifying virus inactivation exclusive of physical removal. Critical analysis of recent discoveries and findings is used to inform recommendations for new research directions for mitigating waterborne viruses by coagulation, including appropriate selection and use of virus surrogates in laboratory and field studies.

2. Physical removal of virions

Physical removal is generally considered to be the dominant form of virus mitigation in coagulation processes. Therefore, many sources do not distinguish between physical removal and overall virus reduction. A summary of virus coagulation studies is provided in Tables 1 and 2. Table 1 summarizes results of studies using a single virus for testing, while Table 2 summarizes studies testing multiple viruses in tandem to facilitate comparison of results among viruses. As shown in the Tables, chemical coagulation has been shown to reduce viruses by 0.5 to 7 $\log_{10}$ (*i.e.*, 90% to 99.99999% reduction), with a typical reduction of approximately 3 $\log_{10}$. In studies of chemical coagulation with post-treatment microfiltration, virus concentrations were reduced up to 8 $\log_{10}$, with a typical reduction of 5 $\log_{10}$. Enhanced coagulation has been shown to reduce virus concentrations by up to 4.5 $\log_{10}$ (ref. 27) and up to 7 $\log_{10}$ (ref. 12) with post-treatment microfiltration. However, enhanced coagulation has not been studied as thoroughly as conventional chemical coagulation and is used for more challenging water sources. Electrocoagulation with post-treatment microfiltration has shown promising results in mitigating bacteriophage MS2, surpassing the EPA's Surface Water Treatment Rule (SWTR) of 4 $\log_{10}$ reduction of viruses.[14,29,30,34]

Table 1 Summary of coagulation studies using a single virus type. Virus reductions are rounded to the nearest 0.5 log_{10}. Reduction values are based on cultural assays. Unless otherwise noted, pH values indicate conditions after coagulant addition. Reduction ranges indicate temporal variation (*e.g.*, in filtration systems)

Coagulation process	**Coagulant**	**Dose (mg Al L^{-1} or mg Fe L^{-1})**	**Bacteriophage**	**log_{10} virus reduction**	**pH**	**NOM (mg L^{-1} DOC)**	**Source**
CC	PACl	1	Qβ	4	7	0	22
	$AlCl_3$			3			
	PACl			4		2.2 (TOC)	
	$AlCl_3$			1.5			
CC + MF	PACl	1.1	Qβ	6–7	6.8	1.1 (TOC)	13
CC + MF	$FeCl_3$	10	MS2	4.5	6.3	0	98
				3.5	8.3		
CC + MF	PACl	0.5	Qβ	4–5	6.8	0.6	11
CC + MF	PACl	1.1	Qβ	7	6.8	0.9	9
				7	7.8		
	Alum	1.1		6.5	6.8		
				0.5	7.8		
CC + UF	PACl, alum	3	MS2	7	7[a]	2.5 (TOC)	15
EnC + MF	PACl	4	MS2	7	5.5[a]	4.2	12
				3–4	6.5[a]		
	$FeCl_3$			3–4	5[a]		
				3–4	6.5[a]		
ElC	Aluminum electrodes	30	MS2	3	6.2	0	28
ElC + MF	Iron electrodes	10	MS2	5	6.3[a]	0	14
				4.5	7.3–8.3[a]		
ElC + MF	Iron electrodes	13	MS2	6.5	6.4[a]	0	30
				1.5		4.9	
		11.5		4.5	7.5[a]	0	
				1		4.9	
ElC + MF	Aluminum electrodes	10	MS2	4	6.4[a]	5.2	29

NOM = natural organic matter, DOC = dissolved organic carbon, TOC = total organic carbon. CC = chemical coagulation, EnC = enhanced coagulation, ElC = electrocoagulation, MF = microfiltration, UF = ultrafiltration. [a] Initial pH value (before coagulant addition).

Table 2 Summary of comparative coagulation studies using multiple virus types. Virus reduction and inactivation data are rounded to the nearest 0.5 $\log_{10}$. Reported inactivation below 1 $\log_{10}$ is shown as "<1", while negligible inactivation is shown as "≪1". Darker shading indicates comparatively greater reduction efficiencies for a given experiment. Unless otherwise noted, reduction values are based on cultural assays, and pH values indicate conditions after coagulant addition. Reduction and inactivation ranges indicate temporal variation (*e.g.*, in filtration systems)

Coagulant	Dose (mg Al L- 1 or mg Fe L- ')	Virus	$\log_{10}$ virus reduction	$\log_{10}$ virus inactivation/ aggregation	pH	NOM(mg L- 1 DOC)	Source
Chemical coagulation							
Alum	1.1	Bacteriophage MS2	6	1	6.8	1	18
		Bacteriophage Qβ	4	2			
PACl		Bacteriophage MS2	6	2			
		Bacteriophage Qβ	7	5			
$FeCl_3$ + polymer	13.8	Adenovirus type 4	1.5	n.r.	8[b]	n.r.	88
		Bacteriophage fr	2				
		Bacteriophage MS2	2				
		Bacteriophage PRD1	1				
		Bacteriophage ΦX174	0.5				
		Feline calicivirus	1.5				
PACl	1.1	Bacteriophage MS2	3	<l	6.8	0.9	20
		Bacteriophage Qβ	6	4			
PACl	1.1	Bacteriophage f1	1.5	n.r.	6.8	0.9	99
		Bacteriophage f2	2.5				
PACl	1	Bacteriophage MS2	4.5	3.5	4.5[b]	0.7	19
		Bacteriophage Qβ	n.r.	3.5			
		Bacteriophage ΦX174	n.r.	<<1			
PACl	1. 9	Bacteriophage MS2	6	3	7[b]	0.8	100
	2.2	Bacteriophage Qβ	5.5	2.5		1.3	
Alum			2	<<1			
Alum	3.2	Bacteriophage MS2	2[a]	1	5.5- 6.0	n.r.	86
		Norwalk virus	1.5 [a]	n.r.			
		Poliovirus 1	1 .5[a]	<1			
$AlCl_3$	2.7	Coxsackievirus BS	1	n.r.	7[b]	2.2	35
		Poliovirus 1	1. 5				
PACl		Coxsackievirus BS	3				
		Poliovirus 1	3				

Chemical coagulation + microfiltration							
PACl	1.1	Bacteriophage MS2	5.5	<l	6.8	0.9	21
		Bacteriophage Qβ	5.5	2.5			
PACl	1.1	Bacteriophage MS2	8	2	6.8	0.76	10
		Bacteriophage Qβ	8	4			
Alum	1.1	Bacteriophage MS2	3-4[a]	n.r.	6.8	0.76	74
		Bacteriophage Qβ	1- 2[a]				
		Norovirus VLP	>3[c]				
PACl	1.1	Bacteriophage MS2	3-4[a]				
		Bacteriophage Qβ	2- 3[a]				
		Norovirus VLP	>3[c]				
Enhanced coagulation							
$FeCl_3$ + polymer	13.8	Adenovirus type 4	2.5	n.r.	5- 6	4.6- 5.6	27
		Feline calicivirus	2.5				
		Bacteriophage fr	2.5			1.8	
		Bacteriophage MS2	2.5				
		Bacteriophage PRD1	2				
		Bacteriophage ΦX174	1. 5				
FeCl, + polymer	13.8	Bacteriophage fr	2	n.r.	5-6.5	4.2	26
		Bacteriophage MS2	0.5				
		Bacteriophage PRD1	0.5				
		Bacteriophage ΦX174	1.5				
		Coxsackievirus B6	3				
		Echovirus 12	2				
		Poliovirus Type 1	2.5				

n.r. = not reported for the given data set, NOM = natural organic matter, DOC = dissolved organic carbon, VLP = virus-like particle. [a]Quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). [b]Initial pH value (before coagulant addition). [c]Quantified by enzyme-linked immunosorbent assay (ELISA).

The physical incorporation of viruses into flocs most likely happens in one of two ways: incorporation in the developing floc (charge neutralization or inter-particle bridging), and/or sorption to surfaces of formed flocs (sweep flocculation). Shirasaki *et al.*[35] found negligible (<0.5 $\log_{10}$) reduction of poliovirus on preformed flocs, compared to approximately 3 $\log_{10}$ reduction during floc formation. In another study, Shirasaki *et al.*[20] found that the physical removal of two bacteriophages occurred during rapid mixing, with little or no additional removal during flocculation and after settling. Kreißel *et al.*[19] similarly found that significant virus mitigation occurred only during floc formation. In a study of virus removal by electrocoagulation, Tanneru *et al.*[29] concluded that sweep flocculation was the dominant mechanism of virus removal based on fluorescent microscopy and virus recovery from flocs. This discrepancy may be due to differences in floc formation between electrocoagulation and chemical coagulation. Chemical coagulation is limited by reaction kinetics and forms dense flocs, while electrocoagulation is limited by coagulant ion diffusion and forms sparse flocs.[36] During electrocoagulation, coagulant is continuously released, so treatment cannot be separated into rapid-mix coagulation and flocculation stages. Therefore, it is difficult to determine whether viruses are incorporated into growing flocs or sorbed to the floc surface.

In the absence of coagulant, viruses at high concentrations may also destabilize to form aggregates due to environmental conditions. Aggregate formation is important from both a theoretical and an experimental perspective. Due to larger size and lower surface charge, aggregates are easier to remove than monodispersed virions. In addition to being more susceptible to treatment processes, aggregates lead to artificially low results when quantifying viruses by cultural methods.[37] Unfortunately, aggregation is often a result of laboratory methods and does not necessarily represent natural conditions.

Numerous factors may influence physical removal of virions in flocs, as described in the following sections. These include the electrostatic and van der Waals forces modeled by the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, as well as non-DLVO factors such as the hydrophobic effect, structural incompatibility between viruses and sorbents (steric hindrance), and interactions with one another (aggregation) and constituents in the water matrix. The impact of these factors depends on the virus itself (*e.g.*, its structure, surface charge, or degree of permeability), the nature of the sorbent (floc characteristics), and the composition of the water matrix. The influence of these phenomena on virus sorption has been studied more extensively for virus transport through porous media.[38–42] Still, many of the lessons learned apply to coagulation as well.

2.1. Electrostatic interactions

Electrostatic forces affect the sorption of virions to surfaces like flocs. Investigators use multiple measures to describe electrostatic forces. Isoelectric point (pI) is the pH at which a particle or surface has a neutral charge in the electrolyte solution. At pH levels above the pI, the surface is negatively charged in solution; below the pI, the surface has a positive charge. Electrophoretic mobility is a measure of particle movement in the presence of an electric field and can be used to infer the electric potential near the particle surface. Electrostatic forces often govern virion sorption due to the long range of electrostatic influence, as well as the low pI of most enteric viruses, indicative of a strong, negative potential near the particle surface at neutral pH. However, electrostatic forces are attenuated at high ionic strength due to screening of the electric field.

2.1.1. The impact of virion permeability on isoelectric point

Research is inconclusive as to whether virion electrostatic charge is solely defined by the capsid surface, or whether deeper capsid functional groups and/or the interior genome compartment also affect electrostatic interactions between the virion and its environment. Models that account for virion permeability have been advanced by Schaldach *et al.*[43] as well as Langlet *et al.*,[44] the latter of which was based on Duval and Ohshima's model for "soft" (permeable) colloids.[45] Both models claim that with increasing virion permeability, the more acidic pI of the genome has greater impact on the overall pI.[43,44,46] To test this hypothesis, Langlet *et*

al.[47] evaluated the removal of bacteriophages MS2 and Qβ on hydrophilic membranes. The two bacteriophages are similar in size and measured pI, but Qβ has a larger genome. MS2 was removed to a greater extent, and Langlet *et al.* concluded that Qβ's genome imparts the virion with a greater negative charge density, which repels the membrane. However, the difference in removal is not necessarily due to the difference in genome size. Qβ has been shown to be more hydrophobic than MS2 (ref. 48) and would therefore be expected to exhibit less sorption to hydrophilic membranes.

Bacteriophage MS2 has been offered as an example of the effect of the viral genome on pI. The theoretical pI of MS2 based on total charged capsid moieties is approximately 7 (ref. 49) or 9,[50] while the pI of MS2's RNA genome is approximately 3.[50] The measured pI of MS2 is generally accepted to be between 3 and 4, closer to the RNA pI than the capsid pI.[51] Using another method to calculate the capsid pI, Penrod *et al.*[38] accurately predicted MS2's measured pI by evaluating only those charged structures exposed on the surface of the capsid. However, Schaldach *et al.*[43] found better correlation with experimental electrophoretic mobility data when allowing for capsid permeability than using the Penrod method.

However, a study by Dika *et al.*[46] comparing MS2 bacteriophages and virus-like particles (VLPs) seems to support Penrod *et al.*'s model for predicting pI. VLPs are assembled by expression of the viral coat proteins in a bacterial host but lack the viral genome. Instead of having a pI between 7 and 9 as predicted, MS2 VLPs had a measured pI between pH 3 and 4. Dika *et al.* hypothesize that negatively-charged host material was trapped within the VLPs during propagation. Considering the intricate, optimized packing of the viral genome into the capsid during normal bacteriophage propagation,[52,53] as well as evidence from electron micrographs,[46] VLPs likely do not contain enough host material in their interior to constitute a negative charge density comparable to whole virions. To accept the interpretation of viruses as soft colloids, we should see at least some increase in the pI of VLPs compared to bacteriophages to reflect the influence of the genome. The permeability model may also be more applicable to some virions than others.

2.1.2. The impact of virion permeability on electrostatic interactions

Several recent investigations have found that accounting for permeability did not yield better predictions of virion sorption or aggregation. Gutiérrez *et al.*[54] determined that the modeled permeability of rotavirus was low enough that a hard colloid formalism would suffice. Yuan *et al.*[55] found that the energy barrier to MS2 adsorption was better predicted by the DLVO model for hard (impermeable) colloids than when permeability was considered. In a study by Nguyen *et al.*,[56] MS2 bacteriophages whose RNA genomes had been degraded at high pH did not significantly differ from intact MS2 bacteriophages in terms of aggregation or adsorption to the water surface at the air–water interface. Nguyen *et al.* concluded that internal RNA had minimal effect on sorption. Dika *et al.*[57] responded with a study showing that the virus purification method used by Nguyen *et al.* (polyethylene glycol precipitation) masks differences between viruses and VLPs.

Researchers have also investigated the impact of the genome on virus–virus sorption phenomena responsible for aggregation. Dika *et al.*[57] found that MS2 aggregates formed at pH 4 did not re-disperse when the solution was then acidified to pH 2. Other experiments of MS2 aggregation using pH titration confirm this trend.[37,46] By contrast, MS2 VLPs aggregated only near the pI value and dispersed at lower pH. The team hypothesized that the difference in aggregation reversibility was due to the attractive influence of the genome. However, VLPs did not aggregate at any pH at high ionic strength, whereas entire virions did. At high ionic strength, the effective distance of electrostatic forces decreases, so VLPs and entire virions should behave more similarly if permeability impacts surface charge. In this study, the MS2 and MS2 VLPs behaved more similarly at low ionic strength. It is also unclear why the relative absence of RNA in VLPs would explain aggregation in this study, while the presence of residual RNA in VLPs was used to explain the VLP pI measured in Dika *et al.*'s previous study.[46]

Other tests found similar patterns of irreversible aggregation for somatic bacteriophages PRD1 and ΦX174 and F-specific bacteriophages Qβ and GA.[48,58,59] Bacteriophages PRD1, Qβ and GA all have measured pI values between 2 and 4 reported in the literature,[51] so aggregation in this pH range is not unusual. From an evolutionary standpoint, enteric viruses and bacteriophages may gain a selective advantage by aggregating to avoid inactivation by proteases in the stomach (pH < 4 (ref. 60)), and dispersing in the near-neutral pH of the intestines for the greatest chance of infection. Aggregation has been shown to inhibit virus inactivation by chemical disinfectants.[61]

However, aggregation below pH 4 is unexpected for ΦX174, which has a generally accepted pI of 6.6 from capillary isoelectric focusing, chromatofocusing and aggregation studies.[51] If the pI of ΦX174 was indeed higher than pH 4, aggregation occurred solely in a pH range where virions should have a positive net charge. Chrysikopoulos and Syngouna[41] and Aronino *et al.*[58] recently reported lower pI values for ΦX174 (4.4 and 2.6, respectively) based on electrokinetic measurements. However, in an extensive review of virus pIs, Michen and Graule[51] discounted Aronino *et al.*'s finding because Aronino *et al.* did not report purifying their bacteriophages for testing. Chrysikopoulos *et al.* also did not report purifying virus stocks.[41,62] Discrepancies in reported pI values for viruses are common in the literature, as differences in electrolyte concentration, composition and purity, and the strain of virus used, may all affect the measured pI.[51] The interpretation of previous experimental results must be based on electrophoretic mobility measurements made under the conditions of a given experiment, rather than on values reported in the literature.

2.2. Non-electrostatic sorption phenomena

When electrostatic interactions are repulsive or neutral, van der Waals and non-DLVO phenomena like the hydrophobic effect, steric hindrance and interactions with constituents in the water matrix may lead to differences in virion sorption. As detailed below, van der Waals and non-DLVO forces tend to modify the effects of electrostatic forces, especially when electrostatic forces are minimized (*e.g.*, by electrostatic screening or at pH values near the pI of the particle).

Arising from electronic resonance between surfaces, van der Waals interactions create an attractive force proportional to the polarizability of the virion and the abiotic surface.[16] In practice, van der Waals forces cannot be measured independently of electrostatic and non-DLVO phenomena.[59] In an extensive study of interactions influencing bacteriophage adsorption to surfaces, Armanious *et al.*[63] found minimal impact of surface polarizability on bacteriophage adsorption. However, the two surfaces compared also differed in hydrophobicity.

The hydrophobic effect arises from hydrogen bonds that preferentially form between water molecules to the exclusion of nonpolar molecules. The hydrophobic effect results in the tendency of nonpolar substances to partition out of the aqueous phase. Armanious *et al.*[63] found that the hydrophobic effect moderated electrostatic repulsion to allow adsorption of bacteriophages fr, GA, MS2 and Qβ to nonpolar surfaces. Armanious *et al.* also established a method for quantifying hydrophobicity based on the size and number of nonpolar patches on the capsid surface, and predicted a pattern of decreasing hydrophobicity: Qβ ≫ fr > GA ≫ MS2. Armanious *et al.*'s method was able to explain broad trends in bacteriophage sorption to hydrophobic surfaces, although completely isolating the hydrophobic effect from other phenomena is not possible. Other researchers[37,48] experimentally determined a relative hydrophobicity of GA > Qβ > MS2.

In two separate studies,[48,59] Dika *et al.* found that surface hydrophobicity could explain differences in the sorption of bacteriophages. Using chemical force microscopy, the hydrophobicity of bacteriophages MS2, Qβ and GA were compared to known hydrophobic and hydrophilic surfaces.[48] Hydrophobicity influenced virus sorption to surfaces even in low ionic strength solution (1 mM $NaNO_3$), where electrostatic forces are expected to dominate.[48] Bacteriophages MS2, PRD1 and ΦX174 were also compared. Despite varying charge densities

among the three bacteriophages in low ionic strength electrolyte, they demonstrated similar electrophoretic mobility at high ionic strength (100 mM).[59] Nevertheless, the bacteriophages differed in their affinities for membranes of varying hydrophobicity. Hydrophobicity has also been determined to favorably impact virus sorption to finely powdered activated carbon.[64]

The molecular-level structure of virus capsids and the sorbent surface may also hinder virion adsorption at close range. This steric hindrance occurs when interactions between the adsorbent and adsorbate are limited by the spatial orientation of their molecular structures. Several studies have found evidence of steric hindrance in virus sorption. Penrod *et al.*[38] found that steric interactions (here considering all non-electrostatic repulsion to be steric in nature) may lead to increased MS2 mobility in porous media when electrostatic forces are screened (*i.e.*, at high ionic strength). Armanious *et al.*[63] also suggested that the variable topography of bacteriophage fr and MS2 capsids, as determined by X-ray crystallography, may have resulted in poor adsorption to a gold surface in comparison to bacteriophages Qβ and GA. Dika *et al.*[48] found that bacteriophages preferentially sorbed to stainless steel over glass, despite similar surface hydrophobicity. This trend was more apparent at high ionic strength, which fits with the theory that surface roughness impacts electrostatic interactions when the roughness is on a scale comparable to the Debye length (a measure of the effective range of electrostatic forces).[48] In all cases, steric hindrance appeared to moderate sorption in conditions of similar electrostatic charge and hydrophobicity, rather than broadly define sorption behavior.

2.3. Impact of water matrix composition on virus sorption

Suspended and dissolved materials in the water matrix, like NOM and dissolved salts, can dramatically impact virion sorption. Because of the heterogeneous charge distribution and polarity of organic matter in the environment, the effect of NOM on virus sorption involves electrostatic forces, hydrophobicity and steric interactions. Generally, NOM contains both polar and nonpolar moieties and has a negative charge at neutral and high pH due to deprotonation of carboxyl and phenyl groups.[39,63] In porous media filtration tests, Zhuang and Jin[39] found that MS2 breakthrough was more rapid in the presence of sorbed or dissolved organic material, while ΦX174 breakthrough was relatively unaffected. MS2 has been shown to be relatively hydrophilic[37,63] and typically more negatively charged at neutral pH than ΦX174.[59] Therefore, MS2 sorption may be expected to be governed by electrostatic forces rather than hydrophobicity, while the hydrophobic effect may mediate ΦX174 sorption. Zhuang and Jin concluded that NOM both competes for sorption sites on the media and enhances sorption of nonpolar virions by creating hydrophobic sorption sites. Armanious *et al.*[63] found high sorption of bacteriophages GA and Qβ at pH 6 on a NOM-coated surface, while MS2 and fr sorption was negligible. GA and Qβ sorption decreased significantly from pH 6 to pH 8, likely due to electrostatic repulsion arising from deprotonation of carboxyl groups on the NOM and capsid surfaces. When ionic strength was increased from 10 mM to 100 mM to screen electrostatic forces, Qβ sorption was high even at pH 8, while MS2 sorption was measureable, though low. These results again illustrate that the hydrophobic effect predominates only when electrostatic forces are weak. Yuan *et al.*[55] found that MS2 deposition on silica was greater than on NOM-coated surfaces, even at ionic strengths high enough to effectively screen electrostatic charges. The team concluded that the results may be due to steric hindrance, by which NOM surface structures prevent binding in contrast to the even surface of silica.

Deposition experiments have shown that cation bridging can significantly increase virion sorption to like-charged surfaces.[54,65,66] In cation bridging, divalent cations (*e.g.*, Ca^{2+} and Mg^{2+}) complex with negatively-charged moieties on both the capsid and the solid surface. The presence of Ca^{2+} and Mg^{2+} ions has been shown to dramatically increase sorption of viruses to repulsive surfaces in comparison to monovalent ions, beyond the expected increase due to screening of electrostatic forces.[54,66] By contrast, rotavirus adsorption to an oppositely-charged (non-repulsive) surface was shown to be independent of Ca^{2+} or Mg^{2+} concentrations.[54] The effect of cation bridging can be significant at Ca^{2+} and Mg^{2+} concentrations typical of drinking water sources.[54] For the

bacterium *Pseudomonas aeruginosa*, cation bridging has been shown to significantly enhance sorption to repulsive surfaces at concentrations as low as 10^{-5} M Ca^{2+} or Mg^{2+}.[67] Interactions between MS2 virions have been shown to transition from repulsive to attractive between 10 mM Ca^{2+} and 50 mM Ca^{2+}.[65]

Ca^{2+} ions have been shown to have a greater positive influence on virus sorption to repulsive surfaces than Mg^{2+} ions.[54,66] Ca^{2+} ions are large and have weakly bound spheres of hydration that allow inner-sphere complexation with carboxyl groups on the virus capsid and the solid surface.[54,66] By contrast, Mg^{2+} ions have tightly-bound spheres of hydration that may allow only outer-sphere complexation. The mechanism for the relatively weak sorption observed in the presence of Mg^{2+} may not be bridging, but rather charge neutralization by complexation with negatively-charged moieties on either the virion or the surface.[66] The ability to form bonds with carboxyl groups makes cation bridging particularly important in the sorption of negatively charged viruses to NOM. In an experiment conducted by Pham *et al.*,[66] Ca^{2+} improved deposition of MS2 on a NOM-coated silica surface to a far greater extent than on a bare silica surface, although the bare silica was more negatively charged than the NOM-coated surface. For comparison, using NOM from the same source but in a monovalent electrolyte, Yuan *et al.*[55] found poorer adsorption of MS2 on a NOM-coated surface than a silica surface. Mylon *et al.*[65] found that a lower concentration of Ca^{2+} was required to destabilize MS2 in the presence of NOM (10 mg L^{-1} total organic carbon; TOC).

2.4. Implications of electrostatic and non-electrostatic phenomena for virus aggregation

Electrostatic repulsion contributes to virion stability, so aggregation typically occurs at high ionic strength or pH ranges near the virion pI.[37] Non-DLVO forces may also impact virus aggregation. Some investigators have suggested that protein loops extending from the capsid surface may contribute to the high stability of virions by steric hindrance.[56,65] Virus aggregation has been shown to be higher in the presence of divalent cations, although not in the typical range of Ca^{2+} and Mg^{2+} concentrations in drinking water.[54,56,65] Hydrodynamic forces may also influence aggregation. Langlet *et al.*[37] suggest that the low electrophoretic mobility of virion aggregates may be due to hydrodynamic drag. Aggregates may show greater hydrodynamic drag due to permeability. Because of this drag, aggregates would tend to stay aggregated once formed.[37] From another perspective, the hydrodynamic drag of individual virions due to capsid permeability may counteract the repulsive electrostatic forces of surfaces and neighboring virions, leading to aggregation.

2.5. Implications of electrostatic and non-electrostatic phenomena for coagulation

While porous media studies provide valuable insights, not all lessons can be assumed to apply to virus coagulation. Unlike sorption to solid surfaces, coagulation may occur by sorption to solid flocs and/or complexation of the virion surface by dissolved coagulant (charge neutralization or inter-particle bridging). In addition, metal oxide flocs differ in structure, charge and polarity from porous media. The challenge lies in determining which parameters are necessary and/or sufficient to describe virion sorption during coagulation/flocculation.

Hydrophobicity is unlikely to have a strong effect on coagulation in many cases, as aluminum and iron hydroxides are polar.[68] However, in the presence of NOM, hydrophobicity may be an important partitioning factor for some viruses. Tanneru *et al.*[29] found that aluminum flocs became more hydrophobic following sorption of NOM. Rebhun *et al.*[69] enhanced removal of hydrophobic ($\log K_{ow} > 4.5$) polyaromatic compounds by adding dissolved humic acid. Therefore, NOM may actually enhance sorption of very hydrophobic virions. Due to the rough, fractal structure of aluminum and iron flocs,[70] steric hindrance may also play a role in sorption to flocs.

To our knowledge, conclusive evidence of the effect of divalent cations on virus sorption to metal hydroxide flocs (as opposed to electrostatically repulsive and/or nonpolar surfaces) does not exist. In 1958, Chang *et*

al.[71] concluded that Ca^{2+} and Mg^{2+} may have inhibited virus mitigation. However, the comparison was made between tests using synthetic *versus* raw water sources, so the difference in virus mitigation cannot be conclusively attributed to divalent cations, as opposed to, *e.g.*, NOM. Chaudhuri and Engelbrecht[72] later showed that alum coagulation of bacteriophage T4 was not affected by Ca^{2+} and Mg^{2+} concentrations up to 330 mg L^{-1} as $CaCO_3$. However, Chaudhuri and Engelbrecht used a synthetic water free of NOM. The hypothesis of cation bridging between NOM and anionic coagulants/polymers has not been tested for viruses. Inhibition of coagulation by NOM has been documented for bacteriophages MS2 (ref. 30) and Qβ.[22] However, neither the influence of divalent cations nor the impact of phage hydrophobicity was tested in these studies. Microbalance experiments of virus deposition on aluminum or iron hydroxide surfaces, similar to those conducted on silica and NOM coated surfaces, could better identify the importance of surface charge, hydrophobicity, and roughness, as well as divalent cations and NOM concentrations.

3. Inactivation during coagulation processes

In addition to physical removal by sorption and co-precipitation, some studies have investigated bacteriophage inactivation by coagulation processes. Viruses may be inactivated by damage to the virion protein capsid and/or the viral genome. Damage to viral proteins manifests as an inability of the virus to attach to the host cell and/or inject the genome, while genomic damage prevents replication and proliferation of the virus in the host.[73] Whether viruses are physically removed or inactivated is not simply a Talmudic question. Coagulation processes increase sludge production, and sludge must be properly handled. If high levels of virus inactivation can be achieved, sludge treatment and handling will be safer and more cost-effective. Safe sludge handling is critical for decentralized water treatment, especially in developing countries that lack the infrastructure for proper disposal. If people risk contact with the sludge, we must be sure coagulation does not just concentrate pathogens.

Evidence of inactivation has been documented for both chemical coagulation and electrocoagulation, as summarized in Table 3. In the case of aluminum coagulants, polynuclear Al_{13} and Al_{30} species are thought to chemically oxidize virions.[19,35] While soluble, monomeric aluminum species are predominantly anionic above pH 6,[16] soluble Al_{13} and Al_{30} species are cationic near neutral pH.[19,35] Since most virions have negative surface charges,[51] the polynuclear cations may interact with and oxidize virions to a greater extent than monomeric anions. PACls produce more polynuclear hydrolytes in solution than simple aluminum salts.[9,35] Correspondingly, the most evidence for virus inactivation has been observed with PACl coagulation.[9,10,17–22] Coagulation with simple aluminum and iron salts (*e.g.*, $Al_2(SO_4)_3$, $AlCl_3$, $FeCl_3$ and $Al(NO_3)_3$) has shown only limited virus inactivation.[17,22,74] Polynuclear iron coagulants have also been developed,[23,24] but these coagulants have not been evaluated for virus mitigation to our knowledge.

Table 3 Summary of methodology for studies reporting inactivation by coagulation. Virus inactivation data are rounded to the nearest 0.5 log_{10}. Reported inactivation below 1 log_{10} is shown as "<1," while negligible inactivation is shown as "≪1"

Coagulation process	Coagulant	Dose (mg Al L^{-1} or mg Fe L^{-1})	Bacteriophage	log_{10} virus inactivation/aggregation	Inactivation quantification method	Aggregation determination method	Source
CC	PACl	1	Qβ	3.5	Plaque assay (with recovery)	Phage recovery efficiency	22
			T4	2			
			MS2	2.5			
			P1	3			
	Alum		Qβ	2			
			T4	<1			
			MS2	1.5			
			P1	1			
CC	PACl	10	Qβ	2	Plaque assay (with recovery)	Phage recovery efficiency	17
	PACl, alum, $AlCl_3$, $AlSO_4$	1	MS2	<1			
			P1	<1			
			Qβ	1–1.5			
			T4	<1			
CC	PACl	1.1	MS2	<1	qRT-PCR (with recovery), plaque assay (with recovery)	DLS	20
			Qβ	4			
CC	Alum	1.1	MS2	1	qRT-PCR, plaque assay	DLS	18
			Qβ	2			
	PACl		MS2	2			
			Qβ	5			
CC	PACl	1.9	MS2	3	qRT-PCR, plaque assay	n.r.	100
		2.2	Qβ	2.5			
	Alum			≪1			
CC	PACl	10	MS2	4	qRT-PCR, plaque assay	DLS	19
			Qβ	6			
			ΦX174	≪1			
EnC	PACl	4	MS2	1–2		n.r.	12

	$FeCl_3$	8		1–2	Plaque assay (with recovery)		
CC + MF	PACl	1.1	MS2	<1	qRT-PCR, plaque assay	Electron micrography	21
			Qβ	2.5			
CC + MF	PACl	1.1	MS2	2	qRT-PCR, plaque assay	n.r.	10
			Qβ	4			
ElC	Aluminum electrodes	30	MS2	< 1	Plaque assay (with recovery)	Phage recovery efficiency	28
		30, 4 h flocculation		3.5			
ElC + MF	Iron electrodes	10	MS2	1–2	Plaque assay (with recovery)	Phage recovery efficiency	30

n.r. = not reported for the given data set. CC = chemical coagulation, EnC = enhanced coagulation, ElC = electrocoagulation, MF = microfiltration. DLS = dynamic light scattering, qRT-PCR = quantitative reverse transcription polymerase chain reaction.

As in sorption studies, multiple investigators found that inactivation by chemical coagulation occurred concurrently to floc formation, with little to no inactivation when viruses were spiked in a solution with pre-formed flocs.[19,22] Kreißel *et al.*[19] found that inactivation was greatest when viruses were exposed to soluble PACl at pH 4.5, indicating that inactivation may be related to soluble species rather than insoluble flocs. Other researchers have suggested alternate mechanisms of inactivation, such as deformation of virions by forces at the interphase boundary,[18] and inhibition of infection by irreversible adsorption of coagulant polymers to the capsid surface (*e.g.*, at binding sites).[17]

Electrocoagulation has been shown to disinfect algae and bacteria, although researchers often do not discern between physical removal and inactivation in their results.[75–78] Few studies have investigated virus mitigation by electrocoagulation,[14,28–30] and to date only Tanneru *et al.*[28,30] have specifically examined virus inactivation by electrocoagulation. Disinfection occurs in electrocoagulation primarily by the oxidation of chloride to free chlorine.[28,79] For this reason, electrocoagulation has only been shown to inactivate viruses in the presence of chloride ions. Because generation of free chlorine is an oxidative process occurring at the anode,[79] chlorine production is a secondary and competing reaction to the oxidative dissolution of the anode itself. Tanneru *et al.*[28] noted that bacteriophage inactivation required a prohibitively long contact time due to the low concentrations of free chlorine generated in the study (<0.1 mg L^{-1}). Inclusion in flocs also shields viruses from inactivation by free chlorine.[28]

Tanneru *et al.*[28] were able to detect damage to both the MS2 genome and proteins after electrocoagulation with extended flocculation times. The team detected conformational changes to proteins and an increase in the concentration of protein oxidation byproducts by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Tanneru *et al.* also used quantitative reverse transcription polymerase chain reaction (qRT-PCR) amplification to directly investigate damage to the MS2 genome. A 77 bp section of the maturation protein coding region[80] was amplified and compared between treated and initial samples. The short length of the amplicon likely makes this method a conservative indicator of total RNA damage. Tanneru *et al.* found a rapid decline in copy number in the first hour after electrocoagulation, comparable to the reduction found using a culture-based plaque assay. Tanneru *et al.* did not find increased genome inactivation with longer contact time, although overall inactivation continued to increase. These results suggest that for electrocoagulation, the mechanism of inactivation may vary over time. Varying mechanisms of inactivation can be at play, even for a given disinfectant. For example, Wigginton *et al.*[73] found that free chlorine attacked MS2 proteins and genome, and inactivation manifested as an inability to inject the viral genome into the host cell. The method used by Wigginton *et al.* may offer a less conservative estimate of RNA damage because approximately half of the viral genome was analyzed.

Due to the different hypothesized inactivation mechanisms (*i.e.*, production of free chlorine during electrocoagulation *versus* polynuclear cations in chemical coagulation), Tanneru *et al.*'s results cannot be extended to chemical coagulation. Wigginton *et al.*[73] established that different chemical oxidants (*i.e.*, $^1O_2$, free chlorine, ClO_2) vary in their mechanisms of inactivation. Likewise, the inactivation mechanism likely differs between free chlorine and the large, polynuclear cations suggested to be responsible for inactivation due to chemical coagulation (*e.g.*, Al_{13} and Al_{30}). Use of an approach similar to that of Wigginton *et al.* or Tanneru *et al.*[28] would help to clarify the mechanism of inactivation by chemical coagulation. As a preliminary hypothesis, polynuclear aluminum species may predominately attack capsid surface proteins, because access to the internal structure would be limited by size and charge (especially as compared to free chlorine).

The following sections discuss difficulties in assessing virus inactivation in the laboratory. Due to the cost and duration of cultural assays, molecular methods seem to be an attractive option. However, research is required to prove the validity of molecular methods for quantifying inactivation. In addition, a given level of inactivation may be important to treatment efficiency, yet difficult to quantify due to its relative insignificance compared to

other treatment fates. Virus aggregation also frustrates attempts to quantify inactivation, and no satisfactory method is available to ensure against aggregation of treated samples.

3.1. Quantification of virus inactivation

Quantification of virus inactivation presents an experimental challenge. Some authors[17,22,28] have employed a cultural plaque assay to quantify the number of infectious viruses in solution and those sorbed to solids using a recovery protocol. This method may be thought of as a "plaque-forming unit (PFU) balance." After gravitational separation, viruses are both sampled in the supernatant and recovered from the floc. The total virus recovery is compared to the untreated, control sample to determine inactivation. A PFU balance is distinct from a mass balance in that it is a discrete count of PFUs, not a continuous measure of mass. Like any plaque assay, the PFU balance can discriminate between infectious and inactive viruses but not between a single virus and an aggregate.[81] Comparison of recovered PFUs to the initial concentration allows determination of virus inactivation, within the expected method recovery efficiency.[28] However, the PFU balance approach requires twice the number of plaque assays to analyze the concentrations of viruses in solution and adsorbed to flocs, thereby increasing cost and time inputs.

Other investigators[9,10,18,19,21] have used qPCR (qRT-PCR for RNA viruses) to compare declines in copy number to declines in PFU counts. Compared to plaque assays, qPCR is comparatively rapid, and aggregation does not affect qPCR results. In contrast to plaque assays, qPCR assesses the total number of intact viral genomes in the sample, regardless of infectivity. This allows a comparison between plaque assay and qPCR results to assess total inactivation. However, there are several concerns with comparing molecular and cultural techniques. For one, molecular methods cannot differentiate between physical removal and inactivation due to genome damage. The copy number of even short amplicons can decrease during inactivation, as described by Tanneru *et al.*[28] When assessing chemical oxidation by soluble PACl, Kreißel *et al.*[19] also showed a decline in copy number of approximately 1 $\log_{10}$ from the initial concentration. Whether the depressed recovery is an artifact of the method or indicative of genome destruction is unclear. In a coagulation study, this reduction in copy number would be indistinguishable from a reduction due to physical removal, as shown in Fig. 1. Therefore, qPCR may overstate the importance of physical removal and understate that of inactivation.

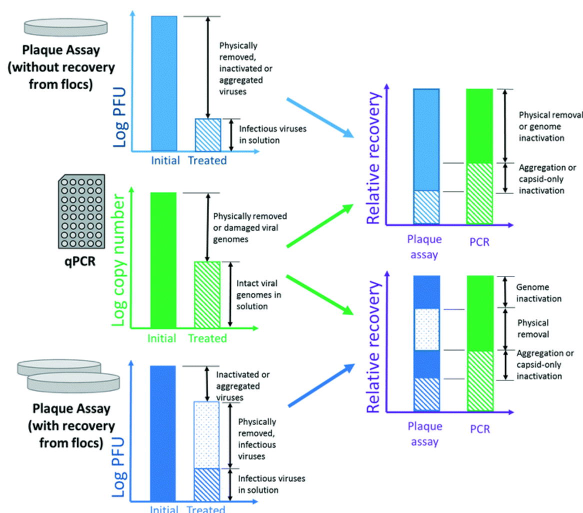


Fig. 1 The most detailed theoretical categorization of fate possible using three different quantification methods independently and in combination. The relative values shown in stacked columns were chosen only for visual clarity. In practice, resolving many of these quantities may be impossible, because quantities may differ in value and variability by several orders of magnitude.

Similarly, some fraction of inactivated viruses are likely removed with the flocs and therefore counted as physical removal. Inactivated viruses could even be disproportionately removed. Destruction of viral proteins can dramatically alter virion structure and genome packing.[82] The effect of morphological changes on sorption cannot be assumed to be negligible. If infectious viruses are more readily removed in flocs, then qPCR analysis of treated water would provide an appropriate means of quantifying total viruses (infectious + inactive). If inactive viruses are readily removed in the floc phase, qPCR analysis would again systematically underreport inactivation.

In one study, Shirasaki *et al.*[20] analyzed both the liquid phase and dissolved floc of treated water by qPCR and plaque assay – essentially performing both a PFU balance and a copy number balance. Despite significant reductions in amplicons in the liquid phase, Shirasaki *et al.* recovered approximately all MS2 and Qβ amplicons from the floc (confidence intervals including 100% efficiency). The high recovery indicates that MS2 and Qβ inactivation by genome destruction was below detection in this study. This lack of genome inactivation may reflect the inactivation mechanism of PACl. However, Qβ inactivation was evident in both the floc and liquid phases, indicating the removal of inactivated viruses in flocs. For both bacteriophages, greater discrepancy between molecular and cultural quantification was observed in the liquid phase than in the floc phase. The greater discrepancy in the liquid phase could possibly be due to aggregation, especially considering that the liquid phase was only centrifuged ($2000 \times g$, 10 min), not dissolved and agitated for resuspension like the floc phase. Regardless, Shirasaki *et al.*'s results suggest that genome inactivation may not significantly impact qPCR results for some bacteriophages and treatment processes. However, using qPCR without recovery from flocs may under-represent inactivation due to sorption of inactivated viruses in the floc, as in the case of Qβ. Shirasaki *et al.* do not report using this same approach in subsequent papers. Application-specific research is required to establish a firm methodological basis before using qPCR and plaque assays without recovery from flocs.

Fig. 1 summarizes the extent of information that could theoretically be learned using the quantification methods discussed above. The combination of plaque assay with recovery from flocs provides the most detailed account of virus fate; however, one or more of these fates are likely to be undetectable in practice. A plaque assay with recovery also provides more relevant information (*i.e.*, the concentration of infectious viruses in the sludge) than qPCR and plaque assay without recovery. If some fates could be considered inconsequential for a particular application (*esp.*, inactivation due to genome damage), the combination of qPCR and plaque assay without recovery would be comparable to plaque assay with recovery.

3.2. Detecting low levels of inactivation

Working with high levels of virus reduction, such that logarithmic representations are customary, also presents an interesting conundrum. Whether quantified by molecular or cultural methods, inactivation is determined by subtracting a concentration of recovered PFUs or amplicons from an initial concentration that may be several orders of magnitude higher. Since the error of each quantity is relative to the concentration, inactivation can only be determined to a statistical degree of certainty when inactivation is a primary mechanism of reduction, as illustrated in Fig. 2.

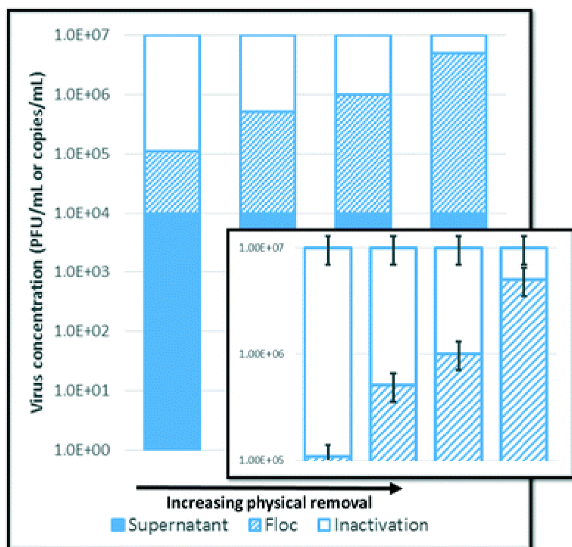


Fig. 2 Confidence in quantifying decreasing amounts of inactivation. In this theoretical case, recovery of bacteriophages from the supernatant remains constant for all bars (10^4 PFU mL^{-1}), while the number of bacteriophages recovered from the floc increases from 10^5 to 5×10^6 PFU mL^{-1}. The standard error of the mean for all measurements is set as 30% (error bars, inset). The quantity recovered from the supernatant has no significant impact on inactivation. However, as inactivation decreases to near 0.5 log_{10} reduction, the confidence intervals begin to overlap, and inactivation cannot be distinguished from the analytical uncertainty.

In addition, inactivation is usually treated as independent from physical removal, rather than additive. If inactivation works as a polishing step, small numbers of inactivated viruses could have a great impact. For example, if an additional 0.09% of the initial virus concentration is inactivated beyond the 99.9% that can be removed in flocs, that minimal reduction means the difference between satisfying the EPA's SWTR requirements or not.[34] The limited information available suggests inactivation might have this polishing effect. As discussed in section 3.1, Shirasaki *et al.*[20] found greater inactivation of MS2 and Qβ in the liquid phase than the floc phase, which indicates that inactivation may contribute to virus reduction beyond the capacity of physical removal alone. In other words, inactivated viruses would not necessarily have been physically removed were they not inactivated. The 'polishing' effect of inactivation would significantly reduce the concentration of viruses remaining in the treated water after physical removal. However, this small virus reduction would be lost on the scale of the original, spiked concentration. Therefore, the amount of inactivation, although important for disinfection, would be difficult to discern experimentally. If the inactivation cannot be accurately assessed, conditions for inactivation cannot be optimized.

3.3. Determination of virus aggregation

In all methods, aggregation remains quantitatively indistinguishable from at least some inactivation, as shown in Fig. 1. Aggregation leads to artificially low plaque counts, because each plaque originates from many viruses instead of one. Based on aggregate size, Langlet *et al.*[81] found that aggregation could be responsible for more than 4 log_{10} reduction in PFUs (from an initial concentration of approximately 10^{11} PFU mL^{-1}). However, an additional control can provide some 'insurance' against aggregation for the plaque assay with recovery method. The method recovery efficiency can be tested under conditions of minimal inactivation (*e.g.*, adsorbing viruses to pre-formed flocs or quenching oxidants with sodium thiosulfate). The recovery efficiency shows not only that viruses can be recovered from the flocs, but also that the viruses in the treated water are no more aggregated than in the initial virus solution. Some investigators[21,46] have also relied on electron micrographs to qualitatively show the presence or absence of aggregates.

Dynamic light scattering (DLS) is a method for determining both electrokinetic response of colloids and the size distribution of particles in solution. Many studies[18–20,59,81] have used DLS analysis to determine conditions in which virions aggregate. However, DLS analysis requires very high virus concentrations (greater than 10^9 PFU mL^{-1})[37] – higher than even the typical spiking concentrations used for testing (usually 10^7–10^8 PFU mL^{-1}). Therefore, researchers cannot directly assess aggregation in the same samples to be tested by plaque assay and/or qPCR. Instead, researchers must try to show whether or not aggregation occurs in conditions similar to those tested.

A significant quantity of virus stock solution is necessary to achieve the required concentrations for DLS. These stock solutions may have higher ionic strengths and differ greatly in composition from natural waters. Electrolyte composition can significantly affect electrokinetic responses like aggregation.[51] Aggregation has been shown to be greater in phosphate-buffered saline (PBS), commonly used for virus stocks, than in deionized water or bicarbonate solution.[55] Preferably, virus stocks should be purified and spiked into the same water matrix used for coagulation tests. However, the method of virus purification may also significantly affect virion properties. As previously mentioned, Dika *et al.*[57] compared three methods of MS2 purification: polyethylene glycol (PEG) precipitation, successive dialyses in deionized water and 1 mM $NaNO_3$, and ultracentrifugation in a CsCl gradient. PEG precipitation resulted in a larger hydrodynamic radius of unaggregated viruses, with aggregation observed at pH 6. Dialysis resulted in aggregation at pH 4, while viruses separated in a CsCl gradient did not aggregate at any pH. Dika *et al.* note that each method has drawbacks: PEG appears to adhere to the capsid surface, dialysis retains viral and non-viral particles based only on membrane exclusion, and cesium ions may permanently deform protein structures. The experiment does not clarify which purification best approximates virus behavior in the environment, however. In addition, Dika *et al.* did not use a solvent wash after PEG precipitation to promote monodispersion, as used by many investigators.[26,27,83–85] A solvent like chloroform or Vertrel™ may be able to strip adhered PEG from the capsid surface. If not, dialysis may best reproduce virus behavior in the environment, because foreign compounds are not introduced. To date, the best course of action is to control against aggregation by testing at pH values and ionic strengths where virions are likely to be stable. Aggregation may then be measured under similar conditions using DLS or qualitatively assessed by electron microscopy.

4. Use of virus surrogates in coagulation studies

Understanding the basis of virion sorption and inactivation is necessary not only for predicting and explaining virus reduction by coagulation technologies, but also for choosing appropriate bacteriophage surrogates for evaluating unit processes. Bacteriophage surrogates are most often used in lab and pilot studies for safety considerations, and because bacteriophage assays require significantly less time and resources than mammalian virus assays. In addition, human viruses are often difficult to propagate in large enough concentrations to show required reductions, *e.g.*, the 4 $\log_{10}$ reduction required by the SWTR.[34]

Very few studies have directly compared the effect of coagulation on both bacteriophage surrogates and the human viruses of interest, as shown in Table 2. Among those studies, there is no common consensus on the relative performance of bacteriophage surrogates and human viruses. For example, Mayer *et al.*[26] found MS2 to be a conservative surrogate for poliovirus 1, while Shin and Sobsey[86] found MS2 reduction to be greater than that of poliovirus 1. However, too many parameters differ between these studies to draw firm conclusions. Numerous factors, including the type and dose of coagulant, ionic strength, pH and composition of the water matrix, play a significant role in the absolute and relative reduction of different viruses.

Clear trends in removal and inactivation between bacteriophages are also difficult to discern, as shown in Table 2. MS2 and Qβ have been compared in the greatest number of coagulation studies. MS2 and Qβ are both commonly used as surrogates in water treatment studies as well. Both bacteriophages infect the F-pilus of *E.*

coli, *i.e.*, they are F-specific.[87] MS2 and Qβ are of similar size (20–30 nm),[37] with similar pI values (2–4) reported in the literature.[51] Both bacteriophages have single-stranded RNA genomes, although Qβ's genome (4217 nt) is about 18% longer than that of MS2 (3569 nt).[87] Out of 8 direct comparisons of reduction of MS2 and Qβ by coagulation (see Table 2), MS2 was reduced to a greater extent in 4 studies, with a negligible overall difference in reduction. However, the mechanism of mitigation was likely different for each bacteriophage. Qβ was inactivated to a greater extent than MS2 in a majority (5 of 7) of direct comparisons, with an average of 1.5 $\log_{10}$ greater inactivation. In two tests using alum as the coagulant, treatment efficiency of MS2 was greater in both, while tests with PACl showed greater average reduction of Qβ. Thus, the suitability of MS2 or Qβ as a surrogate may depend both on the mechanism of reduction and the coagulant used. The variability of these well-studied bacteriophages illustrates the great need for additional head-to-head comparison studies.

Even tests performed using nearly identical conditions can differ significantly. Some tests comparing MS2 and Qβ used the same coagulant type and dose (1.1 mg L^{-1} PACl) with varying results,[10,20–22,74] as shown in Table 2. Similarly, Mayer *et al.*[26] and Abbaszadegan *et al.*[27] used the same methods and source water (though the source water composition varied over time). Mayer *et al.* found relative virus reductions following the order of: fr > ΦX174 > MS2 ≥ PRD1. Abbaszadegan *et al.* found greater reduction for all four bacteriophages with the same coagulant dose, and bacteriophage reduction followed the order of: fr ≥ MS2 > PRD1 > ΦX174. The only clear difference between the conditions of the two studies was that Mayer *et al.*'s source water for bacteriophage tests had a higher NOM concentration, as shown in Table 2. In both tests, the four bacteriophages were considered to be either conservative or representative surrogates for the human viruses of interest. However, in a third test by Abbaszadegan *et al.*,[88] bacteriophages fr and MS2 were reduced to a greater extent than the target viruses, while PRD1 and ΦX174 were conservative surrogates.

When inactivation plays a significant role in virus reduction, the biology of the virus – its morphology and infectious pathway – must be considered in addition to its physical properties. Sigstam *et al.*[89] explain that the easily-oxidized amino acids cysteine and methionine are potential indicators of virus capsid degradation, although protein conformation determines the exposure of those amino acids to disinfectants. Sigstam *et al.* therefore used a complex model to explain cleavage at a particular methionine using the thermodynamics of oxidation in relation to the bonds between capsid proteins, and the "solvent accessible surface area" of that site. The team found that when genome inactivation was the primary disinfection mechanism, inactivation of F-specific bacteriophages fr, GA and MS2 was similar. However when capsid destruction was the primary disinfection mechanism, subtle variations in the protein coat of the four bacteriophages led to differing inactivation.

As with pI, even closely related virus strains can differ in susceptibility to inactivation. For example, Engelbrecht *et al.*[90] measured significantly different free chlorine inactivation rates between serotypes of coxsackievirus (A9 and B5), echovirus (1 and 5) and poliovirus (1 and 2). Virus strains may differ in inactivation depending on the disinfectant as well. In a study by Cromeans *et al.*,[91] echoviruses 1 and 11 had similar free chlorine inactivation rates, but the rate of echovirus 11 inactivation by monochloramine was two orders of magnitude slower than that of echovirus 1. Cromeans *et al.* also found significant differences in inactivation between serotypes of both human adenovirus (2 and 40, 41) and echovirus (1 and 11), although trends were similar between free chlorine and chloramine. Sigstam *et al.*[89] suggest that closely-related viruses may be appropriate surrogates when the primary inactivation mechanism is genome destruction, but not necessarily for capsid-based inactivation. For chemical coagulation, the charge and large size of polynuclear cations may restrict oxidation to only the capsid surface. In this case, conservative surrogates must be chosen by direct comparison with the viruses of interest. For the generation of free chlorine by electrocoagulation, the inactivation mechanism is likely less specific, because both the viral proteins and genome are oxidized by free chlorine.[28,73]

Regarding bacteriophage surrogates, Kreißel *et al.*[19] advance the hypothesis that F-specific bacteriophages may be uniquely susceptible to inactivation. F-specific bacteriophages have only one copy of the maturation protein responsible for binding to and infecting the F-pilus structure. The MS2 maturation protein (A protein) is known to be exposed near one of the five-fold vertices, possibly beneath a pore.[92] Qβ maturation protein (A2 protein) is similarly located near the five-fold vertex, although conformation may be slightly less rigid than in MS2.[92] This orientation may be similar in other F-specific bacteriophages. Kreißel *et al.* argue that the single maturation protein might be easily blocked or damaged, and F-specific bacteriophages would therefore show greater inactivation than somatic coliphages, which have multiple binding sites. Most studies of inactivation by coagulation have used F-specific bacteriophages, as shown in Table 3. Therefore, the use of these surrogates may overstate virus inactivation.

Kreißel *et al.* recommend using the somatic coliphage ΦX174 as a more conservative surrogate for coagulation studies. In their study, ΦX174 was insensitive to inactivation by PACl. The pI values for ΦX174 reported in the literature are higher than the pH 4.5 used in the Kreißel *et al.* study. If polyaluminum cations were responsible for virus inactivation, the coagulant should show little or no attraction to positively charged ΦX174 virions. However, Kreißel *et al.* measured a negative electrophoretic mobility for ΦX174 by DLS. From a practical perspective, ΦX174 forms large, ill-defined plaques if allowed to incubate overnight[19,93] and is therefore not an ideal model virus.

If F-specific bacteriophages are more structurally fragile than somatic bacteriophages, they should also be more susceptible to indiscriminate disinfectants. However, experimental results are inconclusive. ΦX174 may be more sensitive than MS2 to chemical disinfection by $HO^{\cdot}$.[49] In a study of iodine inactivation,[94] MS2 was slightly more sensitive than ΦX174, although neither was nearly as resistant to disinfection as bacteriophage GA, another F-specific bacteriophage. GA also has a relatively high resistance to temperature and pH.[95] In contrast, Sigstam *et al.*[89] found GA to be comparable or more susceptible than MS2 and fr to a range of disinfectants, including free chlorine. Regardless, GA's low susceptibility to some disinfectants and high persistence in the environment casts some doubt on the hypothesis that F-specific bacteriophages are structurally more sensitive to inactivation. In the case of free chlorine oxidation, Wigginton *et al.*[73] noted that extensive damage to the MS2 A protein did not cause an inability to bind to host pili. Rather, damage to the capsid protein was likely responsible for inactivation. Therefore, the A protein may be robust to chemical oxidation. More importantly, the case of bacteriophage GA illustrates that a surrogate may be conservative in one application and overly susceptible in another.

In addition, a conservative surrogate for coagulation must be robust to both physical removal and disinfection. ΦX174 is less hydrophobic than MS2 (ref. 41 and 59) and may therefore be a more conservative surrogate for physical removal in the presence of NOM. ΦX174 and MS2 reduction in raw waters due to $FeCl_3$ coagulation was compared in three studies,[26,27,88] but the results conflicted as to which bacteriophage was reduced to a greater extent. GA has been shown to persist to a far greater degree than MS2 and Qβ in a pilot coagulation/ultrafiltration treatment plant.[96] Jofre *et al.*[97] found that in three water treatment plants, somatic coliphages were found in slightly more samples after prechlorination–flocculation–sedimentation and post-chlorination than F-specific coliphages, but phages of *Bacteriodes fragilis* were yet more resistant. Suffice it to say that not enough is known about common bacteriophage surrogates, let alone the countless other possible bacteriophages available for research.

Resistance to inactivation or physical removal does not automatically make for an excellent surrogate – the surrogate must be tested alongside the actual virus of interest. A good surrogate should be conservative compared to the human virus, but more importantly it must be representative. A 'worst-case scenario' is only valuable insofar as it is remotely possible. By using a surrogate that is especially insensitive, researchers may over-design treatment systems at great cost. Insensitive surrogates may also lead researchers to miss a potential

treatment strategy that could be optimized for insensitive targets. This is particularly true in the case of inactivation due to coagulation. As illustrated in section 3.2, inactivation of less than 1 $\log_{10}$ may be statistically indiscernible when physical removal is dominant. Therefore, the best surrogate is one tailored to the application. If possible, research should employ more than one surrogate, with different electrostatic charge, hydrophobicity and/or resistance to inactivation, as appropriate. Of course, additional research is required to assess these properties for the many possible bacteriophage surrogates.

5. Conclusions

At this stage in coagulation research, viruses can no longer be assumed to be inert nanoparticles. Both the complexity of viruses as bioparticles and the phenomenon of virus inactivation must be embraced. In particular, the role of permeability in virus sorption and aggregation remains unclear. Virion permeability has been estimated by interpreting empirical electrophoretic mobility data.[50] However, to our knowledge, no empirical measures of virion permeability exist, and a clear link between permeability and virion composition and morphology has not been advanced. Furthermore, the direct influence of inner virion structures on surface charge or sorption has not been conclusively demonstrated.

Non-DLVO forces must also be considered to explain and predict virus sorption behavior. Research shows that hydrophobicity is an important contributor to sorption, especially for nonpolar virions. Other forces, such as steric interactions and hydrodynamics, are likely to play a significant role when electrostatic forces are repulsive or minimal (*e.g.*, at high ionic strength or near the virus or floc pI). In addition, the composition of the water matrix is also likely to play a strong role for many viruses. NOM may compete for sorption sites on flocs when repulsive electrostatic charges govern NOM-virion interactions, or NOM may act as a sorbent to enhance flocculation of hydrophobic virions. Ca^{2+} and Mg^{2+} enhance sorption of viruses to similarly-charged species like NOM, either by cation bridging or surface complexation. Most importantly, current research demonstrates that sorption varies by both virion and environmental conditions.

The potential for inactivation in coagulation processes is both a source of frustration and a promising avenue for water treatment research. Inactivation muddles unit treatment performance testing with artificially high reduction rates. However, future coagulation systems could be optimized for inactivation. Applied research should include at least two bacteriophage surrogates with varying susceptibility to physical removal and inactivation. To inform surrogate selection, and to enable design of improved treatment systems, the mechanism of inactivation by chemical coagulation must be determined. If viruses are inactivated by capsid protein damage, determining a surrogate by physical similarities may be inappropriate. This highlights the need for basic research into coagulation that directly compares human viruses of interest and bacteriophages. More comparisons between bacteriophages are also needed. With more systematic comparisons of multiple bacteriophages, researchers could begin to hypothesize about the variability in bacteriophage performance between experiments.

Plaque assays with recovery from flocs remains the gold standard for quantifying inactivation. More research is required to confirm the validity of using a combination of qPCR and plaque assay without recovery from flocs. The combination of qPCR and plaque assays may prove to be both acceptable and cost-saving for some viruses, but only if future research can show that the method does not underreport inactivation. Furthermore, continued research is needed to determine how inactivation impacts total virus reduction by coagulation. If inactivation of viruses acts as a polishing step for coagulation, seemingly insignificant inactivation would be critical for meeting treatment goals. Further studies comparing the recovery of viruses from flocs by both plaque assay and qPCR could help delineate the relationship between coagulation and inactivation. In addition, inactivation must be separated from aggregation, but quantitative assessment of virus aggregation in treated samples is currently not possible.

Ideally, continued research into the physico-chemical properties of viruses will allow us to predict sorption and inactivation behavior. This type of modeling would help to better identify bacteriophage surrogates as well. Currently, surrogates are often selected based on qualities like size and pI. Unfortunately, the complexity of virus sorption and inactivation eludes such simple measures. Therefore, it is essential to begin to draw connections between virus morphology and physical chemistry. Important strides in this direction have been referenced in this review, such as Langlet *et al.*'s model of virus electrokinetics,[44] Sigstam *et al.*'s model of virus capsid susceptibility to inactivation[89] and Armanious *et al.*'s method for assessing hydrophobicity from virion surface structure.[63] However, these models are still under investigation and cannot yet confidently predict behavior of viruses. Through comparisons of morphologically similar bacteriophages, we can learn more about how minor changes in structure impact sorption and inactivation properties. In the future, we may be able to predict virus behavior and identify new bacteriophage surrogates based on subtle aspects like protein structures or genome size and conformation. The benefits of this work would extend far beyond use in coagulation – from filtration systems, to inactivation by nanoparticles, to modeling virus fate and persistence in the environment and the gastrointestinal tract.

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