Impact of virus surface characteristics on removal mechanisms within membrane bioreactors

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ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 6 July 2015
Accepted 13 July 2015
Available online 15 July 2015

Keywords:
Membrane bioreactor
MS2
PhiX174
Bacteriophage fr
Mechanisms
Virus removal

ABSTRACT

In this study we investigated the removal of viruses with similar size and shape but with different external surface capsid proteins by a bench-scale membrane bioreactor (MBR). The goal was to determine which virus removal mechanisms (retention by clean backwashed membrane, retention by cake layer, attachment to biomass, and inactivation) were most impacted by differences in the virus surface properties. Seven bench-scale MBR experiments were performed using mixed liquor wastewater sludge that was seeded with three lab-cultured bacteriophages with icosahedral capsids of ~30 nm diameter (MS2, phiX174, and fr). The operating conditions were designed to simulate those at a reference, full-scale MBR facility. The virus removal mechanism most affected by virus type was attachment to biomass (removals of 0.2 log for MS2, 1.2 log for phiX174, and 3 log for fr). These differences in removal could not be explained by electrostatic interactions, as the three viruses had similar net negative charge when suspended in MBR permeate. Removals by the clean backwashed membrane (less than 1 log) and cake layer (~0.6 log) were similar for the three viruses. A comparison between the clean membrane removals seen at the bench-scale using a virgin membrane (~1 log), and the full-scale using 10-year old membranes (~2–3 logs) suggests that irreversible fouling, accumulated on the membrane over years of operation that cannot be removed by cleaning, also contributes towards virus removal. This study enhances the current mechanistic understanding of virus removal in MBRs and will contribute to more reliable treatment for water reuse applications.

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1. Introduction

A mechanistic understanding of virus removal in membrane bioreactors (MBRs) is essential to determine their effectiveness as components of treatment trains for potable and non-potable water reuse. Pathogens pose an acute public health threat, and minimizing that risk is crucial for further expanding water reuse as an acceptable approach for sustainably managing strained urban water resources. MBRs are becoming popular as a wastewater technology for water reuse because they combine biological treatment and membrane separation (e.g., microfiltration (MF) or ultrafiltration (UF)) into one unit process, and provide better and more consistent effluent quality than conventional activated sludge (Radjenović et al., 2008; Wachinski, 2013). MBRs are expected to have greater removal of larger pathogens, such as protozoa and bacteria, than viruses, because they are larger than the nominal membrane pore sizes (Ottoson et al., 2006), whereas viruses may be capable of passing through membrane pores due to their smaller size. Virus removal by MBRs is thought to occur via four mechanisms: (i) incorporation of viruses into the mixed liquor suspended solids, which are excluded by the membrane, (ii) retention of viruses by the clean backwashed membrane, (iii) retention of viruses by the cake layer formed on the membrane surface after a period of operation, and (iv) inactivation of the viruses within mixed liquor due to extracellular enzymes and predation (Chaudhry et al., 2015). However, it is unclear how viruses with different morphologies and surface characteristics are affected by each of these mechanisms, and whether any differences in treatment through the MBR process for the myriad of viruses present in wastewater is a cause for concern.
Several studies have demonstrated the ability of full-scale MBRs to achieve over 4-log removal of virus surrogates and bacteriophages (De Luca et al., 2013; Francy et al., 2012; Van den Akker et al., 2014; Zanetti et al., 2010) as well as pathogenic viruses (Chaudhry et al., 2015; Da Silva et al., 2007; Kuo et al., 2010; Sima et al., 2011; Simmons et al., 2011) under long-term steady state conditions. Two pilot studies have demonstrated improvement in seeded phage removal after a period of operation (Trussell et al., 2012) and as trans-membrane pressure increased (TMP) (Marti et al., 2011), indicating the build-up of a protective cake layer on the membrane. However, virus removal mechanisms in MBRs have been investigated only by few studies (Chaudhry et al., 2015; Lu et al., 2013; Lv et al., 2006; Shang et al., 2005; Ueda and Horan, 2000; Wu et al., 2010; Zheng and Liu, 2006). The relevant results from studies that reported mechanistic log removal values are summarized in Table 1. Furthermore, due to differences in the scale of experiments, types of target viruses, and operating conditions, it is difficult to determine which removal mechanism is most susceptible to differences in virus morphology and surface characteristics.

Virus interactions with environmental surfaces such as fecal material, clays, and biological flocs are important in determining their澡ment behavior and consequently their removal during treatment. The outer layer of enteric viruses that are typically found in wastewater consists of protein capsids composed of polypeptides that include weakly acidic and basic amino acids, and the pH-dependent interactions of these functional groups imparts a net charge to the capsids. The pH at which the capsid is uncharged is called the isoelectric point (IEP) (Shaw, 2000). Classic DLVO theory for colloid stability has been invoked to model virus behavior and transport. Electrostatic interactions between two charged particles in dispersion are thought to result from a balance between the repulsive electrostatic double-layer interactions and the attractive van der Waals forces (Shaw, 2000). The model assumes a uniformly charged, flat surface, but discrete charges such as ions or a more complex virus particle with a heterogeneous surface may behave differently.

Recent work investigating pathogenic viruses and phages under controlled solution chemistries (divalent cations, ionic strength, natural organic matter, etc.) has demonstrated the importance of individual virus surface properties in determining attachment and aggregation behavior (Armanious, 2014; Gutierrez et al., 2010, 2009; Mylon et al., 2010; Pham et al., 2009). Controlled laboratory studies with viruses also suggest that hydrophobic effects (Gerba, 1984; Templeton et al., 2008) and stearic impacts of structural components (Redman et al., 1997) can also play a significant role in virus-surface interactions. Therefore, viruses with unique surface properties are hypothesized to have different attachment behaviors under the same conditions. However, it is challenging to extrapolate the results of laboratory studies with well-controlled conditions to the complex environment of a real wastewater treatment system.

The objectives of this study were to determine: (i) how viruses with the same size and shape but with different surface characteristics are removed in a bench-scale MBR process using real wastewater; and (ii) which virus removal mechanisms (retention by membrane, retention by cake layer, attachment to biomass, and inactivation) are affected by differences in virus surface characteristics. The experimental design and operational parameters were based on a full-scale study, which serves as a reference (Chaudhry et al., 2015). We used three phages (MS2, phiX174, and fr), which are all approximately 30 nm in diameter with naked icosahedral protein capsids, MS2 and fr are members of the F-colliphages quantified in the reference study, and have single-
stranded RNA. PhiX174 is a somatic coliphage and has single-stranded DNA. All are simply structured viruses with known genomes and protein structures (Sigstam et al., 2013; Smith et al., 2003). Results from this investigation will allow a better understanding of virus removal mechanisms in MBRs, leading to more reliable treatment for water reuse.

2. Materials and methods

2.1. Experimental approach

A summary of all bench reactor experiments conducted in this study is provided in Table 2. Three sets of mixed liquor sludge experiments were conducted to quantify removal of the phages MS2, phiX174, and fr. Experiments 1 and 2 allowed quantification of removal due to a clean chlorine-backwashed membrane only, experiments 3 and 4 allowed determination of the removal contribution due to the membrane with a 3-day cake layer, and experiments 5, 6, and 7 allowed quantification of removal due to the membrane, cake layer, and mixed liquor biomass. The complex fouling layer (cake layer) that develops on the membrane surface is dependent on constituents in the feed solution. The cake layer composition can be assumed to be similar for all real mixed liquor sludge experiments in this study.

2.2. Reactor setup and operation

The bench-scale reactor design and operating parameters were chosen to simulate conditions in a reference, full-scale plant located in the city of American Canyon, California (Chaudhry et al., 2015). The system consisted of a reactor with a 17-L capacity, a virgin polyvinylidene (PVDF) membrane bundle with a nominal pore size of 0.04 μm (Fig. S1) (Puron®, KMS, Wilmington, MA), an aeration unit at the base of the membrane bundle, permeate and aeration pumps, a pH probe, a temperature probe, a heater, and pressure gauges to measure TMP, all controlled through a LabVIEW-based SCADA system (National Instruments Corp., Austin, TX). The permeate line drained back into the main tank so the volume of the system remained unchanged during the experiments. The reactor setup and sampling locations are shown in Fig. 1.

All experiments were conducted under the same operating conditions. Permeate flux was 25 L/m²·h (LMH) (corresponding to a flow rate of 0.18 L/min), compared to 10 LMH at the reference, full-scale plant. A higher permeate flux was used to increase TMP due to fouling and cake layer buildup in a shorter amount of time. Air scour to the membrane was provided at the base of the membrane bundle, alternating between 20 s with air and 20 s without air. Membrane relaxation (zero TMP) was performed for 1 min every hour. Both aeration and membrane relaxation are utilized at full-scale plants to manage membrane fouling. TMP and pH were recorded every 5 s. All experiments were conducted at 20 °C. Typical Ionic composition of the water and wastewater used for the experiments is shown in Table S1. Feed samples were collected through a sampling port at the bottom of the membrane tank and permeate samples were collected directly from the permeate line; both lines were flushed before collecting samples. The membrane was cleaned by backwashing with a sodium hypochlorite solution and dechlorinated with sodium metabisulfite (Sigma Aldrich) before the beginning of each new experiment.

2.3. Experiments with wastewater mixed liquor sludge

2.3.1. Membrane only

Experiments were conducted for all three phages with a cleaned membrane and dechlorinated tap water to quantify the baseline removal by the membrane alone (experiments 1 and 2). The system was operated for 1 h. Membrane feed and permeate samples were collected at 15-min intervals and enumerated in duplicate.

2.3.2. Membrane, cake layer, and biomass

Two three-day sludge experiments were conducted for MS2 and phiX174 (experiments 5 and 7) and one for phage fr (experiment 6). For all sludge experiments, the bench-scale MBR tank was filled with mixed liquor from a demonstration-scale MBR (Vuono et al., 2013) with a mixed liquor solids concentration of approximately 4000 mg/L. The main tank was completely emptied every 24 h.
taking care not to disturb the cake layer on the membrane fibers, and the sludge was replaced (to prevent significant changes in the composition of the biomass due to starvation). A fresh stock of viruses was seeded into the tank after each sludge replacement. Samples of the membrane feed and permeate were collected at 15 min, 1, 3, 5, 10, and 24 h after the addition of the virus stock solution and viruses were enumerated in duplicate.

### 2.3.3. Membrane and cake layer

At the end of each three-day experiment, the sludge was carefully replaced with dechlorinated tap water without disturbing the cake layer. The feed solution was seeded with the appropriate virus and the MBR was operated for one hour. Feed and permeate samples were taken at 15 min intervals to quantify the effect of the cake layer built up on the membrane without the presence of biomass (experiments 3 and 4).

#### 2.4. Bacteriophage stock preparation

Bacteriophages and their corresponding Escherichia coli host strains were purchased from ATCC (Manassas, VA) and propagated according to recommended protocols. MS2 (ATCC 15597-B1) was propagated using E. coli Famp (ATCC 15597, phiX174 (ATCC 13706-B1) using E. coli CN13 (ATCC 13706), and fr (ATCC 15767-B1) using another E. coli strain, designated hostfr for this study (ATCC 19853). As recommended by ATCC, exponential phase bacterial hosts were used for propagation and enumeration of MS2 and fr, and stationary phase host was used for phiX174. Streptomycin sulfate (Fisher Scientific) and ampicillin sodium salt (MP Biochemicals) were used as antibiotics for Famp, each at a final concentration of 0.015 g/L. CN13 was grown with nalidixic acid (Fisher Bioreagents) at a final concentration of 0.01 g/L, and hostfr was grown without antibiotics.

To prepare phage stocks, host bacteria were grown overnight in 30 mL of tryptic soy broth (TSB) with antibiotics (BD Diagnostic Systems) and then inoculated with 50 μL of second-generation phage. If exponential phase host was needed, 1% of the overnight culture was reinoculated into fresh TSB, and phage was introduced 5–8 h later. Phage was propagated for 12–18 h. To harvest phage, chloroform was added to the culture (1:2 by volume) to lyse the host bacteria, vortexed for 2 min, and then centrifuged (2000 g) for 10 min. Phage stock was decanted from the top layer, mixed with antibiotics, 50 μL of bacterial host, the appropriate sample dilution, and poured onto Petri plates with already prepared bottom agar. Typically 100 μL of the feed samples and 1 mL of permeate samples were plated in each dilution. Plates were incubated overnight at 37 °C and virus plaques on a bed of host bacteria were counted after 12–18 h. Modified LB agar components included 10 g of tryptone, 8 g of sodium chloride, 1 g of yeast extract, 1 g of dextrose, 0.3 g of calcium chloride, 15 g of Bacto® Agar (BD) for the bottom agar layer, and 7.5 g for the top agar layer. The appropriate antibiotics were added to the top layer only.

All samples were enumerated in duplicate, using between three to six dilutions, along with host blanks for each bacterial host. The appropriate dilution (containing between 10 and 100 PFU on one Petri plate) from each independent replicate was used to calculate the geometric average concentration and the standard deviation. Error propagation is discussed in section 2.9. Cross-reactivity tests were conducted ahead of the experiments to determine whether a particular phage infected bacterial hosts other than its own. Famp and CN13 were only infected by their own phages in the presence of antibiotics; however, hostfr was infected by all three phages. Thus, MS2 and phiX174 were spiked at the same time for experiments, as the presence of one did not interfere with the enumeration of the other, but dedicated experiments were conducted for phage fr.

#### 2.6. Virus surface characterization

##### 2.6.1. Phage zeta potential measurements

The charge on virus protein capsid functional groups is affected by the chemistry and pH of the surrounding solution. While overall charge of the virus particle does not capture the variation in surface charge or hydrophobicity, this single parameter can provide some insight into virus attachment and removal behavior (Redman et al., 1997). The zeta potential of each type of phage (MS2, phiX174, and fr) was measured in MBR permeate using electrophoretic light scattering on a Malvern Zetasizer Nano-ZS instrument. Phage stocks were prepared as described in section 2.4 and seeded into MBR permeate samples at environmentally relevant pH values (5–8) using hydrochloric acid or sodium hydroxide. The pH of each sample was recorded, and at least four replicate zeta potential measurements were taken for each phage.

##### 2.6.2. Quantification of protein residues on the virus capsids

The number and types of structural amino acid functional groups present on the three different virus capsids were determined, as a starting point for understanding their attachment behavior to mixed liquor biomass. Amino acids (generic formula \( HOOC – R – CHNH₂ \)) were classified as negatively charged, positively charged, hydrophobic, uncharged, and special cases at neutral pH based on the type of functional groups within the side chain \( R \) (Madigan et al., 2012). The external amino acids present on each of the three bacteriophages were counted from three-dimensional computer-generated protein capsid models from the database VIPERdb (Carrillo-Tripp et al., 2009). Details of the amino acid classification and enumeration are given in the Supporting Information (Tables S2 and S3).

#### 2.7. Quality control

##### 2.7.1. Incorporation of seeded viruses into biomass

Phage stocks added to the bench-scale MBR at the beginning of each experiment may not have been in immediate attachment equilibrium with the mixed liquor biomass. To determine whether lab-grown phages mimicked indigenous viruses in terms of attachment to biomass, feed samples were separated into solid and liquid fractions at 1 and 24 h after the virus spike, and each fraction was enumerated separately. Feed samples (5 mL) were centrifuged at 10,000 × g for 1 h, which allowed a theoretical particle size cutoff of 340 nm based on Stokes settling (Merlo et al., 2007). The solid and liquid fractions were enumerated separately for the relevant
phages, in addition to the routine quantification of total feed concentration. Although the seeded phages were primarily in the liquid phase at 1 h (data not shown), over 95% of the seeded phages were attached to the solids component of the mixed liquor after 24 h (Fig. S2). Such fractionation is typical for indigenous viruses (Chaudhry et al., 2015) and demonstrates the relevance of these bench-scale results to real-world applications.

2.7.2. Indigenous and seeded phage comparison

Wastewater mixed liquor sludge contains indigenous phages, many of which may be capable of infecting the host bacteria used for quantification. To ensure that the infectivity assays used for quantification targeted the seeded phage only, we quantified indigenous phage present in the sludge using all three host bacteria. The concentrations of indigenous phage ranged from 10¹ to 10² PFU/mL. Target concentrations for seeded phages were between 10⁵ and 10⁸ PFU/mL, ensuring only the seeded phage were quantified at the relevant dilutions during experiments.

2.8. Data analysis and calculations

2.8.1. Virus decay

Virus inactivation was modeled as a first-order decay process (Shang et al., 2005; Sigstam et al., 2013; Wu et al., 2010). The first-order decay constant \( k \) was determined for each phage in mixed liquor sludge for experiments 5–7 based on Equation (1), where \( C(t) \) is the concentration of the virus at time \( t \) and \( C_{initial} \) is the starting concentration:

\[
C(t) = C_{initial}e^{-kt}
\]  
(1)

Equation (1) was fitted to the 3–24 h time-series feed and permeate data collected in experiments 5–7 by linear regression to determine the decay constants for each phage.

2.8.2. Log removal mechanism contributions

Virus log removal values (LRV) between a starting feed concentration \( C_{feed} \) and a final permeate concentration \( C_{permeate} \) were calculated using Equation (2):

\[
LRV = -\log_{10}\left(\frac{C_{permeate}}{C_{feed}}\right)
\]  
(2)

Membrane only LRV contributions were determined directly from experiments 1 and 2. Experiments 3 and 4 represent the LRV for the membrane plus a 3-day cake layer; therefore, subtracting the membrane only LRV provides the contribution of the sludge cake layer only. The LRVs after 72-h in experiments 5 through 7 represent the total contributions from all three mechanisms (membrane only, cake layer and biomass). Subtracting the LRV for a caked membrane (experiments 3 and 4) from the combined LRV of all mechanisms (experiments 5 and 6) yields the contribution of the biomass.

2.8.3. TMP and log removal values

The TMP is the driving force required to transport liquid through the membrane. An increase in TMP during operation can be used as an indicator of the buildup of fouling (both cake layer and irreversible) on the membrane surface. For experiments 5 through 7, the increase in LRV over each 24-h period of operation was plotted against the increase in TMP (corresponding to the buildup of the cake layer) to elucidate any trends that may be present. The initial value of LRV and TMP was taken to be at one hour after the virus spike for the three phages.

2.9. Quantification of uncertainty

For directly measured concentrations, standard deviation was calculated and the standard error is reported. The error for calculated values was propagated as follows. For a function \( y \), dependent on variables \( x_1, x_2, ..., x_n \) with associated uncertainties \( \delta_{x_1}, \delta_{x_2}, ..., \delta_{x_n} \), function uncertainty \( \delta_y \) can be propagated using the Equation (3):

\[
\delta_y^2 = \left(\frac{\partial y}{\partial x_1}\delta_{x_1}\right)^2 + \left(\frac{\partial y}{\partial x_2}\delta_{x_2}\right)^2 + \ldots + \left(\frac{\partial y}{\partial x_n}\delta_{x_n}\right)^2
\]  
(3)

Based on Equation (3), associated error for log removal values was calculated using Equation (4):

\[
\delta_{LRV}^2 = \left(\frac{\delta y}{\ln 10 \ C_{feed}}\right)^2 + \left(\frac{\delta y}{\ln 10 \ C_{permeate}}\right)^2
\]  
(4)

Each zeta potential measurement from the dynamic light scattering instrument was reported with a mean and standard deviation. Several measurements, \( n \), were taken with fresh samples and the standard deviation of averaged value was determined using Equation (5):

\[
\delta_{ZP}^2 = \left(\frac{\delta y}{n}\right)^2 + \left(\frac{\delta y}{n}\right)^2 + \ldots + \left(\frac{\delta y}{n}\right)^2
\]  
(5)

All other uncertainties reported in this study were also propagated based on Equation (3).

3. Results and discussion

3.1. Phage inactivation within the mixed liquor

The time-series feed and permeate concentrations, and log removals for the experiments with the membrane, the associated cake layer, and mixed liquor biomass (experiments 5 and 6) are shown in Fig. 2. After each seeding event (every 24 h), there was a gradual decrease in phage concentrations in the feed, but the rate decreased over time. We interpreted the decline as the result of two processes: 1) attachment processes of viruses to biomass, reactor walls, tubing, and other surfaces (attachment to biomass would have caused an apparent decrease in phage concentrations if two or more phages remained attached to the same particle during enumeration), and 2) inactivation processes. As described in Section 2.7.1, in separate batch experiments we measured the attachment of viruses to solids and found that it increased dramatically between 1 h and 24 h after seeding; unfortunately we did not measure attachment at intermediate time points. To analyze the data from experiments 5 and 6, we assumed that virus attachment to biomass and reactor components was at equilibrium by 3 h, and that the attachment processes dominated over inactivation during this initial time period. This assumption was based on the observation that the phage feed concentrations decreased rapidly during the first 3 h, at a faster rate than could be explained by first-order decay. For the period 3–24 h after seeding, the decrease in phage concentration followed first-order kinetics. We assumed that inactivation processes were dominant during this time due to enzymes and predation in the mixed liquor. To estimate the virus inactivation rate, we modeled decay as a first-order process between 3 and 24 h after seeding.

The decay constants, \( k \) (hour⁻¹), were found to be 0.02, 0.13, and 0.07 for MS2, phiX174, and fr, respectively (Table 1). The modeled concentrations in Fig. 2 were calculated by assuming first-order decay of the measured feed and permeate concentrations at 3 h. To put these values in perspective, the calculated LRVs that would
occur in an MBR with a typical hydraulic residence time of 10 h are 1.0, 0.6, and 0.3 for MS2, phiX174, and fr, respectively. The calculated decay constants for MS2, phiX174, and fr are lower than those reported previously for other viruses in MBRs (Table 1). It is possible that the mixed liquor biomass did not perfectly replicate the conditions in full-scale plants because of the lack of a constant flux of fresh wastewater. If the activity of the biomass was stunted, processes that contribute to phage inactivation (e.g., extracellular enzymes and higher order predators) may have occurred at slower rates. Also, it is possible that the inactivation rate constant was higher during the initial 3 h, but we did not include this period due to the inability to separate inactivation from attachment. Alternatively, if virus attachment to biomass continued to increase after 3 h, then our calculated decay rate constants may be too high.

3.2. Membrane, cake, and biomass mechanism contributions to log removal

The results for membrane only (experiments 1–2) and caked membrane experiments (3–4) are shown in Fig. 3 as the log removal observed 45 min after seeding the viruses. The log removal was consistent over the course of these hour-long experiments (data not shown). The log removal values due to the combination of membrane, biomass, and the cake layer developed over three days are also shown in Fig. 3; these values were calculated from the 72-h feed and permeate concentrations from experiments 5 and 6. The graphical abstract presents the same results but separated into the individual contributions of each mechanism.

The membrane only contributions were 0.9, 0.4, and 0.5 logs for MS2, phiX174, and fr, respectively. The contribution due to the cake layer appears to be independent of the phage type (0.6 for MS2 and 0.7 for both phiX174 and fr); however, the removal attributed to attachment to mixed liquor biomass was significantly different. Only 0.2 logs could be attributed to biomass for MS2, while the presence of biomass caused a large increase in log removal for phiX174 and fr at 1.2 and 3.0 logs, respectively. The results from the solid–liquid fractionation experiments from section 2.7.1 (360 nm size cutoff) did not offer the resolution to distinguish differences in solid attachment among the seeded phages (Fig. S2), although they suggest that removal by biomass should be more similar than what was observed. Based on experiments 5 and 6, we hypothesize that the differences in the biomass mechanism log removal contribution may be due to the surface composition of the phages. A comparison among these and previously reported mechanism removal contributions results is shown in Table 1.

3.3. Virus surface characteristics and attachment to biomass

Phage particles (MS2, phiX174, and fr) suspended in MBR permeate were all determined to be net negatively charged over the relevant pH range (5–8) using zeta potential measurements (Fig. 4a). There were no statistically significant differences in the
zeta potential of the three phages. Most natural particles in water are also negatively charged (Templeton et al., 2008) so electrostatic interaction at the scale of the whole capsid would not explain the differences in attachment behavior to mixed liquor biomass. This result was surprising because while previous literature has reported MS2 to be net negatively charged (Gerba et al., 2003; Michen and Graule, 2010), phiX174 has been reported to be almost uncharged (Michen and Graule, 2010) and fr was suspected to be positively charged at neutral pH (Gerba et al., 2003). However, a recent study has reported fr to be negatively charged (Armanious, 2014). The large variation of reported isoelectric points in the literature for some viruses suggests that differences in stock preparation, purification protocols, and the specific solution chemistry all affect the net charge of the virus capsid.

Inter-molecular forces (such as van der Waal’s forces, electrostatics, hydrophobic interactions, etc.) are dominant at scales of less than 10 nm, so it is plausible that individual functional groups or regions on the virus capsid (~30 nm diameter) could interact with other surfaces somewhat independently of each other, especially if they are situated far apart on the virus capsid. Fig. 4b shows the total number of negatively charged, positively charged, and hydrophobic amino acid side chains present on the outside of the capsids of the three viruses (based on Tables S2 and S3). Mixed liquor biomass mostly consists of bacterial flocs and extracellular polymeric substances, which contain hydrophobic portions that may encourage phage attachment. Phage fr has the largest number of hydrophobic functional groups on its surface, followed by MS2 and phiX174. Negatively charged functional groups follow a similar trend. All three viruses have a similar number of positively charged groups. Phage fr demonstrated the highest log removal due to attachment to biomass and it also has the highest number of hydrophobic amino acid functional groups on its capsid. However, the number of hydrophobic groups alone cannot explain the differences in attachment behavior because MS2 also has a high number of hydrophobic groups but demonstrated very little removal attributable to attachment to mixed liquor biomass. The behavior of the virus capsid does not appear to be dependent only on its structural composition, but is affected by other materials (such as host cell debris or other foreign particles) that may be deposited on its surface. Based on our analysis, it is difficult to isolate the reasons for the significantly different observed removals of the three phages in the presence of mixed liquor biomass, but a more detailed inquiry into the complex surface—particle interactions may shed some light on the issue.

3.4. Cake layer and TMP

Log removal for all three phages generally improved over the course of the three-day mixed liquor experiments (experiments 5–7; data not shown for replicate experiment 7 in Fig. 2) with the membrane, biomass, and cake layer (Fig. 2). The experiments started with a clean, backwashed membrane and the concentration of the biomass remained consistent; therefore, the increase in log removal can be attributed to the buildup of a fouling layer on the membrane. In Fig. 5 the increase in log removal is shown as a function of the increase in TMP for each 24-h period for MS2, phiX174, and fr (experiments 5 through 7). For all three phages, log removal improved with increasing TMP, although the trend is most prominent for phiX174. Based on these results, and those presented in Fig. 3, no significant differences in LRV of the three phages were observed due to membrane cake layer mechanism.

The observed change in TMP during the experiments was small (<0.5 psi) but it is comparable to the TMP recovery seen at the reference full-scale plant after a chlorine backwash (ranging from 0.1 to 0.6 psi), which suggests that the scale of TMP change in the bench-scale experiments is realistic. SEM images of sacrificial membranes provide visual evidence of fouling (Fig. S3). Experimental conditions with larger TMP changes would be unrealistic because large fluctuations in TMP are undesirable at an MBR plant. TMP increase provides a measure of the amount of fouling on the membrane but cannot distinguish between the buildup of the cake layer, which is removed by chemical cleaning (reversible fouling) and irreversible fouling, which cannot be removed by any cleaning. The membrane bundle used in all experiments was constructed from virgin membranes and contained no irreversible fouling before use. Given the short timespan of the experiments, it is likely that the increase in TMP was due to buildup of reversible cake layer. In contrast, at the reference full-scale plant, which was operating with membranes that were up to 10 years old, the LRV due to the membrane only was higher (2–3 log as reported in Table 1) than in the bench-scale experiments (<1 log). It is likely that irreversible fouling on the field-scale plant membranes resulted in additional virus removal. There was evidence of irreversible fouling in the bench-scale experiments as well, as the TMP
never completely recovered when the membrane was chlorine backwashed after each subsequent experiment.

4. Conclusions

- The three bacteriophages studied have the same size, but have different surface properties. The biomass attachment removal mechanism was the most dependent on virus type. Removals by the clean membrane and the cake layer were similar for all three viruses.
- Virus removal via attachment to biomass was 0.2 log for MS2, 1.2 log for phiX174, and 3.0 log for \( \text{fr} \). Electrostatic interactions could not explain the differences, as there were no statistically significant differences in the zeta potential of the three phages suspended in MBR permeate over the pH range 5–8.
- After 3 days, the cake layer contributed just less than 1 log removal for all three phages, which is consistent with prior observations at a full-scale MBR. Throughout the 3-day operation, the removal of all three phages increased with TMP, which was likely due to an increased contribution by the cake layer over time.
- Long-term operation (years) may increase virus removal, based on the difference in membrane only removal for the bench-scale work reported in this study (less than 1 log) compared to our previous full-scale study (2–3 log; [Chaudhry et al., 2015]). The bench-scale work used virgin membranes, whereas the full-scale membranes had been in operation for up to ten years. The buildup of irreversible fouling over the lifetime of the full-scale membranes might explain the difference in membrane only LRV between the two studies.
- In the previous full-scale study (Chaudhry et al., 2015), the highest removal contributions were from the clean membrane (2–3 log) and cake layer (up to 1.6 log), while attachment to biomass was the least important mechanism (0.5–1 log). Thus, the variability in removal by the biomass mechanisms measured at the bench-scale may have a smaller impact on overall virus removal at full-scale MBRs.

Competing financial interest

The authors declare no competing financial interest.

Acknowledgements

The authors thank Profs. Josh Sharp, John Spear, and the researchers in the GEM lab for hosting R.M.C. at CSM, and for providing lab space, materials and general support. Special thanks are due to Dina Drennan and Jeffrey Ladderud for SEM help, and to Caroline Delaire for assistance with the zeta potential measurements. Financial support for this research was provided by an NSF Graduate Research Fellowship to R.M.C. and the NSF-funded Engineering Research Center for Reinventing the Nation’s Urban Water Infrastructure (ReNUWIt) (EEC-1028968).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.07.020.

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